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

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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, 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.

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Keywords

neuropeptide; resolvins; diabetic retinopathy; treatment

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 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.

Materials and Methods

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 24h. Cells were exposed to VIP (10^{-9} M) for 4h[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 4h, 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.

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 5,000 × *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.

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.

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.

Results

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 Figure 1. As expected, TNF-α protein levels were significantly increased by HREC under high glucose compared to normal glucose conditions (control). This effect was abrogated with VIP treatment, whereby TNF-α levels were similar to controls. No significant effect was observed with VIP treatment in cells cultured in normal glucose.

Effect of VIP on pro-resolving mediators

Our previous research on the cornea has demonstrated VIP's therapeutic effect to be both anti-inflammatory and pro-resolving[17,22–24]. As such, we next addressed a potential mechanism by which VIP might mediate these pro-resolving effects by looking at RvD1 and corresponding receptors, ALX/FPR2 and GPR32. As illustrated in Figure 2A, RvD1 levels were significantly reduced in high glucose versus normal glucose conditions. However, levels were increased with VIP treatment in high glucose. No notable changes were observed in HREC after VIP treatment under normal conditions.

In addition, RvD1 receptors ALX/FPR2 and GPR32 were significantly down-regulated (approximately 50% and 40%, respectively) after high glucose exposure compared to normal glucose controls (Fig. 2B and C, respectively), which is consistent with the changes observed in their ligand, RvD1. Further, VIP treatment enhanced protein levels of both receptors, albeit only GPR32 was significantly increased (28%) over high glucose only. ALX/FPR2 and GPR32 levels after VIP treatment of normoglycemic cells remained comparable to controls.

VIP receptor expression by HREC

As the two predominant receptors of VIP, levels of VPAC1 and VPAC2 were evaluated in HREC. Although described as the major VIP receptor on most cell types[12], VPAC1 was not detected in HREC in either normal or high glucose. In contrast, VPAC2 was constitutively expressed under both high and normal glucose conditions (Fig. 3). However, VPAC2 protein levels were significantly reduced in high glucose versus normal glucose conditions, despite VIP treatment.

VIP-induced effects are carried out by VPAC2 receptors

Despite that VPAC2 was predominantly expressed on HREC, we next confirmed whether the VIP-induced changes in TNF-α levels were directly mediated by this receptor. To do so, HREC were cultured under high glucose conditions followed by exposure to a receptor antagonist for either ALX/FPR2 (WRW4) or VPAC2 (Leu) prior to VIP treatment. As shown in Figure 4, results indicate that VIP-induced down-regulation of TNF-α was abrogated in the presence of the VPAC2 antagonist, but not the ALX/FPR2 antagonist. In fact, TNF-α levels from HREC cultured in HG +VIP+Leu were comparable to HG only and significantly higher than NG, NG+VIP and HG+VIP. While the ALX/FPR2 antagonist, WRW4, did result in a slight increase in TNF-α levels compared to HG+VIP, this difference was not statistically significant.

VIP treatment prevents high glucose-induced increases in VEGF

Considerable clinical effort has been put forth to inhibit VEGF, as it is well known to cause retinal permeability and neovascularization in diabetes. As VIP was previously reported to enhance growth factor production[24], we investigated VEGF protein levels after VIP treatment under both normal and high glucose conditions to evaluate this potential side effect. As depicted in Figure 5, VIP treatment did not have any effect on VEGF levels under normal glucose conditions. Remarkably, high glucose-induced VEGF levels, however, were significantly reduced after VIP treatment.

Discussion

Studies have indicated TNF-α is an important mediator of the retinal pathology observed under hyperglycemic conditions, including leukocyte adherence in retinal blood vessels[25], retinal endothelial cell apoptosis[26], pericyte loss and capillary degeneration[27,28] and vascular permeability and leukostasis[29]. It has been previously shown by Jiang et al. that TNF-α levels are increased in HREC cultured under hyperglycemic conditions[26]. Therefore, as an initial step toward characterizing the retino-protective effects of VIP during DR, we examined whether VIP can regulate levels of this potent pro-inflammatory cytokine. As expected, high glucose significantly induced TNF-α expression in HREC, suggesting its involvement in the diabetic inflammatory response. VIP abrogated this effect, thus returning TNF-α to control levels. This anti-inflammatory effect of VIP has been reported in other diabetic systems; as VIP was shown to inhibit TNF-α induced apoptosis in acinar cells isolated from submandibular glands of non-obese diabetic mice with salivary dysfunction through functional VPAC1 receptors coupled to the protein kinase A signaling pathway[30]. The current study suggests that VIP, as an alternate treatment against DR, may effectively

ameliorate the initial pro-inflammatory cytokine release observed *in vivo*; however this effect appears to be VPAC1-independent and associated with the pro-resolving molecule, RvD1.

The balance between TNF-α and RvD1 appears to be an important factor in disease pathogenesis. Previous research also indicates that TNF-α can suppress RvD1 expression[8]. Using a uveitis model, topical ocular application of RvD1 was shown to reduce levels of TNF-α, resulting in improved disease outcome[31]. Likewise, in a diabetic mouse model, RvD1 expression was enhanced with the reduction of TNF-α following etanercept treatment, leading to a reduction of pathological retinal angiogenesis[8]. This resolvin has been demonstrated to reduce angiogenesis and protect against retinopathy[8]. Therefore, we next sought RvD1 as a potential mechanism by which VIP might mediate its pro-resolving effects. To this end, the current study showed that high glucose conditions decreased RvD1 levels in HREC. More importantly, VIP treatment effectively up-regulated RvD1 production after high glucose exposure similar to observed normal glucose levels. In addition, high glucose-induced reduction of ALX/FPR2 and GPR32 was partially rescued after VIP treatment. Both of these RvD1 receptors are important in carrying out the resolution of acute inflammation by mediating PMN recruitment[31], and promoting D1-miRNA circuits[33]. These data support the idea that VIP's pro-resolving effects are carried out, at least in part, via lipid mediator circuits. Further, the inverse relationship between TNF-α and RvD1 enhance the efficacy of VIP as a potential therapeutic.

Although VPAC1 is broadly expressed on different cell types, most notably immune cells[12], and has received considerable attention for its anti-inflammatory effects, it was not detected in HREC. VPAC1 has been detected in rat brain microvascular endothelial cells[34], as well as transformed murine endothelial cells derived from heart (H5V)[35], indicating that a lack of VPAC1 detection could be unique to retinal endothelial cells. In contrast, VPAC2 appeared to be constitutively expressed under normal glucose conditions, yet decreased after exposure to high glucose. We have previously shown in a bacterial keratitis model that VPAC2 is more strongly correlated with tissue homeostasis and disease resolution[19]. Regarding diabetes, Ma et al. have indicated that VPAC2 activation leads to improved glucose and lipid metabolism, while increasing insulin sensitivity in *db/db* mice[36]. In the current study, we demonstrate that VPAC2, not VPAC1, is expressed by HREC, thus suggesting a potential mechanism by which VIP treatment could ameliorate disease progression of DR. Although VIP treatment was not able to rescue high glucose-induced down-regulation of VPAC2, it is possible that this receptor could be associated with RvD1 expression/activation. In this regard, VIP-induced changes in TNF-α levels were abrogated by a VPAC antagonist. These findings support the notion that the observed VIP-mediated effects regarding this pro-inflammatory mediator are carried out primarily both of this receptor pathway, which will be further explored in future in vivo studies.

In light of our previous research highlighting VIP's ability to enhance growth factor expression during corneal wound healing and reconstitution of the extracellular matrix[23], we next determined whether this neuropeptide increases VEGF levels. VEGF has been implicated as a major causative factor in diabetic macular edema, retinal neovascularization and related complications[37]. Remarkably, HREC expression of VEGF was significantly

down-regulated in high glucose with VIP treatment compared to high glucose only. Similar to the cornea, which must remain clear for accurate visual processing, VIP treatment does not appear to induce angiogenesis via VEGF expression. These findings are essential in moving forward with investigating VIP as an alternative therapy for DR.

Overall, the current study reports the expression of VPAC2, but not VPAC1, on retinal endothelial cells. Additionally, it indicates a novel regulatory role for VIP over RvD1 levels. Taken together, these findings provide rationale to further explore the therapeutic potential of VIP in the development and progression of DR. This neuropeptide not only reduced anti-inflammatory mediators, but is tied to important lipid mediator circuits, as well. In addition, these data suggest that VPAC2 (not VPAC1) and ALX/FPR2 are the major receptors involved in VIP signaling in HREC.

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Highlights

- VIP treatment reduces high glucose-induced TNF-α levels in HREC
- High glucose reduces RvD1 expression by HREC
- VIP treatment restores RvD1 levels in HREC exposed to high glucose
- ALX/FPR2 and GPR32 are expressed on HREC
- VPAC2 is the predominant receptor expressed on HREC

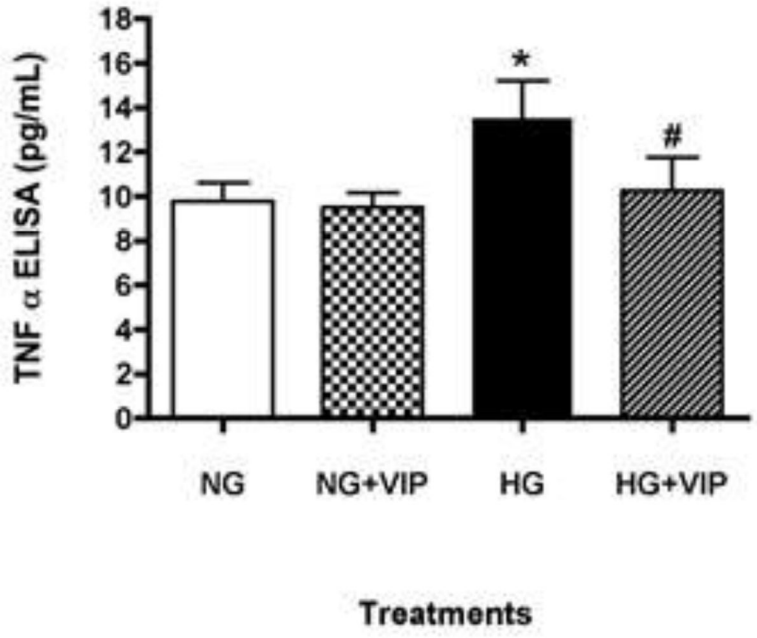

Figure 1.

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 hours. 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.

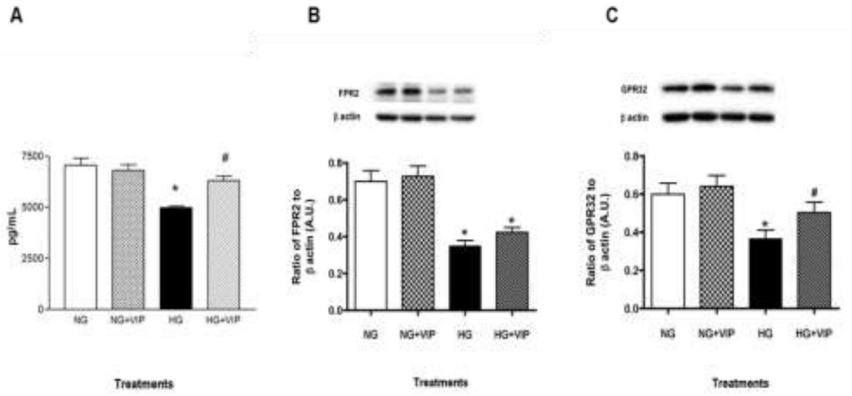

Figure 2.

HREC were cultured under normal glucose (NG, 5 mM) and high glucose (HG, 25 mM) conditions +/− VIP treatment (10^{-9} M) for 4 hours. Protein levels of RvD1 (A) were measured by ELISA and its receptors ALX/FPR2 (B) and GPR32 (C) were detected by Western blot. 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.

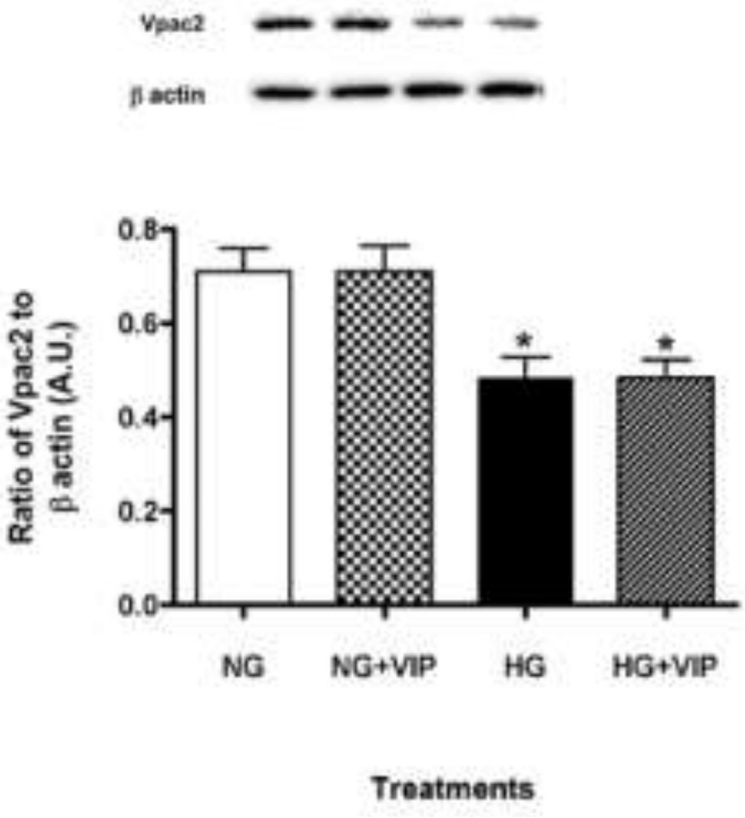

Figure 3.

VPAC2 receptor levels. HREC were cultured under normal glucose (NG, 5 mM) and high glucose (HG, 25 mM) conditions +/− VIP treatment (10^{-9} M) for 4 hours. Protein levels of VPAC2 were examined by Western blot. 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.

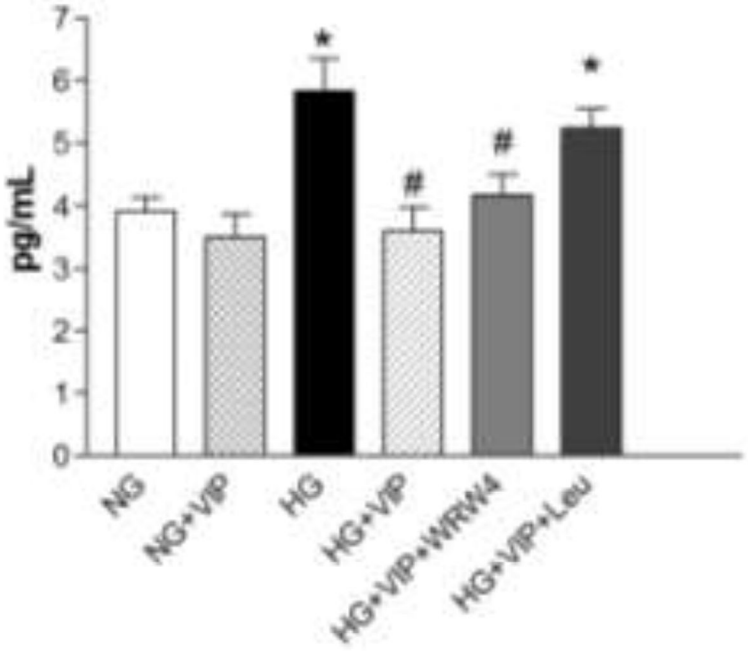

Figure 4.

TNF-α protein levels as detected by ELISA. HREC were cultured under normal glucose (NG, 5 mM) and high glucose (HG, 25 mM) conditions +/− ALX/FPR2 antagonist (WRW4) or VPAC antagonist (Leu) +/− VIP treatment (10^{-9} M). 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.

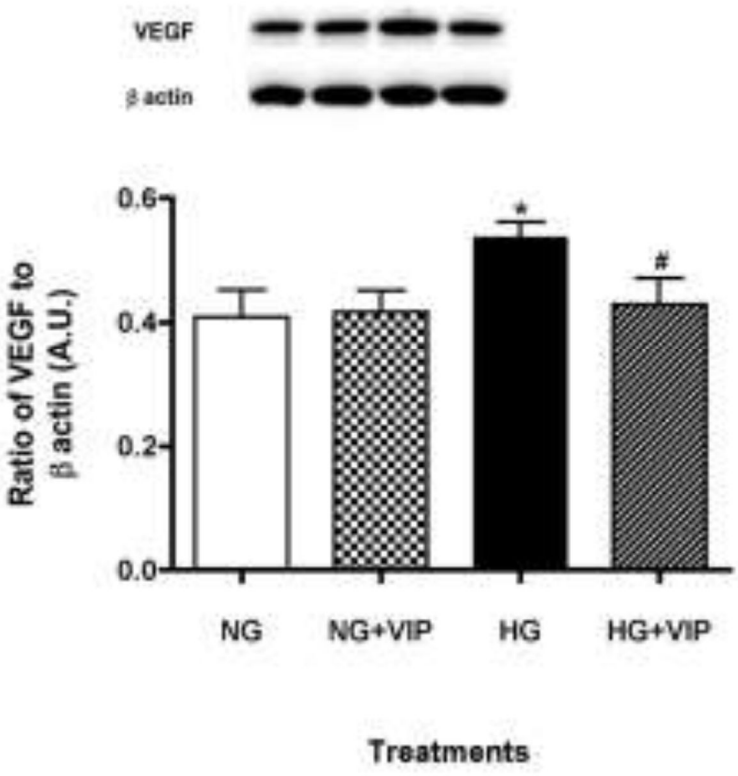

Figure 5.

Protein levels of VEGF as detected by Western blot. HREC were cultured under normal glucose (NG, 5 mM) and high glucose (HG, 25 mM) conditions +/− VIP treatment (10^{-9} M) for 4 hours. 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.