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Prevention of VEGF-induced growth and tube formation in human retinal endothelial cell by aldose reductase inhibition

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Abstract

Objective—Since diabetes-induced vascular endothelial growth factor (VEGF) is implicated in retinal angiogenesis, we aimed to examine the role of aldose reductase (AR) in VEGF–induced human retinal endothelial cell (HREC) growth and tube formation.

Materials and Methods—HREC were stimulated with VEGF and cell-growth was determined by MTT assay. AR inhibitor, fidarestat, to block the enzyme activity and AR siRNA to ablate AR gene expression in HREC were used to investigate the role of AR in neovascularization using cell-migration and tube formation assays. Various signaling intermediates and angiogenesis markers were assessed by Western blot analysis. Immuno-histochemical analysis of diabetic rat eyes was performed to examine VEGF expression in the retinal layer.

Results—Stimulation of primary HREC with VEGF caused increased cell growth and migration, and AR inhibition with fidarestat or ablation with siRNA significantly prevented it. VEGF-induced tube formation in HREC was also significantly prevented by fidarestat. Treatment of HREC with VEGF also increased the expression of VCAM, AR, and phosphorylation and activation of Akt and p38-MAP kinase, which were prevented by fidarestat. VEGF-induced expression of VEGFRII in HREC was also prevented by AR inhibition or ablation.

Conclusions—Our results indicate that inhibition of AR in HREC prevents tube formation by inhibiting the VEGF-induced activation of the Akt and p38-MAPK pathway and suggest a mediatory role of AR in ocular neovascularization generally implicated in retinopathy and AMD.

Keywords

Neovascularization; VEGF; oxidative stress; tube-formation; Aldose reductase

1. Introduction

Neovascularization is one of the key factors in disease induction and progression in many ocular inflammatory diseases, and often leads to severe vision loss (Michels, Schmidt-Erfurth, & Rosenfeld, 2006; Chappelow & Kaiser, 2008; Tolentino, 2009). Potentially blinding ocular diseases such as diabetic retinopathy (DR) and age-related macular

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degeneration (AMD) which are prevalent in developed countries involve neovascularization (Tolentino, 2009; Crawford, Alfaro, Kerrison & Jablon, 2009; Emerson & Lauer, 2007). According to an epidemiological study, approximately 8% of legally blind people between the ages of 20 and 72 in the US have diabetic retinopathy as a cause of their blindness (Klein, Klein, & Moss, 1989). Similarly, the prevalence of neovascular AMD in the US population is estimated to be approximately 1.5% in those aged 40 years and older and is estimated to double by the year 2020 (Friedman et al., 2004). An effective and adequate therapy is still elusive for both pathologies. Although laser therapy is used to obliterate new blood vessels and stop vascular leakage and neovascularization in AMD and DR, it has side effects such as permanent damage to the retina with local visual field loss and is often ineffective in the long term.

Vascular endothelial growth factor (VEGF) has been implicated in the induction, as well as progression of both DR and AMD, making VEGF and its signaling intermediates potential targets for therapeutic intervention in these eye diseases (Michels, Schmidt-Erfurth, & Rosenfeld, 2006; Tolentino, 2009; Andreoli & Miller, 2007). This has resulted in a search for an effective anti-VEGF therapy and led to the finding of many new agents targeting VEGF (Chappelow & Kaiser, 2008; Andreoli & Miller, 2007; Ciulla, & Rosenfeld, 2009). In most cases antibodies against VEGF or VEGF receptor antagonist have been used successfully to prevent neovascularization in experimental models and are at different stages of clinical trials (Emerson & Lauer, 2007; Eyetech Study Group, 2002; Krzystolik et al., 2002; Aisenbrey et al., 2007; Kourlas, & Abrams, 2007; Arevalo, & Garcia-Amaris, 2009; Jardeleza, & Miller, 2009; Dixon, Oliver, Olson, & Mandava, 2009). However, these approaches have inherent side effects, and their long-term effect is not known. Therefore, there is a need to develop novel therapeutic agents, which can effectively block new vessel formation locally in the eye without resulting in systemic side effects.

VEGFs are members of a family of proteins, which include VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PIGF (placental growth factor). These molecules with strong angiogenic potential, which act through the tyrosine kinase receptors VEGFR-1, VEGFR-2, and VEGFR-3 located on the membrane (Shibuya, & ClaessonWelsh, 2006). The induction of pathologic neovascularization has been credited to VEGF-A, which has been studied extensively (Adamis et al., 1994; Shams, & Ianchulev, 2006; Bhisitkul, 2006; Nieves, D'Amore, & Bryan, 2009). Activation of protein kinase C (PKC), due to as varied stimuli as hypoxia, trauma, and inflammation, has been implicated in the increased synthesis of VEGF (Zhou, Yang, Xie, & Yin, 2002; Suzuma et al., 2002; Young et al., 2007; Xia et al., 2007; Xiao, Zeng, Ling, & Lv, 2006; Xu et al., 2008). Once active, VEGF binds to a receptor and initiates a signaling cascade that, in turn, activates the transcription of several genes involved in cell growth and differentiation, which leads to new vessel formation (Guo et al., 1995; Xia et al., 1996; Larrivée, & Karsan, 2000; Gliki, Wheeler-Jones, & Zachary, 2002). Several strategies are being explored to inhibit the action of VEGF, in addition to those aimed at neutralizing anti-VEGF antibodies, receptor antagonists, and soluble receptors. Such strategies include examination of inhibitors of VEGF receptor function and upstream inhibitors of VEGF regulators, such as PKC, PI3K and other upstream kinases (Tolentino, 2009; Wang, Nomura, Patan, & Ware, 2002; Alvarez et al., 2009; Guma et al., 2009). Various investigators have presented evidence that antioxidants and natural polyphenolic compounds with antioxidant properties could prevent neovascularization in experimental animals (Dorrell et al., 2009; Chen, Li, Xing, & Cao, 2008; Tan et al., 2008; Kim et al., 2007; Kim et al., 2009; Keshavarz et al., 2009). Recently our lab has demonstrated that inhibition of aldose reductase (AR), a glucose and lipid aldehyde metabolizing enzyme, has anti-inflammatory and anti-oxidative properties and could prevent cancer angiogenesis in-vitro and in-vivo (Tammali, Reddy, Srivastava, & Ramana, 2011; Yadav et al., 2009a; Yadav et al., 2009b; Yadav, Srivastava, & Ramana, 2010). However, role of AR inhibition

in diabetes-induced retinal neovascularization is not known. Therefore, in the present study we have investigated whether inhibition of AR could prevent human retinal endothelial cell (HREC) growth and tube formation in-vitro, which are essential for new blood vessel formation.

2. Materials and methods

2.1. Materials

Cell-System complete (CS-C) medium containing JetFuel formulated with growth factors and 10% serum, attachment factor and passage reagent group consisting of EDTA-dPBS solution, trypsin/EDTA-dPBS solution and trypsin inhibitor-dPBS solution were obtained from Cell-System (Kirkland, WA). Phosphate-buffered saline (PBS) and gentamicin solutions were purchased from Invitrogen-Gibco (Grand Island, NY). Antibodies against VCAM-1, b-actin and glyceraldehyde phosphate dehydrogenase (GAPDH) were from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against phospho-p38, and total-p38, VEGF-R2 were from Cell Signaling Inc. (Beverly, MA) and rabbit monoclonal antibodies against VEGF receptor 1 were purchased from Abcam Inc (Cambridge, MA). AR inhibitor fidarestat was from Sanwa Kagaku Kenkyusho Co. Ltd, Japan. The transfection reagent (HiPerFect) was from Qiagen (Valencia, CA) and reduced-serum, cell-culturing medium (OptiMEM) were obtained from Invitrogen-Life Technologies (Gaithersburg, MD). Vascular endothelial growth factor (VEGF) was obtained from R&D systems (Minneapolis, MN). The reagents used in Western blot analysis and 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). All other reagents used were of analytical grade.

2.2. Tissue culture of HREC

Primary HREC from the Applied Cell Biology Research Institute (ACBRI) (Kirkland, WA) were thawed, sub-cultured and maintained essentially as prescribed by the supplier by using media, attachment factor and passage reagent group. The cells were grown in a humidified incubator at 37°C and 5% CO_2. To study cell viability, we pretreated the HREC overnight with 20 μM of AR inhibitor, fidarestat, in CS-C medium without growth factors or serum, followed by stimulation with various concentrations (0-100 ng/ml) of VEGF and incubated for 24 h. To investigate the effect of AR inhibition on VEGF-induced activation of MAP kinases and expression of various neo-angiogenic markers, we pre-incubated HREC with AR inhibitor overnight and stimulated the cells with 20 ng/ml VEGF for different time intervals. The cells between the passages of 3-6 were used in this study.

2.3. Cell viability assays

The confluent HREC were harvested and plated in 96-well plates at a density of 5000 cells/well. The cells were growth arrested by incubation in CS-C medium supplemented with 0.1% serum with or without Fidarestat (10 μM) for 24 h. After 24 h, the cells were stimulated with VEGF (0, 10, 20, 50, and 100 ng/ml) and incubated for an additional 24 h. Cell viability was evaluated by using the MTT assay. Briefly, 10 μl of 5 mg/ml MTT in PBS was added to each well and incubated an additional 2 h at 37°C. The medium was removed and the formazan granules obtained were dissolved in 100% dimethyl sulfoxide (DMSO), and absorbance was detected at 562 nm with an ELISA plate reader.

2.4. Cell migration assay

HREC (2×10^5 cells per well) were seeded in 6-well plates, growth-arrested by incubating with AR inhibitor, fidarestat, in 0.1% FBS medium for 24 h. The confluent HREC were wounded with a 200 μL pipette tip and incubated with VEGF (20 ng/ml) without or with AR inhibitor for 24 h or AR siRNA ablation. The wounds were visualized directly through a

light microscope at 0 h and at 24 h and photomicrographs were acquired. The cell migration was quantified and expressed as percent mean distance migration. A representative photomicrograph is shown (Magnification ×100).

2.5. Tube formation assay

Tube formation studies were conducted by using an in vitro angiogenesis assay kit from Trevigen Inc. (Gaithersburg, MD) in a 96-well format. Briefly, HREC were plated at 0.5 to 1.0×10^6 cells per 25 cm^2 flask and cultured overnight. BME solution was thawed in ice-water bath at 2-4°C in a refrigerator overnight, and then 50 μl of BME solution was aliquoted into each well of a 96-well plate and incubated for 60 min at 37°C to gel. For fluorescent monitoring of tube formation, prior to harvesting, HREC were incubated for 30 min with Calcein AM (2 μM) at 37°C. The cells were harvested and counted, and a single cell suspension at 1×10^6 cells / ml was prepared. The cells were diluted in endothelial medium in the presence or absence of VEGF and AR inhibitor. The cells were added at a density of 1×10^4 per 100 μl to each well without disturbing gelled BME and incubated for 16 h in a CO_2 incubator at 37°C. The tube formation was visualized directly through a light microscope, and photomicrographs were acquired. Wherever Calcein AM was used during assay, tube formation was visualized by using a fluorescence microscope (485 nm excitation/520 nm emission).

2.6. Aldose Reductase Assay

The VEGF-stimulated HREC in triplicates in T-150 flasks were washed twice with ice-cold PBS and harvested. The cells were homogenized in 10 mM phosphate buffer (pH 7.0) containing protease inhibitor cocktail (Sigma, Saint Louise, MO) and cleared by centrifugation at 12,000 *g* for 10 min at 4°C. The protein content of the cleared cell homogenate was determine and AR enzyme activity was determined at room temperature in a 1-ml reaction mixture containing 0.1M potassium phosphate buffer (pH 6.0) containing 0.4M Li_2SO_4 and 0.1mM EDTA, 10mM glyceraldehyde, and 0.1mM NADPH at 25 °C. The reaction was monitored by measuring the disappearance of NADPH at 340 nm, using Varian Cary 100 Bio double beam spectrophotometer. One unit of the enzyme activity defined as the amount of enzyme required to oxidize one micromole of NADPH/min.

2.7. Western blot analysis

The VEGF-stimulated HREC in triplicates were washed twice with ice-cold PBS and lysed in ice-cold lysis buffer containing 50 mM HEPES [pH 7.6], 10 mM KCl, 0.5% NP-40, 1 mM DTT, 1 mM phenylmethylsulfonylfluoride (PMSF), 1mM Sodium orthovanadate and a 1:100 dilution of protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma, Saint Louise, MO) for 15 min at 4°C. The crude cells as well as membrane fraction lysates were cleared by centrifugation at 12,000 *g* for 10 min at 4°C. Aliquots of the lysates containing equal amounts of protein (40 μg) were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore, Bedford, MA). The membranes were then incubated in blocking solution containing 5% wt/vol dried fat-free milk and 0.1% vol/vol Tween-20 in tris-buffered saline. Subsequently, the membranes were incubated with specific antibodies against AR, VEGF-R2 and VCAM-1, phospho-Akt, -p38,. The membranes were striped and re-probed with antibodies against total-Akt and -p38, to assess the changes in total proteins. The blots were striped and re-probed with antibodies against a house keeping protein GAPDH or b-actin as a control for equal loading of protein. The membranes were washed and probed with the respective HRP-conjugated secondary antibodies (Southern Biotech, Birmingham, AL) and visualized by chemiluminescence (Pierce biotechnology, Rockford, IL). The densitometry of blots was performed by AlphaImager 2200 software from Cell Biosciences (Santa Carla CA).

2.8. RNA interference ablation of AR

Approximately 2.5×10^5 HREC were seeded per well in a 6-well plate and incubated overnight or to 80% confluence. The cells were incubated with OptiMEM medium containing the AR-siRNA (AACGCAUUGCUGAGAACUUUAUU) or scrambled siRNA (UAAAGUUCUCAGCAAUGCGUUUU; control) to a final concentration of 20 nM and the hIpERfECT transfection reagent (Qiagen) essentially as described by the manufacturer. Briefly, for each well, 300 ng AR siRNA was diluted in serum-free medium to give a final volume of 100 μl and incubated with 18 μl HiPerFect transfection reagent for 10 min at room temperature. The transfection mixture was added to the respective wells containing 700 ul culture medium and incubated for 3 h at 37 C after which 1600 μl complete culture medium was added to each well and cell were incubated for 48 h. Changes in the expression of AR were assessed by Western blot analysis using anti-AR antibodies. For stimulation with VEGF the medium was replaced with 0.1% serum medium 24 h before stimulation.

2.9. Immunohistochemical analysis of VEGF expression in diabetic rat eye

All animal experiments were performed according to the National Institutes of Health Guide for Care and Use of Experimental Animals and approved by University of Texas Medical Branch Animal Care and Use Committee. Diabetes was induced in male Fischer rats (n=4; 180-200 g) by a single injection of streptozotocin (65 mg/kg intraperitoneally). Blood glucose was measured 3 days later and only the rats with blood glucose more than 400 mg/dl were used for further experiments. The diabetic rats were treated with either Fidarestat (10 mg/Kg body wt.; i.p.) or vehicle daily till the end of the experiment. Control rats were not treated. After 7 days of treatment eyes were enucleated, fixed in 10 % buffered formalin and paraffin embedded. Five micrometer sections were deparaffinized, and stained with polyclonal anti-VEGF antibodies Santa Cruz biotechnology (Santa Cruz, CA) using standard protocol. Subsequently, the sections were washed with PBS and incubated with FITC-labeled secondary antibodies, washed and mounted with fluor-save mounting medium (Vector Laboratories Inc, Burlingame, CA). The sections were examined under a Nikon epifluoroscence microscopy. All animal protocols were approved by the institutional animal care committee.

2.10. Statistics

Data presented as mean ± SD and *p* values were determined by using an unpaired Student's t test using Graph-pad prism software. $p<0.05$ was considered as statistically significant.

3. Results

3.1. AR inhibition prevents the VEGF-induced increase in cell growth in HREC

Increased VEGF expression has been reported in the diabetic retina and retinal cells during high glucose (Adamis et al., 1994; Young et al., 2005; Xiao, Zeng, Ling, & Lv, 2006), and we also observed increased VEGF immuno-reactivity in the retina of diabetic rats and treatment with AR inhibitor fidarestat was able to prevented it (Fig. 1). Since VEGF is known to cause a proliferative response in the endothelial cells in-vitro as well as in-vivo, we determined the effect of AR inhibition on the growth factor-induced retinal endothelial cell-growth. As shown in Fig 2A, VEGF (20 ng/ml) caused more than 2-fold cell growth, and AR inhibition significantly blocked the cellular growth induced by VEGF. Further, we used an in vitro migration assay to assess the cell migration characteristics of HREC in the presence and absence of AR inhibitor. In a 6-well plate a wound was scrapped with an aseptic 200-ul pipette tip in a confluent monolayer of HREC across the middle of the plate and incubated with VEGF for 24 h, with the result that HREC migrated and closed most of the wounded area (Fig 2B). In HREC pretreated with AR inhibitor a significant area of the wound remained uncovered after the same period of incubation indicating inhibition of AR

prevented HREC migration. The migration of HREC was quantified and presented as percent mean distance migration. As shown in Fig 2C treatment of HREC significantly ($p<0.01$) prevented the VEGF-induced cell migration. Further, to rule out the non-specific effects of pharmacological inhibitors, we ablated the AR message by transfecting HREC with AR-siRNA and analyzed the cellular migration. HREC covered most of the wounded area in untransfected control (C) and scrambled siRNA (Csi)-transfected HREC, whereas in AR-siRNA transfected HREC, migration was slow and the closing of the wound area was significantly (~65%) incomplete (Fig 3). These findings suggest role of AR in HERC growth and migration and that pharmacological inhibition or genetic ablation of AR could significantly prevented VEGF-induced HREC proliferation and migration.

3.2. AR inhibition prevents tube formation in HREC

Since under the stimulus of VEGF, endothelial cells are known to form tube-like structures leading to neovascularization (Miller et al., 1994), we tested effect of AR inhibition on this phenomenon. Representative photomicrographs shown in Fig 4A, clearly demonstrate that VEGF caused marked increase in tube-formation and branching of those tubes in HREC. However, in HREC treated with AR inhibitor, fidarestat, there was significant inhibition of tube-formation. Similar results were obtained in two separate experiments by utilizing two different methods of observation without (data not shown) and with Calcin M (Fig 4A) fluorescent dye. The tube formation was quantified and expressed as mean number of branch points per viewing field and show that addition of ARI significantly ($p<0.02$) prevented the tube formation by VEGF (Fig 4B). These results suggest that AR inhibition could block the growth factor-induced tube formation by retinal endothelial cells in vitro.

3.4. AR inhibition prevents VEGF-induced phosphorylation/activation of Akt and p-38MAPK in HREC

Although we demonstrated that AR inhibition could prevent cell growth, migration and tube formation, the mechanism as to how inhibition of AR blocks these pathological phenotypes remains unclear. Since mitogen-activated protein kinases (MAPKs) are known to regulate neovascularization (Murata, Kador, & Sato, 2000; Suzuma et al., 2000; Kim et al., 2001), we next investigated the effect of AR inhibition on VEGF-induced activation of Akt and MAPK pathways. As shown in Fig 5, treatment with VEGF caused a time-dependent increase in the phosphorylation of Akt and p38-MAPK in HREC, which was prevented in fidarestat treated cells. There was no significant change in the expression of total Akt and p38 proteins, suggesting that inhibition of AR could prevent HREC growth and neovasculirization by preventing activation of Akt and p-38 mediated signals.

3.5. AR inhibition prevents VEGF-induced expression of angiogenic markers in HREC

The role of various endothelial markers in VEGF-induced neovascularization is well known; therefore we next measured the expression of these marker proteins in HERC in response to VEGF stimulus. As shown in Fig 6A, there was an increased expression of VCAM in response to VEGF stimulation which was inhibited by AR inhibitor treatment. It was interesting to observe that VEGF also induced the expression of AR (Fig 6A). There was approximately 3-fold increase in AR activity associated with the VEGF-induced increased AR expression (Fig 6B) which decreased significantly (>65%) by fidarestat.

In a number of studies VEGF-R2 has been implicated in retinal angiogenesis (Gerhardt et al., 2003; Sarlos et al., 2003). Further, Byeon et al., (2010) have shown that VEGF acts through VEGF-R2/PI3K/Akt pathway to induced cell survival suggesting important role of VEGF-R2. We therefore investigated the effect of AR inhibition or ablation on VEGF-induced expression of VEGF-r2 in HREC. As shown in Fig 6C, both inhibition as well as siRNA ablation of AR suppressed the expression of VEGF-R2 in HREC. These results

indicate that AR inhibition prevents the VEGF-induced expression of endothelial markers, VEGF-R2 and also regulates its own expression in HREC.

4. Discussion

Although angiogenesis mostly seizes in adults except for a few physiological processes, such as the menstrual cycle, pregnancy and wound healing, it is reactivated in many disorders such as tumor development and ocular inflammatory diseases (Gariano, & Gardner, 2005). This reactivation is due to an imbalance in the supply of oxygen and nutrients and results in pathologies that require immediate intervention (Arjamaa, & Nikinmaa, 2006). For example, ocular neovascularization is the key component in diabetic retinopathy and AMD, and understanding of the molecular mechanisms involved in these diseases and targeting of the key molecular event(s) could lead to novel therapy that may prevent new blood vessel formation and thus prevent blindness. It has been convincingly demonstrated that VEGF is involved in the initiation as well as progression of neovascularization in both diabetic retinopathy and AMD (Adamis et al., 1994; Shams & Ianchulev 2006; Bhisitkul 2006), which makes VEGF receptors and VEGF-induced signaling intermediates possible molecular targets to develop potential therapies (Guo et al., 1995; Larrivée, & Karsan, 2000; Gliki, Wheeler-Jones, & Zachary, 2002; Wang, Nomura, Patan, & Ware, 2002; Guma et al., 2009). Indeed, various inhibitors such as anti-VEGF antibodies, e.g. Avastin, VEGF receptor inhibitors and receptor tyrosine kinase inhibitors, have been tested in preclinical studies and currently are at various stages of clinical trials. However, despite the initial successes, anti-VEGF therapy remains at the preclinical stages and the side effects of such therapies, both known and unknown are also a cause of concern. Moreover, emerging evidences suggest that many other angiogenic factors, besides VEGF, could be involved in the neo-vascular pathogenesis (Paques, Massin, & Gaudric, 1997; Simó et al., 2002; Simó et al., 2006). Therefore, novel strategies are required to combat this pathological process.

We have recently shown that AR inhibitors could block the signals of varied stimuli, including high glucose, endotoxins, cytokines, and growth factors and block their cytotoxic effects on multiple cell types (Yadav et al., 2009a; Yadav et al., 2009b; Yadav, Srivastava, & Ramana, 2010; Ramana, Friedrich, Bhatnagar, & Srivastava, 2003; Pladzyk et al., 2006; Yadav, Srivastava & Ramana, 2007). Further, we have also demonstrated recently that inhibition of AR could block tumor angiogenesis in-vitro and in-vivo (Tammali, Reddy, Srivastava & Ramana, 2011), but effects of AR inhibition on the retinal neovascularization remains unkown. Therefore, in the present study, we used primary human retinal endothelial cells (HREC) as a cell culture models to test the effects of AR inhibition on various events such as growth, morphogenesis, i.e., tube formation, angiogenic markers, and activation of various signaling intermediates that are implicated in DR and AMD. It has been shown that high glucose leads to increased AR activity in endothelial cells causing oxidative stress and increased expression of VEGF in the retina during experimental diabetes (Obrosova et al., 2003). In this study, we also observed increased expression of VEGF specific immunostaining in the diabetic rat eye, which was markedly decreased when treated with fidarestat, a potent and specific inhibitor of AR. Further, fidarestat also prevented in-vitro retinal micro-vascular endothelial cell growth and tube formation, which are essential steps in neovascularization. Furthermore, VEGF is known to activate PI3K-Akt signaling pathway through VEGF-R2 receptor and is known to participate in cell survival in retinal pigmented epithelial cells (Byeon et al., 2010), which is a key molecular mechanism in cellular proliferation and neovascularization. Therefore, we next examined the effect of AR inhibition on VEGF-induced activation of Akt and p38-MAPK. Our results indicate that AR inhibition prevented the activation of Akt as well as p38-MAP kinases. Further, we also observed that AR inhibition or ablation decreased the VEGF-induced increased expression

of VEGF-R2 in HREC, indicating that AR inhibition shunts the signaling circuitry that block the autocrine and paracrine effect of VEGF. These changes were associated with VEGF-induced increased AR expression and enzyme activity in HREC, which is decreased following treatment with AR inhibitor, fidarestat (Fig 6A, B). These results thus suggest that using AR inhibitors could be a novel approach to prevent retinal neovascularization and preventing the progression of potentially blinding diseases such as diabetic retinopathy and AMD. Based on our earlier findings and present results it can be safely stated that high glucose–induced increased AR activity is associated with oxidative stress, which induces inflammatory signals through various intermediate kinases leading to the activation of redox-sensitive transcription factors. Once active, the latter transcribe inflammatory cytokines and growth factors, including VEGF, which initiate a vicious cycle of inflammatory signals in an autocrine and paracrine manner resulting in pathogenesis. However, a clear mechanism of AR mediation in oxidative stress-induced pathogenesis is still awaited.

In summary, our results show that AR inhibition blocks the VEGF - induced cell growth and tube formation in HREC. Further, we have also shown that the inhibition of AR prevents VEGF-induced Akt and p38-MAPK protein kinase signaling cascades in HREC. These results suggest that AR inhibitors could be used as potential anti-angiogenic agents in ocular neovascular pathologies such as DR and AMD.

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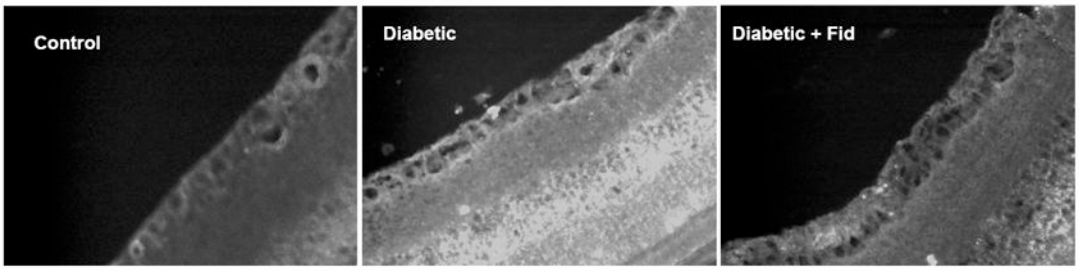

Fig 1. Effect of AR inhibition on VEGF immunoreactivity in diabetic rat retina

Diabetes was induced in Fischer rats (n=4) by a single injection of streptozotocin (65 mg/kg intraperitoneally). After confirmation of diabetes rats were treated with AR inhibitor or carrier for 7 days. Eyes were enucleated, fixed in 4% paraformaldehyde and 5 μM cross-sections were obtained. The eye sections were probed with anti-VEGF antibodies followed by FITC labeled secondary antibodies. A representative photomicrograph from each group is shown. (Magnification 100X).

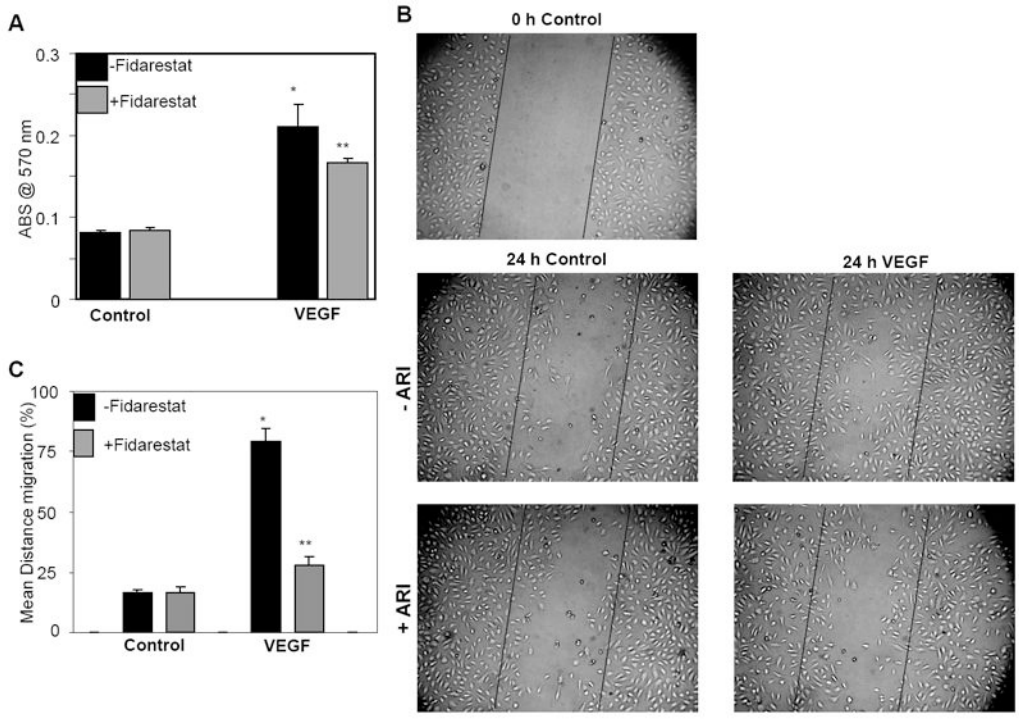

Fig 2. Effect of AR inhibition on VEGF-induced HREC growth and migration

(A). HREC (5000 cells/well) were seeded in 96-well plate and incubated for overnight. The cells were growth-arrested by incubating with 0.1% FBS medium containing fidarestat (10 μM) or carrier for 24 h, followed by incubation with VEGF (20 ng/ml) for 24 h. At the end of incubation cell-growth was assessed by MTT assay. Bars represent mean ± SD (n=6); *p< 0.01 Vs Control; **p<0.05 Vs VEGF. **(B)** HREC (2×10^5 cells/well) were seeded in 6-well plates, growth-arrested by incubating with fidarestat (10 μM) in 0.1% FBS medium for 24 h. The confluent HREC were wounded with a 200 uL pipette tip and incubated with VEGF (20 ng/ml) without or with AR inhibitor for 24 h. The wounded areas were photographed at 0 h and at 24 h. A representative photomicrograph is shown, (Magnification ×100). **(C)** Quantitative assessment of the cell migration as percent mean distance migration is shown. The bars represent mean ± SD (n=4); *p< 0.01 Vs Control; **p<0.01 Vs VEGF.

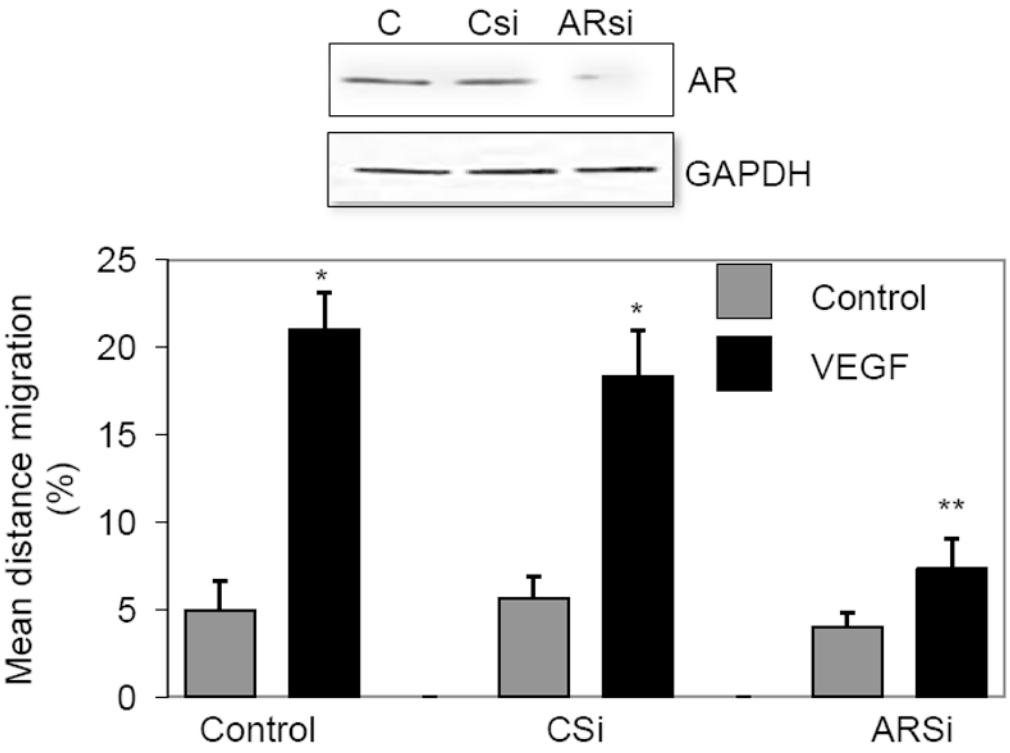

Fig 3. Effect of AR ablation by siRNA on VEGF-induced HREC migration

Approximately 3×10^5 HREC were seeded per well in 6-well plate, transfected with scrambled or AR-siRNA (confirmed by western blotting, inset: C, control, Csi control siRNA, ARsi, AR siRNA) and growth-arrested by incubating in 0.1% FBS medium for 24 h. The confluent HREC were wounded with a pipette tip as in fig 2 and incubated with VEGF for additional 24 h. The cellular migration is depicted by percent mean distance migration (n=4). *p< 0.01 Vs Control or Csi cells; **p<0.025 Vs VEGF treated Control or Csi Cells.

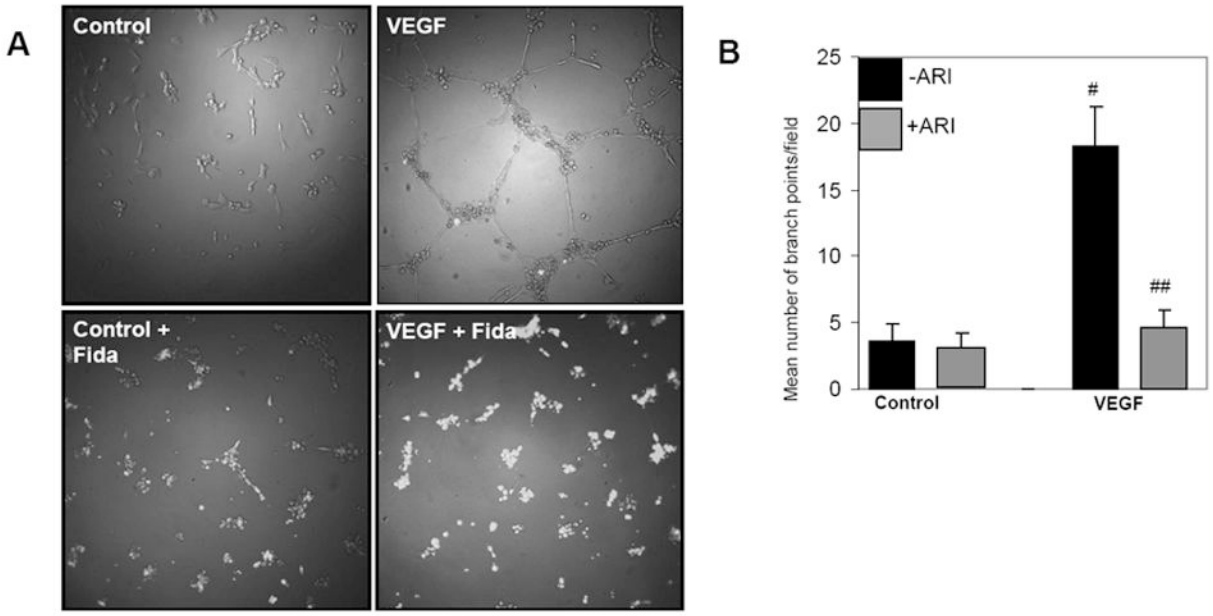

Fig 4. Effect of AR inhibition on VEGF-induced tube formation in HREC

HREC were plated at 0.5 to 1.0 × 10^6 cells per 25 cm^2 flask and cultured overnight. For fluorescent monitoring of tube formation, prior to harvesting, HREC were incubated for 30 min with Calcein AM (2 μM) at 37°C. The cells were harvested, counted, and diluted in endothelial medium in the presence or absence of VEGF and AR inhibitor. The cells were seeded on gelled BME and incubated for 16 h in a 5% CO_2 incubator at 37°C. The tube formation was visualized through a fluorescence microscope (485 nm excitation/520 nm emission), and photomicrographs were acquired. A representative photomicrograph is shown (magnification 100X). **(B)** Quantitative assessment of the tube formation was done by counting the average number of branch points per viewing field. The bars represent mean ± SD (n=4); $^{\#}p< 0.01$ Vs Control; $^{\#\#}p<0.02$ Vs VEGF.

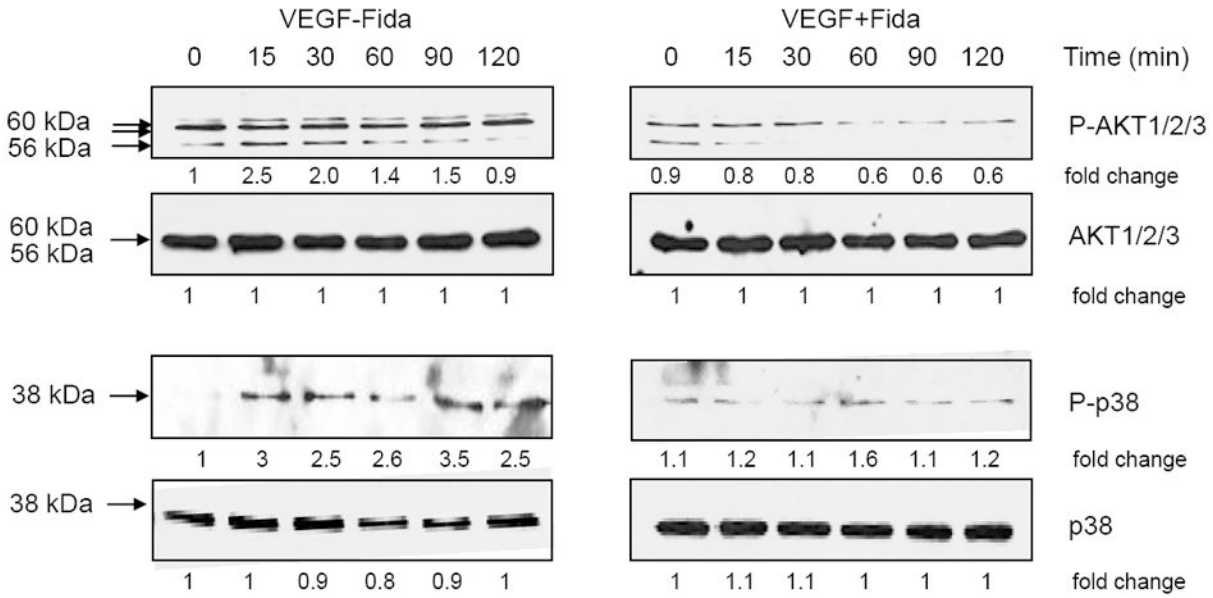

Fig 5. Effects of AR inhibition on VEGF-induced phosphorylation of Akt and p38-MAPKinase in HREC

Growth-arrested HREC were incubated with AR inhibitor or carrier for 24 h followed by treatment with VEGF (20 ng/ml) and incubation for different time periods as indicated. At the end of incubation, cells were washed, lysed and equal amount of protein was separated on SDS-PAGE followed by immunoblotting using antibodies against phospho-Akt1/2/3, and -p38. Immunoblotting of the stripped membranes with antibodies against total Akt1/2/3 and p38 was done on the blots. Numbers below the blots show fold changes in the expression of proteins (n=3).

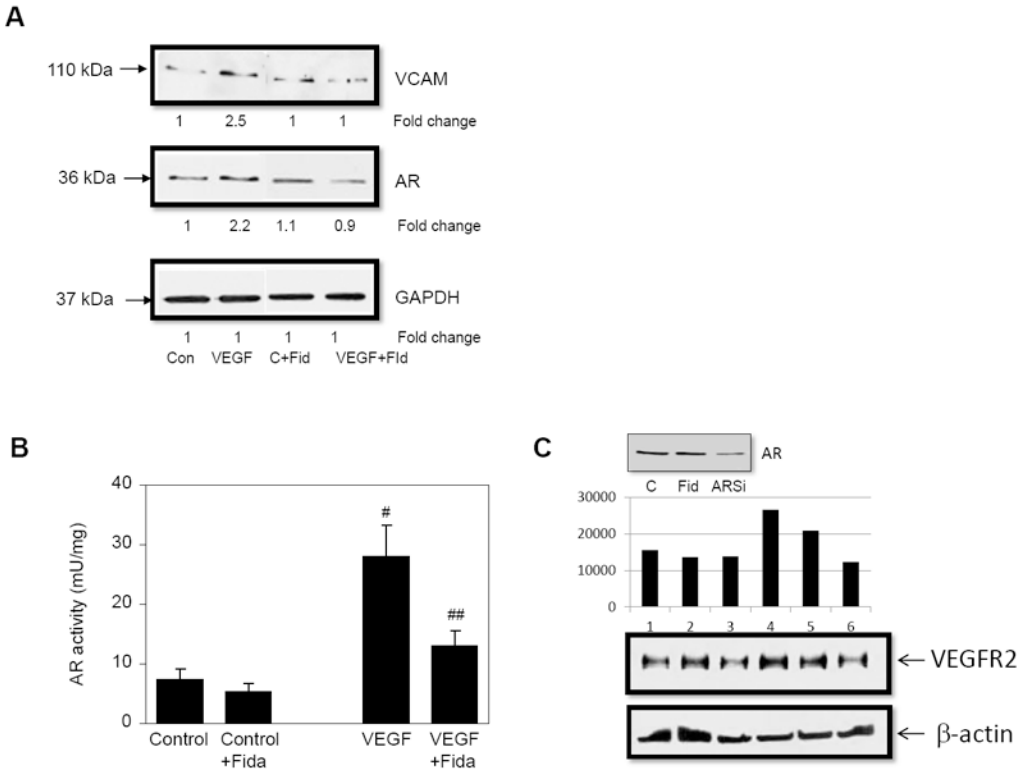

Fig 6. Effect of AR inhibition on VEGF-induced expression of eNOS, VCAM, AR and VEGF-RII

(A) Growth-arrested HREC were preincubated without or with AR inhibitor, fidarestat for 24 h followed by treatment with VEGF (20 ng/ml) and incubation for additional 24 h. At the end of incubation, cells were lyzed and equal amount of protein was separated on SDS-PAGE followed by immunoblotting using antibodies against eNOS, VCAM and AR. Immunoblotting of the stripped membranes with GAPDH antibodies was used to depict equal protein loading. Numbers below the blots show fold-changes in the expression of proteins (n=3). C, control; Fid, Fidarestat. **(B)** For the determination of AR activity, the HREC were incubated with VEGF in the presence or absence of fidarestat for 24 h followed by assessment of enzyme activity (expressed as milliUnits/mg protein) as described in the methods. The bars represent mean ± SD (n=3). $^{\#}p<0.007$ Vs untreated control group; $^{\#\#}p<0.02$ Vs VEGF treated group. **(C)** Effect of AR inhibition or ablation on VEGFRII expression in HREC was determined by Western blots. 2×10^5 HREC were plated in 6-well plates in triplicate and incubated for overnght. Next day (~70% confluent) cells were transfected with AR siRNA and incubated for 48 h. In AR inhibitor treated group Fid (10 uM) was added 24 h before and the cells were treated with VEGF (20 ng/mL) and incubated for additional 24 h. Cell lysate were prepared and 40 ug proteins were subjected to WB analysis using specific antibodies. Same membrane was stripped and reprobed with anti-β-actin antibodies. Bars represent relative density of the respective bands adjusted for β-actin. Lanes 1, control; 2, C+Fid; 3, ARsiRNA; 4, VEGF; 5, Fid +VEGF; 6, ARsiRNA +VEGF. Inset shows AR levels in different conditions. C, control; Fid, Fidarestat; ARSi, Aldose reductase siRNA