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Modulating the hypoxia-inducible factor signaling pathway as a therapeutic modality to regulate retinal angiogenesis

M. DeNiro [a,b,*], O. Alsmadi [c], F. Al-Mohanna [d]

[a] Research Department, King Khaled Eye Specialist Hospital, Riyadh, Saudi Arabia
[b] Department of Comparative Medicine, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia
[c] Department of Genetics, King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia
[d] Department of Biological and Medical Research, King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia

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ABSTRACT

Hypoxia-inducible factor (HIF) signaling cascade plays a critical role in angiogenesis by activating the transcription of genes encoding angiogenic growth factors. This study evaluated the effects of YC-1, a HIF-1 inhibitor, on the morphological, biochemical and molecular changes in human retinal microvascular endothelial cells. We found that YC-1 suppressed vascular endothelial cell proliferation, migration and tube formation, while it significantly increased the proteasome activity. Moreover, YC-1 induced a G_0/G_1 cell-cycle arrest, whereas it exerted only an insignificant proapoptotic effects. Under normoxia or hypoxia, YC-1 did not alter the morphology or the cell viability. Additionally, under hypoxic conditions, YC-1 downregulated HIF-2α, VEGF, EPO, ET-1, and MMP-9 mRNA and protein levels, this was accompanied by a significant decrease in the MMP-9 activity. YC-1 decreased the basal expression of HIF-1α protein under normoxia, whereas it inhibited HIF-1α protein synthesis, stability, and nuclear translocation mechanisms under hypoxia. Furthermore, in a 3D collagen matrix model using mouse retinal explants cultured under normoxic and hypoxic conditions, YC-1; (1) inhibited outgrowth of new vessel sprouts; (2) reduced VEGF expression; (3) dramatically decreased the vessels immunoreactivities for CD31 and von Willebrand Factor (vWF); and (4) was highly effective in reducing the vascular density within the retina, compared to controls. These findings indicate that YC-1 possesses several antiangiogenic properties, both *in vitro* and *ex vivo*, which could be exploited as valuable therapeutic potentials to inhibit formation and the growth of new retinal vessels in the hypoxic retina.

1. Introduction

Diabetic retinopathy (DR) is a leading cause of visual disturbance in adults. In the early stage of the disease, retinal vascular permeability can increase even before the appearance of clinical retinopathy (Sheth, 1999; Russ et al., 2001). Retinal vascular leakage and thickening of the retina lead to diabetic macular edema. In the late stage of DR, abnormal increases in vascular permeability result from retinal ischemia due to nonperfusion of the retina or a decrease in oxygen tension (Barinaga, 1995; Williamson et al., 1993). During this stage, over-proliferation of capillary endothelial cells results in retinal neovascularization (NV), abnormal formation of new vessels in the retina and in the vitreous, leading to proliferative DR (PDR) (Shiels et al., 1998; Stitt et al., 2000). Additionally, during the late stages of DR, the ischemia-induced pathological angiogenesis ultimately causes severe vitreous cavity bleeding and/or retinal detachment, resulting in severe vision loss.

Angiogenic factors, such as vascular endothelial growth factor (VEGF), play a prominent role in promoting retinal NV. Retinal ischemia is the major driving force behind the induction of VEGF, which plays a crucial role in ocular pathogenesis (Michaelson, 1948; Ashton, 1957; Campochiaro, 2000). VEGF has a profound impact on multiple functions in endothelial cells, such as proliferation, migration, survival, tube formation, and vascular permeability (Cross et al., 2003; Matsumoto and Claesson-Welsh, 2001). Previous studies have indicated that VEGF is an important mediator of NV induced by hypoxic retinopathies (Thieme et al., 1995). It has been reported that there is increased VEGF production in both vitreous (Adamis et al., 1994), and ocular fluids (Aiello et al., 1994) of patients with DR. Suppression of VEGF receptor interaction, VEGF expression and VEGF-induced signaling has been shown to inhibit NV in animal models of retinal ischemia.

* Corresponding author at: Research Department, King Khaled Eye Specialist Hospital, Aruba Street, P.O. Box 7191, Riyadh 11462, Saudi Arabia. Tel.: +966 1 482 1234; fax: +966 1 482 9311.
E-mail address: mdeniro@kkesh.med.sa (M. DeNiro).

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Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that controls the hypoxic response and oxygen homeostasis in mammalian cells (Wenger, 2000). HIF-1 plays a critical role by regulating the transcription of the target genes, which are involved in angiogenesis (Pe'er et al., 1995), erythropoiesis (Wang and Semenza, 1996), energy metabolism, glycolysis, and apoptotic and proliferative responses to ischemia and hypoxia (Wenger, 2000; Semenza, 2000; Minet et al., 2000). HIF-1 is a member of the basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) superfamily of transcription factors (Wang et al., 1995). Active HIF-1 is a heterodimer composed of hypoxia-induced α, and constitutively expressed β subunits, both of which are members of the bHLH/PAS family. The regulation of HIF-1 occurs at the transcriptional, translational, post-translational, protein–protein interaction and degradation. Active HIF-1 is primarily expressed during hypoxia. Under normoxic conditions, HIF-1α subunits are very unstable and are rapidly targeted for degradation in the 26S proteasome [Jaakkola et al., 2001; Ivan et al., 2001). Exposure to hypoxia results in a rapid increase of HIF-1α protein in most cells (Jewell et al., 2001) *in vivo* and *in vitro* (Semenza, 2001). The HIF-1β subunit is constitutively expressed and unregulated by oxygen tension (Chilov et al., 1999). The mechanism by which HIF-1α protein is upregulated by hypoxia has been well characterized (Wenger, 2000; Semenza, 2001). When intracellular oxygen reaches a critically low threshold, HIF-1α subunits are rapidly protected from proteosomal degradation, allowing HIF-1α and HIF-1β subunits to associate and form active HIF-1 transcription factor (Jewell et al., 2001). Conversely, under normoxia, proline hydroxylation in the oxygen-dependent degradation domain of HIF-1α mediates HIF-1α binding to the von Hippel–Lindau (pVHL) tumor suppressor protein. In turn, pVHL assembles a complex with E3 ubiquitin ligase that targets HIF for polyubiquitination and subsequent proteosomal degradation (Jaakkola et al., 2001; Ivan et al., 2001). HIF-1 binds to the hypoxia response element (HRE) in hypoxia-responsive target genes, and triggers a global role in the transcriptional regulation and the expression of a variety of angiogenic factors, such as, VEGF (Pe'er et al., 1995), Erythropoietin (EPO) (Wang and Semenza, 1996), and Endothelin-1 (ET-1) (Jing Hu et al., 1998). It has been established that there is a temporal and spatial correlations between the expression of HIF-1 and VEGF in an animal model of retinal NV (Ozaki et al., 1999). There are two other bHLH/PAS proteins that respond to hypoxia; Hypoxia-inducible factor-2α (HIF-2α) (EPAS1) (Gerber et al., 1997), which responds to hypoxia and have restricted tissue expression (Sánchez-Elsner et al., 2002); and Hypoxia-inducible factor-2α (HIF-3α) (Gu et al., 1998).

HIF-2α contains domains similar to those in HIF-1α and exhibit similar biochemical properties, such as heterodimerization with HIF-1β and DNA binding to the same DNA sequence *in vitro*. HIF-2α is also tightly regulated by oxygen tension and its complex with HIF-1β appears to be directly involved in hypoxic gene regulation, as is HIF-1α (Kietzmann et al., 1999). Furthermore, HIF binding to HREs is not sufficient for hypoxic induction for many genes. Synergistic cooperation between HIF-1α or HIF-2α and other transcription factors, such as Smad3, HNF4, ATF1/CREB1, AP1, and Ets-1 have been reported. Additionally, HIF-2 activates transcription and induces hypoxia-mediated gene expression such as VEGF (Liu et al., 1995; Forsythe et al., 1996), Epo (Wang and Semenza, 1993), and ET-1 (Hu et al., 1998). HIF-2α is detectable in normoxia and strongly upregulated in response to hypoxia, alongside with translocation from the cytoplasm to the nucleus.

YC-1 [3-(5′-hydroxymethyl-2′furyl)-1-benzyl indazole] is a small molecule that inhibits cGMP breakdown and potentiates NO-induced soluble guanylyl cyclase (sGC) stimulation (Ko et al., 1994; Friebe and Koesling, 2003). Data reveals that YC-1 suppresses HRE binding by HIF-1 and HIF-1α protein expression (Kim et al., 2006). Furthermore, data have indicated that YC-1 was found to prevent HIF-1α and HIF-1β accumulation in response to hypoxia or mitogen treatment in PC-3 prostate cancer cells (Sun et al., 2007). Under hypoxia, YC-1 reduces the mRNA levels of EPO, VEGF (Chun et al., 2001), and MMP-9 (Liu et al., 2006). Previous data revealed that treatment of primary pulmonary arterial endothelial cells with YC-1 produced significant decreases in ET-1 secretion (Kelly et al., 2004). Moreover, human retinal microvascular endothelial cells (HRMVEC) play a key role in the development and progression of retinal NV. Blocking angiogenesis is an effective strategy for the treatment of microvascular retinal diseases. Therefore, it is highly essential to determine how the HIF/angiogenic factor(s) system is modulated by YC-1 in ocular neovascular diseases. In the present manuscript, we utilized various approaches to explore the influence of YC-1 on the morphological, molecular, biochemical, and enzymatic changes in HRMVEC. This study reveals that YC-1 suppresses retinal new vessel growth and formation via the specific properties, which are demonstrated *in vitro* and *ex vivo* model settings.

2. Materials and methods

2.1. Reagents

YC-1 was purchased from A.G. Scientific (San Diego, CA) and dissolved in sterile DMSO. Mouse anti-human HIF-1α monoclonal antibody (clone H1α67) was purchased from Sigma–Aldrich (St. Louis, MO). Mouse anti-human HIF-2α monoclonal antibody (ep190b) and mouse anti-human β-actin monoclonal antibody were both purchased from Abcam (Cambridge, MA). Mouse anti-human VEGF monoclonal antibody, recombinant human VEGF, monoclonal anti-mouse CD31 antibody, polyclonal anti-mouse von Willebrand factor (vWF) antibody, monoclonal anti-mouse VEGF antibody, and anti-mouse β-actin monoclonal antibody, were all purchased from Chemicon (Temecula, CA). Mouse anti-human-EPO monoclonal antibody, mouse anti-human MMP-9 monoclonal antibody, and recombinant human basic fibroblast growth factor (bFGF) were both purchased from R&D Systems (Minneapolis, MN). Goat Anti-human ET-1 polyclonal antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA). Goat anti-mouse IgG was purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). Recombinant human TNFα was purchased from BD Pharmingen (San Diego, CA).

2.2. Mice

C57BL/6J mice were obtained from Jackson Laboratory (Bar harbor, ME). All animal protocols were approved by the Institutional Review Board and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

2.3. Cell culture conditions and hypoxic protocol

HRMVEC, attachment factor, complete growth medium were purchased from Cell Systems (Kirkland, WA). Cells were cultured in 75-cm2 tissue culture flasks coated with attachment factor maintained in CS-C medium containing 10% FBS and CS-C growth factor at 1× in humidified normoxic conditions (5% CO_2) at 37 °C. To establish hypoxic conditions, cells were placed in airtight chambers (BioSpherix, Redfield, NY) and the O_2 tension was maintained at 1.2% by using Pro-Ox Model 110 O_2 regulator (BioSpherix, Redfield, NY). The chamber was flushed with a gas mixture of 5.32% CO_2, and 93.48% N_2.