·Basic Research·

Rac1 activates HIF -1 in laser induced choroidal neovascularization

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Abstract

• AIM: To study the effect of Rac1 on the induction of HIF-1α in choroidal neovascularization (CNV) in mice.

• METHODS: One hundred C57BL/6J mice were laser photocoagulated to induce CNV, fifty mice of that were selected randomly for intravitreal injection of Rac1 inhibitor NSC23766 solution (1μL). After laser photocoagulation, fundus fluorescein angiography (FFA) was performed to verify the growth of CNV. Immunohistochemistry and Western blot were used to detect HIF-1α and Rac1 in posterior segment of eye globes.

• RESULTS: FFA verified that incidence of CNV was significantly reduced in the eyes with NSC23766 injection comparing with that of eyes without NSC23766 injection ($P<0.01$). Immunohistochemistry detected that HIF-1α and Rac1 mainly expressed in the new fibrovascular tissue. Western blot showed that HIF-1α and Rac1 was highly increased in tissue explants of retinal pigment epithelium (RPE) and choroid without NSC23766 injection. But for tissue explants of RPE and choroid with NSC23766 injection, both the expressions of HIF-1α and Rac1 were inhibited.

• CONCLUSION: Rac1 is crucial to activate HIF-1 regulating the growth of CNV, and its inhibition may have potential therapeutic value.

• KEYWORDS: animal study; choroidal neovascularisation; growth factor; signal transduction; molecular biology
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INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness among people more than 50 years old[1]. The choroidal neovascularization (CNV) accounts for the severely progressive decrease of central visual acuity among 90% of neovascular/exudative (wet) AMD. The pathogenesis of CNV is multifactorial, involving retinal pigment epithelium (RPE) alterations, rupture of Bruch's membrane and angiogenic features. The etiological factors and pathology process of CNV still remain elusive. Vascular endothelial growth factor (VEGF) is a major regulator of angiogenesis and vascular permeability implicated in the development of the CNV, which was upregulated under hypoxia. Inhibiting the expression of VEGF is a effective way of suppressing CNV [2,3]. The upregulated expression of VEGF is related to hypoxia inducible factor-1 (HIF-1), which is an essential global regulator of oxygen homeostasis [4]. The mechanism by which HIF-1 activity is induced under hypoxic conditions remains to be established. HIF-1 is a basic helix-loop-helix/PAS (PER-ARNT-SIM) homology domain protein consisting of HIF-1α and HIF-1β subunits. HIF-1α protein expression and HIF-1 transcriptional activity are precisely regulated by cellular oxygen concentration, whereas HIF-1β protein is constitutively expressed. Although much has been learned about the role of HIF-1 in controlling the expression of hypoxia-inducible genes in RPE cells, such as VEGF, but the status of HIF-1α expression and the underlying mechanisms by which HIF-1α is activated in CNV are largely unknown. In this study, we focused on the *Rho* family small GTPase Rac1 as a potential intermediate in the growth of CNV. Rac1 plays a pivotal role in multiple cellular processes, including cytoskeletal organization, gene

transcription, cell proliferation, and membrane trafficking, through direct or indirect interactions with PI3K, p21-activated kinase (PAK), Ras, and p70 S6 kinase. Rac1 also regulates assembly of the active NAD (P) H oxidase complex. Rac1 is expressed in most cells and is recognized as a critical determinant of intracellular redox status. We demonstrate here that Rac1 plays an essential role in the induction of HIF-1α protein expression in CNV.

MATERIALS AND METHODS

Materials One hundred C57BL/6J mice, age 7-8 weeks, were handled in accordance with institutional guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The mice were anesthetized for all procedures with intramuscular injection of a 1mL/kg mixture (7:1) of ketamine hydrochloride (Zhongxi Pharmaceutical Corp, China) and xylazine hydrochloride (Nanjing Pharma Chemical Plant, China). The pupil was dilated with 5g/L tropicamide and 5g/L phenylephrine hydrochloride eye drops (Santen Pharmaceutical Corp, Japan). In order to induce CNV by laser-induced ruptures of Burch's membrane, 532nm laser (140mW,100ms, 75μm;IRIS Medical OcuLight GL 532nm laser, IRIDEX, USA) was used as described previously [5]. Laser photocoagulation was performed in 2 eyes of each mouse. Each six laser spots were applied in a standardized fashion around the optic nerve using a slit lamp delivery system and a coverslip as a contact lens. Formation of evaporation blebs at the time of laser exposure indicated the effective rupture of Bruch's membrane. The growth of CNV was verified using FFA at day 7 and day 14. Since day 7 after laser photocoagulation, FFA showed CNV had been induced at the site of laser photocoagulation. During the process of angiography, CNV leak fluorescein intensively. At 180 sites of laser photocoagulation in 30 eyes of 15 mice without NSC23766 injection, 161 lesions of CNV (89.44%) were found at day 7. At 180 sites of laser photocoagulation in the 30 eyes of 15 mice with NSC23766 injection, 29 lesions of CNV (16.11%) were found. The results showed that the incidence of CNV was significantly reduced in the laser photocoagulated eyes with NSC23766 injection comparing with that of eyes without NSC23766 injection (P <0.01). No sooner 100 C57BL/6J mice were laser photocoagulated than 50 mice were selected randomly. Rac1 inhibitor NSC23766 solution (1μL) was injected under microscopic visualization into the subretinal space with a blunt 33-gauge needle through a 30-gauge starter, opening just behind the ciliary ruff with the tip angled toward the posterior pole. Caution was taken to avoid the lens material. The blunt needle was advanced into the vitreous cavity for intravitreal injection. Thirty photocoagulated C57BL/6J mice were selected randomly for observation of CNV by fundus fluorescein angiogram (FFA) at day 1, 3, 5, 7 and 14, of which 15 mice undergone NSC23766 injection. Angiography in oculus uterque of each mouse was performed using Heidelberg Retina Angiograph (Heidelberg Engineering, Germany). To adapt the system for the shortage of the mouse eye, a 30-D lens in contact to the fundus camera lens was used. Mice were analyzed under general anesthesia after dilation of the pupil and subsequent intraperitoneal injection of 0.1mL of 25g/L fluorescein sodium (Mingxing Pharmaceutical Corp, China). Each investigation included early phase (1-3 minutes after injection) and late phase (6-8 minutes after injection) images. FFA was performed at day 7 and day 14 after laser photocoagulation.

Methods

Immunohistochemistry for the expression of HIF-1α and Rac1 protein Forty C57BL/6J mice with laser photocoagulation were killed at day 1, 3, 7 and 14, of which 20 mice undergone NSC23766 injection. At each time point, 10 mice were selected randomly as victims, of which 5 mice undergone NSC23766 injection. Eyeballs from each mouse were carefully removed and dissected clean of orbital tissue. Immunohistochemistry was performed as previously reported [6]. Eyeballs were fixed in 40g/L paraformaldehyde at 4℃ overnight. The anterior segments and lenses were removed, the remaining eyecups were cryoprotected with 200g/L sucrose in 0.1mol/L phosphate buffered saline (PBS). Eyecups were then embedded in optimum cutting temperature compound. Sections were cut at 10μm with a cryostat (Leica Microsystems, Germany). Only sections that were cut in the middle of the CNV lesions were included. Five sections from each CNV lesion were selected for immunohistochemistry. The sections were blocked with 30g/L hydrogen peroxide for 25 minutes and with goat's serum for 30 minutes, and then incubated for 40 minutes with rabbit anti-mouse HIF-1α polyclonal antibody (Santa Cruz Biotechnology, USA) or rabbit anti-mouse Rac1 polyclonal antibody (Santa Cruz Biotechnology, USA). After washed in PBS for 3 times, sections were incubated with biotinylated goat anti-rabbit secondary antibodies for 10 minutes, followed by streptavidin-peroxidase conjugate. Sections were incubated with substate-chromogen solution (hydrogen peroxide-DAB), counterstained with hematoxylin, dehydrated with graded series of alcohol, cleared in xylene, sealed with neutral gum. Pictures were taken under a digital microscope (Leica Microsystems, Germany). Eight mice without laser photocoagulation were killed at day 1, 3, 7 and 14, at each time point, eyeballs from 2 mice were removed and immunohistochemistry stained with HIF-1α or Rac1 polyclonal antibody as control.

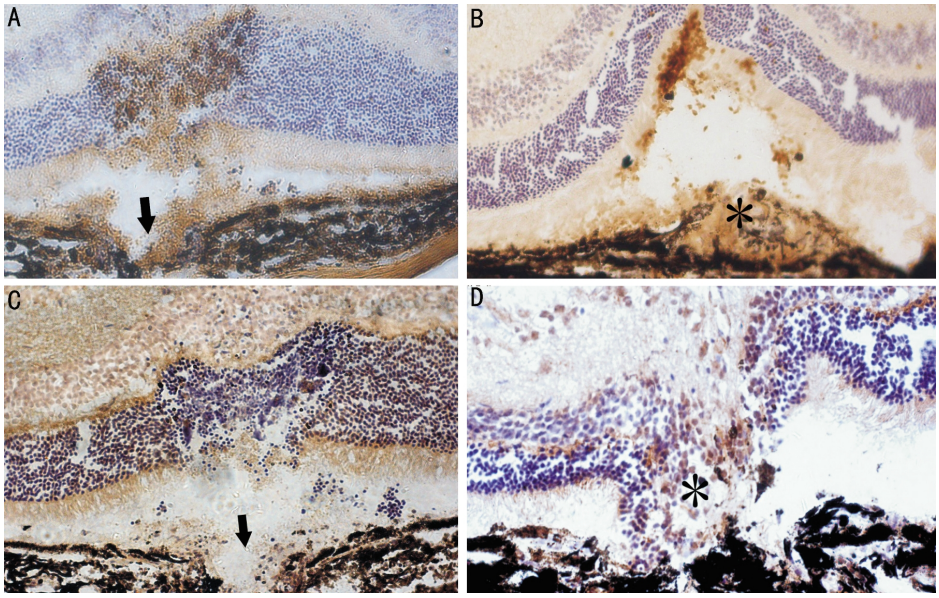

Figure 1 HIF-1α and Rac1 protein expression (SABC×20) A: HIF-1α 1 day after laser; B: HIF-1α 14 day after laser; C: Rac1 1 day after laser; D: Rac1 14 days after laser

Western blot for the expression of HIF-1α and Rac1 protein in choroid and RPE layer Twenty-four C57BL/6J mice with laser photocoagulation were killed at day 7 and 14, of which 12 mice undergone NSC23766 injection. At each time point, 12 mice were selected randomly as victims, of which 6 mice undergone NSC23766 injection. HIF-1α and Rac1 protein expression was semiquantitatively evaluated by western blot analysis of the choroid and RPE layer from C57BL/6J mice with laser photocoagulation at day 7 and day 14. Briefly, the vitreous and sensory retina were removed and the choroid and RPE layer from 3 eyes were lysed for 30 minutes on ice in lysis buffer (10g/L NP-40, 5g/L Deoxycholate, 10g/L SDS, 150mmol/L NaCl, 50mmol/L Tris-HCl,pH=8.0) supplemented with a mixture of protease inhibitors (Cell Signaling Technology, USA). The samples were cleared by microcentrifugation (14 000r/min, 30 minutes, 4℃) and assessed for protein concentration. Twenty micrograms of protein per sample was electrophoresed in a 10% Tris-Glycine gel. Proteins were electrophoretically transferred to nitrocellulose membrane and blocked with a blocking solution (50g/L milk powder insolution). After washes with tris-buffered saline (TBS), comparable blots were probed separately (incubation overnight at 4℃) with rabbit anti-mouse HIF-1α polyclonal antibody or rabbit anti-mouse Rac1 polyclonal antibody (1:1 000, Santa Cruz Biotechnology, USA). After being washed in TBS-Tween 0.05%, respective secondary peroxidase-labeled antibody was applied for 1 hour at room temperature. The blots were then washed 3 times with TBS and processed for chemiluminescence detection of the immunoreactive proteins after incubation for 5 minutes at room temperature with peroxidase substrate (Bio Rad laboratories, USA). Density of the immunoreactive bands was measured by use of the Image-Pro Plus software (Media Cybernetics, USA).

Six mice without laser photocoagulation were killed，the level of HIF-1α or Rac1 protein expression in choroid and RPE layer from 3 eyes were measured by western blot as control. HIF-1α or Rac1 protein expression level was normalized to the corresponding expression of GAPDH control protein and expressed as arbitrary units. Three independent experiments were evaluated.

Statistical Analysis Except for the incidence of CNV in C57BL/6J mice after laser photocoagulation with or without NSC23766 injection were compared using Chi-square test. Other results are expressed as the mean ±SD. The results were compared using one-way ANOVA after homoscedasticity was determined. P <0.05 was considered statistically significant.

RESULTS

HIF-1α and Rac1 Expression One day after laser photocoagulation, immunohistochemistry-stained sections showed disruption of the Bruch's membrane and RPE layer with subretinal exudation and occasional hemorrhage and cellular infiltration into the subretinal space. The presence of HIF-1α and Rac1 in the area of laser photocoagulation was confirmed, in which HIF-1α (Figure 1A) and Rac1 protein (Figure 1C) mainly locating at choroid and surrounding the disruption of Bruch's membrane and RPE layer.

Subretinal vessel ingrowth was first detected at day 3 after photocoagulation at the site of the laser spots. In this impaired areas, HIF-1α (Figure 1B) and Rac1 (Figure 1D)

mainly expressing in the fibrovascular tissue consisting of vessel lumen neovascular membrane.

Semiquantity of HIF-1α and Rac1 Expression of HIF-1α and Rac1 was highly expressed in tissue explants of RPE and choroid after laser photocoagulation without NSC23766 injection at day 7 and day 14 (Figure 2A). Densitometric analysis of three independent experiments showed 2.36±0.27-fold, 4.03±0.73-fold increase statistically (P<0.05) in relative HIF-1α protein expression, and 1.91± 0.08-fold, 2.72±0.03-fold statistically increase (P<0.05) in relative Rac1 protein expression values normalized to the GADPH expression value compared to that of control mice at day 7 and day 14 respectively (Figure 2B). In tissue explants of RPE and choroid with NSC23766 injection, both the expression of HIF-1α and Rac1 were inhibited at day 7 and day 14 (Figure 2C). Densitometric analysis of three independent experiments showed 1.06 ±0.13-fold, 1.11 ± 0.21-fold increase in relative HIF-1α protein expression, and 1.02 ±0.18-fold, 1.08 ±0.21-fold in relative Rac1 protein expression values normalized to the GADPH expression value compared to that of control mice at day 7 and day 14 respectively (Figure 2B). Statistically, both the HIF-1α and Rac1 protein expression are undifferentiated comparing to that of control (P>0.05, Figure 2D).

DISCUSSION

HIF-1 plays crucial roles in angiogenesis [4], which is composed of two proteins, HIF-1α (120kDa) and HIF-1β (91 to 94kDa). There are two transcriptional activation domains in HIF-1α referred to as the N-terminal activation domain (NAD) and the C-terminal activation domain (CAD). Between these two domains is an oxygen-dependent degradation domain (ODD), which, when deleted, confers stability of the protein in the presence of oxygen. HIF-1β expresses constitutively under normoxia or hypoxia. The stability and activity of HIF-1α are regulated by hypoxia. Under normoxia, the HIF-1α subunit is rapidly degraded via the ubiquitin-proteasome pathway. On the contrary, in the hypoxia condition, HIF-1α subunit becomes stable and interacts with HIF-1β subunit to form HIF-1, to modulate its transcriptional activity. Therefore, HIF-1α is the active center of HIF-1. Once activated by hypoxia, HIF-1 binds to the consensus HIF-1 DNA binding site (HBS), which present in the hypoxia-response elements (HRE) of many oxygen-regulated genes, including. HIF-1 target genes include those related to vasomotor control (such as NOS2), angiogenesis (such as VEGF), blood and iron metabolism (EPO, transferrin, transferrin receptor, ceruloplasmin), cell proliferation [Insulin-like Growth Factor(IGF)-1, transforming growth factor (TGF)β, and energy metabolism (glucose transporter-1, -2, and -3, phosphoenolpyruvate carboxylase,

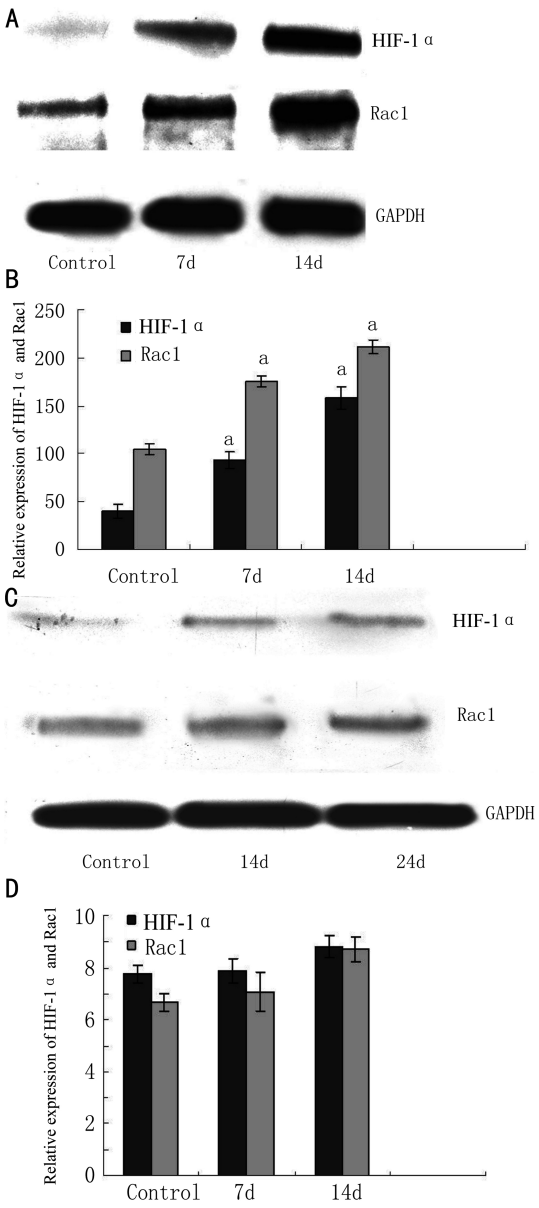

Figure 2 HIF-1α and Rac1 expression in RPE/choroid layer
A: without NSC23766; B: without NSC23766; C: with NSC23766; D: with NSC23766. [a]P<0.05 *vs* control

lactate dehydrogenase A, aldolase, phosphoglucokinase-1, -L and -C, pyruvate kinase, enolase, and many others). Studies have demonstrated that inhibitors of PI3K, serine/threonine protein phosphatase [7], and protein-tyrosine kinase activities blocking hypoxia-induced HIF-1α expression.

In this study, our results showed that upregulated expression of HIF-1α in CNV, which means that HIF-1 was being activated for transcription of VEGF to induce CNV, and maybe laser photocoagulation destoried Bruch's membrane resulting in hypoxia of RPE and choroid. But the mechanism by which HIF-1 is activated for transcription of VEGF to induce CNV is poorly understood. Other study verified that the dramatic inhibitory effects of the

dominant-negative form of Rac1 (Rac1-N17) and the modest stimulatory effects of its constitutively activated form (Rac1-V12) on expression of HIF-1α in hypoxic Hep3B cells, they thought that Rac1-independent signals is necessary for HIF-1 activation. Hypoxia has been considered to play an important role on the formation of CNV. Some studies showed that the relative hypoxia caused by the disturbed balance between the limited blood supply in the macula and the high oxygen demand by the photoreceptors may contribute to the formation of CNV by up-regulating VEGF [8].

Rac1 has previously been shown to mediate the effects of hypoxia-reoxygenation on the activity of transcription factors such as nuclear factor κB and heat shock factor 1 via generation of reactive oxygen intermediates. In a study, hypoxia-reoxygenation, but not hypoxia, was shown to induce heat shock factor 1 activation as a result of Rac1-mediated H_2O_2 generation. In this study, our results verified that the downregulation of expression of HIF-1α by inhibiting Rac1 with NSC23766. The Rac1 inhibitor NSC23766 is a highly soluble and membrane permeable compound, inhibiting Rac1 GTP-loading without affecting Cdc42 or RhoA activity, is a lead small molecule inhibitor of Rac activity and is useful for studying Rac-mediated cellular functions and for modulating pathological conditions in which Rac-deregulation may play a role [9]. Thus, the involvement of Rac1 in hypoxia-induced HIF-1 activation represents a novel pathway relating to the growth of CNV. With these results as a foundation, future studies will be necessary to further delineate the mechanisms and consequences of Rac1 activation in response to HIF-1. We suggest that Rac1 inhibition may have potential therapeutic value, and that will be a novel target other than VEGF and HIF-1 in developing intraocular angiogenesis inhibitors.

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