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Nerve growth factor-mediated vascular endothelial growth factor expression of astrocyte in retinal vascular development

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ABSTRACT

The angiogenic aspect of neurotrophins and their receptors rather than the neuroscientific aspect has been focused. However, their role in retinal vascular development is underdiscovered. The purpose of this study is to understand the role of neurotrophin receptors in retinal vascular development and the mechanisms of their action. To identify the expression of tropomyosin receptor kinase receptor (Trk) in developing retina, tissues of 4, 8, 12, 16 and 26 day-old mice were prepared for experiments. Immunohistochemistry and immunofluorescence double staining against glial fibrillary acidic protein and type IV collagen were performed. TrkA was expressed mainly along the vessel structure in inner part of retina, especially in retinal astrocyte. In cultured primary astrocyte, recombinant nerve growth factor (NGF) was used to activate TrkA. NGF induced the phosphorylation of TrkA, and it also enhanced the level of activated Akt and vascular endothelial growth factor (VEGF) mRNA. Inhibition of phosphoinositide 3-kinase (PI3K) reversed the NGF-induced activation of these two molecules. This study demonstrated that TrkA activation on NGF leads to VEGF elevation by PI3K-Akt pathway and therefore suggested that TrkA could be a stimulator of retinal vascular development.

1. Introduction

Generating initial retinal vasculature begins with emergence of retinal astrocyte. As it transverses inner surface from optic nerve to periphery, it interacts with retinal ganglion cell. By admitting platelet-derived growth factor (PDGF) signals from ganglion cells [1,2], immature astrocyte starts to form a mesh-like network. As crowded astrocytes undergo hypoxic stress, they begin to express vascular endothelial growth factor (VEGF). Superficial vessel plexus sprouting is the consequence of the response of endothelial cells (ECs) to astrocytic VEGF. Retinal astrocyte serves as a vessel template along which retinal ECs can grow. Although in primates, whose vasculature covers almost retinal surface, retinal astrocyte resides in the whole surface, there is no astrocyte in the avascular fovea [3–6]. Filopodial ECs that possess VEGF receptor start to be drawn against the VEGF gradient [7,8], toward avascular region forming primeval superficial vascular plexus. Then, vascular pruning is succeeded. Because EC proliferation occurs at random, selective apoptosis by leukocytes is necessary for patterning. Finally mural cells such as pericytes make the structure mature. Superficial plexus is completed around post-natal one week in mice.

Once superficial plexus is formed, new vessels start to infiltrate perpendicularly. Originating from central retina, they expand toward peripheral area. This time, Müller cells become a guide template [9]. Once they reach to both edges of inner nuclear layer, they make a second vessel plexus [10]. However, little is known about the underlying molecular mechanism.

Neurotrophin and its receptor are well known in neurogenesis and neuroprotection. Furthermore, there is considerable evidence that suggests that they are also linked with vascular biology. Their intrusion is maximized at fetal vascular development or convalescence from vascular injury. While nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and their corresponding receptor, tropomyosin receptor kinase receptor A (TrkA) and TrkB are rarely seen at vascular smooth muscle cell in normal late gestational stage, they were fully expressed during the recovery period following the vascular injury [11,12]. The main field of interest of neurotrophic factors in vascular biology is angiogenesis, the growth of new blood vessels from preexisting capillaries. Therefore, their capability to proliferate ECs has been largely investigated. Best studied is NGF-TrkA signaling. Human dermal microvascular endothelial cell (HDMEC) was the first reported EC

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that reacts to NGF to exert significant angiogenic feature *in vitro* [13]. Human umbilical vein endothelial cell (HUVEC), human choroidal EC, and rat brain EC all show the same feature [14–16]. An angiogenic feature was also tested *in vivo*. Chick embryo chorioallantoic membrane (CAM) assay, the most popular tool for the researching angiogenesis, verified the angiogenic property of NGF [14]. Egg allantoic vessels grew and spread toward membrane in response to NGF. A model of limb ischemia induced neovascularization was also utilized [17]. NGF and TrkA were up-regulated in ischemic muscle caused by mice femoral arterial resection. When NGF was inhibited with neutralizing antibody, density of capillary became sparse and myocytes were collapsed. Conversely, injecting exogenous NGF facilitated new capillary formation and achieved hemodynamic recovery.

A contribution of BDNF-TrkB to angiogenesis is also revealed. The expression of BDNF and TrkB was observed in HUVEC and brain-derived endothelial cell [18,19]. In hypoxia, a greater amount of BDNF mRNA transcript was produced and secreted. This let EC proliferate. Recombinant BDNF promoted angiogenesis *in vitro*. Like that of NGF, the level of VEGF increased when BDNF was given [20].

However, the relevance of Trk's to retinal vascular development has not been investigated. In this study, the role of TrkA of retinal astrocyte in retinal vascular development was addressed. First, immunological staining was done to examine the pattern of Trk's in murine retinal tissue. Then, *in vitro* study was conducted to understand how their signal conveyance was directed to make VEGF was investigated.

2. Materials and methods

2.1. Animals

Experimental C57BL/6J strain mice were used. (Samtako, Gyeonggi-do, Republic of Korea). Breeding temperature was 23 °C indoors and feeding was once a day at morning. Light was given for 12 h daily to maintain circadian rhythm. Every procedure for nurturing and sacrificing mice was strictly confirmed to 'Agreement with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research'.

2.2. Tissue preparation

Mice of post-natal day (P) 4, 8, 12, 16 and 26 were sacrificed by cervical dislocation. Eyes were enucleated after cutting off the optic nerve. It was fixed with 4% paraformaldehyde for 24 h and paraffin embedded. The specimens were sectioned by sagittal plane by 4 μm depth and adhered to slide glass.

2.3. Immunohistochemistry (IHC) staining

All procedures were performed by previous description [21] with small modifications. Anti-TrkA antibody (Santa cruz biotechnology, Santa cruz, CA) and anti-TrkB antibody (Santa cruz biotechnology) were used as primary antibody. Titers were 1:100 and 1:200, respectively.

2.4. Immunofluorescence (IF) staining

Overall procedure was equal to that of IHC mentioned above. Glial fibrillary acidic protein (GFAP, Abcam, Cambridge, United Kingdom), type IV collagen (Milipore, Temecula, CA) were used as primary antibody. Titers were both 1:100. Titer of fluorescence-labeled secondary antibody (Invitrogen, Life technologies, Carlsbad, CA) was 1:400. Cell nuclei were visible with 4′,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich, St. Louis, MO).

2.5. Cell culture

Primary human brain astrocyte cells (Cell systems, Kirkland, WA) were purchased and cultured in Dulbecco's modified eagle medium (DMEM, Thermo fisher scientific, Waltham, MA) at 37 °C in a humidified atmosphere of 5% CO_2. Supplemented are 10% heat-inactivated fetal bovine serum (Gibco, Life technologies) and penicillin–streptomycin (Gibco). N-2 supplement (Invitrogen) for neuronal cells was also added. Reconstituted recombinant human NGF β subunit (R&D systems, Minneapolis, MN) was treated to 70% confluency of astrocyte in serum-free DMEM to activate TrkA. For the maximal effect, 6 h of starvation was introduced prior to NGF treatment. 20 μM of LY294002 (Tocris, Bristol, United Kingdom), the inhibitor of phosphoinositide 3-kinase (PI3K), was administered 30 min before as necessary.

2.6. Western blot analysis

All procedures followed described previously [32] with small modifications. Anti-phospho-TrkA (pTrkA) antibody (Santa cruz biotechnology), anti-Akt antibody (Cell signaling technology, Danvers, MA), anti-phospho-Akt (pAkt) antibody (Cell signaling technology) were used for primary antibody. Primary anti-actin antibody (Sigma–Aldrich) was also used as an internal control. Titers were 1:1000 for all. The densitometric analyses of each band were performed using ImageJ program (NIH, USA).

2.7. Reverse transcription polymerase chain reaction (RT-PCR) analysis

All procedures followed described previously [32] with small modifications. The primers for VEGF were as follow; 5′-ACCACAG TCCATGCCATCAC-3′, 5′-GAGCATGCCCTCCTGCCCGGCTCACCGC-3′. The primers for GAPDH were as follows; 5′-ACCACAGTCCATGCCATCAC-3′, 5′-TCCACCACCCTGTTGCTGTA′. The densitometric analyses were identical to western blotting.

2.8. Statistical analysis

Statistical significance was determined using Student's unpaired *t*-test between control and test group. *P* value under 0.05 was considered significant.

2.9. Computational analysis

Hypoxia responsive elements in the regulatory region of NTRK1 gene (Genbank number: NG_007493.1) were searched using TESS program (CBIL, university of Pennsylvania, PA. http://www.cbil.upenn.edu/cgi-bin/tess/tess). Transcriptional start site of NTRK1 was predicted by PEDB (center for developmental biology, RIKEN, Japan).

3. Results and discussion

3.1. Differential expression of TrkA and TrkB in developing retina

To test the expression of TrkA and TrkB in retinal development, mid-periphery portion of retina was analyzed by immunohistochemistry. The expression of TrkA and TrkB was apparent in various neuronal layers. TrkA expression was observed mainly at inner retinal region (Fig. 1). At P4, TrkA activity was detected at the innermost part of retina, ganglion cell layer and inner part of neuroblastic layer. Primary plexus flows nerve fiber layer and the internal limiting membrane is made up of retinal astrocyte and the end feet of Müller cells. This could suggest that TrkA might participate in not only neuronal but also vascular developmental

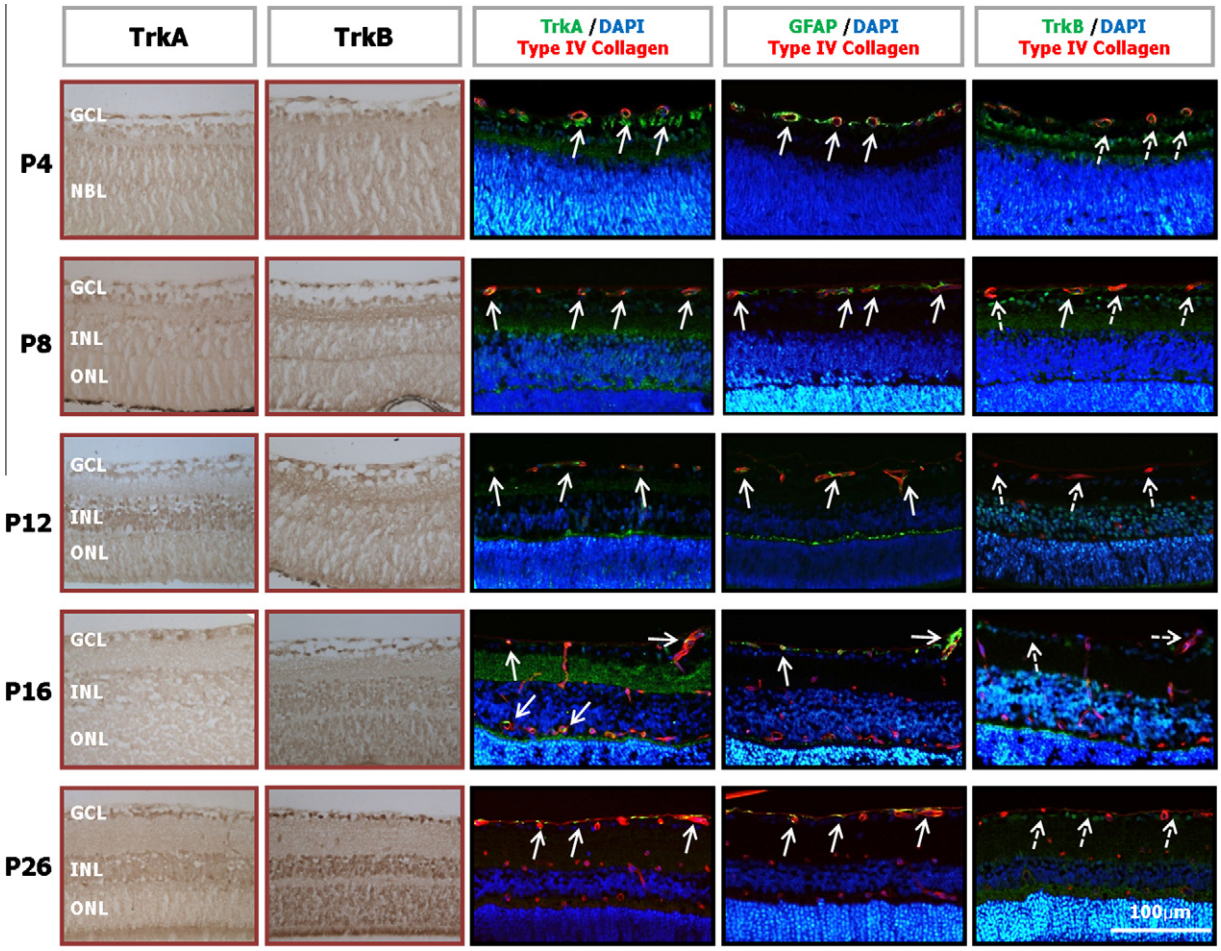

Fig. 1. Differential expression of TrkA and TrkB in developing retina. Retinas from 4, 8, 12, 16 and 28 postnatal days are represented. Vertical retinal sections of developing mouse retina were immunostained for TrkA and TrkB. The expression of TrkA or TrkB in green along the retinal blood vessels was also confirmed through Immunofluorescence by using the antibodies for Collagen Type IV, the endothelial cell marker or GFAP in red. The yellow arrows indicate merged immunofluorescence. Scale bars: 100 μm; GCL: ganglion cell layer; RBL: neuroblast layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

process. TrkA expression was also observed at vessel-like structures near the superficial vessels which again supports its implication in angiogenesis. At P8, the patterns of TrkA were analogous to P4. From P12, the expression signature has spread out toward the outer retina. At P12, P16, and P26 (adult mice), TrkA activity was found commonly at inner part of retina outlining inner nuclear layer. Furthermore, it was also detected along the vessel-like structure at outer retina.

TrkB activity did not differ greatly from TrkA activity. At P4, TrkB activity was detected at the innermost region, ganglion cell layer, inner plexiform layer and inner part of neuroblastic layer (Fig. 1). Here, the role of TrkB in vascularization was also suggested. The TrkB expression at different developmental stages showed a comparable pattern with the TrkA activity. Nevertheless, some disparities were observable. While TrkA was found in deeper vessels from P12 to adulthood, TrkB was not. These result raised the possibility of some contributions of neurotrophin receptors in retinal vascular development and how the two molecules might participate in the developing process differently.

Next, immunofluorescence staining was carried out to search for an exact cell that expresses TrkA or TrkB. Two cell markers that are directly relevant with retinal vessel development, GFAP (astrocyte marker) and type IV collagen (endothelial cell marker), were tested for co-localization with Trk receptors. TrkA was localized where type IV collagen emitted light in the primary plexus (Fig. 1). There certainly were a few points of convergence between TrkA and type IV collagen particularly at deeper vessels of P12, P16, but these points were restricted and thus need to be further investigated. On the other hand, TrkA on superficial retina was intensively expressed around the vessel structure in any period. GFAP was constitutively co-localized with TrkA. TrkA matched extensively with astrocyte near the vessels throughout the whole period examined. These findings intimate that TrkA is expressed in astrocyte. This speculation is also supported by another observation in which certain TrkA expression was detected at peripheral area at P4 when primary plexus formation was not completed (data not shown).

The expression of TrkB was distinctive from TrkA. Although TrkB was apparent around the vessel structure, as the IHC result showed, neither endothelial cell nor astrocyte represented TrkB (Fig. 1). A limited matching signal with GFAP was observed from superficial vessel transiently at P8 only, and TrkB activity was not seen from deeper vessels either. Besides the former two molecules, IF staining of Müller cell and pericyte that are also major contributors in retinal vascular development was tried against both TrkA and TrkB. But there hardly was any meaningful immunoactivities of TrkA and TrkB (data not shown).

TrkA were intensively expressed in astrocyte during the entire developing process. Although the transient astrocytic coincidence with TrkB and the simultaneity of TrkA in deeper endothelium

were reported, the relevance between TrkA and retinal astrocyte was the most evident and confirmed to be reproducible.

3.2. NGF-induced TrkA activation in astrocyte

To clarify the relationship between TrkA and astrocyte, further experiments were performed *in vitro*. To activate the TrkA, its ligand NGF was administered. Of several time points, the effect of phosphorylation was maximized in 10 min from treatments (data not shown). Compared to the basal phosphorylation level, a dramatic activation was checked at the concentration of 25 ng/ml of NGF. At 50 and 100 ng/ml of NGF, their efficacy to stimulate TrkA did not further increase (Fig. 2).

3.3. NGF-induced VEGF production via PI3K/Akt pathway in astrocyte

To evaluate the possibility of astrocytic TrkA induction in vascularization, the level of VEGF, the best-known angiogenic stimulator was investigated. RT-PCR analysis showed a marked increase of VEGF molecule up to two folds. These findings verify that astrocyte acts on endothelial cell as a scouter and a template and suggest that TrkA can be one of the initiators that produce VEGF inducing pathway to draw retinal endothelial cells. VEGF121 and VEGF165, the major isoform variants of VEGF and the essential modulator during retinal vessel formation showed the similar aspect (Fig. 3A).

Among various signaling pathway from TrkA, PI3K – Akt pathway is one of the most unraveled one [23,24]. It is also famous for evoking VEGF secretion. Indeed, Akt reacted to NGF stimulation in a dose dependent manner (Fig. 3B). In 2 h, the level of Akt phosphorylation has risen to the highest (data not shown). These data implicate the participation of NGF/TrkA – Akt – VEGF axis in retinal vascularization.

To further assess the concertation of TrkA, Akt and VEGF on retinal vascularization, LY294002, the pharmacological inhibitor of PI3K, was utilized. Because PI3K is generally accepted as a direct modulator of Akt at its upstream, it is reasonable to assume that TrkA signaling would also pass through PI3K to induce VEGF. So whether LY294002 reverses the aforementioned results was tested, and it completely abolished NGK-induced Akt activation (Fig. 3C). This data demonstrated that PI3K is a liaison between TrkA and Akt. Since LY294002 eliminated the activation of Akt thoroughly, the possibility that PI3K and TrkA independently give signals to Akt is quite remote. Accordingly, this result also consolidates the link between NGF/TrkA and Akt in retinal vascularization. In addition, LY294002 inverted NGF driven VEGF upregulation as well. Transcripts of VEGF121 and VEGF165 were all fairly dropped (Fig. 3D). However, basal levels of VEGF were maintained, implying diverse pathways toward VEGF induction. Our data established the role of NGF/TrkA in VEGF production via PI3K and Akt in astrocyte.

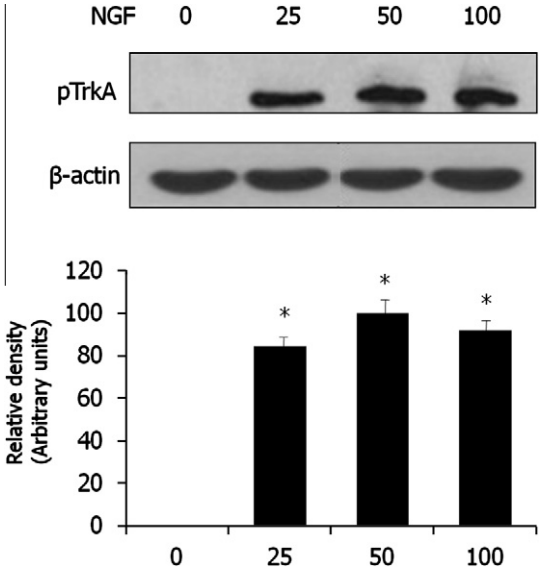

Fig. 2. NGF-induced TrkA activation in astrocyte. Western blot analysis for pTrkA was performed in primary human brain astrocyte cells treated with NGF (25, 50 or 100 ng/ml). β-Actin was served as the loading control. Quantitative analysis was performed by measuring protein expression relative to the control. Figures were selected as representative data from three independent experiments. Each value represents the mean (±SD) of three independent experiments. $^{*}P < 0.05$.

3.4. Positive feedback between TrkA and HIF-1α

Not only did our results say, but various researches reported that Trk receptors upregulated VEGF level in several tissues. As described before, diverse endothelial cells react directly with NGF, and BDNF. Cells other than EC can recognize NGF to produce VEGF such as those in the neuroblastoma cell lines [25], and epithelial cells of ovarian cancer [26]. Various signaling pathways have been established to produce VEGF including RTK signaling, redox system and cellular metabolism status. Trk receptors become one of good driving forces.

The key molecule in producing VEGF is HIF-1α. Many *cis*-acting sequences of *vegf* gene exist like HIF-1, LXR, AP-2, STAT-3, Sp-1,3, and egr-1. HIF-1α is the best characterized. Under the hypoxic condition or its equivalent, HIF-1 gets stabile and binds to hypoxia responsive element (HRE) with HIF-1β. TrkA could be an initiator to give signals toward HIF-1α. Because premature retina is deprived of oxygen, it is likely that HIF-1 is utilized as an oxygen sensor for self propagation, neuroprotection and eventually angiogenesis. The fact that PI3K-Akt pathway is the noticeable positive regulator of HIF-1α even in normoxia lends weight to this hypothesis [27,28]. This concept was supported by other studies as well. NGF induced elevation of VEGF via HIF-1α in neuroblastoma cell was reported [25]. The same group also verified that BDNF facilitated the expression of VEGF and HIF-1α in neuroblastoma cell. Furthermore, interfering with HIF-1 blocked BDNF mediated VEGF expression [20].

There was a report in which HIF-1 is a transcriptional activator of TrkB, taking a diametrical concept [29]. TrkB has many identical HREs ~2 kb upstream of coding gene. At hypoxia, the levels of mRNA and protein of TrkB soared up to 30 folds with a high luciferase promoter activity. Interfering with HIF-1α reserved these results. This result can be explained as a positive feedback system. As a signal from Trk receptor is introduced, HIF-1 gets activated. This, in turn, drives Trk even further letting HIF-1 be expressed more. Experiments support this idea. miR-210 transcribed by HIF-1α reduced the level of glycerol-3-phosphate dehydrogenase 1-like which destabilized HIF-1α. By making HIF-1α more stable, miR-210 helped itself expressed more [30]. Cox-2 also utilized HIF-1 positive feedback by increased level of PGE2 [31]. The positive loop could be explained as a self-saving strategy to escape the dangerous environment.

If the same could be applied to TrkA, the existence and functionality of HRE site of *NTRK1* (gene of TrkA) should be established. Bioinformatics analyses raised the possibility of positive loop system. Regulatory region of *NTRK1* was scanned against conserved HRE sequences, (A/G) CGTG. Five putative HREs were concentrated within 2 kb from transcriptional initiation site, which is unusual for other region (Fig. 4A and B). Two of them have a specific sequence in which two same HRE sequences lie facing each other at both strand (Fig. 4). These resemble HRE of alpha fetoprotein [32], making the hypothesis more plausible although experiments of proof should be conducted.

Just as the role of NGF has been extended to an angiogenic assistant, increasing evidences are challenging the canonical concept of

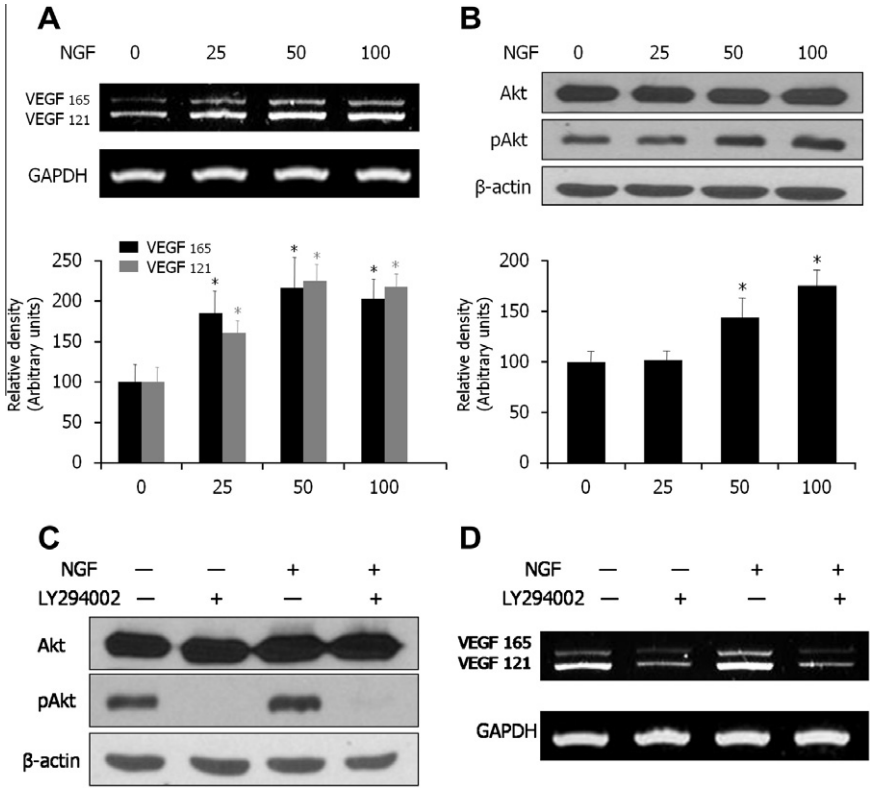

Fig. 3. NGF-induced VEGF production via PI3K/Akt pathway in astrocyte. Primary human brain astrocyte cells were treated with NGF (25, 50 or 100 ng/ml) or 20 μM LY294002. (A, D) RT-PCR for *VEGF* mRNA was performed. GAPDH was used as the loading control. (B, C) Western blot analysis for Akt or pAkt was performed. β-actin was served as the loading control. Quantitative analysis was performed by measuring mRNA or protein expression relative to the control. Figures were selected as representative data from three independent experiments. Each value represents the mean (±SD) of three independent experiments. $^{*}P < 0.05$.

VEGF. One reported that VEGF reinforced the secretion of BDNF from neostriatal EC making neurons outgrow [33]. Our group also showed the administration of VEGF activated TrkA in retinoblastoma cell [22]. Namely, it is certain that neurotrophin and VEGF collaborate together and affect each other. Various cross-talking and shared signaling pathways like PI3K-Akt or MAPK/ERK have been reported [34,35]. Same principles can be applied to astrocyte.

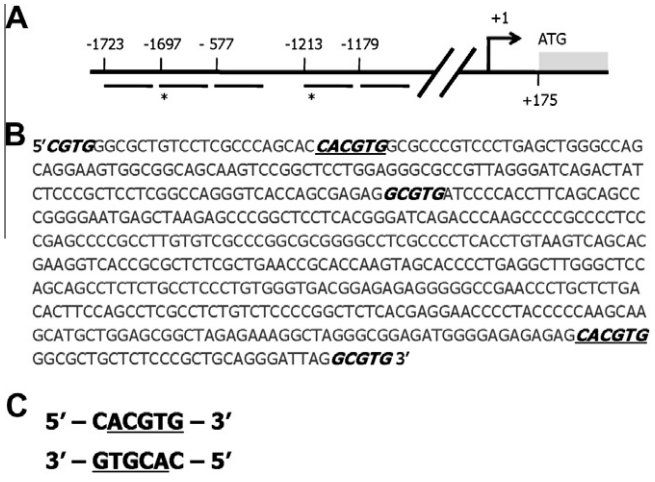

Fig. 4. Positive feedback between TrkA and HIF-1α. (A) Regulatory region of 5′ upstream of NTRK1 gene. Five putative HREs were marked with line at specified locations. Asterisk indicates two distinctive HREs found in promoter of α-fetoprotein. (B) Sequence including HREs in NTRK1 promoter. Putative HREs were marked in bold and italics. Underlined are two putative distinctive HREs. (C) Structure of putative distinctive HRE. Two same sequences lie together. Underlined are conserved HRE sequences.

Because astrocyte has dual duties, supporting neuron and endothelial cells at the same time, it is natural that astrocyte processes and converts two different signals for the maximal effects. On neuronal ischemia, it also secreted several angiogenic and neurotrophic factors [36]. In the subventricular zone of the brain, neural stem cell, the precursor of astrocyte, expressed VEGFR-3 to induce neurogenesis without affecting angiogenesis [37]. If it is deleted, astrocyte disappeared and neurogenesis failed.

In conclusion, this study revealed that activation of astrocytic TrkA could induce VEGF levels via PI3K – Akt pathway in developing retina. Lots of riddles remain unsolved. The relationship between the limited expression such as TrkB in astrocyte or TrkA in deeper plexus and the retinal vascularization was not covered. Also, the participation of HIF-1α in TrkA signaling needs to be investigated. Nevertheless, it is significant in the sense that our study reveals the role of TrkA in astrocyte in retinal vascularization.

Acknowledgments

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