Apelin Is a Crucial Factor for Hypoxia-Induced Retinal Angiogenesis

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Objective—To investigate the role of endogenous apelin in pathological retinal angiogenesis.

Methods and Results—The progression of ischemic retinal diseases, such as diabetic retinopathy, is closely associated with pathological retinal angiogenesis, which is mainly induced by vascular endothelial growth factor (VEGF) and erythropoietin. Although antiangiogenic therapies using anti-VEGF drugs are effective in treating retinal neovascularization, they show a transient efficacy and cause general adverse effects. New therapeutic target molecules are needed to resolve these issues. It was recently demonstrated that the apelin/APJ system, a newly deorphanized G protein–coupled receptor system, is involved in physiological retinal vascularization. Retinal angiography and mRNA expression were examined during hypoxia-induced retinal angiogenesis in a mouse model of oxygen-induced retinopathy. Compared with age-matched control mice, retinal apelin expression was dramatically increased during the hypoxic phase in oxygen-induced retinopathy model mice. APJ was colocalized in proliferative cells, which were probably endothelial cells of the ectopic vessels in the vitreous body. Apelin deficiency hardly induced hypoxia-induced retinal angiogenesis despite the upregulation of VEGF and erythropoietin mRNA in oxygen-induced retinopathy model mice. Apelin small and interfering RNA suppressed the proliferation of endothelial cells independent of the VEGF/VEGF receptor 2 signaling pathway.

Conclusion—These results suggest that apelin is a prerequisite factor for hypoxia-induced retinal angiogenesis. (Arterioscler Thromb Vasc Biol. 2010;30:2182-2187.)

Key Words: angiogenesis ■ VEGF ■ apelin ■ retinopathy

The progression of ischemic retinal diseases, such as diabetic retinopathy, is closely associated with pathological retinal angiogenesis, which is mainly induced by vascular endothelial growth factor (VEGF) and erythropoietin (Epo). Antiangiogenic therapies targeting these factors are effective in treating proliferative diabetic retinopathy. However, these therapies show a transient efficacy and cause the general adverse effects. Furthermore, elevated VEGF levels do not necessarily correlate with pathological retinal angiogenesis in patients with diabetic maculopathy. This suggests that the entire process of pathological retinal angiogenesis includes more than just upregulation of VEGF expression. Therefore, finding other factors involved in pathological retinal angiogenesis is important.

The apelin/apelin receptor (APJ) system is a newly deorphanized G protein–coupled receptor system. Recently, much attention has been focused on the possible roles of the apelin/APJ system in vascular pathophysiology. APJ is expressed in endothelial cells at the leading edge of vessels during early embryogenesis; and apelin, in combination with VEGF, induces the proliferation and assembly of endothelial cells. Moreover, we demonstrated a retardation of physiological retinal vascular development during the early postnatal period and a reduced angiogenic response to VEGF in apelin-knockout (KO) mice. Thus, the apelin/APJ system participates in physiological vascular development in collaboration with VEGF. However, the relationship between the apelin/APJ system and pathological angiogenesis, especially in the retina, is not well defined.

In ischemic retinopathy, hypoxia-inducible factors are upregulated in the retina, leading to alteration of gene expression. Hypoxia-inducible factors are key transcription factors that regulate angiogenesis and the gene expression of angiogenesis-related factors during ischemia. Moreover, consensus sequences for hypoxia-inducible factors are present in the apelin promoter region, and hypoxia-induced apelin expression regulates endothelial cell proliferation and regenerative angiogenesis. Apelin is an ischemia-induced paracrine factor that promotes angiogenesis. We investigated its potential role in pathological retinal angiogenesis in a mouse model of oxygen-induced retinopathy (OIR).
Methods

Animals

Animal experiments were performed in accordance with the guidelines of the Japanese Society for Pharmacology and were approved by the Committee for the Ethical Use of Experimental Animals at Setsunan University, Osaka, Japan. All efforts were made to minimize animal suffering, reduce the number of animals used, and use alternatives to in vivo techniques.

C57BL/6 mice with targeted disruption of the apelin gene (apelin-KO) were generated as previously described by Kidoya et al. These mice were housed in metallic breeding cages in a room with a 12-/12-hour light/dark cycle. The humidity was 55%, the temperature was 23°C, and the mice had free access to food and water.

A Mouse Model of OIR

Ischemic retinopathy was generated in wild-type (WT) and apelin-KO mice according to a previously established protocol for generating the OIR model. In brief, at postnatal day (P) 7, pups and their nursing mothers were exposed to hyperoxic conditions (75% oxygen) for 5 days. On P12, pups were returned to room air (normoxic) conditions for 3, 5, and 8 days until P15, P17, and P20, respectively.

Quantitative PCR to Measure Transcription Levels

Mice were euthanized, and retinas were removed. Total RNA was extracted from the retinas; and reverse transcription of total RNA, 1 μg, was performed as previously described. Quantification of all gene transcripts was conducted using quantitative real-time RT-PCR with ABI Prism 7900-HT (Applied Biosystems, Foster City, Calif). Real-time RT-PCR was performed using SYBR Green fluorescence detection (Takara, Ohtsu, Japan). The primers used are shown in the supplemental Table (available online at http://atvb.ahajournals.org).

Immunohistochemistry

The primary antibodies used were rat monoclonal anti-platelet/endothelial cell adhesion molecule-1 (MEC 13.3) (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) and rabbit polyclonal anti-APJ. To obtain a specific antibody against mouse APJ, a rabbit was immunized with a synthetic peptide (C-PGPMMKGKKGMEIKSPSYSQTLV) derived from the C-terminal region of APJ. Secondary antibodies used were biotinylated anti-rat IgG (DAKO Corp, Carpinteria, Calif) and Alexa 568–conjugated anti-rabbit IgG (Molecular Probes, Eugene, Ore). The binding of biotinylated antibodies was detected with streptavidin–fluorescein isothiocyanate (FITC) (BD Biosciences, San Diego, Calif). Immunostained retinal flat mounts were prepared using fluorescence microscopy (Olympus BX51, Tokyo, Japan). Fluorescence images were captured with a fluorescence microscope (Biorevo, Keyence, Japan).

Measurement of Retinal Vessel Growth

Mice were anesthetized with chloral hydrate, 400-mg/kg body weight IP, and perfused with saline containing 40-mg/mL FITC–labeled dextran (molecular weight, 2,000,000; Sigma) through the left ventricle at the indicated ages. Eyes were removed and fixed for 1 hour in 4% paraformaldehyde/PBS solution. Retinas were dissected and flat mounted, as previously described. For counting of nuclei extending beyond the internal limiting membrane, the eyes of mice were enucleated and fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin at P17. Six sections were selected within 300 μm of the optic nerve in serial sagittal sections (5-μm/L thickness) of whole eyes and stained with hematoxylin-eosin (HE). Photographs were taken with a fluorescence microscope (Biorevo, Keyence, Japan).

Proliferation Assays

Mouse brain endothelioma bEnd.3 cells were maintained in DMEM containing 10% FBS. Apelin and scrambled small interfering RNAs (siRNAs) were purchased (catalog No. 00900627 and 1027284, respectively; Qiagen, Valencia, Calif). To assess the proliferation/viability of endothelial cells, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazium bromide (MTT) assay was also used as previously described. For proliferation, cells were pulse labeled with 10-μmol/L BrdU (Sigma) for 6 hours at 37°C. Cells were fixed in 4% paraformaldehyde for 10 minutes, followed by a denaturation step in 0.5N hydrochloride for 10 minutes and a neutralization step with 0.1-mol/L sodium borate for 10 minutes. Samples were then subjected to immunostaining with an anti–BrdU monoclonal antibody (BD Biosciences) and photographs were taken with a fluorescence microscope (Biorevo).

Statistical Analyses

Statistical analyses of the experimental data were performed by 2-way ANOVA, followed by the Tukey–Kramer test.

Results

The retinal pathological features of OIR model mice were characterized by hypoxia-induced retinal angiogenesis. In brief, pups and their nursing mothers were exposed to 75% oxygen for 5 days from P7 to P12. This period is when the oxygen-induced loss of retinal vessels occurs (hypoxic phase). After returning to the normoxic state in room air during P12 through P17 (hypoxic phase), hypoxia-induced vessel loss turns into retinal angiogenesis.

We first examined the patterns of gene expression in the retinas of OIR model mice. Consistent with a previous report, apelin mRNA expression was upregulated during P7 through P15 in the retinas of mice maintained under normoxic conditions (3.83±0.48-fold at P12 and 4.57±0.47-fold at P15 versus P7) (Figure 1A). Compared with age-matched mice maintained under normoxic conditions, retinal apelin mRNA expression was significantly suppressed during the hypoxic phase in OIR model mice (normoxic control, 3.83±0.48-fold at P12; and OIR, 1.29±0.21-fold at P12) (Figure 1A). During the hypoxic phase, apelin mRNA expression significantly increased compared with that at P7 in OIR model mice (31.18±2.39-fold at P15 versus 18.33±2.37-fold at P17) (Figure 1A). Upregulation of apelin mRNA in OIR model mice was much higher than that of VEGF (3.05±0.14-fold at P15 versus 2.91±0.12-fold at P17) (Figure 1B) and Epo (11.48±1.71-fold at P15 versus 7.85±0.83-fold at P17) (Figure 1C). The expression of the endothelial cell marker tie-2 mRNA in the retina of OIR model mice was significantly lower than that of control mice at P12 in the hypoxic phase but abruptly increased to 4.29±0.38-fold at P17 in the hypoxic phase (Figure 1D). The expression pattern of another endothelial cell marker platelet/endothelial cell adhesion molecule-1 mRNA was also similar to that of tie-2 in the retina of OIR model mice (normoxic control, [mean±SEM] 3.42±0.26-fold at P12 and 4.54±0.16-fold at P17; and OIR, 1.71±0.10-fold at P12 and 5.44±0.39-fold at P17).

To identify the target cells for apelin, we examined the expression of apelin receptor APJ in the retinas of OIR model mice. The expression of APJ mRNA in retinas was unchanged during the hypoxic phase (0.96±0.05-fold at P12 versus P7).
but abruptly increased during the hypoxic phase (from 2.06±0.18-fold at P15 to 2.96±0.13-fold at P17 versus P7). Flat-mount immunostaining of retinas of OIR model mice at P15 showed that APJ was colocalized with platelet/endothelial cell adhesion molecule-1 in retinal vessels, particularly in the vascular plexus, where many endothelial cells were proliferative20 (Figure 2). In addition, APJ expression was not detected in the endothelial cells of vessels that were not accompanied by capillary sprouting (Figure 2, arrowheads).

We also performed HE staining and double labeling of APJ and BrdU in retinal cross sections from mice at P17. Retinal cross sections from OIR model mice, not control mice, showed that many vascular nuclei extended into the vitreous from the retinal surface (Figure 3A and B). APJ was strongly expressed in the ganglion cell layer, the inner nuclear layer, and the intravitreal vessels of retinal cross sections from OIR model mice at P17 (Figure 3C). In addition, APJ colocalized with proliferative cells, which were probably endothelial cells of the ectopic vessels in the vitreous body (Figure 3D, arrows).

![Figure 2. Double immunostaining for platelet/endothelial cell adhesion molecule-1 (PECAM-1) and APJ in flat-mount retina specimens from OIR model mice at P15. Representative pictures are shown. APJ expression was not detected in the endothelial cells of vessels that were not accompanied by capillary sprouting (arrowheads). The bar indicates 100 μm.](image)

Next, using FITC dextran–perfused retinas of OIR model mice, we compared capillary density in the retinal area, where vessels were developed between WT and apelin-KO mice at different stages. Compared with age-matched normoxic controls, vessel loss occurred in the retinas of the 2 genotypes during the hyperoxic phase (Figure 4A). After 5 days of oxygen exposure at P12, capillary density was extremely low and almost similar in WT and apelin-KO mice (Figure 4A). In the hypoxic phase at P17, the retina of WT mice showed a significant increase in capillary density accompanied by abnormal vessel growth, similar to an aneurysm (Figure 4A, arrowheads), which was hardly observed in control retinas. In contrast, there was no increase in capillary density and abnormal vessels in the retinas of apelin-KO mice at P17 (Figure 4B). However, capillary density in apelin-KO mice recovered to a normal level at P28 (supplemental Figure I). Consistent with retinal angiography, retinal tie-2 expression in apelin-KO mice was significantly reduced compared with that in WT mice at P17 (supplemental Figure II). In addition, retinal APJ expression in apelin-KO mice was unchanged during the hypoxic phase (supplemental Figure II). Moreover, there was also significant reduction of capillary density in the retina of OIR model at P17 using apelin heterozygous female mice, in which the physiological retinal vessel growth was normally developed and retinal apelin expression was approximately half compared with WT mice (WT male littersmates, 42.05±0.89%; and heterozygous female mice, 39.14±0.79%; P=0.02 [capillary area by angiography]).
from the retinal surface was significantly reduced in apelin-KO OIR model mice compared with WT OIR model mice (Figure 4C). Furthermore, BrdU-positive cells in apelin-KO OIR model mice at P17 were obviously reduced compared with those in WT OIR model mice (supplemental Figure III).

Next, we examined if the difference in the amount of retinal angiogenesis between WT and apelin-KO mice was dependent on the retinal expressions of VEGF and Epo during the hypoxic phase. VEGF expression was similar in WT and apelin-KO mice at P12, but VEGF expression in apelin-KO mice significantly increased during the hypoxic phase compared with that in WT mice (WT, 3.05 ± 0.14-fold at P15 and 2.91 ± 0.12-fold at P17; and apelin-KO, 3.91 ± 0.31-fold at P15 and 5.44 ± 0.44-fold at P17) (Figure 5A). Epo expression in apelin-KO mice also increased significantly during the hypoxic phase compared with that in WT mice (WT, 18.55 ± 2.25-fold at P15 and 16.97 ± 2.19-fold at P17; and apelin-KO, 31.43 ± 3.69-fold at P15 and 38.59 ± 1.06-fold at P17) (Figure 5B). Moreover, there was no significant difference in the expression of VEGF receptors (VEGFR1 and VEGFR2) during hyperoxic and hypoxic phases between WT and apelin-KO mice (Figure 5C and D). Epo receptor expression in apelin-KO mice was also similar to that in WT mice, except at P15 (Figure 5E).

Finally, to assess the independent effect of apelin and VEGF on the growth of endothelial cells, we examined if inhibition of the apelin/APJ system could suppress the proliferation of endothelial cells independent of the VEGF/VEGFR2 signaling pathway. We confirmed that apelin siRNA reduced apelin mRNA expression to 5% and did not affect VEGF mRNA expression in b.End.3 cells (Figure 6A). We examined the viability/proliferation of endothelial cells by the MTT assay and BrdU incorporation. Apelin siRNA significantly suppressed the cell viability of b.End.3 cells to 80% compared with that of controls exposed to scrambled siRNA; in combination with VEGFR2 inhibitor SU1498% to 55% compared with that of controls exposed to scrambled siRNA, the effect was additive (Figure 6B). Consistent with the MTT assay, apelin siRNA and the SU1498 assay individually suppressed BrdU incorporation (reduced to 60%) compared with that of controls exposed to scrambled siRNA and additively suppressed BrdU incorporation (reduced to 20%) compared with that of controls exposed to scrambled siRNA (Figure 6C).

**Discussion**

To our knowledge, this is the first experimental study addressing the involvement of endogenous apelin in pathological retinal angiogenesis. In the OIR model, vaso-obliteration of the central retinal vessel during the hyperoxic phase was accompanied by subsequent upregulation of VEGF and Epo during the hypoxic phase. The expression of retinal apelin was dramatically increased during the hypoxic phase, and this increase in the expression of apelin was higher than that of VEGF and similar to that of Epo. APJ in the retinas of OIR model mice was highly expressed in capillary endothelial cells, most of which were proliferative. Although the retinas of WT mice showed a significant increase in capillary density accompanied by growth of abnormal vessels in the hypoxic phase, there was no increase in capillary density and abnormal vessels in the retinas of apelin-KO mice despite upregulation of VEGF and Epo mRNA. Furthermore, apelin siRNA suppressed the proliferation of endothelial cells independent of the VEGF/VEGFR2 signaling pathway. These results strongly suggest that the apelin/APJ system is a prerequisite in pathological retinal angiogenesis.

Many factors, such as insulin-like growth factor-1, leptin, and adiponectin, are associated with pathological retinal angiogenesis because they modulate the expression of VEGF or tumor necrosis factor α signaling in the retina. In the present study, we showed that the expression of tumor necrosis factor α, VEGF, and Epo is enhanced in apelin-KO mice (supplemental Figure IV). Moreover, the expression of...
other pathological angiogenesis-related factors, such as H2AX24 and angiopoietin-1,25 was almost similar in both genotypes (supplemental Figure IV). Altogether, the results of the present study strongly suggest that apelin directly affects endothelial cells through the APJ receptor in pathological retinal angiogenesis, not via VEGF or other factors.

In the apelin/ApJ system, the detailed mechanisms by which the apelin/APJ system leads to pathological exuberant angiogenesis remain to be established. Retinal hypoxia after P12 leads to increased proliferation of endothelial cells and pathological exuberant angiogenesis in OIR model mice.26,27 The apelin/APJ system strongly induces proliferation and migration of endothelial cells via p70 S6 kinase.15,28 APJ was expressed in the spreading capillary plexus in retinal angiogenesis and in the ectopic proliferative cells at the retinal surface, which are probably endothelial cells. Compared with the retinas of WT OIR model mice, the number of vascular nuclei extending into the vitreous from the retinal surface was significantly reduced in apelin-KO OIR model mice. Furthermore, no abnormal vessels were formed during generation of the OIR model in apelin-KO mice and during physiological vascular development accompanied by a “mild” upregulation of apelin in WT mice. Altogether, a “dramatic” upregulation of apelin expression may cause exuberant endothelial cell proliferation and pathological angiogenesis via APJ.

Inhibition of the apelin/APJ system significantly suppressed cell viability and BrdU incorporation of endothelial cells. In addition, apelin siRNA synergistically suppressed cell viability and BrdU incorporation in combination with SU1498 (Figure 6). Furthermore, a recent study15 showed that APJ knockdown also inhibits hypoxia-induced endothelial cell proliferation in the presence or absence of SU1498. These data suggest that the apelin/APJ system induced the proliferation of endothelial cells independent of the VEGF/VEGFR2 signaling pathway during hypoxia-induced angiogenesis.

The most important clinical features of ischemic retinopathy are vitreous hemorrhage and/or tractional retinal detachment due to the development of retinal neovascularization. Recently, several VEGF traps have been tested as a potential therapeutic approach to prevent and cure diabetic microvascular complications.31 VEGF initiates blood-retinal barrier breakdown in early diabetes mellitus and leads to macular edema.32,33 On the other hand, a recent clinical study34 reported that the vitreous concentration of apelin was significantly higher in those with proliferative diabetic retinopathy than in controls and was not associated with VEGF. Taken together with our results, inhibition of the apelin/APJ system may prevent abnormal rapid vessel growth.
not normal vessel growth, and offer new therapeutic opportunities against chronic retinal ischemia, such as combination therapy with VEGF inhibitors.

Acknowledgments
We thank Masanori Yoneyama, PhD, and Kiyokazu Ogita, PhD, for their help with BrdU staining.

Sources of Funding
This study was supported by Grants-in-Aid for Young Scientists (Start-up) 19890247 and (B) 21790098 (Dr Kasai).

Disclosures
None.

References
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Arterioscler Thromb Vasc Biol. 2010;30:2182-2187; originally published online August 12, 2010;
doi: 10.1161/ATVBAHA.110.209775
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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## Supplemental Material

Table I. Primer sets used for real-time PCR

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Supplemental Fig. I. The retinal angiography of the OIR model at P28. Representative pictures show normalized retinal vessels at P28 in both wild-type and apelin-KO mice.
Supplemental Fig. II. Expression of Tie-2 and APJ in retina of OIR model using apelin-KO mice was examined by real-time PCR (n = 4–5). Data are mean ± SEM. **p < 0.01 vs. wild-type mice. †p < 0.05 vs. P7 values for each genotype.
Supplemental Fig. III. Immunohistochemistry for BrdU (green) and APJ (red) in retinal cross-sections at P17. Representative pictures show that the number of BrdU-positive cells above the GCL was fewer in the KO-OIR mice than in the WT-OIR mice.
Supplemental Fig. IV. Expression of TNF-α, angiopoietin-1, and H2AX in retina of OIR model using apelin-KO mice was examined by real-time PCR (n = 4–5). Data are mean ± SEM. †p < 0.05 vs. P7 values for each genotype.
Supplemental Fig. V. Effect of VEGFR2 inhibitor, SU1498, on hypoxic-induced retinal angiogenesis. (A) Representative pictures show P17 retinal angiographies of WT-OIR mice treated with vehicle or SU1498 (9 mg/kg, i.p. at P13 and 15). (B) Capillary density in the retinas of OIR model mice (n = 4 – 6). Data are mean ± SEM.
Supplemental Fig. VI. Effect of antagonistic antibodies to VEGFR1 or 2 on hypoxic-induced retinal angiogenesis. Capillary density in the P17 retinas of WT-OIR model mice treated with control (PBS), anti-VEGFR1 (AP-MAB0702, Angioproteomie), or anti-VEGFR2 (AP-MAB0701, Angioproteomie) antibodies (10 μg/eye, intravitreal injection at P13 and 15). Data are mean ± SEM.