Suppression of Choroidal Neovascularization by Inhibiting Angiotensin-Converting Enzyme: Minimal Role of Bradykinin

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PURPOSE. Angiotensin-converting enzyme (ACE), also known as kininase II, functions not only to convert angiotensin I to angiotensin II, but also to cleave bradykinin into inactive fragments. Thus, ACE inhibition causes the tissue accumulation of bradykinin, exerting either of two opposite effects: anti- or proangiogenic. The purpose of the present study was to investigate the role of bradykinin in the development of choroidal neovascularization (CNV), with or without ACE inhibition.

METHODS. Laser photocoagulation was used to induce CNV in wild-type C57BL/6J mice and angiotensin II type 1 receptor (AT1-R)-deficient mice. Wild-type mice were pretreated with the ACE inhibitor imidapril, with or without the bradykinin B2 receptor (B2-R) antagonist icatibant daily for 6 days before photocoagulation, and the treatment was continued daily until the end of the study. CNV response was analyzed by volumetric measurements using confocal microscopy 1 week after laser injury. The mRNA and protein levels of vascular endothelial growth factor (VEGF), intercellular adhesion molecule (ICAM)-1, and monocyte chemotactic protein (MCP)-1 in the retinal pigment epithelium–choroid complex were examined by RT-PCR and ELISA, respectively.

RESULTS. ACE inhibition led to significant suppression of CNV development to the level seen in AT1-R-deficient mice. B2-R blockade together with high-dose but not low-dose ACE inhibition resulted in more potent suppression of CNV than did ACE inhibition alone. B2-R blockade alone exhibited little or no effect on CNV. VEGF, ICAM-1, and MCP-1 levels, elevated by CNV induction, were significantly suppressed by ACE inhibition. VEGF but not ICAM-1 or MCP-1 levels were further attenuated by B2-R blockade with ACE inhibition.

CONCLUSIONS. These results suggest a limited contribution of the kallikrein-kinin system to the pathogenesis of CNV, in which the renin–angiotensin system plays more essential roles for facilitating angiogenesis. The present study indicates the possibility of ACE inhibition as a novel therapeutic strategy to inhibit CNV. (*Invest Ophthalmol Vis Sci.* 2007;48:2321–2326) DOI:10.1167/iovs.06-1296

Age-related macular degeneration (AMD) is the most common cause of blindness in developed countries.[1] It is complicated by choroidal neovascularization (CNV), leading to severe vision loss and blindness. During CNV, new vessels from the choroid invade the subretinal space through Bruch's membrane, resulting in the formation of fibrovascular tissues containing vascular endothelial cells, retinal pigment epithelial cells, fibroblasts, and macrophages.[2] Bleeding and lipid leakage from the immature vessels in the proliferative tissue cause damage to the retinal functions. Molecular and cellular mechanisms for promoting CNV are not fully elucidated. CNV seen in AMD develops with chronic inflammation adjacent to the retinal pigment epithelium (RPE), Bruch's membrane and choriocapillaris. Inflammatory processes including macrophage infiltration[3–6] and the cytokine network[5–7] are associated with CNV, as well as the pathologic angiogenesis seen in solid tumor. Vascular endothelial growth factor (VEGF), a potent proinflammatory and angiogenic cytokine, has been shown to play a central role in CNV.[5–9] VEGF were expressed in both the rodent model of laser-induced CNV[5] and human CNV tissues surgically removed from patients with AMD.[6] VEGF signaling blockade leads to significant suppression of experimental CNV.[7] In accordance with these experimental results, recent clinical trials revealed that intravitreal administration of VEGF antagonists ameliorated the visual outcome compared with sham injections.[8,9] In addition, CNV tissues from both human surgical samples and the rodent laser-induced model express molecules responsible for macrophage infiltration, including intercellular adhesion molecule (ICAM)-1[10,11] and monocyte chemotactic protein (MCP)-1.[2] Genetic ablation of ICAM-1[11] and CCR-2,[4] a receptor for MCP-1, inhibits CNV in a murine model.

The kallikrein-kinin system (KKS) is implicated in various pathologic and physiologic processes, including inflammation, allergy, blood coagulation, and fibrinolysis, and the lowering of systemic blood pressure due to vessel dilation and diuretic action. Bradykinin, the central molecule of the KKS generated by kallikrein from kininogen, induces inflammation via bradykinin B2 receptor (B2-R; Fig. 1) including vessel dilation and leakage.[12] Recent reports[12–15] have demonstrated bradykinin as a positive regulator for angiogenesis, which is shown to depend on B2-R-induced expression of VEGF.[16,17] To date, however, no data have been shown on the role of the KKS in CNV.

The renin–angiotensin system (RAS) plays an important role in the elevation of systemic blood pressure. Angiotensin II, the final product of the RAS generated by angiotensin-converting enzyme (ACE) from angiotensin I, has two cognate receptors, angiotensin II type 1 receptor (AT1-R) and AT2-R.[18] Since the

FIGURE 1. Scheme showing the RAS and the KKS components contributing to CNV. Note that inhibition of ACE, also known as kininase II, causes the tissue accumulation of bradykinin, exerting two opposite effects of ACE inhibition: the RAS deactivation leading to antiangiogenic action and the KKS activation leading to proangiogenic action.

major pathogenic signaling of angiotensin II is mediated by AT1-R, ACE inhibitors and AT1-R blockers are widely used in patients with hypertension. In addition, various functions of the RAS have been pointed out, including angiogenesis, inflammation, and tumor growth.[19–21] Recently, we demonstrated that AT1-R, but not AT-2R, blockade led to significant suppression of CNV,[22] providing the first biological evidence of the critical role of the RAS in the pathogenesis of CNV (Fig. 1).

Of note, ACE proved to be an identical molecule with kininase II, which degrades bradykinin to inactive fragments,[12] showing the direct interaction between the RAS and the KKS (Fig. 1). ACE inhibitors, which cause the tissue accumulation of bradykinin,[12] have been shown to be proangiogenic by enhancing the KKS.[23–27] In contrast, ACE inhibitors have also been reported to be antiangiogenic by suppressing the RAS,[28–31] suggesting the tissue specificity of the treatment. However, whether the effect of ACE inhibition on CNV is pro- or antiangiogenic has not been investigated. In the present paper, we defined the effect of ACE inhibition in a murine model of laser-induced CNV. Moreover, the current data are the first to show the role of the KKS in CNV with or without ACE inhibition, together with underlying molecular mechanisms.

MATERIALS AND METHODS

Animals

C57BL/6J mice (7–10 weeks old; CLEA, Tokyo, Japan) and AT1-R-deficient mice (based on the C57BL/6J strain and donated by Tanabe Seiyaku Co., Ltd., Osaka, Japan) were used. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of CNV

Laser-induced CNV is widely used as an animal model for neovascular AMD and reflects the pathogenesis of CNV seen in AMD. In this model, new vessels from the choroid invade the subretinal space after photo-

coagulation. Laser photocoagulation was performed around the optic disc at a wavelength of 532 nm, power of 200 mW, duration of 100 ms, and spot size of 75 μm, using a slit lamp delivery system (Novus Spectra; Lumenis, Tokyo, Japan), as described previously.[3]
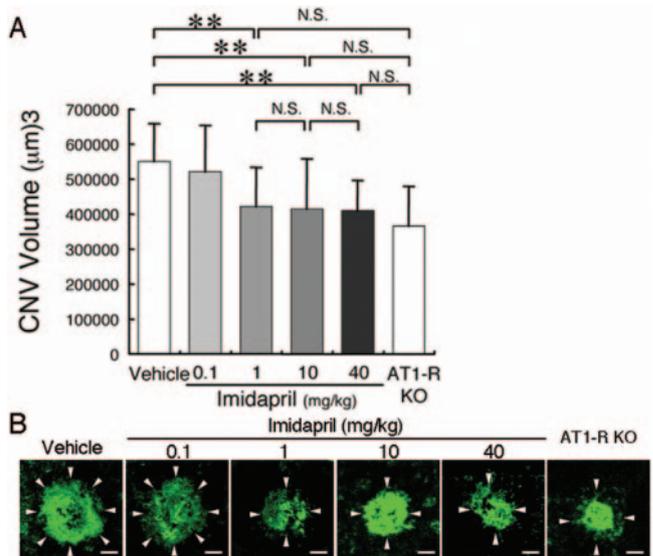
ACE Inhibition

Animals were pretreated with the ACE inhibitor imidapril or phosphate-buffered saline (PBS) daily for 6 days before photocoagulation, and the treatments were continued daily until the end of the study. Imidapril was the kind gift of Tanabe Seiyaku Co., Ltd. Imidapril was orally administered to mice at a dose of 0.1, 1, 10, or 40 mg/kg body weight.
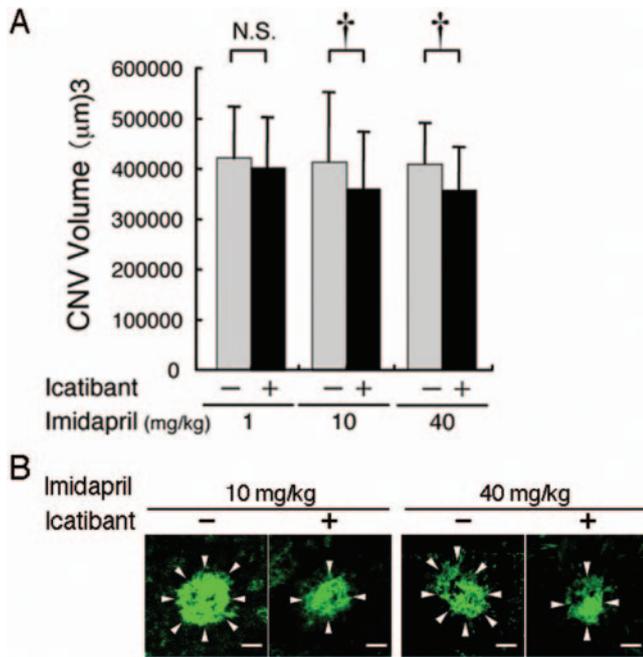
B2-R Blockade

The B2-R antagonist icatibant (Hoe140; Sigma, St. Louis, MO) was intraperitoneally administered at a dose of 0.1 mg/kg body weight, together with a high (10 mg/kg) or low (1 mg/kg) dose of the ACE inhibitor imidapril, and the CNV volume was analyzed. In addition, icatibant alone at a dose of 0.01, 0.1, or 0.5 mg/kg was administered to mice with CNV. Recently, the phase III clinical trial of icatibant, expected to reduce vascular permeability, for the treatment of hereditary angioedema was successfully completed.

Quantification of Laser-Induced CNV

One week after laser injury, the eyes were enucleated and fixed with 4% paraformaldehyde (PFA). Eye cups obtained by removing anterior segments were incubated with 0.5% fluorescein-isothiocyanate (FITC)-isolectin B4 (Vector, Burlingame, CA). CNV was visualized with a blue argon laser (wavelength, 488 nm) on a scanning laser confocal microscope (FV1000; Olympus, Tokyo, Japan). Horizontal optical sections of CNV were obtained every 1-μm step from the surface to the deepest focal plane. The area of CNV-related fluorescence was measured by NIH Image (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). The summation of the whole fluorescent area was used as the volume of CNV, as described previously.[3]

FIGURE 2. Suppression of CNV with ACE inhibition. (**A**) CNV volume. (**B**) Flatmounted choroids from vehicle- and imidapril-treated and AT1-R knockout mice. ACE inhibition with imidapril led to significant suppression of CNV to the level of that in the AT1-R knockout mice. *Arrowheads*: lectin-stained CNV tissues. *n* = 13–15. **P < 0.001. Scale bar, 50 μm.

FIGURE 3. Partial role of B2-R in CNV with ACE inhibition. (**A**) CNV volume. (**B**) Flatmounted choroids from imidapril- and icatibant-treated mice. B2-R blockade with icatibant, together with high-dose ACE inhibition with imidapril, resulted in more potent suppression of CNV. *Arrowheads*: lectin-stained CNV tissues. $n = 13$–15. †$P < 0.05$. Scale bar, 50 μm.

RT-PCR Analyses for *VEGF*, *ICAM-1*, and *MCP-1*

Total RNA was isolated from the RPE-choroid complex 3 days after photocoagulation using an extraction reagent (TRIzol; Invitrogen, Carlsbad, CA) and reverse-transcribed with a cDNA synthesis kit (First-Strand; GE Healthcare, Buckinghamshire, UK). PCR was performed with *Taq* DNA polymerase (Takara Bio, Ohtsu, Japan) in a thermal controller (Gene Amp PCR system; Applied Biosystems, Foster, CA). The primer sequences and the expected size of amplified cDNA fragments are as follows: 5′-GAA GTC CCA TGA AGT GAT CCA G-3′ (sense) and 5′-TCA CCG CCT TGG CTT GTC A-3′ (antisense) (319 bp and 451 bp) for *VEGF120* and *VEGF164*, respectively; 5′-GTG TCG AGC TTT GGG ATG GTA-3′ (sense) and 5′-CTG GGC TTG GAG ACT CAG TG-3′ (antisense) (505 bp) for *ICAM-1*, 5′-CCC CAC TCA CCT GCT GCT ACT-3′ (sense) and 5′-GGC ATC ACA GTC CGA GTC ACA-3′ (antisense) (380 bp) for *MCP-1*; and 5′-ATG TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3′ (sense) and 5′-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3′ (antisense) (837 bp) for *β*-actin.

ELISA for VEGF, ICAM-1, and MCP-1

The RPE-choroid complex was carefully isolated from the eyes 3 days after photocoagulation and placed into 200 μL of lysis buffer supplemented with protease inhibitors and sonicated. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C, and the protein levels of VEGF, ICAM-1, and MCP-1 were determined with the mouse VEGF, ICAM-1, and MCP-1 ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's protocols.

Morphometric and Statistical Analyses

All results are expressed as the mean ± SD. The data were processed for statistical analyses (Mann-Whitney test). Differences were considered statistically significant at $P < 0.05$.

RESULTS

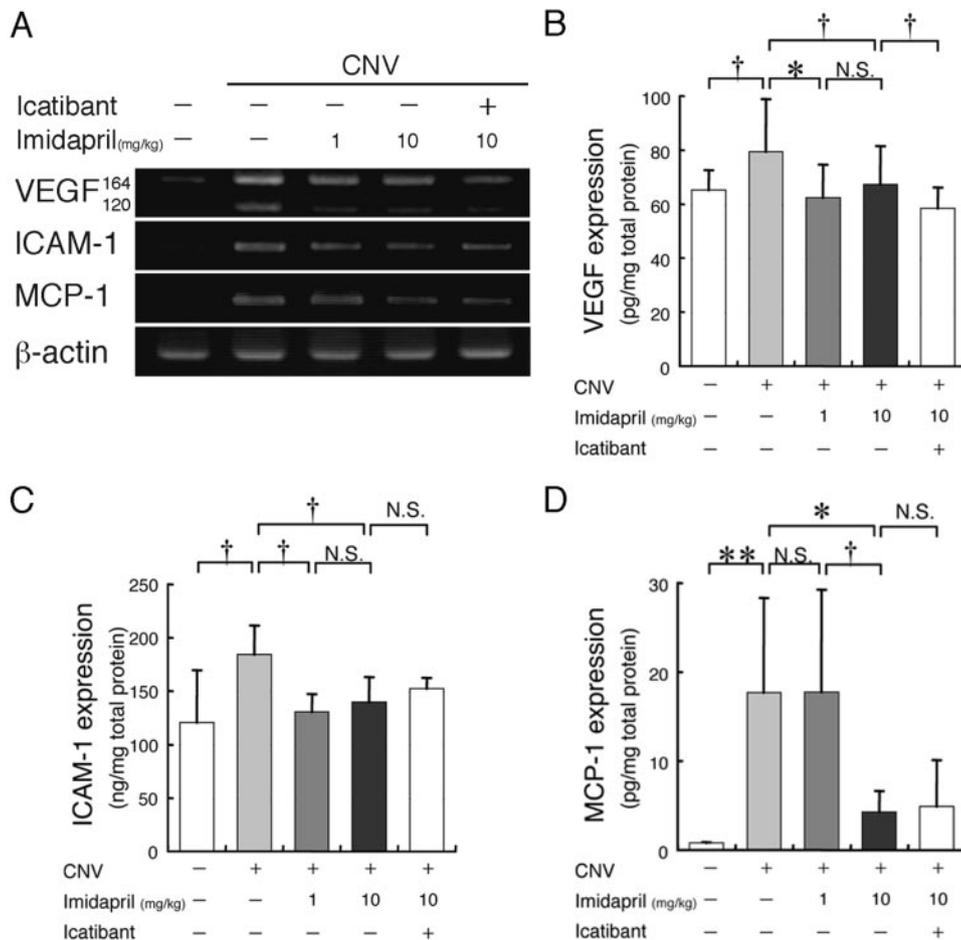### Suppression of CNV with ACE Inhibition

The CNV volume was measured to evaluate the effects of ACE inhibition with imidapril on the development of CNV (Fig. 2). Imidapril-treated mice at a dose of 1, 10, or 40 mg/kg showed a significant ($P < 0.001$) decrease in the CNV volume ($421{,}630 \pm 101{,}857$ μm^3 for 1 mg/kg, $415{,}041 \pm 137{,}207$ μm^3 for 10 mg/kg, and $409{,}669 \pm 87{,}086$ μm^3 for 40 mg/kg), compared with vehicle-treated mice ($550{,}345 \pm 108{,}015$ μm^3). The difference in the CNV volume between any dose (1, 10, or 40 mg/kg) of ACE inhibition and AT1-R deficiency ($389{,}132 \pm 114{,}635$ μm^3) was not statistically significant.

Partial Role of B2-R Signaling in CNV Development with ACE Inhibition

As the ACE inhibitor-induced suppression of CNV was not in a dose-dependent fashion (Fig. 2), we predicted the possible role of the KKS upregulated by ACE inhibition. Mice receiving the high (10, 40 mg/kg body weight) or low (1 mg/kg body weight) dose of imidapril, both of which significantly suppressed CNV (Fig. 2), were simultaneously administered with the B2-R antagonist icatibant (0.1 mg/kg body weight) (Fig. 3). Icatibant treatment significantly suppressed the CNV volume when cotreated with the high, but not low, dose of imidapril. The CNV volume reduced by the high dose of imidapril ($415{,}041 \pm 137{,}207$ μm^3 for 10 mg/kg, $409{,}669 \pm 87{,}086$ μm^3 for 40 mg/kg) was significantly ($P < 0.05$) further attenuated by the simultaneous administration of icatibant ($360{,}936 \pm 112{,}920$ μm^3 for 10 mg/kg and $357{,}958 \pm 97{,}821$ μm^3 for 40 mg/kg).
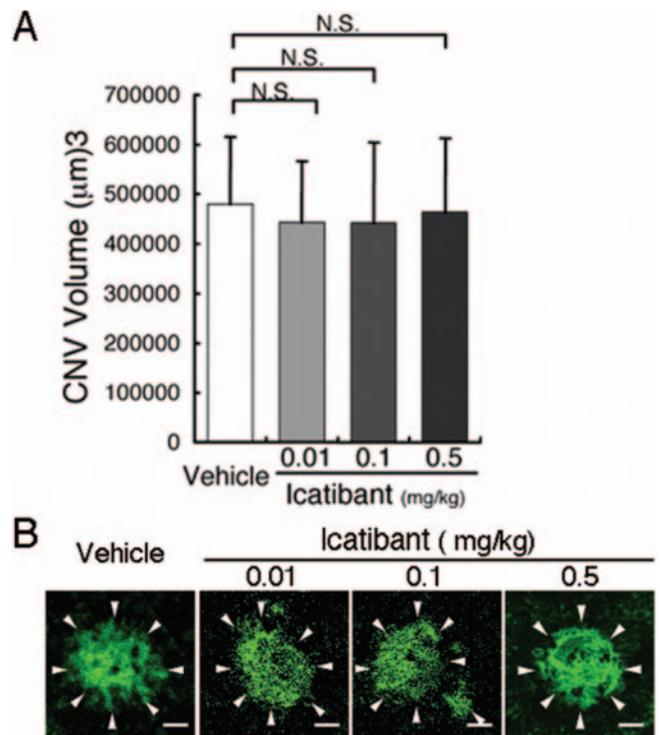
FIGURE 4. Negligible role of B2-R in CNV without ACE inhibition. (**A**) CNV volume. (**B**) Flatmounted choroids from vehicle- and icatibant-treated mice. B2-R blockade with icatibant alone exhibited little or no effect on CNV. *Arrowheads*: lectin-stained CNV tissues. $n = 11$–15. Scale bar, 50 μm.

FIGURE 5. Additively suppressive effect of B2-R blockade with ACE inhibition on VEGF, but not ICAM-1 or MCP-1, expression analyzed by semiquantitative RT-PCR (**A**) and ELISA (**B–D**). The mRNA expression and protein levels of VEGF, ICAM-1, and MCP-1, elevated by inducing CNV, were significantly suppressed by imidapril. VEGF, but not ICAM-1 or MCP-1, protein levels were further attenuated by the simultaneous administration of imidapril and icatibant. $n = 8$–10. *$P < 0.01$, †$P < 0.05$.

Negligible Role of B2-R Signaling in CNV Development without ACE Inhibition

Administration of icatibant alone without ACE inhibition did not significantly change the volume of CNV ($450,685 \pm 120,616$ μm^3 for 0.01 mg/kg, $441,961 \pm 162,050$ μm^3 for 0.1 mg/kg, and $463,653 \pm 148,311$ μm^3 for 0.5 mg/kg, $P > 0.05$), compared with vehicle-treated animals ($479,393 \pm 135,885$ μm^3; Fig. 4).

Additively Suppressive Effect of B2-R Blockade with ACE Inhibition on VEGF, But Not ICAM-1 or MCP-1, Expression

To analyze molecular mechanisms in the partial contribution of B2-R signaling to CNV with ACE inhibition, we analyzed the mRNA and protein levels of CNV-related molecules including VEGF, ICAM-1, and MCP-1 in the RPE-choroid complex by semiquantitative RT-PCR (Fig. 5A) and ELISA (Figs. 5B–D), respectively. The mRNA expression and protein levels of VEGF, ICAM-1, and MCP-1 in the RPE-choroid complex were upregulated by inducing CNV. ACE inhibition with systemic administration of imidapril at the dose of 1 or 10 mg/kg body weight significantly reduced the mRNA expression and protein levels of VEGF, ICAM-1, and MCP-1. The B2-R antagonist icatibant further reduced the mRNA expression and protein levels of VEGF, but not ICAM-1 or MCP-1, in the RPE-choroid complex.

DISCUSSION

The RAS and the KKS, each playing an antagonistic role in the regulation of systemic blood pressure, are recently shown as

positive modulators of angiogenesis. The present study revealed, for the first time to our knowledge, several important findings concerning the dual contribution of the RAS and the KKS to CNV with ACE inhibition. ACE inhibition led to significant suppression of CNV development to the level seen in AT1-R knockouts (Fig. 2). Notably, the ACE inhibitor-induced suppressive effect on CNV was not in a dose-dependent fashion (Fig. 2). B2-R blockade, together with ACE inhibition resulted in more potent suppression of CNV than ACE inhibition alone (Fig. 3). B2-R blockade alone exhibited little or no effect on CNV (Fig. 4), suggesting a limited contribution of the KKS to the pathogenesis of CNV, in which the RAS plays more essential roles for facilitating angiogenesis. As possible molecular mechanisms, B2-R-mediated upregulation of VEGF, but not ICAM-1 or MCP-1, expression blunted the CNV-reducing effect of ACE inhibition which suppressed these CNV-related molecules (Fig. 5).

Inhibition of ACE, also known as kininase II, has proved to be either proangiogenic by activating the KKS or antiangiogenic by deactivating the RAS, depending on the disease models. ACE inhibition was reported to enhance neovascularization after surgically induced hindlimb ischemia in rabbits,[26] spontaneously hypertensive rats,[27] and mice with[25] or without[23] streptozotocin-induced diabetes. The ACE inhibitor-induced enhancement of hindlimb neovascularization was abolished in B2-R-deficient mice.[23,25] ACE inhibitor treatment also increased cardiac capillary density in spontaneously hypertensive rats, which was diminished by B2-R blockade with icatibant.[24] In contrast, ACE inhibition was reported to suppress neovascularization in the rodent model of oxygen-induced ischemic retinopathy.[28–30] In the murine model of hepatocel-

lular carcinoma, ACE inhibitor treatment also reduced tumor development and angiogenesis.[31] In the current model of laser-induced CNV, ACE inhibition with imidapril was found to be antiangiogenic (Fig. 2), consistent with previous reports[28–30,32] showing the antiangiogenic effect of ACE inhibitors on human and rodent ischemic retinopathies. In these ocular disorders treated with ACE inhibitors, the RAS deactivation is likely to overwhelm the KKS activation, leading to antiangiogenic action in the treatment. Indeed, significant suppression of neovascularization in the choroid and the retina via inhibition of the RAS was already confirmed in our recent data from the use of AT1-R blockers.[22,33]

Of note, the ACE inhibitor-induced suppressive effect on CNV was not dose dependent (Fig. 2). This finding led us to hypothesize the possible role of bradykinin accumulated by ACE inhibition in CNV. B2-R blockade with icatibant, together with the high-dose ACE inhibition, resulted in an additively suppressive effect on CNV (Fig. 3). The data are the first to show the KKS as a positive regulator of CNV. Indeed, the major KKS component including kininogen, kallikrein, kininase II (ACE), and B2-R was shown to be present in the choroid and the retina.[34,35] Although ACE inhibition in our present data was antiangiogenic in total, the ACE inhibitor-induced activation of the KKS was suggested to play a proangiogenic role, at least in part, in diluting the suppressive effect on CNV mediated by the RAS deactivation.

Accordingly, our next focus was to examine whether the KKS contributes to CNV without ACE inhibition. The KKS has been reported to function as a positive regulator of angiogenesis.[13–17] In the murine model of hindlimb ischemia, intramuscular delivery of adenovirus containing the human kallikrein gene enhanced the ischemia-induced neovascularization.[15] In the rodent models of sarcoma and carcinoma, pharmacologic blockade of B2-R[17] and genetic depletion of kininogen or B2-R[14] reduced tumor angiogenesis and growth. Surprisingly, our present data demonstrated that B2-R blockade did not affect the development of CNV (Fig. 4). This is compatible with the data showing that the low-dose ACE inhibition failed to induce B2-R-mediated bioactivity (Fig. 3), suggesting a partial role of the KKS in the pathogenesis of CNV. The KKS activation in the development of CNV is probably minimal but is inducible when kininase II bioactivity is potently inhibited by the excessive dose of ACE inhibitors.

The molecular analyses showed that the ACE inhibitor treatment reduced the expression of CNV-related molecules including VEGF, ICAM-1, and MCP-1, whereas the B2-R blocker-induced additive decrease was observed only in VEGF expression (Fig. 5). Recent in vivo and in vitro data using pharmacologic inhibition[17] and genetic ablation[14] of B2-R have suggested that bradykinin induces VEGF via B2-R. Reasonably, B2-R-mediated VEGF expression (Fig. 5) was one of the molecular mechanisms by which the ACE inhibitor-induced activation of the KKS blunted its suppressive effect of CNV (Fig. 3). In contrast, ACE inhibitors have been shown to suppress AT1-R-mediated VEGF expression via reducing angiotensin II production.[36–38] Consistently, the present data on the ACE inhibitor-induced suppression of VEGF (Fig. 5) are supported by our recent report,[22] showing AT1-R-mediated VEGF expression in the current CNV model.

The present data showing that the ACE inhibitor imidapril was antiangiogenic in the treatment of CNV (Fig. 2) suggest that the pathogenesis of CNV is closely associated with the RAS, but minimally with the KKS. In the eye, the KKS was activated only when ACE was potently inhibited with an excessive dose. Recent clinical trials have indicated the advantages of ACE inhibitors for the treatment of hypertension and cardiovascular diseases,[39,40] also known as risk factors predisposing to AMD.[41] Reasonably, ACE inhibition, which not only

reduces neovascularization in the eye but also improves the systemic background, is likely to be a novel therapeutic strategy as a preventive, early, and additive treatment for AMD. A large-scale, prospective and randomized clinical trial is awaited to validate the suppressive effect of ACE inhibition on CNV.

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