β3-Adrenergic Receptors Regulate Retinal Endothelial Cell Migration and Proliferation

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Sympathetic nerves may play a role in vascular disorders of the eye. In the present study, we hypothesized that activation of β3-adrenergic receptors on retinal endothelial cells would promote migration and proliferation of these cells, two markers of an angiogenic phenotype. We show, for the first time, expression of β3-adrenergic receptors on cultured retinal endothelial cells. Activation of these receptors with BRL37344, a specific β3-adrenergic receptor agonist, promoted migration that was blocked by inhibitors of phosphatidylinositol 3-kinase (PI3K), the mitogen activated protein kinase component MEK, and matrix metalloproteinases (MMPs) 2 and 9. BRL37344 stimulated proliferation, which could be blocked by inhibitors of Src, PI3K, and MEK. These cells also express the β1-adrenergic receptor with no β2-adrenergic receptor expression observed. Stimulation of the β3-adrenergic receptor with xamoterol, a specific partial agonist, did not promote proliferation or migration. These results support the hypothesis that β3-adrenergic receptors play a role in proliferation and migration of cultured human retinal endothelial cells.

Diabetic retinopathy is the number one cause of blindness in humans between the ages of 20 and 74 years (1). Age-related macular degeneration is the predominant cause of blindness in the elderly population (2, 3). Although both diseases involve abnormal blood vessel growth in the eye, the mechanisms underlying this growth remain unknown. Vascular endothelial growth factor (VEGF) levels may be increased, and this up-regulation could initiate neovascularization (4). However, it appears that these disorders are much more complicated. Both diseases also have a potential for dysfunction of sympathetic nerve function. Patients with diabetes mellitus or advanced aging show both structural and functional indices of sympathetic neuropathy (5–7) It has been suggested that diabetic neuropathy may contribute to dysfunctional wound healing (8) and diabetic retinopathy (9). Few studies have attempted to determine whether changes in sympathetic innervation may result in vascular complications, such as retinopathy or dysfunctional wound healing.

Ocular angiogenesis in the posterior region likely comes from either choroidal or retinal vascular beds. The current opinion is that age-related macular degeneration results from choroidal neovascularization, which destroys retinal pigmented epithelial cells, and blood vessels that invade the photoreceptor layer (3). Diabetic retinopathy, on the other hand, is thought to result from the abnormal growth of retinal blood vessels (1). Both, however, produce a significant loss of vision or blindness. Although many hypotheses have been put forward to explain the abnormal vascular growth in both of these diseases, a definitive answer is not yet available. Most will agree that both of these diseases result from an imbalance of angiogenic versus angiostatic factors.

While investigating sympathetic nerve regulation of choroidal blood flow, we found that blood flow to the treated eye increased over 4-fold following superior cervical ganglionectomy and was not altered in other cranial structures (9). Sympathectomy also produced significant increases in the number of choroidal venules and arterioles and the retinal capillaries in the outer nuclear layer (9). Additionally, we determined that the β-adrenergic receptor antagonist propranolol (which blocks β1- and β2-adrenergic receptors) produced changes in the choroid similar to those after a sympathectomy (10). Because the β3-adrenergic receptor was thought to be found primarily in white and brown adipose tissues (11), we did not characterize the role of this receptor after sympathectomy.

β-adrenergic receptor subtypes have been located on some types of endothelial cells (12, 13). In the present study, we sought to determine which subtypes of β-adrenergic receptors were present on retinal endothelial cells. We also characterized the signaling pathways associated with the activation of these receptors. Finally, we determined whether activation of β-adrenergic receptors in cultured retinal endothelial cells could mediate proliferation and migration, two markers of an angiogenic phenotype.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human retinal endothelial cells were purchased from Cell Systems (Kirkland, WA). Cells were used at passages 3–6 for all experiments. Cells were grown in attachment factor-coated dishes and maintained in serum-free medium (Cell Systems) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. After the cells reached 80–90% confluence, they were passaged with the use of a passage reagent group (Cell Systems). Starvation medium contained all of the above ingredients except that 0.1% bovine serum albumin was substituted for 10% serum. Human microvascular endothelial cells were isolated by enzymatic digestion of blood vessels taken from the mesentery of the small bowel and cloned by limiting dilution (designated MM1 cells). Endothelial cell identity was verified by positive staining for a factor VIII-related antigen.

**Pharmacological Activators and Inhibitors**—Stimulation of the β3-adrenergic receptor was achieved by application of the specific receptor...
agonist BRL37344 (10 μM). Xamoterol (10 μM) was used as a specific β₂-adrenergic receptor agonist. Both activators were obtained from Toecis (Ellisville, MO).

Protein kinases A and G were blocked with KT5720 (1 μM) and KT5823 (1 μM). Src was blocked by PP2 (1 μM), and the inactive isoform PP3 (1 μM) served as a negative control. Wortmannin (100 nM) or LY294002 (2 μM) were used to inhibit phosphatidylinositol-3-kinase activity; the inactive isoform LY294011 (100 μM) served as a negative control. The phosphatidylinositol ether analog IL-6-hydroxymethylchiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (10 μM) was blocked as Akt-I in the remainder of the text, was used as a potent and selective inhibitor of Akt (protein kinase B). The MAP kinase pathway was blocked using the specific MEK inhibitor PD98059 (10 μM). Matrix metalloproteinases (MMPs) 2 and 9 were blocked with MMP-2/9 inhibitor III (1 μM), abbreviated as MMP-I in the remainder of the text. All inhibitors were obtained from Calbiochem, except wortmannin (Toecis, Ellisville, MO).

β₂-Adrenergic Receptor Protein Expression—Western blotting was conducted to determine which β₂-adrenergic receptor subtypes were present on retinal endothelial cells (REC; passage 2–6), choroidal endothelial cells (ChEC; passage 10–15), Cells in 60-mm dishes were lysed (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin, leupeptin, and pepstatin and 1 mM Na₃VO₄, 1 mM NaF, and 0.1% SDS), and 50 μg of protein was separated on a 4–12% pre-cast polyacrylamide gel (Invitrogen), blotted onto a nitrocellulose membrane, and blocked with Super Block™ (Pierce) for 1 h at room temperature. Primary antibodies to the β₂-AR (A-20; 5 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA), β₁-AR (H-73; 5 μg/ml, Santa Cruz Biotechnology), or β₂-adrenergic receptor (C-20; 5 μg/ml; Santa Cruz Biotechnology) were applied overnight at 4 °C. Membranes were probed with horseradish peroxidase-conjugated anti-rabbit secondary antibodies applied at a 1:10,000 dilution for 2 h at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence (LumiGlo, Cell Signaling, Beverly, MA) using Kodak BioMax ML film and scanned into the computer using reflectance scanning.

Western blots were used to evaluate the phosphorylation states of MAPK (phospho-p42/44, 1:1000, ERK1/2, Cell Signaling), Src (phospho-Src-416, 1:1000, Cell Signaling), or Akt (phospho-Akt, Ser473, 1:1000, BIO SOURCE) were done as described above following stimulation of the β₂-adrenergic receptor.

Radioligand Binding Assays—β₂-adrenergic subtype levels were quantified on intact human retinal endothelial cells by modification of previously described methods (14, 15) using the hydrophilic β₂-adrenergic receptor ligand (−)[5,7,3H]CGP 12177 (33 Ci/mmol; PerkinElmer Life Sciences). To prevent receptor internalization, binding studies were performed at 4 °C. Cells were grown in 12-well plates and serum-starved for 24 h. For determination of saturation binding, cells were incubated in 0.3 ml of serum-free medium containing 1% bovine serum albumin, 25 mM HEPES (pH 7.4 at 4 °C), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B and increasing amounts of [3H]CGP 12177 per well. To assess displacement of [3H]CGP 12177 binding by the β₂-adrenergic receptor agonist BRL37344 and the β₂-receptor antagonist propranolol, cells were incubated with 25 nM [3H]CGP 12177. Three wells of cells were used per condition, and all binding assays were performed three times. Binding was carried out for 6 h, as preliminary studies established that equilibrium binding was reached at that time. Afterward, the binding medium was aspirated, and the cells were washed twice with 0.5 ml of ice-cold Hank's balanced salt solution for 1.5 h and then read on a fluorescent plate reader (Bio-Tek, Winooski, VT, Model FL600, gain of 100) at 485/530 nm. Migration of cells was expressed as a percentage of control (those receiving only starvation medium) after background fluorescence was subtracted. Data were analyzed using Prism software (GraphPad, San Diego, CA) and presented with significance at p < 0.05.

Proliferation Assay—Endothelial cell proliferation was assessed using an assay based on the cleavage of the tetrazolium salt WST-1 to formazan. The number of viable cells results in an increase in the overall activity of the mitochondrial dehydrogenases in the sample. The augmentation in enzyme activity leads to an increase in the formazan dye formed. The formazan dye produced by viable cells can be quantified by a multwell spectrophotometer by measuring the absorbance of the dye solution at 440 nm.

To perform the experiments, an aliquot of 50,000 retinal endothelial cells was added to each well of a 96-well tray in medium with 10% fetal bovine serum. After cells attached to the 96-well tray, the cells were washed, and high serum medium was replaced with starvation medium overnight. All wells were rinsed with phosphate-buffered saline. Negative control wells received starvation medium, and positive control wells received agonist only. Inhibitors were added 30 min prior to application of an agonist to allow for complete blockade. Controls treated with agonist alone were also included to determine their effect on proliferation. Cells were allowed to incubate for 48 h. At this time, the WST-1 reagent, dissolved in Electro Coupling Solution (Chemicon), was added for 4 h to measure cell proliferation. The plates were read on a spectrophotometer, and data were presented as a percentage of negative control proliferation with p < 0.05 being significant.

RESULTS

β₂-Adrenergic Receptor Expression in Endothelial Cells—The β₂-adrenergic receptor protein was detected in lysates of human retinal endothelial cells but not in human microvascular mesenteric endothelial cells (Fig. 1A). No protein expression of β₂-adrenergic receptor protein was observed in retinal endothelial cells, whereas choroidal endothelial cells had β₂-adre-
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Fig. 2. A, specific binding of [3H]CGP 12177 to human retinal endothelial cells as a function of ligand concentration. The results shown are representative of three experiments and are expressed as a fentomole of ligand bound per microgram of total cell protein. B, Scatchard analysis of the data of a representative experiment.

nergic receptor expression (Fig. 1B). The β3-adrenergic receptor protein was noted in four different human retinal endothelial cell lysates (Fig. 1C).

Binding of [3H]CGP 12177 to Human Retinal Endothelial Cells—Saturation radioligand binding studies were performed to quantify the levels of β3-adrenergic receptor subtypes on human retinal endothelial cells. Fig. 2A shows the specific binding of the hydrophilic β-adrenergic receptor ligand [3H]CGP 12177 to human retinal endothelial cells. Analysis of the saturation binding results revealed the presence of two populations of binding sites with high and low affinity for [3H]CGP 12177. The Kd of the high affinity site was determined to be 0.06 ± 0.02 nM (n = 3), and the Kd of the low affinity site was 25 ± 4 nM (n = 3). The presence of two populations of [3H]CGP 12177 binding sites of high and low affinity was confirmed by Scatchard analysis (Fig. 2B). The total number (Bmax) of high and low affinity binding sites was determined to be 0.03 ± 0.003 and 0.07 ± 0.023 fmol/μg protein, respectively. Based on comparable Kd values reported by others (15), the high and low affinity sites would represent binding of [3H]CGP 12177 to β1- and β3-adrenergic receptors, respectively. Using a concentration of [3H]CGP 12177 equal to the Kd of the low affinity binding site (25 nM), the β3-adrenergic receptor agonist BRL37344 at 10 μM displaced 37.0 ± 5.3% of specific radioligand binding. The remainder of the specific binding was displacable with the β1-adrenergic antagonist, propranolol.

Stimulation of β3-Adrenergic Receptors with BRL37344 Phosphorylates Several Known Signaling Molecules—Administration of BRL37344 produced significant phosphorylation of Akt versus non-treated retinal endothelial cells (Fig. 3, A and B). Similarly, activation of β3-adrenergic receptors also increased phosphorylation of both ERK1/2 (Fig. 3, C and D) and Src (Fig. 3, E and F; *, p < 0.05 versus non-treated controls). β3-Adrenergic Receptor Stimulation Increases Retinal Endothelial Cell Migration—Activation of the β3-receptor produced a 43 ± 7% increase in retinal endothelial cell migration (p < 0.001, Fig. 4A). This could be attenuated by inhibition of phosphatidylinositol 3-kinase (PI3K) with either LY294002 (p < 0.001) or wortmannin (p < 0.001), whereas the non-active isoform LY303511 had no effect. Retinal endothelial cell migration elicited by β3-adrenergic receptor activation was also diminished by inhibition of Akt (p < 0.05), MMP2/MMP9 (p < 0.001), or MEK (p < 0.01). Migration induced by BRL3744 was not diminished in the presence of blockers of protein kinase A, protein kinase G, or Src. With the exception of MMP-I, the inhibitors alone had no significant effect on migration (Fig. 4B). Treatment of retinal endothelial cells with the β3-adrenergic agonist xamoterol did not produce a significant increase in migration over control values.

β3-Adrenergic Receptor But Not β1-Receptor Activation Promotes Retinal Endothelial Cell Proliferation—Stimulation of β3-adrenergic receptors on retinal endothelial cells with BRL37344 produced a 28 ± 4% increase in proliferation (p < 0.001, Fig. 5A). This proliferation could be completely blocked by inhibitors of Src (p < 0.01), PI3K (LY294002, p < 0.01), wortmannin, p < 0.001), or MEK (p < 0.01). The administration of Akt-I, KT5720, or KT5823 did not abolish the BRL37344-stimulated retinal endothelial cell proliferation. No pharmacological agents altered proliferation when given in the absence of BRL3744 (Fig. 5B). Stimulation of β3-adrenergic receptors with xamoterol did not significantly affect retinal endothelial cell proliferation (Fig. 5C).

DISCUSSION

β3-Adrenergic Receptors in Retinal Endothelial Cells—β3-adrenergic receptors have been reported to be located primarily in thermogenic or fat tissues. Because we had previous data to support a role for β-adrenergic control of ocular blood vessel growth (10), we wanted to determine which β-adrenergic subtypes were present in cultured retinal endothelial cells. In this
study, we demonstrate protein expression for $\beta_3$-adrenergic receptors in human retinal endothelial cells (Fig. 1). Protein expression for the $\beta_1$-adrenergic receptor was observed; however, no expression of $\beta_2$-adrenergic receptor by Western blotting was noted. The observation of both $\beta_1$- and $\beta_3$-adrenergic receptors on retinal endothelial cells was supported by radioligand binding studies (Fig. 2). These results led us to question whether activation of $\beta_2$- or $\beta_1$-receptors would mediate retinal endothelial cell migration and proliferation.

$\beta_3$-Adrenergic Receptors in Retinal Endothelial Cell Proliferation—Others have noted that $\beta_3$-adrenergic receptors linked to G-coupled proteins promote phosphorylation of Src and the subsequent activation of ERK1/2 of the MAPK pathway (18) in adipose tissue. It was assumed that this activation was responsible for lipolysis and thermoregulation. Activation of these receptors in retinal endothelial cells by BRL37344 promoted a similar response (Fig. 5A), which was comparable with that noted in response to Eph B4 stimulation. Since LY294002 and wortmannin prevented retinal endothelial cell proliferation, it is clear that PI3K is involved. LY294002 and wortmannin prevented cell proliferation, it is clear that PI3K is involved. It has been reported that Src can phosphorylate PI3K in addition to activating ERK1/2 (19) in receptors linked to G-proteins. Therefore, this may be the pathway for ERK1/2 regulation of retinal endothelial cell proliferation after the administration of BRL37344. Although ERK1/2 can be stimulated via nitric oxide production and protein kinase G activation after Akt phosphorylation, we observed that activation of ERK1/2 was not mediated by Akt activation.

Fig. 4. A, migration assay for the effects of BRL37344 alone or with various inhibitors given 30 min prior to BRL37344. B, migration assay for the effects of the various inhibitors in the absence of BRL37344. C, migration assay for the effects of xamoterol (10 $\mu$m, $\beta_1$-adrenergic receptor agonist) alone or with the same inhibitors as those in Fig. 3A. *, $p < 0.05$ versus control; #, $p < 0.05$ versus BRL37344 or xamoterol treatment, $n = 4$ for each treatment.
This is not likely in these retinal endothelial cells, because neither the Akt-inhibitor or KT5823 blocked proliferation. Furthermore, the protein kinase A inhibitor KT5720 did not affect proliferation, suggesting that cAMP is not involved in retinal endothelial cell proliferation following β3-adrenergic receptor stimulation. Stimulation of cultured retinal endothelial cells with xamoterol (β3-adrenergic receptor agonist, 10 µM) given 30 min after various inhibitors used in Fig. 4, A and B. * p < 0.05 versus control; #, p < 0.05 versus BRL37344 or xamoterol treatment alone, n = 4–6 for each treatment.

β3-Adrenergic Receptor-mediated Migration of Retinal Endothelial Cells—Relative to proliferation, much less has been demonstrated for the role of G-protein-coupled receptors in cell migration. Using BRL37344 to stimulate the β3-adrenergic receptors on retinal endothelial cells, we showed that these receptors could regulate migration. Stimulation increased migration through a Matrigel-coated membrane by >37%. The increased migration in response to BRL37344 is similar to that noted after Eph B4 treatment of this same cell type.3 The BRL37344-mediated migration could be antagonized by prior administration of inhibitors of PI3K, Akt, MEK, and MMP-2/MMP-9. PI3K appears to be directly linked to the β3-adrenergic receptor. Similar signaling designs have been reported previously in models of heart disease and β-adrenergic receptors (21). β-adrenergic receptors may exhibit kinase activity and are designated as βARK. In this situation, βARK recruits PI3K to the plasma membrane of the myocardium and leads to its phosphorylation (21). PI3K is then able to phosphorylate Akt and lead to the induction of matrix metalloproteinase 2 and 9 (20). Additionally, Akt can activate ERK1/2 of the MAPK pathway, as occurs in retinal endothelial cells stimulated with Eph B4.2 The Src inhibitor PP2 did not alter retinal endothelial cell migration. Unlike what was observed with proliferation, G-protein-coupled phosphorylation of Src does not initiate the signaling for migration. As was noted in retinal endothelial cell proliferation, activation of the β3-adrenergic receptors does not appear to modulate cell migration. Thus, it appears that β3-adrenergic receptors, not β1-adrenergic receptors, are involved in retinal endothelial cell migration via PI3K, Akt, MMP 2 or 9, and ERK1/2.

The results from these studies demonstrate for the first time that β3-adrenergic receptors exist on human retinal endothelial cells. Alterations in sympathetic nerve activity may contribute...
to vascular complications of diabetes. It may be that diabetic retinopathy results from changes in $\beta_3$-adrenergic receptor signaling. If these in vitro studies on cultured cells can be extrapolated to the intact retinal circulation, then the signaling of $\beta_3$-adrenergic receptors may be capable of initiating retinal endothelial cell proliferation and migration, both of which are critical stages of angiogenesis.

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