Characterization of Endothelin A (ET<sub>A</sub>) and Endothelin B (ET<sub>B</sub>) Receptors in Cultured Bovine Retinal Pericytes

D. M. McDonald, J. R. Bailie, D. B. Archer, and U. Chakravarthy

**Purpose.** The endothelins are a family of structurally similar vasoactive peptides. It has been shown recently that cultured retinal microvascular endothelial cells secrete endothelin-1 (ET-1) and that corresponding pericytes bear receptors and are responsive to this peptide. These findings suggest a role for ET-1 in the autoregulation of retinal blood flow. There are at least two known subtypes of ET receptors, ET<sub>A</sub> and ET<sub>B</sub>. The purpose of this study was to characterize endothelin receptor subtypes on cultured bovine retinal pericytes (BRP).

**Methods.** To characterize the specific binding sites for ET-1 and ET-3 on monolayers of BRP, a radioligand binding assay was performed using [125I] ET-1 and [125I] ET-3. Competition binding studies with ET-1 and ET-3 were used to assess the heterogeneity of the ET-receptor population on BRP. Also, [125I] ET-1 and ET-3 were covalently linked to their corresponding receptors and analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography.

**Results.** [125I] ET-1 and [125I] ET-3 showed specific binding to BRP and subsequent Scatchard analysis for both labels showed upward concavity, implying two-site ligand binding. Unlabeled ET-1 was found to displace [125I] ET-1 with greater efficiency than ET-3, indicating the presence of the ET<sub>A</sub> receptor subtype. Conversely, [125I] ET-3 was displaced by ET-1 and ET-3 with equal potency, indicating a component of ET<sub>B</sub> in the receptor population. Preincubation with BQ123, an ETA selective antagonist, decreased the binding of [125I] ET-1 but had no effect on [125I] ET-3 binding curves. Affinity cross-linking of the receptors showed two distinct protein bands on SDS-PAGE of 66 and 45 kd, corresponding to ET<sub>A</sub> and ET<sub>B</sub>.

**Conclusions.** These results show that BRP possess ET<sub>A</sub> and ET<sub>B</sub> receptor subtypes. The function of ET<sub>B</sub> on BRP may be to modulate the vasoconstrictive effect of ET-1 caused through ET<sub>A</sub>. Invest Ophthalmol Vis Sci. 1995;36:1088-1094.

Endothelin-1 (ET-1) belongs to a family of structurally similar bicyclic peptides (ET-1, ET-2, and ET-3) and is the most potent vasoconstrictor yet discovered, with a potency 10 times that of angiotensin II. Receptors for ET-1 are found in the vascular smooth muscle of tissues including brain, heart, aorta, eye, liver, and kidney, indicating a key role for ET-1 in the control of vascular tone throughout the body.

Pharmacologic and ligand-binding studies of ET-induced responses in a variety of cells indicate the presence of two types of ET receptors, designated ET<sub>A</sub> and ET<sub>B</sub>, a finding confirmed by cDNA cloning. ET<sub>A</sub> receptors are highly specific for ET-1, mediate vasoconstriction and cellular proliferation, and are the predominant receptor type on vascular smooth muscle cells. ET<sub>B</sub> is a nonselective subtype that binds ET-1, ET-2, and ET-3 with equal affinity. ET<sub>B</sub> receptors are found on endothelial cells and are thought to mediate vasodilation through the generation of endothelium-derived relaxing factor (nitric oxide) and prostacyclin.

The retinal microvasculature is devoid of extrinsic innervation and is therefore dependent on, as yet, poorly defined autoregulatory mechanisms for the maintenance of vascular tone and control of blood flow. It is likely that autoregulation in the retinal vasculature is mediated by the action of endothelium-derived vasoactive agents on local smooth muscle or...
cyte cells. Recently, it was shown that cultured retinal microvascular endothelial cells secrete the vasoconstrictor, ET-1, and that corresponding pericytes bear receptors to this peptide, suggesting a role for ET-1 in the regulation of capillary blood flow. However, an in vivo study by Butryn et al. showed that ET-1, though constricting retinal arteries and arterioles, did not exert such an effect on the capillary bed, suggesting heterogeneity of response. Different classes of endothelin receptors have been documented in the renal mesangial cell, which is functionally classified as a pericyte. In this study, Martin et al. showed that mesangial cells possess both ET_A and the ET_B receptor; therefore, the possibility that retinal pericytes express ET_A and ET_B receptors was considered.

MATERIALS AND METHODS

Cell Culture
Bovine retinal capillary pericytes (BRP) were isolated and cultured by the method of Gitlin and D'Amore. Bovine eyes were obtained after death from a local abattoir. The pericytes were maintained in Dulbecco's modified eagle's medium (DMEM, Gibco; Paisley, UK) supplemented with 20% fetal calf serum (Gibco), antibiotics, and fungizone, and they were subcultured for up to five passages. The phenotype of these cells was confirmed by immunocytochemical staining for muscle actins, which was always >95%, and the absence of endothelial cell contamination was shown by the absence of staining for anti-factor VIII-related antigen. In addition, the absence of glial cells was confirmed by glial fibrillary acidic protein staining.

Iodination of ET-1 and ET-3

Radiolabeled ET-1 and ET-3 were prepared by incubating approximately 10 ml of peptide with Iodogen (Pierce & Warriner, Chester, UK) and Na\(^{125}\)I (Amersham; Buckinghamshire, UK) for 15 minutes at room temperature in 50 mMmol phosphate buffer, pH 7.2. The reaction mixture was loaded onto a Waters C18 Bondapak column (Millipore, Milford, MA) and purified by reverse-phase high-pressure liquid chromatography using a linear gradient of acetonitrile 0% to 50% (vol/vol) in water developed over 50 minutes at a flow rate of 1 ml/minute. Suitable fractions were collected and stored at -20°C. The specific activity of the stored aliquots was estimated to be 2000 Ci/mmole for \(^{125}\)I ET-1 and 1780 Ci/mmole for \(^{125}\)I ET-3 using the method of Morris.

Receptor Binding Assays

Pericytes from passages 2 and 3 were used for all experiments. Cells were grown in DMEM supplemented with 20% fetal calf serum and plated at a seeding density of 1.5 \times 10^5 cells/well in 24-well tissue culture plates. After 3 days, monolayers were serum starved for 48 hours in basal DMEM containing antibiotics, with a daily change of media. Cells were washed twice with binding buffer (Hanks' balanced salt solution containing 11 mM glucose, 5 mM Hepes [pH 7.4], and 0.1% bovine serum albumin) and incubated at 4°C for 16 hours in the same buffer containing \(^{125}\)I ET-1 or \(^{125}\)I ET-3 (40 to 80 pM) with appropriate dilutions of competing cold peptide (ET-1 or ET-3; Cambridge Research Biochemicals, Cambridge, UK) in a final volume of 250 ml. To inhibit selectively the ET_A receptor, cultures were preincubated with BQ123 (10^{-7} M; Neosystem) for 2 hours at 4°C. At the end of the desired incubation time, any free ligand was removed by washing the intact attached cells three times with ice-cold phosphate-buffered saline (PBS). Cells were then solubilized with 0.1 N NaOH and transferred to tubes for counting on an NE 1600 gamma counter (Nuclear Enterprises, Edinburgh, Scotland). Nonspecific binding was defined as cell-associated radioactivity in the presence of saturating concentrations of ET-1 (10^{-7} M) or ET-3 (10^{-7} M) and was always <5% of total binding. Specific binding was calculated as total binding minus nonspecific binding.

To determine the specific binding of ET-1 and ET-3 to BRP, cells were incubated with increasing concentrations of \(^{125}\)I ET-1 or \(^{125}\)I ET-3 following the protocol described. Specific binding was determined as total minus nonspecific binding, at each of the concentrations used, in the presence of 10^{-7} M unlabeled ET-1 or ET-3. Results were analyzed using the method of Scatchard to calculate K_d and B_{max}.

Covalent Labeling of Endothelin Receptors

Confluent monolayers of BRP in 25-cm² tissue culture flasks were washed twice with ice-cold PBS before the addition of binding buffer (as described) containing \(^{125}\)I ET-1 or \(^{125}\)I ET-3 (80 pM) in the presence and absence of 10^{-7} M unlabeled ET-1 or ET-3. After incubation at 4°C for 4 hours, the attached cells were washed four times with ice-cold PBS, and the bound ligand was covalently cross-linked to the receptor with disuccinimidyl suberate (DSS, 2.5 mM; Pierce & Warriner, Chester, UK) for 1 hour at 4°C. The reaction was stopped by the addition of an equal volume of ice-cold Tris HCl (1 M) ethylenediamine tetraacetic acid (400 mM), pH 6.8. The cells were scraped using a rubber policeman and centrifuged at 1600 rpm for 10 minutes. After two washes in PBS, the pellet was resuspended in 100 ml of sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris, 5% SDS, 5% glycerol, 0.01% bromophenol blue, 2% b-mercaptoethanol, 5 mM ethylenedi-
amine tetraacetic acid, pH 6.8), which resulted in cellular lysis.

**SDS–Polyacrylamide Gel Electrophoresis**

The lysed cell samples were boiled for 5 minutes, centrifuged at 13,000 rpm for 10 minutes to remove particulate matter, and electrophoresed by tricine SDS–polyacrylamide gel electrophoresis. The gels were stained using Coomassie brilliant blue (0.1%). Autoradiography was performed by exposing the dried gel to a Kodak X-OMAT film (Eastman Kodak; Rochester, NY) with an intensifying screen at —70°C for 2 to 4 weeks.

**RESULTS**

To characterize the specific binding sites for ET-1 and ET-3 in cultured BRP, radioligand binding assays were performed using [125I] labeled ET-1 and ET-3. Specific binding for ET-1 was >90% of total binding at any concentration of labeled ET-1 tested and was a saturable process. Scatchard analysis of the data did not yield a linear relationship but showed upward concavity (Fig. 1A), suggesting the presence of two classes of binding site. Apparent dissociation constant (Kd) for each class of binding site was calculated. Binding of [125I] ET-1 revealed one class of binding site with a Kd of 6.25 x 10⁻¹⁰ M and 97,860 binding sites/cell and another with a Kd of 1.56 x 10⁻⁹ M and 28,605 binding sites/cell (Fig. 1A), possibly corresponding to the ETA and ETB receptors, respectively.

Previous studies have shown that the ETA receptor subtype binds ET-1 and ET-2 with approximately equal affinity and ET-3 with much less affinity, whereas the ETB subtype binds ET-1, ET-2, and ET-3 with relatively equal affinity. To characterize further the type of ET receptor present in BRP, competition studies were performed using ET-1 and ET-3 in the presence and absence of the ETA specific antagonist, BQ123. ET-1 inhibited the binding of [125I] ET-1 to intact BRP with an IC₅₀ value of 1.35 nM (Fig. 2, Table 1). By contrast, ET-3 failed to compete for [125I] ET-1 binding except at concentrations in the micromolar range, indicating the presence of the ETA receptor subtype.

[125I] ET-1 binds to ETA and ETB receptors, whereas [125I] ET-3 binds predominantly to ETB receptors with extremely low affinity for the ETA subtype. Therefore, to determine if ETA receptors are present in BRP, receptor binding studies using [125I] ET-3 were performed. Binding of ET-3 was a saturable process with specific binding > 90% of total binding. Scatchard analysis of ET-3 binding showed a biphasic pattern (Kd values of 4.37 x 10⁻¹¹ M and 4.09 x 10⁻¹⁰ M, corresponding to 9033 (ETA) and 36,130 (ETB) binding sites/cell, respectively [Fig. 1B]), suggesting two separate receptor populations. To confirm that these binding sites correspond to ETA and ETB, competition binding assays were performed using labeled ET-3 competing with cold ET-1 and ET-3. As shown in Figure 3, the binding of [125I] ET-3 to BRP. Total amount of ligand bound was considerably lower than for ET-1. As with ET-1, the upward concavity suggested by the Scatchard plot was borne out by the fitting of two independent lines to the data, confirming the presence of two receptor populations.

BQ123 is an ETA-selective antagonist and caused an 85% reduction in the binding of [125I] ET-1 to BRP.

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>ETA (fmol)</th>
<th>ETB (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[125I] ET-1</td>
<td>9033</td>
<td>36,130</td>
</tr>
<tr>
<td>[125I] ET-3</td>
<td>9033</td>
<td>36,130</td>
</tr>
</tbody>
</table>

---

**FIGURE 1.** (A) Scatchard plot showing specific binding of [125I] endothelin (ET)-1 to bovine retinal pericytes (BRP). Cells in duplicate wells were incubated with increasing concentrations of labeled ET-1 for 16 hours at 4°C, and cell-bound radioactivity was determined. Nonspecific binding was defined as the amount of label bound in the presence of an excess (10⁻⁷ M) of unlabeled peptide. The plot shows upward concavity, indicating heterogeneous receptor populations. The best fit line by linear least square fitting was extrapolated to determine Kd. The equation and Kd are given for each best fit line. (B) Scatchard plot showing specific binding of [125I] ET-3 to BRP. Total amount of ligand bound was considerably lower than for ET-1. As with ET-1, the upward concavity suggested by the Scatchard plot was borne out by the fitting of two independent lines to the data, confirming the presence of two receptor populations.
Endothelin Receptors in Retinal Pericytes

FIGURE 2. Competition inhibition of [I25I] ET-1 binding by unlabeled ET-1, ET-3, and ET-1 + BQ123. Inhibition (B/Bo) is expressed as the ratio of radioactivity bound in the presence of competitor to that bound in its absence. Each point is the mean of duplicate treatments, and the graph is typical of at least three independent experiments. -E7 = ET-1; -0- = ET-1 + BQ123; - - = ET-3.

The remaining 15% could have represented residual binding of ET-1 to ET A receptors. The binding of [I25I] ET-3, however, was unaffected by preincubation with BQ123 (Fig. 3), providing further evidence for the presence of ETA receptors on retinal pericytes.

Affinity cross-linking studies of [I25I] ET-1 in retinal pericytes showed two labeled bands with apparent molecular masses of approximately 66 kd and 45 kd (Fig. 4). The 66-kd and 45-kd bands reflect binding of labeled ET-1 to ETA and ETB receptors, respectively. Because the ETA receptor is a high-affinity receptor for ET-1, incubation with [I25I] ET-1 in the presence of an excess of cold ET-3 only inhibited labeling of the lower (ETB) band, whereas an excess cold ET-1 inhibited both bands. Furthermore, when affinity cross-linking studies were performed with [I25I] ET-3, although both bands labeled, they were both inhibited by an excess of cold ET-3 and cold ET-1. These findings were similar under reducing and nonreducing conditions (data not shown).

DISCUSSION

Previous studies published by our laboratory have shown that at a concentration of 0.1 nM, ET-1 is capable of contracting bovine retinal pericytes in vitro.12 Recently, Ramachandran et al10 also reported an ET-1-mediated rise in free intracellular Ca2+ levels in BRP, indicating the presence of functional ET receptors on BRP. The present study provides evidence for the existence of the two ET-receptor subtypes, ETA and ETB, on BRP, with ETA predominating. ETA receptors bind ET-1 with a higher affinity than ET-3. ET-1 caused 50% displacement of labeled ET-1 at a concentration of 1.5 nM (IC50 value), whereas ET-3 was only capable of displacing [I25I] ET-1 at concentrations in the micromolar range. These results are similar to those of Takahashi et al,11 who reported IC50 values for ET-1 and ET-3 at 0.56 nM and >1 μM, respectively. Takahashi’s findings, though providing evidence for the presence of ETA receptors on BRP, did not examine binding of labeled ET-3 to retinal pericytes. Because [I25I] labeled ET-1 has a similar affinity for each of the two receptor subtypes, without the use of competition studies with [I25I] ET-3, it is difficult to detect heterogeneity within the ET-receptor population as reported here.

The present study has shown that BRP also expresses ETB receptors that bind both peptides with equal affinity. In this regard, it is notable that ETA and ETB receptors have been demonstrated on renal mesangial cells, which represent the pericytes of the glomerulus.20 Our results are not in accordance with the observations of Yamagishi,21 who reported the presence of ET A but the absence of ET B in BRP by Northern blot analysis. However, another recent report has demonstrated the presence of ETB mRNA in cultured VSMC, which are analogous to microvascular pericytes.22 Such discrepancies emphasize the diffi-

<table>
<thead>
<tr>
<th>Radiolabeled Ligand</th>
<th>IC50 Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ET-1</td>
</tr>
<tr>
<td>[I25I]ET-1</td>
<td>1.3 ± 1.4  (nM) (n = 5)</td>
</tr>
<tr>
<td>[I25I]ET-3</td>
<td>2.2 ± 2.8  (nM) (n = 4)</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SE of n independent experiments.
FIGURE 3. Competition inhibition of [125I] ET-3 binding by unlabeled ET-1, ET-3, and ET-3 + BQ123. Inhibition (B/Bo) is expressed as the ratio of radioactivity bound in the presence of competitor to that bound in its absence. Each point is the mean of duplicate treatments, and the graph is typical of at least three independent experiments. - ET-3; - ET-1; - ET-3 + BQ123.

Affinity labeling with bifunctional cross-linking reagents is useful in determining the presence of multiple receptor subtypes, and our findings are similar to those of Martin et al. who showed that cultured rat mesangial cells contain ET_A and ET_B receptors. The bovine ET_B receptor isolated from a variety of tissues has been shown to have an apparent molecular weight varying between 50 kd and 52 kd, which is slightly larger than our results indicated. This disparity may be attributed to proteolysis of the receptor, use of tissues from disparate origins, or both.

The role of the ET_B receptor in contractile cells in particular is subject to some speculation. Intravenous bolus injection of ET-1, ET-2, or ET-3 causes an initial transient depressor effect followed by a prolonged systemic pressor response. The initial depressor response is endothelium dependent and has been attributed to nitric oxide release from the endothelium by ET-1, acting through the ET_A receptor. In this regard, it is noteworthy that the depressor response is strongest with ET-3, which is more likely to act through the ET_A receptor owing to the differences in affinity that these peptides exhibit for their receptors. In isolated blood vessels denuded of endothelial cells, all three peptides (ET-1, ET-2, and ET-3) elicit slow-developing and long-lasting vasoconstriction.

Accumulating evidence shows that ET_B receptors on smooth muscle may contribute to vasoconstriction in some vascular tissues, including rabbit pulmonary artery, porcine coronary artery, and dog, monkey, and rabbit saphenous vein. Functional evidence includes potent vasoconstrictor activity by ET_A-selective agonists (ET-3, [Ala 1,3,11,15] ET-1, and S6c) and the inability of BQ-123, an ET_A selective antagonist, to block vasoconstrictor activity. Batra et al. have suggested that the Ca^{2+} response to ET-1 in rat cultured aortic smooth muscle cells was due to the accumulation of effects caused through ET_A and ET_B receptor subtypes. On the other hand, a recent study has linked ET-3 stimulation of renal mesangial cells to the production of nitric oxide, a potent vasodilator and a relaxant of vascular smooth muscle. The authors considered this stimulation to be mediated through ET_B receptors because the stimulation was not inhibited by BQ123. They postulated that the ET-3-induced activation of the nitric oxide–cyclic guanosine 3',5'-monophosphate system acts to buffer the contractile effects of ET-1, especially when local levels of ET are inappropriately high. In the retinal pericytes, Ramachandran et al. have shown that ET-1 produces a rapid rise in the free/bound Ca^{2+} ratio at concentrations as low as 1 × 10^{-12} M. ET-2 was less effective at eliciting such a response, and ET-3 had no effect at all. This suggests that the rise in intracellular Ca^{2+} was mediated by ET_A receptors; however, when
Endothelin Receptors in Retinal Pericytes

10^{-7} M ET-3 was applied to the pericytes, subsequent application of ET-1 at the same concentration produced no response. Our results showing the presence of the ETA receptor in pericytes allows us to suggest that ET-3, acting through ETB, may block the effects caused by ET-1. A recent in vivo study reported that ET-1 caused retinal arteries and arterioles to constrict but had no effect on the capillary bed, suggesting that this mechanism may also occur in vivo.

In conclusion, this study has demonstrated the presence of the two endothelin-receptor subtypes, ETA and ETB, on BRP. Any imbalance between the expression of the two receptor types could represent an important contributing factor in pathologic conditions, such as hypertension and diabetes. This is especially important in the capillary bed of the retina in which the fine control of blood flow is achieved by autoregulation.

Key Words

bovine retinal pericytes, receptors, ETA, ETB, retinal circulation

Acknowledgments

The authors thank the Department of Medicine for the use of their gamma counter and Dr. T. Gardiner for his excellent editorial advice.

References