Rapid Publication

Production of Endothelin 1 by Cultured Bovine Retinal Endothelial Cells and Presence of Endothelin Receptors on Associated Pericytes

KAZUHIRO TAKAHASHI, ROGER A. BROOKS, SANDIP M. KANSE, MOHAMMAD A. GHATEI, EVA M. KOHNER, AND STEPHEN R. BLOOM

Endothelinlike immunoreactivity was detected by radioimmunoassay in medium conditioned by cultured endothelial cells obtained from bovine retinal microvessels (9.2 ± 6.5 pM, *n* = 4). Sephadex G-25 column chromatography and fast-protein liquid chromatography revealed that most of the endothelinlike immunoreactivity was eluted in an identical position to synthetic endothelin 1. Retinal capillary pericyte–conditioned medium contained 2.9 pM endothelinlike immunoreactivity. In contrast to endothelial cells, retinal pericytes were found to bind endothelin. The dissociation constant and binding capacity were 0.14 nM and 1.5 × 10^5 sites/cell (*n* = 3), respectively. These findings suggest that endothelin produced by the retinal endothelial cells binds to the pericytes, adding support to the suggestion that pericytes in the retina may have a musclelike function. ***Diabetes*** **38:1200–202, 1989**

Endothelin 1 is a potent vasoconstrictor peptide that was originally isolated and structurally characterized from the conditioned medium of porcine aortic endothelial cells (1). Endothelin binding sites have been shown in cultured rat vascular smooth muscle cells (2). Three endothelin genes (for endothelin 1, 2, and 3) exist in the human, porcine, and rat genome (3). Endothelin is thought to be produced by endothelial cells of the major arteries, but it has not been established whether endothelin is produced in microvascular circulation.

To help clarify the physiological role of endothelin in retinal microcirculation and its involvement in the genesis of diabetic retinopathy, we investigated the production of endothelin and the presence of endothelin binding sites on cultured bovine retinal endothelial cells and pericytes.

From the Department of Medicine, Royal Postgraduate Medicaf School, Hammersmith Hospital, London, United Kingdom.

Address correspondence and reprint requests to Professor S.R. Bloom, Department of Medicine, Francis Fraser Laboratory, 2nd Floor, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK.

Received for publication 19 June 1989 and accepted 28 June 1989.

RESEARCH DESIGN AND METHODS

Cell culture. Bovine retinal endothelial cells (BREC) were isolated and cultured as previously described (4). Briefly, retinas were dissected from freshly slaughtered cattle, homogenized in minimum essential medium, and filtered through an 85-μm filter. The trapped microvessels were digested in a 1-mg/ml collagenase-dispase (Boehringer Mannheim, Mannheim, FRG) solution for 65 min at 37°C and plated onto a fibronectin substrate in 75-cm^3 tissue-culture flasks. The culture medium was 8% Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Tryptose phosphate broth (Gibco, Paisley, UK) and 10% plasma-derived serum. All culture mediums were purchased from Gibco and tissue-culture plastics from Falcon (Becton Dickinson, Oxford, UK). Bovine retinal pericytes (BRP) were cultured from microvessels prepared like the endothelial cells, but the vessels were plated at a lower density onto the plastic substrate of a 75-cm^3 tissue-culture flask. Supplemented DMEM was also used for BRP, but 20% fetal calf serum replaced the plasma-derived serum. Both cell types were grown to confluence in sealed flasks gassed with 95% air/5% CO_2.

Both cell types were characterized by their morphology. BREC displayed contact inhibition and had a typical cobblestone appearance. BRP had many cytoplasmic processes and overgrew at confluence. BREC showed uniform staining for factor VIII–related antigen, whereas BRP did not stain.

At confluence, the cells were trypsinized. After washing twice with Hank's buffered salt solution (HBSS), 0.25% trypsin was added. When the cells had detached from the substrate (1.5 min for BREC and 10 min for BRP), the trypsin was neutralized by the addition of serum-containing medium. For preparation of conditioned medium, trypsinized cells were plated into 75-cm^3 tissue-culture flasks and grown to confluence as described. At confluence, medium was removed, the cells were washed with HBSS, and 5 ml of either serum-containing DMEM or serum-free DMEM was added. After 2 days, this medium was harvested and used for measurement and characterization of endothelin.

For binding studies, trypsinized cells were seeded into 24-well tissue-culture plates that had been coated with fibronectin for BREC but not for BRP. They were grown to confluence in 0.8 ml serum-containing DMEM/well before use.

Endothelin characterization. Endothelinlike immunoreactivity (ELI) in conditioned medium was extracted by Sep-Pak C18 cartridges (Waters, Milford, MA). Eight milliliters of conditioned medium acidified with 4 ml of 4% acetic acid was loaded onto a cartridge, and ELI was eluted with 2 ml of 60% (vol/vol) acetonitrile/water containing 0.026 M ammonium acetate. The eluate was dried in a Savant vacuum centrifuge, the resulting pellet reconstituted in assay buffer (60 mM PO_4 buffer, pH 7.4, containing 10 mM EDTA, 7 mM sodium azide, and 0.3% (wt/vol) bovine serum albumin [BSA]), and the aliquot assayed in duplicate. The mean ± SD recovery of this extraction procedure was 95 ± 8.9% (n = 6).

The antiserum to endothelin 1 was raised in a rabbit with synthetic endothelin 1 (Nova Biochem, Nottingham, UK) conjugated with BSA by the diazo technique (5). The antiserum produced was used in the assay at a final dilution of 1:35,000. Synthetic endothelin 1 (Peptide Institute, Osaka, Japan) was used as standard and for iodination with ^{125}I-labeled sodium by the chloramine-T method (6) and purified by high-performance liquid chromatography as previously described (7). After 3 days of incubation at 4°C, antibody-bound and free fractions were separated by charcoal adsorption of the free fraction.

The sensitivity of this assay was 0.5 fmol/tube at 95% confidence. The assay showed 23% cross-reaction with endothelin 2 and 20% with endothelin 3 (Peptide Institute) but no cross-reaction with other known peptides. Intra- and interassay coefficients of variation were 12% (n = 9) and 19% (n = 7), respectively.

Fractionation of ELI in the medium extract was carried out by Sephadex G-25 column chromatography (0.9 × 58 cm, superfine) and fast-protein liquid chromatography (FPLC) with a high-resolution reverse-phase (Pep RPC HR 5/5) C-18 column (Pharmacia, Uppsala, Sweden) with a gradient of acetonitrile from 15 to 35% (vol/vol) in water over 1 h at 1 ml · min^{-1} · $fraction^{-1}$.

Binding experiments. Confluent 24-well plates (7 × 10^4 BREC/well or 4 × 10^4 BRP/well) were used for binding assays. Cells were incubated at 37°C for 90 min with 0.05 nM ^{125}I-labeled endothelin 1 in Earl's balanced salt solution containing 10 mM HEPES and 0.3% BSA. After incubation, cells were extensively washed and solubilized in 1 M NaOH. The cell-bound radioactivity was determined in a γ-counter. Specific binding was obtained by subtracting from total binding the nonspecific binding in the presence of an excess (0.1 μM) of unlabeled endothelin 1. Scatchard plots were obtained by adding increasing amounts of labeled endothelin, and the results were analyzed by linear regression. IC_{50} values were obtained by adding increasing concentrations of endothelin 1, 2, and 3.

RESULTS

ELI was detected in the BREC-conditioned culture medium (mean ± SE 9.2 ± 6.5 pM, n = 4), whereas no ELI was detected in the unconditioned medium (<0.3 pM). The ELI in the BRP-conditioned culture medium was 2.9 pM.

Column chromatography and FPLC showed that most ELI eluted in an identical position to synthetic endothelin 1, whereas there was none in the position of endothelin 2 and little in the position of endothelin 3 (Fig. 1). The recovery of ELI in the conditioned medium from column and liquid chromatography was 107 and 73%, respectively.

Endothelin binding to BREC and BRP reached equilibrium between 90 and 120 min (data not shown). The binding sites in BRP were saturable (Fig. 2*A*). The number of binding sites in BRP was 1.5 × 10^5 sites/cell, and the K_d was 0.14 nM (n = 3). Compared with BRP, binding in BREC was very low (<2000 sites/cell), and reliable measurements of B_{max} and K_d could not be made (data not shown). Binding was specific in BRP and was not displaced by unrelated peptides. Endothelin 2 and 3 showed a lower affinity for the receptors compared with endothelin 1. The IC_{50} of endothelin 1 and 2 was 0.56 and 1.53 nM (n = 3), respectively. Endothelin 3 caused only 55% displacement of label at concentration of 1 μM (Fig. 2*B*).

DISCUSSION

This study showed that endothelin 1 is present in the conditioned medium of cultured BREC, and endothelin binding

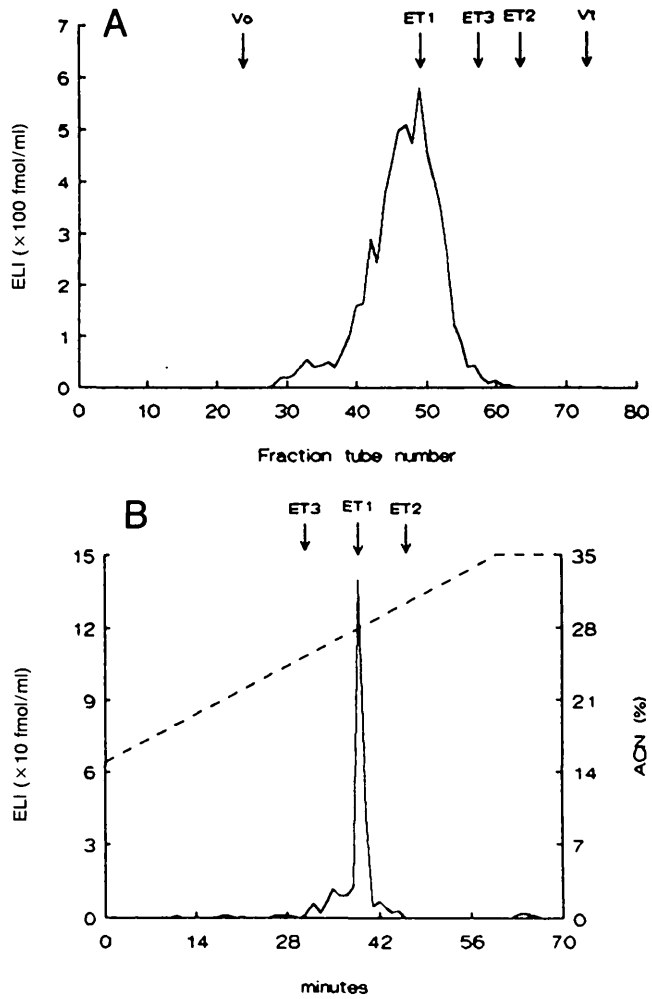

FIG. 1. Sephadex G-25 column chromatography (*A*) and fast-protein liquid chromatography (*B*) of conditioned medium of cultured bovine retinal endothelial cells. ELI, endothelinlike immunoreactivity; Vo, void volume; Vt, total volume; ET1, ET2, and ET3, elution positions of endothelin 1, 2, and 3, respectively; ACN, acetonitrile.

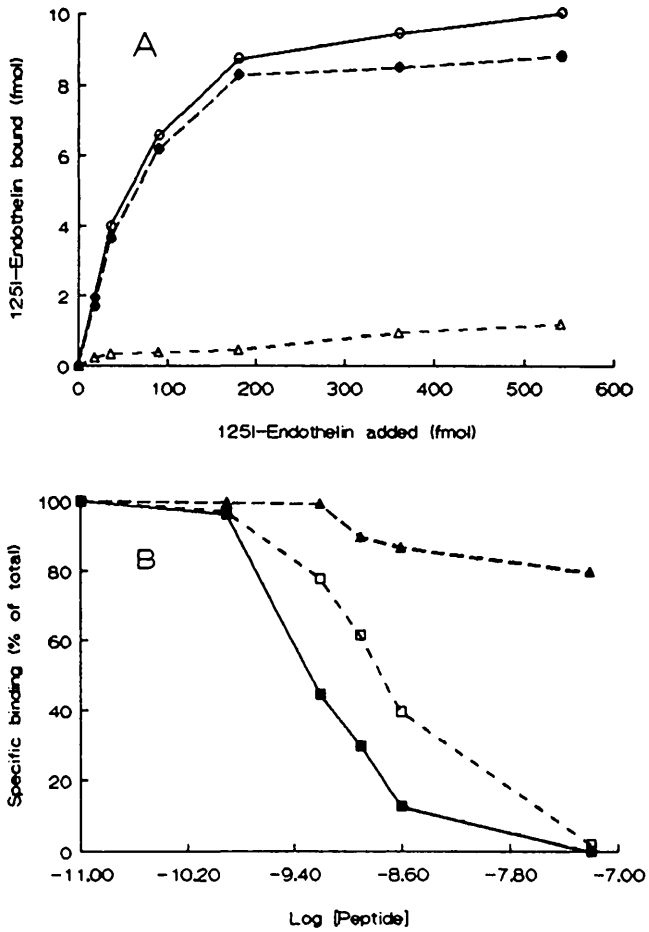

FIG. 2. *A*: saturation of endothelin binding sites in bovine retinal pericytes by adding increasing concentration of ^{125}I-labeled endothelin. ○, Total binding; ●, specific binding; △, nonspecific binding. *B*: displacement of ^{125}I-labeled endothelin binding sites in bovine retinal pericytes by increasing concentration of endothelin 1 (■), endothelin 2 (□), and endothelin 3 (▲).

sites are present in cultured BRP. FPLC revealed that most of the ELI in the BREC-conditioned culture medium eluted in an identical position to synthetic endothelin 1, and little eluted in the position of endothelin 3. The antiserum to endothelin 1 used in this study showed 20% cross-reaction with endothelin 3, therefore it is possible that a greater amount of endothelin 3 may be present in the conditioned medium than was shown by FPLC analysis.

The finding of endothelin was initially unexpected, because embryologically the retina is an outgrowth of the brain, and preproendothelin mRNA has not yet been reported in brain microvessels (1). If endothelin is present in human retinal microvessel endothelial cells, it may be important in the evolution of some retinal vascular diseases, especially diabetic retinopathy.

The exact role of pericytes in the retina has not been clearly established, but a recent study by Orlidge and D'Amore (8) suggested that pericytes have a controlling influence on endothelial cell growth and multiplication. A muscle cell–like action for pericytes was put forward by Kuwabara and Cogan (9) in the 1960s who suggested that capillary dilation with endothelial proliferation and shunt-vessel formation seen in diabetic retinopathy after pericyte loss was due to the loss of this action (10). This possibility was strengthened by Hohman et al. (11), who showed the presence of muscle actin in human retinal pericytes, and Henkind (12), who suggested that pericytes may be capable of differentiating into smooth muscle cells under certain conditions.

In most tissues, endothelin acts on the smooth muscle cells of the vascular wall. The fact that in the retina it may act on pericytes would further emphasize the musclelike function of these cells and explain the dilation of capillaries seen in early diabetic retinopathy by the time pericyte loss occurs (13). Loss of endothelin action could therefore play an important part in the development of diabetic retinopathy.

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