Characterization of Endothelin Receptors and Effects of Endothelin on Diacylglycerol and Protein Kinase C in Retinal Capillary Pericytes

TIAN-SHING LEE, KANG-QUAN HU, TING CHAO, AND GEORGE L. KING

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Retinal capillary pericyte is a cell type selectively lost in early diabetic retinopathy. The physiological function of pericytes is not yet clearly identified, although it probably has contractile properties. We determined the specific binding of endothelin 1, a 21–amino acid peptide with potent vasoconstrictive action, and the stimulation of diacylglycerol/protein kinase C (DAG/PKC) pathway in cultured retinal capillary pericytes by endothelin. A single specific binding site for ^{125}I-labeled endothelin was identified, with an apparent K_d of 1.3 nM and a maximal binding capacity of ~1–2 × 10^5 sites/cell. Endothelin (100 nM) increased total cellular DAG content by 15% at 5 min and 24% at 10 min. When pericytes were labeled isotopically with [^{3}H]glycerol, endothelin stimulated [^{3}H]DAG formation by 100% at 10 min and 88% at 30 min. After 10 min of endothelin treatment, PKC activities were increased by 60 and 100% in the membranous and cytosolic pools, respectively. We conclude that bovine retinal capillary pericytes possess numerous high-affinity specific binding sites for endothelin that mediate the action of endothelin by the stimulation of the DAG/PKC pathway in pericytes. These findings suggest that endothelin is a regulator of the contractile properties of pericytes, which may be adversely affected in diabetic retinopathy. *Diabetes* 38:1643–46, 1989

The main histopathological site of abnormalities in diabetic retinopathy is in the retinal capillaries, which under normal conditions are composed of endothelial cells surrounded by an equal number of pericytes (1). In the early phase of diabetic retinopathy, pericytes are selectively lost, with an increase of capillary diameter and permeability (1–3). Because pericytes contain contractile elements similar to those in the vascular smooth muscle cells (4), it has been proposed that the alteration of the response of pericytes to vasoactive regulators may be responsible for these early findings (3). We characterized the response of pericytes to endothelin 1, a newly discovered vasoconstrictor peptide (5). This study may aid our knowledge of the physiological role of retinal capillary pericytes.

Endothelin is a 21–amino acid peptide secreted by vascular endothelial cells (5). Its pressor effect lasts for >40–60 min and is one of the most potent vasoconstrictors known (5). Because the vasoconstrictive action of endothelin can be observed in both macro- and microvascular systems (6–8), it is possible that endothelin can also act on the pericytes of retinal capillaries. The molecular mechanisms for endothelin-induced vascular contraction include phosphoinositide turnover and activation of diacylglycerol/protein kinase C (DAG/PKC) pathway (9,10). To test the hypothesis that endothelin can act on pericytes, we examined the specific binding of ^{125}I-labeled endothelin and the stimulation of DAG/PKC pathway by endothelin on cultured bovine retinal capillary pericytes.

RESEARCH DESIGN AND METHODS

All lipids were purchased from Avanti (Pelham, AL). All solvents were of high-performance liquid-chromography grade from Fisher (Fair Lawn, NJ). Silica gel G thin-layer chromatography (TLC) plates were from Merck (Darmstadt, FRG). Synthetic human endothelin was from Peninsula (Belmont, CA).

Bovine retinal capillary pericytes were isolated by a series of homogenization and filtration steps described previously (11) and subsequently cultured with Dulbecco's modified Eagle's medium (DMEM) with 15% fetal bovine serum (Gibco, Grand Island, NY). The identity of the pericytes was positively confirmed by staining with 3G5 monoclonal antibody, which has recently been shown to be specific for pericytes among the microvascular cells (12).

From the Research Division, Joslin Diabetes Center; Department of Medicine, Brigham and Women's Hospital; and Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to George L. King, MD, Research Division, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215.

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Confluent pericytes on 6-well plates (~2 × 10^5 cells/well) were washed three times with binding medium (DMEM, pH 7.4, with 2 mg/ml bovine serum albumin and 25 mM HEPES) and incubated at 15°C with 1 ml binding medium containing 3 × 10^4 counts/min of ^{125}I-endothelin (Amersham, Arlington Heights, IL; sp act 1860 Ci/mmol). At the end of indicated times, the cells were washed three times with ice-cold phosphate-buffered saline and solubilized in 0.1% sodium dodecyl sulfate, and the bound radioactivity was measured. Nonspecific binding was determined in parallel and defined as binding in the presence of 800 nM unlabeled endothelin. Specific binding was calculated as total binding minus nonspecific binding.

After preincubation with [^{3}H]glycerol (Amersham) for 2 h, cells were treated with various concentrations of endothelin for indicated intervals. Reactions were terminated by aspirating the medium and adding 1 ml of methanol, and cellular lipids were extracted as described by Bligh and Dyer (13). DAG was resolved on silica gel G TLC plates developed together with standards in hexane/diethyl ether/acetic acid (60/40/1) (14). Spots of lipids on TLC plates were visualized by charring with H_2SO_4, and individual spots, identified by the standards, were scraped into vials. Radioactivity was then counted.

Total DAG levels were measured by DAG kinase assay as described by Preiss et al. (15). DAG kinase was from Lipidex (Westfield, NJ). The standard curve showed a linear slope from 50 to 600 pmol DAG.

Pericytes were fractionated into cytosolic and membranous portions, and PKC was partially purified by DEAE chromatography as previously described (16). The activity of PKC was measured by its ability to transfer ^{32}P from [γ-^{32}P]ATP (Du Pont-NEN, Boston, MA) into histone H1 in the presence or absence of 0.5 mM Ca^{2+}, 6.4 μg/ml 1,2-dioleoyl-*sn*-glycerol, and 96 μg/ml phosphatidylserine as previously described (17). The PKC activities were corrected for protein content, which was determined by the method of Bradford (18).

RESULTS

Specific binding of ^{125}I-endothelin to bovine retinal pericytes was time and temperature dependent. Binding reached an apparent equilibrium between 30 and 60 min at 37°C and between 3 and 4 h at 15°C, respectively (Fig. 1*A*). Therefore, in all subsequent binding studies, incubation of pericytes with endothelin was performed at 15°C for 6 h. The equilibrium binding at 15°C was ~60% of that at 37°C. The binding of ^{125}I-endothelin was inhibited by unlabeled endothelin with a half-maximal inhibition concentration of 1.6 nM (Fig. 1*B*). Scatchard analysis showed a single class of binding sites with an apparent K_d of 1.3 nM and a maximal binding capacity (B_{max}) of 1–2 × 10^{11} pmol/mg protein or 1–2 × 10^5 binding sites/cell (Fig. 1*C*).

Addition of 100 nM endothelin to pericytes prelabeled for 2 h with [^{3}H]glycerol resulted in an increase of [^{3}H]DAG formation (Fig. 2*A*). The maximal increase was reached at 10 min (100 ± 15%; $P < .001$) and remained elevated for at least 30 min (88 ± 19%; $P < .02$). Fig. 2*B* shows the dose dependence of the effect of endothelin on [^{3}H]DAG formation. The plateau was approached at an endothelin concen-

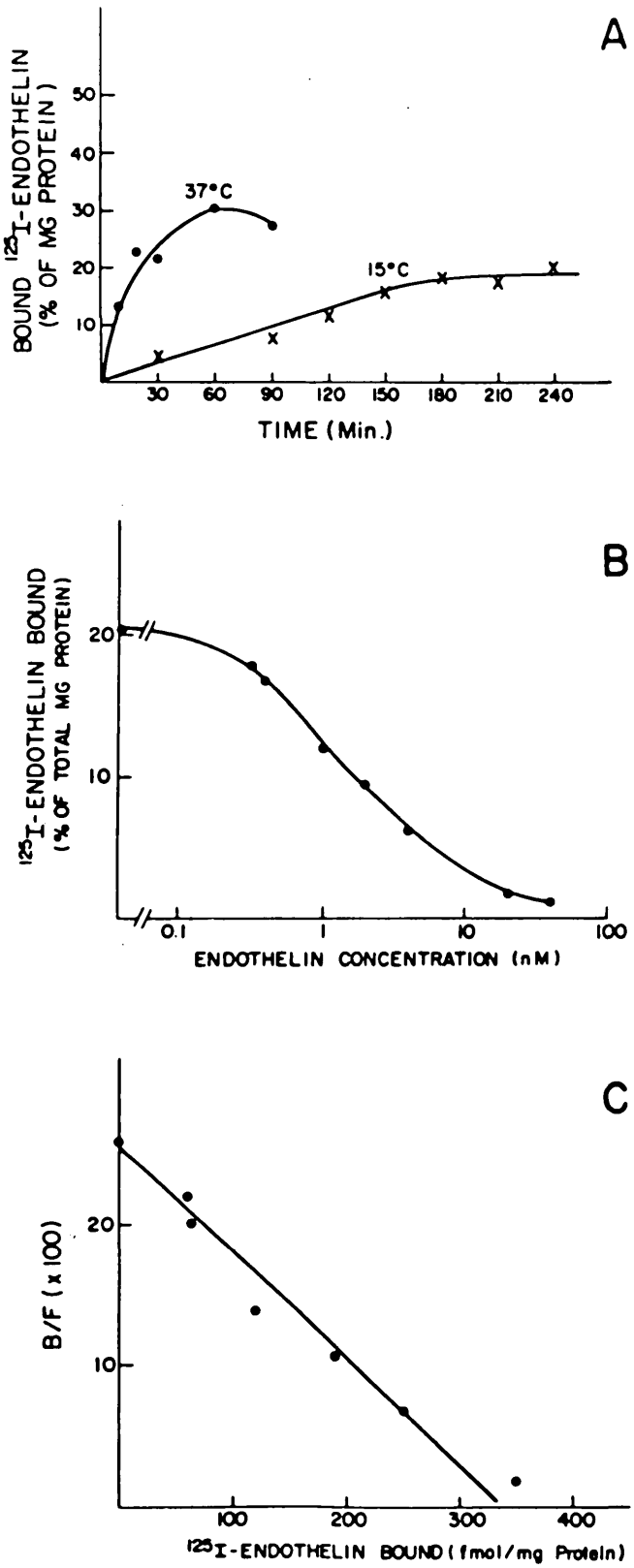

FIG. 1. *A*: time course of ^{125}I-labeled endothelin binding. Confluent cells were incubated with 3 × 10^4 counts/min of ^{125}I-endothelin at 37°C (●) or 15°C (X) for indicated time. Specific binding was obtained by subtracting nonspecific binding in presence of 800 nM unlabeled endothelin. Each point is mean of duplicate or triplicate experiments. *B*: displacement curve of ^{125}I-endothelin binding. Confluent cells were incubated with 3 × 10^4 counts/min ^{125}I-endothelin in presence of increasing concentrations of unlabeled endothelin. Each point is mean of triplicate experiments. *C*: Scatchard analysis of ^{125}I-endothelin binding data shown in *B*. B/F, bound/free.

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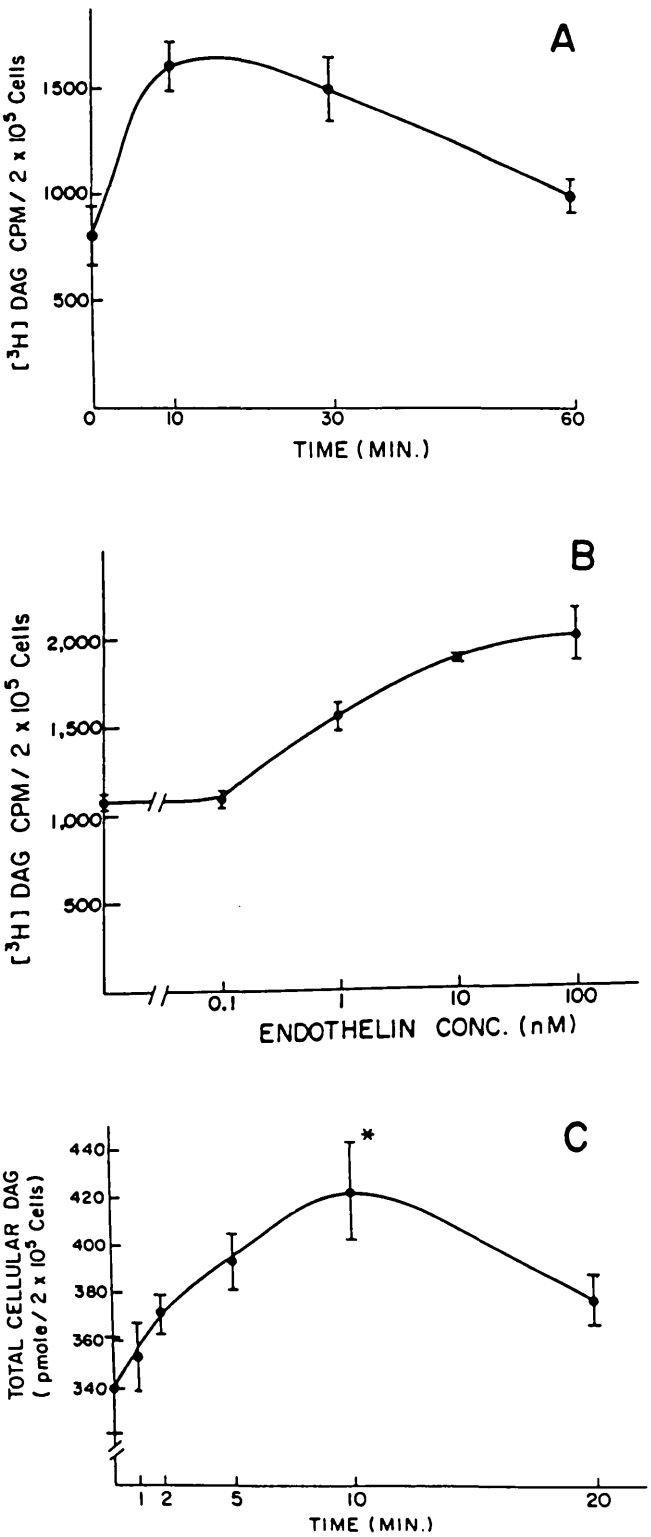

FIG. 2. *A*: time course of endothelin-induced [^{3}H]-diacylglycerol (DAG) accumulation. Cells were incubated with [^{3}H]glycerol (2 μCi/ml) for 2 h, washed, and exposed to 100 nM endothelin for indicated time. [^{3}H]DAG accumulation was measured as described in RESEARCH DESIGN AND METHODS. *B*: dose dependence of endothelin-induced [^{3}H]DAG accumulation. Cells were labeled, washed, and exposed to various concentrations of endothelin for 10 min, and then [^{3}H]DAG accumulation was measured. *C*: time course of endothelin-induced total DAG accumulation. Cells were exposed to 100 nM endothelin for indicated time, then total cell DAG was measured as described in RESEARCH DESIGN AND METHODS. Values are means ± SE from at least 3 experiments for *A* and *B* and means ± SD from 4 experiments for *C*. **P* < .05. cpm, Counts per minute.

tration of ~100nM, and half-maximal stimulation was reached at 1 nM.

Addition of 100 nM endothelin to pericytes induced a time-dependent increase in total cellular DAG content (Fig. 2*C*). An increase of total DAG level was detected at 1 min, which reached the maximum at 10 min (24 ± 6%, $P < .05$) and was sustained above baseline for at least 20 min.

After exposure to 100 nM endothelin for 10 min, cytosolic PKC activity increased by 100% (from 27 ± 0.3 to 54 ± 13 pmol P_i transferred · mg^{-1} protein · min^{-1}, $P < .05$; Fig. 3). PKC activity in the membranous pool also increased over the same period from 1010 ± 120 to 1610 ± 20 pmol P_i transferred · mg^{-1} protein · min^{-1}, $P < .01$), a 60% increase (Fig. 3).

DISCUSSION

This study demonstrates the presence of a specific receptor for endothelin and the simulation of DAG/PKC pathway by endothelin in cultured bovine retinal capillary pericytes. The endothelin receptors in pericytes appear to be a single class of high-affinity and high-capacity binding sites. The binding

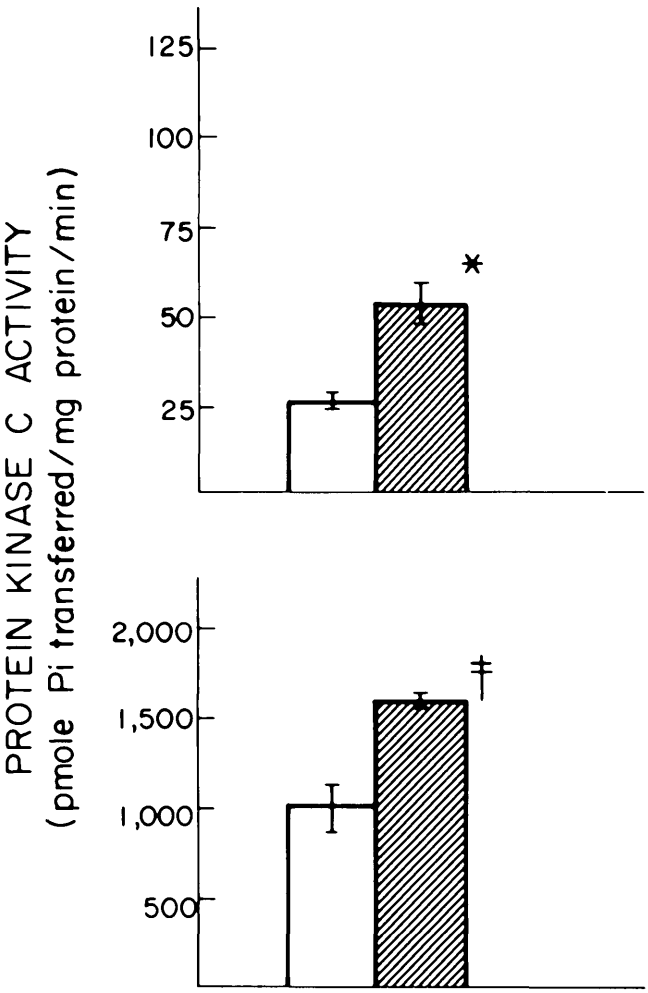

FIG. 3. Stimulation of protein kinase C activity in control (*open bars*) and endothelin-treated (*shaded bars*) cells from cytosol (*top*) or membrane (*bottom*). Treated cells were exposed to 100 nM endothelin for 10 min and then subfractionated. Protein kinase C activity was measured as described in RESEARCH DESIGN AND METHODS. Values are means ± SD with $n = 4$. **P* < .05; ‡*P* < .01.

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affinity of endothelin receptors in pericytes is comparable to that of cultured rat cardiocytes (K_d 0.6–0.9 nM; 19), and glomerular mesangial cells (K_d 0.76 nM; 20). It is greater than that of human vascular smooth muscle cells (K_d 0.13 nM; 21). The B_{max} of endothelin receptors in pericytes is comparable to that of cultured rat cardiocytes (5–8 × 10^4 sites/cell; 22) and is far greater than those of human vascular smooth muscle cells (169 sites/cell; 21) and rat vascular smooth muscle cells (1.1–1.3 × 10^4 sites/cell; 22). The presence of many high-affinity receptors for endothelin in pericytes would suggest a role for endothelin in regulating pericyte function. Interestingly, the number and affinity of endothelin receptors appear to be very different between retinal pericytes and arterial smooth muscle cells. These differences may be due to changes in culture conditions, species studied, and/or inherent receptor subpopulations in various tissues. Recent studies of endothelin receptors seem to support the last possibility (23).

The cellular transduction pathways for endothelin-induced contraction involve both phosphoinositide turnover and activation of DAG/PKC pathway (9,10). This study provides direct evidence that endothelin activates DAG/PKC pathway in pericytes. The time course of DAG accumulation in pericytes (Fig. 2*A*) is similar to the reported time course of endothelin-induced DAG accumulation in cultured bovine aortic smooth muscle cells (9) and endothelin-induced contraction in arterial strips (5), suggesting that these vascular cells may have a very similar signaling pathway for endothelin.

It seems reasonable to suggest that endothelin-induced increase in membranous PKC activity is due to increases in DAG. However, it is possible that factors other than DAG may modulate PKC activity and influence its subcellular distribution, e.g., phosphorylation and dephosphorylation of PKC, proteolytic activation of PKC, conformational changes in the enzyme by various ligands, and possible changes in membrane phospholipid composition induced by endothelin. The mechanisms for the increase in cytosolic PKC activity are less clear. It is possible that endogeneously produced DAG may be bound to cytosolic PKC and contribute to its activation. Other factors may also be involved in increasing cytosolic PKC activity (e.g., phosphorylation, limited proteolysis). Note that insulin has been reported to increase membrane and cytosolic PKC activity in myocytes (24).

In summary, our data suggest that endothelin may stimulate a sustained contractile response in retinal pericytes. This possibility is supported by a recent report that messenger RNA for endothelin has been reported in cultured human retinal capillary endothelial cells (25), suggesting a source of endothelin in the retina. If retinal capillary pericytes contract in vivo in response to endothelin, it is possible that the changes of endothelin secretion from retinal capillary endothelial cells in response to various chemical and physical stimuli can act as a regulatory system for regional blood flow in the retina. Because the growth and metabolism of pericytes are altered by hyperglycemia (26), the response of pericytes to various vasoconstrictive substances such as endothelin may thus be changed. These changes may lead to some hemodynamic disturbance in the retina and may eventually contribute to the development of diabetic retinopathy.

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