Endothelin-Induced Changes of Second Messengers in Cultured Human Ciliary Muscle Cells

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Purpose. To characterize the pharmacology of endothelin-induced changes in phospholipase C (PLC) activity, intracellular calcium concentration ([Ca²⁺]i), and cyclic adenosine monophosphate (cAMP) in cultured human ciliary muscle (HCM) cells.

Methods. Changes in PLC activity of HCM cells were determined by production of [³H]inositol phosphates. [Ca²⁺]i was determined by single-cell dynamic fluorescence ratio imaging. Radioimmunoassays were used to determine cAMP and prostaglandin E₂ (PGE₂) concentrations.

Results. Endothelin-1 (ET-1) stimulated PLC (mean EC₅₀ = 335 pmM) and activated calcium mobilization in HCM cells. These effects were mediated by the endothelin ETₐ receptor subtype because an ETB receptor-selective agonist, sarafotoxin S6c, was ineffective. Additionally, effects of ET-1 were inhibited by pretreatment with a selective ETA antagonist, BQ610 (mean pKᵢ = 9.96 for PLC). ET-1 also stimulated the production of PGE₂ (mean EC₅₀ = 12.0 nM) and cAMP (mean EC₅₀ = 5.2 nM) by these cells. PGE₂ appeared to mediate the stimulatory effect of ET-1 on adenylyl cyclase because blockade of ET-1-induced PGE₂ production by 10 μM indomethacin also completely blocked the ET-1-activated cAMP production.

Conclusions. ET-1 stimulated PLC and increased [Ca²⁺]i in HCM cells by the ETₐ receptor subtype. ET-1 also increased cAMP production, an effect likely mediated by the enhanced production of prostaglandins. Invest Ophthalmol Vis Sci. 1996;37:1058–1066.

Endothelin-1 (ET-1) and endothelin-3 (ET-3), potent peptide vasoconstrictors,¹ are found in various human ocular structures, including the retina, choroid, iris, ciliary body,² ciliary epithelium,³ and aqueous humor.⁴ When injected intravitreally into the rabbit eye, 4 pmol or less of endothelins caused a dose-dependent increase in intraocular pressure,⁵ but, at doses higher than 1 nmol, they initially increased and subsequently lowered intraocular pressure.⁶ The difference in pharmacologic effects of low and high doses of endothelins is interesting. It is currently believed that the ocular hypertension produced by endothelins is mediated by prostaglandins, because pre-treatment of rabbits with systemic indomethacin, a cyclooxygenase inhibitor that inhibits the production of prostaglandins, eliminated this effect.⁵

The mechanism of the lowering of intraocular pressure by endothelins is unclear. However, Lepple-Wienhues et al⁹ showed that ET-1 caused contraction of ciliary muscle strips of the bovine eye. Because tension on the ciliary muscle regulates aqueous outflow and accommodation,¹⁰ ET-1-induced contraction of the ciliary muscle should induce accommodation and increase aqueous outflow. In fact, when perfused into the anterior chamber of the anesthetized monkey eye, ET-1 significantly increased the outflow facility and affected accommodation as predicted.¹¹ Thus, effects of endothelins on the ciliary muscle may lead to the modification of intraocular pressure.

Two distinct endothelin receptor subtypes have been cloned and expressed. They are named ETₐ¹² and ETₐ.¹³ The ETₐ receptor selectively interacts with ET-1, whereas the ETₐ receptor is not selective. Thus, ET-1 has a much higher affinity than ET-3 for the ETₐ receptor, whereas both peptides have similar affinity
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for the ET₈ receptor.¹⁴ Recently, a third endothelin receptor, designated ET₉, was identified.¹⁵ In radioligand-binding studies, the cloned ET₇ receptor has a slightly higher affinity for ET-3 than for ET-1. It is currently unknown which of these receptor subtypes is/are involved in ocular actions of endothelins.

Activation of ET₈ and ET₉ receptors has been shown to stimulate phospholipase C (PLC), increase turnover of phosphoinositides, and increase intracellular calcium concentration ([Ca²⁺]i).¹⁴ These actions are thought to lead to endothelin-induced contraction in smooth muscles.¹⁴ Therefore, it is interesting to explore whether ET-1-induced contraction of ciliary muscle also is mediated by similar second messengers. In this article, using cultured human ciliary muscle cells, we have demonstrated that endothelins, through the ET₈ receptor, stimulated PLC activity and increased [Ca²⁺]i. In addition, we showed that ET-1 increased the production of prostaglandin E₂ (PGE₂), which in turn elevated the intracellular concentration of cyclic adenosine monophosphate (cAMP) in these cells. All these endothelin actions could affect the contractility of the ciliary muscle and are potentially important in regulating intraocular pressure.

MATERIALS AND METHODS

Chemicals

ET-1, ET-3, and homopiperidinyl-carbonyl-D-formyltryptophyl-D-tryptophan (BQ610) were purchased from Peptides International, Inc. (Louisville, KY). Fura-2 acetoxymethyl ester was obtained from List Biological Laboratories, Inc. (Campbell, CA). Myo-[2-³H]-inositol (specific activity of 370–740 GBq/mmol) was obtained from Amersham (Arlington Heights, IL). Radioimmunoassay kits for cAMP and PGE₂ were obtained from Biomedical Technologies, Inc. (Stoughton, MA) and New England Nuclear Research (Boston, MA), respectively. Cell culture supplies were obtained from Gibco/BRL (Grand Island, NY). Sarafotoxin S6c, indomethacin, LiCl, 3-isobutyl-1-methylxanthine, and all buffer chemicals were purchased from Sigma (St. Louis, MO).

Human Ciliary Muscle Cell Culture

The cultured human ciliary muscle (HCM) cell strain was a kind gift of Drs. Ernst Tamm and Elke Lütjen-Drecoll. Culture conditions for these cells were described previously.¹⁷ Briefly, HCM cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum, 4 mM L-glutamine, and 50 µg/ml gentamicin, and they were subcultured by trypsinization. Cells of passages 10 to 16 were used in the following experiments.

Phospholipase C Assay

Phospholipase C activity assay in HCM cells was described previously.¹⁷ Confluent monolayers of HCM cells were incubated with myo-[2-³H]-inositol 5 µCi/ml per well in serum-free DMEM in a 24-well plate for 2 days. On the day of experiment, the medium was exchanged into DMEM–F12 (1:1) nutrient mixture with 10 mM LiCl. After 10 minutes of incubation, 10 µl of drugs were added to each well. In antagonist experiments, 10 µl of agonists were added 12 minutes after the addition of antagonists. A standard agonist incubation time of 1 hour was chosen because pilot studies showed that ET-1-induced accumulation of inositol phosphates was time dependent and linear up to 2 hours (data not shown). After the incubation, the assay was terminated by replacing the medium with 1 ml of 0.1 M formic acid. The cell lysate was then loaded onto an anion exchange column (AG 1-X8 anion exchange resin in formate form, 1 ml; BioRad, Hercules, CA) and eluted. The radioactivity in the eluate was counted with a β-scintillation counter. Phospholipase C activity was estimated from the production of radioactive inositol phosphates.

Intracellular Ca²⁺ Concentration Measurement

Details of the experimental procedure have been described.¹⁷ In brief, HCM cells grown on a coverslip were incubated for 60 minutes in a loading buffer (NaCl 125 mM, KCl 5 mM, CaCl₂ 1.8 mM, MgCl₂ 2 mM, NaH₂PO₄ 0.5 mM, NaHCO₃ 5 mM, HEPES 10 mM, glucose 10 mM, bovine serum albumin 0.1%, fura-2 acetoxymethyl ester 5 µM, pH 7.2). After washing the coverslip with an assay buffer (loading buffer without bovine serum albumin and fura-2 acetoxymethyl ester), the coverslip was mounted in a chamber on the stage of a microscope. The chamber was filled with 3 ml of the assay buffer and kept at 37°C during the experiment. Intracellular fluorescence intensity at 510-nm emission wavelength, excited by alternating 340 and 380 nm excitation wavelengths, was measured by a single cell video imaging method using Image-1/F Fl Quantitative Fluorescence System (Universal Imaging, West Chester, PA). Intracellular Ca²⁺ concentration was calculated from the intensity ratio of fluorescence at these two excitation wavelengths according to the equation of Grynkiewicz et al.¹⁸ Various drugs were added into the chamber as indicated. The chamber was washed five times with assay buffer between additions of agonists.

Cyclic Adenosine Monophosphate Assay

Assay of cAMP was performed as described.¹⁹ Upon confluence, HCM cells in 24-well plates were incubated with 1 ml of DMEM–F12 nutrient mixture supplemented with 0.8 mM ascorbate and 1 mM 3-isobu-
tyl-1-methylxanthine for 10 minutes at room temperature. Then, 10 μl of drug was added into each well, and the cells were incubated for another 8 minutes. In antagonist experiments, agonists were added 8 minutes after the addition of antagonists. Cyclic AMP production induced by ET-1 was shown to be time dependent and linear up to 20 minutes after the addition of the agonist (data not shown). Thus, for convenience, a standard agonist incubation time of 8 minutes was used. After incubation, the reaction was stopped by replacing the medium with 0.2 ml of ice-cold 0.1 M acetic acid (pH 3.5). Acetic acid was neutralized with 0.3 ml of ice-cold 0.1 M sodium acetate (pH 11.6). The concentration of cAMP in an aliquot of cell lysate was determined by radioimmunoassay according to the procedure recommended by the manufacturer.

Prostaglandin E₂ Assay
Confluent HCM cells in 24-well plates were used. Cells were rinsed with 4 × 0.5 ml of DMEM–F12. Five minutes after the rinse, 10 μl of ET-1 was added to each well, and the cells were incubated at room temperature for 10 minutes. A 10-minute incubation was selected because pilot time course studies indicated that the production of PGE₂ was time dependent and linear up to 20 minutes (data not shown). At the end of incubation, aliquots of medium were transferred into polypropylene tubes, and the PGE₂ concentration in each aliquot was determined by a radioimmunoassay according to the manufacturer’s recommended procedure.

RESULTS

Endothelin-1 (100 nM) activated PLC in HCM cells by 10.9 ± 1.0-fold (mean ± SEM, n = 10) with a mean EC₅₀ of 335 pM (log [EC₅₀] = −9.48 ± 0.16, n = 10) (Fig. 1A). Its analog, ET-3, also stimulated PLC in these cells. However, ET-3 was 1000-fold less potent and was a partial agonist. Mean EC₅₀ of its stimulation was 304 nM (log [EC₅₀] = −6.52 ± 0.35, n = 3), and its maximum efficacy was only approximately 50% that of ET-1 (Fig. 1A). Thus, efficacy and potency profiles of these two endothelin peptides suggest that their effects on the HCM cells were mediated by the endothelin ETₐ receptor subtype. Indeed, sarafotoxin S6c, an endothelin ETₐ receptor subtype-selective agonist,²⁰ did not stimulate PLC in these cells (Fig. 1A). Furthermore, the PLC stimulation induced by ET-1 was inhibited completely by pretreatment with BQ610, an ETₐ receptor subtype-selective antagonist,²¹ with a mean pKi of 9.96 ± 0.47 (n = 4) (Fig. 1B).

Stimulation of PLC in smooth muscle usually leads to activation of calcium mobilization. Endothelin-1 also activated calcium mobilization in HCM cells. Figure 2 is an example of selected pseudocolor HCM cell images showing calculated values of [Ca²⁺], before and after the addition of 1 nM of ET-1. It is clear that all ciliary muscle cells responded to ET-1. These images could be quantified and expressed in relation to time (Fig. 3). The mean resting [Ca²⁺], in these cells was 73.4 ± 9.3 nM (n = 40). Addition of 1 nM ET-1 caused an increase in [Ca²⁺], in these cells, with a peak [Ca²⁺], of 888 ± 116 nM (n = 21) followed by a sustained elevation until the peptide was removed (Fig. 3A). This ET-1-induced increase in intracellular calcium concentration reached its peak 2 to 4 minutes after the addition of the peptide.

The effect of ET-1 on calcium mobilization evidently was mediated also by the ETₐ receptor subtype because sarafotoxin S6c, at concentrations as high as 100 nM, did not activate calcium mobilization (Fig. 3B).
FIGURE 2. Pseudocolor ratio images of fura-2 loaded human ciliary muscle cells showing
time-dependent changes of intracellular calcium concentration after 1 nM endothelin-1
treatment. The peptide was administered at time 0. The spectrum on the right indicates
calculated calcium concentration represented by each color.

3B). Accordingly, pretreatment of the cells with 1 nM of BQ610 completely blocked increase in [Ca^{2+}], induced by 10 nM of ET-1 (data not shown).

In addition to activation of PLC and calcium mobilization, we further found that, in HCM cells, ET-1 stimulated adenylyl cyclase (AC) by 2-fold to 7-fold (mean = 3.0 ± 0.5), with a mean EC_{50} of 5.2 nM (log EC_{50} = -8.29 ± 0.09, n = 7) (Fig. 4A). Similar to its effects on PLC and [Ca^{2+}], ET-1 activation of AC seemed to be mediated by the ETA receptor subtype, as indicated by the fact that sarafotoxin S6c, at concentrations as high as 1 μM, did not affect AC activity (Fig. 4A). Additionally, the AC stimulation induced by ET-1 was antagonized by the pretreatment with BQ610 (pK_{A} = -11.19 ± 0.33, n = 3) (Fig. 4B).

Endothelins are not known to stimulate directly the activity of adenylyl cyclases.\textsuperscript{14} We tested whether the stimulative effect of ET-1 on AC in these cells was mediated by the production of PGE_{2}. Figure 5A shows that addition of PGE_{2} onto HCM cells stimulated cAMP accumulation by 21.8 ± 0.5-fold, with a mean EC_{50} of 239 nM (log [EC_{50}] = -6.62 ± 0.28, n = 4). More important, ET-1 increased the concentration of PGE_{2} in culture medium of HCM cells in a concentration-dependent manner. Under the conditions used, the resting concentration of PGE_{2} in the medium was 2.1 ± 0.2 nM (mean ± SEM, n = 6). At 100 nM, ET-1 increased PGE_{2} concentration to 4.1 ± 0.2 nM (n = 6). The stimulatory effect of ET-1 had a mean EC_{50} of 12.0 nM (log [EC_{50}] = -7.92 ± 0.18, n = 3) (Fig. 5B), which approximates the EC_{50} of ET-1 for AC stimulation.

To clarify further the involvement of PGE_{2} in the ET-1-stimulated cAMP accumulation, HCM cells were treated with indomethacin (10 μM), a cyclooxygenase inhibitor, to inhibit the ET-1-induced production of PGE_{2}; concentrations of second messengers, as well as of PGE_{2}, were then measured. As demonstrated in

In HCM cells, ET-1 increased the production of inositol phosphates and [Ca^{2+}],. These results agree with those described by Stahl et al., who observed that 100 nM ET-1 induced calcium mobilization in HCM cells. We further discovered that both the PLC and calcium effects of ET-1 apparently were mediated by the endothelin ETA receptor subtype because ET-1 was much more potent and efficacious than ET-3 in the activation of PLC; even at high doses, sarafotoxin S6c, an ETB receptor subtype-selective agonist, failed to mimic ET-1; and BQ610, an ETA-receptor subtype-selective antagonist, potently and completely inhibited the effects of ET-1. These stimulatory effects of endothelins compare very well with those reported in many smooth muscles, as well as in several ocular cells and tissues. For example, ET-1 was shown to stimulate PLC in the iris sphincter of the rabbit, dog, cat, and pig, but not

Figure 6, indomethacin pretreatment completely blocked PGE2 production caused by ET-1 as predicted (Fig. 6B). Similarly, it completely suppressed the increase in cAMP production resulting from ET-1 treatment (Fig. 6C). The suppression in AC by indomethacin was most likely an outcome of its inhibition of PGE2 production because indomethacin did not directly affect AC activity, as indicated by its lack of effect on isoproterenol-activated AC (Fig. 6D). Indomethacin, at the concentration used, also did not affect the stimulatory effect of ET-1 on PLC in HCM cells (Fig. 6A), suggesting that indomethacin did not nonspecifically modify the actions of ET-1.

DISCUSSION

In HCM cells, ET-1 increased the production of inositol phosphates and [Ca^{2+}]. These results agree with
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bovine, rhesus monkey, or human. The peptide also increased intracellular calcium in cultured bovine trabecular meshwork cells. Functionally, activation of PLC and calcium mobilization by ET-1 are related to contraction of these tissues. Abdel-Latif and Zhang demonstrated that ET-1 caused contraction of both iris sphincter and dilator muscles of the rabbit. Similarly, Lepple-Wienhues and colleagues showed that ET-1 was a potent contracting agent for bovine trabecular meshwork. Considering that a similar signal transduction pathway is likely to be present in human ciliary muscles and that contraction by ET-1 was observed in bovine ciliary muscle strips, our results suggest that endothelins, by activation of the ET_A receptor, should give rise to contraction of human ciliary muscles. Indeed, by using a cell contraction assay described previously, ET-1 (10 nM or 100 nM) caused a 50% decrease in cross-sectional surface area of the HCM cells (Pang I-H, unpublished observation, 1993). This finding is consistent with the above hypothesis. Further experiments are underway to confirm this observation.

FIGURE 5. Involvement of prostaglandin E2 (PGE2) on endothelin (ET)-1-activated adenylyl cyclase (AC). (A) Representative concentration–response curve of PGE2-induced AC activation in human ciliary muscle cells. Similar results were obtained in four independent studies. (B) Representative concentration–response curve of ET-1-induced PGE2 formation. Three independent experiments generated similar results. Each symbol represents datum from a single sample.

Activation of calcium mobilization by the ET_A receptor and subsequent contraction of the muscles should increase aqueous outflow by traction on the scleral spur and thus lower IOP. Our results predict that an ET_A receptor-selective agonist will be ocular hypotensive in humans and will mimic the outflow effect of ET-1 in the anesthetized monkey eye. Whether activation of the ET_B receptor affects intracellular pressure in humans is not established. However, Haque et al reported that the ET_B receptor-selective agonist, sarafotoxin S6c, lowered intraocular pressure in rabbits, but with a clearly slower time of onset when compared to ET-1. They suggested that the ET_A receptor probably mediates the early phase, and that the ET_B receptor plays a key role in the later phase of the endothelin effect. We speculate that the ET_B receptor-mediated hypotensive effect probably involves tissues other than the ciliary muscle.

In addition to the stimulation of PLC and [Ca^{2+}]), we showed that ET-1 increased production of PGE2 and cAMP in a dose-dependent manner in HCM cells. The increase in cAMP was inhibited by BQ610 and was not mimicked by sarafotoxin S6c, indicating that activation of AC by ET-1 in HCM cells similarly was mediated by the ET_A receptor subtype. It is interesting to note that in bovine iris sphincter muscle, ET-1 activation of cAMP formation apparently was mediated by the ET_B receptor subtype. Because direct stimulation of AC by endothelins is not known to occur, this enzyme probably was stimulated by ET-1 indirectly, that is, AC was stimulated by product(s) downstream of PLC activation or other endothelin effects in these cells.

Endothelins were reported to release arachidonic acid and prostaglandins by stimulating phospholipase A2 in cultured vascular smooth muscle and endothelial cells. Abdel–Latif et al observed the same phenomenon in rabbit iris sphincter smooth muscle. Certain prostaglandins can activate AC and increase intracellular cAMP. Consequently, ET-1-activation of AC in HCM cells could be a result of enhanced prostaglandin production. Of the various prostaglandins tested, only PGE2 was found to be effective in increasing cAMP in these cells. Other prostaglandins, such as PGD2 and PGF2a, did not affect AC (data not shown). The finding that PGE2 concentration in the cell medium was increased by ET-1 further provides a probable mechanism for the cAMP effect of ET-1. Yet, it may be argued that the change in
FIGURE 6. Effect of indomethacin on (A) phospholipase C activation by endothelin (ET)-1, (B) ET-1-induced increase in prostaglandin E₂ production, (C) ET-1-activated adenylyl cyclase, and (D) isoproterenol-activated adenylyl cyclase. Concentrations of compounds used in these studies: ET-1, 100 nM; indomethacin, 10 μM; isoproterenol, 1 μM. All data are presented as mean value ± SEM. Responses of ET-1 (A to C) or isoproterenol (D) alone define 100%, and response of vehicle alone defines 0%. Indo = indomethacin; Iso = isoproterenol.

concentrations of PGE₂ induced by ET-1 was not sufficient to account for the ET-1-activated cAMP production. The maximum amount of PGE₂ in culture medium after ET-1 treatment was approximately 4 nM, a very low concentration compared to the level of PGE₂ required to stimulate AC (EC₅₀ = 239 nM) in HCM cells. However, we could only measure the PGE₂ level in the culture supernatant, which theoretically represents a significant dilution when compared to the prostaglandin level at the proximity of the cell monolayer where PGE₂ was formed and where the cells should encounter a much higher concentration, perhaps as high as 100-fold. If this assumption is correct, the discrepancy between the measured PGE₂ level and that required to activate AC is not a major concern, and the data presented do not disagree with the above hypothesis.

Thus, the findings in this study are consistent with the proposed hypothesis that ET-1 increases production of PGE₂, which subsequently stimulates AC and enhances the accumulation of cAMP. To confirm this hypothesis, a cyclooxygenase inhibitor, indomethacin, was used to block the ET-1-induced production of PGE₂. Blockade of PGE₂ production specifically inhibited endothelin-induced cAMP formation without affecting other ET-1 actions or isoproterenol-induced cAMP formation. These cumulative results suggest that stimulation of AC by ET-1 was mediated indirectly by the enhanced PGE₂ production. However, it is not clear whether ET-1 increases prostaglandin production by increasing intracellular calcium or directly by a parallel pathway that is independent of PLC and [Ca²⁺].

Currently, the physiological and pharmacologic significance of ET-1-induced PGE₂ and cAMP production in HCM cells is uncertain. Increased concentration of cAMP in the ciliary muscle causes relaxation (see ref. 31, for example), which probably compromises the contractile effect of endothelins. In bovine ciliary muscles, the maximum contraction generated by ET-1 was only 52% of that generated by carbachol. It is possible that a prostaglandin-mediated increase
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in cAMP acts as a modulator of the contractile effects of endothelins in a classical second messenger “cross-talk” mechanism.

Prostaglandins have been implicated to be responsible for the initial ocular pressure rise after intravitreal injection of ET-1. The exact prostaglandin species involved in this action is still unknown. Because we did not quantify prostaglandins other than PGE_2 in this study, the inventory of prostaglandins released by ET-1 from the HCM cells is yet to be established. It will not be surprising if the ciliary muscle, together with the iris sphincter, contributes to the ET-1-induced production of ocular hypertensive metabolite(s) of arachidonic acid.

The current study demonstrated that endothelins activated phospholipase C, calcium mobilization, PGE_2, and cAMP production in human ciliary muscle cells. All these actions apparently were mediated by the ET_A receptor. Their contribution in ciliary muscle functions and subsequently on ocular biology could be significant. Further studies are being conducted to clarify some of these interesting issues.

Key Words
ciliary muscle cell, cyclic adenosine monophosphate, endothelin, intracellular calcium, phospholipase C

Acknowledgments
The authors thank Drs. Ernst Tamm and Elke Lütjen-Drecoll for providing the human ciliary muscle cells.

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