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Mitogenic activity of endothelin on human cultured prostatic smooth muscle cells

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Abstract

The effects of endothelins on human prostatic smooth-muscle cell growth were examined. Endothelin-1 and endothelin-3 induced a concentration-dependent increase in DNA synthesis and also promoted cell growth. Use of subtype selective antagonists BQ-123 ((cyclo(D-Trp-D-Asp(ONa)-Pro-D-Val-Leu); endothelin ET_A receptor selective) and BQ-788 ((*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methyl Leu-D-Trp-(COOMe)-D-Nle-ONa); endothelin ET_B receptor selective), indicated that mitogenic effects of endothelin were mediated through activation of both endothelin ET_A and ET_B receptors. The mitogenic effects of endothelin-1 and endothelin-3 were significantly inhibited by pretreatment of the cells with pertussis toxin. However, mitogenesis due to basic fibroblast growth factor was not affected. In conclusion, endothelin has mitogenic effects on human prostatic smooth muscle cells through activation of both endothelin ET_A and ET_B receptors via different signalling pathways from basic fibroblast growth factor. This may contribute to smooth muscle hyperplasia associated with benign prostatic hyperplasia.

Keywords: Prostate; Smooth muscle cell; Endothelin receptor; Proliferation

1. Introduction

Endothelin was initially identified as a potent vasoconstricting peptide isolated from culture medium of porcine vascular endothelial cells (Yanagisawa et al., 1988). Endothelin consists of three isopeptides, endothelin-1, endothelin-2 and endothelin-3. The endothelins have a variety of important physiological functions associated with the cardiovascular, renal, pulmonary and nervous systems (Masaki et al., 1992; Rubanyi and Polokoff, 1994). These activities are exerted through cell surface endothelin receptors. Endothelin receptors are thought to be divided into two subtypes, endothelin ET_A and ET_B. The endothelin ET_A receptor has higher affinity for endothelin-1 and endothelin-2 than endothelin-3, whereas the endothelin ET_B receptor has no isopeptide selectivity (Arai et al., 1990; Sakurai et al., 1990). It is reported that endothelin receptors are present in the human prostate and mediate contractile response of the tissue (Kobayashi et al., 1994a,b).

The prostate is composed of stromal and epithelial components. Smooth muscle cells in the stroma represent 22% of total prostate area (Shapiro et al., 1992b) and mediate prostate contraction. Benign prostatic hyperplasia, which is the most common benign tumor in males, results from hyperplasia of the cellular elements of the prostate gland. The stromal components of the prostate play a major role in the pathogenesis of benign prostatic hyperplasia (Shapiro et al., 1992a). Approximately 30% of men will undergo surgical resection or enucleation of the prostate by age 70 owing to the development of prostatism or bladder outlet obstruction resulting from hyperplasia (Barry, 1990). However, the etiology of human benign prostatic hyperplasia is not well characterized.

Endothelin has been reported to induce proliferation of various cells (Battistini et al., 1993). The mitogenic effects of endothelin on human prostatic smooth muscle cells may play an important role in the pathophysiology of benign prostatic hyperplasia. Nevertheless, there have been no previous reports on the effects of endothelin as a growth

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factor in prostatic smooth muscle cells. We recently reported that both endothelin ET_A and ET_B receptors are present in human prostatic smooth muscle cells (HP cells) and stimulation of both subtypes produced an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) via pertussis toxin sensitive pathway (Saita et al., 1997). In order to elucidate the function of endothelin ET_A and ET_B receptors in HP cells, we investigated both the mitogenic effect of endothelins on human prostatic smooth muscle cells and their signalling pathway using pertussis toxin.

2. Materials and methods

2.1. Drugs

The following drugs were used: [^{3}H]thymidine (83.2 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA); endothelin-1 and endothelin-3 were from the Peptide Institute (Osaka, Japan) and, pertussis toxin and basic fibroblast growth factor (basic FGF) were from Sigma (St. Louis, MO, USA). BQ-123, (cyclo(D-Trp-D-Asp(ONa)-Pro-D-Val-Leu) and BQ-788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methyl Leu-D-Trp-(COOMe)-D-Nle-ONa), were synthesized by Yamanouchi Pharmaceutical (Tokyo, Japan). Other chemicals used were of analytical grade.

2.2. Cell culture

Human prostate tissue was obtained by radical prostatectomy from 72-year-old patient with benign prostatic hyperplasia. A primary culture of smooth muscle cells from human prostate tissue (HP cell) was obtained by an explant method as described previously (Yazawa et al., 1994). Cells were cultured in phenol red free RPMI 1640 medium supplemented with 15% fetal bovine serum, 2.5 μg/ml testosterone propionate, 1 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μ/ml human transferrin, 5 ng/ml sodium selenite, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. Cells from passages 6 to 20 were used in this study. There was no significant difference in results from cells used in any passage below number 20.

2.3. [^{3}H]thymidine incorporation

HP cells were seeded at a density of 2×10^4 cells in 1 ml of medium in 24-well plates and grown for 48 h. Cells were deprived of serum and all supplements for 48 h in phenol red free RPMI 1640 medium to arrest the cell growth. Agonists were then added, and after a 20-h incubation, the cells were pulse-labelled for 4 h with 1 μCi/ml [^{3}H]thymidine (16.8 mCi/mmol). Radiolabeled DNA was extracted by washing the cells twice with cold phosphate-buffered saline, precipitating them with 10% trichloroacetic acid for 1 h at 4°C, and lysing them with 0.5 M NaOH. The radioactivity was measured using liquid scintillation counting. In experiments with endothelin antagonists, the cells were incubated with antagonists 30 min before addition of agonists. The pertussis toxin was added 3 h before addition of the agonists and maintained in culture medium throughout the assay.

2.4. Cell proliferation assay

HP cells were seeded at a density of 1.5×10^5 cells in 25 cm^2 flasks to grow for 48 h. Cells were deprived of serum and all supplements for 48 h in phenol red free RPMI 1640 medium to arrest the cell growth. Agonists were then added, and after a 72-h incubation, the cells were detached by trypsinization, and counted using a hemocytometer.

2.5. Analysis of data

Results are expressed as the mean $\pm$ S.E. Statistical significance was assessed by a one-way analysis of variance followed by Dunnet's multiple comparison test. Probabilities of less than 5% ($P < 0.05$) were considered significant.

3. Results

3.1. Mitogenic activity of endothelin on HP cells

Endothelin-1 induced a concentration dependent increase in DNA synthesis, as assessed by [^{3}H]thymidine incorporation, in human prostatic smooth muscle cells (HP cells). Endothelin-3, a selective endothelin ET_B receptor agonist, also induced an increase in DNA synthesis (Fig. 1). Endothelin-1 and endothelin-3 at 10 nM stimulated DNA synthesis to 2.8- and 2.2-fold from basal level, respectively. Another endothelin ET_B receptor agonist sarafotoxin S6c gave a similar magnitude of response as endothelin-3 (data not shown). Basic FGF, a mitogen to human prostatic stromal cells (Kassen et al., 1996; Sherwood et al., 1992), also induced an increase in DNA synthesis in HP cells. A submaximal response was reached at 10 ng/ml (a 2.1-fold increase from basal level; data not shown). Since DNA synthesis may occur without cell division, the HP cell number was counted after stimulation by either endothelins or basic FGF (Fig. 2). Endothelin-1 and endothelin-3 at 10 nM significantly increased cell number comparable to basic FGF at 10 ng/ml.

Increase in DNA synthesis and cell number by endothelin-1 was inclined to be more potent than that by endothelin-3.

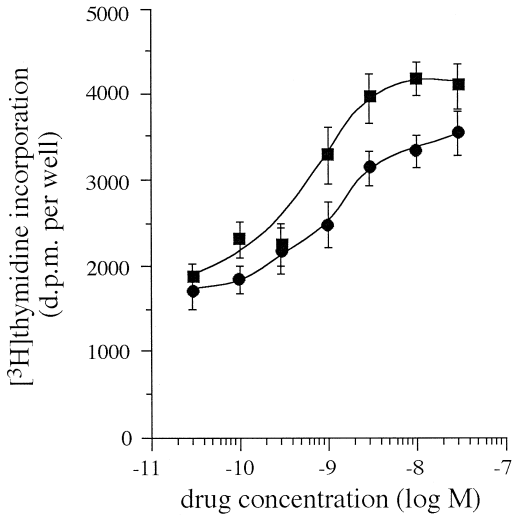

Fig. 1. Concentration-response curves for endothelin-1-(■)- and endothelin-3-(●)-induced DNA synthesis in human cultured prostatic smooth muscle cells. Growth-arrested cells were stimulated with increasing concentrations of endothelin-1 or endothelin-3 for 20 h. As described in Section 2, [^{3}H]thymidine incorporation was measured after 20 h stimulation with agonists. Basal [^{3}H]thymidine incorporation was 1495 ± 76 dpm. Each point shows the mean of at least six separate experiments in duplicate; vertical lines indicate S.E.

3.2. Endothelin receptor subtypes which mediate mitogenic activity

We used BQ-123, a selective endothelin ET_A receptor antagonist, and BQ-788, a selective endothelin ET_B receptor antagonist, to characterize which endothelin receptor subtypes mediate mitogenic activity. Fig. 3 shows the effects of increasing concentrations of BQ-123 and BQ-788

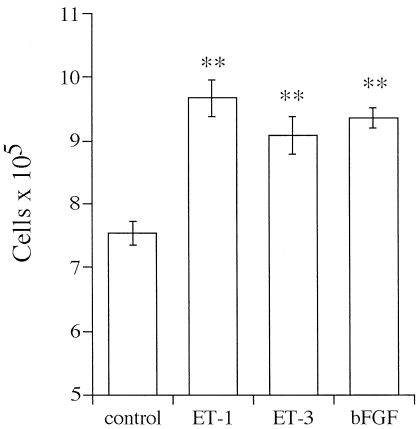

Fig. 2. Effects of endothelin-1 (ET-1), endothelin-3 (ET-3) and basic FGF (bFGF) on proliferation of human cultured prostatic smooth muscle cells. Growth-arrested cells were stimulated with 10 nM endothelin-1, endothelin-3 or 10 ng/ml basic FGF for 72 h. Cells were counted as described in Section 2. Each datum represents mean ($n = 9$); vertical lines indicate S.E. $**P < 0.01$ compared with the data in the absence of agonist (control).

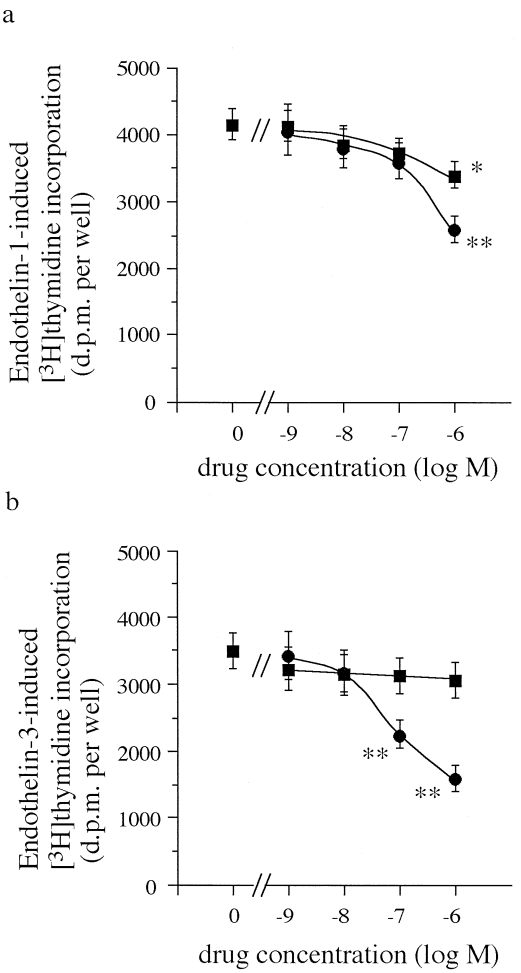

Fig. 3. Effects of BQ-123 (■) and BQ-788 (●) on (a) endothelin-1 and (b) endothelin-3 induced DNA synthesis in human cultured prostatic smooth muscle cells. [^{3}H]thymidine incorporation was measured in growth-arrested cells as described in Section 2. Increasing concentrations of BQ-123 or BQ-788 were applied to the cells 30 min before stimulation with 10 nM endothelin-1 or 10 nM endothelin-3. Basal [^{3}H]thymidine incorporation was 1392 ± 62 dpm. Each point shows the mean of at least 6 separate experiments in duplicate; vertical lines indicate S.E. $*P < 0.05$, and $**P < 0.01$, compared with the data in the absence of antagonist.

on endothelin-1- and endothelin-3-induced [^{3}H]thymidine incorporation. Up to 1 μM of these antagonists were used. BQ-123 at 1 μM significantly inhibited endothelin-1-induced increase in DNA synthesis, but had no effect on endothelin-3-induced increases. BQ-788 at 1 μM significantly inhibited both endothelin-1- and endothelin-3-induced increase in DNA synthesis. However, these antagonists had weaker activity than was expected from binding studies (Saita et al., 1997). Either BQ-123 or BQ-788 alone had just a marginal effect on basal and basic FGF-induced [^{3}H]thymidine incorporation (data not shown). These results indicate that the mitogenic effect by endothelin in HP cells is mediated via activation of both endothelin ET_A and ET_B receptors.

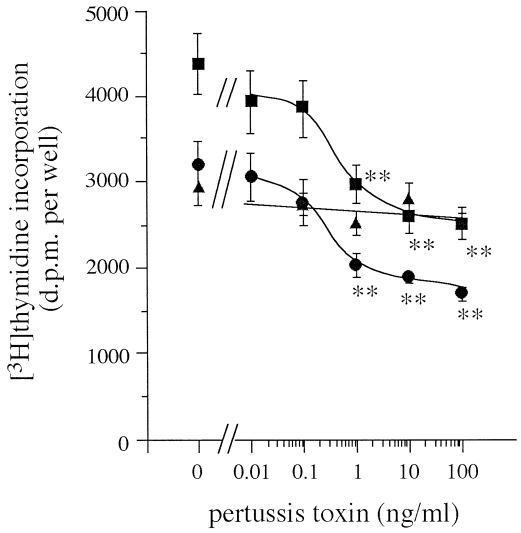

Fig. 4. Effects of pertussis toxin on endothelin-1-(■), endothelin-3-(•) and basic FGF-(▲) induced DNA synthesis in human cultured prostatic smooth muscle cells. Pertussis toxin was added to growth-arrested cells for 3 h before stimulation with 10 nM endothelin-1, endothelin-3 or 10 ng/ml basic FGF and maintained throughout the assay. [^{3}H]thymidine incorporation was measured as described in Section 2. Basal [^{3}H]thymidine incorporation was 1342 ± 43 dpm. Each point shows the mean of at least 6 separate experiments in duplicate; vertical lines indicate S.E. $**$ $P < 0.01$ compared with the data in the absence of pertussis toxin.

3.3. Pertussis toxin sensitivity for endothelin and basic FGF-induced DNA synthesis

Pertussis toxin catalyzes the ADP-ribosylation of some GTP-binding protein, and uncouples them from their linked receptors (Gilman, 1987). We used pertussis toxin to examine the intracellular mitogenic signalling pathways activated by endothelin-1, endothelin-3 and basic FGF. A 3-h pretreatment of the cells with increasing concentrations of pertussis toxin induced a concentration dependent reduction in endothelin-1- and endothelin-3-induced increase in DNA synthesis (Fig. 4). Inhibition of endothelin-1-induced increase in DNA synthesis by pertussis toxin was partial, but significant at more than 1 ng/ml pertussis toxin, and endothelin-3-induced increase was inhibited by pertussis toxin to nearly basal level. However, pertussis toxin did not affect mitogenesis induced by basic FGF. These results indicate that the signal transduction pathways for the endothelins are different from those of basic FGF.

4. Discussion

The major findings of the present study are: (1) endothelin-1 and endothelin-3 exert mitogenic effects on human prostatic smooth muscle cells; (2) mitogenic effects due to endothelin-1 are mediated through activation of both endothelin ET_A and ET_B receptors; and (3) experiments using pertussis toxin showed that mitogenic signalling of the endothelins is different from that of basic FGF. These results suggest that endothelins may play a role in mediating human prostatic smooth muscle hyperplasia seen in patients with benign prostatic hyperplasia.

The mitogenic effects of endothelin in vascular smooth muscle (Bobik et al., 1990; Eguchi et al., 1992) pulmonary artery smooth muscle (Hassoun et al., 1992; Zamora et al., 1993) and airway smooth muscle (Panettieri et al., 1996) have been well documented. In these systems, the effects of endothelin appear due to stimulation of the endothelin ET_A, but not the ET_B, receptor subtype. This report is the first to demonstrate that endothelin has mitogenic activity in human prostatic smooth muscle cells. Endothelin-1 and endothelin-3 at concentrations of 10 nM produce a 2.8- and 2.2-fold increase in DNA synthesis, respectively, and stimulate cell proliferation (Figs. 1 and 2). Endothelin receptor subtypes responsible for the mitogenic activity were characterized using the selective ligands endothelin-3 (endothelin ET_B receptor agonist), BQ-123 (endothelin ET_A receptor antagonist) and BQ-788 (endothelin ET_B receptor antagonist). Endothelin-3 induced a concentration-dependent increase in DNA synthesis. These effects were inhibited by BQ-788, but not BQ-123. The effects of endothelin-1 on DNA synthesis were inclined to be more potent than that of endothelin-3. Endothelin-1 was antagonized by both BQ-123 and BQ-788 (Figs. 1 and 3). These data indicate that both endothelin ET_A and ET_B receptors mediate their mitogenic effects by endothelin. The endothelin receptor subtypes mediating mitogenic effects of endothelin in human prostatic smooth muscle cells were shown to be different from those in vascular, pulmonary artery and airway smooth muscle cells.

Higher concentrations of antagonists would be required to block mitogenic response as opposed to receptor binding (Fig. 3). One explanation for this relative inefficiency of endothelin antagonists in blocking mitogenic effect by endothelin is due to reversal binding of antagonists to the receptors and irreversal binding of endothelin. Endothelin antagonists may not effectively compete with endothelin for the binding to the receptors in longer time incubations. Vigne et al. (1993) showed BQ-123 forms less stable complexes with endothelin ET_A receptors than the complexes formed by endothelin and the receptors. Another explanation is that endothelin antagonists were degraded into inactive form by the cells. However, this is unlikely because high performance liquid chromatographic analysis revealed that no degradation of antagonist was observed under our experimental condition (data not shown).

Langenstroer et al. (1993) reported that endothelin-1 immunoreactivity in human prostate was present primarily in the glandular epithelium. Consequently, endothelin released from glandular epithelium may cause contraction of smooth muscle in stroma in a paracrine manner, and also may play a role in cell growth. Derangement of cell growth by endothelin-1 has been implicated in several pathophysiologic conditions including atherosclerosis, car-

diac hypertrophy, and glomerulosclerosis (Masaki, 1993; Simonson, 1993). Kondo et al. (1995) reported that significant increases was observed in endothelin receptors with benign prostatic hyperplasia compared to normal prostate. It may be possible that inappropriate endothelin secretion from glandular epithelium and/or overexpression of endothelin receptors in prostate cells eventually leads to benign prostatic hyperplasia. Unfortunately, comparison of tissue endothelin levels between normal prostate and benign prostatic hyperplasia has not yet been examined. Further investigation is need to clarify the relevance between endothelin and benign prostatic hyperplasia.

Physiologic and pharmacologic studies provide compelling evidence that the tension of human prostatic smooth muscle contributes to urethral obstruction (Caine et al., 1976). Therefore, smooth muscle cell relaxants such as tamuslosin (YM617), a selective alpha1A receptor antagonist, can be successfully used to improve BPH symptoms (Kawabe et al., 1990; Yazawa et al., 1992). In the human prostate, both endothelin ET_A and ET_B receptors mediate contraction of prostate smooth muscle in a manner roughly comparable to that mediated through α_1 receptors (Kobayashi et al., 1994a; Langenstroer et al., 1993). Use of combined endothelin ET_A/ET_B receptor antagonists rather than one selective for endothelin ET_A or ET_B receptors may be effective therapy for benign prostatic hyperplasia symptoms in a fashion similar to alpha1 blockers. Furthermore, it is possible that combined endothelin antagonists will be ideal drugs to suppress cell growth in the prostate, and inhibit prostate hyperplasia. However, it is important to investigate whether endothelin plays a central role in prostate cell growth, because other growth factors such as androgen and basic FGF could also contribute to it. It will prove interesting to investigate the action of endothelin in synergism with other growth factors.

Basic FGF is known to activate receptor tyrosine kinase, and to be a mitogen for prostatic stromal cells (Sherwood et al., 1992). It has been demonstrated that basic FGF is also mitogenic to prostatic smooth muscle cell in this experiment. We used pertussis toxin to investigate the signalling pathways of both endothelin and basic FGF. The effects of basic FGF were not inhibited by pertussis toxin, while those of endothelin-3 were inhibited to a basal level. This suggests that mitogenic response via activation of the endothelin ET_B receptor is sensitive to pertussis toxin. Endothelin-1-induced increase in DNA synthesis was partially sensitive to pertussis toxin, suggesting mitogenic response by endothelin-1 may be mediated by more than one pathway. The inhibitory action of pertussis toxin was not a consequence of toxicity, because basic FGF induced mitogenic response was not affected. These results suggest that the mitogenic activity of endothelins are mediated via pertussis toxin-sensitive GTP-binding protein. Growth of prostatic smooth muscle cells is shown to be regulated by several growth factors and also several signalling pathways.

It has been reported that mitogenesis is induced by mechanisms that are apparently dependent on either the activation of phospholipase C or tyrosine kinase (Chambard et al., 1987). There are several reports that the endothelin receptor elicits the activation of both phospholipase C and tyrosine–kinase pathways (Imokawa et al., 1996; Simonson et al., 1996). It was previously reported that both endothelin ET_A and ET_B receptor subtypes in HP cells evoke $[Ca^{2+}]_i$ increase via phospholipase C activation and this increase is sensitive to pertussis toxin (Saita et al., 1997). We are now investigating the involvement of phospholipase C or tyrosine-kinase pathways in growth stimulation by endothelin.

In summary, the present data indicate that endothelins induce human prostate smooth muscle cell proliferation. Use of subtype selective ligands indicate that mitogenic effect of endothelin is mediated via activation of both endothelin ET_A and ET_B receptors. Pertussis toxin sensitivity of DNA synthesis clearly demonstrates that mitogenic signalling by endothelin was different from that of basic FGF. These findings may have pathophysiologic significance on benign prostatic hyperplasia.

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