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EXPERIMENTAL STUDY

Potentiation response of cultured human uterine leiomyoma cells to various growth factors by endothelin-1: role of protein kinase C

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Abstract

Objective: Factors responsible for the abnormal proliferation of myometrial cells that accompanies leiomyoma formation are unknown, although steroid hormones and peptide growth factors have been implicated. We hypothesized that endothelin-1 (ET-1) is a physiological regulator of tumor growth.
Design: In this study, we investigated the role of ET-1 on growth of human leiomyoma cells and its synergistic effect with growth factors, as well as the signaling pathway involved in this interaction.
Methods: Leiomyoma cell proliferation was assayed by [^{3}H]thymidine incorporation and cell number. Protein kinase C (PKC) isoforms were analyzed by Western blot using specific antibodies.
Results: ET-1 on its own was unable to stimulate DNA synthesis but potentiated the leiomyoma cell growth effects of basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), IGF-I and IGF-II. The failure of a protein tyrosine kinase (PTK) inhibitor, tyrphostin 51, to affect the potentiating effect of ET-1, supports the hypothesis of non-involvement of PTK in this process. The inhibition of PKC by calphostin C or its down-regulation by phorbol 12,13-dibutyrate (PDB) eliminated the potentiating effect of ET-1, but did not block cell proliferation induced by the growth factors alone. Five PKC isoforms (α, β1, ϵ, δ and ζ) were detected in leiomyoma cells, but only phorbol ester-sensitive PKC isoforms (PKCα, ϵ and δ) contribute to the potentiating effect of leiomyoma cell growth by ET-1.
Conclusions: We have demonstrated that ET-1 potentiates leiomyoma cell proliferation to growth factors through a PKC-dependent pathway. These findings suggest a possible involvement of ET-1 in the pathogenesis of leiomyomas.

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Introduction

Uterine leiomyomas are benign smooth muscle tumors of the myometrium arising in as many as 30% of women over 35 years old. Leiomyomas are the commonest indication for hysterectomy due to menorrhagia and pelvic pain. Factors responsible for enlargement of leiomyomas have been poorly understood. Steroid hormones are strongly implicated because these tumors appear during the reproductive years, increase in size during pregnancy and stabilize or regress after menopause. Traditionally, estrogen has been considered as the major promoter of leiomyoma growth, but clinical observations also suggest that progesterone plays a critical role in the pathogenesis of uterine leiomyomas (for a review see ref. 1). The mitogenic effects of estrogens are likely mediated by local production of growth factors and their receptors (2, 3). Leiomyoma cells produce more of several growth factors such as epidermal growth factor (EGF), insulin-like growth factors (IGF-I, IGF-II) and basic fibroblast growth factor (bFGF) than do the normal myometrial cells and more of their corresponding receptors (4–8). Attention has recently been drawn to the possibility that endothelin-1 (ET-1) might be a promoter of myometrial cell growth because this peptide may be involved in the development of a variety of neoplasms, acting in an autocrine/paracrine fashion during tumor development and metastasis (9). The exclusive presence of ET_A receptors functionally coupled to phospholipase C in leiomyomas (10–12), together with the establishment of ET-1 growth properties in cultured human myometrial cells (13), led to the postulation that ET-1 might be a new regulator of the growth of leiomyomas.

Despite accumulating evidence of the pathophysiological significance of ET-1 as a mitogen, there is still no confirmation that this peptide has growth properties in human leiomyoma cells. Conventionally, calcium, protein kinase C (PKC) and protein tyrosine kinase (PTK) signaling pathways are thought to contribute to ET-1-induced cell growth and differentiation. However, in normal human myometrial cells, substantial evidence indicates that only the activation of PKC is required for ET-1-induced proliferation (14). PKCs constitute an expanding multigene family, so far with 12 known isoforms classified into three subfamilies on the basis of their structure and ability to bind cofactors. These are: the conventional PKC isoforms (α, β1, β2, γ), which can be activated by calcium and diacylglycerol (DAG) or phorbol esters; the novel PKC isoforms (δ, ϵ, η, θ), which can be activated by DAG and phorbol esters, but in a calcium-independent manner; and the atypical PKC isoforms (ζ, ι, λ). This last group is unresponsive to calcium and DAG or phorbol esters, but is regulated by other phospholipidic mediators (for a review see ref. 15). However, it was not then possible to say whether PKC is responsible for growth factor-induced proliferation in cultured leiomyoma cells, or is necessary and/or sufficient for the synergistic effect of ET-1 with growth factors.

Accordingly, the first aim of this study was to determine whether ET-1 by itself is mitogenic for cultured human leiomyoma cells, or if it acts as a comitogen with growth factors to induce DNA synthesis and cell proliferation. The second aim of the study was to examine whether PKC and PTK are coupled to downstream signaling pathways that regulate leiomyoma cell growth by ET-1.

Materials and methods

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM) with or without phenol red and fetal calf serum (FCS) were from GIBCO Life Technologies (Cergy, Pontoise, France). [methyl-^{3}H]thymidine (50 Ci/mmol), Hybond-C membranes, the enhanced chemiluminescence detection [system (ECL), and X-rays films were obtained from Amersham International (Chalfont, Buckinghamshire, UK). ET-1, sarafotoxin 6c (S6c) were from Neosystem Laboratoire (Strasbourg, France). Antibodies against PKCα, PKCϵ and PKCζ were from Sigma (St Louis, MO, USA). They were raised in rabbits against peptides 659–672, 726–737 and 577–592 respectively. Antibodies against PKCβ1 and PKCδ were from Santa Cruz Biotechnology (Le Perray-en-Yvelines, France). They were raised in rabbits against peptides corresponding to amino acid sequences 656–671 and 656–673 respectively. The second antibody, donkey anti-rabbit IgG conjugated to horseradish peroxidase, was purchased from Amersham International, and pre-stained molecular-weight markers were from Bio-Rad (Richmond, CA, USA). EGF, IGF-I, IGF-II and bFGF were purchased from Chemicon International (Temecula, CA, USA). Phorbol 12,13-dibutyrate (PDB), leupeptin, Nonidet P-40, phenylmethylsulfonylfluoride (PMSF), calphostin C, tyrphostin 51, and other drugs and chemicals used were of the highest quality available from Sigma.

Leiomyoma cell culture

Tissue specimens were obtained from four cycling women aged 39–45 years undergoing hysterectomy for symptomatic uterine leiomyomas. All women were in the follicular phase of the menstrual cycle. None had received hormone treatment for at least 3 months before surgery. The uteri were examined by a pathologist to exclude adenomyosis or malignant change. The samples of intramural leiomyoma were all of similar size (2 cm diameter) to standardize the experimental protocol. To avoid contamination with endometrium, no submucosal leiomyoma was collected. After collection, the samples of leiomyoma were placed in DMEM supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. This study was approved by the Comité Consultatif de Protection des Personnes pour la Recherche Biomédicale (Paris-Cochin, France).

Leiomyoma cells were prepared using the explant method. Cells were cultured in DMEM supplemented with antibiotics and 10% FCS. For cell growth experiments, leiomyoma cells were used between *passages* 3 and 6, with no noticeable difference in results observed with cells from individual *passages* or with cells isolated from different uteri. Confluent leiomyoma cells were identified by their positive reaction with monoclonal antibodies against smooth muscle α-actin, smooth muscle-1 (SM1) and SM2 myosin heavy chains and by the typical 'hill and valley' microscopic findings. Each population of leiomyoma cells studied came from a separate patient.

Assessment of proliferation

Leiomyoma cells (3000–4000 cells/well) were cultured to subconfluence in 96-well dishes in the presence of 10% FCS for 48 h. The cells in exponential growth were then transferred to serum-deprived media for 72 h to achieve quiescence. Quiescent cells were incubated for 48 h in serum-free media in the presence of various concentrations of ET-1 and/or growth factors, as previously described (13). In combination experiments, protein kinase inhibitors were added 30 min before incubation with ET-1 and/or growth factors. None of these drugs tested was toxic to human leiomyoma cells under our conditions as controlled by trypan blue exclusion. Serum-free DMEM was used as a negative control and DMEM with 10% FCS as a positive control. [^{3}H]thymidine (0.4 μCi/well) was added during

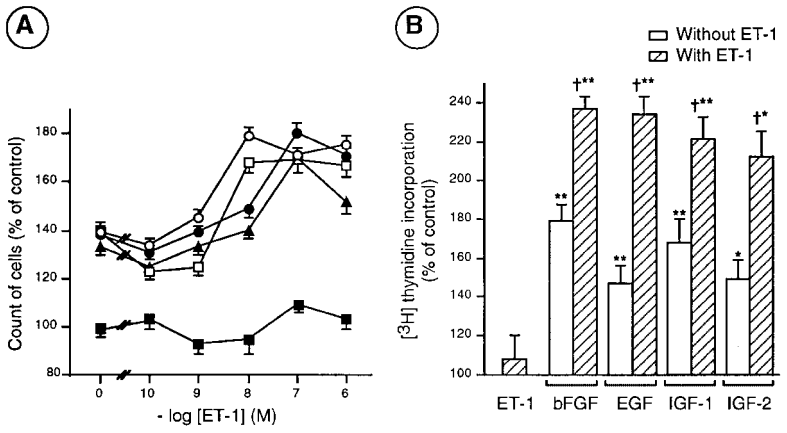

Figure 1 (*A*) Effect of increasing concentrations of ET-1 on leiomyoma cell proliferation with and without growth factors. Quiescent leiomyoma cells (4000 cells/well) were incubated with the indicated concentrations of ET-1 without (■) or with 15 nmol/l bFGF (□), EGF (●), IGF-I (○), IGF-II (▲). This experiment was repeated twice independently. (*B*) [^{3}H]Thymidine incorporation induced by 100 nmol/l ET-1 and/or bFGF, EGF, IGF-I or IGF-II (15 nmol/l) and were determined as in Materials and Methods and are expressed as a percentage of serum-free control. Basal [^{3}H]thymidine incorporation was 6802 ± 964 d.p.m./well. Data are means ± S.E. of quadruplicate determinations from 5–17 independent experiments, each performed on a different population of leiomyoma cells (*subcultures* 3–6) isolated from four different uteri. $^*P < 0.05$ and $^{**}P < 0.001$ as compared with serum-deprived cells. †$P < 0.05$ as compared with growth factor alone.

the final 24 h of incubation. After incubation, cells were washed twice with phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+}, fixed with 5% trichloroacetic acid, washed twice with 100% ethanol, and solubilized with 0.5 N sodium hydroxide. Cell-associated radioactivity was measured by scintillation counting. Cell numbers were determined in separate experiments. Quiescent human leiomyoma cells were incubated with similar treatments. After 72 h, cells were trypsinized and counted with a hemocytometer. The viability of cells was controlled by trypan blue exclusion. All experiments were performed in quadruplicate ([^{3}H]thymidine incorporation) or sextuplicate (determination of cell number) and repeated at least three times on cell from different tissue specimens. All the experiments could not be performed on the same batches.

Western blot analysis of PKC

The experiments were carried out at as previously described with cultured human myometrial cells (14). Subconfluent leiomyoma cells were left untreated or treated with 0.1 μmol/l PDB for 48 h in serum-free medium and subsequently washed twice with PBS. The cells were scraped into 20 mmol/l Tris–HCl buffer, pH 7.5, containing 250 mmol/l sucrose, 1 mmol/l EGTA, 2 mmol/l EDTA, 50 mmol/l β-mercaptoethanol, 2 mmol/l PMSF, 5% glycerol, and 40 μg/ml leupeptin and were sonicated twice for 10 s. Equal amounts of protein lysates (40 μg) were separated by 8% SDS-PAGE and after electrophoretic separation, proteins were transferred to a nitrocellulose membrane. Non-specific binding sites were blocked by incubating the membrane with 5% fat-free dried milk in TBST (10 mmol/l Tris–HCl, pH 7.5, 0.15 mol/l NaCl, 0.1% Tween 20). Anti-PKC antibodies were added at the appropriate concentration and incubated for 45 min at room temperature. The membrane was washed with TBST and incubated for 30 min with the secondary antibody. The blots were developed with ECL reagents and visualized on Kodak X-ray films, and the intensities of the immunoreactive bands were analyzed densitometrically by the NIH Image 1.60 Software Package (NIH, Bethesda, MD, USA). The specificity of the immunobands was tested by competition with appropriate peptides used as immunogens.

Statistical analysis

Data are shown as means ± S.E. We used the two-tailed Wilcoxon paired nonparametric signed-rank test to analyze the data from the five to ten separate experiments. A P value <0.05 was accepted as significant.

Results

Effect of ET-1 on DNA synthesis and proliferation of cultured leiomyoma cells

ET-1 alone (up to 1 μmol/l) did not significantly increase leiomyoma cell number (Fig. 1A) or [^{3}H]thymidine incorporation, whereas bFGF, EGF, IGF-I or IGF-II (15 nmol/l) caused leiomyoma cell proliferation, with increases in DNA synthesis of 179 ± 11%, 147 ± 11%, 168 ± 14% or 149 ± 12% of the serum-free control respectively (Fig. 1B). The increase in cell number in response to bFGF, EGF, IGF-I or IGF-II (15 nmol/l) was potentiated by ET-1 (0.1 nmol/l to 1 μmol/l) in a concentration-dependent manner (Fig. 1*A*). Similarly, the increase in DNA synthesis in response to co-addition of ET-1 (100 nmol/l) with each growth factor (15 nmol/l) to leiomyoma cells resulted in maximal stimulation of 237 ± 8% (bFGF),

Table 1 Effect of PTK inhibition by tyrphostin 51 on the potentiation of growth factors by ET-1. Results are expressed as a percentage of serum-free control (100%). Data are means ± S.E.

	None	Tyrphostin 51
ET-1	107±15	102±13
bFGF	139±14*	113±11
bFGF+ET-1	181±13*†	133±12*
EGF	138±17*	108±7
EGF+ET-1	192±18*†	110±13
IGF-I	139±15*	112±11
IGF-I+ET-1	177±15*†	110±7
IGF-II	133±12*	114±8
IGF-II+ET-1	170±14*†	133±10*

*$P < 0.05$ vs control, †$P < 0.05$ vs growth factor alone.

234 ± 11% (EGF), 221 ± 14% (IGF-I) and 212 ± 15% (IGF-II) of the serum-free control (Fig. 1B).

Under the same conditions, the receptor ET_B agonist, sarafotoxin 6c (S6c) (0.1 nmol/l–1 μmol/l) on its own or together with each of the growth factors did not stimulate either DNA synthesis or cell proliferation (data not shown).

Effect of PTK and PKC inhibition on the potentiating effect of ET-1

To further evaluate whether PTK activation was required for mitogenic signaling by both ET-1 and growth factors, we examined the effect of tyrphostin 51 (Tyr 51), the most potent EGF-receptor kinase inhibitor. We confirmed that in the presence of Tyr 51, none of the growth factors bFGF, EGF, IGF-I or IGF-II was able to induce a significant proliferative effect. At a concentration of 100 nmol/l, Tyr 51 reduced by 78–86% the increase in [^{3}H]thymidine incorporation induced by 15 nmol/l of growth factor (Table 1). The co-addition of ET-1 and bFGF, under Tyr 51 treatment, did not significantly increase DNA synthesis beyond that obtained with the growth factor alone, however the levels of [^{3}H]thymidine incorporation were consistently greater than those in serum-free conditions. Comparable results were obtained with co-application of ET-1 and IGF-II. When exposed to Tyr 51, leiomyoma cells lost the responsiveness to the mitogenic activity of co-addition of ET-1 and EGF or IGF-I. All these results suggest that treatment of leiomyoma cells with Tyr 51 prevents the potentiating effect of ET-1.

We next examined the effect of calphostin C, a PKC inhibitor, to assess the contribution of PKC to ET-1-potentiated leiomyoma cell proliferation with growth factors. Calphostin C has a dose-dependent inhibitory effect on cell proliferation induced by ET-1 (100 nmol/l) with each of the growth factors tested (Fig. 2A). Pretreatment of cells with 100 nmol/l calphostin C significantly attenuated the enhanced DNA synthesis induced by a combination of ET-1 and growth factors. By contrast, calphostin C did not significantly decrease [^{3}H]thymidine incorporation induced by bFGF, EGF, IGF-I or IGF-II alone (Fig. 2B).

The role of PKC on the potentiating effect of ET-1 on cell proliferation was further investigated by examining the effects of PKC down-regulation on this response. Leiomyoma cells were pretreated for 72 h with 100 nmol/l PDB to deplete phorbol ester-sensitive PKC isoforms. The PDB treatment did not change the basal leiomyoma cell number. Whereas the cell proliferation induced by growth factor alone was unaffected by PDB treatment (data not shown), the increase in cell number induced by simultaneous addition of ET-1 and growth factors was reduced and returned to level obtained with the growth factor alone (Fig. 3A). We next investigated which phorbol ester-sensitive PKC isoforms are present in leiomyoma cells. Western blot analysis revealed the presence of at least five PKC isoforms: PKCα, PKCβ1, PKCδ, PKCε and PKCζ (Fig. 3B). Long term-treatment with 100 nmol/l PDB

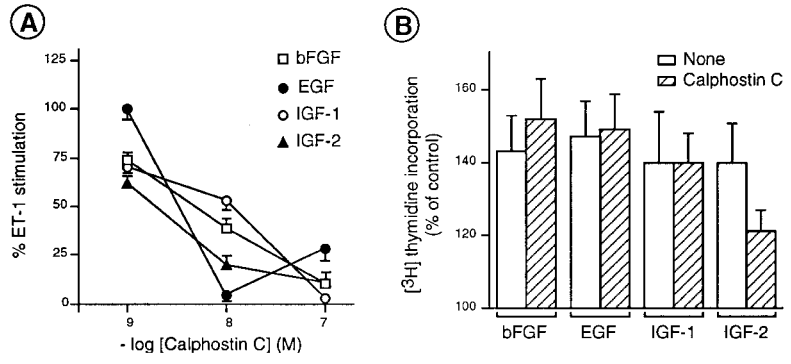

Figure 2 Effect of PKC inhibition by calphostin C on the potentiation of growth factors by ET-1. (*A*) Quiescent leiomyoma cells (4000 cells/well) were pre-incubated with increasing concentrations of calphostin C for 30 min before adding 100 nmol/l ET-1 with 15 nmol/l bFGF (□), EGF (●), IGF-I (○) or IGF-II (▲). Data shown are percentage of maximal cell number increase observed with ET-1 and growth factors and are from a single experiment representative of the three giving similar results (*subcultures* 3–5). (B) Effect of 100 nmol/l calphostin C on 15 nmol/l growth factor-induced increase in [^{3}H]thymidine incorporation. Results are expressed as percentage of serum-free control. Data are means ± S.E. of quadruplicate determinations from four independent experiments, each performed on a different population of leiomyoma cells (*subcultures* 3–6) isolated from two different uteri.

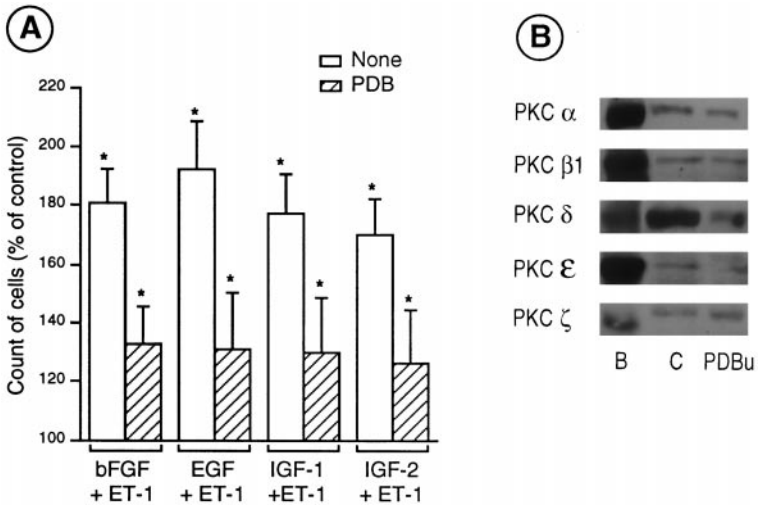

Figure 3 Effect of prolonged treatment with PDB: (A) on the potentiation of growth factors by ET-1. Quiescent leiomyoma cells were treated with 100 nmol/l PDB for 72 h and then stimulated with co-administration of 100 nmol/l ET-1 and 15 nmol/l bFGF, EGF, IGF-I or IGF-II. Results are expressed as percentage of serum-free control. Data are means ± S.E. of sextuplicate determinations from six independent experiments on a different population of leiomyoma cells (*subcultures* 3–6) isolated from three different uteri. $*P < 0.05$ as compared with serum-deprived cells. (B) The immunodetection of PKCα, PKCβ1, PKCδ and PKCζ in leiomyoma cells. Cells (*subculture* 4) were incubated without (control, C) and with 100 nmol/l PDB for 72 h. Samples (40 μg of protein) were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunodetected with specific antibodies. Rat brain extract (B) was used as positive control.

induced a decrease in the total immunoreactive amounts of PKCα, PKCδ and PKCε but not PKCβ1 and PKCζ. The isoforms PKCα and PKCδ were the most sensitive as 45% and 49% were respectively lost after this treatment. Under the same conditions, PKCε immunoreactivity was decreased by only 25%.

Discussion

The present study demonstrated that ET-1 potentiated the proliferative effect of growth factors through a PKC-dependent pathway in cultured human leiomyoma cells.

Whereas ET-1 on its own is not mitogenic, this peptide is an effective co-mitogen with polypeptide growth factors such as bFGF, EGF, IGF-I and IGF-II in leiomyoma cells. This contrasts with the absence of a requirement for growth factors to elicit an ET-1-induced growth response in normal human myometrial cells grown in culture (13). A question arises over the role of specific autocrine growth factors in leiomyoma cell proliferation. An abnormal elevated expression of different growth factors and/or their receptors has been demonstrated to be involved in the pathogenesis of leiomyomas. Thus, leiomyoma cells are a more important endogenous source of bFGF, EGF, IGF-I and IGF-II than normal myometrial cells (4–8). As expected, exogenous bFGF, EGF and IGFs stimulated the DNA synthesis and proliferation of leiomyoma cells. Synergism between ET-1 and growth factor effects is a common phenomenon in vascular smooth muscle. Numerous investigations have revealed that ET-1 is either not at all a proliferative agent or only a very weak proliferative one but that it potentiates PDGF-(platelet-derived growth factor-), EGF- and bFGF-induced mitogenic effect in human vascular smooth muscle cells (16–18).

In addition, the failure of the selective ET_B agonist S6c to initiate DNA synthesis in the absence or presence of growth factors argues against an ET_B receptor being linked to these proliferative responses. Although further experiments are necessary to confirm the presence of ET_A receptors in cultured leiomyoma cells, our findings agree with our recent results that demonstrated functional ET_A and ET_B receptors in normal human myometrium, while only functional ET_A receptors were detected in human uterine leiomyomas (11, 12). In contrast to peptide growth factor receptors, which possess intrinsic protein tyrosine kinase activities, G-protein coupled receptors (GPCRs), such as ET_A receptors, have not been demonstrated to possess endogenous tyrosine kinase. Next, we have investigated which PTK or PKC pathway was responsible for cooperative action between ET-1 and growth factors to induce leiomyoma cell proliferation. Tyrosine kinase activation may be sufficient for growth factors to stimulate proliferation of cultured leiomyoma cells, whereas the potentiating effect of ET-1 appears to require additional signals. In addition, our present data indicate that the mitogenic response of leiomyoma cells to bFGF, EGF and IGFs is not dependent on PKC activation. Neither [^{3}H]thymidine incorporation nor the cell number increase in response to each growth factor was significantly blocked by PKC inhibition. By contrast, most of the potentiating effect of ET-1 was clearly inhibited by the PKC inhibitor, calphostin C. The loss of the ET-1 effect when co-administrated with bFGF, EGF and IGFs in PKC-depleted leiomyoma cells obtained after prolonged treatment with PDB further supports a crucial role for phorbol ester-sensitive isoforms of PKC in this process. We next identified the pattern of expression of PKC isoforms in cultured human leiomyoma cells. Among the five isoforms of PKC (α, β1, ε, δ and ζ) found in uterine leiomyoma cells, we believe that either PKCα, PKCδ and PKCε

alone or in combination, rather than atypical phorbol ester-insensitive PKCζ, are involved in the potentiating effect of ET-1. However, the surprising resistance of PKCβ1 to being down-regulated by PDB in cultured leiomyoma cells supports the hypothesis that this isoform should not be required for eliciting potentiating effect of ET-1. The reason for the retention of membrane PKCβ1 in PDB-down-regulated leiomyoma cells is not presently clear, and caution must be exercised in interpreting such data. We cannot rule out the possibility that prolonged treatment by a phorbol ester might result in nuclear localization of this isoform, without any down-regulation as previously reported in human T-cells (19). The existence of multiple PKC isozymes in leiomyoma cells may reflect the various specialized cellular functions mediated by this kinase (20). However, although the roles of individual isozymes in migration, invasion and in growth control have only recently begun to be explored, the type of isoform involved appears to depend on the cell type (21). By identifying the specific role(s) of each of these PKC isoforms, insights may be gained into how ET-1 regulates leiomyoma cell proliferation, and how it contributes to leiomyoma formation. Further work is required to identify the signaling pathway downstream of PKC in leiomyoma cells.

Taken together, our data have demonstrated a growth-potentiating effect of ET-1 in human uterine leiomyoma cells in response to growth factors through a PKC-dependent pathway. It is likely that ET-1 acts in an autocrine–paracrine fashion as a mediator of growth factors in promoting tumor enlargement. Whether this mechanism has any part to play in the pathogenesis of leiomyomas, remains to be determined.

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