Published in final edited form as:
Endocrinology. 2004 February ; 145(2): 706–715. doi:10.1210/en.2003-1063.

Involvement of Protein Kinase C-Dependent Mitogen-Activated Protein Kinase p44/42 Signaling Pathway for Cross-Talk between Estradiol and Transforming Growth Factor-β3 in Increasing Basic Fibroblast Growth Factor in Folliculostellate Cells

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Abstract

We have recently shown that TGF-β3, in the presence of estradiol, increases the release of basic fibroblast growth factor (bFGF) from folliculostellate (FS) cells in the pituitary. We determined the interactive effects of TGF-β3 and estradiol on bFGF production and release from FS cells, and the role of the MAPK pathway in TGF-β3 and estradiol interaction. We found that TGF-β3 and estradiol alone moderately increased cell content and release of bFGF from FS cells; but together, they markedly increased the peptide. Estradiol and TGF-β3 alone moderately activated MAPK p44/42; together they produced marked activation of MAPK p44/42. Pretreatment of FS cells with an MAPK kinase 1/2 inhibitor or with protein kinase C inhibitors suppressed the activation of MAPK p44/42, bFGF release, and protein level increases, all of which were induced by TGF-β3 and estradiol. Estradiol and TGF-β3, either alone or in combination, increased the levels of active Ras. Furthermore, bFGF induction by TGF-β3 and estradiol was blocked by overexpression of Ras N17, a dominant negative mutant of Ras p21. Estrogen receptor blocker ICI 182,780 failed to prevent estrogen's and TGF-β3's effects on bFGF. These data suggest that an estradiol receptor-independent protein kinase C-activated Ras-dependent MAPK pathway is involved in the cross-talk between TGF-β3 and estradiol to increase bFGF production and/or release from FS cells.

Estrogen exposure has been considered a risk factor for the development of prolactin-secreting pituitary tumors known as prolactinomas in humans (1-3). Experimentally, prolactinomas can be induced by estradiol treatment in laboratory animals (4,5). It has been shown that estradiol's mitogenic action on lactotropes is regulated by a variety of hormones and growth factors, including TGF-β3 and basic fibroblast growth factor (bFGF) (6). Using the Fischer-344 rat strains for *in vitro* and *in vivo* studies, we have shown that estradiol regulates the production and secretion of TGF-β1 and TGF-β3 in the anterior pituitary (6). These two growth regulatory polypeptides are derived from a TGF-β family of peptides and are quite homologous. These peptides have been shown to regulate cell growth and differentiation in a number of cells with different biological origins (7-11). Within the pituitary gland, TGF-β1 and TGF-β3 are produced primarily in lactotropes of the anterior lobe and in melanotropes of the intermediate lobe (12-14). In the lactotropes, TGF-β3 stimulates cell proliferation; however, TGF-β1 inhibits it. Their opposing actions are related to their different sites of actions. TGF-β1 inhibits

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cell proliferation by acting directly on lactotropes, whereas TGF-β3 increases lactotropic cell proliferation indirectly by acting on folliculostellate (FS) cells. Estradiol inhibits TGF-β1 production, but it stimulates TGF-β3 production in lactotropes. Cell supernatant from FS cell cultures, stimulated with estradiol and TGF-β3 or with exogenous bFGF, increases lactotrope proliferation (15). These data suggest a key role of TGF-β3 and estradiol interaction in lactotropic cell proliferation through bFGF production from FS cells. The peptide bFGF has been shown to regulate cell proliferation, differentiation migration, angiogenesis, and wound healing in various tissues, including the pituitary (reviewed in Refs. 6,16, and 17).

TGF-β1–3 are known to exert their effects by first binding to their cell surface TGF-β type II (TβRII) receptors. This ligand binding induces TβRII to associate with the TGF-β type I receptor (TβRI), which leads to a unidirectional phosphorylation event in which TβRII phosphorylates TβRI. This phosphorylation of TβRI activates its kinase domain, which further phosphorylates smad 2/3 proteins (reviewed in Ref. 18). The proteins are brought to the membrane through the smad anchor for the receptor activation protein (19). Activated smad 2/3 binds to smad 4 and further translocates to the nucleus to activate various transcription factors (20,21). In addition to this smad-mediated TGF-β signaling pathway, evidence over the past few years suggests that TGF-β might signal through several MAPKs, including signal-regulated kinases [extracellularly regulated kinases (Erks); p44/42], c-jun terminal kinases (JNKs), and p38 MAPKs (22-27). Although TβRII and smad signaling in the pituitary has been demonstrated, the effects of TGF-βs on MAPKs in the pituitary cells have not been well studied (for a review, see Ref. 6).

The MAPK p44/42 has also been shown to be regulated by estrogen (28,29). The genomic effects of estrogen occur primarily through interaction with estrogen receptors (30). The nongenomic actions of estrogen include activation of Ras (31), which, in turn, activates MAPK p44/42, Raf-1 (32), protein kinase C (PKC) (33), protein kinase A (34), and Maxi-K channels (35) and also causes increases in intracellular calcium levels and nitric oxide production (36). These actions of estrogen may be mediated by a plasma membrane-associated estrogen receptor or by nonclassical estrogen receptors (37). In light of the recent evidence for similar actions of both TGF-β-related peptides and estradiol on MAPK (28,38), it was of interest to determine whether there is cross-talk between estradiol and TGF-β3 to regulate bFGF production from FS cells. In the present study, using an FS cell line, we found that TGF-β3 and estradiol, either alone or in combination, increased bFGF production and release via activation of a PKC-Ras-MAPK kinase (MEK)-MAPK p44/42 pathway in FS cells.

Materials and Methods

Cell culture and reagents

We established an FS cell line from primary cultures of anterior pituitary cells obtained from cyclic female Fischer-344 rat pituitaries (15). The cell line was maintained in DMEM/F-12 media with 10% fetal bovine serum. During experimentation, cells were maintained in DMEM/F12 containing serum supplement (consisting of 100 μ_M human transferrin, 5 μ_M insulin, 1 μ_M putrescine, and 30 n_M sodium selinite). In a study verifying the effectiveness of antiestrogen ICI 182 780, PR1 cell line is used. This cell line is derived from rat pituitary lactotrope (13) and was maintained in DMEM/F12 media with 10% fetal bovine serum. TGF-β3 was purchased from R&D Systems (Minneapolis, MN), and 17β-estradiol was obtained from Sigma (St. Louis, MO). The MAPK p44/42 inhibitor (U0126) and monoclonal and polyclonal antibodies, directed against phosphorylated and total MAPK p44/42, respectively, were bought from Cell Signaling (Beverly, MA). The PKC inhibitors [chelerythrine chloride and Bisindolylmalemide (Bis)] and p38 inhibitor (SB202190) were purchased from Calbiochem (San Diego, CA). A plasmid containing a dominant negative mutant of Ras p21 (Ras N17) was purchased from Clontech Laboratories (Palo Alto, CA). The estrogen receptor blocker, ICI 182 780 (Zenecia

Pharmaceuticals, Macelesfield, Cheshira, UK), was a kind gift from Dr. Carol Bagnell (Rutgers University). SB 203580 (Calbiochem), a p38/JNK inhibitor, was a kind gift from Dr. Wendie Cohick (Rutgers University). A Ras activation assay kit was purchased from Upstate Biotechnology (Waltham, MA).

bFGF immunoassay

FS cells (250,000/well) were grown in 24-well plates in serum-containing medium. After 2 d ofplating, the medium was changed to a serum-free medium. On the following day, cells were treated with vehicle (control), TGF-β3 (0.1–10 ng/ml), estradiol (1–100 nM), or TGF-β3 and estradiol in serum-supplemented media for either 2 h or 24 h. Cell supernatants were collected and assessed for bFGF levels using Quantikine Immunoassay Kits (R&D Systems). Cells were lysed in 100 μl of a lysis buffer containing 150 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% Triton-X, and a protease inhibitor cocktail (Sigma). Ten microliters of cell lysate were used to assess bFGF levels using immunoassay as described previously in this article. For blocking studies, cells were pretreated with inhibitors for 1 h, followed by TGF-β3 and estradiol treatment either alone or in combination. Control cells in these inhibitor studies were treated with vehicle [dimethylsulfoxide (DMSO)]. The total protein concentration in each cell lysate was determined using the BioRad assay (Bio-Rad, Hercules, CA) to calculate the bFGF release or cellular content of bFGF per microgram or per milligram of total protein.

Phosphorylation of MAPKs

Monolayers of FS cells (1×10^6/well) were grown in six-well plates. They were incubated in serum-supplemented media and then treated with TGF-β3 (1 ng/ml) and/or estradiol (10 nM) for 2 h or 24 h. Cells were lysed and analyzed for total and phosphorylated MAPK p44/42 with immunoblotting. Monoclonal and polyclonal antibodies, specific for phospho MAPK and total MAPK, were used. For blocking studies, cells were pretreated with inhibitors for 1 h, followed by TGF-β3 and estradiol treatment alone or in combination. Control cells in these inhibitor studies were treated with vehicle (DMSO).

Western blot

Cell monolayers were treated with vehicle or tested agents, washed with Tris buffer saline, and lysed in a lysis buffer containing 150 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM $MgCl_2$, 1% Triton-X, 10% glycerol, and a protease inhibitor cocktail (Sigma) for 1 h on ice. Cellular debris was cleared by centrifugation (10,000 × *g* for 10 min). The protein concentration in the supernatant was determined using a Bio-Rad protein assay reagent (Bio-Rad). Equal amounts of protein from each sample were resolved on SDS/PAGE and transferred to immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were incubated with primary antibody for 1 h at room temperature in 5% milk, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20. Membranes were washed and incubated with alkaline phosphatase conjugated with either mouse or rabbit secondary antibody for 1 h and developed using CDP star Western blot chemiluminescence reagent (Perkin-Elmer Life Sciences, Foster City, CA). For quantification of MAPK p44/42 activity, band intensities of phospho MAPK p44/42 were determined using Scion Image software and normalized to the corresponding total MAPK p44/42.

Transient transfection

Dominant negative Ras N17 or a control vector plasmid was transiently transfected into FS cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 24-well plates, according to the manufacturer's protocol. After 18 or 24 h of transfection, cells were treated with estradiol (10 nM) and/or TGF-β3 (1 ng/ml) for 6–24 h, for bFGF induction. Subsequent procedures for cell lysis for Western blots and bFGF assays were performed as described previously in this article.

Ras activity assay

Cells numbering 5×10^6 per treatment were stimulated with estradiol or TGF-β3, alone or in combination, for 1 h and lysed for 30 min, on ice, in 500 μl lysis buffer (included with the Ras activation assay kit). The lysates were then centrifuged, and supernatants were mixed with 30 μl glutathione-*S*-transferase (GST) fusion protein containing the Ras binding domain of Raf, immobilized in glutathione-agarose beads. The samples were incubated for 90 min at 4 C with gentle rotation. The beads were then washed three times with lysis buffer. The bound GTP Ras protein was eluted in gel loading buffer and subjected to 12.5% SDS/PAGE. The levels of active Ras were assessed by immunoblotting with specific anti-Ras antibody. For positive and negative controls, untreated cell lysates were loaded with GTPγS or GDP, respectively, before mixing with beads as per the kit's instructions.

Intracellular levels of prolactin

PR1 cells (200,000 cells/well) were plated in 12-well plates for 24 h. Cells were then maintained in media containing serum supplement for 18 h, followed by treatment with vehicle, estradiol (10 nM), ICI 182, 780 (10^{-7} and 10^{-6} M), or estradiol and ICI 182,780 for 96 h at 24-h intervals. Cells were lysed in lysis buffer, and prolactin levels were analyzed by Western blotting using antibody for prolactin (NIDDK antirat PRL-S-9; 1:200,000) and actin (Oncogene Research Products; San Diego, CA). Actin levels were used to normalize the loading errors.

Statistical analysis

Data shown in the figures are mean ± SEM of the indicated number of experiments performed independently. Data were analyzed using one-way or two-way ANOVA, as appropriate. *Post hoc* analysis after ANOVA employed the Newman-Keuls test. $P < 0.05$ was considered significant.

Results

TGF-β3 and estradiol interactive actions on cellular levels of bFGF in FS cells

Previously we have shown that TGF-β3 dose-dependently increases the release of bFGF from FS cells in the presence of estradiol (15). To determine whether an interactive effect of TGF-β3 and estradiol on bFGF exists, dose-response studies were performed using various concentrations of estradiol or TGF-β3 alone or by fixing the concentration of one agent and changing the concentration of the other.

An estradiol dose ranging from 1–100 nM increased bFGF levels in FS cells in a concentration-dependent manner (Fig. 1A). The maximum response was observed at an estradiol concentration of 100 nM, but there was no statistical significant difference between 10- and 100-nM concentrations. TGF-β3 alone also dose-dependently increased bFGF levels at a dose range of 0.1–10 ng/ml (Fig. 1B). The maximal bFGF response was observed at the 10-ng/ml dose of TGF-β3.

The estradiol dose-response effect was significantly magnified by the presence of TGF-β3, and the TGF-β3 effect was magnified by the presence of estradiol. These data suggested that TGF-β3 and estradiol interacted to increase bFGF levels in FS cells. Estradiol (10 nM) and TGF-β3 (1 ng/ml) together showed an additive effect on bFGF levels in FS cells. The additive effect was also confirmed by a time kinetic study in the presence of TGF-β3 and estradiol either alone or in combination (Fig. 1C). A stimulatory and additive effect of TGF-β3 and estradiol was also found on bFGF release from FS cells, similar to that found for bFGF levels just described (Fig. 1D). These data suggested a cooperative model in which estradiol and TGF-β3 interacted, resulting in a maximum increase of bFGF levels and release from FS cells.

Activation of MAPK p44/42 by estradiol and TGF-β3 in FS cells

MAPKs are key signal-transducing proteins that transmit signals within cells and are involved in various biological effects of hormones and growth factors (39,40). Hence, the role of MAPKs in TGF-β3 and estradiol interaction was investigated. To determine the total and phosphorylated form of MAPK p44/42, using Western blot analysis, FS cells were treated alone or in combination with the dose of TGF-β3 (1 ng/ml) or estradiol (10 n_M) that produced the maximal bFGF response. Time-course studies, using a combined treatment of TGF-β3 and estradiol, indicated that MAPK p44/42 is optimally activated between 2 and 3 h [phosphorylation of p44/42 (fold-change of control): 1 h (3.5 ± 0.19); 2 h (5.82 ± 0.6); 3 h (5.49 ± 0.4); n = 5; $P < 0.05$, 1 h *vs.* 2 h or 3 h]. Using the 2-h treatment paradigm, we found that, alone, TGF-β3 and estradiol each moderately activated MAPK p44/42; but together, these agents produced significantly more phosphorylation of MAPK (Fig. 2, A and B). The magnitude difference between the MAPK activation response to these two agents alone and in combination was smaller than those for bFGF release (compare Figs. 1 and 2), suggesting that a small change in signal may result in a large change in effect.

Involvement of MAPK p44/42 in estradiol- and/or TGF-β3-induced bFGF production and release from FS cells

To analyze the contribution of the MAPK p44/42 cascade in the increase in bFGF production and release from FS cells, we used MEK1/2 kinase p44/42 inhibitor U0126, which blocks the function of MEK1/2 kinases (41,42). These kinases are upstream from MAPK p44/42 and are known to phosphorylate MAPK p44/42 (39,43). FS cells were preincubated with various concentrations of U0126 or vehicle for 1 h and then treated with TGF-β3 and/or estradiol for 2 h to determine MAPK p44/42 activation, and for 24 h to measure bFGF production. The inhibitor at 1 and 10 μm concentrations significantly inhibited the TGF-β3- and/or estradiol-induced phosphorylation of MAPK p44/42 (Fig. 3, A and B). U0126 treatment also concentration-dependently reduced estradiol and TGF-β3-induced bFGF levels in FS cells (Fig. 4A). The maximal effective dose of the U0126 that blocks estradiol and TGF-β3-induced bFGF levels was also able to completely block estradiol-alone- and TGF-β3-alone-induced bFGF release. This dose also partially blocked the bFGF release induced by a combined treatment of TGF-β3 and estradiol (Fig. 4B).

To further confirm the specificity of the MAPK p44/42 pathway and to rule out the possibility of involvement of other MAPKs such as p38 or JNK, we determined the effect of MAPK p38 inhibitor SB202190 and p38/JNK inhibitor SB203580 on bFGF levels in FS cells. MAPK p38 inhibitor SB202190 (350–750 n_M) did not produce any significant effect on basal bFGF levels or on TGF-β3 and/or estradiol-induced bFGF levels (data not shown), nor did various higher concentrations of these blockers (Fig. 5A). SB203580, an inhibitor of p38 known to inhibit JNK pathway at concentrations of 25–50 μ_M (44), had no significant effect on bFGF levels induced by TGF-β3 and estradiol alone or in combination (Fig. 5B). These results suggest that p38 and JNK MAPK pathways are not involved in estradiol and/or TGF-β3-induced increases in bFGF levels in FS cells. The blocker SB203580 was found to be effective in inhibiting JNK and p38 activation in mammary cell systems (Dr. Wendie Cohick's laboratory, unpublished data), which confirms the effectiveness of this inhibitor.

Involvement of the Ras-activated MAPK pathway in TGF-β3- and/or estradiol-regulated bFGF expression in FS cells

Because the MAPK inhibitor blocked the estradiol and TGF-β3 effects on bFGF in FS cells, it was of interest to find out whether the classical pathway of the Ras-MEK-MAPK cascade is involved with bFGF expression in FS cells. Ras is a small G protein that becomes activated when it binds to GTP. Hence, we evaluated the levels of active (GTP-bound) Ras by pull-down experiments using an immobilized GST-fusion protein containing the binding domain of Raf.

As shown in Fig. 6A (i), the levels of Ras-GTP were increased, parallel to increases in phospho MAPK p44/42 after estradiol and/or TGF-β3 treatments. Fig 6A (ii) shows the data of positive and negative control studies for Ras activity assay. For positive and negative controls, FS cell lysate was loaded with GTP-γ-S (which causes persistent activation of G proteins) or GDP (which replaces all GDP and inactivates G proteins), respectively, and levels of active Ras were assessed.

Ras N17, a dominant negative mutant of Ras p21, is a convenient tool for studying the Ras-related signal transduction pathway (45). We transiently transfected FS cells with vehicle or a Ras N17 plasmid, using the lipofectamine reagent. After 24 h, cells were treated with TGF-β3 and/or estradiol for 2 h or 24 h, forMAPK activation or bFGF induction, respectively. Ras N17 vector expresses a dominant negative form of Ras protein that contains a serine-to-asparagine mutation at residue 17. The expression of this variant is known to knock out endogenous Ras expression in mammalian cells (45,46). Western blot analysis showed that the expression of Ras N17 in FS cells blocked the TGF-β3- and/or estradiol-induced activation of MAPK p44/42 (data not shown). Control vector-transfected cells showed increased bFGF cell content in the presence of estradiol and/or TGF-β3 (Fig. 6B). Ras N17-transfected cells showed reduced basal levels as well as estradiol- and/or TGF-β3-induced bFGF levels. The inhibitory effect of Ras N17 overexpression on the basal levels of bFGF made it difficult to identify its effect on growth factor-induced levels of bFGF. A similar effect of overexpression of Ras N17 was reported previously (47). Possibly, the long-term overexpression of Ras N17 is shutting down other pathways, which may be indirectly dependent on Ras. To address this issue, cells were transiently transfected for 24 h, followed by estradiol and TGF-β3 treatment for a period of 6 h, instead of 24 h, and analyzed for bFGF levels. Data from this study suggested that short-term over-expression of Ras N17 did not affect the basal levels but did reduce the estradiol- and/or TGF-β3-induced levels of bFGF (Fig. 6C).

Requirement of PKC for phosphorylation of MAPK and bFGF production

Phosphorylation of MAPK p44/42 can be induced by the activation of PKCs (48). To identify whether the PKC system participates in the estradiol/TGF-β3 interaction leading to MAPK p44/42 activation, we determined the action of a PKC blocker on TGF-β3- and/or estradiol-induced MAPK activation and bFGF levels in FS cells, as described in *Materials and Methods.* In an initial screening, treatment of FS cells with various concentrations of a general PKC inhibitor, chelerythrine chloride, blocked both the phosphorylation of MAPK p44/42 and the estradiol- and/or TGF-β3-induced increase in bFGF levels in FS cells (data not shown). Using a specific general PKC inhibitor Bis (49), we found that the estradiol and/or TGF-β3-induced increases in MAPK activation were suppressed at both 2.5- and 5-μ_M concentrations (Fig. 7, A and B). These same doses of Bis also suppressed the effect of estradiol and/or TGF-β3 on bFGF levels (Fig. 7C).

Effect of estrogen receptor blocker ICI 182,780 on estradioland TGF-β3-induced bFGF expression

To find out whether the additive effect of estradiol and TGF-β3 on FS cells' bFGF production requires activation of an estrogen receptor, we determined the effect of an estrogen receptor blocker on bFGF cell content. Cells were pretreated with ICI 182,780 (0, 10^{-7}–10^{-6} $_M$) for 1 h and then treated with estradiol alone or with both estradiol and TGF-β3 for 24 h. Immunoassay for bFGF cell content did not show any significant difference between inhibitor-treated and vehicle-treated groups (Fig. 8A). Basal bFGF levels were not affected by the blocker treatment. Because estradiol blocker failed to prevent estradiol action on bFGF production, the effectiveness of the blocker was verified by determining its action on estradiol-induced increase in intracellular prolactin in PR1 cells. ICI 182,780 has been shown to block estrogen's action on prolactin production from lactotrope-derived PR1cells (50). As shown in Fig. 8B, we could

not detect any PRL signal in either control or antiestrogen-treated cells during the experimental period. However, in estradiol-treated cells, we detected a PRL signal, which was blocked by the estrogen antagonist, which confirmed the effectiveness of the reagent. These data suggested that an estradiol receptor-independent action in estradiol and TGF-β3 interaction occurred to regulate FS cells' bFGF production.

Discussion

The data presented here demonstrate, for the first time, that estradiol and the peptide growth factor, TGF-β3, interact in an additive manner to increase the production and release of bFGF from FS cells. We found that this increase is associated with increased phosphorylation of MAPK p44/42. We also noted that a MAPK p44/42 inhibitor, PKC inhibitors, and a dominant negative mutant of Ras p21 all prevented the effects of TGF-β3 and estradiol on the phosphorylation of MAPK p44/42 and bFGF production and release from FS cells. A MAPK p38 inhibitor did not. Furthermore, the additive effect of estradiol and TGF-β3 was not affected by an estradiol receptor blocker. These data suggest that TGF-β3 and estradiol interact to increase bFGF production and release from FS cells via the estradiol receptor-independent PKC–Ras-MEK-MAPK p44/42 signaling pathway.

The interaction of estradiol and TGF-β3 may be critical for estradiol-induced cell-proliferating action on lactotropes. This interaction increases bFGF release maximally, thereby producing a maximal effect on cell proliferation. Previous studies have demonstrated that estradiol stimulates lactotropic cell production of TGF-β3, which causes lactotropic cell proliferation by increasing bFGF release from FS cells (6). Hence, it seems that estradiol not only increases the release of bFGF-inducing TGF-β3 from lactotropes but also increases its action on FS cells, to produce maximal bFGF release and lactotrope proliferation.

Synergistic interactions of IGF-I or insulin and estradiol have been demonstrated (51). It has been found that IGF-I and estradiol synergistically activate protein kinase B and Akt to increase proliferation of MCF-7 cells (52). The interaction between the IGF receptor and the estradiol receptor was found to be dependent on MAPK activation (53). Interestingly, the interaction of TGF-β3 and estradiol to increase bFGF in FS cells is also mediated by MAPK p44/42.

The data presented here identify a mediatory role of a specific MAPK, p44/42, in the interaction of TGF-β3 and estradiol on bFGF of FS cells. Previously it has been shown that estradiol alone rapidly activates phosphorylation of MAPK p44/42 in the brain tissue (54). Studies of GH3 cells, which secrete pituitary prolactin, revealed that antiestrogen receptor antibodies, directed against the hinge region of the estrogen receptor, blocked rapid activation of MAPK p44/42 and estradiol-induced prolactin secretion (55). In some cell systems, estradiol has been shown to activate a rapid MAPK p44/42 response within 3–5 min of treatment (29), as well as a delayed sustained response (28,29). For rapid effects of estrogen, the receptors must be located in the plasma membrane. Delayed effects could be attributed to classical nuclear receptor and genomic action, as well as paracrine action of estradiol through the secretion of growth factors (28). There has been increasing evidence in recent years supporting the existence of an alternative receptor that is both genetically and pharmacologically different from a classical estrogen receptor (37) and insensitive to the estrogen receptor blocker ICI 182, 780 (56). We found a slow (but persistent) activation of MAPK p44/42 by both estradiol and TGF-β3 for bFGF production from FS cells. The estrogen receptor blocker ICI 182,780 did not block bFGF levels in FS cells induced by estradiol or by TGF-β3 and estradiol, suggesting the possibility of a classical estrogen receptor-independent mechanism to increase bFGF production. There is evidence that estrogen receptor-independent pathways could be activated in estradiol receptor-negative cells (57), or that a nonclassical estrogen receptor could be involved in estrogen's effects on the endocrine pancreas (58).

TGF-β isoforms 1–3 are known to elicit a wide variety of biological responses by binding to TβRII/TβRI and further activating smad proteins (18-21). It is becoming increasingly clear that smads may not be solely responsible for the entire effect of TGF-βs. A possible interaction between smad and MAPK pathways for TGF-β-stimulated collagen gene expression has been documented (38). Blanchette *et al.* (47) showed, for fur gene expression, that the activation of MAPK p44/42 by TGF-β1 targets smad 2 for increased translocation to the nucleus. More recently, it has been shown that, in differentiated chondrocytes, rapid and transient activation of both MAPK p44/42 and MAPK p38 is required for high levels of aggrecan gene expression (59). However, it has also been shown that, in bovine endothelial cells, the MAPK JNK pathway, but not the MAPK p44/42 or MAPK p38 pathways, activates smad 2-mediated transcription (60). It has also been reported that the Ras-MAPK pathway is essential for the action of TGF-β1 in lung and intestinal epithelial cells, whereas smads contribute indirectly to TGF-β1's action on these cells (61). Although some studies have shown TGF-β1's effect on MAPK activation, this is the first report showing TGF-β3's effect on MAPK p44/42 for bFGF production. In the present study, the MEK1/MEK2 blocker U0126 blocked not only the estradiol- and/or TGF-β3-induced MAPK activation but bFGF protein production and release from FS cells as well. The MAPK p38 inhibitor did not have any effect. It is possible that the additive effect of estradiol and TGF-β3 on bFGF production requires cross-talk between MAPK and smad proteins (47). We are currently investigating the possible interaction of smad and MAPK pathways for estradiol and TGF-β3 interaction on bFGF production.

The data presented here also provide evidence for a mediatory role of Ras p21 in the interaction between TGF-β3 and estradiol. Ras p21 is a small G protein that transduces its signal by binding to its effector proteins, such as Raf-1 and MEKK1 (39,47). Ras activation has been shown to be a prerequisite for the TGF-β1- or estradiol-induced activation of MAPK p44/42 in many cell types (28,62). It has also been shown that the Ras-dominant negative mutant is not effective in blocking MAPK p44/42 induced by TGF-β1 in COS cells (63). In FS cells, estradiol and TGF-β3 action on MAPK is Ras p21 dependent.

In this study, we found that the PKC inhibitors suppressed the phosphorylation of MAPK p44/42 as well as the bFGF expression induced by TGF-β3 and estradiol in FS cells. These data are consistent with previous findings that PKC activates MAPK p44/42 through a Ras- or Raf-dependent pathway (64-66), and that PKC regulates bFGF production in rat dermal fibroblast cells (67) and adrenal chromaffin cells (68). Keshamouni *et al.* (28) also reported the predominant role of PKC in estradiol-induced p44/42 MAPK activation. PKC is known to activate the MAPK p44/42 pathway by directly phosphorylating c-Raf-1 or by activating the Ras-GTP-Raf complex (63,69). Hence, the present data, showing a PKC inhibitor blockade of MAPK p44/42 activation and bFGF levels, suggest the involvement of the PKC-Ras-MEK-MAPK p44/42 cascade in the TGF-β3/estradiol interaction in bFGF expression and release.

In conclusion, the results from this study provide evidence that estradiol and TGF-β3 cross-talk to activate a PKC-dependent MAPK pathway to increase bFGF production and release from FS cells. The MAPK pathway that is activated by estradiol and TGF-β3 belongs to the Ras-MEK-MAPK p44/42 cascade. We provide evidence that the interaction between estradiol and TGF-β3 is critical for a maximal response of lactotropes to estradiol's challenge. Estradiol has been shown to magnify the action of a GnRH on gonadotropic cells in the pituitary by promoting the priming action of the peptide (70). Estradiol may magnify TGF-β3's action on FS cells by a similar mechanism. It is also possible that the interaction is the result of cross-talk of MAPK p44/42 with other pathways regulating bFGF release, and is activated attributable to a high level of phosphorylated MAPK p44/42 after combined treatments with estradiol and TGF-β3. Further experimental evidence is required to establish these views.

Acknowledgments

This work was supported by NIH Grants CA 77550 and AA 11591 (to D.K.S.).

Abbreviations

bFGF	Basic fibroblast growth factor
Bis	Bisindolylmalemide
DMSO	dimethylsulfoxide
FS	folliculostellate
GST	glutathione-*S*-transferase
JNK	c-jun terminal kinase
MEK	MAPK kinase
PKC	protein kinase C
TβR	TGF-β receptor

References

1. Gooren LJ, Assies J, Asscheman H, de Slegte R, van Kessel H. Estrogen-induced prolactinoma in a man. J Clin Endocrinol Metab 1988;66:444–446. [PubMed: 3339116]
2. Garcia MM, Kapcalam LP. Growth of a microprolactinoma to a macro-prolactinoma during estrogen therapy. J Endocrinol Invest 1995;18:450–455. [PubMed: 7594240]
3. Carol W, Lauterbach H, Klinger G, Unger A, Michels W. Prolactin stimulation using the metoclopramide test in females taking oral contraceptives. Zentralbl Gynakol 1998;110:1515–1521. [PubMed: 3149100]
4. Ishbashi, MY.; Yamaji, T. Functional heterogeneity of human prolactin producing pituitary adenoma cells. In: MacLeod, RM.; Scapagnni, U.; Thorner, MO., editors. Prolactin basic and clinical correlates. Springer-Verlag; New York: 1985. p. 641-702.
5. Sadoul JL, Thyss A, Freychet P. Invasive mixed growth hormone/prolactin secreting pituitary tumour: complete shrinking by octreotide and bromocriptine, and lack of tumour growth relapse 20 months after octreotide withdrawal. Acta Endocrinol (Copenh) 1992;126:179–183. [PubMed: 1543025]
6. Hentges S, Sarkar DK. Transforming growth factor-β regulation of estradiol-induced prolactinomas. Front Neuroendocrinol 2001;22:340–363. [PubMed: 11587556]
7. Shipley GD, Pittelkow MR, Wille JJ Jr, Scott RE, Moses HL. Reversible inhibition of normal human prokeratinocyte proliferation by type β transforming growth factor-growth inhibitor in serum-free medium. Cancer Res 1986;46:2068–2071. [PubMed: 2418960]
8. Reiss M, Sartorelli AC. Regulation of growth and differentiation of human keratinocytes by type β transforming growth factor and epidermal growth factor. Cancer Res 1987;47:6705–6709. [PubMed: 2445478]
9. Battegay EJ, Raines EW, Seifert RA, Bowen-Pope DF, Ross R. TGF-β induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. Cell 1990;63:515–524. [PubMed: 2171777]
10. Massague J. The transforming growth factor-β family. Annu Rev Cell Biol 1990;6:597–641. [PubMed: 2177343]
11. Roberts B, Sporn MB. Peptide growth factors and their receptors. In: Sporn MB, Roberts AB, eds. Handbook of experimental pharmacology. Heidelberg: Springer-Verlag; 1990;95:419–472.
12. Burns G, Sarkar DK. Transforming growth factor β 1-like immunoreactivity in the pituitary gland of the rat: effect of estrogen. Endocrinology 1993;133:1444–14449. [PubMed: 8365375]

13. Pastorcic M, De A, Boyadjieva N, Vale W, Sarkar DK. Reduction in the expression and action of transforming growth factor β1 on lactotropes during estrogen-induced tumorigenesis in the anterior pituitary. Cancer Res 1995;55:4892–4898. [PubMed: 7585526]
14. Hentges S, Pastorcic M, De A, Boyadjieva N, Sarkar DK. Opposing actions of two transforming growth factor-β isoforms on pituitary lactotropic cell proliferation. Endocrinology 2000;141:1528–1535. [PubMed: 10746660]
15. Hentges S, Boyadjieva N, Sarkar DK. Transforming growth factor-β3 stimulates lactotrope cell growth by increasing basic fibroblast growth factor from folliculo-stellate cells. Endocrinology 2000;141:859–867. [PubMed: 10698159]
16. Trinkaus-Randall V, Nugent MA. Biological response to a synthetic cornea. J Control Release 1988;53:205–214. [PubMed: 9741928]
17. Nugent MA, Iozzo RV. Fibroblast growth factor-2. Int J Biochem Cell Biol 2000;32:115–120. [PubMed: 10687947]
18. Attisano L, Wrana JL. Signal transduction by the TGF-β superfamily. Science 2002;296:1646–1647. [PubMed: 12040180]
19. Tsukazaki T, Chiang TA, Davison AF, Attisano L, Wrana JL. SARA, a FYVE domain protein that recruits Smad2 to the TGF receptor. Cell 1998;95:779–791. [PubMed: 9865696]
20. Wrana JL, Attisano L, Wieser R, Ventura F, Massague J. Mechanism of activation of the TGF-β receptor. Nature 1994;370:341–347. [PubMed: 8047140]
21. Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K, ten Dijke P. TGF-β receptor-mediated signaling through Smad2, Smad3 and Smad4. EMBO J 1997;16:5353–5362. [PubMed: 9311995]
22. Yonekura A, Osaki M, Hirota Y, Tsukazaki T, Miyazaki Y, Matsumoto T, Ohtsuru A, Namba H, Shindo H, Yamashita S. Transforming growth factor-β stimulates articular chondrocyte cell growth through p44/42 MAP kinase (ERK) activation. Endocr J 1999;46:545–553. [PubMed: 10580747]
23. Engel ME, McDonnell MA, Law BK, Moses HL. Interdependent SMAD and JNK signaling in transforming growth factor-β-mediated transcription. J Biol Chem 1999;274:37413–37420. [PubMed: 10601313]
24. Hanafusa H, Ninomiya-Tsuji J, Masuyama N, Nishita M, Fujisawa J, Shibuya H, Matsumoto K, Nishida E. Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-β-induced gene expression. J Biol Chem 1999;274:27161–27167. [PubMed: 10480932]
25. Hocevar BA, Brown TL, Howe PH. TGF-β induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway. EMBO J 1999;18:1345–1356. [PubMed: 10064600]
26. Sano Y, Harada J, Tashiro S, Gotoh-Mandeville R, Maekawa T, Ishii S. ATF-2 is a common nuclear target of Smad and TAK1 pathways in transforming growth factor-β signaling. J Biol Chem 1999;274:8949–8957. [PubMed: 10085140]
27. Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL. Transforming growth factor-β1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol Biol Cell 2001;12:27–36. [PubMed: 11160820]
28. Keshamouni VG, Mattingly RR, Reddy KB. Mechanism of 17-β-estradiol-induced Erk1/2 activation in breast cancer cells. A role for HER2 AND PKC-delta. J Biol Chem 2002;277:22558–22565. [PubMed: 11960991]
29. Santen RJ, Song RX, McPherson R, Kumar R, Adam L, Jeng MH, Yue W. The role of mitogen-activated protein (MAP) kinase in breast cancer. J Steroid Biochem Mol Biol 2002;80:239–256. [PubMed: 11897507]
30. van der Burg B, van Selm-Miltenburg AJ, de Laat SW, van Zoelen EJ. Direct effects of estrogen on c-fos and c-myc protooncogene expression and cellular proliferation in human breast cancer cells. Mol Cell Endocrinol 1989;64:223–228. [PubMed: 2507374]
31. Weigel NL. Steroid hormone receptors and their regulation by phosphorylation. Biochem J 1996;319:657–667. [PubMed: 8920964]
32. Reddy KB, Yee D, Hilsenbeck SG, Coffey RJ, Osborne CK. Inhibition of estrogen-induced breast cancer cell proliferation by reduction in autocrine transforming growth factor α expression. Cell Growth Differ 1994;5:1275–1282. [PubMed: 7696176]

33. Arteaga CL, Coronado E, Osborne CK. Blockade of the epidermal growth factor receptor inhibits transforming growth factor α-induced but not estrogen-induced growth of hormone-dependent human breast cancer. Mol Endocrinol 1988;2:1064–1069. [PubMed: 3221874]
34. Dickson RB, Lippman ME. Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. Endocr Rev 1987;8:29–43. [PubMed: 3549276]
35. Sirbasku DA. Estrogen induction of growth factors specific for hormone-responsive mammary, pituitary and kidney tumor cells. Proc Natl Acad Sci USA 1978;75:3786–3790. [PubMed: 278988]
36. Sporn MB, Roberts AB. Autocrine growth factors and cancer. Nature 1985;313:745–747. [PubMed: 3883191]
37. Nadal A, Ropero AB, Fuentes E, Soria B. The plasma membrane estrogen receptor: nuclear or unclear. Trends Pharmacol Sci 2001;22:597–599. [PubMed: 11730951]
38. Hayashida T, Poncelet AC, Hubchak SC, Schnaper HW. TGF-β1 activates MAP kinase in human mesangial cells: a possible role in collagen expression. Kidney Int 1999;56:1710–17120. [PubMed: 10571779]
39. Davis RJ. The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 1993;268:14553–14556. [PubMed: 8325833]
40. Pearson G, Robinson F, Beere GT, Xu B, Karandikar M, Berman K, Cobb MH. Mitogen activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 2001;22:153–183. [PubMed: 11294822]
41. Duncia JV, Santella JB 3rd, Higley CA, Pitts WJ, Wityak J, Frietze WE, Rankin FW, Sun JH, Earl RA, Tabaka AC, Teleha CA, Blom KF, Favata MF, Manos EJ, Daulerio AJ, Stradley DA, Horiuchi K, Copeland RA, Scherle PA, Trzaskos JM, Magolda RL, Trainor GL, Wexler RR, Hobbs FW, Olson RE. MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products. Bioorg Med Chem Lett 1998;20:2839–2844. [PubMed: 9873633]
42. Seger R, Seger D, Lozeman FJ, Ahn NG, Graves LM, Campbell JS, Ericsson L, Harrylock M, Jensen AM, Krebs EG. Human T-cell mitogen-activated protein kinase kinases are related to yeast signal transduction kinases. J Biol Chem 1992;267:25628–25631. [PubMed: 1281467]
43. Robbins DJ, Zhen E, Owaki H, Vanderbilt CA, Ebert D, Geppert TD, Cobb MH. Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 *in vitro*. J Biol Chem 1993;268:5097–5106. [PubMed: 8444886]
44. Han Z, Boyle DL, Aupperle KR, Bennett B, Manning AM, Firestein GS. Jun N-terminal kinase in rheumatoid arthritis. J Pharmacol Exp Ther 1999;291:124–130. [PubMed: 10490895]
45. Feig LA, Cooper GM. Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. Mol Cell Biol 1988;8:3235–3243. [PubMed: 3145408]
46. Szeberenyi J, Cai H, Cooper GM. Effect of a dominant inhibitory Ha-ras mutation on neuronal differentiation of PC12 cells. Mol Cell Biol 1990;10:5324–5332. [PubMed: 2118994]
47. Blanchette F, Rivard N, Rudd P, Grondin F, Attisano L, Dubois CM. Cross-talk between the p42/p44 MAP kinase and Smad pathways in transforming growth factor β 1-induced furin gene transactivation. J Biol Chem 2001;276:33986–33994. [PubMed: 11448947]
48. Liebmann C. Regulation of MAP kinase activity by peptide receptor signaling pathway: paradigms of multiplicity. Cell Signal 2001;3:777–785. [PubMed: 11583913]
49. Bell D, Schluter KD, Zhou XJ, McDermott BJ, Piper HM. Hypertrophic effects of calcitonin gene-related peptide (CGRP) and amylin on adult mammalian ventricular cardiomyocytes. J Mol Cell 1995;27:2433–2443.
50. Chun T-Y, Gregg D, Sarkar DK, Gorski J. Differential regulation by estrogens of growth and prolactin synthesis in pituitary cells suggests that only a small pool of estrogen receptors is required for growth. Proc Natl Acad Sci USA 1998;95:2325–2330. [PubMed: 9482884]
51. Dupont J, Le Roith D. Insulin-like growth factor 1 and oestradiol promote cell proliferation of MCF-7 breast cancer cells: new insights into their synergistic effects. Mol Pathol 2001;54:149–154. [PubMed: 11376126]
52. Ahmad S, Singh N, Glazer RI. Role of AKT1 in 19-β estradiol- and insulin-like growth factor I (IGF-I)-dependent proliferation and prevention of apoptosis in MCF-7 breast carcinoma cells. Biochem Pharmacol 1999;58:425–430. [PubMed: 10424760]

53. Hamelers IH, van Schaik RF, van Teeffelen HA, Sussenbach JS, Steenbergh PH. Synergistic proliferative action of insulin-like growth factor I and 17 β-estradiol in MCF-7S breast tumor cells. Exp Cell Res 2002;273:107–117. [PubMed: 11795951]
54. Norfleet AM, Clarke CH, Gametchu B, Watson CS. Antibodies to the estrogen receptor-α modulate rapid prolactin release from rat pituitary tumor cells through plasma membrane estrogen receptors. FASEB J 2000;14:57–165.
55. Watson CS, Norfleet AM, Pappas TC, Gametchu B. Rapid actions of estrogens in GH3/B6 pituitary tumor cells via a plasma membrane version of estrogen receptor-α. Steroids 1999;64:5–13. [PubMed: 10323667]
56. Gu Q, Korach KS, Moss RL. Rapid action of 17β-estradiol on kainate-induced currents in hippocampal neurons lacking intracellular estrogen receptors. Endocrinology 1999;140:660–666. [PubMed: 9927291]
57. Obrero M, Yu DV, Shapiro DJ. Estrogen receptor-dependent and estrogen receptor-independent pathways for tamoxifen and 4-hydroxytamoxifen-induced programmed cell death. J Biol Chem 2002;277:45695–45703. [PubMed: 12244117]
58. Ropero AB, Soria B, Nodal M. A nonclassical estrogen membrane receptor triggers rapid differential actions in the endocrine pancreas. Mol Endocrinol 2002;16:497–505. [PubMed: 11875108]
59. Watanabe H, de Caestecker MP, Yamada Y. Transcriptional cross-talk between Smad, ERK1/2, and p38 mitogen-activated protein kinase pathways regulates transforming growth factor-β-induced aggrecan gene expression in chondrogenic ATDC5 cells. J Biol Chem 2001;276:14466–14473. [PubMed: 11278290]
60. Brown JD, DiChiara MR, Anderson KR, Gimbrone MA Jr, Topper JN. MEKK-1, a component of the stress (stress-activated protein kinase/c-Jun N-terminal kinase) pathway, can selectively activate Smad2-mediated transcriptional activation in endothelial cells. J Biol Chem 1999;274:8797–8805. [PubMed: 10085121]
61. Russell M, Lange-Carter CA, Johnson GL. Direct interaction between Ras and the kinase domain of mitogen-activated protein kinase kinase kinase (MEKK1). J Biol Chem 1995;270:11757–11760. [PubMed: 7744823]
62. Hartsough MT, Frey RS, Zipfel PA, Buard A, Cook SJ, McCormick F, Mulder KM. Altered transforming growth factor signaling in epithelial cells when Ras activation is blocked. J Biol Chem 1996;271:22368–223675. [PubMed: 8798398]
63. Marais R, Light Y, Mason C, Paterson H, Olson MF, Marshall CJ. Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. Science 1998;280:109–112. [PubMed: 9525855]
64. Nishibe S, Wahl MI, Hernandez-Sotomayor SM, Tonks NK, Rhee SG, Carpenter G. Increase of the catalytic activity of phospholipase C-γ 1 by tyrosine phosphorylation. Science 1990;250:1253–1256. [PubMed: 1700866]
65. Chen X, Rubock MJ, Whitman M. A transcriptional partner for MAD proteins in TGF-β signaling. Nature 1996;383:691–696. [PubMed: 8878477]
66. Wood KW, Sarnecki C, Roberts TM, Blenis J. Ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell 1992;68:1041–1050. [PubMed: 1312393]
67. Lowe WL Jr, Yorek MA, Karpen CW, Teasdale RM, Hovis JG, Albrecht B, Prokopiou C. Activation of protein kinase-C differentially regulates insulin-like growth factor-I and basic fibroblast growth factor messenger RNA levels. Mol Endocrinol 1992;6:741–752. [PubMed: 1603084]
68. Stachowiak MK, Moffett J, Joy A, Puchacz E, Florkiewicz R, Stachowiak EK. Regulation of bFGF gene expression and subcellular distribution of bFGF protein in adrenal medullary cells. J Cell Biol 1994;127:203–223. [PubMed: 7929563]
69. Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, Rapp UR. Domains of protein kinase C α activates RAF-1 by direct phosphorylation. Nature 1993;364:249–225. [PubMed: 8321321]
70. Pickering AJ, Fink G. Do hypothalamic regulatory factors other than luteinizing hormone releasing factor exert a priming effect? J Endocrinol 1979;81:235–238. [PubMed: 224131]

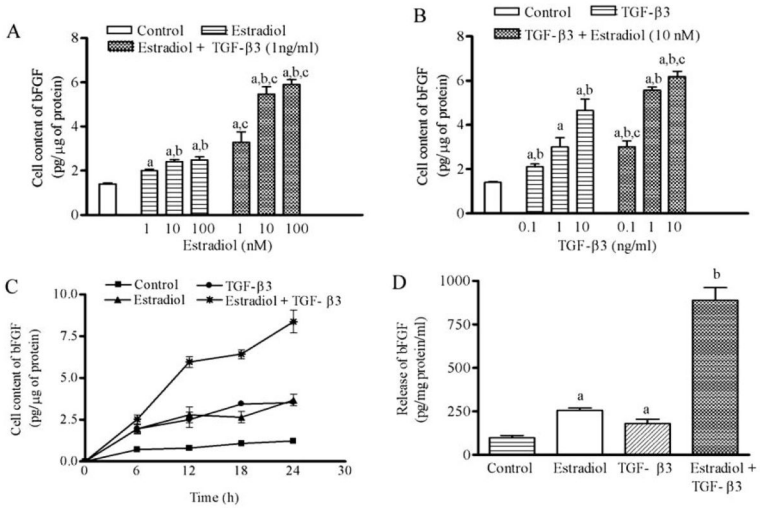

Fig. 1.

Effects of TGF-β3 and/or estradiol on cell content and release of bFGF from FS cells. A and B, Dose-dependency of the effect of estradiol and TGF-β3, either alone or in combination, on bFGF levels in FS cells. a, $P < 0.01$, compared with control untreated group; b, $P < 0.05$, compared with 1 nM estradiol or 1 ng/ml TGF-β3 treatment of the respective group; c, $P < 0.01$, compared with estradiol- or TGF-β3-alone-treated group of respective treatment. C, Time-dependency of the effects of TGF-β3 and estradiol. FS cells were treated with estradiol (10 nM) and TGF-β3 (1 ng/ml), alone or in combination, for various time periods. Statistical analysis of data, using two-way ANOVA, showed significant differences at: 1) $P < 0.001$, among control *vs.* estradiol or TGF-β3 or estradiol+TGF-β3-treated groups for all time points; and 2) $P < 0.001$, estradiol or TGF-β3 *vs.* estradiol TGF-β3-treated group for all time points. No significant difference was found between estradiol- and TGF-β3-treated groups at any time period. D, TGF-β3 and estradiol effects on bFGF release from FS cells that were treated with estradiol (10 nM) and TGF-β3 (1 ng/ml), either alone or in combination, for 24 h. a, $P < 0.01$, compared with untreated control; b, $P < 0.01$, compared with all other groups. Data are mean ± SEM of three to six individual experiments, carried out separately, in triplicate.

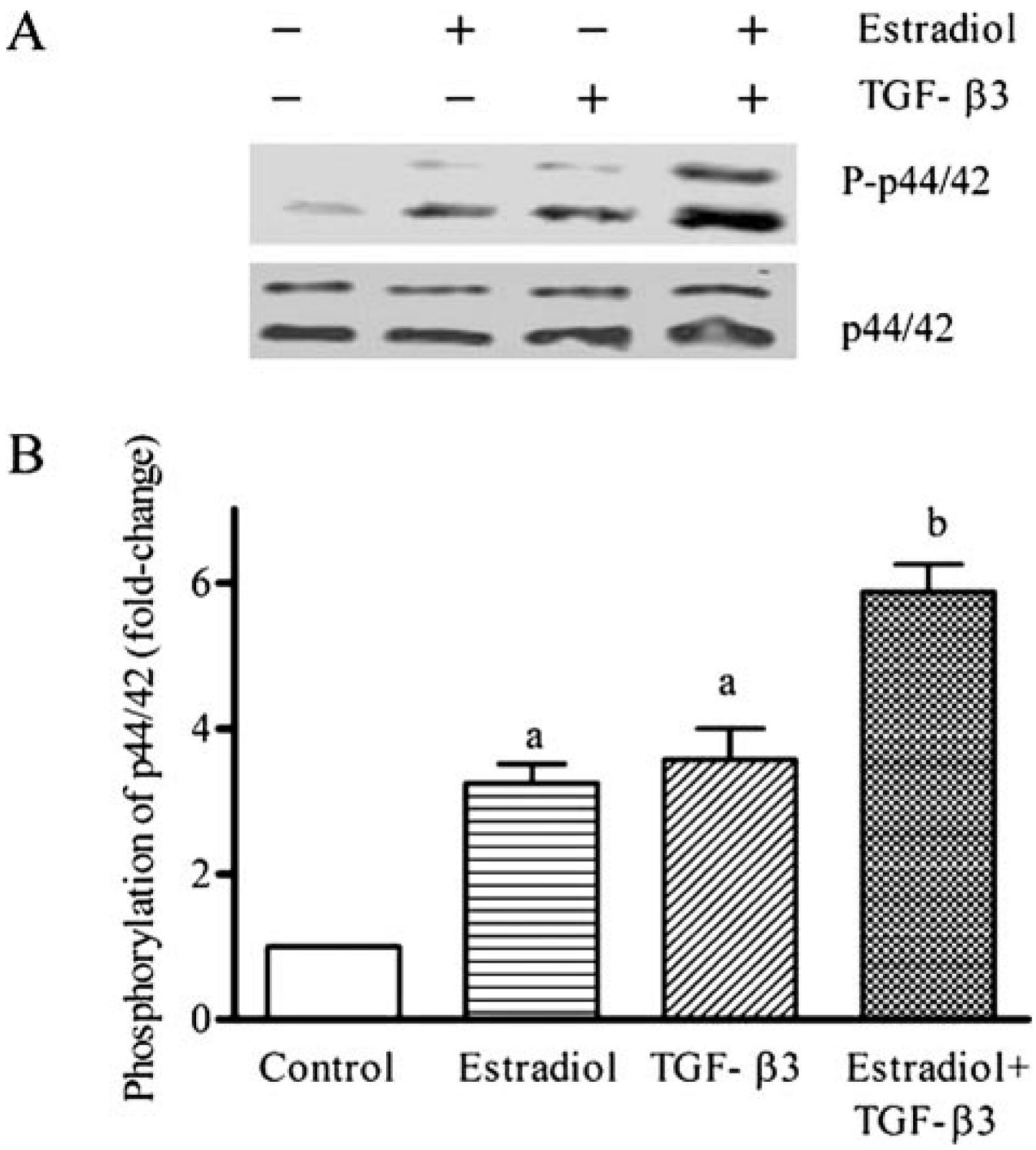

Fig. 2.

Effect of TGF-*β*3 and/or estradiol on MAPK activation in FS cells. FS cells were treated with estradiol (10 nM) and TGF-*β*3 (1 ng/ml), either alone or in combination, for 2 h, to determine MAPK p44/42 phosphorylation by Western blot. A, Representative Western blot showing changes in phosphorylation of MAPK p44/42 activation. B, Densitometric analysis of phosphorylated *vs.* total MAPK p44/42. The data represent the fold-increase over controls. Each *bar* represents mean ± SEM of three independent experiments. a, $P < 0.05$, compared with control; b, $P < 0.01$, compared with all other groups.

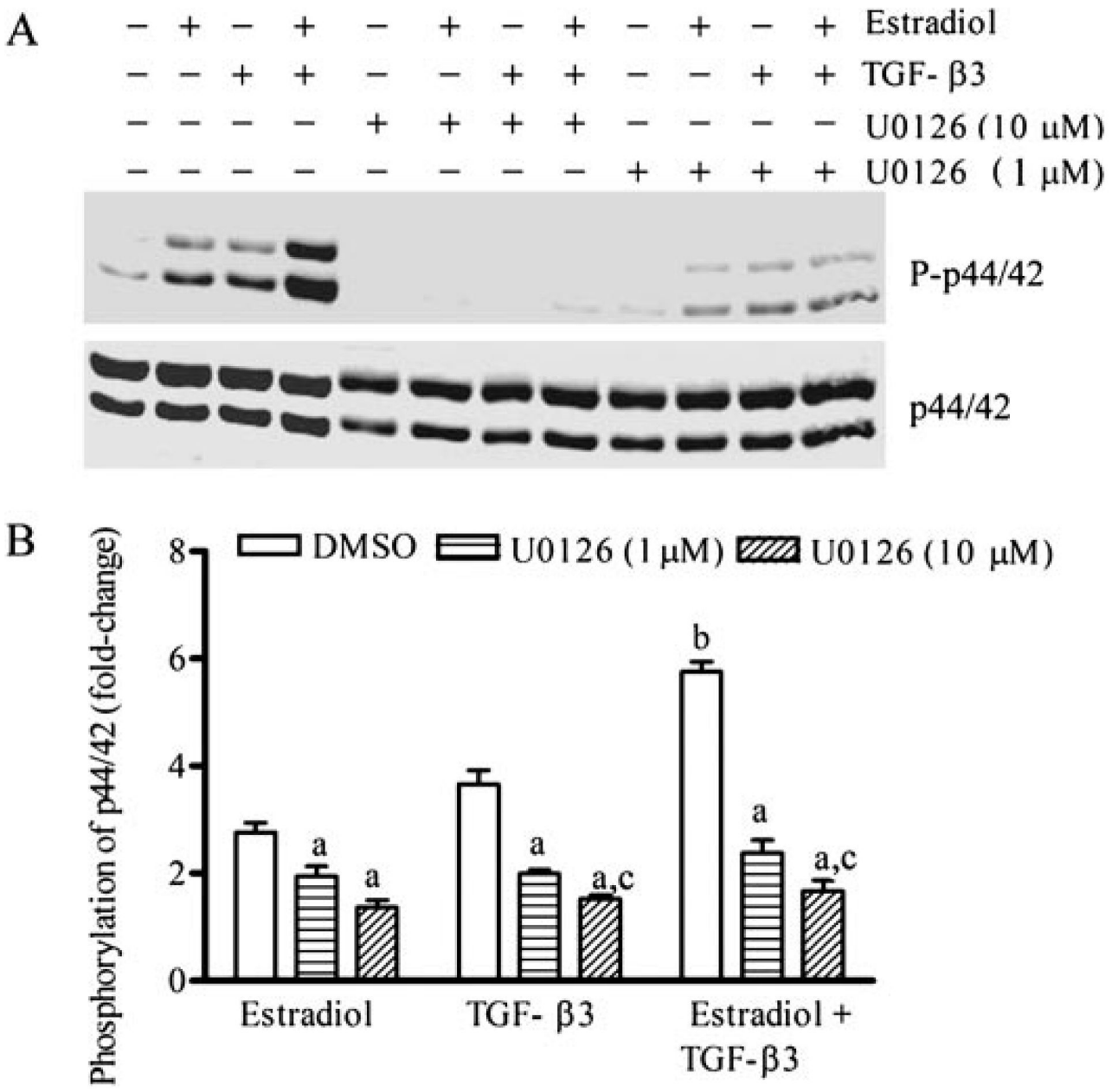

Fig. 3.

Effect of U0126, a MEK1/2 kinase inhibitor, on TGF-*β*3-and/or estradiol-induced increase in MAPK activity in FS cells. FS cells were incubated with U0126 or vehicle for 1 h and then treated with estradiol (10 nM) and TGF-*β*3 (1 ng/ml), either alone or in combination, for 2 h. A, Representative blot showing the effect of the MAPK inhibitor. B, Densitometric analysis of phosphorylated *vs.* total MAPK. The data represent the fold-increase over controls. a, $P < 0.05$, compared with the respective DMSO-treated group; b, $P < 0.05$, compared with all other groups; c, $P < 0.05$, compared with respective 1-*μ*M U0126 treatment. Each *bar* represents mean ± SEM of three individual experiments.

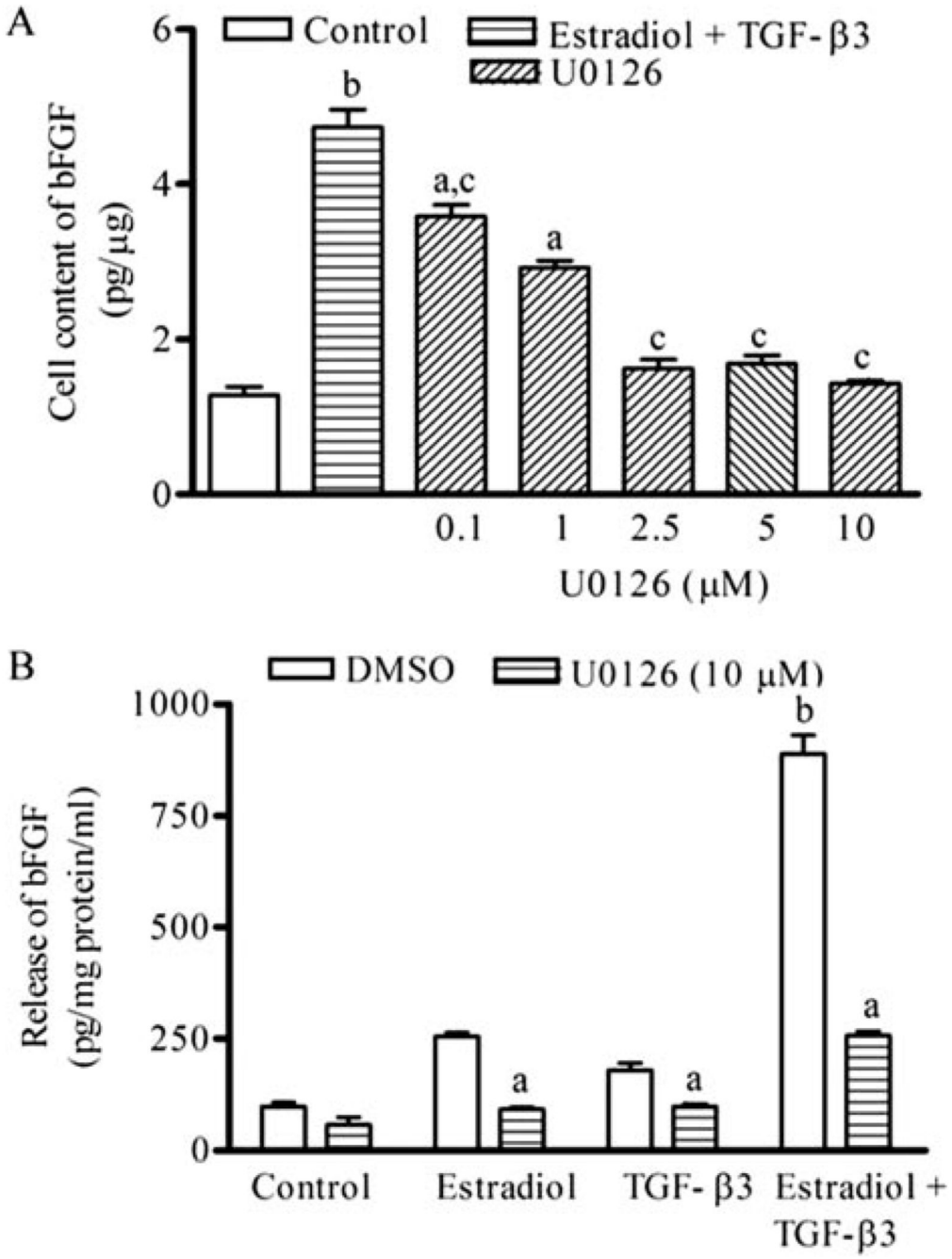

Fig. 4.

Effects of U0126, a MEK1/2 kinase inhibitor, on TGF-*β*3-and/or estradiol-induced increase in the cell content and release of bFGF from FS cells. FS cells were treated with U0126 (0.1–10 μ_M) or vehicle for 1 h, then with estradiol (10 n_M) and TGF-*β*3 (1 ng/ml), either alone or in combination, for 24 h. A, Dose-response effects of U0126 on cellular levels of bFGF. a, $P < 0.01$, compared with control untreated group; b, $P < 0.01$ compared with all other groups; c, $P < 0.01$, compared with 1-μ_M-U0126-treated group. B, The effect of U0126 on bFGF release (the values shown here for DMSO-treated groups are from Fig. 1B for comparison). a, $P < 0.01$, compared with the respective controls; b, $P < 0.01$, compared with all other groups. Data are mean ± SEM of three to five individual experiments, carried out separately, in triplicate.

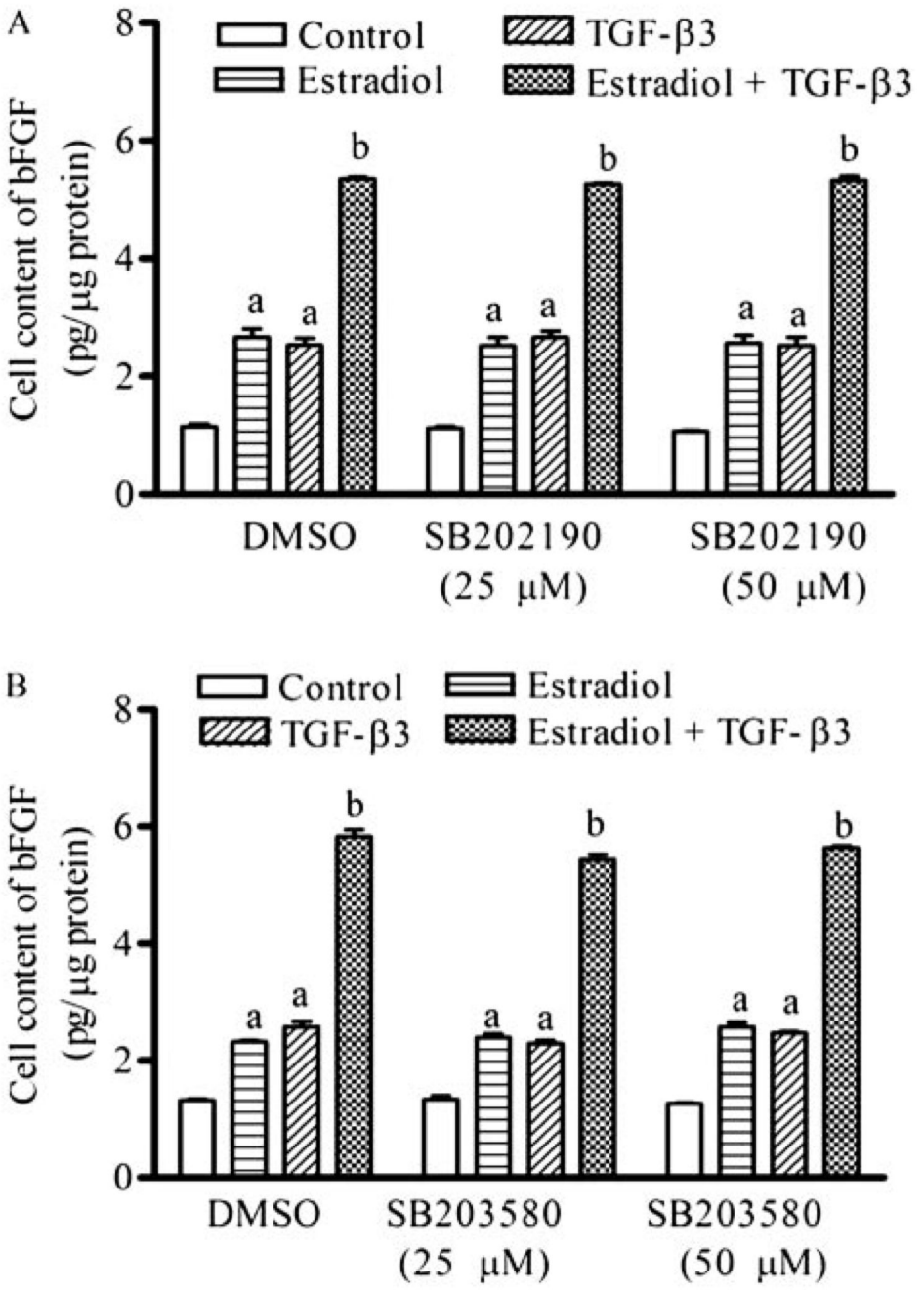

Fig. 5.

Effects of MAPK p38 or p38/JNK inhibitors on TGF-*β*3-and/or estradiol-induced increase in cellular levels of bFGF in FS cells. FS cells were preincubated with cell-permeable inhibitor of MAPK p38, SB202190, or inhibitor of p38/JNK pathway, SB203580, or vehicle in serum-supplemented media for 1 h followed by treatment with estradiol (10 nM) and TGF-*β*3 (1 ng/ml), either alone or in combination, for 24 h. A. Effect of p38 inhibitor SB202190. B. Effect of p38/JNK inhibitor SB203580. a, $P < 0.01$, compared with respective inhibitor or control. b, $P < 0.01$, compared with all other treatments in same group. Each *bar* represents mean ± SEM of three individual experiments performed separately, in triplicate.

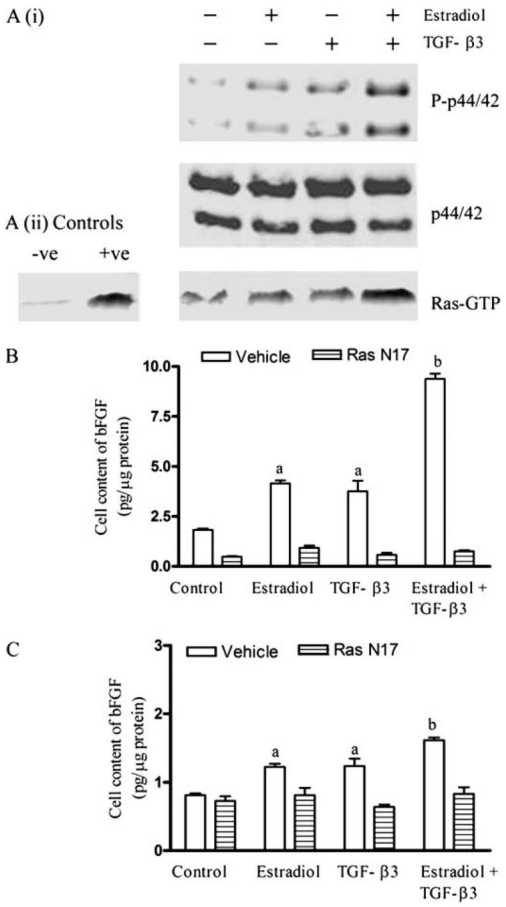

Fig. 6.

Role of Ras-MAPK pathway in TGF-*β*3- and/or estradiol-induced increases in bFGF levels. A, Determination of Ras activity. FS cells were stimulated with estradiol (10 nM) and TGF-*β*3 (1 ng/ml), either alone or in combination, for 2 h, and lysed in lysis buffer. Cell lysates were then subjected to pull-down assay with GST-RBD-agarose followed by SDS/PAGE and immunoblotting with anti-Ras antibody to detect active (GTP-bound) Ras. Aliquots of the same cell lysate (without pull-down assay) were independently analyzed by immunoblotting, using anti-MAPK (to control loading) and the phospho-MAPK antibody to evaluate activation of MAPK. A (i), A representative experiment is shown. This experiment was repeated three times with similar results; (ii), −ve and +ve control for Ras activity assay. B and C, Effects of over-expression of Ras N17 on the changes of FS cell content of bFGF. FS cells were transiently transfected with Ras N17, a dominant negative mutant of Ras p21, or vehicle. Eighteen hours after transfection, cells were incubated with estradiol (10 nM) and TGF-*β*3 (1 ng/ml), either alone or in combination, for 24 h (B) or 6 h (C), for cellular bFGF levels. Each *bar* represents mean ± SEM of three individual experiments, performed separately, in triplicate. a, $P < 0.05$, compared with controls; b, $P < 0.05$, compared with all other groups.

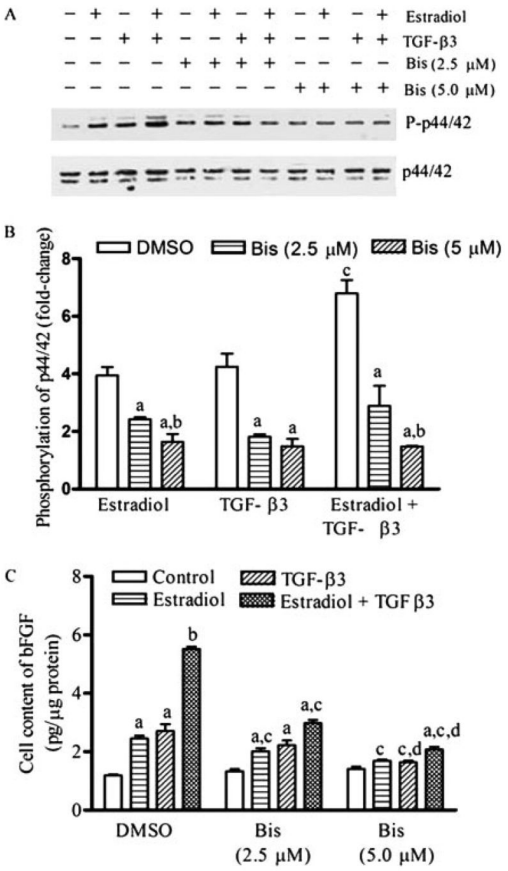

Fig. 7.

Effect of Bis, a PKC inhibitor, on TGF-*β*3- and/or estradiol-induced increase in phosphorylation of MAPK p44/42 and cell content of bFGF. FS cells were preincubated with the PKC inhibitor Bis (2.5 and 5 μ_M) or vehicle, in serum-supplemented media, for 1 h, and then treated with estradiol (10 n_M) and TGF-*β*3 (1 ng/ml), either alone or in combination, for 2 h (to determine phosphorylation of MAPK p44/42) or for 24 h (to measure cell content of bFGF). A, Representative blot showing the effect of this PKC inhibitor on phosphorylation of MAPK p44/42. B, Densitometric analysis of phosphorylated *vs.* total MAPK p44/42 in the presence and absence of Bis. The data express the fold-increase over DMSO-only-treated controls. a, $P < 0.01$, compared with the respective DMSO-treated group; b, $P < 0.0$, compared with respective 2.5-μ_M-Bis-treated group; c, $P < 0.01$, compared with all other groups. C, Effect of the PKC inhibitor Bis on bFGF content induced by TGF-*β*3 (1 ng/ml) and estradiol (10 n_M). a, $P < 0.01$, compared with respective inhibitor-only-treated group; b, compared with all other groups; c, $P < 0.05$, compared with respective DMSO-treated group; d, $P < 0.05$, compared with respective 2.5-μM-Bis-treated group. Each *bar* represents mean ± SEM of three individual experiments, performed separately, in triplicate.

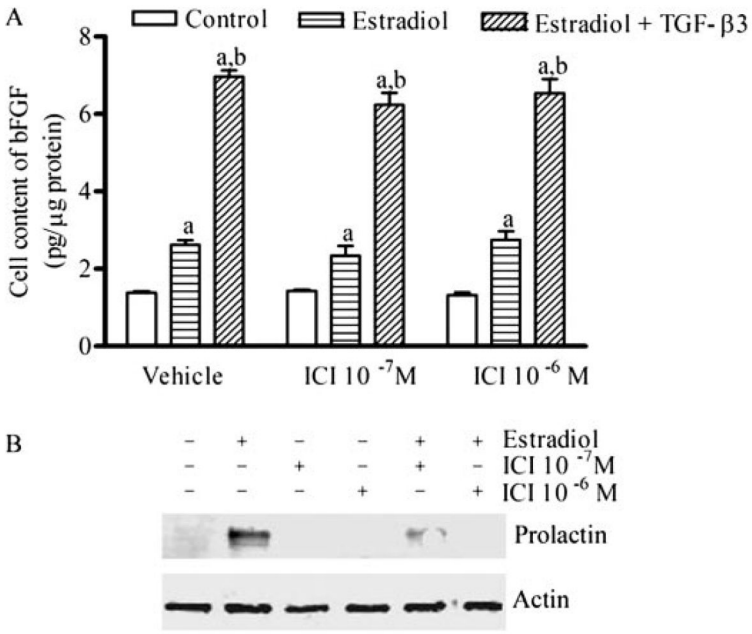

Fig. 8.

Effects of estrogen receptor blocker on estradiol- or TGF-β3+estradiol-induced increase in cellular levels of bFGF in FS cells. A, FS cells were preincubated with estrogen receptor blocker ICI 182,780 (ICI) (10^{-7}–10^{-8} M) or vehicle, in serum-supplemented media for 1 h, followed by treatment with TGF-β3 (1 ng/ml) and estradiol (10 nM) or estradiol (10 nM) alone for 24 h. Each *bar* represents the mean ± SEM of three individual experiments, performed separately, in triplicate. a, $P < 0.01$, compared with respective untreated group; b, $P < 0.01$, compared with respective estradiol-treated group. B. Representative blot verifying the well-known antagonistic effects of ICI 182,780 on estradiol-induced increase in intracellular prolactin in PR1 cells (50).