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Role of PKC–Ras–MAPK p44/42 in ethanol and TGF-β3-induced bFGF release from folliculostellate cells

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Running title: Ras-MAPK p44/42 mediated action of ethanol and TGF-β3

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Abbreviations: bFGF, basic fibroblast growth factor; TGF-β3, transforming growth factor beta; MAPK, mitogen activated protein kinase; PKC, protein kinase C

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Abstract

In the present study, we determined the interactive effects of ethanol and transforming growth factor beta-3 (TGF-β3) on basic fibroblast growth factor (bFGF) release from folliculostellate (FS) cells, and the role of the mitogen-activated protein kinase (MAPK) pathway in this interaction. We found that TGF-β3 and ethanol alone increased release of bFGF from FS cells, but together they showed markedly increased levels of bFGF compared to the individual effect. Ethanol and TGF-β3 alone moderately increased activation of MAPK p44/42, but together they produced marked activation of MAPK p44/42. TGF-β3 alone increased the activation of smad2. Ethanol did not activate smad2 or alter TGF-β3-activation of smad2. Pretreatment of FS cells with a MEK1/2 inhibitor or with a protein kinase C (PKC) inhibitor suppressed the TGF-β3 and ethanol actions on MAPK p44/42 activation and bFGF release. Ethanol and TGF-β3, either alone or in combination, increased the levels of active Ras. Furthermore, the MAPK p44/42 activation by TGF-β3 and ethanol was blocked by overexpression of Ras N17, a dominant negative mutant of Ras p21. These data suggest that the PKC-activated Ras-dependent MAPK p44/42 pathway is involved in the cross-talk between TGF-β3 and ethanol to increase bFGF release from FS cells.

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Introduction

Alcohol drinking and estrogen exposure have both been considered risk factors for the development of prolactin-secreting pituitary tumors known as prolactinomas in humans and laboratory animals (Gooren et al., 1988; Garcia and Kapcala, 1995; De et al., 2002). There are several reports showing evidence of high levels of prolactin in chronic alcoholic men and women (Mello et al., 1988; Välimäki et al., 1990; Seki et al., 1991; Teoh et al., 1994; Gavaler, 1994). Alcoholics also show elevated blood levels of estrogens (Valimaki et al., 1990), which are believed to be due to peripheral conversion of weak androgens of estrogens. We have previously shown that, like estradiol, ethanol induces hyperprolactinemia by increasing prolactin release from lactotropes and by increasing the number of lactotropes in the anterior pituitary (De et al., 1995, 2000, 2002; Hentges and Sarkar, 2001). We have also shown that ethanol and estradiol act similarly on the dopaminergic regulation of prolactin release (Oomizu et al., 2003). Recently, we have found that estrogen and transforming growth factor beta 3 (TGF-β3) interact to increase production of the basic fibroblast growth factor (bFGF) from folliculostellate (FS) cells (Hentges and Sarkar, 2001; Chaturvedi and Sarkar, 2004). bFGF acts on lactotropes to increase their proliferation (Hentges et al., 2000). Whether ethanol interacts with TGF-β3 to increase bFGF production from FS cells is not known.

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 TGF-βII to associate with the TGF-β3 type I receptor (TβRI), which leads to a unidirectional phosphorylation event in which TβRII phosphorylates TβRI. This phosphorylation of TβRI activates its kinase

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domain, which further phosphorylates smad2 and smad3 proteins (Attisano and Wrana 2002). Activated smad2 and 3 bind to smad4 and further translocate to the nucleus to activate various transcription factors (Wrana et al., 1994; Tsukazaki et al., 1998). In addition to this smad-mediated TGF-β signaling pathway, evidence over the past few years suggests that TGF-β might signal through several mitogen-activated protein kinases (MAPKs) (Engel et al., 1999; Hanafusa et al., 1999; Chaturvedi and Sarkar, 2004).

The various MAPKs have also been shown to be activated by ethanol, depending on the cell type (See review by Arror and Shukla, 2004). Ethanol can activate MAPK p44/42 by increasing the activation of protein kinase C (PKC) (Luo and Miller, 1999; Washington et al., 2003). We have recently found that estrogen elevates TGF-β3-induced bFGF release from FS cells by activating the PKC-dependent MAPK p44/42 pathway (Chaturvedi and Sarkar, 2004). However, the role of the MAPK p44/42 pathway in ethanol's and TGF-β3's interactive action on bFGF release from FS cells was not determined.

In this present study we determined whether, like estradiol (Chaturvedi and Sarkar 2004), ethanol also promotes TGF-β3 action by activating MAPK p44/42 signaling in FS cells. We also determined if ethanol also modulates the levels of TGF-β3-activated smad2. Our data suggest a role of PKC–Ras–MAPK p44/42 activation without the involvement of smad2 in ethanol and TGF-β3 interaction for bFGF release from FS cells.

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Material and Methods

Cell Culture and Reagents. We have previously established an FS cell line (F344-FS) from anterior pituitary cells of Fischer-344 (F344) female rats (Hentges et al., 2000). The FS cell line was maintained in DMEM/F-12 with 10% fetal calf serum (FCS). These cells were used in between generations 20 and 30. During experimentation, cells were maintained in DMEM/F-12 containing serum supplement (consisting of 30 nM selenium, 100 μM iron-free human transferrin, 1 μM putrescine and 5 μM insulin) but no FCS. The MEK inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) was purchased from Cell Signaling (Beverly, MA); the PKC inhibitor Bisindolylmalemide (Bis) was purchased from Calbiochem (San Diego, CA); TGF-β3 was purchased from R&D Systems (Minneapolis, MN), and Ras N17, a plasmid containing a dominant negative mutant of Ras p21, was purchased from Clontech Laboratories (Palo Alto, CA).

bFGF Assay. FS cells (250,000/well) were grown in 24-well plates in serum-containing medium. After 2 d of plating, the medium was changed and fresh medium containing serum supplement was added in the culture. On the following day, cells were treated with vehicle (control) or TGF-β3 (0.001–10 ng/ml) and ethanol (6.25–100 mM), alone or in combination, in medium containing serum supplement for 24 h. Media was collected, and levels of bFGF were determined using an immunoassay kit (R&D Systems, Minneapolis, MN.) Total protein concentrations in cell lysates were determined using the Bio-Rad assay (Bio-Rad, Hercules, CA) to normalize the levels of bFGF per mg of total protein.For blocking studies, cells were pre-treated with inhibitors for 1 h, followed by

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TGF-β3 and ethanol treatments for various time periods. Control cells were treated with vehicle (DMSO) alone.

Phosphorylation of MAPK p44/42. FS cells (0.5 x 10^6/well) were grown in 6-well plates followed by incubation in medium containing serum supplement. Cells were then treated with vehicle, TGF-β3 (1 ng/ml) and ethanol (50 mM), alone or in combination for 2 h. These doses of ethanol and TGF-β3 were used because these are physiological doses and showed significant effect on bFGF release. Cells were lysed, and the cell extracts were analyzed for total and phosphorylated MAPK p44/42 by immunoblotting. For blocking studies, cells were pre-treated with inhibitors for 1 h, followed by TGF-β3 and ethanol treatments for another 2-h period. Control cells were treated with vehicle alone.

Western Blot. Cells were lysed in sample gel loading buffer containing (Tris-HCl pH 6.8, 2% glycerol, 0.05% bromophenol blue, 10% glycerol, 5% of 14.4 M beta-mercaptoethanol, and phosphatase inhibitor cocktail I and II; Sigma, St. Louis, MO) and heated at 95°C for 5–6 min. Each sample was resolved on SDS/PAGE and transferred to immobilon-P PVDF membranes (Millipore, Bedford, MA). Total MAPK p44/42 and phosphorylated MAPK p44/42 proteins were detected together in one blot at the same time. For this purpose, membranes were incubated with both a rabbit anti-MAPK p44/42 antibody (Upstate Biotechnology, Waltham, MA) and a mouse anti-phosopho MAPK p44/42 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer (Licor Biotechnology, Lincoln, NE). Afterward, membranes were washed and incubated with fluorescent-labeled secondary anti-mouse and anti-rabbit antibodies for 1 h. Membranes were scanned at 700 nm (anti-rabbit) and 800 nm (anti-mouse) wavelengths using an Infrared Imaging System (Licor Biotechnology) to determine the fluorescence of the

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bands for phosphorylated and total MAPK p44/42. For quantification of MAPK p44/42 activity, band intensities of phosphorylated MAPK p44/42 were determined using Scion Image software and normalized to the corresponding total MAPK p44/42. Levels of active Ras and phosphorylated smad2 were measured by immunoblotting using the mouse anti-Ras antibody (Upstate Biotechnology) and rabbit anti-phospho-smad2 antibody (Cell Signaling), respectively. For these assays, 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 a peroxidase-conjugated anti-mouse or anti-rabbit antibody for 1 h. Then they were developed using an ECL Western blot chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ). Band intensities were quantified using Scion Image software.

Transient Transfection. FS cells were transfected with a plasmid encoding for a dominant negative mutant of Ras p21, Ras N17, or with a vector plasmid using the LipoFECTAMINE 2000 reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol, in 24-well plates as described by us previously (Chaturvedi and Sarkar, 2004). After 24 h, cells were treated with TGF-β3 (1 ng/ml) and/or ethanol (50 mM) for 1 h. Cells were lysed and analyzed for total and phosphorylated MAPK p44/42 by immunoblotting.

Ras Activity Assay. Cells numbering 5 x 10^6 per treatment were stimulated with TGF-β3 (1 ng/ml) and ethanol (50 mM), alone or together, for 1 h and lysed for 30 min on ice in 500 µl of lysis buffer (provided with the Ras activation assay kit). The lysates were then centrifuged, and supernatants were mixed with 30 µl of Glutathione S transferase (GST)

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fusion protein containing the Ras binding domain of Raf, and immobilized in glutathione-agarose beads. The samples were incubated for 90 min at 4°C with gentle rotation. The beads were then washed 3 times with lysis buffer. The GTP-bound Ras p21 protein was eluted in gel loading buffer and subjected to 12.5 % SDS/PAGE. Levels of active Ras were assessed by immunoblotting with a specific anti-Ras antibody.

Statistical Analyses. Data shown in the figures are mean ± SEM of the indicated number of experiments performed independently. Data were analyzed using one-way ANOVA followed by Newman-Keuls test for post-hoc analysis. The Student t-test was used to compare two groups. A probability value less than 0.05 was considered significant.

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Results

Effects of ethanol and TGF-β3, alone or together, on bFGF release from FS cells. In order to determine whether ethanol interacts with TGF-β3 to increase bFGF production from FS cells, we evaluated the effects of various concentrations of ethanol and TGF-β3, alone or together, on bFGF release from FS cells. The concentration-response curves of ethanol and of TGF-β3 on bFGF release from FS cells are shown in Figs. 1A and 1B, respectively. Ethanol, at a concentration range of 12.5–100 mM, concentration-dependently increased bFGF release from FS cells; a 100-mM concentration of ethanol produced the maximum effect (Fig. 1A). TGF-β3 increased bFGF release at a concentration range of 0.1–10 ng/ml; the maximal bFGF response was observed at the 10 ng/ml concentration (Fig. 1B). The interactive action of TGF-β3 and ethanol was studied by comparing the changes in the TGF-β3 concentration-dependent effect on bFGF release in the presence and absence of ethanol. Fig. 2 shows that the TGF-β3 concentration-dependent effect on bFGF release was significantly enhanced by ethanol at 50- and 100-mM doses, but not at the 25-mM dose. However, 100 mM dose of ethanol and 10 ng/ml dose of TGF-β3 that induced that maximal bFGF release did not further increase the bFGF release when combined together. These results suggest that ethanol and TGF-β3 may interact to produce an additive effect on bFGF release from FS cells. The dose of 1ng/ml for TGF-β3 and 50 mM for ethanol (50 mM) were used in all signaling experiments.

Activation of MAPK p44/42 by ethanol and TGF-β3 in FS cells. MAPKs are key signal-transducing proteins that transmit signals within cells and are involved in various

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biological effects of hormones and growth factors (Davis 1993; Pearson et al., 2001). We have previously found that the effects of TGF-β3 and estradiol on bFGF levels are mediated by MAPK p44/42. Hence, the role of MAPK p44/42 in TGF-β3 and ethanol interaction was investigated. Activation of MAPK p44/42 was determined by Western blot analysis. Time-course studies, using a combined treatment of TGF-β3 (1 ng/ml) and ethanol (50 mM), indicated that MAPK p44/42 is optimally activated by 1–2 h (phosphorylation of MAPK p44/42; fold change of control: 0.5 h: 2.5 ± 0.09; 1 h: 4.82 ± 0.5; 2 h: 5.29 ± 0.6; n=4). Using the 2-h treatment paradigm, we found that alone TGF-β3 and ethanol moderately activated MAPK p44/42, but together these agents produced significantly more phosphorylation of MAPK (Figs. 3A and 3B).

Involvement of MAPK p44/42 in ethanol- and/or TGF-β3-induced bFGF release from FS cells. To analyze the contribution of the MAPK p44/42 cascade in ethanol- and/or TGF-β3-increased bFGF release from FS cells, we used an MEK1/2 kinase inhibitor, U0126, which blocks the function of MEK1/2 kinases (Seger et al., 1992; Duncia et al., 1998). These kinases are upstream to MAPK p44/42 and are known to phosphorylate MAPK p44/42 (Davis 1993; Robbins et al., 1993). FS cells were pre-incubated with various concentrations of U0126 or vehicle for 1 h and then treated with TGF-β3 and/or ethanol for 2 h to determine MAPK p44/42 activation, and for 24 h to measure bFGF release. The inhibitor at 0.1–10 μM concentrations significantly inhibited the TGF-β3- and/or ethanol-induced phosphorylation of MAPK p44/42 (Figs. 4A and 4B). U0126 treatment also concentration-dependently reduced ethanol- and/or TGF-β3-induced bFGF levels in FS cells (Fig. 4C).

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Involvement of the Ras-activated MAP kinase pathway in TGF-β3- and/or ethanol-regulated bFGF expression in FS cells. Since the MAPK p44/42 pathway inhibitor blocked the effects of ethanol and TGF-β3 on bFGF release from FS cells, it was of interest to find out whether the classical pathway of the Ras–MEK–MAP kinase cascade is involved with bFGF expression in FS cells. 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. The levels of Ras–GTP were increased (densitometric analysis of Ras activity; fold change of control: ethanol: 2.1 ± 0.2, TGF-β3: 2.5 ± 0.3, ethanol+TGF-β3: 5.0 ± 0.4; $p<0.05$, ethanol or TGF-β3 alone vs ethanol+TGF-β3 group; n=4) parallel to increases in phosphorylated MAPK p44/42 after ethanol and/or TGF-β3 treatments (Fig. 5A).

To further confirm the role of Ras p21, we transiently transfected FS cells with vehicle or a Ras N17 plasmid, a dominant negative mutant of Ras p21, using the LipoFECTAMINE reagent. After 24 h, cells were treated for 2 h with TGF-β3 and/or ethanol for MAPK p44/42 activation. The Ras N17 vector expresses a dominant negative form of the Ras protein that contains a serine-to-aspargine mutation at residue 17. Western blot analysis showed that the expression of Ras N17 in FS cells inhibited the TGF-β3- and/or ethanol-induced activation of MAPK p44/42 (Fig. 5B). Results of the densitometric analysis of Western blots for phosphorylation of MAPK p44/42 in a vehicle-treated set were: fold change of control: ethanol: 2.4 ± 0.38; TGF-β3: 3.5 ± 0.34; ethanol+TGF-β3: 5.5 ± 0.44 ($p<0.05$, ethanol or TGF-β3 vs ethanol+TGF-β3 group; n=4), and phosphorylation of

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MAPK p44/42 in a Ras N17-expressed set were: fold change of control: ethanol: 1.3 ± 0.10; TGF-β3: 1.8 ± 0.34; ethanol+TGF-β3: 2.03 ± 0.44; (n=4). Activation of MAPK p44/42 in Ras N17-expressing cells was significantly ($p<0.05$) different from its respective vehicle-treated group (Fig. 5B).

Requirement of PKC for phosphorylation of MAPK p44/42 and bFGF production. To identify whether the PKC system participates in the ethanol/TGF-β3 interaction leading to MAPK p44/42 activation, we determined the action of a PKC blocker on TGF-β3- and/or ethanol-induced activation of MAPK p44/42 and increase of bFGF levels in FS cells as described in the Materials and Methods section. Using a specific PKC inhibitor, Bis, (Bell et al., 1995), we found that the activation of MAPK p44/42 by ethanol, with or without TGF-β3, was suppressed at both the 2.5- and 5-µM concentrations (Figs. 6A and 6B). The same doses of Bis also suppressed the stimulatory effects of ethanol and TGF-β3, either alone or in combination, on bFGF levels (Fig. 6C).

Effect of ethanol and TGF-β3 on Smad 2 activation. It has recently been shown that TGF-β1 can target smad2 as well as the MAPK p44/42 pathway (Blanchette et al., 2001). Hence, we determined the effect of ethanol and TGF-β3 interaction on smad2 activation. FS cells were treated with ethanol and TGF-β3, alone or together, for 1 h, and the activation of smad2 in these cells was determined using Western blotting. As shown in Figs. 6A and 6B, TGF-β3 alone increased activation of smad2; ethanol alone or with TGF-β3 didn't affect smad2 activation (Figs. 7A and 7B).

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Discussion

The data presented here demonstrate, for the first time, that ethanol and the peptide growth factor TGF-β3 interact in an additive manner to increase the release of bFGF from FS cells. We found that both ethanol- and TGF-β3-induced bFGF release as well as simultaneously increased phosphorylation of MAPK p44/42. We also showed that a MAPK p44/42 inhibitor and PKC inhibitors prevented the effects of TGF-β3 and ethanol on the phosphorylation of MAPK p44/42 and on bFGF release from FS cells. Ethanol and TGF-β3 together increased the activation of Ras p21, whereas the overexpression of Ras p21 inhibited MAPK p44/42 activation. TGF-β3 alone increased the phosphorylation of smad2 while ethanol alone or together with TGF-β3 did not show any effect on the activation of this protein. These data suggest that TGF-β3 and ethanol interact to increase bFGF production and its release from FS cells via the PKC–Ras–MEK–MAPK p44/42 signaling pathway. The interaction of ethanol and TGF-β3 may be critical for ethanol-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 ethanol promotes estradiol cell proliferating action on lactotropes (De et al., 1995, 2002). Estradiol is known to increase lactotropic cell production of TGF-β3, which causes lactotropic cell proliferation by increasing bFGF release from FS cells (Hentges et al., 2000). Hence, ethanol may promote estradiol action on lactotropes proliferation by increasing FS cells' bFGF response to TGF-β3.

Estradiol and ethanol both have been found to increase the levels of prolactin mRNA in the pituitary and to modify alternative splicing of the dopamine receptor in the pituitary

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(Oomizu et al., 2003). In this study we showed that, like estradiol, ethanol interact with TGF-β3 to increase bFGF release from FS cells. These data suggest that both agents might act similarly on various signaling cascades regulating functions of pituitary cells.

The effects of ethanol on bFGF release and MAPK activation bear many similarities with those of estradiol. For example, like estradiol (Chaturvedi and Sarkar, 2004), ethanol interacts with TGF-β3 in an additive manner to increase the prod
uction and release of bFGF and the phosphorylation of MAPK p44/42 in FS cells. Furthermore, like estradiol, a MAPK p44/42 inhibitor, PKC inhibitors, and a dominant negative mutant of Ras p21 all prevented the combined effects of TGF-β3 and estradiol on the phosphorylation of MAP kinase p44/42 and bFGF in FS cells. Hence, these data suggest that ethanol and estradiol may affect bFGF release similarly by interacting with TGF-β3 and activating the PKC–Ras–MEK–MAPK p44/42 signaling pathway. However, future studies using estrogen receptor activators and inactivators are required to establish the estradiol and ethanol interactive action on bFGF release.

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 (Wrana et al., 1994; Tsukazaki et al., 1998). 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 (Hayashida et al., 1999). Blanchette and colleagues showed, for fur gene expression, the activation of MAPK p44/42 by TGF-β1 targets smad2 for increased translocation to the nucleus

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(Blanchette et al., 2001). However, we did not find any effect of ethanol, alone or with TGF-β3, on smad2 activation, suggesting that ethanol interacts with TGF-β3 without activating smad2.

Ethanol and TGF-β3's interactive effect on bFGF release and MAPK p44/42 phosphorylation was blocked by a PKC inhibitor, suggesting the possibility of a mediatory role of PKC in bFGF release. PKC has been shown to activate MAPK p44/42 by activating Ras p21 (Wood et al., 1992). Eleven PKC isoforms have been identified and classified into three groups based on their ability to be activated by Ca^{2+} and DAG (Ron et al., 1999) The classical PKC-α, -ßI, -ßII, and -γ isoforms are activated by calcium (Ca^{2+}) and diacylglycerol (DAG); the novel PKC-θ-, η-, δ-, -ε are Ca^{2+} -independent but DAG-dependent; finally, the atypical PKC -ζ, -ι (λ in murine cells) are Ca^{2+}- and DAG-independent (Jaken 1996). Bis, a blocker of PKC, is known to act as a competitive inhibitor for the ATP-binding site of PKC (Toullec et al., 1991), and shows high specificity for PKCα-, β_I-, β_{II}-, γ-, δ-, and ε- isozymes of PKCs (Gekeler et al., 1996). Because the PKC inhibitor Bis blocked the effect of ethanol and TGF-β3 on bFGF release, it can be postulated that ethanol and TGF-β3 action on bFGF release is mediated by calcium- or DAG-dependent PKC isoforms rather than atypical PKCs.

We found that overexpression of a dominant negative mutant of Ras p21 inhibited ethanol's and TGF-β3's effects on MAPK p44/42 activation. The expression of this variant is known to knockout endogenous Ras expression in mammalian cells (Feig and Cooper 1988; Szeberenyi et al., 1990). The data presented here identify the role of the

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PKC-dependent Ras-MAPK p44/42 pathway in the interaction of TGF-β3 and ethanol on FS cells for bFGF release.

In conclusion, the results from this study provide evidence that ethanol and TGF-β3 cross-talk to activate a PKC-dependent MAP kinase pathway to increase bFGF release from FS cells. The MAP kinase pathway that is activated by ethanol and TGF-β3 belongs to the Ras-MEK-MAP kinase p44/42 cascade. The interaction between ethanol and TGF-β3 appear to be critical for a maximal response of FS cells to ethanol's challenge. Ethanol has been shown to increase lactotropic cell growth and magnify the action of estradiol on lactotropic cell proliferation in the pituitary. Therefore, the interaction of TGF-β3 and ethanol on FS cells for maximal bFGF release might be important in ethanol's control of lactotropic cell proliferation.

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Figure Legends

Fig. 1. Effect of TGF-β3 or ethanol on bFGF release from FS cells. FS cells were treated for 24 h with various concentrations of ethanol or TGF-β3. Levels of bFGF in the culture media were assayed using an immunoassay. A. Line diagram showing the concentration-dependent effects of ethanol on bFGF release. a, $p<0.01$ compared to the control group; b, $p<0.01$ compared to the 12.5- or 25-mM ethanol treatment.; c, $p<0.01$ compared to the 50-mM ethanol treatment. B. Line diagram showing the concentration-dependent effects of TGF-β3 on bFGF release. a, $p<0.01$ compared to the control group; b, $p<0.01$ compared to the 0.1-ng/ml TGF-β3 treatment.; c, $p<0.01$ compared to the 1-ng/ml TGF-β3 treatment. Each point represents mean ± SEM of 3–4 experiments.

FIGURE 2. Combined effect of TGF-β3 and ethanol on bFGF release from FS cells. FS cells were treated with various concentrations of TGF-β3 (1–10 ng/ml) alone or with either 25 mM, 50 mM or 100 mM of ethanol for 24 h, and bFGF release was determined in supernatant. A. 25 mM of ethanol alone or in combination with TGF-β3. a, $p<0.01$ compared to control group. b, $p<0.01$ compared to 25 mM alone and to the respective TGF-β3 concentration. B. 50 mM ethanol alone or in combination with TGF-β3. a, $p<0.01$ compared to control untreated group. b, $p<0.01$ compared to 50 mM alone and respective TGF-β3 concentration. C. 100 mM ethanol alone or in combination with TGF-β3. a, $p<0.01$ compared to control untreated group. b, $p<0.01$ compared to 100 mM alone and respective TGF-β3 concentration. Each bar represents mean ± SEM of 3 experiments.

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FIGURE 3. Effects of ethanol and TGF-β3 on phosphorylation of MAPK p44/42 (phosphorylation of p44/42). FS cells were treated for 2 h with TGF-β3 (1 ng/ml) and ethanol (50 mM) alone or in combination. The p-MAPK level was determined by Western blotting as described in the Materials and Methods section. A. Representative Western blots showing changes in p-MAPK. B. Densitometric analysis of p-MAPK versus total MAPK. Data express the fold increase over unstimulated cells. Each bar represents mean ± SEM of 6 separate experiments. a, $p<0.05$ compared to respective control group; b, $p<0.05$ compared to all other groups.

FIGURE 4. Effect of U0126 on TGF-β3- and ethanol-induced increase in phosphorylation of MAPK p44/42 (phosphorylation of p44/42) and bFGF release. FS cells were pre-incubated for 1 h with various concentrations of U0126, an inhibitor of the MAPK pathway, or with vehicle. Cells were then treated with TGF-β3 (1 ng/ml) and ethanol (10 nM), alone or in combination, for 2 h or 24 h for MAPK p44/42 activation or for bFGF assay respectively. A. Representative blot showing the effect of U0126 on p-MAPK increased by ethanol or TGF-β3 alone and in combination. B. Densitometric analysis of p-MAPK versus total MAPK in the presence and absence of U0126. Data express the fold increase over control. C. The effect of various concentrations of U0126 on bFGF release increased by ethanol and TGF-β3. Each bar represents mean ± SEM of 3 individual experiments. a, $p<0.05$ compared to respective control group; b, $p<0.05$ compared to all other groups; c, compared to respective DMSO (vehicle)-treated groups; d, $p<0.05$ compared to respective 0.1-µM U0126-treated group.

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FIGURE 5. Role of Ras–MAPK p44/42 pathway in ethanol- and TGF-β3-induced increase in phosphorylated MAPK (p-MAPK). FS cells were stimulated with ethanol (50 mM) and TGF-β3 (1 ng/ml) alone or together. Cell lysates were then subjected to pull-down assay with GST-RBD-Agarose followed by SDS/PAGE and immunofluorescent blotting with an anti-Ras antibody to detect active (GTP-bound) Ras. Aliquots of the same cell lysate were independently analyzed by immunofluorescent blotting for MAPK (to control loading) and p-MAPK (to evaluate activation of MAPK). A. Representative blot showing Ras activity and changes in p-MAPK. B. Representative blots showing the effect of expression of Ras N17 on p-MAPK increased by ethanol and TGF-β3. FS cells were transiently transfected with Ras N17, a dominant negative mutant of Ras p21, or vehicle. Eighteen hours after transfection, cells were incubated for 2 h with TGF-β3 (1 ng/ml) and ethanol (50 mM), alone or in combination, for activation of MAPK. Experiments were repeated 3–4 times with similar results.

FIGURE 6. Effect of a PKC inhibitor (Bis; 2.5 or 5 μM) on TGF-β3- and ethanol-induced increase in phosphorylation of p44/42 (p-MAPK) and bFGF release. FS cells were pre-incubated for 1 h with the PKC inhibitor Bis or vehicle. Afterward, cells were treated with TGF-β3 (1 ng/ml) and ethanol (50 mM) alone or in combination for 2 h for the phosphorylated MAPK or for 24 h for bFGF release. A. Representative blots showing the effect of the PKC inhibitor Bis on p-MAPK. B. Densitometric analysis of p-MAPK versus total MAPK in the presence and absence of Bis. Data express the fold increase over control cells. C. Effect of the PKC inhibitor Bis on bFGF release induced by TGF-β3 (1 ng/ml) and ethanol (50 mM). Each bar represents mean ± SEM of 3 individual

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experiments. a, $p<0.05$ compared to the respective control group; b, $p<0.05$ compared to all other groups; c, compared to the respective DMSO (vehicle)-treated group; d, significantly different from respective 2.5-μM Bis-treated groups.

FIGURE 7. FS cells were treated for 2 h with TGF-β3 (1 ng/ml) and ethanol (50 mM) alone or in combination. A. Representative blots showing changes in phosphorylation of smad2 and phosphorylation of MAPK p44/42 (p-MAPK). Membranes were blotted with a total MAPK p44/42 antibody to confirm equal amounts of protein in all the samples. B. Densitometric analysis of phosphorylated smad2 versus total MAPK. The data express the fold increase over control cells. Each bar represents mean ± SEM of 4 separate experiments. a, $p<0.05$ compared to the respective control group.

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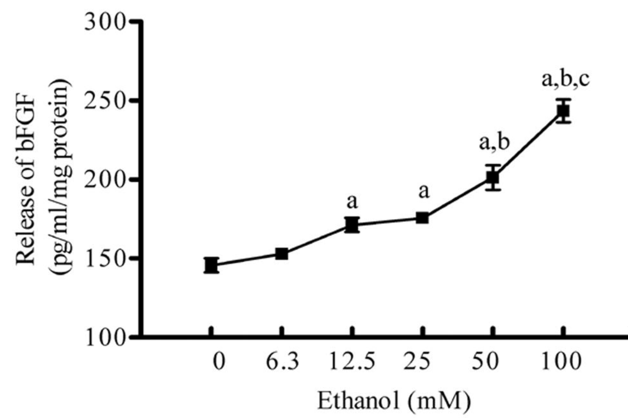
Figure 1



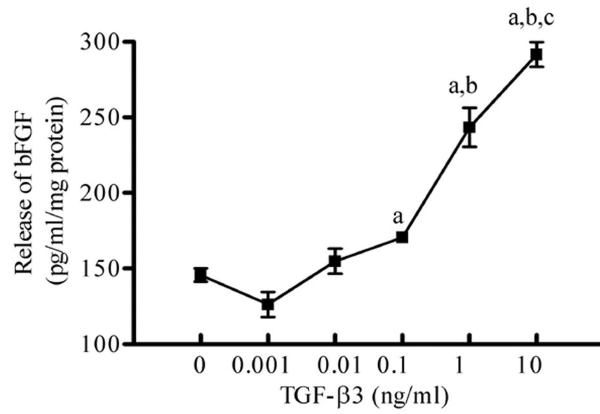


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Figure 2

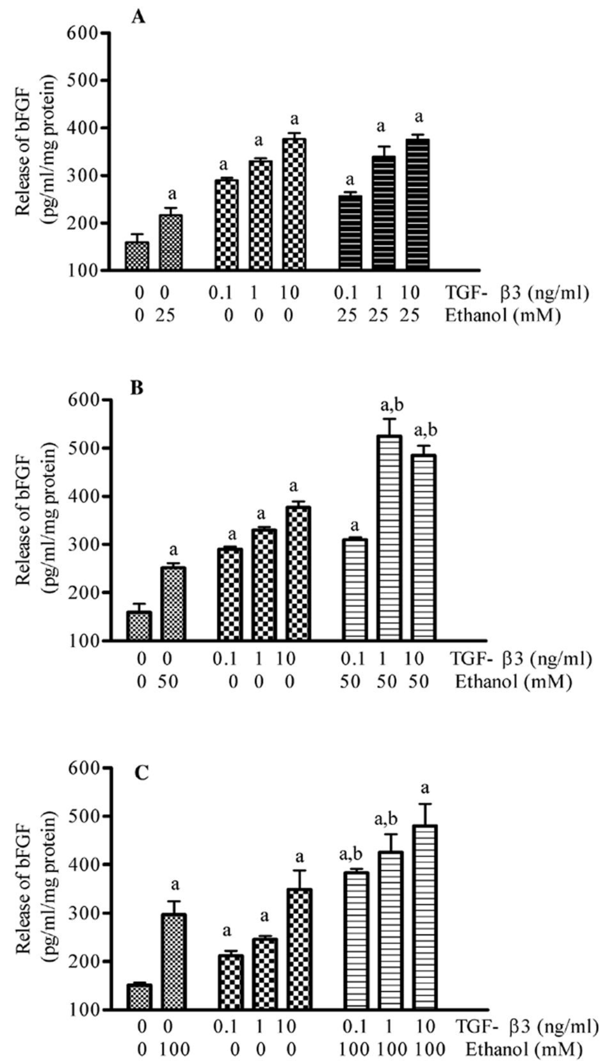

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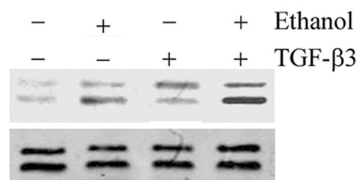
Figure 3

A



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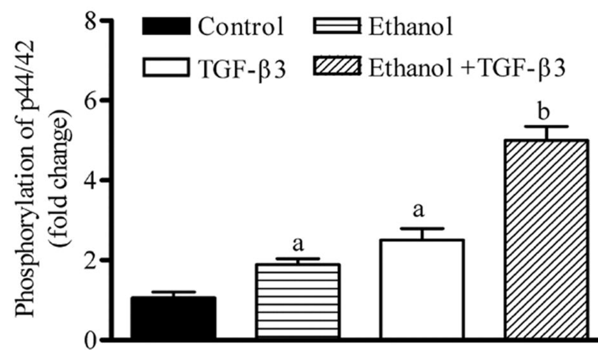

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Figure 4

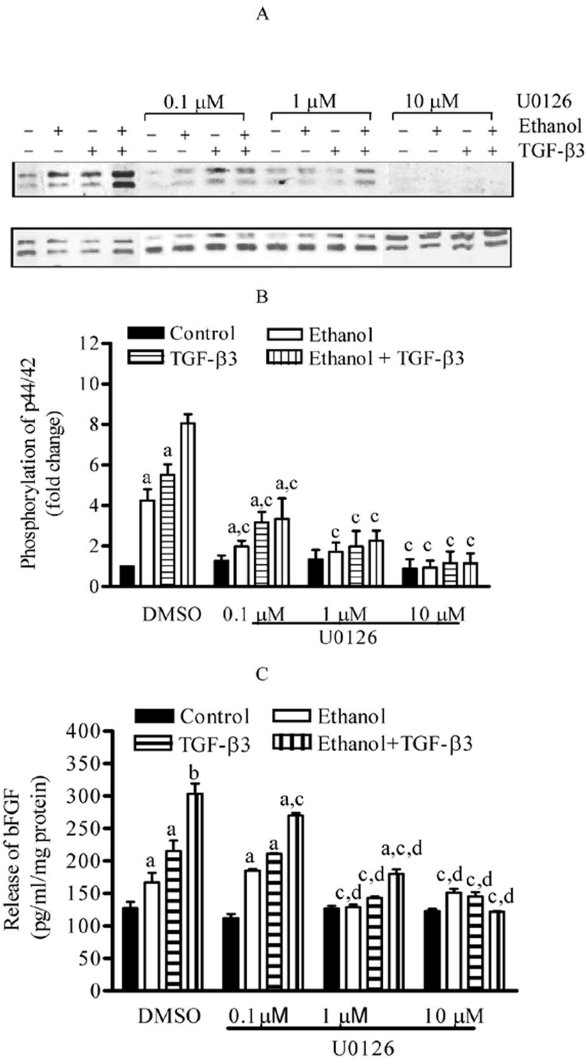

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Figure 5

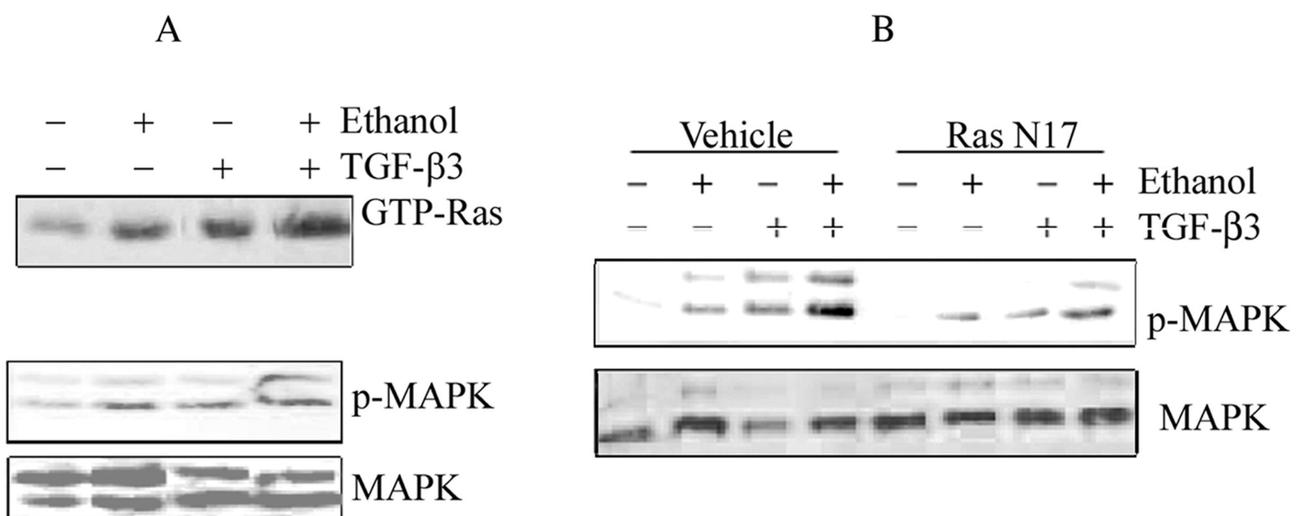

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Figure 6

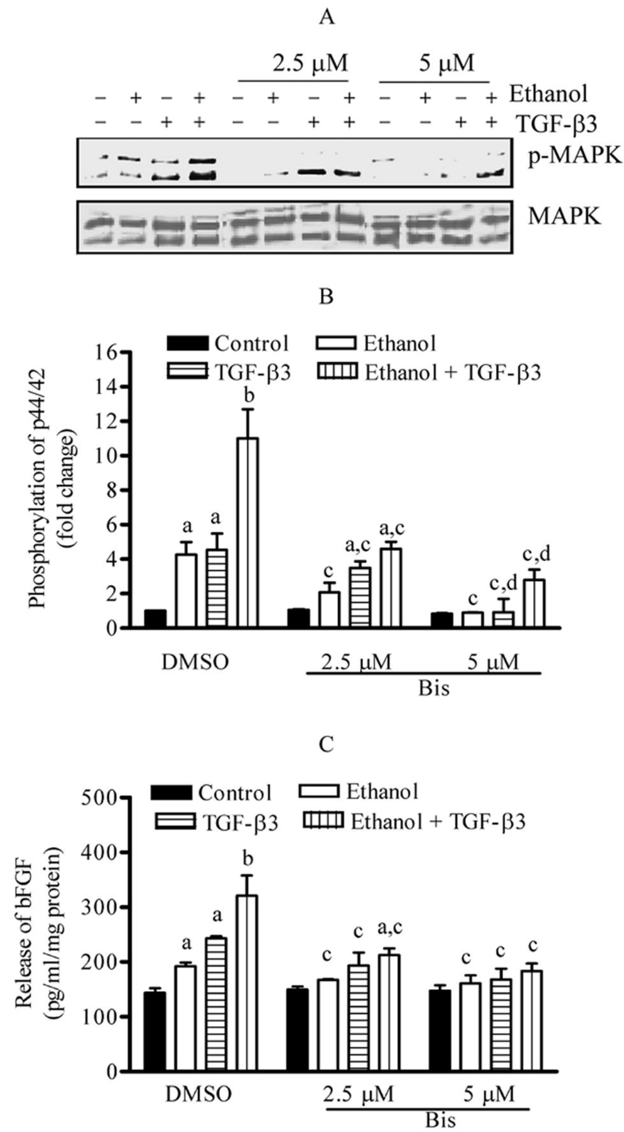

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Figure 7