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The role of epidermal growth factor receptor in ethanol-mediated inhibition of activator protein-1 transactivation

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Abstract

A potential mechanism underlying ethanol-induced alterations in gene expression is the disruption of transcription factor activity. Growth factor receptors, particularly receptor tyrosine kinases, play an important role in modulating many biological effects of ethanol. We demonstrated here that the expression of epidermal growth factor receptor (EGFR) mediated the effect of ethanol on the activity of transcription factor activator protein-1 (AP-1). Ethanol had little effect on AP-1 activity in the fibroblast cells devoid of EGFR (B82); however, it significantly suppressed AP-1 activity in B82 cells that were stably transfected with either a wild-type EGFR (B82L) or a kinase-deficient receptor (B82M721) in a concentration-dependent manner. EGF activated AP-1 only in B82L cells; the activation was mediated primarily by Akt and ERK. Ethanol inhibited EGF-induced EGFR autophosphorylation, phosphorylation of ERK as well as Akt and its substrate GSK-3β, and subsequently blocked EGF-stimulated AP-1 activation in B82L cells. On the other hand, ethanol had little effect on EGF-stimulated JNK activation. Phorbol ester 12-*O*-teradecanoyl-phorbol-13-acetate (TPA) activated AP-1 in B82L and B82M721 cells, but not B82 cells. TPA-induced activation of ERK and PKCδ was dependent on the expression of EGFR although the intrinsic kinase activity of EGFR was not required. In contrast, TPA-induced phosphorylation of p38 MAPK, JNKs and other PKC isoforms was independent of EGFR. Ethanol selectively inhibited TPA-induced phosphorylation of ERK and PKCδ, and modestly suppressed TPA-stimulated AP-1 activation in B82L and B82M721 cells. Thus, EGFR plays a critical role in the interaction between ethanol and AP-1.
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Keywords: Alcohol; Cell signaling; Gene transcription; Receptor tyrosine kinases

1. Introduction

Alcohol abuse produces profound damages to various organ systems. At cellular and molecular levels, ethanol exposure may modify the expression/function of target proteins by altering gene transcription, translation, or post-translational regulation. A potential mechanism underlying ethanol-mediated alteration in gene transcription is to disrupt the activity of transcription factors. Activator protein-1 (AP-1) is a transcription factor that controls the transcription of many genes responsible for the regulation of cell proliferation, differentiation, transformation and survival. AP-1 regulates the transcription of genes with a consensus DNA recognition sequence TGA/(C/G), designated as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-responsive element (TRE) in the promoter regions [1]. AP-1 consists of a family of Jun/Fos dimers that include different Jun proteins (c-Jun, JunB and JunD) and Fos proteins (c-Fos, FosB, Fra-1, Fra-2 and FosB2) [2–4]. Each of these proteins consists of a ''leucine zipper,'' which permits Jun proteins to form homodimers or heterodimers among themselves or with Fos proteins [2].

The expression of growth factor receptors is critical in determining the cellular susceptibility to ethanol; cells expressing high levels of receptor tyrosine kinases are

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usually more sensitive to ethanol [5–8]. Particularly, the epidermal growth factor receptor (EGFR) family plays a pivotal role in modulating many biological effects of ethanol [5–8]. EGFR contains a cell-surface EGF binding domain of 621 amino acids separated from a cytoplasmic 542 amino acid domain by a 23 amino acid hydrophobic transmembrane sequence [9]. EGFR is not only responsible for the pleotropic effects triggered by its ligands, but is also a part of signaling networks activated by stimuli that do not directly interact with the EGFR ectodomain [10–13]. To investigate the role of EGFR in ethanol-induced alteration in AP-1 activity, we examined the effect of ethanol on AP-1 in cells with different status of EGFR (cells with wild-type EGFR, kinase-inactive EGFR or devoid of EGFR). B82 cells are a mouse fibroblast line that is devoid of EGFR, as assessed by EGF binding as well as by RNA blot analysis [14,15]. B82L and B82M721 are stable transfectants of B82 cells generated by introducing a wild-type human EGFR and a tyrosine kinase-deficient human EGFR (contains a lysine 721 to methionine 721 mutation), respectively [14]. These two variants express 350,000–450,000 human EGFRs per cell and have a similar affinity for EGF binding. Membranes prepared from untransfected B82 cells showed no EGF-dependent phosphorylation of exogenous substrates [14]. Although EGFR is present, cell membranes from B82M721 cells exhibited neither basal level nor EGF-stimulated protein tyrosine kinase activity [14]. We have previously shown that EGF failed to stimulate AP-1 activity in B82M721 cells, indicating that intrinsic kinase activity was required for EGF-induced AP-1 activation [11]. TPA also activates AP-1; the activation also requires the presence of an EGFR protein. However, intrinsic EGFR tyrosine kinase appears non-essential for this activation because TPA can activate AP-1 in both B82L and B82M721 cells, but not in B82 cells [11]. The B82 cells and their derivatives provide a unique model system to assess the role of EGFR in ethanol-mediated alteration of AP-1 activity.

In the present study, we examined the AP-1 activity in B82 cells and their derivatives by measuring the activity of the luciferase reporter driven by a promoter sequence containing AP-1 binding sites. We have previously shown that the measurement accurately determines the transactivation of AP-1 [16,17]. Here, we demonstrated that the expression of EGFR mediated the effect of ethanol on AP-1 activity. Ethanol significantly inhibited AP-1 activity in fibroblast cells that express either a wild-type EGFR or a kinase-inactive receptor, but had little effect on AP-1 activity in the fibroblast cells devoid of EGFR. Ethanol blocked EGF-induced AP-1 activation in cells expressing wild-type EGFR; it inhibited EGFR autophosphorylation and down-stream signaling pathways that regulate AP-1 activation. Ethanol also inhibited TPA-induced AP-1 activation in an EGFR-dependent manner, but to a much lesser extent. Furthermore, we delineated signaling pathways that account for ethanol-induced inhibition.

2. Materials and methods

2.1. Cell culture and reagents

Mouse fibroblasts B82, B82L and B82M721 stably expressing AP-1-luciferase reporter were established by transfection with an AP-1-luciferase reporter construct [16]. The construct was generated by the insertion of a sequence of the collagenase promoter region [−73/+63 containing one AP-1 binding site (TGAGTCA)] into the luciferase reporter vector PGL2-basic (Promega, Madison, WI). The expression of AP-1 reporter was verified in these transfected cell masses (B82 AP-1 mass1, B82L AP-1 mass1 and B82M721 mass1) [11]. The cells were grown in Eagle's MEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C with 5% CO_2. EGF, lithium and an inhibitor for PI3-kinase (LY294002) were purchased from Sigma Chemical Co. (St. Louis, MO). Inhibitors for MEK1 (PD98059), p38 MAPK (SB202190), GSK-3β (TDZD-8), protein kinase C (Bisinodolylmaleimide I) and EGFR (AG1478) were purchased from Calbiochem (La Jolla, CA). An inhibitor for JNK (D-JNKI1) was obtained from Alexis Biochemicals (San Diego, CA). The luciferase assay kit was purchased from Promega.

2.2. Ethanol exposure protocol

Because of ethanol's volatility, a method utilizing sealed containers was used to maintain ethanol levels in the culture medium [6]. With this method, ethanol concentrations in the culture medium can be accurately maintained.

2.3. Assay for AP-1 activity

AP-1 transactivation was determined by assaying the activity of the luciferase reporter [16,17]. Briefly, cells were cultured in 96-well plates and grown in a medium containing 10% FBS. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2. For assaying AP-1 activity, sub-confluent cultures were maintained in a medium containing 0.1% FBS for 24 h and subsequently treated with ethanol with/without kinase inhibitors for specified durations. After treatment, cellular protein was extracted with a lysis buffer supplied in the luciferase assay kit (Promega), and luciferase activity was measured with a Monolight luminometer (3010, Analytical Luminescence Laboratory, Sparks, MD). AP-1 activity (luciferase activity) was calculated and expressed relative to the untreated cultures.

2.4. Western blot analysis

Cell lysates (40–60 μg of protein) were resolved by SDS–PAGE on 8–12% polyacrylamide gels, and separated proteins were transferred to nitrocellulose membranes.

After blocking with either 5% non-fat milk or 5% BSA in TPBS (0.01 M PBS, pH 7.4 and 0.1% Tween 20) at room temperature for 1 h, the membranes were probed with various antibodies directed against EGFR or intracellular signaling proteins. Antibodies directed against phospho-Akt (Ser-473 and Thr-308), phospho-GSK-3β (Ser-9), phospho-panPKC, phospho-PKCα/β, phospho-PKCδ (Thr-505 and Ser-643) and phospho-PKCθ were purchased from Cell Signaling Technology Inc. (Beverly, MA). Antibodies directed against ErbBs (EGFR, ErbB2, ErbB3 and ErbB4), phospho-EGFR (Tyr-1173 and Tyr-1068) and phospho-p38 MAPK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies directed against phospho-ERK1/2 and phospho-JNKs were obtained from Promega. An antibody directed against actin was from Sigma. The antibody–antigen complexes were visualized by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

2.5. Statistical analysis

Differences among treatment groups were tested using an analysis of variance (ANOVA). In cases where significant differences were detected ($p < 0.05$), specific post-hoc comparisons between treatment groups were examined with Student–Newman–Keuls tests.

3. Results

3.1. Effect of ethanol on the basal AP-1 activity of cells with different status of EGFR

The EGFR family includes four closely related members: EGFR (ErbB1), ErbB2, ErbB3 and ErbB4. The expression of the EGFR family in B82, B82L and B82M721 cells was examined by immunoblots (Fig. 1). B82 cells were verified devoid of EGFR. All three cells expressed similar levels of ErbB2 and ErbB3, while ErbB4 was undetectable in these cells by immunoblot. Ethanol had little effect on the expression of all three ErbBs (Fig. 1). The basal AP-1 activity (the activity not stimulated by exogenous reagents) in B82L and B82M721 cells was 21–25% higher than B82 cells (Fig. 2A). We examined the effect of ethanol on the basal AP-1 activity in B82, B82L and B82M721 cells. Ethanol significantly inhibited basal AP-1 activity in B82L and B82M721, but not B82 cells in a concentration-dependent manner (Fig. 2B). The suppression of AP-1 activity by ethanol was not caused by an alteration in cell number; ethanol exposure at these concentrations (100–400 mg/dl) and durations (3–12 h) did not significantly affect cell viability and cell numbers (data not shown). Various signaling pathways that potentially are involved in the regulation of AP-1 activity were examined. The levels of phosphorylated Akt and GSK-3β in B82L and B82M721 cells were higher than B82 cells,

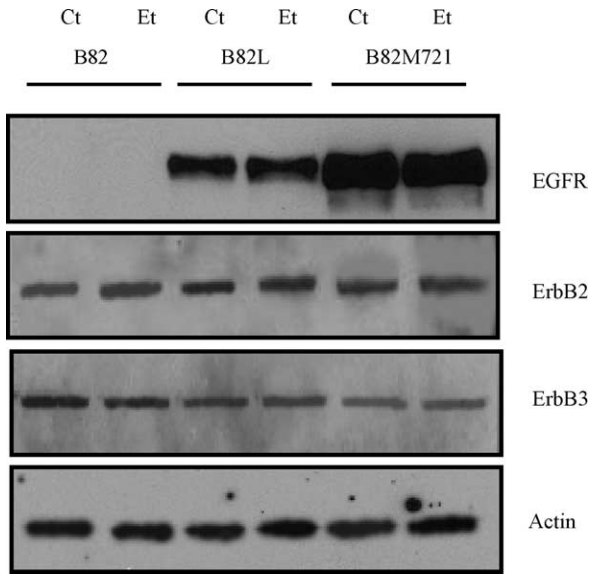

Fig. 1. Expression of the EGFR family in B82, B82L and B82M721 cells. B82, B82L and B82M721 cells were exposed to ethanol (0 or 400 mg/dl) for 24 h, and the expression of the EGFR family (EGFR, ErbB2, ErbB3 and ErbB4) was examined with immunoblots. After detection of ErbB proteins, the blots were stripped and re-probed with an anti-actin antibody. The experiment was replicated three times. Ct: control group; Et: ethanol-treated groups.

although the total amounts of these proteins were similar (Fig. 3A). GSK-3β is a substrate of Akt and a negative regulator of AP-1 activity; phosphorylation of GSK-3β at Ser-9 by Akt renders GSK-3β inhibition [18]. The levels of phosphorylated forms of other signaling components, such as p-ERK, or p-JNKs, p-p38 MAPK or p-PKCs, were quite weak and relatively constant among these three cells (data not shown). An inhibitor for PI-3K (LY294002) significantly suppressed basal AP-1 activity in B82L and B82M721 cells, but not in B82 cells (Fig. 3B). In contrast, inhibitors for GSK-3β (TDZD-8 and $LiCl_2$) significantly increased AP-1 activity. It was noted that GSK-3β inhibitors induced a modest up-regulation of AP-1 activity in B82 cells. Only the data using TDZD-8 was presented here. These results indicate that PI-3K/Akt and GSK-3β signaling pathways regulate AP-1 activation in these cells. Ethanol reduced Akt phosphorylation at both Ser-473 and Thr-308 (data on Thr-308 not shown) and GSK-3β (Ser-9) in B82L and B82M721 (Fig. 3C), suggesting that the inhibition of AP-1 by ethanol may result from a blockage of Akt activation, which subsequently activates GSK-3β. Consistent with its effect on AP-1 activity, ethanol had little effect on the phosphorylation of Akt and GSK-3β in B82 cells (3C).

3.2. EGFR regulates AP-1 activity through multiple signaling pathways

We have previously demonstrated that EGF stimulated AP-1 activation in B82L cells that express wild-type EGFR, but not in cells devoid of EGFR (B82) or cells

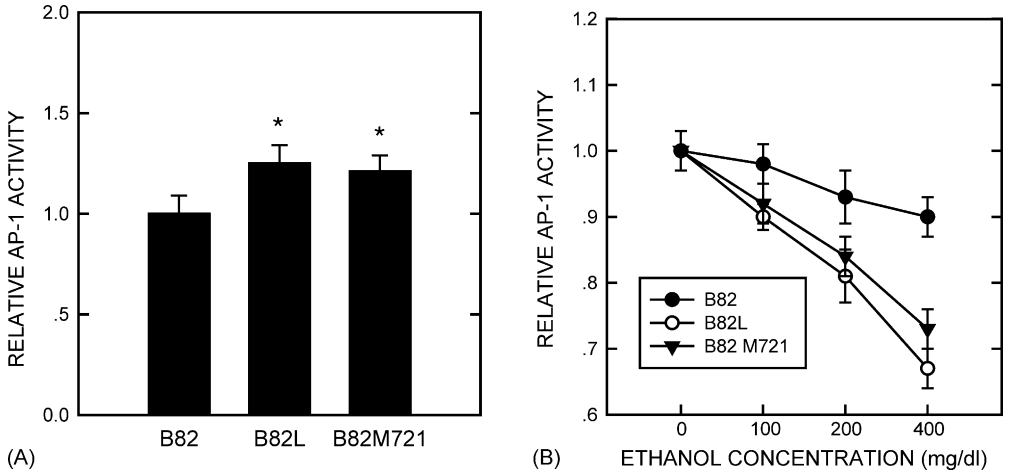

Fig. 2. Effect of ethanol on basal AP-1 activity in B82, B82L and B82M721 cells. (A) Basal AP-1 activity in B82, B82L and B82M721 cells was determined by assaying the luciferase activity as described in Section 2. The AP-1 activity in B82L and B82M721 cells was expressed relative to that of B82 cells. The result was the mean ± S.E.M. of four replicates. $^{*}p < 0.05$, a statistically significant difference from AP-1 activity in B82 cells. (B) B82, B82L and B82M721 cells were exposed to ethanol (0–400 mg/dl) for 12 h, and AP-1 activity was determined as described above. The levels of AP-1 activity in ethanol-exposed cultures were expressed relative to untreated controls. The result was the mean ± S.E.M. of four replicates.

expressing tyrosine kinase-deficient EGFR (B82M721) [11]. Several signaling pathways that potentially regulate AP-1 activity were examined using specific kinase inhibitors. As shown in Fig. 4, inhibitors of MEK1 (PD98059) and PI-3K (LY294002) dramatically inhibited EGF-stimulated AP-1 activation. Bisinodolylmaleimide-I (Bis-I) is a pan-PKC inhibitor [19]. Bis-I also offered a modest blockage of AP-1 activation. EGF-mediated AP-1 activation was

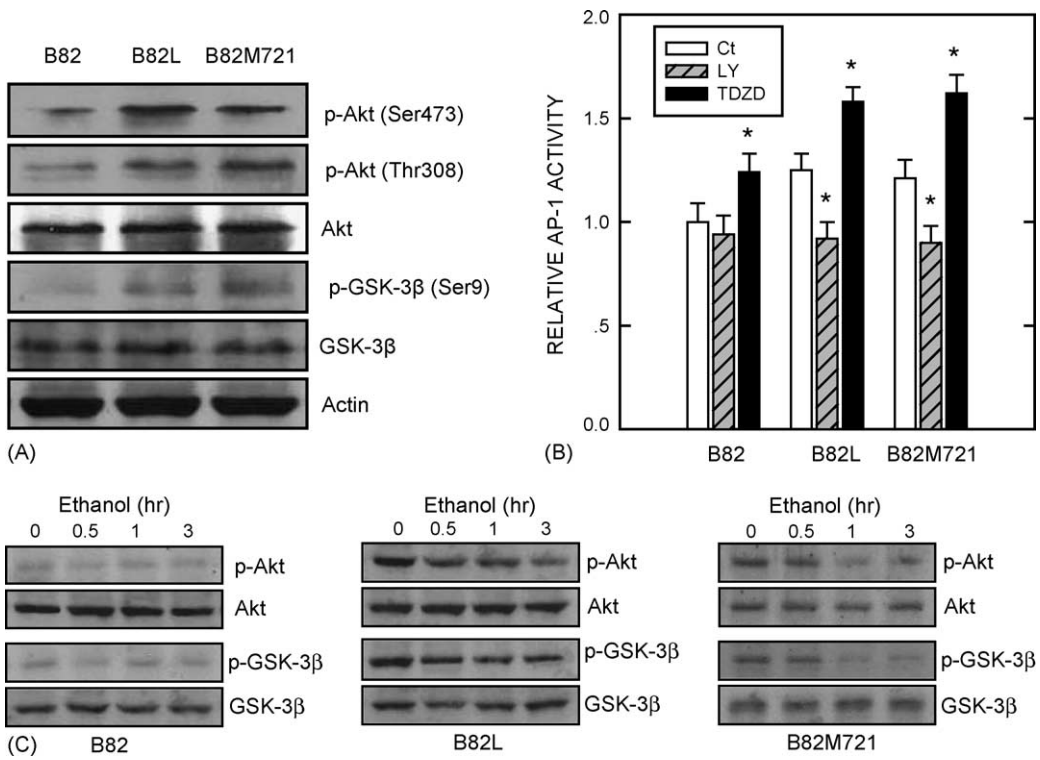

Fig. 3. Expression and phosphorylation of Akt and GSK-3β in B82, B82L and B82M721 cells. (A) The expression and phosphorylation of Akt and GSK-3β was determined by immunoblots using specific antibodies as described in Section 2. After detection of Akt and GSK-3β, the blots were stripped and re-probed with an anti-actin antibody. Triplicate experiments were performed independently. (B) B82, B82L and B82M721 cells were treated with LY294002 (10 μM) or TDZD-8 (10 μM) for 12 h, and AP-1 activity was determined as described above. The AP-1 activity in all treatment groups was expressed relative to that of untreated B82 cells. The result was the mean ± S.E.M. of three independent experiments. (C) B82, B82L and B82M721 cells were exposed to ethanol (400 mg/dl) for indicated durations. The phosphorylation of Akt (Ser-473) and GSK-3β (Ser-9) was determined by immunoblots as described in Section 2. The blots were stripped and re-probed with antibodies directed against non-phosphorylated forms of Akt and GSK-3β. The experiment was replicated three times.

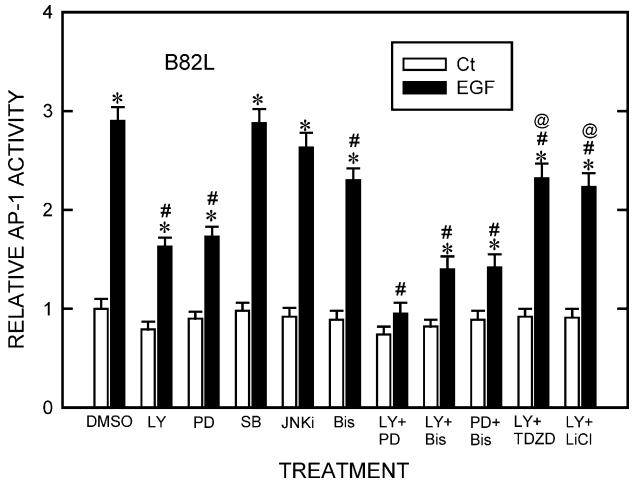

Fig. 4. The signaling pathways that mediate EGF-stimulated AP-1 activity in B82L cells. B82L cells were pre-treated with various protein kinase inhibitors for 30 min, then exposed to EGF (30 ng/ml) for 12 h. AP-1 activity was determined as expressed relative to DMSO-treated controls. Selective inhibitors employed were as follows: LY294002 (LY, 10 μM) is a PI-3K inhibitor; PD98059 (PD, 50 μM) is a MEK1 inhibitor; SB203580 (SB, 10 μM) is a p38 MAPK inhibitor; D-JNKI1 (JNKI, 1 μM) is a JNK inhibitor; Bisinodolylmaleimide-I (Bis, 1 μM) is a pan-PKC inhibitor; TDZD-8 (TDZD, 10 μM) and LiCl (20 mM) are GSK-3β inhibitor. $^{*}p < 0.05$, significant difference from paired- and non-EGF-treated cultures; $^{\#}p < 0.05$, significant difference from DMSO- and EGF-treated cultures; $^{@}p < 0.05$, significant difference from LY- and EGF-treated cultures. The result was the mean ± S.E.M. of four independent experiments.

completely blocked by simultaneous administration of PD98059 and LY294002, suggesting that PI-3K/Akt and ERK pathways were major signaling components that regulated EGF-induced AP-1 activation. GSK-3β is a negative regulator of AP-1 activity in these cells (Fig. 3B). GSK-3β inhibitors (LiCl or TDZD-8) significantly mitigated the inhibitory effect of LY294002 on EGF-induced AP-1 activation, suggesting that GSK-3β mediates PI-3K/Akt regulation of AP-1 activation (Fig. 4). EGF-induced activation of these signaling pathways was verified by the subsequent Western blot analysis that examined the phosphorylation of these components (Fig. 6B).

3.3. Effect of ethanol on EGF- and TPA-stimulated AP-1 activity

Ethanol significantly inhibited EGF-stimulated AP-1 activation in a concentration-dependent manner in B82L cells (Fig. 5A). To determine whether ethanol also affected AP-1 activation induced by other stimuli, we examined the effect of ethanol on TPA-stimulated AP-1 activity. Although ethanol also suppressed TPA-induced AP-1 activation, the extent of inhibition was much less than its effect on EGF-stimulated activity. For example, ethanol inhibited TPA-induced AP-1 activation at concentrations of 400 mg/dl or higher, while it significantly decreased EGF-stimulated AP-1 activation at 100 mg/dl in B82L cells (Fig. 5A). AG1478, a specific ATP binding site inhibitor of EGFR tyrosine kinase, blocked EGF-stimulated AP-1 activation; however, it had little effect on TPA-induced AP-1 activation in B82L cells (Fig. 5B). Similarly, ethanol at 400 mg/dl inhibited TPA-stimulated AP-1 activation in B82M721 cells (Fig. 5C). Together, the results validate that intrinsic kinase activity of EGFR is essential for EGF-stimulated AP-1 activation, but not required for TPA-mediated AP-1 activation. To determine whether ethanol affects EGFR phosphorylation stimulated by EGF, we examined the effect of ethanol on two major EGFR autophosphorylation sites, Tyr-1173 and Tyr-1068 in B82L cells. Ethanol inhibited EGF-stimulated phosphorylation of EGFR on both Tyr-1173 and Tyr-1068 (Fig. 6A) (data on Tyr-1068 not shown). As a result, ethanol inhibited EGFR down-stream signaling pathways, including major pathways for AP-1 activation, namely Akt/GSK-3β and ERK (Fig. 6B). Interestingly, ethanol had little effect on EGF-mediated JNK activation. Both EGF and ethanol did not induce noticeable alterations in the phosphorylation of PKCs and p38 MAPK (data not shown).

We have previously demonstrated that the presence of EGFR is required for TPA-induced AP-1 activation, but the intrinsic tyrosine kinase activity is not essential [11]. We have shown that TPA stimulated AP-1 activity in B82L and B82M721 cells, but not in B82 cells. Here, we showed that TPA had little effect on the phopshorylation of EGFR (Fig. 7A). However, TPA-induced phosphorylation of ERK and PKCδ was dependent on the expression of EGFR since TPA dramatically increased ERK and PKCδ phosphorylation in B82L and B82M721 cells, but only weakly in B82 cells (Fig. 7B). The intrinsic kinase activity appeared unnecessary for TPA-regulated phosphorylation of ERK and PKCδ since TPA-induced phosphorylation was observed in both B82L and B82M721 cells. TPA-induced phosphorylation of p38 MAPK, JNK and other PKC isoforms was independent of EGFR; the levels of TPA-stimulated phosphorylation were similar in B82, B82L and B82M721 cells (Fig. 7B). On the other hand, TPA did not significantly promote the phosphorylation of Akt and GSK-3β in these cells (data not shown). Ethanol significantly inhibited TPA-stimulated phosphorylation of ERK and PKCδ in B82L and B82M721 cells; however, it had little effect on the phosphorylation of p38 MAPK and other PKC isoforms. This result was consistent with ethanol modestly effecting TPA-induced AP-1 activation in B82L and B82M721 cells. Although initial exposure to ethanol (5–30 min) did not alter TPA-mediated JNK phosphorylation, longer treatment (60 min) produced a modest inhibition (Fig. 7B), suggesting that the effect of ethanol on JNKs may be indirect.

4. Discussion

The present study demonstrates that ethanol at physiologically relevant concentrations inhibits AP-1 activity.

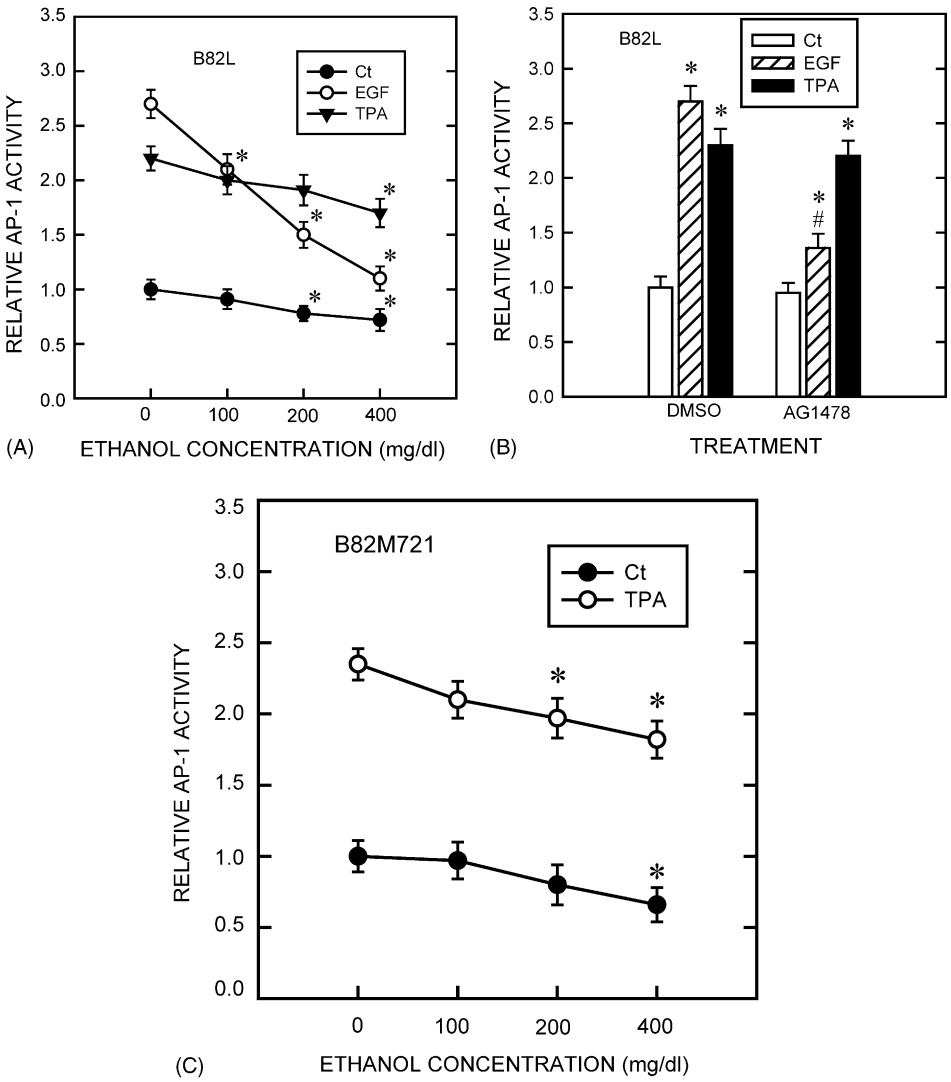

Fig. 5. Effect of ethanol on EGF- and TPA-mediated AP-1 activation. (A) B82L cells were treated with EGF (30 ng/ml) or TPA (30 ng/ml) with or without ethanol (100–400 mg/dl) for 12 h. AP-1 transactivation (luciferase activity) was measured and expressed relative to that of non-ethanol-treated cultures. The result was the mean ± S.E.M. of four independent experiments. $^{*}p < 0.05$, significant difference from non-ethanol-treated cultures. (B) B82L cells were pre-treated with either DMSO or AG1478 (200 nM) for 30 min, and then exposed to EGF (30 ng/ml) or TPA (30 ng/ml) for 12 h. AP-1 transactivation (luciferase activity) was measured and expressed relative to the paired-cultures that received neither EGF nor TPA treatment. The result was the mean ± S.E.M. of four independent experiments. $^{*}p < 0.05$, significant difference from non-EGF and non-TPA-treated cultures. $^{\#}p < 0.05$, significant difference from DMSO- and EGF-treated cultures. (C) B82M721 cells were treated with TPA (30 ng/ml) with or without ethanol (100–400 mg/dl) for 12 h. AP-1 transactivation (luciferase activity) was measured as described above. The result was the mean ± S.E.M. of four independent experiments. $^{*}p < 0.05$, significant difference from non-ethanol-treated cultures.

Interestingly, ethanol-induced inhibition only occurs in cells expressing either wild-type or kinase-deficient EGFR (B82L or B82M721), indicating the effect of ethanol requires the presence of EGFR. Mutation of lysine-721 at an EGFR kinase domain (B82M721 cells) results in a loss of intrinsic kinase activity and autophosphorylation in response to EGF [11,14]. B82M721 cells display a similar response to ethanol as do the cells expressing wild-type EGFR (B82L), suggesting that the intrinsic kinase is not required for the response. It has been shown that intrinsic kinase activity is critical for many biological effects mediated by EGFR [13]. However, some functions of EGFR apparently do not require intrinsic kinase activity [14,20]. For example, although EGFR is required for TPA-induced AP-1 activation, the intrinsic kinase activity of EGFR is not essential for TPA-mediated activation [11].

Enhanced phosphorylation of Akt (Ser-473 and Thr-308) and GSK-3β (Ser-9), but not total amount of these proteins, is observed in B82L and B82M721 cells; however, it is not enhanced in B82 cells, suggesting that EGFR facilitates PI-3K/Akt activation. It is currently unknown how EGFR, especially a kinase-defective EGFR, activates PI-3K/Akt. GSK-3β is a substrate of Akt. Activated Akt phosphorylates GSK-3β at Ser-9; the activity of GSK-3β is negatively regulated by its phosphorylation at Ser-9 [18]. It has been demonstrated that activation of GSK-3β sup-

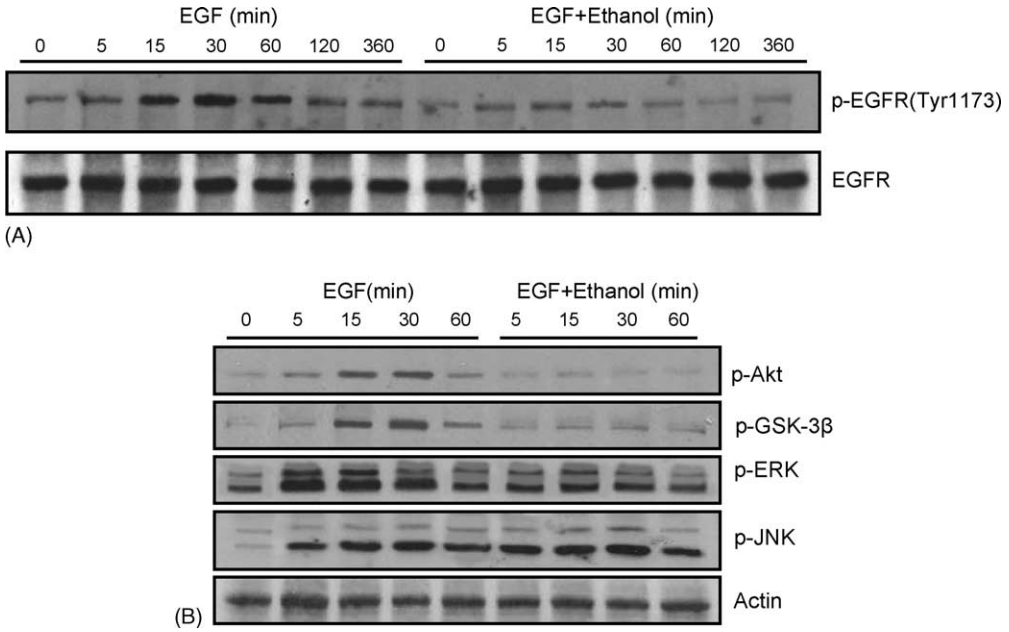

Fig. 6. Effect of ethanol on EGF-stimulated signal transduction in B82L cells. B82L cells were cultured in serum free medium overnight, and then treated with EGF (30 ng/ml) with or without ethanol (400 mg/dl) for 5–360 min. The autophosphorylation (Tyr-1173) and expression of EGFR (A) and the phosphorylation of other signaling components (B) were examined by immunoblots. The blots were stripped and re-probed with an anti-actin antibody. The experiment was replicated three times.

presses AP-1 transactivation [18]. Treatment of GSK-3β inhibitors increases AP-1 activity in B82, B82L and B82M721 cells, verifying that GSK-3β is a negative regulator of AP-1 activity in these cells. Therefore, higher levels of basal AP-1 activity in B82L and B82M721 cells may result from an inhibition of GSK-3β. Ethanol decreases the phosphorylation of Akt (Ser-473 and Thr-308) and GSK-3β (Ser-9) in B82L and B82M721, leading to GSK-3β activation. Taken together, these results suggest that ethanol-induced inhibition of AP-1 activity is mediated by the activation of GSK-3β. The mechanisms underlying ethanol-induced inhibition of Akt activation in B82L and B82M721 cells are currently unknown. A recent study suggests that ethanol may decrease Akt phosphor-

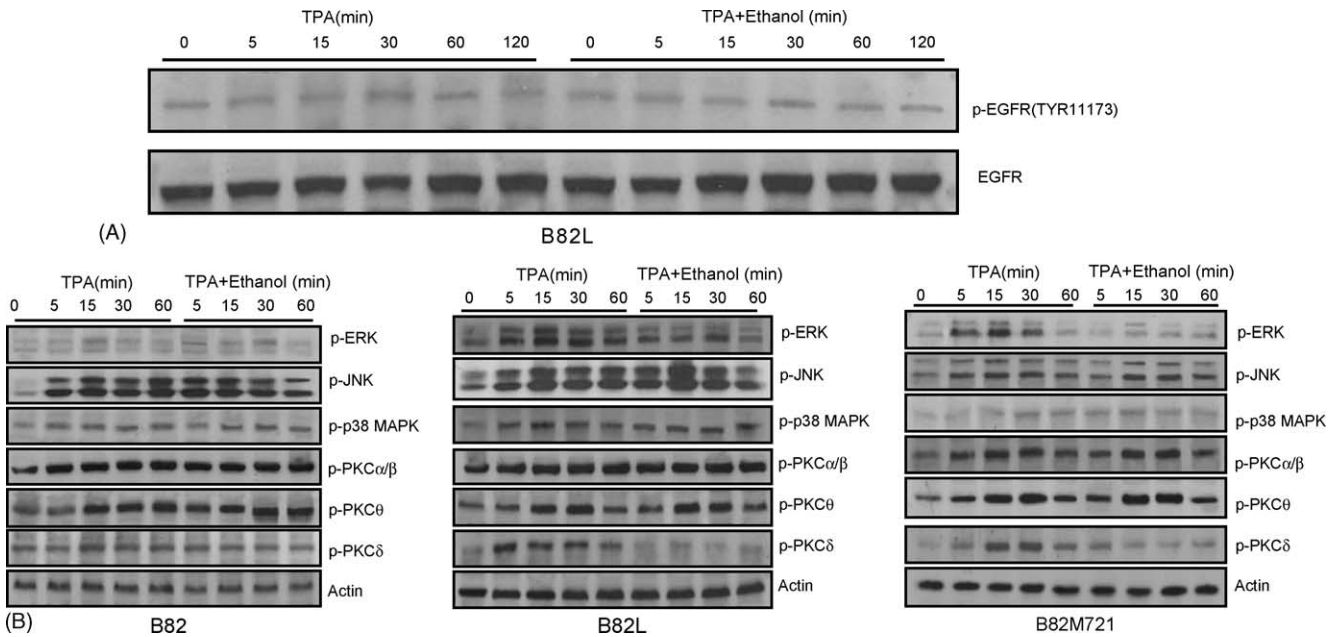

Fig. 7. Effect of ethanol on TPA-induced signal transduction in B82, B82L and B82M721 cells. (A) B82L cells were cultured in serum free medium overnight, and then treated with TPA (30 ng/ml) with or without ethanol (400 mg/dl) for 5–120 min. The autophosphorylation (Tyr-1173) and expression of EGFR were examined by immunoblots. The experiment was replicated three times. (B) B82, B82L and B82M721 cells were cultured in serum free medium overnight, and then treated with TPA (30 ng/ml) with or without ethanol (400 mg/dl) for 5–60 min. The phosphorylation of signaling components was examined by immunoblots. The blots were stripped and re-probed with an anti-actin antibody. The experiment was replicated three times.

ylation by up-regulating the expression of the lipid phosphatase PTEN in HepG2E47 cells [21].

EGF-induced AP-1 activation is observed only in B82L cells; a selective inhibitor of EGFR tyrosine kinase (AG1478) blocks EGF-stimulated AP-1 activity, indicating that a fully functional EGFR is required [11]. Since simultaneous administration of PD98059 and LY294002 completely blocks EGF-stimulated AP-1 activation in B82L cells, PI-3K/Akt and ERK pathways are major signaling components that regulate EGF-induced AP-1 activation. Using GSK-3β inhibitors (LiCl or TDZD-8), we have verified that GSK-3β is a negative regulator of AP-1 activity. Furthermore, these GSK-3β inhibitors significantly mitigate the inhibitory effect of LY294002 on EGF-induced AP-1 activation, suggesting that GSK-3β is a critical down-stream component of PI-3K/Akt that regulates AP-1 activity.

Ethanol dramatically inhibits EGF-stimulated AP-1 activation in B82L cells; the inhibition is likely mediated by its effect on EGFR activation. Ethanol inhibits EGF-elicited EGFR autophosphorylation as well as phosphorylation of several down-stream signaling pathways, including Akt and ERK, the major signaling components that regulate AP-1 activity in these cells. The molecular mechanisms by which ethanol impairs EGFR-mediated signaling are not clear. We have demonstrated that ethanol can induce the production of reactive oxygen species (ROS) [22,23]. A recent study indicates that ROS may block EGFR-mediated intracellular signaling by decreasing EGFR autophosphorylation and phosphorylation of its down-stream effectors [24]. Therefore, oxidative stress is a potential mechanism underlying the inhibitory effect of ethanol on EGFR signaling. EGF also increases JNK phosphorylation in B82L cells, and the phosphorylation is not affected by ethanol exposure. JNK is minimally involved in EGF-mediated AP-1 activation in B82L cells because blocking JNK activation does not affect EGF-stimulated AP-1 activity (Fig. 4). It is not clear why ethanol does not affect EGF-stimulated JNK phosphorylation, yet it blocks EGFR activation (Fig. 6A). JNK is a stress-responsive kinase; it is likely ethanol-induced cellular stress overrides the inhibition of JNK activation that is controlled by an EGFR signaling system. TPA-induced AP-1 activation is dependent on the presence of EGFR, but independent of EGFR intrinsic kinase activity. This conclusion is supported by the following observations: TPA activates AP-1 in both B82L and B82M721 cells, but not B82 cells (Fig. 5C) [11]; AG1478 does not affect TPA-induced AP-1 activation (Fig. 5B). Although ethanol also interferes with TPA-induced AP-1 activation, the profile of inhibition is different from its effect on EGF action. The inhibitory effect of ethanol on TPA-mediated AP-1 activation is modest and requires higher ethanol concentrations. For example, ethanol at 100 and 200 mg/dl fails to inhibit TPA-induced AP-1 activation, while it significantly decreases EGF-stimulated AP-1 activation by 22 and 45%, respectively. Ethanol of 400 mg/dl or higher is necessary to inhibit TPA-mediated AP-1 activation. At this concentration, ethanol inhibits TPA-stimulated AP-1 activation by 23%, while it reduces EGF-mediated AP-1 activation by 59%. TPA-mediated phosphorylation of ERK and PKCδ is observed in both B82L and B82M721 cells, but only weakly in B82 cells (Fig. 7B). Thus, TPA-stimulated activation of ERK and PKCδ is dependent on EGFR, but the intrinsic kinase is not required. ERK is an important regulator of AP-1 activity. A recent study indicates that the activation of PKCδ also causes AP-1 activation [25]. Therefore, ERK and PKCδ are likely the major mediators of TPA-induced AP-1 activation in B82L and B82M721 cells. Ethanol selectively blocks TPA-stimulated activation of ERK and PKCδ. As a result, suppression of TPA-stimulated AP-1 activation by ethanol in B82L and B82M721 cells is likely caused by the inhibition of ERK and PKCδ activation. It is unclear, however, how an EGFR, especially a kinase-defective EGFR, participates in a signaling network. It has been shown that kinase-inactive EGFR can interact with other members of the EGFR family, such as ErbB2. ErbB2 possesses a functional tyrosine kinase although a specific ligand has not been identified [26]. The interaction between kinase-defective EGFR and ErbB2 induces ErbB2 enzymatic activation and binding to a down-stream signal transducer, resulting in the activation of several intracellular signaling pathways including Akt and ERK1/2 [27,28]. It remains to be determined whether TPA can facilitate this interaction. The inhibition of EGF- and TPA-induced AP-1 activation by ethanol is only partial, suggesting that there may be other signaling pathways regulating AP-1 activation. There is considerable cross-talk between EGFR and G-protein coupled receptors. G-proteins participate in the regulation of AP-1 transactivation [29,30]; therefore, it is important to evaluate the role of G-proteins in EGFR-mediated AP-1 activation in future.

TPA-induced activation of JNK, p38 MAPK, PKCα/β and PKCθ is independent of EGFR; the activation is observed in all three cells. Interestingly, ethanol does not affect TPA-mediated phosphorylation of these signaling components. This result implies that the target of ethanol action is on TPA/EGFR interaction. JNK and PKCα/β have been shown to participate in the regulation of AP-1 activation in other model systems [31,32]. Although TPA activates JNK and PKCα/β in B82 cells, it fails to stimulate AP-1 activity. This result suggests that the extent of TPA-induced activation in B82 cells is not sufficient to activate AP-1. Alternatively, other ‘‘co-factors’’ necessary for JNK and PKCα/β regulation of AP-1 activation are missing in B82 cells.

It has been demonstrated that EGFR plays a critical role in intracellular signaling elicited by various environmental stimuli that do not directly interact with an EGFR ectodomain, such as radiation, osmotic shock and exposure to heavy metal and radical-generating agents [33–35]. The present study shows that EGFR is a critical signaling

component, which determines cellular susceptibility to ethanol exposure. Ethanol exposure induces profound damage to the CNS, and AP-1 is a known target of ethanol exposure during neuronal development [36–39]. The expression of the EGFR family is developmentally regulated [40]. Elucidating the role of EGFR in ethanol's disruption of AP-1 activity provides an important insight into ethanol-induced damage at the molecular level.

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