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Overexpression of ErbB2 enhances ethanol-stimulated intracellular signaling and invasion of human mammary epithelial and breast cancer cells *in vitro*

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Both epidemiological and experimental studies indicate that ethanol is a tumor promoter and may promote metastasis of breast cancer. However, the molecular mechanisms underlying ethanol-mediated tumor promotion remain unknown. Overexpression of ErbB proteins in breast cancer patients is generally associated with poor prognosis. The ErbB proteins are a family of receptor kinases that include four closely related members: epidermal growth factor receptor (EGFR/ErbB1), ErbB2/neu, ErbB3, and ErbB4. Particularly, ErbB2 plays a pivotal role in ErbB-mediated activities. Here we demonstrated that amplification of ErbB2 expression sensitized a specific cellular response to ethanol. Human breast cancer cells or mammary epithelial cells with a high expression of ErbB2 exhibited an enhanced response to ethanol-stimulated cell invasion *in vitro*. Ethanol also stimulated cell proliferation; however, this stimulation was independent of ErbB2 levels. Ethanol triggered divergent intracellular signaling among cells expressing different ErbB2 levels. In the cells overexpressing ErbB2, ethanol was more effective in the activation of c-Jun NH_2 terminal protein kinases (JNKs) and p38 mitogen-activated protein kinase (p38 MAPK) as well as the induction of reactive oxygen species (ROS) than the cells with normal ErbB2 expression. Blockage of either JNKs or p38 MAPK activation eliminated ethanol-mediated cell invasion. In contrast, the reduction of hydrogen peroxide concentration by catalase exposure had little effect on ethanol-induced cell invasion. These results indicated that ethanol-induced cell invasion was primarily mediated by JNKs and p38 MAPK, whereas the involvement of ROS formation might be minimal. Our study suggests that overexpression of ErbB2 may augment ethanol-elicited signaling and promote ethanol-stimulated tumor metastasis.
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Introduction

Breast cancer is a leading cause of morbidity and mortality in women (Kelsey and Horn-Ross, 1993). The endogenous and environmental factors that contribute to its etiology remain elusive. Alcohol, tobacco, and diet are the three major human cancer risk factors (Doll and Peto, 1981; Mufti, 1993). Today, over 50 epidemiological studies have examined the relation between ethanol consumption and breast cancer. Most of the studies agree that there is a positive correlation between ethanol intake and the risk of breast cancer (see review by Hiatt, 1990; Plant, 1992; Rosenberg *et al*., 1993; Longnecker, 1994; Singletary and Gapstur, 2001). In general, studies show that risk is directly correlated with the duration of drinking, that is, the greater the total number of years drinking, the greater the risk (Bowlin *et al*., 1997; Swanson *et al*., 1997; Smith-Warner *et al*., 1998; Kuper *et al*., 2000). Furthermore, epidemiological data indicate that alcohol consumption is associated with advanced and invasive breast tumors (Weiss *et al*., 1996; Vaeth and Schlessinger, 1998). These epidemiological data are supported by recent experimental studies using animal models and cell culture systems. These experimental studies show that ethanol can induce mammary tumor and stimulate proliferation as well as invasion of breast tumor cells (Singletary, 1997; Luo and Miller, 2000; Meng *et al*., 2000; Watabiki *et al*., 2000; Singletary *et al*., 2001; Izevbigie *et al*., 2002). The data imply that the most likely mechanism of ethanol action involves tumor growth and metastasis. It has been suggested that bioactivation of ethanol to acetaldehyde or formation of free radicals may be involved in ethanol-mediated breast cancer promotion (Wright *et al*., 1999; Castro *et al*., 2001). The molecular mechanisms underlying ethanol action, however, remain to be determined.

The ErbB family of receptor kinases includes four closely related members: epidermal growth factor receptor (EGFR or ErbB1), ErbB2/neu, ErbB3, and ErbB4. Among the family, ErbB2 is most directly related to breast cancer. Amplification of the ErbB2/neu is found in 20–30% of breast cancer patients and is associated with poor prognosis (Slamon *et al*., 1987;

McCann *et al*., 1991; Paterson *et al*., 1991). In addition, the overexpression of ErbB2 is positively correlated with lymph node metastasis in breast cancer patients (Slamon *et al*., 1987; Lacroix *et al*., 1989; Tauchi *et al*., 1989). Although no known ligand has been identified, ErbB2 is the preferred heterodimerization partner for all ErbB family members, and it plays a pivotal role in the ErbB receptor-mediated signaling (Plowman *et al*., 1993; Sliwkowski *et al*., 1994; Carraway *et al*., 1995; Tzahar *et al*., 1996; Graus-Porta *et al*., 1997). Recent studies indicate that the status of ErbB2 expression in a given cell is critical in determining the cellular response to growth factors or environmental stimuli (Schmidt-Ullrich *et al*., 1999; Spencer *et al*., 2000; Bowers *et al*., 2001; Sun *et al*., 2002).

We have previously demonstrated that the sensitivity of neuronal cells to ethanol was associated with the expression levels of mitogenic growth factor receptors (Luo and Miller, 1997). The present study sought to determine whether the status of ErbB2 expression in human breast cancer cells and mammary epithelial cells determines cellular response to ethanol.

Results

Ethanol enhances the invasive potential of breast cancer and mammary epithelial cells overexpressing ErbB2

Ethanol stimulated the invasion of breast cancer and mammary epithelial cells (Figure 1a). The extent of ethanol-stimulated invasion was correlated to the levels of ErbB2 expression; cells expressing high levels of ErbB2 displayed an enhanced response to ethanol-mediated cell invasion. For example, human breast cancer cells, MDA-MB-435, transfected with ErbB2 (MDA-MB-435^{ErbB2}) were more sensitive to ethanol-mediated cell invasion than their parental cells. The invasive potential of HS578 T cells, a breast cancer line with high levels of ErbB2, was dramatically increased by ethanol exposure (Figure 1a and b). In contrast, BT-20 cells, a breast cancer line with low expression of ErbB2, did not respond to ethanol.

Four stable clones of HB2 mammary epithelial cells transfected with ErbB2 were examined. These clones confirmed overexpression of ErbB2 (Ye *et al*., 1996). All these stable ErbB2 transfectants displayed significantly more invasive potential and were more responsive to ethanol than their parental cells (data not shown). Among these stable clones, HB2^{ErbB2} cells expressed highest ErbB2 levels and exhibited significantly higher invasive potential than parental cells (Figures 1b and 2a). At physiologically relevant concentrations, ethanol induced a dose-dependent increase in invasive potential of HB2^{ErbB2} cells (Figure 2b). In contrast, ethanol did not significantly promote the invasion of HB2 cells. Ethanol treatment also induced a modest increase in the growth of HB2 and HB2^{ErbB2} cells (Figure 3a). Although the basal growth rate was different, ethanol-stimulated cell growth was similar between these cell lines. Ethanol did not affect the viability of these cells (Figure 3b). We, therefore, concluded that ethanol-mediated alteration of cell number resulted from changes in cell proliferation. Since HB2^{ErbB2} cells displayed high ErbB2 expression and rigorously responded to ethanol-mediated cell invasion, the subsequent studies focused on comparing the effects of ethanol on HB2^{ErbB2} cells to parental HB2 cells.

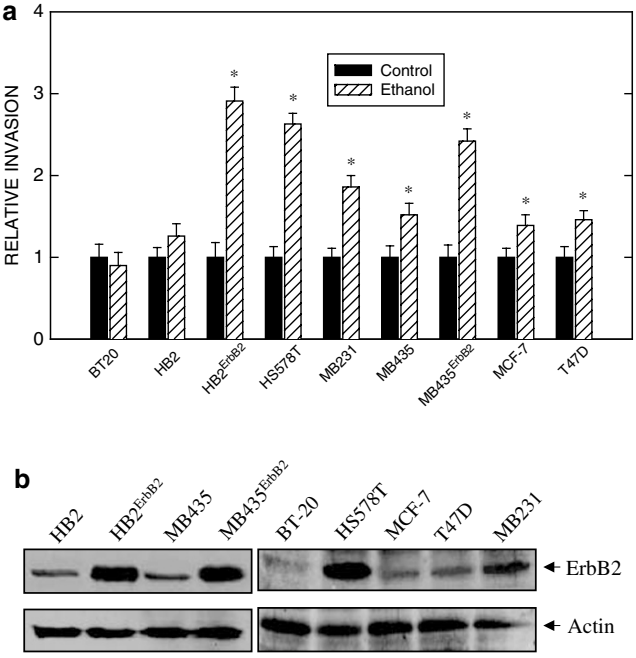

Figure 1 Correlation between ethanol-stimulated cell invasion and the expression of ErbB2. (**a**) Effects of ethanol on cell invasion. Human breast cancer cells or mammary epithelial cells were plated to the upper compartments of the invasion chambers and exposed to ethanol (0 or 400 mg/dl) for 48 h. Following ethanol exposure, invasive capacity (percentage of invaded cells) was assayed. The invasive capacity of all cells was arbitrarily designated as one and ethanol-mediated alteration was expressed as an arbitrary value relative to nonethanol-treated controls. Each data point (±s.e.m.; bars) is the mean of three to four independent trials. *Denotes a statistically significant difference between control and ethanol-treated cells ($P<0.05$). (**b**) Expression of ErbB2 in various human breast cancer cells. Cellular proteins (40 μg) was loaded into each lane. The expression of ErbB2 was examined with immunoblot using a specific anti-ErbB2 antibody (top panel). The same blot was stripped and reprobed with an anti-actin antibody (bottom panel)

Ethanol triggers divergent signal pathways in the cells expressing different ErbB2 levels

Ethanol activated three members of mitogen-activated protein kinases (MAPKs), namely, extracellular signal-regulated kinase (ERK), c-Jun NH_2 terminal protein kinase (JNK1/2) and p38 mitogen-activated protein kinase (p38 MAPK), and Akt, a substrate of PI-3 kinase (Figure 4a). The Western blot analyses of these kinases of HB2 and HB2^{ErbB2} cells were performed in the same blot. As shown in Figure 4a, the basal and ethanol-stimulated ERK activation was similar between HB2 and HB2^{ErbB2} cells, except that ethanol-mediated activation in HB^{ErbB2} cells lasted a little longer than in HB2

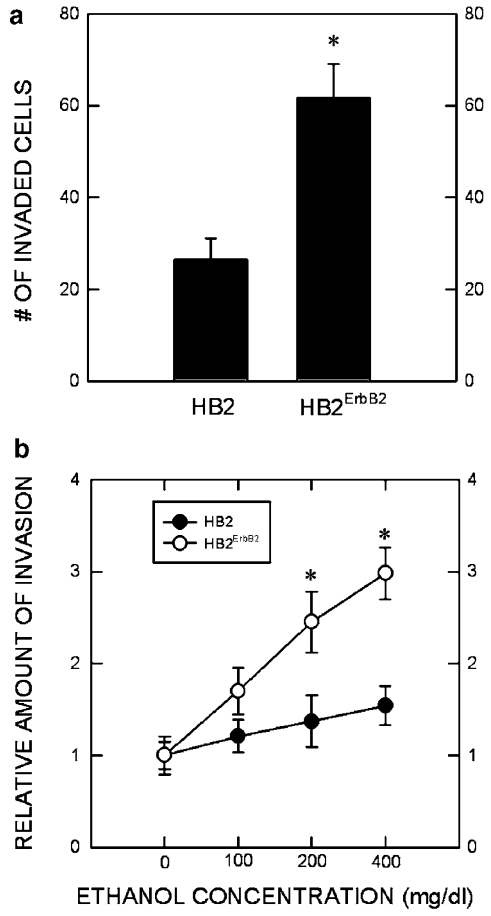

Figure 2 Invasive capacity of human mammary epithelial cells *in vitro*. (**a**) Invasive capacity of HB2 and $HB2^{ErbB2}$ cells. Equal amounts of HB2 and $HB2^{ErbB2}$ cells were plated to the upper compartments of the invasion chambers. The invasive potential of these cells was assayed as described in Figure 1a. (**b**) Concentration-dependent effect of ethanol on the invasion of HB2 and $HB2^{ErbB2}$. Cells were exposed to ethanol (0, 100, 200, and 400 mg/dl) for 48 h. Ethanol-stimulated invasion was determined as described in Figure 1a. Each data point (±s.e.m.; bars) is the mean of three independent trials. *Denotes a statistically significant difference between control and ethanol-treated cells ($P<0.05$)

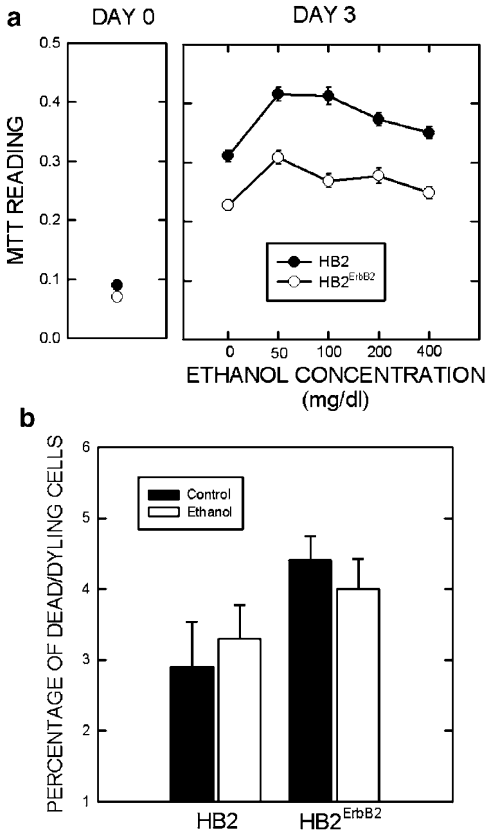

Figure 3 Effects of ethanol on the growth and survival of human mammary epithelial cells. (**a**) Cell number. Equal amounts of HB2 and $HB2^{ErbB2}$ cells were plated into 96-well culture trays and maintained in a medium containing 10.0% serum. Cells were exposed to ethanol (0, 100, 200, and 400 mg/dl) for 3 days. The numbers of viable cells were determined with MTT assay. (**b**) Cell survival. Cells were exposed to ethanol (400 mg/dl) for 3 days. The dead/dying cells were determined using trypan blue exclusion method and the ratio of dead/dying cells to total cells was calculated. Each data point (±s.e.m.; bars) is the mean of four independent trials

cells. In contrast, both the basal and ethanol-stimulated activation of p38 and JNK1/2 in $HB2^{ErbB2}$ cells was much stronger than in HB2 cells. Ethanol also induced a modest activation of Akt, a substrate of PI3-K. However, the extent of activation did not differ between HB2 cells $HB2^{ErbB2}$ cells. Ethanol-activated intracellular signaling may be transmitted by the effect of ethanol on ErbB2 activation. We examined the effect of ethanol on ErbB2 expression and autophosphorylation in HB2 and $HB2^{ErbB2}$ cells. As shown in Figure 4b, ethanol exposure had little effect on either the expression or the autophosphorylation of ErbB2 in both cell lines. Therefore, the effect of ethanol was downstream of ErbB2.

Activation of JNK and p38 MAPK is required for ethanol-induced cell invasion

Since ethanol triggered divergent intracellular signaling in HB2 and $HB2^{ErbB2}$ cells, we sought to determine which signaling pathway was involved in ethanol-mediated cell invasion by using specific inhibitors (PD98059, SB202190, D-JNKI1, and LY294002) for MAPKs and PI3-K. As shown in Figure 5a, these inhibitors were effective in blocking the activity of respective kinases. As ethanol did not significantly promote *in vitro* invasion of HB2 cells, we only examined the effect of these inhibitors on ethanol-induced invasion in $HB2^{ErbB2}$ cells. As shown in Figure 5b, these inhibitors had little effect on basal invasion of $HB2^{ErbB2}$ cells; however, blockage of activation of either JNKs or p38 MAPK by D-JNK1 or SB202190 significantly inhibited ethanol-stimulated cell invasion. In contrast, inhibition of ERK and PI3-K pathway by PD98059 and LY294002 had little effect on ethanol-mediated invasion. The effect of these inhibitors did not result from cellular toxicity, because at the concentrations applied they did not significantly affect cell viability as assayed by MTT assay (data not shown).

Role of reactive oxygen species in ethanol-induced cell invasion

It has been suggested that ethanol can stimulate the formation of reactive oxygen species (ROS) and some

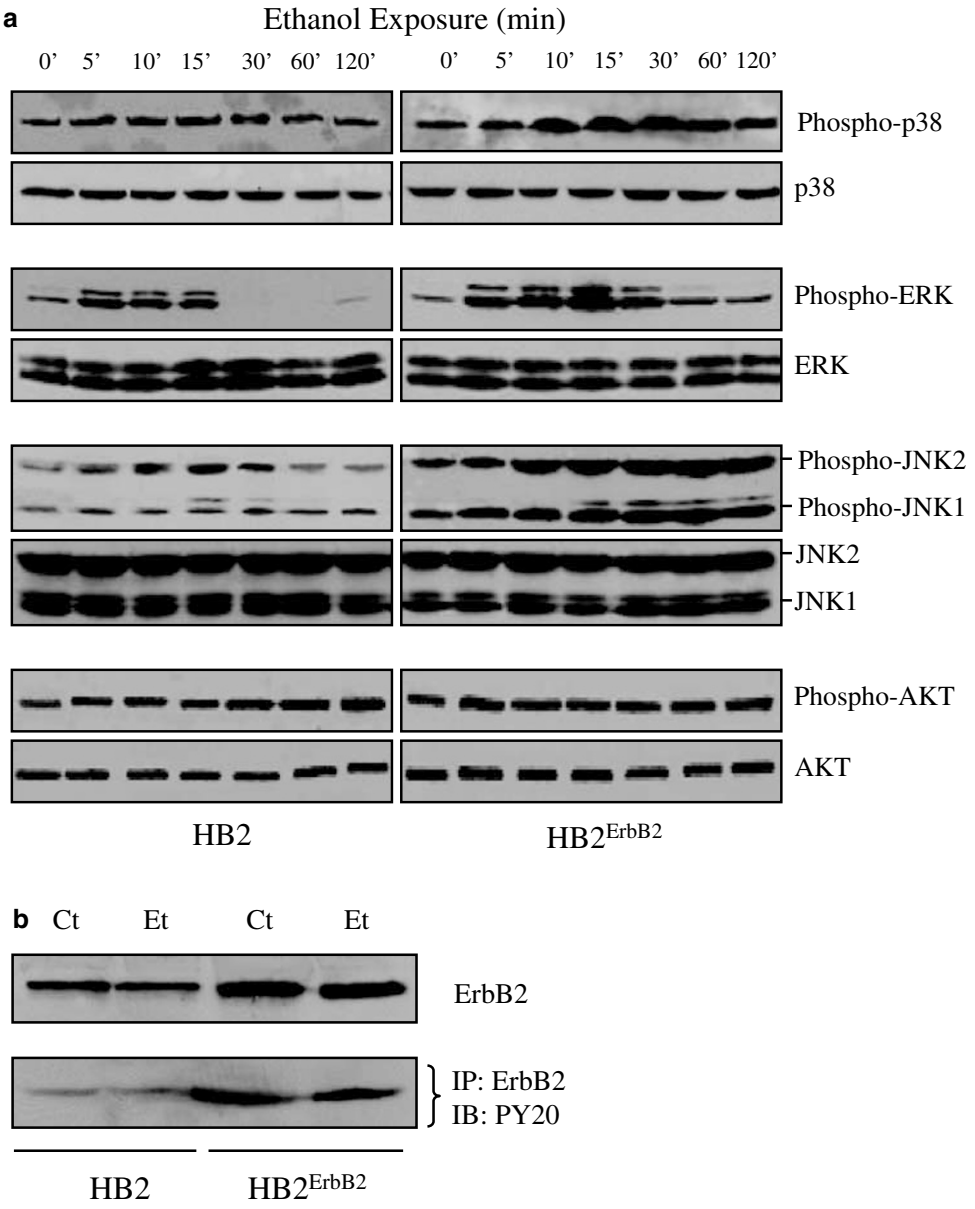

Figure 4 Effects of ethanol on intracellular signaling and ErbB2 expression/autophosphorylation in HB2 and $HB2^{ErbB2}$ cells. (**a**) Intracellular signaling. Cells were grown in a serum-free medium for 24 h. After serum starvation, cells were treated with ethanol (400 mg/dl) for various times (5–120 min). The phosphorylation and expression of MAPKs and Akt were determined with immunoblot using specific antibodies against either phosphorylated or regular forms of MAPKs and Akt. The experiments were replicated three times. (**b**) ErbB2 expression/autophosphorylation. Cells were exposed to ethanol (400 mg/dl) for 24 h. The expression of ErbB2 was determined by immunoblot using a specific anti-ErbB2 antibody. For assaying autophosphorylation, ErbB2 was immunoprecipitated and phosphorylation was detected by immunoblot using an anti-phosphotyrosin antibody

biological effects of ethanol are mediated by ROS (Bailey and Cunningham, 1998; Wright *et al*., 1999; Li *et al*., 2001; Hoek and Pastorino, 2002; Reinke, 2002). We investigated the ability of ethanol to generate ROS in HB2 and $HB2^{ErbB2}$ cells using a spin trapping method with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as the spin trap. As shown in Figure 6a, ethanol treatment generated a typical ESR spectrum of free radicals. The spectrum consists of a 1:2:2:1 quartet with splittings of $a_H = a_N = 14.9$ G, where a_N and a_H denote hyperfine splitting of the nitroxyl nitrogen and α-hydrogen, respectively. Based on these splittings and the 1:2:2:1 line shape, this spectrum was assigned to the DMPO/•OH adduct, which is evidence for the •OH radical generation. The intensity of the •OH radical in ethanol-treated $HB2^{ErbB2}$ cells was much stronger than in HB2 cells. The addition of catalase, a specific H_2O_2 scavenger, completely eliminated the •OH radical, indicating that H_2O_2 was generated in ethanol-stimulated cells and was precursor of •OH generation. To further verify the •OH radical generation, we performed the competition experiments in which the •OH radical abstracts a hydrogen atom from formate, with the trapping of a new radical. As expected, addition of

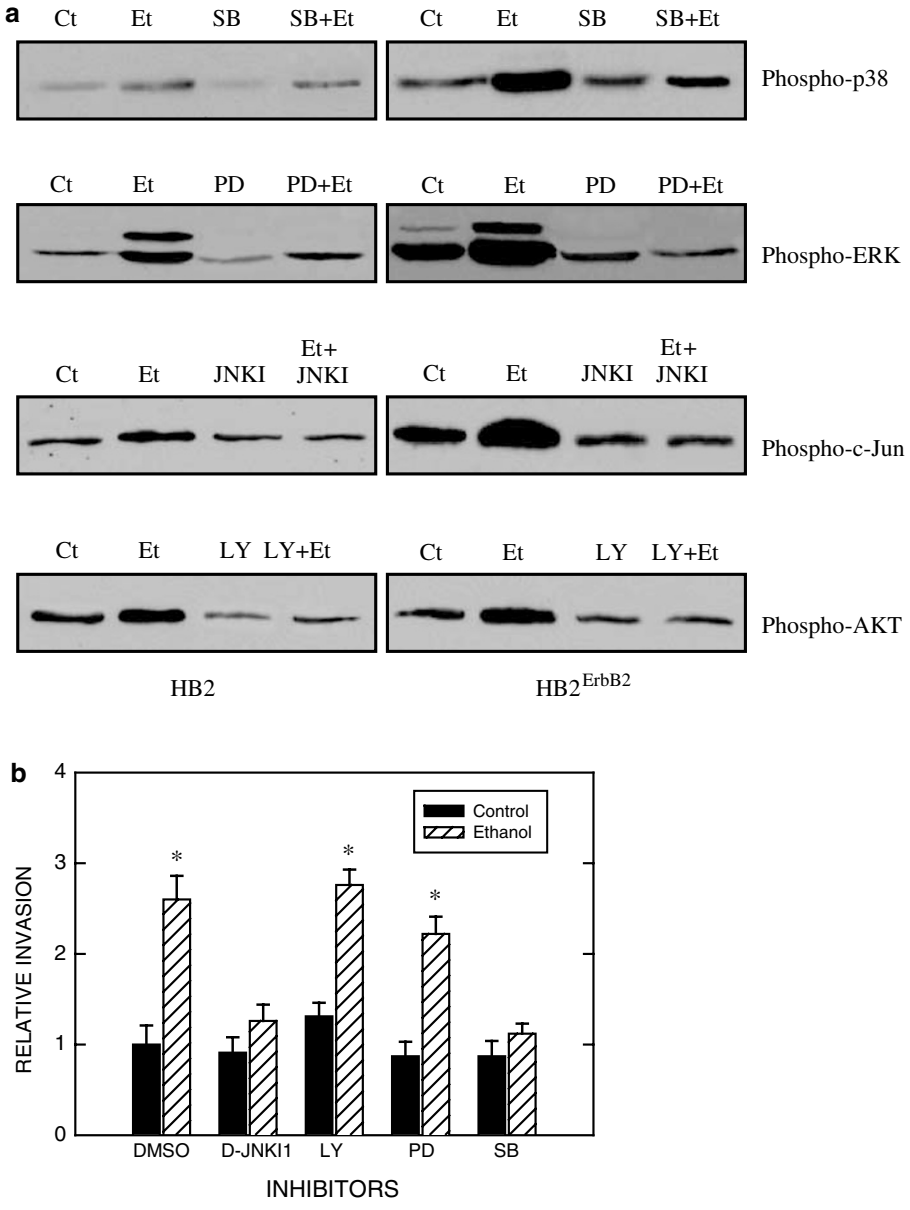

Figure 5 Effects of kinase inhibitors on ethanol-mediated invasion. (**a**) Effect of inhibitors on ethanol-triggered kinase activation. HB2 and $HB2^{ErbB2}$ cells were pretreated with various inhibitors for 30 min prior to ethanol exposure. Cells were exposed to ethanol (400 mg/dl) for 15 min and the phosphorylation of kinases was determined as described in Figure 4. (**b**) Effect of inhibitors on ethanol-mediated invasion. Cells were plated to the upper compartments of the invasion chambers and pretreated with various inhibitors 30 min before exposure to ethanol. Cells were exposed to ethanol (0 or 400 mg/dl) and the invasive capacity was assayed as described in Figure 1a. The final concentrations for inhibitors were: D-JNKI1, 1.0 μM; LY294002, 10.0 μM; PD98059, 50.0 μM; SB202190, 10.0 μM. Each data point ($\pm$s.e.m.; bars) is the mean of three independent trials. *Denotes a statistically significant difference between matched control and ethanol-treated groups ($P<0.05$)

sodium formate, a scavenger of •OH radical, decreased the intensity of the DMPO/•OH signal and resulted in the appearance of new spin adduct signal. These results verified that •OH radicals were produced. The results were similar in three independent experiments. The findings indicated that ethanol was able to generate a whole spectrum of ROS in mammary epithelial cells and $HB2^{ErbB2}$ cells were more sensitive to ethanol-mediated ROS production than HB2 cells. Next, we sought to determine whether ROS was involved in ethanol-activated intracellular signaling and cell invasion. As shown in Figure 6b, catalase, a hydrogen peroxide scavenger, did not affect ethanol-stimulated activation of JNKs and p38 MAPK in $HB2^{ErbB2}$ cells. Similarly, catalase had little effect on ethanol-mediated invasion in $HB2^{ErbB2}$ cells (Figure 6c).

Discussion

This is the first report demonstrating that the levels of ErbB2 expression determine a cellular response to

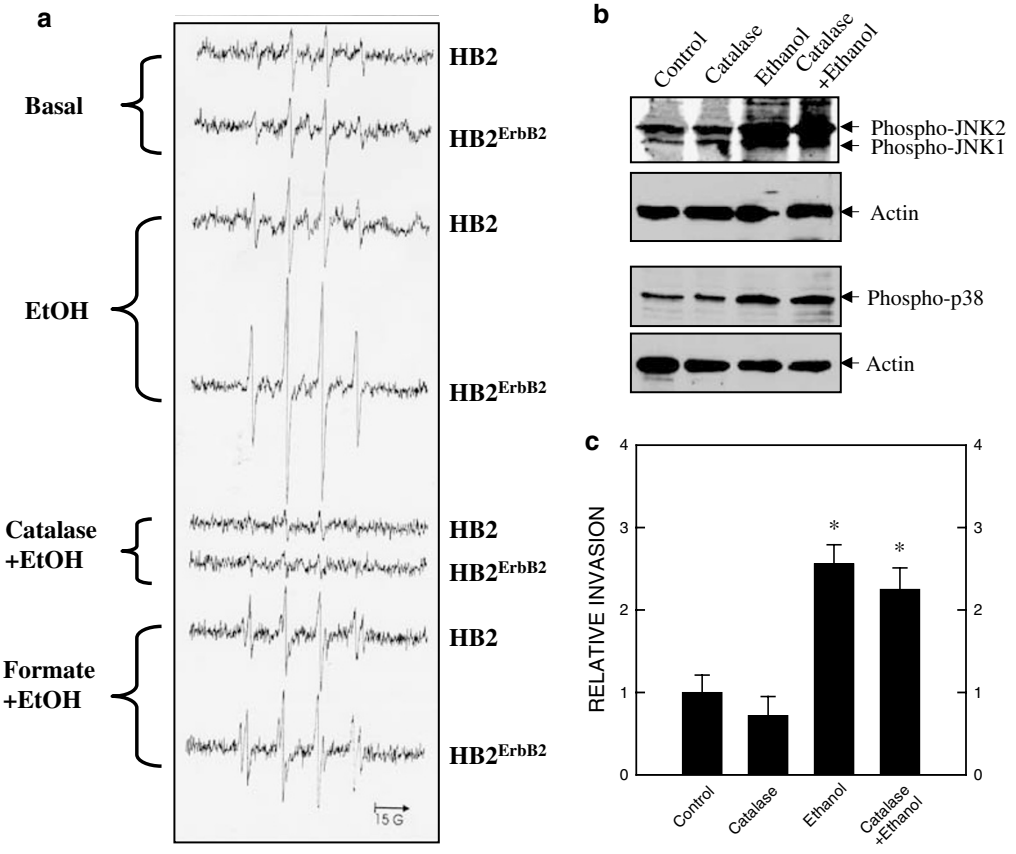

Figure 6 Ethanol-induced ROS formation and the effect of ROS scavenger on cell signaling and invasion. (**a**) Measurement of ethanol-induced ROS generation by ESR. HB2 and $HB2^{ErbB2}$ cells (1×10^6) were incubated in PBS containing 100.0 mM DMPO, 400 mg/dl ethanol with or without different ROS scavengers as indicated. ESR spectra were recorded for 30 min. The final concentrations for ROS scavengers were: catalase, 2000 U/ml; formate, 50.0 mM. (**b**) Effect of catalase on MAPK activation. $HB2^{ErbB2}$ cells were pretreated with H_2O_2 scavenger catalase (2000 U/ml) 30 min prior to exposure to ethanol. Cells were exposed to ethanol (400 mg/dl) for 15 min. The phosphorylation of JNKs and p38 MAPK was determined as described in Figure 4. (**c**) Effect of catalase on cell invasion. $HB2^{ErbB2}$ cells were pretreated with catalase 30 min prior to ethanol exposure (400 mg/dl). The invasive capacity was assayed as described in Figure 1a. Each data point ($\pm$s.e.m.; bars) is the mean of three independent trials. * Denotes a statistically significant difference from controls ($P<0.05$)

ethanol and high expression of ErbB2 enhance an ethanol-mediated *in vitro* invasion of human mammary epithelial and breast cancer cells. Amplification of ErbB2 occurs in a variety of tumors, including 20–30% of breast cancer patients (Hynes and Stern, 1994). In fact, ErbB2 amplification is used as an independent prognostic indicator of patient survival and is correlated with a number of adverse prognostic factors in breast cancer, including an increased occurrence of metastasis and micrometastatic bone marrow disease (Slamon *et al*., 1987; Pantel *et al*., 1993). Our study implies that the levels of ErbB2 expression may predict the susceptibility to ethanol exposure.

Ethanol, particularly at a low concentration (50 mg/dl), also enhances proliferation of human mammary epithelial cells. This stimulation, however, is independent of ErbB2 levels. Previous studies show that ethanol promotes proliferation of breast cancer cells *in vitro* (Singletary *et al*., 2001; Izevbigie *et al*., 2002). It appears that the stimulation is dependent on the status of estrogen receptors and is mediated by ERK activation, that is, ethanol-mediated growth promotion only occurs in estrogen receptor-positive cells (Singletary *et al*., 2001; Izevbigie *et al*., 2002). Ethanol enhances estrogen receptor signaling in breast cancer cells (Fan *et al*., 2000). Therefore, it is likely that ethanol-stimulated cell proliferation is mediated by estrogen receptor signaling.

The mechanisms underlying ErbB2-induced sensitization to ethanol are currently unknown. ErbB2 is the preferred heterodimerization partner of all activated ErbB family members (Graus-Porta *et al*., 1997). Although no identified ligand, ErbB2 can be transactivated by dimerization with ErbB family members, and its transactivation allows the recruitment of distinct effector molecules leading to diversification of signal transduction response (Olayioye *et al*., 1998). ErbB2 amplification in breast cancer cells leads to an association with adaptor proteins and downstream effectors (Olayioye *et al*., 1998; Spencer *et al*., 2000). Like ours, previous studies demonstrate that overexpression of ErbB2 promotes migration and invasion of breast cancer cells *in vitro* (Tan *et al*., 1997; Spencer *et al*.,

2000). In addition to its pivotal role in ErbB signaling, our study indicates that ErbB2 enhances ethanol-stimulated signaling, and it appears that this enhancement has some specificity. For example, ErbB2 promotes ethanol-elicited activation of JNKs and p38 MAPK without affecting ethanol-mediated activation of ERK and Akt. Our data indicate that ethanol does not alter either the ErbB2 expression or its phosphorylation; therefore, the interaction should be downstream of the ErbB2 receptor. ErbB2 associates with distinct adaptor proteins and downstream effectors, such as Chk, Grb2, Shc, p130CAS, c-CrkII, and Src family kinases (Spencer *et al*., 2000; Olayioye *et al*., 2001). Src family kinases play an essential role in ErbB2-mediated MAPK activation (Olayioye *et al*., 2001). It is possible that ethanol may facilitate the association of ErbB2 to certain adaptor protein or effectors. This possibility is currently under investigation.

MAPKs play an instrumental role in the transmission of signals from cell surface receptors and environmental cues to diverse biological activities. Three major MAPKs have been identified: the ERKs, the JNKs, and the p38 MAPK. ERKs are mainly activated by growth factors and are involved in the regulation of cell proliferation, while JNKs and p38 MAPK are most potently activated by environmental stresses (Marshall, 1995). Our results show that blocking the activation of JNK and p38 MAPK does not affect basal invasion; however, it eliminates ethanol-stimulated invasion. JNKs and p38 MAPK have been shown to participate in the regulation of cell mobility and the invasion in various cell types (Lakka *et al*., 2000; Hauck *et al*., 2001; Shin *et al*., 2001; Park *et al*., 2002; Denkert *et al*., 2002). Here we clearly demonstrate that JNKs and p38 MAPK also play an important role in ethanol-mediated invasion of mammary epithelial cells. ERK is minimally involved in ethanol-mediated invasion. In contrast, a previous study shows that ERK is critical for ErbB ligand-induced migration/invasion (Spencer *et al*., 2000). In that study, ErbB ligands, such as EGF or heregulin, are shown to induce an ErbB2-dependent and sustained activation of ERK (longer than 2 h); blockage of activation of either ErbB2 or ERK eliminates ErbB ligand-stimulated migration/invasion. Our result indicates that ethanol exposure produces a transient activation of ERK (within 30 min). It has been demonstrated that dependent on cell types or the profile of activation, ERK activation may lead to different and sometimes opposite biological activity. For example, sustained activation causes growth arrest whereas acute activation induces cell proliferation (Qiu and Green, 1991; Tombes *et al*., 1998; Luo and Miller, 1999).

ROS has been suggested to contribute to carcinogenesis (Jurgensmeier *et al*., 1997; Dhar *et al*., 2002). ROS formation is implicated for ethanol-increased risk for breast cancer (Wright *et al*., 1999; Castro *et al*., 2001). We demonstrate here that ethanol differentially regulates ROS formation in the cells overexpressing ErbB2 and the cells with normal ErbB2 levels; cells with high levels of ErbB2 expression are more susceptible to ethanol-induced ROS formation. ROS may act as second messengers and activate intracellular signal cascade, including JNKs and p38 MAPK (Lo *et al*., 1996; Mansat-de Mas *et al*., 1999; Sano *et al*., 2001; Chen *et al*., 2001; Kulisz *et al*., 2002). However, our study indicates that catalase (a H_2O_2 scavenger) does not affect either the activation of JNK/p38 MAPK or the cell invasion induced by ethanol. Lack of effect of catalase suggests that ROS may not be a key mediator of intracellular signaling in this system. Alternatively, catalase may not effectively remove the accumulation of intracellular H_2O_2. Our recent study demonstrates that vanadate-induced formation of ROS does not result in the activation of JNKs and ERKs in cerebellar neurons (Luo *et al*., 2003). Therefore, the biological effect of ROS may be cell-type specific. Even ROS is not involved in ethanol-mediated cell invasion *in vitro*, its role in other events related to ethanol-induced tumorigenic transformation remains to be investigated.

The molecular mechanisms for ethanol-stimulated cell invasion are not known. Activation of MAPKs leads to the recruitment of transcriptional factors and gene transcription. Several studies have shown that ethanol can upregulate gene transcription in breast cancer cells (Verma and Davidson, 1999; Zhu *et al*., 2001a, b). Ethanol may increase the expression and/or activity of proteins that regulate cell mobility and invasion. For example, matrix metalloproteinases (MMPs) are a family of proteinases that degrade the extracellular matrix. Overexpression and high activity of MMPs are found in malignant breast carcinomas, and are associated with shortened survival (Talvensaari-Mattila *et al*., 1998; Duffy *et al*., 2000). Both the expression and the activation of MMPs are under tight control of JNKs and p38 MAPK (Lakka *et al*., 2000; Simon *et al*., 2001; Reunanen *et al*., 2002; Shin *et al*., 2002). We are currently investigating whether ethanol-induced activation of JNKs and p38 MAPK leads to upregulation of MMPs.

Materials and methods

Cell culture and treatment

Human breast cancer cell lines, BT-20, MDA-MB-231, MCF-7 and T47D, were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and breast cancer cells HS578 T were kindly provided from Dr Donald A Sens at West Virginia University, Morgantown WV. These cells were grown in Eagle's MEM containing 10.0% fetal bovine serum (FBS), 2.0 mM L-glutamine, and 25.0 μg/ml gentamycin at 37°C with 5.0% CO_2. HB2 line is a clonal derivative of a non-tumorigenic mammary epithelial cell line MTSV1-7, and expresses many of the markers typical of luminal mammary epithelial cells (Alford *et al*., 1998). Stable clones of ErbB2 overexpression were established by transfection of HB2 with a full-length cDNA of human ErbB2 (Ye *et al*., 1996). HB2 cells and their transfectants were grown in the same medium as above, except that 5.0 μg/ml hydrocortisone and 10 μg/ml bovine insulin were added. Human breast cancer cells, MDA-MB-435 and MDA-MB-435^{ErbB2} (MDA-MB-435 cells overexpressing ErbB2), were a generous gift from Dr Dihua Yu at

the University of Texas M.D. Anderson Cancer Center, Houston, TX. These cells were cultured in DMEM/F12 medium containing 4.5 g/l glucose and 10.0% FBS. Cells were exposed to ethanol for a specified duration. To block a specific signaling pathway, cells were treated with a protein kinase inhibitor 30 min prior to ethanol exposure. Inhibitors for p38 MAPK (SB202190) and MEK1 (PD98059) were purchased from Calbiochem (La Jolla, CA, USA), selective JNK inhibitor (D-JNKI1) was purchased from Alexis Biochemicals (San Diego, CA, USA), and PI3-kinase inhibitor (LY294002) was obtained from Promega (Madison, WI, USA).

Ethanol exposure protocol

As a result of ethanol's volatility for chronic exposure (e.g., 48 h), a method utilizing sealed containers (Luo and Miller, 1997) was used to maintain ethanol levels in the culture medium. With this method, ethanol concentrations in the culture medium can be accurately maintained.

Cell proliferation and viability

MTT assay The MTT assay was employed to determine the number of viable cells in culture (Cat. #: 1 465 007, Roche Molecular Biochemicals. Indianapolis, IN, USA) (Sun *et al*., 2002). The assay is based on the cleavage of the yellow tetrazolium salt MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] to purple formazan crystals by metabolically active cells. Briefly, the cells were plated into 96-well microtiter plates and exposed to ethanol for 3 days. Following ethanol exposure, 10 μl of MTT labeling reagent was added to each well and the plates were incubated at 37°C for 4 h. The cultures were then solubilized and spectrophotometric absorbance of the samples was detected by a microtiter plate reader. The wavelength to measure absorbance of formazan product is 570 nm, with a reference wavelength of 750 nm.

Trypan blue staining Cell viability was determined by a trypan blue exclusion method (Luo *et al*., 1999). Dead/dying (trypan blue-positive) and viable (trypan blue-negative) cells were counted with a hemacytometer. The ratio of dead/dying cells to total cells was calculated.

In vitro *cell invasion*

A bioassay for *in vitro* cell invasion using Matrigel Invasion Chambers (Fisher Scientific; Cat#: 08-774-122) was performed as described previously (Luo and Miller, 2000).

Immunoprecipitation and immunoblotting

ErbB2 immunoprecipitates were generated with a previously described method (Luo and Miller, 1999). Briefly, the cell lysate was collected, and an aliquot containing 200 μg of protein was incubated with monoclonal antibody against ErbB2 (Santa Cruz Biotech. Santa Cruz, CA, USA; 1 : 50 in PBS) for 1 h at 4°C. A volume of 20 μl of Protein A/G conjugated to agarose (Santa Cruz Biotech.) was added to the lysate, and the mixture was incubated overnight at 4°C. Immunoprecipitates were collected by centrifugation at 10 000 *g* for 10 min. The pellet was washed three times with 0.5 ml RIPA buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 1.0% Nonidet P-40, 0.10% sodium dodecylsulfate (SDS), 0.50% deoxycholic acid sodium, 0.10 mg/ml phenylmethylsulfonyl fluoride, 1.0 mM sodium orthovanadate, and 3.0% Aprotinin (Sigma)]. The immunoblotting procedure for protein expression was performed as previously described (Luo and Miller, 1999). Briefly, cells were washed with PBS and lysed with RIPA buffer for 10 min, solubilized cells were centrifuged, the supernatant was collected, and the protein concentration was determined. Aliquots of the protein (40 μg) were loaded onto the lanes of a SDS 10.0% polyacrylamide gel. The proteins were separated by electrophoresis, and the separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with either 5.0% nonfat dry milk or 2.5% BSA (for detection of phosphorylation) in 0.010 M phosphate-buffered saline (pH 7.4) and 0.05% Tween-20 (TPBS) at room temperature for 1 h to block nonspecific immunoreactivity. Subsequently, the filters were incubated with primary antibodies directed against phosphorylated MAP kinases or Akt for 1 h at room temperature. Antibodies against phospho-p38 MAP kinase and ErbB2 were from Santa Cruz Biotech (Santa Cruz, CA, USA), and antibodies against phospho-ERK1/2 and phospho-JNK were from Promega. Anti-phospho-Akt (Ser473) antibody was obtained from New England Biolabs, Inc. (Beverly, MA, USA). Anti-phosphotyrosin antibody (PY20) was purchased from Zymed Laboratories Inc. (South San Francisco, CA, USA). After two quick washes in TPBS, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington Hts. IL, USA) diluted at 1 : 2000 in TPBS for 1 h. The immune complexes were detected by the enhanced chemiluminescence method (Amersham). In some cases, the blots were stripped and reprobed with an anti-actin antibody (Santa Cruz Biotech).

Electron spin resonance (ESR) measurements

ESR spin trapping technique with DMPO as the spin trap was used to detect free radical generation. This technique involves an additional reaction of a short-lived radical with a diamagnetic compound (spin trap) to form a relative long-lived free radical product (spin adduct), which can be studied by conventional ESR. The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped, while the hyperfine couplings of the spin adduct are characteristics of trapped radicals. The spectra were recorded using a Varian E9 ESR spectrometer and a flat cell assembly as previously described (Shi and Dalal, 1991). Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl as reference standards. A volume of 500.0 μl of cell suspension (1×10^6/0.5 ml) was mixed with 500.0 μl PBS containing 100.0 mM DMPO and 400 mg/dl ethanol with/without ROS scavengers. The reaction mixture (500.0 l) was transferred to a flat cell for ESR measurement.

Statistical analysis

Differences among treatment groups were tested using a one-way analysis of variance (ANOVA). Differences in which P was less than 0.05 were considered statistically significant. In cases where significant differences were detected, specific *post hoc* comparisons between treatment groups were examined with Student–Newman–Keuls tests.

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