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INDUCTION OF THE c-*jun* PROTO-ONCOGENE BY A PROTEIN KINASE C-DEPENDENT MECHANISM DURING EXPOSURE OF HUMAN EPIDERMAL KERATINOCYTES TO ETHANOL

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Abstract—The present work demonstrates that ethanol induces expression of the c-*jun* proto-oncogene in human keratinocytes. Increased c-*jun* mRNA levels were detectable at 1 hr of exposure to 1% ethanol and at 24 hr remained above that in control keratinocytes. An increase in c-*jun* expression was also detectable at ethanol concentrations of 0.1 and 0.5%. Similar findings were obtained for the related *jun-B* and c-*fos* early response genes. The results also demonstrate that ethanol exposure is associated with increases in protein kinase C activity in both the cytosol and membrane fractions. This increase was detectable at 5 min and maximal at 30–60 min. The finding that induction of c-*jun* expression by ethanol was inhibited by the isoquinolinesulfonamide derivative H7, but not by HA1004, suggested that this effect is mediated by protein kinase C. Furthermore, down-regulation of protein kinase C by prolonged exposure to 12-*O*-tetradecanoylphorbol-13-acetate was associated with a block in ethanol-induced c-*jun* expression. We also demonstrated that ethanol exposure is associated with rapid (5–30 min) increases in intracellular levels of diradylglycerol. Taken together, these findings demonstrate that the exposure of keratinocytes to ethanol results in the activation of protein kinase C and c-*jun* expression.

The c-*jun* gene is induced as an immediate early response to certain growth factors and phorbol esters [1–3]. The product of this gene, Jun/AP-1, binds to the heptameric DNA consensus sequence $TGA^{G}/_{C}TCA$ and functions in the transcriptional regulation of gene expression [4]. Studies of genes, including c-*jun*, induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA)† have demonstrated activation by Jun/AP-1 binding to this *cis*-acting response element (TRE) [5, 6]. These and other findings have demonstrated that protein kinase C (PKC) regulates the DNA-binding activity of this factor [7]. The affinity of binding to the TRE is also related to the formation of Jun/Jun homodimers or heterodimers with other transcription factors, such as the c-*fos* gene product, that contain a leucine zipper motif [8–10]. *Jun-B* is another member of the Jun family that forms dimers and binds to the TRE, although the properties of c-*jun* and *jun-B* differ in the regulation of gene transcription [11–13].

The present work has examined whether exposure of human epidermal keratinocytes to ethanol is associated with induction of c-*jun* expression. The results demonstrate that ethanol induces c-*jun*, as well as *jun-B* and c-*fos*, gene expression. The findings also demonstrate that ethanol activates PKC and induces c-*jun* mRNA levels by a protein kinase C-dependent signaling mechanism.

MATERIALS AND METHODS

Cell culture. Normal human epidermal keratinocytes (Clonetics, San Diego, CA) were grown in keratinocyte growth medium (KGM) containing 0.1 ng/mL epidermal growth factor (EGF), 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 0.15 mM calcium, 0.4% (v/v) bovine pituitary extract and gentamycin/amphotericin B. The cells were maintained in keratinocyte basal medium (EGF-free KGM) for 24 hr and then treated with various concentrations of ethanol (absolute ethyl alcohol; Aapeer Alcohol Co., Shelbyville, KY), H7 (Seikagaku America Inc., St. Petersburg, FL), HA1004 (Seikagaku) or TPA (Sigma Chemical Co., St. Louis, MO).

Isolation and analysis of RNA. Total cellular RNA was purified by the guanidine isothiocyanate-cesium chloride technique [14]. The RNA was analyzed by electrophoresis through 1% agarose-formaldehyde gels, transferred to nitrocellulose filters and hybridized to the following ^{32}P-labeled DNA probes: (1) the 1.8-kb *Bam*HI/*Eco*RI insert of a human c-*jun* gene purified from a pBluescript SK(+) plasmid [5]; (2) the 1.8-kb *Eco*RI fragment of the murine *jun-B* cDNA from the p465.20 plasmid [11]; (3) the 0.9-kb *Sca*I/*Nco*I insert of a human c-*fos* gene purified from the pc-fos-1 plasmid [15]; and (4) the 2.0-kb *Pst*I insert of a chicken β-actin gene purified from the pA1 plasmid [16]. Hybridizations were performed at 42° for 24 hr in 50% (v/v) formamide, 2× SSC, 1× Denhardt's solution, 0.1% sodium

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† Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; EGF, epidermal growth factor; DAG, diradylglycerol; PA, phosphatidic acid; PI, phosphoinositide; PKC, protein kinase C; IP, inositol phosphate; and SSC, 0.15 M sodium chloride + 0.015 M sodium citrate.

dodecyl sulfate (SDS), and 200 μg/mL salmon sperm DNA. The filters were washed twice in 2× SSC, 0.1% SDS at room temperature and then in 0.1× SSC, 0.1% SDS at 60° for 1 hr.

PKC assays. Keratinocytes (10^7) were suspended in 0.4 mL of ice-cold lysis buffer [20 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM ethyleneglycolbis(β-aminoethylether)tetra-acetate (EGTA), 10 mM β-mercaptoethanol, 0.5% Triton X-100, 25 μg/mL leupeptin and 25 μg/mL aprotinin]. The cells were disrupted in a Dounce homogenizer, incubated on ice for 30 min and then centrifuged at 15,000 *g* for 2 min. The supernatant was applied to DEAE-cellulose (Whatman DE52) ion exchange columns and the DEAE eluate diluted in TEM buffer (20 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA and 10 mM β-mercaptoethanol) containing 200 mM NaCl to obtain a range of three different enzyme concentrations.

To determine the subcellular distribution of PKC, keratinocytes (10^7) were suspended in lysis buffer without Triton X-100. The cells were disrupted by passage through a 25-gauge needle and then centrifuged at 15,000 *g* for 20 min at 4°. The supernatant (cytosolic fraction) was applied to DEAE cellulose for partial purification of PKC. The pellet (membrane fraction) was resuspended in lysis buffer with 0.5% Triton X-100, sonicated for 15 sec on ice, incubated on ice for 60 min, and then centrifuged at 15,000 *g* for 10 min at 4°. The supernatant (detergent-solubilized membrane fraction) was used to partially purify PKC on DEAE-cellulose columns.

PKC activity was determined as described ([17] and Protein Kinase C Assay System, GIBCO BRL, Grand Island, NY). The partially purified protein fractions were incubated for 5 min at 30° in phospholipid (phosphatidylserine and phorbol ester in Triton X-100 mixed micelles; GIBCO BRL), [γ-^{32}P]ATP (6000 Ci/mmol; NEN, Boston, MA) and PKC synthetic peptide from myelin basic protein (GIBCO BRL). All assays were also performed in the presence of a PKC inhibitor peptide (GIBCO) to monitor for specificity of the phosphorylation reaction. The samples were dried on phosphocellulose, washed in 1% H_3PO_4 and assayed by scintillation counting. PKC activity was determined as described in the Protein Kinase C Assay System (GIBCO BRL).

Diradylglycerol (DAG) measurements. DAG mass was quantitated as described by measuring the formation of phosphatidic acid (PA) in the presence of ATP and DAG kinase [18]. Lipids were extracted from keratinocytes with $CHCl_3$/methanol/1 N HCl/1 mM EGTA (5:5:2:2, by vol.), lyophilized and redissolved in a 100-μL reaction mixture containing 50 μg/mL DAG kinase (Lipidex, Westfield, NJ), 15% β-octylglucoside, 2.5 mM 1,2-dioleoyl-*sn*-3-phosphatidylglycerol, 1 mM ATP and 1.8×10^6 cpm [γ-^{32}P]ATP (3000 Ci/mmol). After incubation for 30 min at room temperature, the reaction was terminated by adding 600 μL $CHCl_3$/methanol (1:2, v/v). The lipids were extracted by adding 600 μL of 1% HCl/$CHCl_3$ (2:1, v/v) and then analyzed by TLC using $CHCl_3$/acetone/methanol/acetic acid/H_2O (10:4:3:2:1, by vol.) as a solvent system. PA was cochromatographed as authentic standard. The TLC plate was subjected to autoradiography and the band corresponding to PA was scraped and counted.

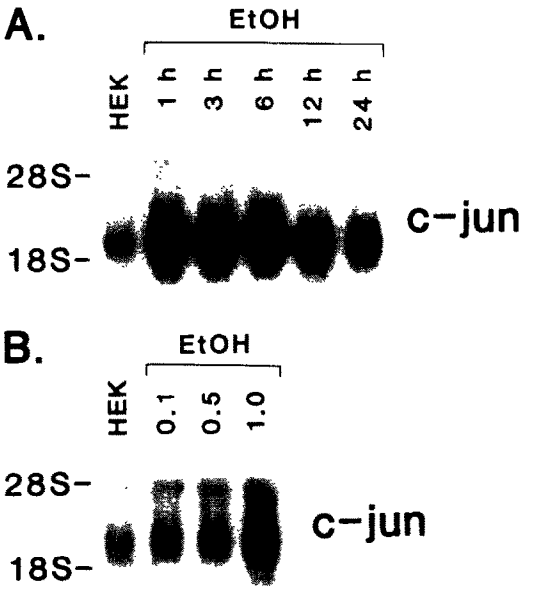

Fig. 1. Ethanol-induced c-*jun* expression in keratinocytes. Human epidermal keratinocytes (HEK) were treated with (A) 1% ethanol (EtOH) for the indicated times, and (B), the indicated concentrations of ethanol for 6 hr. Total cellular RNA (20 μg) was hybridized to the ^{32}P-labeled c-*jun* probe. Hybridization to a labeled actin probe demonstrated equal loading of the lanes.

RESULTS

While c-*jun* is induced in a variety of different cell types, little is known about the expression of this gene in keratinocytes. Human epidermal keratinocytes were cultured in serum-free medium containing insulin, EGF and bovine pituitary extract. Cells were deprived of EGF for 24 hr. Under these experimental conditions, there were low to undetectable levels of c-*jun* transcripts (Fig. 1A). In contrast, growth of these cells in the presence of EGF was associated with increases in c-*jun* mRNA levels (data not shown). These findings were in concert with the previous demonstration that EGF induces c-*jun* transcripts in fibroblasts [1] and indicate that this gene is also expressed in response to growth factor stimulation of keratinocytes.

Keratinocytes were exposed to 1% ethanol and then monitored for c-*jun* mRNA levels. This nontoxic concentration of ethanol rapidly (< 1 hr) induced c-*jun* transcripts by 7.5-fold (Fig. 1A). While the induction of c-*jun* expression is often transient in growth factor and phorbol ester stimulated cells [1–3], ethanol treatment was associated with persistent increases in c-*jun* mRNA levels at 24 hr (Fig. 1A). This induction of c-*jun* expression was detectable at ethanol concentrations of 0.1% (17 mM) to 1.0% (172 mM) (Fig. 1B). While treatment with 0.1 and

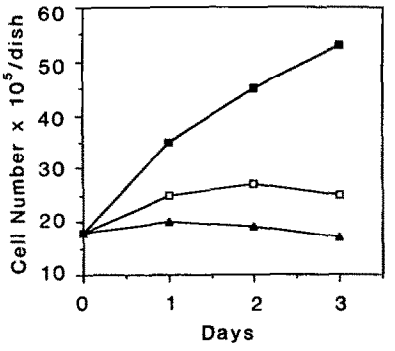

Fig. 2. Effects of ethanol on keratinocyte growth and viability. Keratinocytes were grown in complete medium (■) and in the presence of 0.1% (□) or 1.0% (▲) ethanol. Cell counts were monitored at the indicated times. Viability as determined by trypan blue exclusion was over 95% at each time point.

Fig. 3. Induction of the *jun-B* and c-*fos* genes in ethanol-treated keratinocytes. Keratinocytes (HEK) were treated with 1% ethanol for the indicated times. Total cellular RNA (20 μg) was hybridized to the ^{32}P-labeled *jun-B* (A) and c-*fos* (B) probes. Actin signals demonstrated equal loading of the lanes.

1% ethanol was associated with inhibition of keratinocyte growth, there was little if any toxicity during exposures of up to 72 hr (Fig. 2).

Although the c-*jun* gene codes for the major forms of AP-1, other related factors, such as *jun-B*, contribute to the formation of this complex. *Jun-B* mRNA levels were at low to undetectable levels in the human keratinocytes, while exposure to 1% ethanol was associated with induction of these transcripts by 5-fold at 6 hr (Fig. 3A). In contrast to the more rapid effects of ethanol on c-*jun* expression, increases in *jun-B* mRNA levels were first detectable at 3 hr. Moreover, *jun-B* gene expression remained above that in control cells for at least 12 hr (Fig. 3A). Since both c-*jun* and *jun-B* can form heterodimers with c-*fos*, we also studied the effects of ethanol on c-*fos* mRNA levels. Although c-*fos* mRNA was present at low levels in untreated keratinocytes, treatment with ethanol was associated with a rapid increase in these transcripts (Fig. 3B); c-*fos* expression was maximal at 6 hr (9-fold) and then down-regulated by 24 hr of ethanol exposure (Fig. 3B). Comparable results were obtained in keratinocytes treated with 32 nM TPA. TPA treatment was associated with increases in c-*jun* transcripts which were detectable at 6 hr and persisted at 24 hr (Fig. 4). Similar findings were obtained for *jun-B* (Fig. 4) and c-*fos* (data not shown). The finding that both ethanol and TPA increase expression of c-*jun*, as well as *jun-B* and c-*fos*, would indicate that diverse AP-1 complexes may be formed in these cells.

The demonstration that activation of PKC is associated with induction of c-*jun* expression [2, 7, 19] suggested that the effects of ethanol on c-*jun*

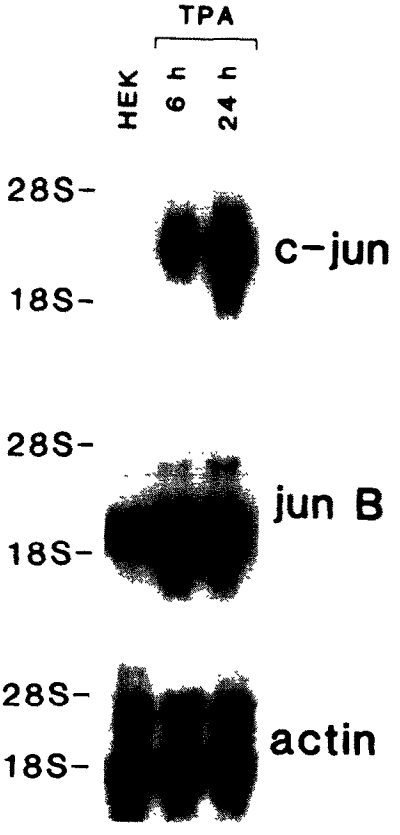

Fig. 4. TPA-induced c-*jun* and *jun-B* expression in keratinocytes. Keratinocytes (HEK) were treated with 32 nM TPA for the indicated times. Total cellular RNA (20 μg) was hybridized to the labeled c-*jun*, *jun-B* and actin probes.

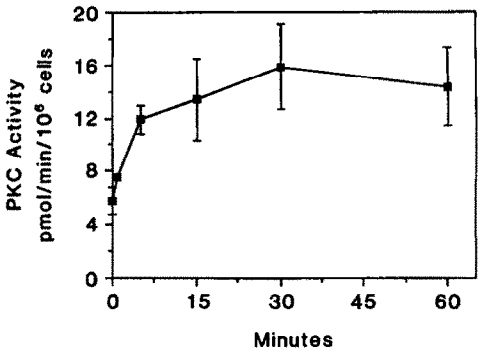

Fig. 5. Activation of PKC in ethanol-treated keratinocytes. Keratinocytes were treated with 1% ethanol. At the indicated times, cells were harvested, lysed and assayed for phosphorylation of a synthetic peptide derived from myelin basic protein. Six replicates were performed at the different time points in each experiment. The results represent the means ± SD of two separate experiments. Addition of a pseudosubstrate inhibitor in the reaction at each time point demonstrated a decrease in enzyme activity to less than 10% of control.

mRNA levels could be mediated by this enzyme. To address this issue, we first studied the effects of ethanol on PKC activity in keratinocytes. These studies were performed with a synthetic peptide derived from myelin basic protein which serves as a specific substrate for this enzyme [17]. The level of total enzyme activity was increased after ethanol treatment (Fig. 5). This effect was maximal by 30 min and remained above control levels with longer drug exposures (Fig. 5). These experiments were also performed in the presence of a pseudosubstrate inhibitor of protein kinase C and under these conditions there was less than 10% of the activity obtained in the absence of inhibitors (data not shown). Similar studies were performed after separation of the cell extract into cytoplasmic and membrane fractions. Under these conditions, there was an increase in PKC activity in both fractions, although this effect of ethanol was more pronounced in the membranes than in the cytoplasm (Fig. 6A). These results were compared to those obtained with TPA. Treatment with TPA for 30 and 60 min was associated with increases in PKC activity in the membrane fraction and a concomitant decrease in cytoplasmic activity (Fig. 6B). Taken together, these findings indicated that treatment of keratinocytes with ethanol is associated with activation of PKC, although the subcellular distribution of this activity differs from that in TPA-treated cells.

The involvement of PKC in ethanol-induced c-*jun* expression was addressed by treating keratinocytes with 50 μM H7, an isoquinolinesulfonamide derivative that inhibits this enzyme [20]. While H7 had no detectable effect on c-*jun* mRNA levels in otherwise untreated keratinocytes, this agent completely inhibited ethanol-induced c-*jun* expression (Fig. 7A). Since H7 can inhibit other protein kinases, we used similar concentrations of HA1004, a more selective inhibitor of cAMP- and cGMP-dependent kinase activity [21]. HA1004 had no detectable inhibitory effect on c-*jun* mRNA levels induced by ethanol (Fig. 7A). Similar findings were obtained with 25 μM H7 and HA1004 (data not shown). Keratinocytes were also pretreated with TPA for 36 hr to down-regulate PKC activity [22]. The treatment of cells with ethanol after TPA removal was associated with a 6.2-fold increase in c-*jun* expression. In contrast, the addition of ethanol to cells maintained in the presence of TPA resulted in over 75% inhibition of ethanol-induced c-*jun* mRNA levels (Fig. 7B). Taken together with the H7 studies, these results supported the involvement of PKC in the induction of c-*jun* expression by ethanol.

Previous work has demonstrated that DAG functions as an endogenous activator of PKC [22]. Consequently, we determined the effects of ethanol on DAG levels in the keratinocytes. These studies

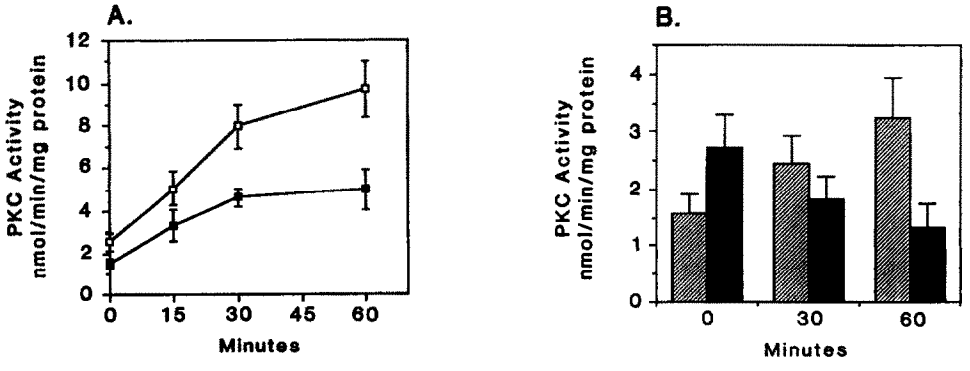

Fig. 6. Subcellular distribution of PKC in ethanol- and TPA-treated keratinocytes. Keratinocytes were exposed to 1% ethanol (A) or 32 nM TPA (B) for the indicated times. The cells were harvested, lysed and separated into membrane and cytosolic fractions. PKC activity was determined by phosphorylation of the synthetic peptide derived from myelin basic protein. The results represent the means ± SD of two separate experiments each performed in triplicate. Key: (A) membrane (□), and cytosol (■); (B) membrane (hatched), and cytosol (closed).

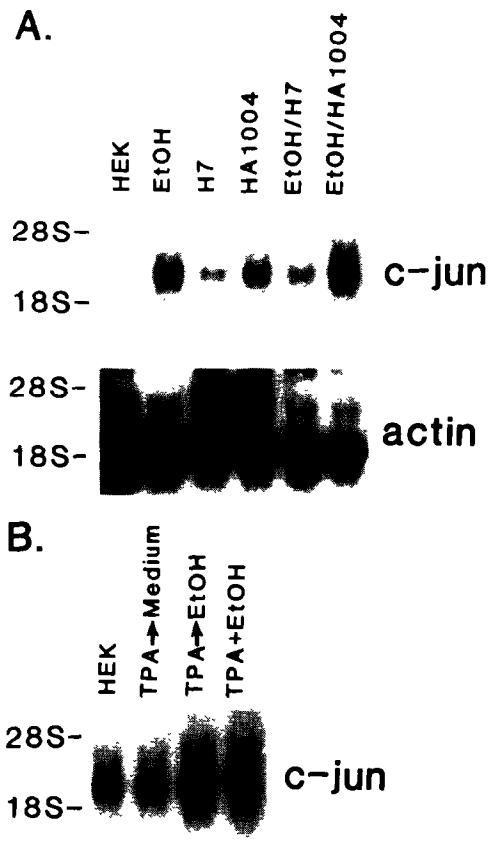

Fig. 7. Inhibition of ethanol-induced c-*jun* expression by down-regulation of PKC. (A) Keratinocytes were treated with 1% ethanol for 6 hr, 50 μM H7 for 2 hr and 50 μM HA1004 for 2 hr. The H7 and HA1004 were also added for the last 2 hr of the 6-hr ethanol incubation. (B) Keratinocytes were exposed to 32 nM TPA for 36 hr, washed and incubated in KBM (TPA → medium) or KBM with 1% ethanol for an additional 8 hr (TPA → EtOH). Other cells were treated with TPA for 36 hr and 1% EtOH was added without washing for an additional 8 hr (TPA + EtOH). Total cellular RNA (20 μg) was hybridized to the ^{32}P-labeled c-*jun* probe. Hybridization to the actin probe demonstrated equal loading of the lanes.

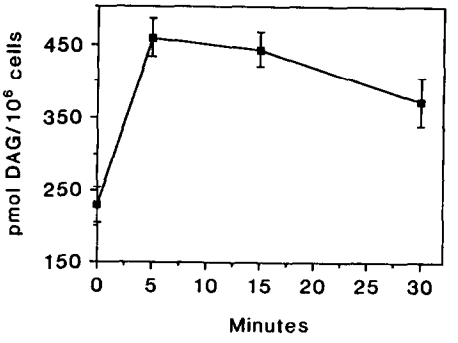

Fig. 8. Effect of ethanol on intracellular DAG levels. Keratinocytes were treated with 1% ethanol for the indicated times. Lipids were extracted and incubated in the presence of DAG kinase and [γ-^{32}P]ATP. DAG mass was determined by the formation of [^{32}P]PA. The results represent the means ± average deviation of two experiments.

were performed with an assay that measures DAG mass [18]. Untreated keratinocytes had 230 pmol DAG/10^6 cells (Fig. 8). In contrast, exposure to ethanol for 5 min was associated with an approximately 2-fold increase in these levels (Fig. 8). The DAG level at 30 min of ethanol exposure was also above control, but was somewhat lower than that obtained at 5 min (Fig. 8). These findings thus indicated that ethanol increases DAG levels and that this increase is in concert with the associated activation of PKC.

DISCUSSION

In addition to tobacco smoking, alcohol consumption represents a primary cause of oral and pharyngeal cancer [23–29]. This effect is probably related to direct contact between this agent and the mucosa of the oropharynx. Indeed, more recent studies have demonstrated an increased risk of oral cancer among users of mouthwash which is high in alcohol content [30]. However, few insights are available regarding the mechanisms responsible for this effect. Since ethanol has not been shown to be carcinogenic in laboratory animals, it has been postulated that this agent acts as a solvent which enhances the penetration of carcinogens [30, 31]. Such a solvent action might account for increased cancer risk when used with tobacco smoking, but perhaps not the independent effects of ethanol on the development of oropharyngeal tumors. Other possible mechanisms include the induction of microsomal enzymes which enhance activation of certain carcinogens [32]. The present studies have used epidermal keratinocytes, the normal counterpart of squamous cell carcinomas [33], to study the effects of ethanol on intracellular signaling events.

The present results demonstrate that exposure of epidermal keratinocytes to ethanol is associated with induction of c-*jun* gene expression. This effect was detectable at ethanol concentrations as low as 0.1 to 1%. Blood ethanol levels of 0.4% can be achieved in humans, while epithelial cells in the oropharynx are chronically exposed to even higher local concentrations following ingestion of this agent. Indeed, local mucosal tissue exposure could be up to 50-fold higher than the concentrations used in our studies when drinking a 100-proof (50% ethanol) alcoholic beverage. Furthermore, swishing with a mouthwash containing 25% ethanol would also result in much higher concentrations than that required for induction of c-*jun* expression. These effects of ethanol were not limited to the c-*jun* gene since similar findings were obtained for the related *jun-B* and c-*fos* genes. Thus, ethanol induces several genes which code for products that contribute to formation of the AP-1 complex. These genes appear to be regulated non-coordinately since *jun-B* expression was delayed compared to that for c-*jun* and c-*fos*.

The *jun-B* protein can exhibit negative regulatory effects on genes induced by AP-1 [13], although c-*jun* and *jun-B* were co-expressed in both ethanol and TPA-treated keratinocytes.

Tumor promoters, such as certain phorbol esters, bind to PKC and activate this enzyme [22]. Other structurally unrelated tumor promoters have also been shown to activate PKC [34]. Moreover, the finding that overproduction of PKC mimics the response of treating cells with TPA has indicated that this enzyme mediates at least in part the effects of tumor promoters [35]. The present results demonstrate that exposure of keratinocytes to ethanol is associated with activation of PKC. This effect was detected with a synthetic peptide substrate for this enzyme. Increases in the phosphorylation of this substrate following ethanol treatment were also blocked with a pseudosubstrate inhibitor of PKC. These findings indicated that ethanol increases both cytoplasmic and membrane-associated PKC in keratinocytes. Furthermore, activation of PKC provided a potential explanation for the effects of this agent on induction of c-*jun* expression. Indeed, H7, a non-specific inhibitor of PKC, blocked alcohol-induced c-*jun* expression. While the findings with H7 could be attributed to inhibition of other protein kinases, down-regulation of PKC with prolonged TPA exposure also blocked the effects of ethanol on this gene. Activation of PKC is known to function as a second messenger in the induction of c-*jun* expression by phorbol esters [19]. The involvement of this enzyme in regulation of the c-*jun* gene by ethanol would thus support signaling by a similar pathway.

Previous studies have demonstrated that ethanol activates phosphoinositide (PI)-specific phospholipase C in hepatocytes [36]. Stimulation of phospholipase C is associated with DAG production and thereby the potential for activation of PKC. The present results demonstrate that ethanol treatment of keratinocytes results in increases in DAG levels and that this effect is temporally related to activation of PKC. However, we were unable to detect an increase in inositol monophosphate (IP_1), diphosphate (IP_2) or triphosphate (IP_3) levels (data not shown). These findings suggest that ethanol-induced increases in DAG are not the consequence of the induction of a PI-specific phospholipase C in keratinocytes, but rather the hydrolysis of phospholipids by another mechanism, perhaps by a phosphatidylcholine-specific phospholipase C. Another possibility is that the sphingoid homologs of diglyceride could be serving as phosphoryl group acceptors in the DAG assay. While further studies will be needed to more precisely define the signaling events induced by ethanol, the demonstration that this agent activates PKC and c-*jun* expression in keratinocytes provides a molecular basis to address these issues.

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