Journal of Cellular Biochemistry 74:278–291 (1999)

Ethanol Inhibits Prolactin-Induced Activation of the JAK/STAT Pathway in Cultured Astrocytes

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Abstract Alcohol consumption has multiple effects in the central nervous system (CNS). Whereas, alcohol is an immunosuppressive drug the effect of alcohol on the neuroimmune system, remains unclear. In cultured astrocytes, prolactin (PRL) induces mitogenesis and the expression of inflammatory cytokines, including tumor necrosis factor-α (TNFα). We have recently shown that whereas ethanol does not inhibit PRL receptor binding, it markedly inhibits PRL-induced mitogenesis and TNFα secretion in cultured astrocytes. It is clear that PRL activates the tyrosine phosphorylation of several proteins, including members of a novel family of protein tyrosine kinases, the Janus Kinases (JAKs). The aims of this study were to characterize PRL-induced activation of the JAK/STAT (signal transducers and activators of transcription) pathway, and to determine if ethanol affects JAK/STAT activation in cultured astrocytes. We found that PRL specifically increases the tyrosine phosphorylation of JAK2, but not JAK1, JAK3, or Tyk2, and the subsequent phosphorylation of STAT1α, STAT5a, and STAT5b. Preincubation of astrocytes with ethanol markedly inhibited phosphorylation of JAK2, STAT1α, STAT5a, and STAT5b. In PRL-stimulated astrocytes, ethanol inhibited binding of nuclear proteins to oligonucleotides corresponding to the gamma-interferon activated sequence (GAS). Further, ethanol blocked PRL-induced increases in interferon regulatory factor-1 (IRF-1) mRNA, a PRL/cytokine inducible transcription factor involved in the regulation of a number of cytokine inducible genes. The inhibition of tyrosine phosphorylation by ethanol was not a general effect, however, as we found that ethanol increased basal and NGF-induced tyrosine phosphorylation of extracellular signal-activated protein kinase-1 (ERK-1). These data indicate that ethanol inhibits PRL-induced tyrosine phosphorylation of the JAK/STAT pathway resulting in decreased nuclear GAS DNA binding and inhibition of the PRL inducible gene, IRF-1. Thus, suggesting that ethanol-induced inhibition of JAK2 phosphorylation may be one mechanism though which ethanol could alter the brain's response to injury or infection. J. Cell. Biochem. 74:278–291, 1999.

Key words: ethanol; astrocytes; JAK/STAT pathway

The immunosuppressive effects of chronic alcohol use have been well documented [Szabo, 1993; Blank et al., 1993; Ewald and Shao, 1993; Nelson et al., 1989]. Despite the clear relationship between alcohol and impaired CNS function, there is little information concerning the effect of alcohol exposure on the molecular and cellular mechanisms involved in the neuroimmune response. In the CNS, the response to injury and disease involves interactions among various cell types (neurons, astrocytes, oligodendroglia, microglia, and infiltrating inflammatory cells), cytokines, growth factors, and membrane associated proteins [Hatten et al., 1991; Eng et al., 1992]. Although astrocytes comprise as much as 25% of the cells in the CNS, astrocytes have received little attention until recently. Unlike neurons, astrocytes retain the ability to divide and multiply. In response to injury or infection, astrocytes undergo hypertrophy and proliferation and can function as immunocompetent cells by secreting cytokines, expressing MHC class I and II antigens, and by presenting antigens to T cell clones in a MHC-restricted response [Chung et al., 1991]. This process, termed astrogliosis, is the most frequent cellular reaction to CNS injury or infection, and is found in many neurological disorders, including multiple sclerosis (MS), acquired immune deficiency syndrome dementia complex, Alzheimer's disease, and the animal model for MS, experimental allergic encephalomyelitis [Hickey et al., 1985; Benveniste, 1992; Martin et al., 1992].

Presented in part at the 80th Annual Meeting of the Endocrine Society.

Grant sponsor: National Institute on Alcohol Abuse and Alcoholism; Grant number: AA11277.

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Received 7 January 1999; Accepted 17 February 1999

Originally considered a reproductive hormone, it is now clear that prolactin (PRL) also plays a role in the regulation of humoral, cell-mediated, and auto-immune responses [Gala, 1991; Nagy and Berczi, 1978; Nagy et al., 1983; Bernton et al., 1988]. Using a homogeneous population of cultured astrocytes, we have shown that PRL stimulates astrocyte growth and the expression of several cytokines [DeVito et al., 1992, 1993, 1995, 1995]. Further, we found that ethanol (10 to 50 mM) inhibits PRL-induced mitogenesis and stimulation of TNFα production [DeVito et al., 1997]. Competitive inhibition binding studies and western blot analysis, revealed that the inhibitory effect of ethanol was not due to a decrease in the binding of PRL to its receptor or diminished PRL receptor expression. In the rat, three different forms of the prolactin receptor (PRLR) have been cloned, each differing in the length of the cytoplasmic domain [Kelly et al., 1991]. A notable characteristic of PRLRs, as well as other members of the hematopoietic receptor superfamily, is the absence of a consensus sequence indicative of catalytic function, such as protein kinase activity. It is now clear, however, that one of the signal transduction pathways activated by PRL, and a variety of polypeptide hormones and cytokines, involves members of a novel family of protein tyrosine kinases, the Janus kinases (JAK-1, JAK-2, JAK-3, and Tyk-2). In Nb2 rat lymphoma cell line, PRL induces the rapid tyrosine phosphorylation of several proteins, including JAK-2 [Rui et al., 1992; Dusanter-Fourt et al., 1994; Gilmour and Reich, 1994; David et al., 1994; Lebrun et al., 1994; Rui et al., 1994], and the subsequent activation and nuclear translocation of cytoplasmic signal transducers and activators of transcription (STATs). In the present study, we performed a step-by-step analysis of the effect of ethanol on PRL-induced activation of the JAK/STAT pathway in cultured rat astrocytes. We found a marked inhibitory effect of ethanol on the tyrosine phosphorylation of JAK2, STAT1α, STAT5a, and STAT5b in cultured rat astrocytes. This decrease JAK/STAT phosphorylation was associated with a decrease in IRF-1 gene expression, suggesting that ethanol may inhibit the neuroimmune response to infection or injury.

MATERIALS AND METHODS

Cell Culture

Astrocytes were prepared from cerebral hemispheres of 1-day-old rat pups as previously described [DeVito et al., 1992]. Cells were originally seeded at 2×10^5 cell/cm^2 in Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum and cultured for 7 days at 37°C, under an atmosphere of 5% CO_2, 95% air. Cells were subcultured every 7 days and were used between the second and fifth passage. Prior to use, cells were dispersed by trypsin, counted in a hemocytometer and cell viability determined by exclusion of trypan blue.

Immunoprecipitation and Immunobloting

Cultured astrocytes were solubilized in 0.5 ml of lysis buffer containing 10 mM Tris-HCl pH 7.6, including 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 ug/ml, and 5 ug/ml leupeptin on ice. Insoluble material was pelleted at 12,000*g* for 30 min at 4°C. Clarified lysates were incubated rotating end over end overnight with polyclonal antisera to individual JAK or STAT proteins as specified. Antibodies were capture by incubation for 60 min with protein-A sepharose beads and, washed, subjected to SDS PAGE, and transferred to nitrocellulose. Blots were blocked, probed with anti-phosphotyrosine antibody, and tyrosine phosphorylated proteins were detected by enhanced chemiluminescence as previously described [DeVito et al., 1995]. Identification of proteins were verified by reprobing the blot with polyclonal antisera to individual JAK or STAT proteins as specified. Blots were then extensively washed with PBS, developed using ECL-detection reagents from Amersham Life Sciences (Arlington Heights, IL) according to the manufactures' instructions, and exposed to Fugi Rx film.

Extraction of Soluble Nuclear Proteins

Soluble nuclear proteins were obtained as described by van Wijnen et al. [1992]. In brief, astrocytes were washed twice with ice-cold PBS, and harvested in 1 ml hypotonic buffer containing 10 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid; HEPES)-KOH, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.l mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 ug/ml aprotinin, 10

μg/ml leupeptin, and 1 mM sodium orthovanadate. After incubation on ice for 15 min, cells were vortexed for 10 sec in hypotonic buffer containing 0.1% NP-40, followed by incubation on ice for 10 min. Nuclei were pelleted by centrifugation, washed twice with hypotonic buffer, and then the nuclear pellets were incubated at 4°C for 30 min in hypertonic buffer containing 20 mM HEPES-KOH, pH 7.9, 400 mM KCI, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, and 20% glycerol. Supernatants were collected after centrifugation and stored at –80°C until use. Protein concentrations were measured by the Bradford method [Bradford, 1976].

Electrophoretic Mobility Shift Assay (EMSA)

The synthetic oligonucleotide probes used in EMSA were as follows; for the GAS element of the IRF-1 promoter 5′-GATCCATTTCCCCGAAATGA-3′ and for NFκb 5′-CAACGGCAGGGGAATCTCCCTCTCCTT-3′. The double-stranded probes were end-labeled using the Klenow-DNA polymerase and [^{32}P]α-dATP. Nuclear proteins (5 μg) were incubated in a final volume of 20 μl with 40 fmol [^{32}P]-labeled probe at 22°C for 30 min in buffer containing 10 mM HEPES-KOH, pH 7.9, 100 mM KCl, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol, 0.05% NP-40, and 2 μg poly (dIodC). The nucleoprotein complexes were resolved by nondenaturing electrophoresis in a 5% polyacrylamide gel for 3 h at 4°C in buffer containing 45 mM Tris-HCl, pH 8.0, 45 mM boric acid, and 1 mM EDTA. Gels were dried and exposed to Fuji RX film. For competition experiments, a 100-fold molar excess of the unlabeled oligonucleotides was added 15 min before incubation of nuclear extracts with radiolabeled probes.

Isolation of Total Cellular RNA and Northern Blot Analysis

Cells were washed twice with ice cold PBS and total cellular RNA was isolated by the acid guanidium-thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi [1987], using materials obtained from Cinna/Biotex Lab, Inc. (Houston, TX). Total cellular RNA concentration was quantified by UV absorption at 260 nm. For Northern blot analysis, equal amounts of RNA were fractionated by electrophoresis in 1.2% agarose gel containing 2.2 M formaldehyde and blotted on Duralon-UV filters (Stratagene, La Jolla, CA). Filters were cross-linked by UV and baked. After prehybridization for 6 h at 42°C in a solution containing 10% dextran sulfate, 50% deionized formamide, 1% SDS, 200 mg/ml denatured salmon sperm DNA, and 1 M NaCl, the filters were hybridized to a ^{32}P-labeled IRF-1 cDNA probe (Kindly provided by Dr. Yu-Lee, Baylor College of Medicine, Houston, TX) overnight at 42°C. Blots were washed in 2 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate), 0.l% SDS three times at room temperature for 15 min, and then in 0.5 × SSC, 0.1% SDS, for 30 min at 65°C. Filters were exposed to Fuji RX film (Fuji Photo Film Co., Tokyo, Japan) at −70°C using double fluorescent-intensifying screens. Quantification of the autoradiogram was performed by densitometry. The relative amounts of mRNA were normalized with GAPDH RNA content.

Prenatal Alcohol Exposure (PAE)

All animal studies were approved by the University of Massachusetts Animal and Care and Use Committee. Nulliparous females (150 to 210 g) were individually housed each evening with a male until a vaginal smear indicated day 1 of pregnancy. They were then housed individually under controlled lighting, lights on between 0600 and 1900 h. Prospective mothers were fed a chow diet (Purina 5008) until gestational (G) day 7. At this time the rats were weight-matched and separated into the three dietary groups: a chow (Ch) diet, a control (Ct) diet, or a ethanol diet (Et). The Ct and Et diets were high-protein, liquid diets (Lieber-DeCarli) which are nutritionally balanced to meet the specific needs of pregnant rats (Bioserve, Frenchtown, NJ). Both diets were fortified with Vitamin Diet Fortification Mixture and Salt Mixture. For the establishment of primary astrocyte cultures from ethanol treated rats the Et diet contained 2.2% (v/v) ethanol at day 7 and 4.5% v/v ethanol at day 14 ethanol so that 13 and 27%, respectively, of the total caloric value of the diet were ethanol derived. In the Ct diet, an isocaloric amount of dextrins and maltose was substituted for the ethanol. The Ch-diet consisted of standard rat chow and water ad libitum. Liquid diets were provided in 120 ml graduated feeding tubes. All Et-fed rats were paired by weight with a rat fed the Ct diet, and the two were pair fed. That is, each Ct-fed rat received the same volume of liquid diet as

that consumed the previous day by the weight-matched Et-fed rat. Thus, during their pregnancies both the Ct- and Et-fed rats consumed diets of identical caloric content. The amount of liquid ingested by the Ct- and Et-fed females was determined at 9:00 AM each morning and at 17:00 PM each evening. If a Et-fed rat required more than the estimated 100 ml of diet, additional diet was added at 17:00 to insure that each Et-fed rat receives an adequate supply of diet to meet an individual animals needs. After recording the past day's food consumption, the Et-fed and Ct-fed rats received their daily food allotments. Throughout the gestation period, Ch-fed rats were fed chow and water ad libitum. All pregnant rats were weighed on alternative days, and only those weight-matched Ct-fed and Et-fed rats that maintained a weight within 10 percent of the Ch-fed rats were kept in the study. At birth the pups were counted and weighed. Maternal blood samples were collected by orbital venipuncture under general anesthesia (pentobarbital 50 mg/kg, IP) on the morning of birth from Ct- and Et-fed rats. Pooled blood samples was collected from the pups of each litter during the preparation of the primary cultures. The concentration of ethanol in the blood samples was measured using a Sigma diagnostic kit (#332 UV).

RESULTS

Effect of Ethanol on Prolactin-Induced Tyrosine Phosphorylation of JAK Kinases

In Nb2 cells, prolactin stimulates the tyrosine phosphorylation of JAK2. We therefore tested the effect of prolactin on Jak kinases in cultured rat astrocytes. Quiescent astrocytes were treated with prolactin for 5, 15, and 30 min. JAK2 was immunoprecipitated from cell lysates, separated by SDS-PAGE, and immunoblot blot analysis was performed using a anti-phosphotyrosine antibody (anti-pY). Immunoprecipitation with anti-JAK2 revealed a marked tyrosine phosphorylation response to prolactin of a protein with a molecular weight of 120 kDa (Fig. 1). Reblotting of the samples with anti-JAK2 confirmed the identity of the protein as JAK2. Kinetic analysis showed prolactin induced a rapid tyrosine phosphorylation of JAK2 to levels that were easily detectable after 5 min of stimulation and remained detectable for at least 30 min. Pre-incubation of astrocytes with ethanol for 18 h did not affect JAK2 content immunoprecipitated form cell lysates (Fig. 1, lower row). In contrast, in cells pre-exposed to ethanol for 18 h, prolactin-induced tyrosine phosphorylation of JAK2 was modest and only detectable after 15 min of incubation (Fig. 1,

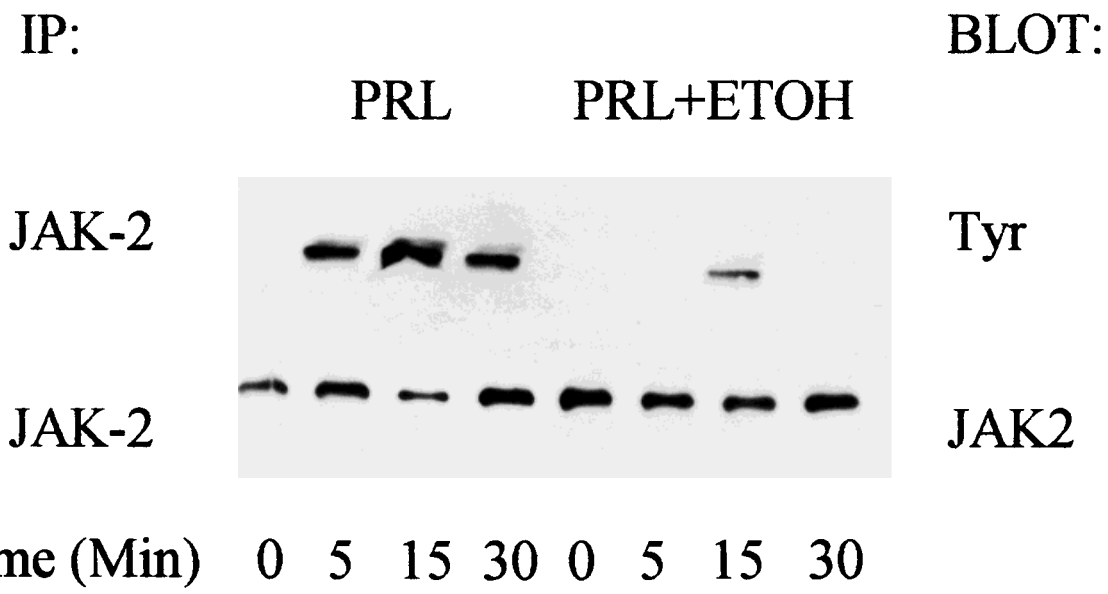

Fig. 1. Immunoblot analysis of the effect of ethanol on prolactin-induced tyrosine phosphorylation of JAK2 in cultured astrocytes. Astrocytes were prepared from cerebral hemispheres of 1-day-old rat pups and seeded at 2×10^5 cell/cm^2 in Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum and cultured for 7 days at 37°C, under an atmosphere of 5% CO_2, 95% air. Cells were subcultured every 7 days were used between the second and sixth passage. Quiescent cells were preincubated which DMEM or DMEM+50 mM ethanol for 18 h prior to stimulation with PRL (10 nM).

upper row). Immunoprecipitation of with JAK1 or JAK3 antisera revealed the presence of these kinases in the astrocyte cell lysates, however, immunoblot analyses with anti-pY revealed no tyrosine activation of JAK1 or JAK3 (Data not presented). Tyk2 was not detected in the cell lysates.

To determine if lower ethanol concentrations inhibited prolactin-induced tyrosine phosphorylation of JAK2, cells were pre-incubated with ethanol ranging from 0.5 to 50 mM for 18 h prior to treatment with prolactin for 15 min. Immunoblot analysis revealed that exposure of cells to ethanol for 18 h resulted in a dose dependent decrease in JAK2 tyrosine phosphorylation (Fig. 2). Densitometric analysis revealed that ethanol concentrations of 10 mM and 25 mM resulted in 54% and 83% decreases in the relative abundance (Anti-Py/JAK) of tyrosine phosphorylation of JAK2 when compared to prolactin stimulated cell in the absence of ethanol. To determine the duration of ethanol preexposure required to inhibit prolactin-induced phosphorylation, cells were stimulated with prolactin in the absence of ethanol or following pre-incubation with ethanol for 1 to 24 h (Fig. 3). Immunoblot blot analysis revealed that pre-exposure of astrocytes for 8 h was required to inhibit prolactin-induced tyrosine phosphorylation of JAK2.

To determine if ethanol resulted in a general decrease in tyrosine phosphorylation, we examined the effect of ethanol on the phosphorylation of extracellular signal-related protein kinase-1 (ERK-1). In quiescent astrocytes, in the presence, and absence, of ethanol low levels of ERK-1 were detected (Fig. 4). Incubation of cells with nerve growth factor (50 ng/ml) for 5 or 20 min resulted in a marked increase in ERK-1 tyrosine phosphorylation of ERK-1. Ethanol had no inhibitory effect on the relative increase in tyrosine phosphorylation (Tyr/ERK-1). Rather there was a small increase in the relative amount of tyrosine phosphorylation in the ethanol treated cells.

Effect of Ethanol on Prolactin-Induced Tyrosine Phosphorylation of STAT Proteins

We next examined the effect of ethanol on prolactin induced tyrosine phosphorylation of STAT transcription factors, the substrates for JAK tyrosine kinases. Immunoblot analysis of cell lysates revealed that stimulation of astrocytes with prolactin for 30 min resulted in a marked increase in STAT 1 tyrosine phosphorylation (Fig. 5). Densitometric analysis of the immunoblott revealed that pre-incubation of cells with ethanol for 18 h, resulted in a 89 ± 2.6% decrease in JAK2 tyrosine phosphorylation. Furthermore, kinetic analysis revealed that treatment of quiescent rat astrocytes with prolactin induced rapid and robust tyrosine phosphorylation of STAT1, STAT5a, and STAT5b (Fig. 6; first, third and fifth panels).

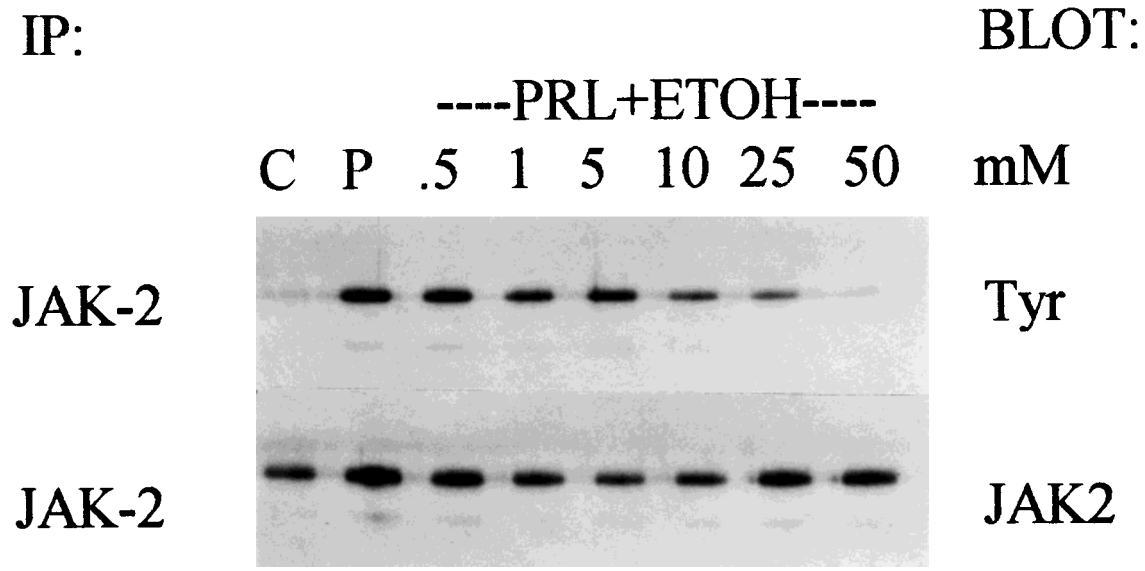

Fig. 2. Immunoblot analysis of the effect of ethanol concentration on prolactin-induced tyrosine phosphorylation of JAK2 in cultured astrocytes. Cells were cultured as described in Figure 1. Immunoblot analysis of the same blot with anti-JAK2 confirmed the identity of the protein as JAK2.

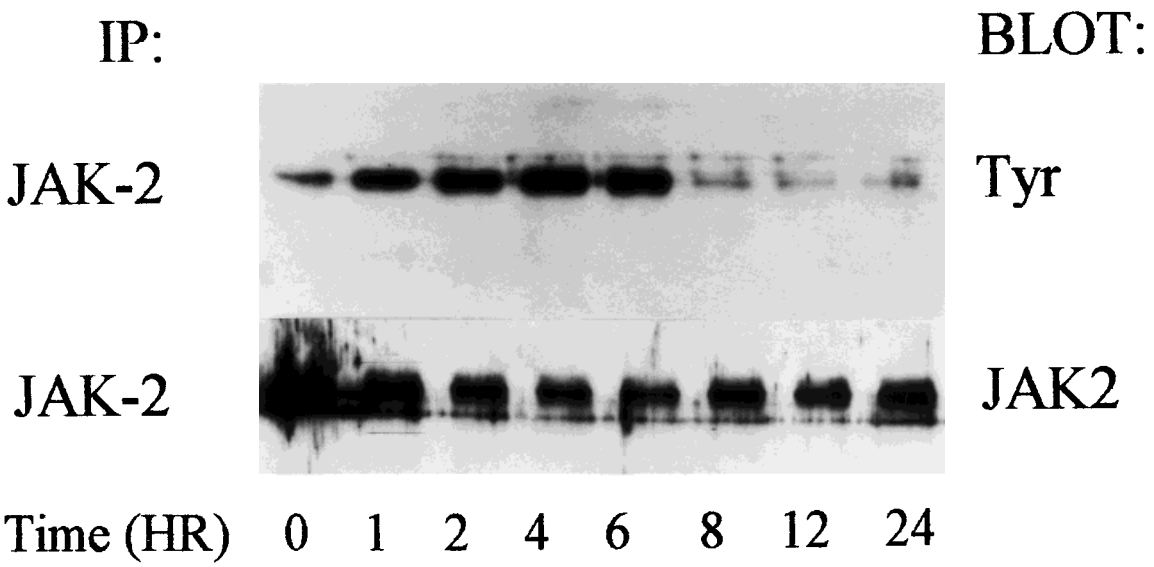

Fig. 3. Effect of the duration of ethanol exposure on PRL-induced activation of JAK2. Cells were cultured as described in Figure 1. Quiescent cells were preincubated in the presence, or absence, of ethanol (50 mM) for the times indicated and then stimulated with PRL (10 nM) for 15 min.

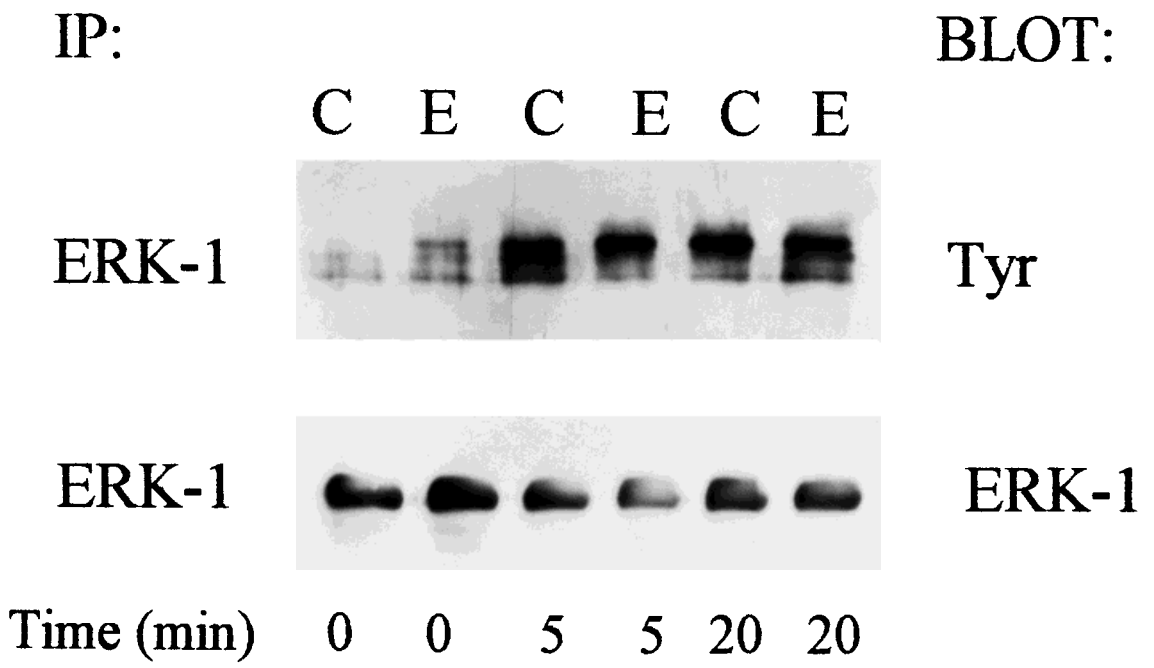

Fig. 4. Immunoblot analysis of the effect of ethanol on tyrosine phosphorylation of ERK1-kinase in cultured astrocytes. Cells were cultured as described in Figure 1, and incubated with ethanol for the times indicated. Immunoblot blot analysis revealed that ethanol increased tyrosine phosphorylation of ERK1-kinase. Immunoblot analysis of the same blot with anti-ERK-1 confirmed the identity of the protein as ERK-1.

Reblotting of the samples with corresponding STAT antibodies confirmed the identities of the proteins as STAT1, STAT5a, and STAT5b (Fig. 6; second, fourth and sixth panels). In rat astrocytes pretreated with ethanol for 18 h, however, tyrosine phosphorylation of STAT1 and STAT5b were markedly delayed and decreased. Further, tyrosine phosphorylation of STAT5a was not detected in astrocytes pretreated with ethanol.

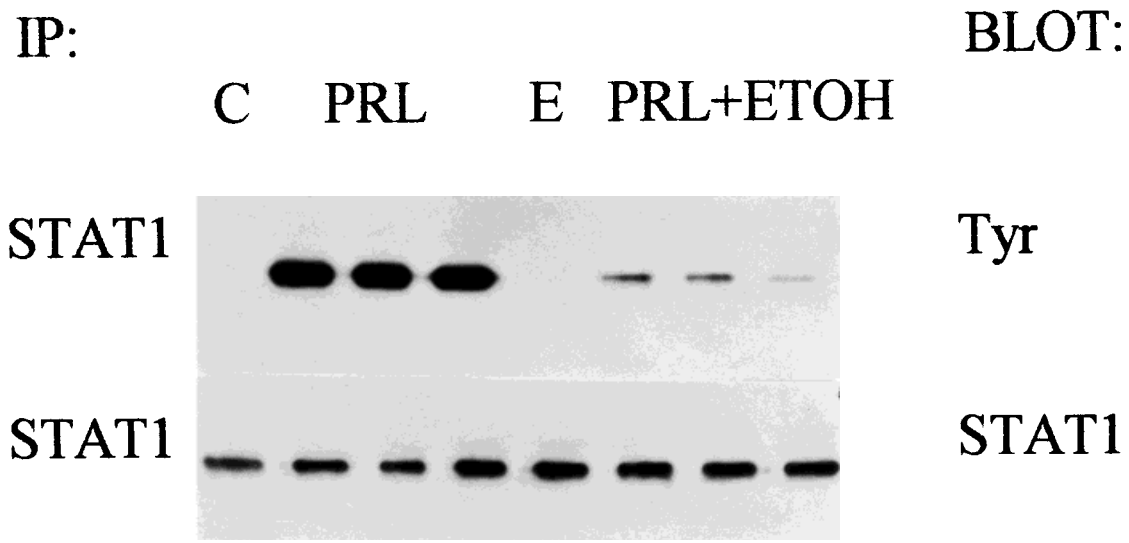

Fig. 5. Immunoblot analysis of the effect of ethanol on prolactin-induced tyrosine phosphorylation of STAT1 in cultured astrocytes. Cells were cultured as described in Figure 1. Quiescent cells were preincubated in the presence, or absence, of ethanol (50 mM) for 18 h and then stimulated with PRL (10 nM) for 15 min. Immunoblot analysis of the same blot with anti-STAT1 confirmed the identity of the protein as STAT1.

Effect of Ethanol on Prolactin-Induced STAT Binding to DNA

To establish whether ethanol inhibits STAT binding to DNA we examined the effect of ethanol on the ability of STAT proteins to interact with the GAS element in IRF-1 promotor. Nuclear proteins from quiescent astrocytes, or astrocytes stimulated with prolactin in the presence, or absence, of ethanol were analyzed for binding to a ^{32}P-labeled gamma-activated site probe. Electrophoretic mobility shift assay of nuclear proteins from astrocytes stimulated in the absence of ethanol revealed that prolactin induced the formation of a single complex (Fig. 7). In contrast, in the presence of ethanol prolactin failed to induce the formation of the protein-DNA complex. Addition of a 100-fold excess of unlabeled oligonucleotides to the binding reaction completely decreased prolactin-induced formation of the complex. To determine if prolactin induced the formation of STAT1 and STAT5 DNA complexes supershift analyses were performed and revealed that the complex was completely supershifted by antiserum to either STAT1 of STAT5 (Fig. 8). Antiserum to STAT5b also blocked protein/DNA binding (data not presented). To determine if the decreased nuclear binding of STAT proteins was associated with a decrease in prolactin responsive genes we examined the effect of ethanol on prolactin-induced increase transcription factor, IRF-1 mRNA. In the absence of ethanol, prolactin induced the rapid induction of IRF-1 mRNA levels in cultured rat astrocytes (Fig. 9). In contrast, in the presence of ethanol, prolactin-induced increase in IRF-1 mRNA levels were markedly decreased.

Effect of PAE on Prolactin-Induced Tyrosine Phosphorylation of JAK2

To determine if the inhibitory effect of in vitro ethanol exposure on JAK2 activation in cultured astrocytes reflects the effect of in vivo exposure we examined the effect of prenatal alcohol exposure on PRL-induced JAK activation. Figure 10 presents the relative intensity of JAK2 tyrosine phosphorylation in primary astrocyte cultures prepared from pair fed controls and PAE treated rats. PRL resulted in a marked increase in the relative amount of tyrosine phosphorylation in cultures prepared from pair fed controls. In the presence of ethanol (25 mM) there was a decrease in the relative amount of tyrosine phosphorylation when compared to controls. In primary cultures prepared from PAE rats, and cultured in the absence of ethanol there was a decrease in the relative amount of tyrosine phosphorylation of JAK2 when compared to pair fed controls. In primary astrocyte cultures prepared from PAE treated rats which were pre-incubated with ethanol (25 mM) for 18 h prior to PRL stimulation, there

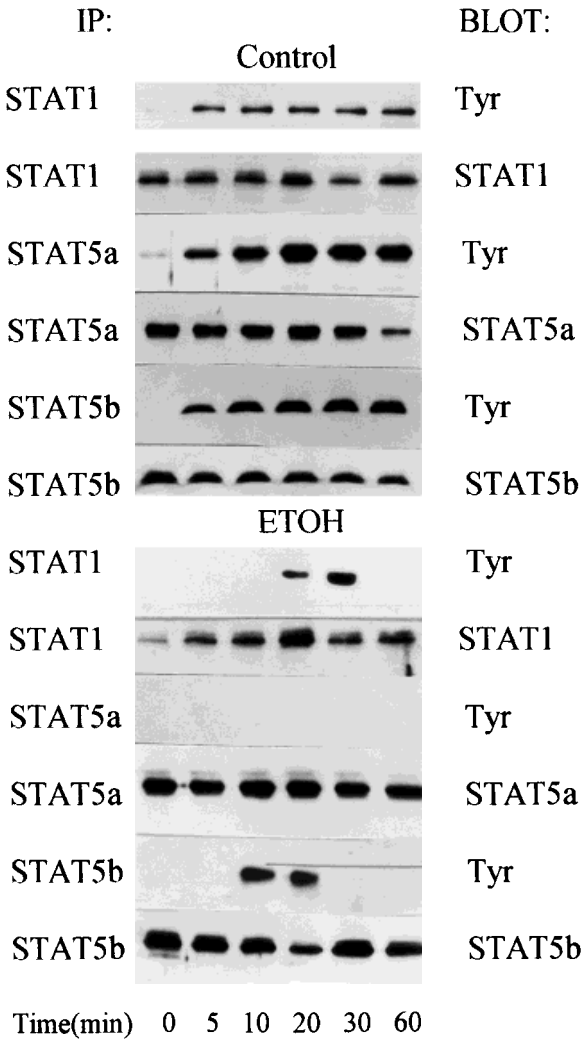

Fig. 6. Immunoblot analysis of the effect of ethanol on prolactin-induced tyrosine phosphorylation of STAT proteins in cultured astrocytes. Cells were cultured as described in Figure 1. Quiescent cells were preincubated in the presence or absence of ethanol (50 mM) for 18 h and then stimulated with PRL (10 nM) for the times indicated. Samples were immunoprecipitated and blotted with antiserum to Tyr and then with anntiserum to STATs 1α, 5a, 5b, or Tyr as indicated.

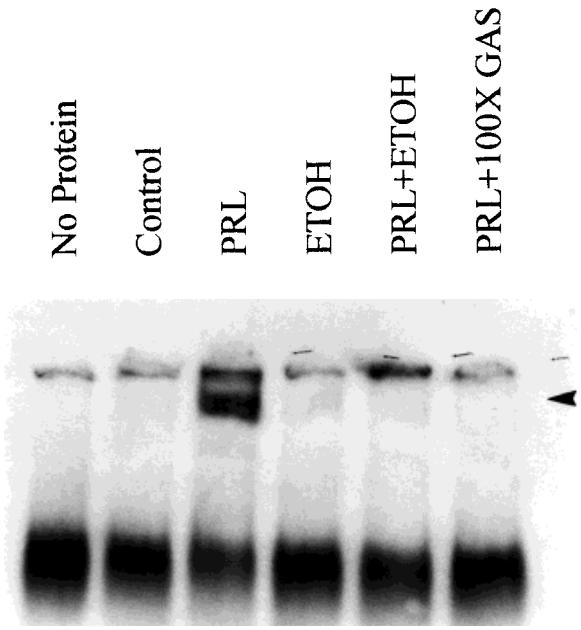

Fig. 7. The effect of ethanol on DNA binding activity of STAT factors was determined by gel-mobility shift assays using an oligonucleotide probe containing a high affinity target sequence for STAT factors. Astrocytes were grown in the presence and absence of ethanol, and nuclear protein extracts were prepared from control (0) and PRL-stimulated (30 min) astrocytes. Nuclear proteins were incubated with ^{32}P-labeled double stranded oligonucleotide sequences (5'-CATTTCCCGTAAATC-3).

was a further decrease in JAK2 tyrosine phosphorylation.

DISCUSSION

The major finding of this paper is that exposure of cultured rat astrocytes to low concentrations of ethanol results in a marked inhibition of the JAK/STAT pathway. These studies provide a mechanism for how ethanol may inhibit astrogliosis, and provide the first evidence of an effect of ethanol on a key set of transcription factors involved in the regulation of cellular immunity, cell growth, transformation, and cell death.

In the rat, three different forms of the PRLR have been cloned which differ in the length of the cytoplasmic domain [Kelly et al., 1991]. A notable characteristic of PRLRs, as well as other members of the hematopoietic receptor superfamily, is the absence of a consensus sequence indicative of catalytic function, such as protein kinase activity. It is now clear, however, that the regulation of gene expression occurs through intercellular signals resulting from prolactin-induced aggregation of the PRLR resulting in the activation of the receptor associated JAK2 tyrosine kinase [Rui et al., 1992, 1994; Dusanter-Fourt et al., 1994; Gilmour and Reich, 1994; David et al., 1994; Lebrun et al., 1994]. Downstream events following JAK2 activation involve the tyrosine phosphorylation of STAT transcription factors forming homo- or heterocomplexes which translocate into the nucleus, bind to cognate DNA elements, and activate gene expression.

The effect of PRL on the activation of the JAK/STAT pathway in astrocytes has not been examined. We therefore tested the effect of

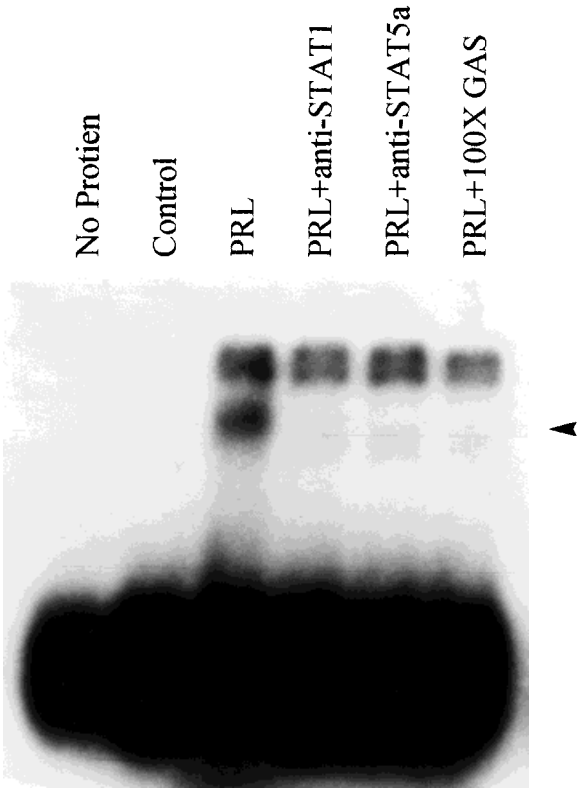

Fig. 8. Antibody interference of DNA binding activity by gel-mobility shift assays (EMSA) of PRL-stimulated GAS binding activities. Cells were treated as described in Figure 5 and EMSAs were performed after the addition of STAT antiserum as indicated.

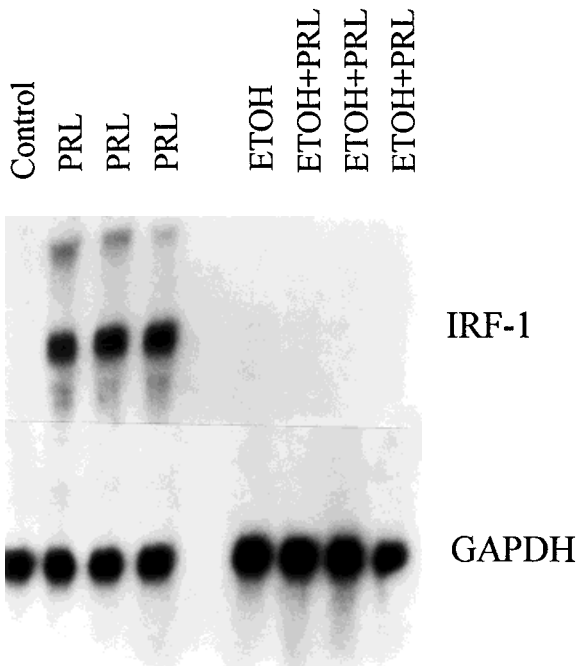

Fig. 9. Effect of ethanol on IRF-1 mRNA levels in cultured rat astrocytes. Cells were cultured as described in Figure 1, and then stimulated with PRL (10 mM) for 120 min. Blots were hybridized with ^{32}P-labeled IRF-1 probe, then stripped and rehybridized with ^{32}P-labeled GAPDH probe. The results are expressed as mean ± SEM, n = 3.

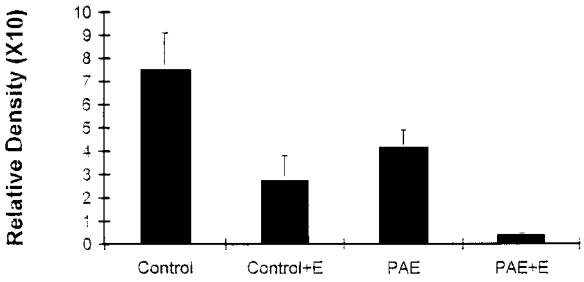

Fig. 10. Effect of prenatal ethanol exposure on PRL-induced increase JAK2 tyrosine phosphorylation in primary astrocyte cultures. Primary astrocyte cultures were obtained from control rats and PAE exposed during the last 10 days of gestation. All cultures were grown in the absence of ethanol for 7 days and then cultured in the presence or absence of ethanol (50 mM) for 18 h. Cell were stimulated with vehicle or PRL (1 nM) for 15 min and harvested. The results are expressed as the mean ± SEM, n = 3.

PRL on the activation of JAK kinases in cultured rat astrocytes. Individual JAK kinases were immunoprecipitated from cell extracts, separated by SDS PAGE, and immunoblotted with anti-phosphothyrosine antibodies. We found that JAK2, but not JAK1, JAK3, or TYK2 showed a rapid and robust tyrosine phosphorylation response to PRL. In the presence of ethanol, however, PRL-induced activation of JAK-2 was markedly inhibited. Further, immunoblot analyses with anti-JAK2 revealed that the inhibitory effect of ethanol on tyrosine phosphorylation was not the result of an ethanol-induced decrease in the cytosolic concentration of JAK2. Having established that ethanol inhibits the activation of JAK2 in cultured astrocytes, we determined if the inhibition of JAK2 activation by ethanol was sufficient to inhibit tyrosine phosphorylation, dimerization, and nuclear translocation of STAT proteins. Here we show that in cultured rat astrocytes, PRL-induced a marked and sustained increase in STAT-1, STAT-5a, and STAT-5b phosphorylation. In the presence of ethanol, however, the degree and duration of PRL-induced STAT phosphorylation was markedly decreased. Consistent with the inhibitory effect of ethanol on JAK2 activation, the ethanol-induced inhibition of STAT activation could not be explained by an ethanol-induced decrease in cytosolic STAT concentrations. Further, the degree and duration of the

inhibitory effect of ethanol was different for the individual STATs. For STAT1 and STAT5b, ethanol delayed the onset and the duration of tyrosine phosphorylation. In contrast, ethanol decreased tyrosine phosphorylation of STAT5a to undetectable levels. To determine if the decrease in STAT activation resulted in decreased STAT binding to cognate DNA elements gel-mobility shift assays were performed. We found that PRL induced the binding of activated STAT factors to the IRF GAS target sequence. In the presence of ethanol, however, binding to IRF GAS target sequences was inhibited. This ethanol-induced decrease in SIE binding activities in nuclei obtained from PRL-stimulated astrocytes provides evidence supporting the hypothesis that ethanol inhibits the PRL-induced dimerization and nuclear translocation of STAT proteins. To identify which STAT proteins are involved, suppershift experiments were performed which show that both STAT1 and STAT5 transcription factors are translocated to the nuclei in PRL stimulated astrocytes. Together, our data clearly indicate that ethanol markedly inhibits PRL-induced activation of the JAK/STAT pathway in cultured rat astrocytes. Thus, our data suggest that ethanol should result in the inhibition of PRL inducible genes which are regulated by STAT/DNA binding. To test this hypothesis, we examined the effect of ethanol on PRL-induced regulation of IRF-1, a transcriptional activator originally shown to regulate IFN inducible genes. We show here that ethanol markedly inhibited PRL-induced IRF-1 gene expression. Together, our studies demonstrate for the first time that ethanol can inhibit the activation of JAK2 and that such inhibition results in a decrease in STAT activation, dimerization, and nuclear translocation, resulting in the inhibition of the transcription factor IRF-1. Thus, one mechanism of ethanol-induced inhibition of astrogliosis may involve uncoupling of cytokine-induced signal transduction pathways utilizing the JAK pathway. Our results are also consistent with previous reports that ethanol inhibits insulin-induced tyrosine phosphorylation in several tissues, including the CNS [Xu et al., 1995; Resnicoff et al., 1993, 1994; Sasaki and Wands, 1994]. For example, ethanol inhibits growth of NIH 3T3 fibroblasts and C6 glioblastoma cells through a decrease in autophosphorylation of the IGF receptor [Resnicoff et al., 1993, 1994]. Further, in the CNS, ethanol inhibits insulin-induced neuronal thread protein gene expression which is associated with a decrease in tyrosine phosphorylation of the insulin receptor β-subunit and insulin receptor substrate-1 [Xu et al., 1995]. In the liver, ethanol inhibits insulin receptor substrate-1 tyrosine phosphorylation resulting in a decrease in phospatidylinositol-3 kinase, a Src homology 2 domain containing signal transduction molecule [Sasaki and Wands, 1994].

Our results clearly indicate that ethanol inhibits the activation of the JAK/STAT pathway in cultured rat astrocytes. The mechanism(s) involved in the ethanol-induced inhibition of JAK2 activation, however, are unclear. Several possible mechanisms can be suggested: 1) Ethanol could result in a general decrease in tyrosine phosphorylation. To test this hypothesis we examined the effect of ethanol on NGF-induced ERK-1 tyrosine phosphorylation. We found that ethanol not only failed to inhibit NGF-induced tyrosine phosphorylation of ERK-1, but resulted in a slight enhancement of ERK-1 phosphorylation, which is consistent with the upregulation of ethanol on NGF- and bFGF-induced ERK-1 phosphorylation in PC12 cells [Roivainen et al., 1995]. In addition, in A431 cells, a human epidermal carcinoma, ethanol has a biphasic effect of tyrosine kinase activity with low concentrations of increasing kinase activity and high ethanol concentrations inhibiting kinase activity [Thurston and Shukla, 1992]; 2) The downregulation of the erythropoietin-induced JAK/STAT pathway occurs through the dephosphorylation of JAK2 kinase by the Sar homology 2 (SH2) domain-containing protein tyrosine phosphates (PTPase), SHP-1 [Marrero et al., 1998]. Accordingly, ethanol could increase SHP-1 activity in cultured astrocytes resulting in the downregulation of JAK1. To date, however, we are unaware of any studies examining the effect of ethanol on phosphatase activity; 3) PRL binding to the PRL-receptor results in receptor dimerization, or oligomerization, which activates PRL-receptor associated JAK2 activation and autophosphorylation of the PRL-receptor. We have shown that ethanol does not interfere with the binding of PRL to its receptor in intact growing cells [DeVito et al., 1997]. Further, we show here that an ethanol pretreatment of 8 h is required for ethanol-induced inhibition of JAK2 activation. Thus, it could be hypothesized that the active confirmation of the PRL-receptor is not maintained in the presence of ethanol, irrespective of ligand

binding, by disruption of protein hydrophobic interactions, or possibly through the induction of protein synthesis. It remains to be determined, if ethanol inhibits PRL-receptor dimerization, JAK2 association or PRL-receptor autophosphorylation.

IRF-1 was originally cloned as a transcription factor involved in the regulation of IFN gene expression [Miyamoto et al., 1988]. The IRF-1 gene is an immediate early response gene that is transcriptionally regulated by PRL [Yu-Lee et al., 1990], and other cytokines such as gamma-IFN and TNFα [Taniguchi et al., 1995]. IRF-1 and IRF-2 were originally identified as a transcriptional activator, and repressor, respectively, of IFN inducible genes. Several lines of evidence, however, suggest that IRF-1 regulates immune responses and apoptosis, and acts as an anti-oncogenic factor [Taniguchi et al., 1997]. Activation of IRF-1 results in enhanced expression of inducible nitric oxide synthase (iNOS), class II transactivator (CIITA), and MHC class I and II antigens [Flodstrom and Eizirik, 1997; Gobin et al., 1997; Hobart et al., 1997]. IRF-proteins are involved in the regulation of various immune processes, as demonstrated by several studies showing that mice deficient in IRF-1 have reduced levels of CD_4^- $CD8^+$ T cells, while IRF-2 deficient mice suffer from bone marrow suppression of hematopoiesis and B-lymphopoiesis and die following virus infection [Taniguchi, 1997]. Further, targeted disruption of the IRF-1 gene in mice results in reduced incidence and severity of antigen-induced autoimmune diseases [Tada et al., 1997] and a dominant negative mutant of IRF-1 prevents the expression of gamma-IFN inducible genes [Thornton et al., 1996].

It is now clear that in addition to modulating the cellular responses to cytokines and viral infection, IRFs are involved in the regulation of cell growth, susceptibility to transformation by oncogenes, induction of apoptosis, resistance to bacterial infection, and the development of T and B cell repertoires. Studies have established a role of IRF-1 as a tumor suppressor. That is, the IRF-1 gene is localized on chromosome 5q31.1, a region which is deleted in patients with leukemia and preleukemic myelodysplasia [Willman et al., 1993]. Compelling evidence that IRF-1 functions as a tumor suppressor has been provided by Tanaka el al. who demonstrated that embryonic fibroblasts (EFs) from IRF-1 nude mice undergo cell transformation by expressing a single oncogene, *c*H-*ras* [Tanaka et al., 1996]. This phenotype is also found in mice lacking the tumor suppressor p53. In addition, both IRF-1 and p53 are essential for DNA damage induced apoptosis of EFs expressing *c*H-*ras* [Tanaka et al., 1996]. Analysis of bone marrow and peripheral mononuclear cells from patients with leukemia and preleukemic myelodysplastic syndrome (MDS) or leukemia secondary to MDS have revealed preferential expression of a skipped IRF-1 mRNA which lacks exons 2 and 5 resulting in no tumor suppressor activity [Harada et al., 1994]. Thus, current studies indicate that IRFs provide a molecular link between cytokines and the regulation of cellular immunity, cell growth, transformation, and cell death. Our observation, that low concentrations of ethanol inhibit PRL-induced IRF-1 mRNA levels suggest that an ethanol-induced decrease in IRF-1 gene expression may be on mechanism through which ethanol exerts its immunosuppressive effects.

Animal models clearly show debilitating effects of PAE on CNS development, including gross malformation, microencephaly, heterotropias, errors of migration, neuronal depletion, abnormal cell death, and cerebrovascular damage [West et al., 1994]. Primary astrocyte cultures obtained from animals prenatally exposed to ethanol, and cultured in the absence of ethanol, show morphological, and biochemical changes similar to those of astrocyte cultures prepared from nonethanol treated controls, but grown in the presence of ethanol [Renau-piqueras et al., 1988, 1989; Davies and Vernadakis, 1984; Guerri et al., 1990; Saez et al., 1991]. We have recently shown that astrocytes from PAE rats, cultured in the absence of ethanol, exhibit marked inhibition of PRL-induced mitogenesis, and TNF-α expression [DeVito et al., 1997]. Further, when astrocytes obtained from PAE rats were switched from ethanol free media, to media containing low concentrations of ethanol PRL-induced mitogenesis and TNF-α expression were completely blocked. Consistent with these effects we now show that PRE decreases JAK2 activation in primary astrocyte cultures, and sensitizes astrocytes to the inhibitory effect of ethanol on JAK2 activation. Thus, our results suggest that ethanol-induced inhibition of the JAK/STAT pathway is one mechanism through which ethanol can inhibit astrogliosis.

Most of the studies described in this report used an ethanol concentration of 50 mM. This concentration was chosen because it results in maximal inhibition of PRL-induced cell growth, without decreasing cell viability [DeVito et al., 1997]. This concentration, while slightly greater then those associated with impaired mental capabilities and coordination (10 mM) or ataxia (20 mM) in humans is within the physiological relevant range. That is, this ethanol concentration, is similar to, or less, then the blood alcohol levels achieved in animal models of binge-like alcohol exposure [Thomas et al., 1996; Maier et al., 1997; Goodlett et al., 1997]. Further, studies in drinking women have reported blood alcohol concentrations above 50 mM [Urso et al., 1981; Church and Gerkin, 1988; Hammond et al., 1973; Wells and Barhill, 1996], including a report of one women with a blood alcohol level of approximately 330 mM (1.5%) [Johnson et al., 1982]. It is important to note, however, that in this study we observed inhibitory effects of ethanol on JAK2 activation with ethanol concentrations within the physiological relevant range. Specifically, we observed inhibition of PRL-induced tyrosine phosphorylation of JAK2 in cells exposed to 10 mM ethanol, which is less the legal threshold of blood alcohol concentration of 0.1% (17 mM) in most states. Thus, suggesting the possibility that moderate alcohol exposure may have a negative impact on the neuroimmune response.

In summary, our observation that ethanol disrupts the activation of one of the major pathways involved in the regulation of a number immune responses in cultured astrocytes strongly suggests that alcohol ingestion could result in an impaired immune response. More importantly, our observation that primary astrocytes prepared from rats exposed to ethanol before birth have decreased JAK2 activation, and are more sensitive to the inhibitory effects of ethanol suggests that PRE may have a long term effects on the CNS response to infection.

ACKNOWLEDGMENTS

This work was supported in part by funds from the Diabetes Endocrinology Research Center, University of Massachusetts Medical Center, Worcester, MA, and by the National Institute on Alcohol Abuse and Alcoholism.

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