Platelet-Derived Growth Factor-Mediated Signal Transduction Underlying Astrocyte Proliferation: Site of Ethanol Action

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Platelet-derived growth factor (PDGF) is a critical regulator of cell proliferation. Because ethanol inhibits cell proliferation *in vivo* and *in vitro*, we hypothesize that ethanol-induced inhibition results from differential interference with signal transduction pathways activated by PDGF. Cultured cortical astrocytes were used to examine the effects of ethanol on PDGF-mediated signal transduction, on the expression of two PDGF monomers (A- and B-chains), and on the expression of two PDGF receptor subunits (PDGF α and PDGF β). PDGF-B chain homodimer (PDGF-BB), and to a lesser extent PDGF-A chain homodimer (PDGF-AA), stimulated the proliferation of astrocytes raised in a serum-free medium. Ethanol attenuated these actions in a concentration-dependent manner. Ethanol inhibited both PDGF-AA- and PDGF-BB-mediated phosphorylation of PDGF α , but it had little effect on PDGF β autophosphorylation. Likewise, ethanol abolished the association of PDGF α to Ras GTPase-activating protein (Ras-GAP), but it did not affect the binding of Ras-GAP to PDGF β . PDGF stimulated the activities

of mitogen-activated protein kinase (MAPK) in protein kinase C (PKC) independent and dependent manners. Ethanol inhibited the PKC-independent, acute activation of MAPK; however, it stimulated the PKC-dependent, sustained activation of MAPK. The expression of neither ligand was altered by exposure to ethanol for 3 d. Moreover, such treatment specifically upregulated PDGF α expression in a concentration-dependent manner. It did not, however, affect the binding affinity of either receptor. Thus, the signal transduction pathways initiated by PDGF-AA and PDGF-BB were differentially affected by ethanol. This differential vulnerability resulted from the preferential effects of ethanol on PDGF α autophosphorylation. Hence, ethanol-induced alterations are transduced through specific receptors of mitogenic growth factors.

Key words: alcohol; cell proliferation; cerebral cortex; fetal alcohol syndrome; glia; MAP kinase; phosphorylation; protein kinase C; Scatchard analysis

Platelet-derived growth factor (PDGF) is mitogenic for many cells (Kohler and Lipton, 1974; Ross et al., 1974; Westermarck and Wasteson, 1976; Uren et al., 1994). High amounts of PDGF and their receptors are expressed in the immature brain (Westermarck and Wasteson, 1976; Reddy and Pleasure, 1992; Valenzuela et al., 1997). PDGF consists of two monomers: an A-chain (PDGF-AA) and a B-chain (PDGF-BB). In its active form, PDGF is a disulfide-bound dimer of two monomers, and all dimeric combinations (i.e., PDGF-AA, PDGF-AB, and PDGF-BB) exist naturally (Bowen-Pope et al., 1989; Hart et al., 1990; Claesson-Welsh, 1994).

PDGF elicits its biological activity through interactions with transmembrane high-affinity receptors (Claesson-Welsh, 1994). There are two receptor subunits for PDGF: the PDGF α receptor (PDGF α) and the PDGF β receptor (PDGF β). Each subunit has a molecular weight of between 170 and 190 kDa. The binding of PDGF ligands with their receptors induces the dimerization of the subunits (Ullrich and Schlessinger, 1990). The PDGF α binds to either the A- or B-chain, whereas the PDGF β only binds to the B-chain (Inui et al., 1994). Thus, PDGF-AA induces the

formation of PDGF α –PDGF α homodimers, PDGF-AB can induce PDGF α –PDGF α homodimers and PDGF α –PDGF β heterodimers to form, and PDGF-BB induces the formation of any of the three possible dimers (PDGF α –PDGF α , PDGF α –PDGF β , and PDGF β –PDGF β) (Claesson-Welsh, 1994).

PDGF initiates a defined signal transduction pathway. Both PDGF α and PDGF β are tyrosine kinases. Binding of a PDGF ligand to either PDGF α or PDGF β induces receptor autophosphorylation (Ek and Heldin, 1982). In turn, stimulation of the PDGF receptor activates an enzyme cascade that includes various phosphorylating enzymes, i.e., protein kinase C (PKC), Ras, Raf, and mitogen-activated protein kinase (MAPK), and ultimately triggers cell division (Bornfeldt et al., 1995; Hart et al., 1995).

Cell proliferation is profoundly depressed by ethanol. In fact, ethanol-induced reductions in cell proliferation are a major cause of the microcephaly characterizing fetal alcohol syndrome (Miller, 1992; Luo and Miller, 1998; Mooney and Miller, 1999). Such inhibitory effects of ethanol may result from interference with mitogenic growth factors, specifically with the PDGF. *In vitro* studies of various neuroblastoma cells show that ethanol can block the PDGF-mediated proliferation (Luo and Miller, 1997a,b).

Three hypotheses were tested in the present study. (1) Ethanol does not universally affect all PDGF-mediated activities. That is, one PDGF receptor subunit is more susceptible to ethanol toxicity than the other. (2) Ethanol inhibits PDGF-regulated cell proliferation by promoting a state of chronic activation of

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MAPK. (3) Ethanol-induced inhibition of PDGF signaling results in a compensatory alteration of receptor expression.

The present studies focused on immature astrocytes for three reasons. (1) Their proliferation is adversely affected by ethanol (Davies and Vernadakis, 1984; Kennedy and Mukerji, 1986; Guerri et al., 1990; Snyder et al., 1992). (2) Astrocytes respond to PDGF and express PDGF receptors (Nagano et al., 1993; Prins et al., 1996; Valenzuela et al., 1997). (3) Their numbers are significantly reduced *in vivo* after prenatal exposure to ethanol (Miller and Potempa, 1990). Thus, we used primary cultures of cortical astrocytes to determine the effect of ethanol on the PDGF system.

MATERIALS AND METHODS

Astrocyte cultures

Astrocytes were obtained from the neocortices of 4- or 5-d-old rats. Pups were decapitated, and the dorsolateral cortices were carefully removed. Each cortex was immersed in H-EBSS solution (13.8 mM NaCl, 5.0 mM KCl, 25.0 mM HEPES, 4.2 mM NaHCO₃, 1.0 mM NaH₂PO₄, and 0.010% phenol red). The meninges were removed by rotating tissues on frosted glass, and the tissue was minced with a sterile razor blade. The mash was suspended in 10 ml of a trypsin solution (0.25 mg/ml trypsin in H-EBSS) for 15 min at 37°C. An equal volume of a solution of DNase (130 Kunitz U/ml; Sigma, St. Louis, MO) and a trypsin inhibitor (0.75 mg/ml; Sigma) was added to halt trypsin activity. A brief (5 sec) centrifugation sedimented the blocks. The tissue was triturated through a Pasteur pipette to produce a cell suspension, and the suspension was centrifuged through a 4.0% bovine serum albumin (BSA) solution for 5 min at 1000 rpm. The pelleted cells were resuspended in Eagle's MEM supplemented with 1.0 mM glutamine, 33 mM glucose, 10% fetal calf serum, and 180 μ M gentamycin.

The primary astrocytes were plated in 150 cm² culture flasks and purified. Briefly, the cells were incubated at 37°C in a humidified environment containing 5.0% CO₂ for 4–6 d. The cultures were shaken at 250 rpm for 6–18 hr at 37°C, and the medium was removed. This procedure eliminated the oligodendrocytes from the culture because astrocytes remained attached to the plate, whereas the oligodendrocytes were shaken loose and removed with medium. The result was cultures that contained >95% astrocytes, as identified by immunostaining with an anti-glial fibrillary acidic protein antibody. After the cultures were purified, they were grown for an additional 7–10 d in a serum-free medium (equal volumes of MEM and F12 medium with 5.0 mg/l insulin, 5.0 mg/l transferrin, and 5.0 μ g/l selenium; Collaborative Biomedical, Bedford, MA).

Ethanol was added to the medium of many of the cultures. Ethanol is a volatile liquid. To maintain stable ethanol concentrations in the culture medium, a method using sealed containers was used (Adickes et al., 1988; Pantazis et al., 1992; Luo and Miller, 1997b). Briefly, ethanol was added to the medium, and the culture dishes were placed in a bath containing ethanol in the same concentration as that in the medium. Carbon dioxide was added to the sealed container to maintain the volume of CO₂ at 5.0%. Ethanol concentration was assayed using a kit that relies on the conversion of NAD to NADH, which is catalyzed by alcohol dehydrogenase (UV332, Sigma). Using this procedure, we have shown that the ethanol concentration in the culture medium was stable and maintained for 3 d at >90% of the original concentration (Luo and Miller, 1997b).

Cell proliferation assays

Cell counting. Astrocytes (60,000/ml) were plated in the wells of 24-well culture trays and incubated in a serum-free medium. Twenty-four hours after plating (day 0), growth factor and/or ethanol treatment was initiated. The number of cells was counted in untreated cultures on day 0 and in cultures treated for 3 d with a PDGF ligand (0 or 30 ng/ml; Santa Cruz Biotechnology, Santa Cruz CA) (>97% purity) and/or ethanol (0, 200, 400, or 800 mg/dl). The medium was carefully removed from the culture well with a Pasteur pipette before counting.

To ensure that no cells remained floating in the medium or were accidentally drawn off, the medium was examined with an inverted light microscope. No floating cells were found, even for cultures treated with high concentrations of ethanol (800 mg/dl) for 3 d. Thus, the counts of the cells attached to the floor of the well appeared to be accurate measures of total cell number.

Viable and dead/dying cells were distinguished using a trypan blue

exclusion assay. Trypan blue was used to identify viable cells, because only living cells can prevent the dye from passing through their cell membranes. After the medium was removed, a solution containing 0.15 ml of 0.25% trypsin in 0.10% EDTA and 0.15 ml of 0.40% trypan blue in 0.010 M PBS, pH 7.4, was added to the well. The solution was gently triturated to detach the cells. Using this procedure, the cells were simultaneously detached from the floor of the plate and labeled. The numbers of viable (trypan blue-negative) and dead/dying (trypan blue-positive) cells were counted with a hemocytometer. Two tallies per well were taken, and the mean for three wells was used as a data point.

[³H]thymidine incorporation. A second independent assay of cell proliferation was determined by measuring the amount of [³H]thymidine ([³H]dT) incorporated during a 1 hr exposure. Cells were grown for 3 d in 35 mm Petri dishes that contained 2.0 ml of serum-free culture medium. The medium contained one of the two PDGF ligands (0 or 30 ng/ml) and/or ethanol (0 or 400 mg/dl). Thus, there were six treatment groups: (1) untreated cells, (2) cells treated with ethanol alone, (3) cells treated with PDGF-AA alone, (4) cells treated with PDGF-BB alone, (5) cells treated with PDGF-AA and ethanol, and (6) cells treated with PDGF-BB and ethanol.

After 3 d of PDGF and/or ethanol treatment, 10 μ l of [³H]dT (New England Nuclear, Boston MA; specific activity ~80 Ci/mmol) was added to a culture dish. One hour later, the radioactive medium was removed. Cells were detached from the dish by adding 0.30 ml of a solution containing 0.25% trypsin and 0.10% EDTA. The cells were mixed and incubated with 4.0 ml of 10% ice-cold trichloroacetic acid (TCA) in PBS for 20 min. TCA-lysed cells were poured into a Millipore chimney with a Whatman GF/A glass-fiber filter. The precipitated DNA remained on the filter, and to ensure a quantitative recovery and to reduce background, the filter was washed three times with fresh, cold 5.0% TCA. The filter was dried with 95% ethanol and placed into a scintillation vial containing 15 ml of a scintillation cocktail (DuPont NEN, Boston, MA). The amount of [³H] incorporated in the samples was counted with a model LS3801 scintillation counter (Beckman, Brea, CA).

Signal transduction

Receptor autophosphorylation. The activation of a receptor kinase is one of the initial steps in PDGF-mediated signal transduction. An index of receptor kinase activity is ligand-triggered receptor autophosphorylation. PDGF is mitogenic for various neural cells (Valenzuela et al., 1997), whereas ethanol is a potent anti-proliferative agent (Miller, 1992; Luo and Miller, 1998). In fact, ethanol can antagonize PDGF-mediated cell proliferation in various neuroblastoma cells (Luo and Miller, 1997a,b). It is critical to know whether ethanol alters PDGF-mediated receptor phosphorylation.

Astrocytes were prepared for receptor phosphorylation studies by growing them to 80–90% confluency in 60 mm dishes in a medium containing 10% serum. The medium was removed, and the cells were washed twice with a serum-free medium and then maintained in the serum-free medium for 24 hr. After serum starvation, the cells were treated with 30 ng/ml of either PDGF-AA or PDGF-BB (Santa Cruz Biotechnology) for 5, 10, 20, 40, or 60 min, and the medium was removed. Cells were washed twice with cold PBS, then incubated in 0.50 ml of RIPA buffer at 4°C for 10 min. Cells were removed by scraping them from the dish, transferred to 1.5 ml Eppendorf tubes, and spun at 10,000 $\times$ g at 4°C for 15 min.

Receptor immunoprecipitates were generated as follows. The cell lysate was collected, and an aliquot containing 200 μ g of protein was incubated with a rabbit polyclonal antibody against either PDGF α r or PDGF β r (1:50 in PBS) for 1.0 hr at 4°C. Twenty microliters of Protein A/G conjugated to agarose (Santa Cruz Biotechnology) was added to the lysate, and the mixture was incubated overnight at 4°C. Immunoprecipitates were collected by centrifugation at 10,000 $\times$ g for 10 min at 4°C. The pellet was washed three times with 0.50 ml RIPA buffer. After a final wash, the supernatant was aspirated. The pellet was resuspended in 20 μ l of electrophoresis sample buffer, and the suspension was boiled for 5 min.

The samples were electrophoretically separated and transferred to nitrocellulose membranes for immunoblotting. Nonspecific binding was blocked by washing the filter with 5.0% BSA in PBS and 0.10% Tween-20 (TPBS). The filters were probed with a monoclonal antibody against phosphorylated tyrosine (PY20; 1:20,000 in PBS; Transduction Laboratories, Lexington KY). The immunocomplexes were detected with the enhanced chemiluminescence (ECL) method. To assure that alterations in receptor tyrosine phosphorylation did not result from variations in receptor content, the nitrocellulose membranes were stripped of the

PY20 antibody and reprobed with an anti-receptor subunit antibody. For stripping, nitrocellulose membranes were submerged in a solution of 100 mM β -mercaptoethanol, 2.0% SDS, 62.5 mM Tris-HCl, pH 6.7, for 30 min at 50°C or overnight at 4°C. After being stripped, a membrane was washed with TPBS, blocked with 5.0% milk, and reprocessed with either an anti-PDGFR α or an anti-PDGFR β antibody.

Association of Ras–GAP. The phosphorylated receptor binds to an SH2 domain effector molecule, e.g., Ras–GAP, that in turn feeds into the Ras–Raf–MAPK pathway. Therefore, the effects of PDGF and ethanol on the binding of a PDGF receptor with Ras–GAP was examined. Astrocytes were maintained in a serum-free medium for 24 hr and then treated with PDGF-AA or PDGF-BB (30 ng/ml) for 10 min. The cells were lysed with a lysis buffer (1.0% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1.0 mM EDTA, 1.0 mM EGTA, 0.20 mM sodium vanadate, 0.20 mM PMSF, and 0.50% Nonidet P-40). Cell lysates were centrifuged at $10,000 \times g$ for 10 min, and supernatants were collected. The solubilized protein in the supernatant (200 μ g) was immunoprecipitated with either an anti-PDGFR α or an anti-PDGFR β antibody. Ras–GAP associated with PDGF receptors was co-immunoprecipitated. Immunocomplexes were electrophoretically separated on 10.0% polyacrylamide gel and transferred to nitrocellulose membranes by the method described above. The nitrocellulose membranes were probed with monoclonal antibody directed against Ras–GAP (1:800 dilution; Transduction Laboratories).

MAPK activity. MAPK is considered to be a gateway of mitogenic activity. Therefore, the activity of this kinase was examined in near-confluent cultures of purified cortical astrocytes. Astrocytes were grown in a serum-free medium for 24 hr and then treated with ethanol (400 mg/dl) and/or a PKC inhibitor, 1-(5-isoquino-linesulfonyl)-2-methylpiperazine (H-7; 50 μ M; Calbiochem, San Diego, CA). At this concentration, H-7 specifically affects the activity of all PKC isoforms without altering the activity of other enzymes such as receptor kinases (Hidaka et al., 1984; Choudhury et al., 1993). One hour after ethanol and/or H-7 treatment, the cultures were incubated with a growth factor (PDGF-AA or PDGF-BB; 30 ng/ml) for 5, 10, 20, 60, or 90 min. After growth-factor stimulation, the cells were rinsed three times with ice-cold PBS (0.01 M) and solubilized with 0.30 ml lysis buffer (50 mM β -glycerophosphate, pH 7.2, 100 mM sodium vanadate, 5.0 mM magnesium chloride, 1.0 mM EGTA, 0.50% Triton X-100, 1.0 mM 1,4-dithiothreitol, 3.0% aprotinin, and 10 μ g/ml leupeptin) at 4°C for 20 min. Cell lysates were collected and centrifuged at $10,000 \times g$ for 10 min. Cellular proteins (600 μ g) in the supernatants were used for immunoprecipitating MAPK. MAPK was immunoprecipitated with an antibody directed against extracellular signal-regulated kinase (ERK) (1:50 dilution; Transduction Laboratories), which recognizes both the 44 kDa and 42 kDa ERKs. MAPK activity in the immunoprecipitates was assayed by the phosphorylation of myelin basic protein (MBP) using a commercial kit (Upstate Biotechnology, Lake Placid, NY). MAPK-mediated transfer of the γ -phosphate of [γ - 32 P]ATP to MBP was quantified with a scintillation counter. The experiment was replicated four times.

PKC activity. PKC is a key component of receptor-initiated activities. Ethanol is known to alter PKC activity (Messing et al., 1991; DePetrillo and Liou, 1993; Roivainen et al., 1995). Therefore, the effects of ethanol on growth factor-mediated changes in PKC activity were explored.

Cultured astrocytes were grown in a serum-free medium for 24 hr, treated with ethanol (0 or 400 mg/dl) for 1 hr, and then exposed to either PDGF-AA or PDGF-BB (30 mg/dl) for 10 min. The cells were washed in PBS and solubilized with 0.30 ml lysis buffer, and cell lysates were collected. Cell lysates containing 600 μ g protein were chilled, sonicated for 30 sec, and centrifuged at $100,000 \times g$ for 30 min at 4°C. The supernatants and pellets were processed separately for cytosolic and membranous PKC activity, respectively. This step was necessary because activation by growth factors induces PKC to translocate from the cytosol to the plasma membrane (Nishizuka, 1992; Inui et al., 1994). Additional steps were required to prepare the pellets. They were solubilized with 40 μ l of the lysis buffer described above except that the concentration of Triton X-100 was increased to 1.0%, and then centrifuged at $14,000 \times g$ for 10 min at 4°C. This new supernatant was used in our assay of PKC activity in the cell membranes.

The phosphotransferase activity of PKC of the cytosolic and membranous fractions was measured with a commercial kit (Upstate Biotechnology). This assay was based on the PKC-mediated transfer of the γ -phosphate of [γ - 32 P]ATP to a specific substrate peptide (OKRPSQR-SKYL). In some cases, H-7 (50 μ M) was applied to block PKC activity. H-7 did not affect the autophosphorylation of PDGF receptors

(Choudhury et al., 1993). As a negative control, PKC activity was blocked by pretreating the cells for 1 hr with H-7 before performing the PKC activity assay.

Ligand and receptor expression

Immunoblots. Although the other parts of this investigation examined the acute effects of ethanol on signal transduction, it was important to determine whether ethanol had more lasting effects on the upstream effectors: the PDGF ligands and their receptors. The expression of these proteins was determined using Western immunoblots.

Primary astrocytes were grown with a serum-free medium in 60 mm culture dishes. The cells were treated with ethanol (0, 200, 400, or 800 mg/dl) for 3 d. The medium was removed, and the cells were washed twice with ice-cold PBS. The cells were lysed by a 10 min wash in 1.0 ml ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1.0% Nonidet P-40, 0.10% SDS, 0.50% deoxycholic acid sodium, 0.10 mg/ml phenylmethylsulfonyl fluoride, 1.0 mM sodium orthovanadate, 10.0 μ g/ml leupeptin, 2.0 μ g/ml pepstatin A, and 3.0% aprotinin). Lysates were centrifuged at $10,000 \times g$ for 15 min. The supernatants, which contained cellular proteins, were collected, and the protein concentration of each sample was determined (Lowry et al., 1951). Proteins were mixed in an equal volume of electrophoresis sample buffer (1.0 ml glycerol, 0.50 ml β -mercaptoethanol, 3.0 ml 10% SDS, 1.25 ml 1.0 M Tris-HCl, pH 6.7, and 2.0 mg bromophenol blue) and boiled for 3 min. For comparison, some samples were not heated. The amount of expression and the size of the bands were apparently unaffected by boiling.

The proteins were separated by gel electrophoresis. Thirty micrograms of protein were loaded on each lane of an SDS-polyacrylamide gel (10% acrylamide) and electrophoresed. After being separated, the samples were transferred to 0.20 μ m nitrocellulose filters. Nonspecific antigenicity was blocked by washing the filters for 1 hr at room temperature with TPBS. After two washes (10 min each) with TPBS, the filters were then incubated for 1 hr at room temperature with a polyclonal antibody directed against PDGF A-chain, PDGF B-chain, PDGFR α , or PDGFR β (diluted 1:500, Santa Cruz Biotechnology). Subsequently, the filters were washed in TPBS and incubated with a secondary antibody conjugated to horseradish peroxidase (1:2000 dilution; Amersham, Arlington Heights, IL). The immunocomplexes were detected with an ECL method (Amersham). Three replicate immunoblots were processed for each primary antibody.

Two controls for nonspecific immunoreactivity were performed. In these trials, filters were processed without the primary or secondary antibody. The results of the controls were consistently negative.

Scatchard analysis. The effects of ethanol on the numbers of PDGF-AA and PDGF-BB binding sites were determined using a Scatchard saturation binding assay (Seifert et al., 1993). This study complements the receptor expression study described above. Although the immunoblotting study provides data on the total expression of PDGF receptors (membrane-bound as well as cytosolic pools), the saturation binding assay generates information only on the extracellularly directed membrane-bound receptors. Thus, the data from the Scatchard and immunoblotting analyses can be used to determine the effects of ethanol on the translocation of the receptors. Furthermore, the Scatchard analysis provides valuable data on the ligand-binding affinities.

Astrocytes were raised in 24-well culture trays containing a medium supplemented with 10% fetal calf serum and ethanol (0 or 400 mg/dl) for 3 d. The cells were rinsed twice with cold binding buffer (Ham's F-12, 25 mM HEPES, pH 7.4, and 0.25% BSA). [125 I]PDGF-AA or [125 I]PDGF-BB (specific activities 76 cpm/pg and 220 cpm/pg, respectively; iodinated at the Iowa City Veterans Affairs Diabetes Research Center) was added to wells at one of a range of concentrations (0.625–20 ng/ml) in 0.20 ml binding buffer. The cells were incubated for 3.5 hr at 4°C. The radioactive binding buffer was removed, and the cells were washed three times with fresh, cold binding buffer. Cells were extracted by the addition of PBS containing 1.0% Triton X-100 and 0.25% BSA, and cell-associated radioactivity (bound ligands) was quantified using an Auto-Gamma 5000 gamma counter (Packard, Downers Grove, IL).

The amounts of bound [125 I]ligand and free [125 I]ligand were determined. The concentration of free PDGF was calculated by subtracting the amount of PDGF bound to the cells from the amount of [125 I]PDGF originally added to the wells. Nonspecific binding was determined by preincubation with 200 ng/ml of unlabeled ligands. Nonspecific binding ranged from 10 to 15% of the total binding. Specific binding for PDGF receptors was determined by subtracting nonspecific binding from total binding. The Scatchard plot (the amount of bound [125 I]ligand vs the

Figure 1. PDGF-mediated astrocyte growth. Astrocytes were maintained in a serum-free medium and treated with either PDGF-AA or PDGF-BB (30 ng/ml) for 3 d. The *left panel* shows the mean number of cells in a culture ($\pm$ SEM) before PDGF or ethanol (200–800 mg/dl) was added. The *plot* depicts the original (concocted) ethanol concentrations. The *middle panel* describes the number of cells in a culture after 3 d of treatment with PDGF and/or ethanol. The amount of ethanol-induced inhibition (expressed as a percentage) was measured against the amount of PDGF stimulation in the absence of ethanol (*right panel*). The formula for calculating the percent inhibition (PI) is $PI = [(PDGF+)_0 - (PDGF+)_x] / [(PDGF+)_0 - (PDGF-)_x]$ (100%), in which (PDGF+) and (PDGF-) are the numbers of cells in cultures treated with and without PDGF, respectively, after being treated with a particular concentration of ethanol (i.e., 0 or x mg/dl). Each data point is based on six trials.

ratio of the concentration of bound [¹²⁵I]ligand to the amount of free [¹²⁵I]ligand) was drawn. The number of binding sites (B_{max}) and the receptor affinity (K_d) were calculated (Rosenthal, 1967) using the software LIGAND (Munson and Rodbard, 1980). Cells in parallel wells that were not treated with a radiolabeled ligand were harvested, and the number of viable cells was determined (see above). These numbers were used to calculate the number of binding sites per cell. All data points were based on quadruplicate samples.

Statistical analysis

Differences among the treatment groups (e.g., growth factor- or ethanol-treated) were examined by two-way ANOVA. Student–Newman–Keuls tests were used for *post hoc* comparisons. Tukey B tests were used to examine changes over time. Statistical analyses were performed using the software Sigmapstat 2.0 (Jandel, San Rafael, CA).

RESULTS

PDGF-mediated cell proliferation

Astrocytes did not grow in a nonsupplemented, serum-free medium. That is, the number of cells in the untreated cultures did not change significantly over a 3 d experiment.

PDGF ligands were potent mitogens for astrocytes. After the addition of PDGF-AA or PDGF-BB (30 ng/ml), the number of astrocytes increased significantly ($p < 0.05$) (Fig. 1). The stimulatory effect of PDGF-BB was greater than that for PDGF-AA. After 3 d treatment, PDGF-AA and PDGF-BB increased the number of cells over controls by 1.5- and 2.4-fold, respectively. The mitogenic effects of the PDGF ligands were further assessed by measuring PDGF-stimulated [³H]dT incorporation (Fig. 2). As with the changes in cell number, both PDGF-AA and PDGF-BB treatment significantly ($p < 0.05$) increased [³H]dT uptake, and PDGF-BB had a stronger stimulatory effect.

Cell survival (as indicated by the incidence of trypan blue-positive cells) was not affected by either PDGF ligand. The incidence of astrocyte death was between 6.6 ± 0.9 and $7.9 \pm 1.3\%$ in the control and growth-factor treated cultures. No statistically significant differences were detected. Therefore, the changes in cell number and [³H]dT incorporation can be attributed solely to the effects of growth factors on the cell proliferation.

Ethanol treatment attenuated PDGF-mediated growth in a

Figure 2. Effects of PDGF and ethanol on [³H]dT incorporation. Astrocytes were maintained in a serum-free medium. [³H]dT incorporation was measured after 3 d of treatment with PDGF (0 or 30 ng/ml) and/or ethanol (0 or 400 mg/dl). Data represent the means ($\pm$ SEM) for four independent experiments. Asterisks denote statistically significant differences ($p < 0.05$) in comparisons with the untreated (control) cultures.

concentration-dependent manner (Fig. 1). Furthermore, ethanol differentially affected the action of PDGF-AA and PDGF-BB. At concentrations of 200 mg/dl or higher, ethanol completely blocked PDGF-AA-mediated cell growth, but at 200 mg/dl, ethanol only partially (65%) inhibited a PDGF-BB-mediated increase in cell number. The inhibitory effect of ethanol on cell proliferation was confirmed in a study of PDGF-affected [³H]dT uptake. Ethanol (400 mg/dl) completely blocked PDGF-AA-mediated [³H]dT uptake and partially attenuated PDGF-BB-stimulated [³H]dT incorporation (Fig. 2). Cell death apparently was not affected by treatment with ethanol or ethanol and a growth factor. The numbers of dead/dying (trypan blue-positive)

Figure 3. Time sequence of PDGF receptor autophosphorylation. Glia were isolated from cortices of 5-d-old rats, purified, and cultured in serum-supplemented medium for 2 weeks. Subsequently, the cells were grown in a serum-free medium for 24 hr and treated with PDGF-AA or PDGF-BB for 0, 5, 10, 20, 40, or 60 min. PDGF α and PDGF β were immunoprecipitated separately and assayed for tyrosine phosphorylation. The experiment was repeated three times.

cells in these cultures were not significantly different from the numbers in the untreated cultures. The frequency of trypan blue-positive neurons was 7.6 ± 1.3 to $9.2 \pm 1.6\%$.

Receptor autophosphorylation

PDGF receptors are tyrosine kinases. When they bind a ligand, these kinases autophosphorylate and set a cascade of events into motion. The tyrosine phosphorylation of a specific receptor subunit was determined by an immunoprecipitation procedure.

Receptor activation was a fast process (Fig. 3); increased receptor autophosphorylation was detected within 5 min of adding the PDGF. It peaked at 10 min after growth factor administration and then declined. PDGF-AA-stimulated phosphorylation was detected only in samples immunoprecipitated with an anti-PDGF α antibody (Fig. 4). This indicates that PDGF-AA induced the phosphorylation of only the PDGF α and not the PDGF β . On the other hand, PDGF-BB induced the phosphorylation of samples immunoprecipitated with either an anti-PDGF α or an anti-PDGF β antibody. Thus, PDGF-BB induced the phosphorylation of both receptor subtypes.

In the presence of ethanol (400 mg/dl), both PDGF-AA- and PDGF-BB-mediated phosphorylation of the PDGF α was re-

duced; however, PDGF-BB-stimulated phosphorylation of PDGF β was not affected. Therefore, ethanol inhibited only the tyrosine phosphorylation of the PDGF α .

Association of Ras-GAP

Activated tyrosine kinase receptors bind to downstream effectors with an SH2 domain. One such effector is Ras-GAP. PDGF-AA induced the association of Ras-GAP to PDGF α , whereas PDGF-BB resulted in Ras-GAP binding to either receptor isoform (Fig. 5). Ethanol exposure (1 hr) significantly inhibited both PDGF-AA- and PDGF-BB-mediated association of Ras-GAP to PDGF α , but it had little effect on the binding between Ras-GAP and PDGF β .

MAPK activity

Both PDGF-AA (30 ng/ml) and PDGF-BB (30 ng/ml) rapidly and transiently stimulated MAPK activity (Fig. 6). Significant ($p < 0.05$) stimulation appeared within 5 min, was maximal after 10 min, and fell to control levels within 60 min. Of the two ligands, PDGF-BB had a stronger stimulatory effect on MAPK activity. After 10 min of treatment, PDGF-BB and PDGF-AA increased MAPK activity by 357 and 165%, respectively.

Ethanol treatment (400 mg/dl) significantly ($p < 0.05$) increased basal MAPK activity (i.e., the activity induced in the absence of growth factors) by 74.0%. Ethanol also affected PDGF-mediated MAPK activity. In the presence of ethanol, PDGF-AA and PDGF-BB (10 min) significantly ($p < 0.05$) increased MAPK activity 219 and 482%, respectively, beyond the amount of MAPK activity in the untreated controls. The ethanol-induced potentiation abated within 20 min after the growth factor was added. This abatement was complete for the cultures treated with PDGF-AA; however, it was incomplete for the cultures treated with PDGF-BB. In the latter, MAPK activity was 51.9% higher after 90 min of PDGF stimulation than it was at the beginning of the experiment. Nevertheless, for both PDGF ligands, the amount of activation was maintained at a level signifi-

Figure 4. Effect of ethanol on PDGF receptor autophosphorylation. *Top*, Astrocytes were maintained in a serum-free medium for 24 hr, then exposed to ethanol (0 or 400 mg/dl) for 1 hr. Subsequently, the cells were treated with PDGF-AA or PDGF-BB (30 ng/ml) in the presence or absence of ethanol for 10 min. Cell lysates were collected, and an equal amount of cellular protein (200 μ g) was immunoprecipitated with an anti-PDGF α or anti-PDGF β antibody. The immunoprecipitates were electrophoretically separated and probed with an antibody (PY20) directed against phosphorylated tyrosine. *Bottom*, After the nitrocellulose papers were stripped, they were reprobbed with either anti-PDGF α or anti-PDGF β antibody. These blots show that not only did the phosphorylation signal align with the receptor signal, but the loading of the protein on each lane was consistent. Note that protein expression of the PDGF α was unaffected by 1 hr ethanol treatment; changes in the expression of the protein PDGF α required a chronic exposure, e.g., 3 d (Fig. 8). Three repeats of the experiment were performed.

Figure 5. Association of Ras-GAP. After the isolation, purification, and growth in a medium containing 10% fetal calf serum, astrocytes were maintained in a serum-free medium for 24 hr. The cells were exposed to ethanol (0 or 400 mg/dl) for 1 hr and treated with PDGF-AA or PDGF-BB (30 ng/ml) in the presence or absence of ethanol for 10 min. Cell lysates were collected and immunoprecipitated with an anti-PDGF α r or anti-PDGF β r antibody. The immunoprecipitates were electrophoretically separated and probed with a monoclonal antibody directed against Ras-GAP. The experiment was performed three times.

icantly ($p < 0.05$) greater than that evident in the control cultures.

The effects of PDGF on MAPK activity were further assessed in astrocytes that were treated with H-7. H-7 had little effect on basal MAPK activity; however, H-7 pretreatment significantly ($p < 0.05$) reduced PDGF-enhanced MAPK activity (from 165 to 100% for PDGF-AA treatment and from 357 to 225% for PDGF-BB treatment). It is important to note that neither PDGF-AA- nor PDGF-BB-mediated kinase activity was totally eliminated by H-7. That is, regardless of whether the cells were exposed to H-7, the MAPK activity in cells treated with a PDGF ligand remained significantly ($p < 0.05$) greater than in control cells.

H-7 pretreatment completely blocked ethanol-stimulated MAPK activity (Fig. 6, *inset*). In fact, there was no significant difference in the activity expressed by control and ethanol-treated cells. Likewise, in the presence of H-7, the PDGF-AA-mediated stimulation of MAPK activity was eliminated by ethanol. In contrast, PDGF-BB-regulated MAPK activity was only partially, but significantly ($p < 0.05$), inhibited by ethanol.

PKC activity

PKC activity in the membranes was significantly ($p < 0.05$) increased by ethanol (400 mg/dl) and PDGF (30 ng/ml) treatment (Fig. 7). In contrast, ethanol and PDGF had little effect on PKC activity in the cytosolic fraction. The ratio of PKC activity in membranes to total activity (cytosolic plus membranous) was significantly ($p < 0.05$) increased by ethanol and PDGF.

Ethanol potentiated the growth factor-stimulated PKC activity. That is, PKC activity in the membranes of cells treated with PDGF-AA or PDGF-BB and ethanol was significantly ($p < 0.05$) greater (31.3 and 48.8%, respectively) than it was with a growth factor alone. H-7 pretreatment blocked all ethanol- and growth factor-induced stimulation.

Expression of PDGF ligands and receptors

The expression of PDGF ligands and receptors was examined in cells raised in a serum-free medium using Western immunoblots. Cultured astrocytes expressed both ligand isoforms (Fig. 8). Chronic exposure (3 d) to ethanol (up to 800 mg/dl) did not affect the expression of either ligand. The astrocytes also expressed both receptor subunits. The expression of PDGF α r was upregulated by ethanol in a concentration-dependent manner; however, the expression of PDGF β r was not affected by ethanol. Note that similar data were obtained for cells raised in a medium containing 10% fetal calf serum.

Ethanol-induced alterations in PDGF receptor characteristics were examined with a Scatchard analysis. Ethanol did not alter the binding affinity (K_d) for either PDGF-AA or PDGF-BB (Fig. 9, Table 1). On the other hand, ethanol significantly ($p < 0.05$) increased the density of PDGF-AA binding sites by 46%. In contrast, the density of PDGF-BB binding sites was not significantly altered by ethanol. Thus, the results of the PDGF binding study were consistent with those of the immunoblotting studies showing that PDGF α r expression was selectively increased by 3 d treatment with ethanol.

DISCUSSION

PDGF-mediated cell proliferation

Astrocytes do not grow in a serum-free medium; however, after supplementation with PDGF, astrocytes do proliferate (as shown by changes in cell number and [3 H]dT incorporation). Cell death among astrocytes maintained in a serum-free medium occurs at a low rate, and the incidence of this death is unaffected by either PDGF homodimer. Therefore, we conclude that PDGF acts purely as a mitogen. These findings concur with reports that PDGF is a potent mitogen for cultured astrocytes (Prins et al., 1996; Valenzuela et al., 1997). Interestingly, PDGF-AA is a less effective mitogen than PDGF-BB. Similar differential effects have been described for neuroblastoma cells (Luo and Miller, 1997a,b).

Ethanol inhibits PDGF-mediated proliferation of astrocytes in a concentration-dependent manner; i.e., increasing concentrations of ethanol produce progressively stronger inhibition. Ethanol differentially affects the action of two PDGF ligands. PDGF-AA-mediated cell proliferation is more susceptible to the action of ethanol. At a concentration of 200 mg/dl, ethanol completely blocks PDGF-AA-stimulated cell proliferation, but it only partially inhibits PDGF-BB-mediated astrocyte growth. A similar differential effect occurs in neuroblastoma cells (Luo and Miller, 1997a,b). In contrast to the effects of ethanol on cell proliferation, astrocyte survival is not compromised either by ethanol alone or in combination with PDGF. This implies that ethanol acts on the signal transduction system mediating growth factor-stimulated cell proliferation.

PDGF-mediated signal transduction

Receptor activation

The activities of PDGF receptor tyrosine kinases are essential to the mitogenic properties of the ligands. Blocking the receptor kinases eliminates PDGF-mediated cell proliferation (Kovalenko et al., 1994; Buchdunger et al., 1995). Both our study and one by Zhang and Hutchins (1997) show that PDGF treatment induces receptor phosphorylation in cultured neurons and astrocytes. PDGF-AA induces the phosphorylation of only the PDGF α r, whereas PDGF-BB activates both receptor subtypes. These findings are consistent with data on vascular smooth muscle cells

Figure 6. Effects of PDGF, ethanol, and H-7 on time sequence of MAPK activity. MAPK activity was determined for astrocytes pretreated with ethanol (0 or 400 mg/dl) and/or H-7 (0 or 50 μ M) for 1 hr in a serum-free medium (designated as time 0 min). The cells were then incubated with either PDGF-AA (30 ng/ml) or PDGF-BB (30 ng/ml) for as long as 90 min. *Insets.* The histograms compare MAPK activity for cultures treated with ethanol (0 or 400 mg/dl) and/or H-7 (0 or 50 μ M) at time 0 min (black bars) or at time 90 min (white bars). Each data point represents the mean of four trials ($\pm$ SEM). Asterisks denote statistically significant ($p < 0.05$) differences relative to the control cultures at time 0 min.

showing the specificity of PDGF-AA for the PDGF α r and the promiscuous ability of PDGF-BB to induce tyrosine phosphorylation of either the PDGF α r or PDGF β r (Inui et al., 1994).

Ras–GAP association

Receptor activation is accompanied by the formation of multimeric complexes between the receptors and downstream SH2-containing proteins (Bornfeldt et al., 1995; Hart et al., 1995). The complexes are formed by the binding of phosphorylated tyrosine residues in receptors to the SH2 domains in the effectors (Fig. 10). This association, in turn, regulates the activity of the effectors. Ras–GAP (an SH2-containing protein) is an important modulator of the Ras–Raf–MAPK signal pathway that eventually results in cell proliferation (Satoh et al., 1993; Zhang et al., 1993; Li et al., 1995).

PDGF-AA induces the association of PDGF α r to Ras–GAP, whereas PDGF-BB recruits Ras–GAP to bind to both PDGF α r and PDGF β r. This result is consistent with the finding that PDGF-AA specifically activates the PDGF α r and that PDGF-BB induces the phosphorylation of either the PDGF α r or the PDGF β r. Therefore, downstream receptor signaling by

PDGF-AA and PDGF-BB is mediated solely by the PDGF α r and by the PDGF α r or PDGF β r, respectively.

MAPK activation

Activation of the Ras–Raf–MAPK cascade ultimately induces cell proliferation (Bornfeldt et al., 1995) (Fig. 10). Thus, MAPK plays a critical role in the control of cell growth and differentiation. The present study shows that both PDGF-AA and PDGF-BB stimulate MAPK in cultured astrocytes and that PDGF-BB has a stronger effect than PDGF-AA. These data concur with those of Prins et al. (1996).

An alternative pathway to stimulate MAPK activity is through PKC (Sözei et al., 1992; Kolch et al., 1993; Li et al., 1995). The present study shows that pretreatment with H-7 completely blocks PDGF-stimulated PKC activity. Furthermore, both PDGF-AA- and PDGF-BB-mediated MAPK activities are partially inhibited by H-7 pretreatment. This incomplete inhibition implies that PDGF can affect MAPK via two pathways: a PKC-dependent and a PKC-independent pathways (Fig. 10). The latter is likely to be the Ras–Raf–MAPK pathway. Dual pathways have been de-

Figure 7. PKC activity. The effects of four substances (PDGF-AA, PDGF-BB, ethanol, and H-7) on PKC activity in the membranal (*top*) and cytosolic fractions (*middle*) was determined. The *graph* at the *bottom* describes the effects of the four substances on the ratio of PKC activity in the membrane to the combined PKC activity in the membrane and cytosol. Statistically significant differences ($p < 0.05$) in comparisons relative to the untreated (control) cells are noted by an asterisk. Differences caused by H-7 pretreatment (i.e., comparing cells that were similarly treated with PDGF and/or ethanol) are identified by #. Ethanol-induced differences for cells that were treated with the same growth factor are labeled with +. Each experiment was performed four times.

scribed in human mesangial cells (Choudhury et al., 1993) and in smooth myocytes (Inui et al., 1994).

There is considerable cross-talk between the PKC and the Ras–Raf–MAPK pathways (Fig. 10). That is, activation of PKC can stimulate MAPK via a Ras–Raf-dependent or Ras–Raf-independent manner. For example, stimulation of PKC in lymphocytes inhibits Ras–GAP activity and increases the formation of Ras–GTP (Downward et al., 1990), and PKC can directly phosphorylate and activate Raf-1 in insect cells (Sözei et al., 1992) and fibroblasts (Kolch et al., 1993). In contrast, renal mesangial cells use a Ras–Raf-independent pathway; PKC activates neither Ras nor Raf-1, yet still it can affect MAPK (Li et al., 1995). Likewise, PKC can activate MAPK in 3T3 cells by a mechanism independent of Raf (Schönwasser et al., 1998).

The presentation of the PKC is important in PDGF-mediated signaling. PDGF treatment significantly increases membranal PKC activity but has little effect on cytosolic activity. The relative amounts of PKC activity in the membrane and cytosol are important because they indicate the state of PKC mobilization. To become fully active, PKC must translocate from cytosolic pools to the membrane where it can interact with its phospholipid substrates (Kikkawa et al., 1982; Kraft et al., 1982; Neary et al., 1988). As most PKC activity in unstimulated astrocytes resides in the cytosol, PDGF must promote the translocation of PKC from the cytosol to the membrane. Similarly, both PDGF-AA and PDGF-BB induce a significant translocation of PKC from cytosol to membrane in vascular smooth muscle cells (Inui et al., 1994).

Figure 8. Expression of PDGF ligands and receptors. *Top*, The effect of ethanol on the expression of the 18 kDa PDGF-AA and 16 kDa PDGF-BB was analyzed with Western immunoblots. Astrocytes were treated with ethanol (0–800 mg/dl) for 3 d. Thirty micrograms of cellular protein were loaded onto each lane. *Bottom*, Expression of the two PDGF receptor subunits was examined with immunoblots. Both receptors are 180 kDa but could be distinguished using specific antibodies that had no cross-reactivity with the other subunit.

Figure 9. PDGF binding characteristics. The kinetics of PDGF-AA and PDGF-BB binding was assessed by a Scatchard analysis. Astrocytes were maintained in a medium containing 10% serum and treated with a PDGF ligand (0 or 30 ng/ml) and/or ethanol (0 or 400 mg/dl) for 3 d. Each data point is the mean of four independent trials.

Table 1. Scatchard analysis of PDGF binding

	Untreated	Ethanol (400 mg/dl)
$K_{dPDGF-AA}$	459 ± 34 pM	503 ± 27 pM
$K_{dPDGF-BB}$	570 ± 53 pM	502 ± 48 pM
$B_{maxPDGF-AA}$	$42,600 \pm 2400$ /cell	$62,300 \pm 4400$ /cell*
$B_{maxPDGF-BB}$	$28,000 \pm 1700$ /cell	$32,900 \pm 2400$ /cell

Binding parameters were determined from the data of the binding assay described in Figure 9. The differences in PDGF binding parameters of ethanol-treated and untreated astrocytes were analyzed by a Student's *t* test for independent samples. The asterisk denotes a statistically significant difference ($p < 0.05$) in a comparison relative to the untreated cultures.

Effect of ethanol on PDGF signaling

Receptor phosphorylation

Ethanol inhibits the autophosphorylation of PDGF receptors, primarily the PDGF α r (Fig. 10). This is consistent with the selective ethanol-induced elimination of PDGF-AA-mediated cell proliferation. The effects on PDGF β r-mediated activities are partial. This results from the ability of PDGF-BB to bind to either receptor isoform. The implication from these data is that the receptor kinase is the primary target of ethanol. This conclusion is supported by the finding that short-term treatment with ethanol has no effect on the amount of receptor expression.

The selective effect of ethanol on specific growth-factor receptors has been described for neural and non-neural cells. For example, ethanol inhibits the tyrosine phosphorylation of insulin-like growth factor I receptor (IGF-Ir) in NIH 3T3 fibroblast cells; however, it does not affect the tyrosine phosphorylation of the structurally and functionally similar insulin receptor (Resnicoff et al., 1993). In fact, these authors posit that all ethanol-induced damage to cell proliferation is regulated by the IGF-Ir. The present results argue against the privileged position of the IGF-Ir and show that various receptors can mediate ethanol-induced damage. On the other hand, we must emphasize that ethanol does not affect all the activity of all mitogenic receptors (cf. the effects on the PDGF α r and the PDGF β r). Furthermore, ethanol also affects the tyrosine phosphorylation of the epidermal growth factor (EGF) receptor in human epidermoid carcinoma cells (Thurston and Shukla, 1992) but does not alter the tyrosine kinase activity of nerve growth factor (NGF) and basic fibroblast

growth factor (bFGF) receptors in pheochromocytoma (PC12) cells (Roivainen et al., 1995). Thus, ethanol does not universally affect all receptors.

The specific target of ethanol currently is unknown. That is, a loss of PDGF-stimulated receptor tyrosine activity can result from one of three types of mutation: (1) a point mutation of the ATP-binding site, (2) a deletion of the C-terminal region, and (3) replacement of the membrane-spanning sequences (Escobedo et al., 1988). Direct ethanol-induced damage to the receptor at any of these loci may inhibit receptor kinase activity. Alternatively, ethanol may indirectly change tyrosine kinase activity by altering other protein kinases or phosphatases that regulate PDGF receptor tyrosine kinase.

Ras-GAP association

The association between the phosphorylated PDGF α r and Ras-GAP is inhibited by ethanol. In contrast, PDGF β r-Ras-GAP association is unaffected by ethanol. These specific effects mirror the ethanol-induced alterations in PDGF receptor phosphorylation. Hence, blocking of the receptor kinase is transmitted through the next step of the signal transduction pathway.

MAPK activation

The present study shows that ethanol stimulates MAPK activity in cultured cortical astrocytes. It does so by effectively raising MAPK activity 74.0%, i.e., increasing the set-point of "basal" activity. This change is achieved within 1 hr of ethanol administration and maintained for at least 90 min more. A stimulatory effect of ethanol on MAPK has been also reported for PC12 cells (Roivainen et al., 1995) and embryonic hepatocytes (Reddy and Shukla, 1996). Such results are paradoxical in that stimulation of MAPK activity is considered a pro-proliferative event, yet ethanol, which is an anti-proliferative agent, also stimulates MAPK activity.

PDGF receptor autophosphorylation is inhibited by ethanol. Theoretically, if the receptor kinase-Ras-Raf pathway is the only way to effect MAPK activity, then ethanol-induced inhibition of receptor phosphorylation should depress PDGF-mediated signal transduction and PDGF-stimulated MAPK activity. Our empirical data, however, show that treatment with ethanol increases MAPK activity and potentiates PDGF-stimulated MAPK activity. On the basis of these findings, we conclude that ethanol can activate MAPK via a pathway that bypasses the PDGF receptors. Possibly, activation of this "alternative" pathway masks the ethanol-mediated inhibition of PDGF signaling.

Candidate key components in the ethanol-induced stimulation of MAPK are PKCs. Not only do PKCs regulate MAPK activity (see above), but they are activated by ethanol (Messing et al., 1991; DePetrillo and Liou, 1993; Roivainen et al., 1995). The present data indicate that ethanol promotes the translocation of PKC from the cytosol to the membrane. It is only after the growth factor has promoted the PKC to associate with the membrane that it can interact with its substrate. Likewise, Skwish and Shain (1990) show that ethanol increases the PKC activity in the membrane.

Ethanol increases MAPK activity in control and PDGF-treated astrocytes; such increases are transduced by the activation of PKC (Fig. 10). After all, H-7 completely eliminates the ethanol-induced stimulation of MAPK activity. Thus, ethanol chronically activates MAPK via a PKC-dependent pathway. On the other hand, ethanol inhibits the transient PDGF receptor-regulated activation of MAPK via a PKC-independent pathway. In fact,

Figure 10. Schematic of signal transduction pathways of MAPK activation initiated by PDGF ligands. PDGF-AA induces only the tyrosine phosphorylation of PDGF α r, whereas PDGF-BB activates either receptor subunit. Receptor phosphorylation causes the activation of downstream effector molecules that contain an SH2 domain (e.g., Ras-GAP) and the sequential activation of Ras, Raf-1, MAPK kinase (MEK), and eventually MAPK. Alternatively, receptor phosphorylation can activate PKC, which in turn, can stimulate MAPK by either by a direct effect on Ras–Raf-1 or through an unknown intermediate molecule(s). Ethanol can affect receptor phosphorylation and PKC activity in the MAPK cascade. These positive (dashed lines) and negative (dotted lines) effects can alter the signal transduction cascade that ultimately leads to the stimulation or inhibition of MAPK.

when PKC activity is blocked, ethanol totally eliminates PDGF-AA-mediated activation of MAPK and partially inhibits PDGF-BB-regulated MAPK activity. This differential effect parallels the effects of ethanol on the activation of PDGF receptor isoforms. We conclude, therefore, that (1) complete inhibition of PDGF-AA-mediated MAPK activation results from interference with the activity of PDGF α r—ethanol selectively inhibits the autophosphorylation of the PDGF α r and the subsequent signal transduction—and (2) the incomplete blockade of PDGF-BB signaling on MAPK results from the ability of PDGF-BB to activate either the PDGF α r or PDGF β r and that PDGF β r is not affected by ethanol.

MAPK can be activated by mitogenic and growth inhibitory agents. The activation of MAPK may not be an all-or-none event; rather the critical event may be defined by the duration of activation. For example, NGF and bFGF, inhibitors of PC12 cell proliferation, induce chronic MAPK activation for several hours. In contrast, EGF, a stimulator of PC12 cell proliferation, induces only transient (<30 min) MAPK activation (Qiu and Green, 1992; Tombes et al., 1998). Depending on cell types, NGF may induce either acute or chronic activation of MAPK. When NGF acutely activates the MAPK of rat hepatocytes, it stimulates DNA synthesis in these cells (Tombes et al., 1998). In contrast, when MAPK is chronically activated in hepatocytes via infection with an inducible estrogen receptor–Raf-1 fusion protein, DNA synthesis is inhibited. Furthermore, both transforming growth factor β 1 (TGF β 1) and ethanol are potent growth inhibitory agents for B104 neuroblastoma cells (Luo and Miller, 1999). They induce a chronic state of increased MAPK activity. Blocking MAPK activity in these cells completely reverses TGF β 1- and ethanol-mediated growth inhibition. Therefore, acute activation of ERK promotes cell proliferation, and chronic activation of ERK inhibits this process. It can be deduced that ethanol impairs PDGF-mediated cell proliferation by inhibition of acute MAPK activation and a concurrent promotion of chronic activation.

Ligand and receptor expression

Our results and those of Silberstein et al. (1996) show that cultured astrocytes express both PDGF monomers. Chronic ethanol exposure does not affect ligand production. Moreover, it does not affect the affinity of the ligand to bind with its receptor(s). This lack of an ethanol-induced effect contrasts with the rapid effects of ethanol on signal transduction.

Astrocytes express both PDGF receptor isoforms. Ethanol exposure selectively alters the expression of PDGF α r. The expression of a PDGF receptor can be influenced by the availability of PDGF (Eriksson et al., 1991; Bejcek et al., 1993). Ethanol alters neither ligand expression nor binding affinity. Therefore, we must conclude that the altered receptor expression is not a response to changed ligand availability, but rather it reflects a direct effect of ethanol on the receptor. Interestingly, only the expression of the PDGF α r is affected by ethanol. Ethanol upregulates PDGF α r expression, as is evident in immunoblots and saturation binding assays. It is appealing to speculate that this is a compensatory response to the ethanol-induced inhibition of this receptor subtype because (1) ethanol preferentially inhibits the tyrosine phosphorylation of the PDGF α r and PDGF α r-mediated signal transduction and (2) chronic ethanol exposure (3 d) is required to induced receptor upregulation; acute ethanol exposure (1 hr) does not affect the expression of PDGF α r. Other data also indicate that the PDGF α r is more susceptible to environmental modulation than the PDGF β r. For example, TGF β 1, a potent anti-mitogenic agent, selectively reduces the expression of PDGF α r without affecting PDGF β r (Paulsson et al., 1993; Bonner et al., 1995).

Conclusions

Various *in vivo* and *in vitro* data show that growth factor receptors are targets of ethanol toxicity. High- and low-affinity neurotrophin receptors (TrkA, TrkB, and p75) are sensitive to ethanol *in vivo* (Aloe and Tirassa, 1992; Valles et al., 1994; Baek et al., 1996;

Dohrman et al., 1997). Ethanol exposure also alters the *in vitro* expression of growth factor receptors such as the bFGF receptor, EGF receptor, insulin receptor, IGF-Ir, and p75 (Rifkin et al., 1983; Wang et al., 1992, 1994; Resnicoff et al., 1993; Luo et al., 1996; Luo and Miller, 1997b; Seabold et al., 1998).

PDGF and PDGF receptors are widely expressed in neurons and glia in both the developing and mature nervous systems (Valenzuela et al., 1997). In addition to its mitogenic effect on glia, recent studies show that PDGF is also an important neurotrophic and neuroprotective agent for neurons in the CNS (Valenzuela et al., 1997). The present study shows that ethanol inhibits PDGF-mediated astrocyte proliferation and alters PDGF signaling by blocking receptor kinase activity. The results show that (1) ethanol-induced interference with the action of this important growth factor is a critical mechanism underlying ethanol-induced disruptions in cell proliferation, (2) ethanol raises the set-point for basal MAPK activity, thereby changing growth factor-mediated increases in MAPK activity into an anti-proliferative signal, and (3) ethanol is a valuable tool for dissecting the mechanism(s) by which PDGF regulates cell proliferation.

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