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Ethanol inhibits mitogen activated protein kinase activity and growth of vascular smooth muscle cells in vitro

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Abstract

The aim of this study was to determine the effect of ethanol on vascular smooth muscle cell proliferation and mitogen activated protein kinase (MAPK) signaling. Rat aortic smooth muscle cell growth in vitro was determined by measuring cell counts and [^{3}H]thymidine incorporation. MAPK signaling was determined by assessing MEK (also referred to as MAPK kinase) activity by measuring phosphorylated extracellular signal-regulated kinase ($_{pp}$44ERK − 1 and $_{pp}$42ERK − 2) expression, and ERK activity by measuring ERK-2-dependent phosphorylation of myelin basic protein (MBP). In quiesced smooth muscle cells, ethanol treatment (24 h) inhibited serum-stimulated mitogenesis in a dose-dependent manner, (IC_{50} = 60 mM), in the absence of any effect on smooth muscle cell viability. In addition, ethanol treatment caused a significant shift to the right in the smooth muscle cell growth curve, extending the population doubling time from ~48 h (control) to ~70 h (ethanol). Acute (15 min) ethanol treatment reduced serum-stimulated $_{pp}$44ERK − 1 and $_{pp}$42ERK − 2 expression in a dose dependent fashion; 24.5 ± 1.5% and 77.6 ± 3.2% inhibition for 20 mM and 160 mM ethanol, respectively. Furthermore, there was a significant dose-dependent decrease in ERK2 activity in ethanol treated smooth muscle cells as compared to control smooth muscle cells. These data demonstrate an inhibitory effect of ethanol on smooth muscle cell proliferation and MAPK signalling in vitro. It is tempting to speculate that these actions of ethanol may contribute to its cardiovascular effects in vivo. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Alcohol; Smooth muscle cell proliferation; MAPK (mitogen activated protein kinase)

1. Introduction

A biphasic effect of alcohol on the incidence of cardiovascular disease has been documented, where moderate consumption of ethanol exerts a protective effect (Rimm et al., 1996; Doll et al., 1994) while chronic alcohol abuse is associated with a higher incidence of cardiovascular disorders such as stroke, hypertension and coronary artery disease (Dyer et al., 1981; Klatsky et al., 1992). The precise mechanism whereby ethanol elicits these effects is unclear.

Arterial smooth muscle cell proliferation plays an important role in the normal development of blood vessels, the pathogenesis of atherosclerosis and the arterial response to injury (for review Schwartz et al., 1995; Schwartz and Liaw, 1993). In addition, accelerated smooth muscle cell proliferation is a characteristic feature in arteries of hypertensive patients and animals (Dzau and Gibbons, 1993; Cho et al., 1997). Consequently, there has been extensive interest in defining both positive and negative regulators of smooth muscle cell growth and many factors have been identified that may play a role in this process. An ethanol-induced reduction in neointimal formation following balloon injury has been reported in both rabbit and pig models (Merritt et al., 1997; Liu et al., 1997). However, the precise mechanism of this effect was not determined. While ethanol has been shown in vitro to inhibit the proliferation of glial cells (Luo and Miller, 1996; Guizzetti and Costa, 1996), its effect on vascular smooth muscle cells has not been defined.

Several studies have provided compelling evidence for a role of mitogen activated protein kinases (MAPKs) in regulating smooth muscle cell growth (Mii et al., 1996).

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MAPKs, (also called extracellular signal-regulated kinases or ERKs) are a family of proline-directed protein kinases activated as part of a cascade by phosphorylation on both threonine and tyrosine residues. MAPKs are rapidly activated in response to ligand binding by both growth factor receptors with intrinsic tyrosine kinase activity, such as the platelet derived growth factor and epidermal growth factor receptor, and receptors that are coupled to heterotrimeric guanine nucleotide binding proteins (G proteins) such as the thrombin receptor (Pelech and Sanghera, 1992; Boulton et al., 1991; L'Allemain et al., 1991). Members of the MAPK family include the ERKs (ERK-1 and ERK-2) which are activated by MEK (also referred to as MAPK kinase). In proliferating cells it has been postulated that activated ERK-MAPKs phosphorylate specific cytoplasmic and nuclear proteins needed for passage through certain checkpoints in the cell cycle (e.g., G1/S and G2/M) (Tamemoto et al., 1992; Pages et al., 1993).

In the present study we demonstrate that ethanol, at physiologically relevant concentrations, inhibits serum-stimulated growth and MAPK activity in cultured smooth muscle cells.

2. Materials and methods

2.1. Materials

[^{3}H]Thymidine (~100 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from New England Nuclear (Boston, MA). A specific antibody raised to pick up the phosphorylated forms of ERK1 and ERK2 ($_{pp}$44ERK1, $_{pp}$42ERK2) was purchased from Promega (Madison, WI). Anti-rabbit IgG (horseradish peroxidase linked) and ECL detection system were obtained from Amersham (Arlington Heights, IL). 4-Methylpyrazole was from Sigma (St. Louis, MO). Myelin basic protein (MBP), RPMI 1640 and fetal calf serum (FCS) were purchased from Gibco BRL (Gaithersburg, MD). All other chemicals were of the highest purity commercially available.

2.2. Cell culture

Rat vascular smooth muscle cells were isolated and cultured as previously described (Cahill et al., 1990). Briefly, thoracic aortae of male Sprague–Dawley rats (150 and 175 g, Crl:CD(SD)BR-CD, Charles River Labs, MA) were stripped of fat and connective tissue and digested in minimal essential medium (MEM) containing 0.7 mg/ml collagenase (Type IA, Sigma), 0.25 mg/ml elastase (type III, Sigma), 0.4 mg/ml soybean trypsin inhibitor and 1 mg/ml bovine serum albumin for 30 min at 37°C. Following adventitia removal and further incubation in the enzyme solution, dissociated vascular smooth muscle cells were seeded into conventional plastic tissue culture plates (Falcon) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (SMC growth media) in a humidified atmosphere of 5% CO_2, 95% air. Cells were routinely subcultured after treatment for 5 min with 0.125% trypsin-EDTA at 37°C. Cells between passage 5–9 were used for these studies.

2.3. Ethanol treatment

In ethanol treated cells, 200 proof ethanol was diluted and added to the medium to achieve desired concentrations for the specified time. Media ethanol concentration was determined over time, in the absence or presence of smooth muscle cells, using a commercially available ethanol assay kit (Sigma, St. Louis, MO). Cell viability, in the presence of ethanol, was evaluated using the trypan blue exclusion assay and by comparing gross morphology to that of control cells.

2.4. Thymidine incorporation

Smooth muscle cells were plated on 24 well plates (Beckton Dickinson, Franklin Lakes N.J.) at a density of 50,000 cells/well and allowed to grow for 3–4 days in smooth muscle cell growth media. Smooth muscle cells were then placed in serum-depleted media (RPMI 1640 medium supplemented with 0.2% heat-inactivated fetal bovine serum, plus 100 U/ml penicillin and 100 μg/ml streptomycin) for 48 h. Cells were then exposed to 5% fetal calf serum media which causes a robust increase in [^{3}H] thymidine incorporation in these cells, with or without ethanol (0.08–320 mM) for 24 h, unless otherwise specified. Cells were pulsed with [^{3}H] thymidine (1 μCi/well)

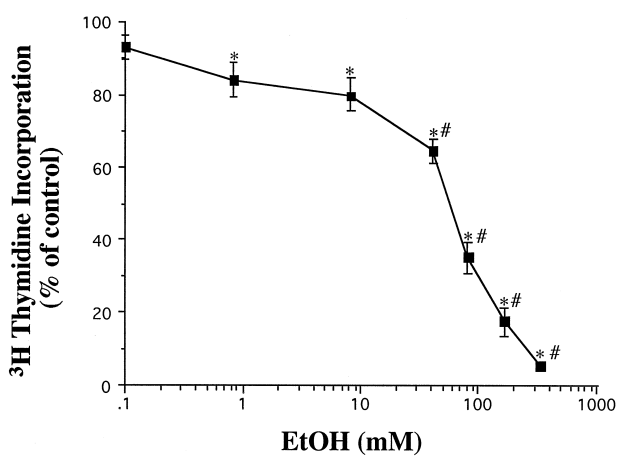

Fig. 1. The effect of ethanol on smooth muscle cell [^{3}H]thymidine incorporation. Quiesced smooth muscle cells in 24 well plates were treated with 5% fetal calf serum (FCS) media with or without ethanol (0.1–320 mM EtOH) for 24 h. ^{3}H-Thymidine incorporation was determined as described in Section 2. Data are expressed as percentage of the control (5% FCS media) and are the means ± S.E.M., $n = 5$. $*$ $P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. previous ethanol concentration. Control incorporation of [^{3}H]Thymidine was 11,470 ± 986 cpm/well.

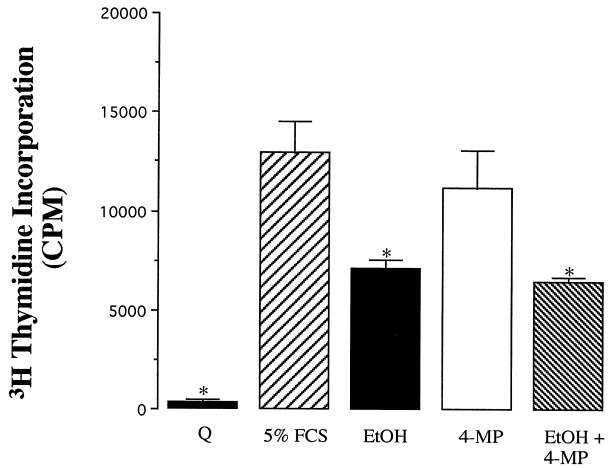

Fig. 2. The effect of 4-methyl pyrazole (4-MP) on ethanol inhibition of smooth muscle cell mitogenesis. Cells were treated for 24 h in the absence (5% fetal calf serum media, control) or presence of 80 mM ethanol (EtOH), with or without 4-methylpyrazole (4-MP, 10 μM), before ^{3}H-Thymidine incorporation was determined as described in Section 2. Data are the means ± S.E.M., $n = 3$. $* P < 0.05$ vs. 5% FCS. Q = quiesced cells.

between hour 18–22 and cells processed 2 h later for DNA incorporated [^{3}H] thymidine as previously described (Cahill and Hassid, 1993).

2.5. Cell counts

The change over time in the number of cells in a well was used as an index of cell proliferation. Smooth muscle cells were plated on 24 well plates (Beckton Dickinson, Franklin Lakes N.J.) at a density of 20,000 cells/well. Following a 48 h period in serum-depleted media, cells in parallel wells were exposed to growth media (RPMI-1640 containing 10% FCS and antibiotics) with or without ethanol (80 mM). Cell counts of parallel triplicate wells were made on a daily basis using a light microscope and a hemocytometer (Advanced Neubaumer). Media with or without ethanol was replaced daily.

2.6. Preparation of cell lysates

Harvested cells were placed in ice cold lysis buffer containing 25 mM HEPES, 300 mM NaCl, 1.5 mM $MgCl_2$, 200 μM EDTA, 1% Triton X-100, 20 mM β-glycerophosphatase, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 0.5 mM dithiothreitol, 100 mM Na_3VO_4, 100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin and adjusted to pH 7.5. Lysates were subjected to ultrasonification using a sonic dismembrator (Fisher Scientific, Pittsburgh, PA). Samples were then aliquoted and stored at −80°C prior to use for Western Blot analysis and in myelin basic protein (MBP) phosphorylation assays.

2.7. MAPK activity

(i) Smooth muscle cells were placed in serum-depleted media (RPMI 1640 medium supplemented with 0.2% heat-inactivated fetal calf serum, plus 100 U/ml penicillin and 100 μg/ml streptomycin) for 48 h. They were then stimulated for 15 min with 5% or 0.5% fetal calf serum as indicated, in the absence or presence of ethanol. 5% fetal calf serum caused a more robust increase in MAPK activity when compared to 0.5% fetal calf serum. The two serum concentrations were used to determine if the effect of ethanol could be overcome by increasing the percent serum, similar to changing the concentration of antagonist titered against an agonist. Cell lysates were prepared and MAPK activity determined by measuring specific phosphorylated $_{pp}$44ERK − 1 and $_{pp}$42ERK − 2 expression by immunoblot as described previously (McKillop et al., 1997). Equal protein loading was confirmed by India-ink staining of protein in each lane of the same blot. The signal intensity (integral volume) of the appropriate bands on the autoradiogram was analyzed using a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA) and the Imagequant software package (Biosoft, Indianapolis, IN).

(ii) To determine the functional activity of ERK2 proteins in control or ethanol treated SMC, a phosphorylation reaction was performed using myelin basic protein (MBP) as a substrate for ERK2, as described previously (McKil-

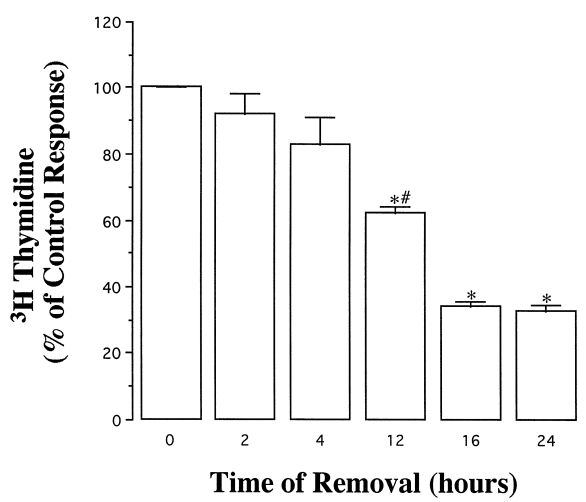

Fig. 3. Time course of the effect of ethanol on smooth muscle cell mitogenesis: 'delayed removal experiment'. Quiesced cells were incubated in the presence of 5% fetal calf serum media for 24 h. Ethanol (EtOH, 80 mM) was present for the first 2, 4, 12, or 16 h or for the whole 24 h period. Data are expressed as percentage of control (5% FCS only, 24 h) and are the means ± S.E.M., $n = 3$. $* P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. 24 h ethanol response.

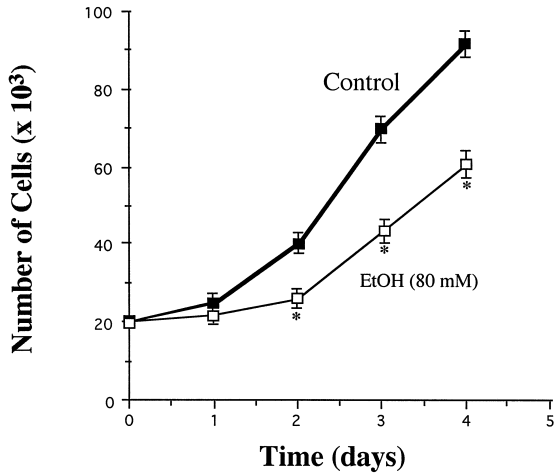

Fig. 4. The effect of ethanol on smooth muscle cell proliferation: cell counts. Smooth muscle cells were seeded at a density of 20,000 cell/well in a 24 well plate and treated with growth media (RPMI 1640 containing 10% fetal calf serum; control), in the absence or presence of ethanol (EtOH, 80 mM) as described in Section 2. Cell counts of parallel triplicate wells were made on a daily basis. Data are means ± S.E.M. of a representative experiment. ∗ $P < 0.05$ vs. control.

lop et al., 1997). Briefly, 50 μg cell lysate protein was incubated with 0.1 μg anti-ERK2 antibody (Santa Cruz, La Jolla, CA) for 60 min at 4°C to facilitate immunoprecipitation. Following incubation, gamma-bind G-sepharose beads (Pharmacia Biotech) were added and the mixture was allowed to incubate for a further 15 min at 4°C. The sepharose beads were then precipitated at 5000 × *g* for 60 s at 4°C. The kinase reaction was performed on the pellet fraction by the addition of 10 μCi [γ-^{32}P] adenosine triphosphate, 20 μmol/l adenosine triphosphate, 3.3 mmol/l dithiothreitol, and 40 μg myelin basic protein, followed by incubation for 30 min at 30°C. Reactions were terminated by the addition of 10 μl of Laemmli buffer and boiling samples for 5 min. Samples were resolved on a 15% Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE), covered in saran wrap and vacuum-dried for 2 h at 80°C. Gels were then exposed to X-ray film with an intensifying screen for 4–6 h, phosphorylated myelin basic protein being detected at 21 kDa. Densitometric analysis was performed as described above.

2.8. Statistics

The data shown are the mean ± S.E.M. n = number of individual experiments, each performed in triplicate. Statistical significance was estimated using the following analy-

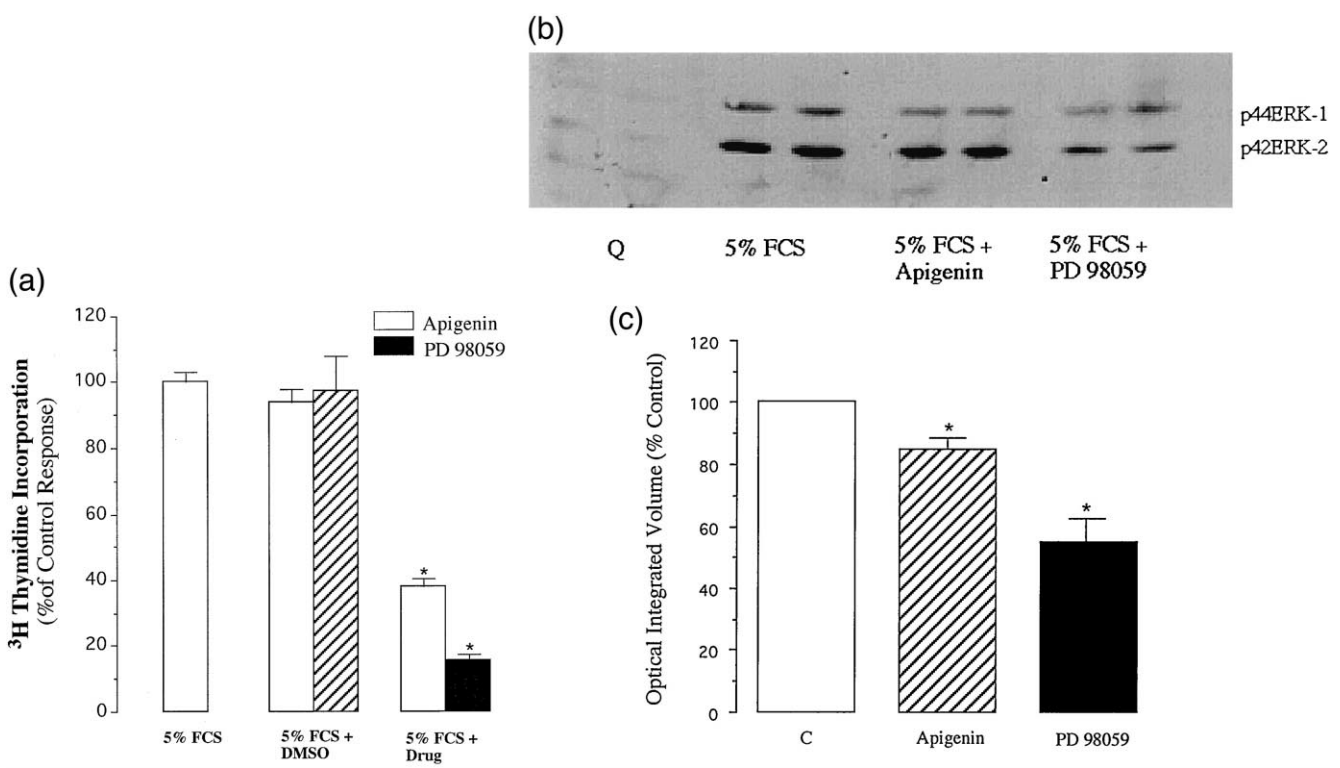

Fig. 5. (a)The effect of MAPK inhibitors on serum-stimulated smooth muscle cell mitogenesis. Quiesced cells were treated for 24 h with 5% fetal calf serum media, in the absence or presence of apigenin (25 μM) or PD 98059 (10 μM) before [^{3}H]thymidine incorporation was determined. Data are expressed as percentage of control (5% FCS) and are the means ± S.E.M., $n = 3$. ∗ $P < 0.05$ vs. control. DMSO was the vehicle control. (b) The effect of Apigenin and PD 98059 on MAPK activity. Quiesced SMC were stimulated for 15 min with or without 5% FCS media, in the absence or presence of apigenin (25 μM) or PD 98059 (10 μM), and MAPK activity determined by measuring specific phosphorylated ERK1 and ERK2 ($_{pp}$44ERK − 1 and $_{pp}$42ERK − 2) by immunoblot. A representative Western blot is shown (top panel; Q = quiesced cells) together with the cumulative densitometric data of at least 3 separate experiments (lower panel). ∗ $P < 0.05$ vs. control (FCS alone).

sis: Unpaired Student's *t*-test for comparison of two groups; Wilcoxon–Signed Rank test for the densitometric data. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Effect of ethanol on smooth muscle cell [^{3}H] thymidine incorporation

To determine the effect of ethanol on the proliferation of vascular smooth muscle cells in vitro, we measured [^{3}H] thymidine incorporation into cell DNA as one index of cell growth. Ethanol treatment (0–320 mM for 24 h) potently inhibited serum-stimulated mitogenesis in a concentration-dependent manner with significant inhibition observed at 0.8 mM ($16 \pm 1\%$, $p < 0.05$, $n = 8$), and a maximal inhibition ($95 \pm 18\%$, $n = 8$) observed at 320 mM (Fig. 1). The IC_{50} (i.e., the concentration required to cause 50% inhibition of maximal response) for ethanol was ~ 60 mM. Ethanol, at the concentrations used, had no significant effect on smooth muscle cell viability as assessed by trypan blue exclusion (data not shown). Ethanol concentration in 24-well plates over the experimental period (24 h) was $> 90\%$ of the original concentration. Thus, evaporation was not considered to be a confounding factor. The concentration was the same in the absence or presence of smooth muscle cells suggesting that there was no significant metabolism of ethanol occurring.

To confirm that the observed inhibition by ethanol was caused by the alcohol itself and not by its metabolites, the alcohol dehydrogenase inhibitor 4-methylpyrazole was used. SMC were treated for 24 h in the absence or presence of ethanol (80 mM), with or without 4-methylpyrazole (10 μM). As demonstrated in Fig. 2, 4-methylpyrazole did not significantly affect the inhibitory effect of ethanol on smooth muscle cell ^{3}H-thymidine incorporation.

The temporal relationship between ethanol and inhibition of serum-stimulated smooth muscle cell mitogenesis was assessed following different ethanol incubation times in 'delayed removal' experiments. Ethanol was present for various times, then removed and the incubation continued up to 24 h in the presence of serum alone. When ethanol was present for 12 h, then removed, it elicited a significant

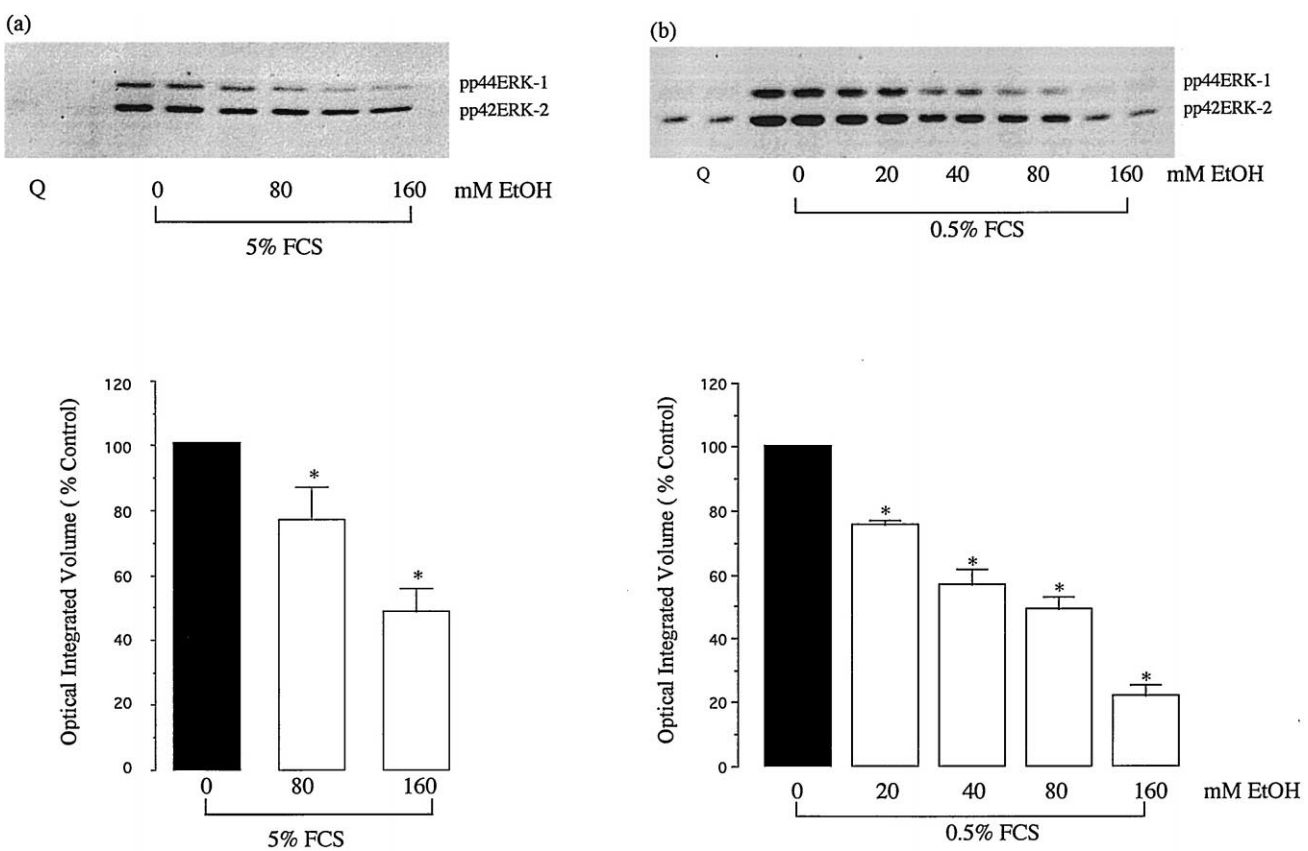

Fig. 6. The effect of ethanol on MAPK activity. Smooth muscle cells were quiesced following 48 h in serum-depleted media. They were then stimulated for 15 min with or without (a) 5% serum media or (b) 0.5% serum media, in the absence or presence of the indicated concentrations of ethanol (EtOH), and MAPK activity determined by measuring specific phosphorylated ERK1 and ERK2 ($_{pp}$44ERK − 1 and $_{pp}$42ERK − 2) by immunoblot. Representative Western blots are shown (top panel a,b), together with the cumulative densitometric data of at least 3 separate experiments (lower panel a,b). $* P < 0.05$ vs. control (FCS alone).

inhibition of ^{3}H-thymidine incorporation (Fig. 3). The maximal inhibition of proliferation by ethanol was reached after incubation for 16–24 h (Fig. 3).

3.2. Effect of ethanol on cell growth

The growth of smooth muscle cells in media containing 10% fetal calf serum (growth media), in the absence (control) or presence of ethanol, was determined by daily cell counts of trypsinized cells. The number of SMC in control media, following a lag phase, rose exponentially (Fig. 4). Ethanol treatment (80 mM) caused a significant shift to the right in the growth curve for these cells; population doubling time was approximately 48 h and 70 h for control and ethanol treated cells, respectively (Fig. 4).

3.3. Effect of ethanol on MAPK activity

3.3.1. (i) phosphorylated ERK1 / ERK2 expression

Several studies support a key role for mitogen activated protein kinases (MAPKs) in regulating SMC growth (Mii et al., 1996). In quiesced cells, serum (5% fetal calf serum) addition stimulated smooth muscle cell mitogenesis and MAPK activity, an effect that was inhibited with specific MEK inhibitors (Apigenin 25 μM, and PD 098059 10 μM); ~60% and ~85% inhibition of ^{3}H-thymidine incorporation (Fig. 5a), and ~15% and ~55% inhibition of MAPK activity (Fig. 5b) for apigenin and PD 098059, respectively. In separate experiments, MAPK activity in control and ethanol treated cells was determined by measuring specific phosphorylated ERK1 and ERK2 ($_{pp}$44ERK − 1 and $_{pp}$42ERK − 2) expression by immunoblot. Serum (5% fetal calf serum) rapidly stimulated MAPK activity in a time-dependent fashion, with a maximum increase at 5–15 min (data not shown). Ethanol significantly reduced serum-stimulated MAPK activity; 23.4 ± 9.2% and 51.8 ± 7.2% inhibition for 80 and 160 mM ethanol, respectively (Fig. 6a). In addition, when the serum concentration was reduced from 5% to 0.5%, ethanol dose-dependently inhibited MAPK activity to an even greater extent; 24.5 ± 1.5%, 43 ± 4.6%, 50.7 ± 3.4% and 77.6 ± 3.2% inhibition for 20 mM, 40 mM, 80 mM and 160 mM ethanol, respectively (Fig. 6b).

3.3.2. (ii) Functional activity of ERK2; myelin basic protein assay

MAPK (ERK2) activity was assessed as a measure of the ability of immunoprecipitated ERK2 to phosphorylate myelin basic protein. Using this technique, a single protein band was detected at 21 kDa (Fig. 7). The detection of this

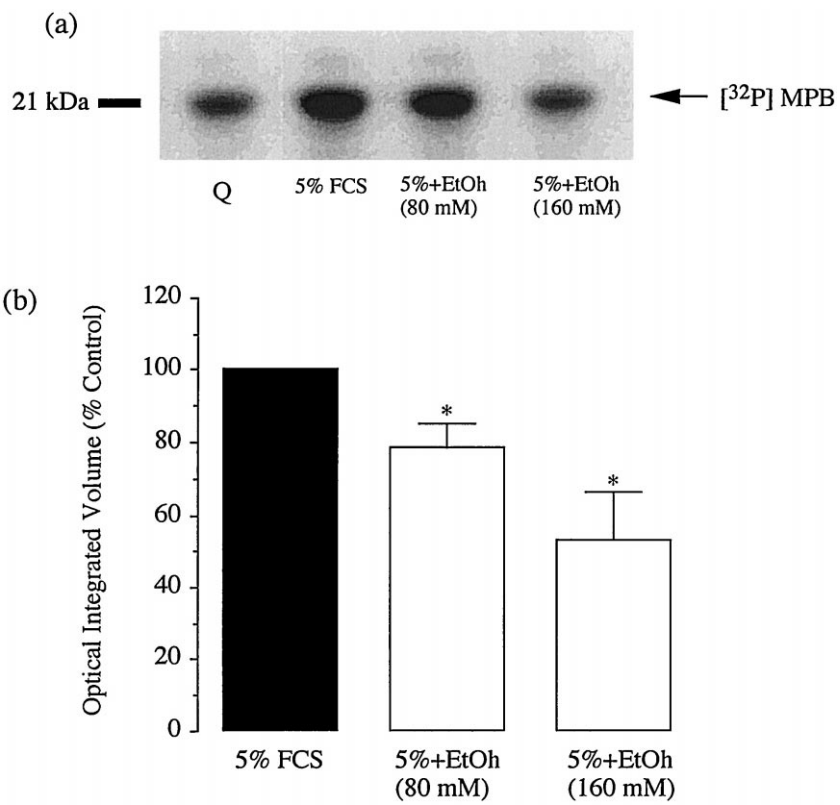

Fig. 7. (a) Representative gel illustrating functional activity of immunodetectable ERK2 proteins as assessed by the ability to catalyze myelin basic protein (MBP) phosphorylation. Following immunoprecipitation with an ERK2 specific antibody, samples were incubated with MBP and [^{32}P]-ATP and resolved on an 15% SDS-PAGE gel. Subsequent exposure of the gel to X-ray film revealed one major band in the 21 kDa molecular weight range identified as [^{32}P]-MBP. (b) Cumulative densitometric data of 3 separate experiments. $* P < 0.05$ vs. 5% FCS (control).

antigen was linear with respect to protein concentration. There was a significant decrease in detection of [^{32}P] myelin basic protein in ethanol treated smooth muscle cell as compared to control cells (Fig. 7); 21 ± 6% and 47 ± 15% inhibition for 80 and 160 mM ethanol, respectively.

4. Discussion

The current studies define for the first time an inhibitory effect of ethanol on MAPK signalling and smooth muscle cell proliferation in vitro. Our results clearly demonstrate that treatment of cultured vascular smooth muscle cells with ethanol decreased MAPK activity and growth of these cells in a dose-dependent manner. Because of the important role the MAPK pathway plays in controlling smooth muscle cell growth, decreased activity of MAPK's may be of critical importance to the antimitogenic response of ethanol in these cells.

Previous studies have demonstrated the antiproliferative effects of ethanol in several diverse cell types including several glial cell lines (Luo and Miller, 1996), primary hepatocytes (Carter and Wands, 1988), fibroblasts (Resnicoff et al., 1993), HL-60 myeloid leukemia cells (Cook et al., 1990), human bone cells (Friday and Howard, 1991), human T cells (Imperia et al., 1984), and rat hepatoma cells (Higgins, 1987). While ethanol has been shown to inhibit neointimal hyperplasia in coronary arteries following balloon injury (Merritt et al., 1997; Liu et al., 1997) few studies have addressed the direct effects of ethanol on vascular smooth muscle cell growth. Alcohol feeding attenuated postinjury cell proliferation following balloon angioplasty in rabbits (Merritt et al., 1997). The preservation of arterial lumen diameter was achieved by decreasing neointimal proliferation, in part, by decreasing low density lipoprotein (LDL) oxidation in these animals (Merritt et al., 1997). However, the main pathogenesis of neointimal formation is smooth muscle cell migration, proliferation and extracellular matrix production. Moreover, in a recent study using rabbit iliac arteries following balloon angioplasty, significant inhibition of phenotype conversion from contractile to synthetic was observed following ethanol treatment, indicative of an inhibition of smooth muscle cell proliferation (Liu et al., 1996). Our results in vitro support such a contention since ethanol directly inhibited smooth muscle cell mitogenesis and proliferation, an effect that was independent of metabolites of ethanol since 4-methylpyrazole, an alcohol dehydrogenase inhibitor, did not significantly alter the antimitogenic effect of ethanol in these cells. That ethanol had no effect on cell number after 24 h, in contrast to its effect on [^{3}H] thymidine incorporation and MAPK activity, is not surprising since 10% serum did not significantly increase cell number after 24 h. However, following 48 h treatment when there was a significant increase in cell number, ethanol had a significant anti-proliferative effect.

Epidemiological data suggests that the effect of alcohol consumption on the incidence of cardiovascular disease (encompassing coronary artery disease, atherosclerosis, stroke, hypertension etc.) is biphasic or J-shaped; i.e., low to moderate consumption (generally considered as 1–3 drinks/day) is beneficial, whereas heavy consumption (> 4 drinks/day) is harmful (Doll et al., 1994; Klatsky et al., 1992). However, the effect of ethanol on smooth muscle cell proliferation and MAPK activity reported here is concentration-dependent. There are many possible reasons for this apparent discrepancy. While higher doses of ethanol may still potently inhibit smooth muscle cell proliferation, other deleterious effects of ethanol at these concentrations may predominate in vivo. Indeed, several studies have demonstrated a strong dose–response relation between increasing alcohol consumption and decreasing incidence of coronary heart disease (Pearson, 1996) whereas a J-shaped relation exists between alcohol consumption and total mortality indicating that heavier drinkers have a greater risk of death from non-cardiovascular causes including several kinds of cancer, cirrhosis, and suicide (Pearson, 1996). Furthermore, in this study the effect of a 24 h exposure to ethanol was examined whereas epidemiological data generally reflects alcohol exposure over years.

One key regulatory enzyme pathway for control points within the cell cycle has been identified as mitogen-activated protein kinases (MAPK's) (Mii et al., 1996; Pelech and Sanghera, 1992; Boulton et al., 1991; L'Allemain et al., 1991). Several studies have provided compelling evidence for a role of MAPK's in regulating smooth muscle cell growth (Mii et al., 1996; Pelech and Sanghera, 1992; Boulton et al., 1991; L'Allemain et al., 1991). MAPK signaling, in particular ERK activity, is increased in rat carotid arteries following balloon injury concomitant with enhanced medial cell proliferation (Koyama et al., 1998). Furthermore, inhibition of MAPK signaling with PD 098059, a MAPK kinase (MEK) inhibitor, reduced medial cell replication following injury (Koyama et al., 1998). In the present study, we confirm that inhibition of MAPK signaling in vitro with PD 098059 or another MEK inhibitor, apigenin, resulted in a dramatic decrease in smooth muscle cell mitogenesis. Moreover ethanol, at concentrations that significantly inhibited smooth muscle cell mitogenesis and growth, acutely inhibited MAPK signaling. Specifically, ethanol inhibited MEK activity since phosphorylated $_{pp}$44ERK − 1 and $_{pp}$42ERK − 2 expression, a function of MEK activity (Daaka et al., 1998), was significantly reduced in these cells. Moreover, ethanol decreased $_{pp}$42ERK − 2 activity since phosphorylation of myelin basic protein, a function of $_{pp}$42ERK − 2 activity, was also significantly reduced in these cells following acute exposure to ethanol. The temporal relationship between ethanol and inhibition of serum-stimulated smooth muscle cell mitogenesis suggests that ethanol may be acting at G1 or at the G1/S interface of the cell cycle. While most studies have shown that MAPKs are activated

at the G0/G1 boundary, they are also activated in cells progressing from G1 to S and are re-activated late in mitosis (Edelmann et al., 1996). Moreover, peak *ras* activation following serum stimulation, an upstream regulator of MAPK signaling and a potential target for ethanol, occurred at mid G1 phase of the cell cycle (Taylor and Shalloway, 1996). Taken together the temporal association between ethanol's antimitogenic effects and inhibition of ERK activity suggests that ethanol inhibits smooth muscle cell mitogenesis, in part, by decreasing MAPK signaling in these cells.

The mechanism of ethanol's induced changes in smooth muscle cell MAPK signaling and growth remains unclear. The decreased phosphorylation of ERK-1 and ERK-2 by MEK in cells exposed to ethanol suggests that MEK inhibition determines ERK inhibition by ethanol in these cells. Similar conclusions were reported recently for agonist-stimulated MEK activity in that the efficacy of agonist stimulated Mek activation determined the susceptibility of ERK to inhibition in smooth muscle cells (Plevin et al., 1996). The importance of Mek activation and phosphorylation of ERK to the growth of cells is evident from studies using novel MEK inhibitors, PD 098059 and apigenin (Dudley et al., 1995). Ethanol has also been shown to interact with several membrane-associated signal transduction systems resulting in both positive and negative regulation of adenylyl cyclase (Hoffman and Tabakoff, 1990), phosphoinositide-specific phospholipase C (Hoek and Rubin, 1990), and phospholipase A_2 (Rubin, 1989). In many cases the effects of ethanol appear to be at the level of intermediary GTP-binding proteins (Sara and Hall, 1990) or on the enzyme itself as is the case for ethanols' effect on protein kinase C (Slater et al., 1993). It is generally assumed that ethanol exerts its effects by perturbation of the membrane lipid bilayer, other hydrophobic sites such as protein-membrane interfaces, or proteins themselves (Taraschi and Rubin, 1985). Indeed, several studies have demonstrated that ethanol can inhibit the proliferation of cells at the level of growth factor receptor expression (Resnicoff et al., 1993). Ethanol inhibited basic fibroblast growth factor (bFGF)-induced proliferation of C6 astrocytoma cells (Luo and Miller, 1996). In addition ethanol, at concentrations similar to those used in the current study, markedly inhibited cell growth stimulation in response to insulin like growth factor-1 (IGF-1) and depressed tyrosine phosphorylation of the IGF-1 receptor (Resnicoff et al., 1993). Since ethanol generally induces G1 phase arrest in diverse cell types in vitro, an effect that is also observed in SMC, it is possible that inhibition of specific growth factor receptor autophosphorylation may in part account for the antiproliferative effects of ethanol in SMC. Similar effects on other tyrosine kinase-linked receptors such as epidermal growth factor receptors have been reported (Thurston and Shukla, 1992), despite no effect of ethanol on the platelet derived growth factor receptor pathway (Resnicoff et al., 1993).

In conclusion, the current studies demonstrate for the first time an inhibitory effect of physiologically relevant concentrations of ethanol on smooth muscle cell MAPK signaling and growth in vitro. Changes in MAPK signaling may represent an important mechanism whereby ethanol can regulate vessel wall function and structure following injury and thereby contribute to the cardiovascular effects of alcohol.

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