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# Ethanol differentially modulates the expression and activity of cell cycle regulatory proteins in rat aortic smooth muscle cells

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**Abstract**

The aim of this study was to determine the effect of ethanol on cell cycle events during the $G_1$ and S phases in cultured vascular smooth muscle cells (VSMC). Flow cytometric analysis for the DNA content in rat aortic VSMC indicated that following ethanol treatment, the cell population in the $G_0/G_1$ phase increased; 57.8 ± 1.6% vs. 72.3 ± 1.2%, concomitant with a decrease in cells in the S phase; 12.7 ± 1.4% vs. 3.67 ± 0.6%, for control vs. ethanol, respectively. Western blot analysis on VSMC lysates demonstrated that ethanol (10–160 mmol/l) dose-dependently inhibited serum-induced retinoblastoma (pRb) hyperphosphorylation. While having no effect on Cdk2 protein expression, ethanol dose-dependently decreased ($IC_{50}$ ~ 60 mmol/l) Cdk2 activity, assessed by histone H1 phosphorylation. Furthermore, ethanol induced the expression of the cyclin-dependent kinase (Cdk) inhibitor $p21^{waf1/cip1}$, and inhibited the induction of cyclin A. These data demonstrate that modulation of the expression and activity of key cell cycle regulatory molecules may be a mechanism by which ethanol inhibits VSMC proliferation. These actions of ethanol may be relevant to its cardiovascular protective effect in vivo. 

*Keywords:* Alcohol; Vascular smooth muscle; Proliferation; Cell cycle; Atherosclerosis

## 1. Introduction

Chronic alcohol abuse is associated with increased morbidity and mortality owing to hypertension, cardiomyopathy, hepatic cirrhosis and certain cancers as well as accidents and suicides (Tunstall-Pedoe et al., 1999; Klatsky et al., 1992; Thun et al., 1997). In contrast, epidemiological studies have associated moderate alcohol consumption with a reduced incidence of cardiovascular disease (Tunstall-Pedoe et al., 1999; Klatsky et al., 1992; Thun et al., 1997; Friedman and Kimball, 1986; Goldberg et al., 2001). While the favorable effect of ethanol on plasma lipoprotein levels is believed to be a predominant protective mechanism, potentially beneficial effects of ethanol on platelets, endothelial cells, and vascular smooth muscle cells have been more recently recognized (Rubin, 1999; Davda et al., 1993; Zhang et al., 1992; Hendrickson et al., 1998; Redmond et al., 2000).

In addition to playing an important role in the normal development of blood vessels, the migration and proliferation of vascular smooth muscle cells play a key role in the progression of atherosclerosis and in the development of restenosis (Ross, 1993; Schwartz et al., 1995). In the arterial media, vascular smooth muscle cells are normally quiescent, proliferate at low indices (< 0.05%), and remain in the $G_0/G_1$ phase of the cell cycle (Gordon et al., 1990). After vessel injury, vascular smooth muscle cells migrate into the intimal layer of the arterial wall, where they leave their quiescent state and reenter the cell cycle (Ross, 1993). Progression through the mammalian cell cycle is directed by multiple holoenzymes comprising a catalytic cyclin-dependent kinase (Cdk) and a cyclin regulatory subunit. Functional Cdk/cyclin complexes are presumed to phosphorylate target proteins that facilitate cell cycle progression. Different cyclin/Cdk complexes are required for direction through various stages of the cell cycle (Li and Brooks, 1999). The kinase activities of the cyclin/Cdk complexes are negatively regulated by Cdk inhibitors. Two families of Cdk inhibitors have been identified: the INK4 family and the Kip/Cip family. The Kip/Cip family includes $p21^{waf1/cip1}$, $p27^{kip1}$, and $p57^{kip2}$; and the INK4 family includes p14, $p15^{INK4B}$,

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$p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$. The Kip/Cip family displays a broader specificity than the INK4 family and is referred to as general inhibitors (Li and Brooks, 1999). It has been demonstrated that $p21^{waf1/cip1}$ is upregulated in arteries after balloon catheter injury and overexpression of $p21^{waf/cip1}$ in vascular smooth muscle cells results in $G_1$ arrest and inhibition of cell growth (Yang et al., 1996; Chang et al., 1995). In quiescent cells, retinoblastoma protein (Rb) is present in a hypophosphorylated state (pRb) that enables it to bind to and sequester members of the E2F family of transcription factors (Weinberg, 1995). Cyclin/Cdk complexes can phosphorylate pRb and thus release the sequestered E2F transcription factors. This enables E2F to activate the transcription of genes required for further cell cycle progression (Nevins, 1992).

Ethanol has been shown to inhibit neointimal hyperplasia in coronary arteries following balloon angioplasty in ethanol fed rabbits (Merritt et al., 1997). Local delivery of ethanol by balloon catheters in balloon-injured coronary arteries has also been shown to inhibit intimal hyperplasia (Liu et al., 1997). We have previously demonstrated a direct inhibitory effect of ethanol on vascular smooth muscle cell proliferation in vitro (Hendrickson et al., 1998). To elucidate further the mechanism underlying the ethanol-induced inhibition of vascular smooth muscle cell proliferation, we examined the effects of ethanol on cellular events during the $G_1$ and S phases, including pRB phosphorylation, Cdk2 activity and the expression of cyclin A, Cdk2, and of the Cdk inhibitor $p21^{waf1/cip1}$.

## 2. Materials and methods

### 2.1. Smooth muscle cell isolation and culture

Rat vascular smooth muscle cells (VSMC) were isolated and cultured as previously described (Cahill et al., 1990). Briefly, thoracic aortae of male Sprague–Dawley rats (150 and 175 g, Cr1: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, St. Louis, MO), 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 and 37 °C. Following adventitia removal and further incubation in the enzyme solution, dissociated VSMC were seeded into conventional plastic tissue culture plates (Falcon, Becton Dickenson, Franklin Lakes, NJ) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biosource Int., Camarillo, CA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin, in a humidified atmosphere of 5% $CO_2$, 95% air. Cells were routinely subcultured after treatment for 2 min with 0.125% trypsin–EDTA at 37 °C. Cells between passages 11 and 18 were used for these studies.

### 2.2. Ethanol treatment

In ethanol-treated cells, 200 proof ethanol (Pharmco Products, Brookfield, CT) was diluted and added to VSMC medium to achieve desired concentrations. Cultures were then incubated for various times at 37 °C. To avoid evaporation of ethanol, Petri dishes were immediately sealed in Parafilm. Cell viability, in the presence of ethanol, was evaluated by trypan blue exclusion assay and by comparing gross morphology to that of control cells.

### 2.3. Thymidine incorporation

VSMC were plated on 24 well plates (Corning, Corning, NY) at a density of 25,000 cells/well and allowed to reach approximately 70% confluence. Cells were then placed in serum-depleted media for 48 h. Cells were then exposed to 5% fetal calf serum for 24 h, in the absence or presence of ethanol at various concentrations. Cells were pulsed with [$^3$H] thymidine (1 μCi/well) between hours 18 and 22 and cells processed 2 h later for DNA incorporated [$^3$H] thymidine as previously described (Hendrickson et al., 1998).

### 2.4. Flow cytometry

Cellular DNA content was assessed by flow cytometry. VSMC were seeded into 100-mm plates and grown to approximately 70% confluence. The cells were quiesced for 48 h and then exposed to 5% fetal calf serum in the presence or absence of ethanol for 24 h. The cells were harvested and adjusted to a concentration of $1 \times 10^6$ cells/ml. Cells were fixed with ice-cold 70% ethanol and stained with 50 μg/ml of propidium iodide (PI). Samples were analyzed with a flow cytometer (FACSCalibur cytometer, Cell Quest software; Becton Dickinson).

### 2.5. Western blotting

Cells were lysed with ice-cold lysis buffer (pH 8.0) containing (in mmol/l) Tris–HCl 50, NaCl 150, EDTA 1, dithiothreitol 1, $Na_3VO_4$ 0.2, phenylmethylsulfonyl fluoride 1, and leupeptin 0.1; 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1% NP-40. Western blot analysis was performed as described previously (Redmond et al., 1999). Immunoblotting was performed with monoclonal or polyclonal antibodies against retinoblastoma protein (14001A, Pharmingen, San Diego, CA), cyclin A (C-19), Cdk2 (M2), and $p21^{waf1/cip1}$ (H-164) (Santa Cruz Biotechnology, Santa Cruz, CA). Equal protein loading in each lane was confirmed by staining membranes with Ponceau S red. Autoradiogram bands were quantitated by NIH Image 1.60.

### 2.6. Immunoprecipitation and kinase assay

Lysates were prepared as above and then clarified by centrifugation at $10,000 \times g$ for 10 min at 4 °C. Approx-

imately 200 μg of protein lysate was added to 1 μg of anti-Cdk2 antibody and incubated for 1 h at 4 °C. The immunocomplexes were precipitated by addition of Protein A Agarose (Santa Cruz Biotechnology). Immunoprecipitated proteins on beads were washed three times with lysis buffer and then twice with kinase buffer (50 mM Tris, 10 mM $MgCl_2$, 20 μM ATP, 1 mM NaF, 0.1 mM $Na_3V0_4$, and 1 mM dithiothreitol, pH 7.5). Kinase activity of the immunopellet was assayed using histone H1 (Life Technologies) as substrate (3 μg histone H1 and 5 μCi [γ-$^{32}$P] ATP in kinase buffer) for 30 min at 37 °C. The reaction was terminated by addition of Laemmli sample buffer and separated on 12.5% SDS-polyacrylamide gel. The gel was dried and autoradiography performed. Autoradiogram bands were quantitated by NIH Image 1.60.

### 2.7. Statistics

The data shown are the mean ± S.E.M. $n$ = number of individual experiments, with a minimum of three independent experiments performed. Statistical significance was estimated using the following analysis: unpaired Student's $t$-test for comparison of two groups; and 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 DNA synthesis

To determine the effect of ethanol on the proliferation of VSMC, we measured [$^3$H] thymidine incorporation into cell DNA as one index of cell growth. We confirmed our previous findings (Hendrickson et al., 1998) that ethanol treatment (0.1–320 mmol/l, 24 h) potently inhibited serum-stimulated mitogenesis in a concentration-dependent manner with an $IC_{50}$ = 60 mmol/l (data not shown). Ethanol, at the concentrations used, had no significant effect on VSMC viability as assessed by trypan blue exclusion. Morphologically, the cells were the same as control cells as determined by light microscopy.

### 3.2. Ethanol inhibits $G_1 \rightarrow S$ progression of VSMC

The effect of ethanol on cell cycle progression was determined by flow cytometry. Subconfluent VSMC were synchronized by serum starvation for 48 h, with 79.4 ± 3.6% of cells arrested in $G_0/G_1$ phase and 2.1 ± 0.18% present in S phase (Fig. 1A). Quiescent VSMC were induced to enter S phase by stimulation with 5% fetal calf serum. The population of $G_0/G_1$ cells decreased significantly (57.8 ± 1.6%), with a concomitant rise in S phase cells (12.7 ± 1.4%) (Fig. 1B). Treatment with ethanol (160 mmol/l, 24 h) inhibited fetal calf serum-stimulated $G_1 \rightarrow S$ progression, as shown by the increase in $G_0/G_1$ cells (72.3 ± 1.2%) accompanied by a concurrent decrease in S phase cells (3.67 ± 0.6%) (Fig. 1C).

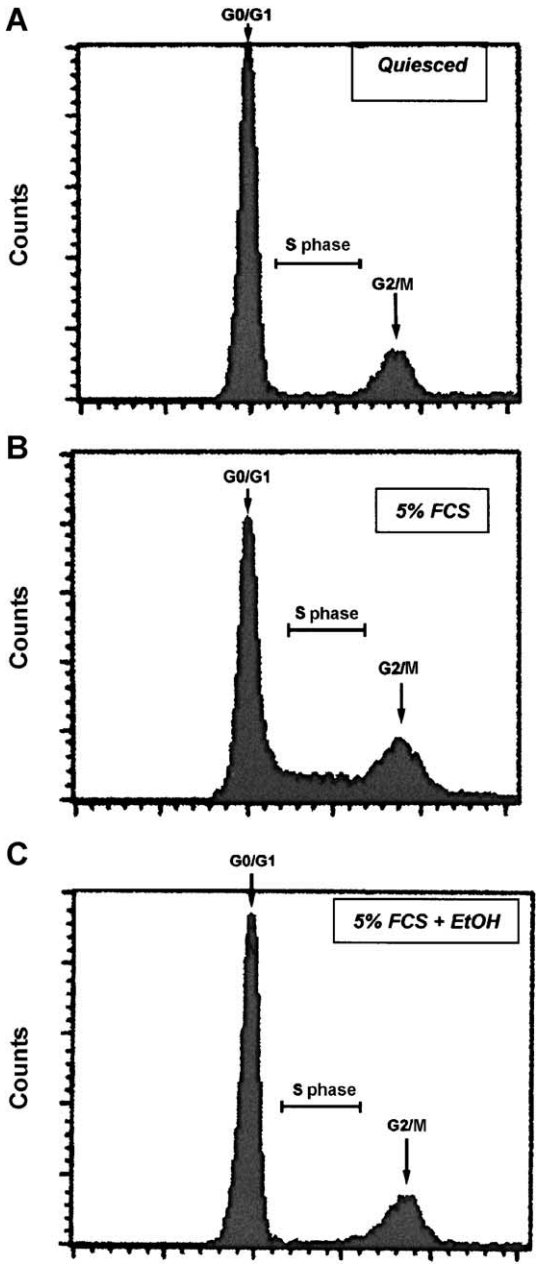


Fig. 1. The effect of ethanol on the cell cycle. Quiesced VSMC (serum starved for 48 h) were stimulated (24 h) with 5% fetal calf serum (FCS) in the absence or presence of ethanol. DNA was stained by propidium iodide (PI) and the fluorescence evaluated by flow cytometry. The $x$- and $y$-axes represent the intensity of PI fluorescence and cell number, respectively. (A) Quiescent VSMC, (B) 5% FCS, and (C) 5% FCS + ethanol (160 mmol/l).

### 3.3. Effect of ethanol on phosphorylation of pRb protein

Hyperphosphorylation of pRb is required for $G_1 \rightarrow S$ transition in most mammalian cells. Therefore, to interpret the mechanism by which ethanol inhibits proliferation of VSMC, we examined its effect on pRb phosphorylation. Rb

migrates in an SDS-polyacrylamide gel as multiple, closely spaced bands reflecting varying degrees of phosphorylation. In quiescent cells, only the hypophosphorylated form of Rb protein (pRb) was detectable (Fig. 2). After 16 h of mitogenic stimulation with 5% fetal calf serum, a mobility shift of Rb was observed which is indicative of increased phosphorylation (ppRb). Ethanol inhibited this serum-induced phosphorylation in a dose-dependent manner (Fig. 2). As calculated from densitometric measurements, doses of 10, 20, 40, and 80 mmol/l ethanol led to 20.5 ± 5%, 34.7 ± 9%, 50.9 ± 6%, and 80.1 ± 2% inhibition, respectively, of pRb phosphorylation compared with serum stimulated controls ($n = 3$). Ethanol at 160 mmol/l or greater resulted in essentially complete inhibition of pRb phosphorylation.

### 3.4. Effect of ethanol on mitogen-induced activation of Cdk2 activity

To elucidate the mechanism by which ethanol inhibits pRb phosphorylation, we measured the expression and activity of Cdk2 for which pRb is a putative physiological substrate. Cdk2 levels were low in quiescent cells and were increased after 16 h of mitogenic stimulation with 5% fetal calf serum. Ethanol (1–160 mmol/l) had no significant effect on Cdk2 expression as determined by Western blot analysis (Fig. 3A). We next measured the activity of Cdk2 using histone H1 as a phosphorylation substrate. The activity of Cdk2 was low in quiescent and in early $G_1$ cells. A 15–20-fold increase was evident following 16 h of stimulation with 5% fetal calf serum. Treatment of VSMC with ethanol resulted in a dose-dependent decrease in Cdk2 activity (Fig. 3B); ethanol: 10 mmol/l, 14.4 ± 1.5% inhibition; 40 mmol/l, 40.5 ± 6.18% inhibition; 80 mmol/l, 55.6 ± 4.7% inhibition; and 160 mmol/l, 83.9 ± 9.6% inhibition.

### 3.5. Effect of ethanol on the expression of $p21^{waf1/cip1}$

The activities of Cdks are controlled by the Cdk inhibitor proteins, which include Cip/Kip family members, such as $p21^{waf1/cip1}$, and INK4 family members, such as $p15^{INK4b}$ and $p16^{INK4a}$ (Liu et al., 1997). Because $p21^{waf1/cip1}$ is known to inhibit the activities of many cyclin/Cdk complexes, we investigated the effect of ethanol on $p21^{waf1/cip1}$ expression.

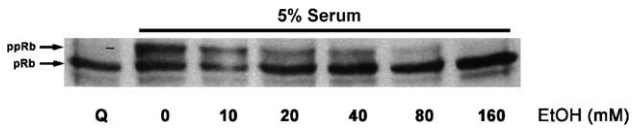


Fig. 2. Ethanol inhibits phosphorylation of retinoblastoma protein (Rb). Quiesced (Q) VSMC were treated with 5% fetal calf serum (FCS) in the absence or presence of ethanol (10–160 mmol/l) for 16 h. Hypophosphorylated (pRb) and hyperphosphorylated (ppRb) states of Rb are indicated by arrows. A Western blot representative of three individual experiments is shown.

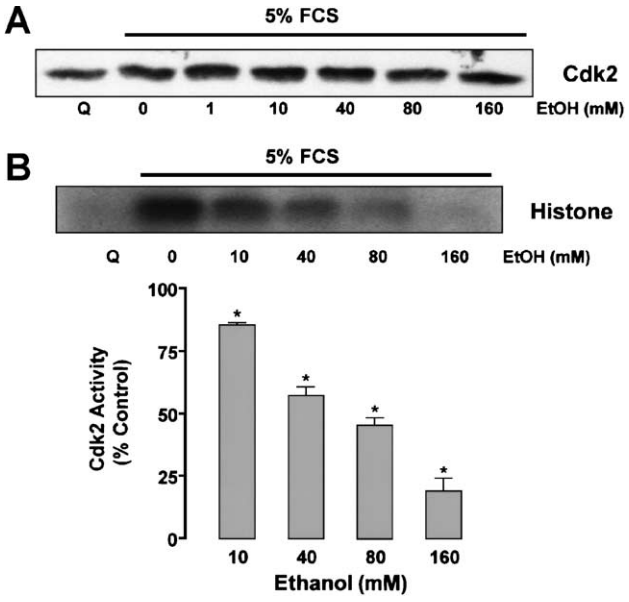


Fig. 3. Effect of ethanol on Cdk2 expression and activity. Quiesced (Q) VSMC were treated with 5% fetal calf serum (FCS) in the absence or presence of ethanol (1–160 mmol/l) for 16 h. (A) Representative Western blot showing no significant effect of ethanol on Cdk2 protein expression. (B) Representative blot showing dose-dependent inhibition of Cdk2 activity, as determined by histone H1 phosphorylation (top panel), together with the cumulative densitometric data of at least three separate experiments (lower panel). * $P < 0.05$ vs. control (FCS alone).

Abundant $p21^{waf1/cip1}$ protein expression was detected in quiescent VSMC (serum depleted for 2 days). Following 5% fetal calf serum stimulation, $p21^{waf1/cip1}$ expression decreased substantially (Fig. 4). Addition of ethanol (10 or

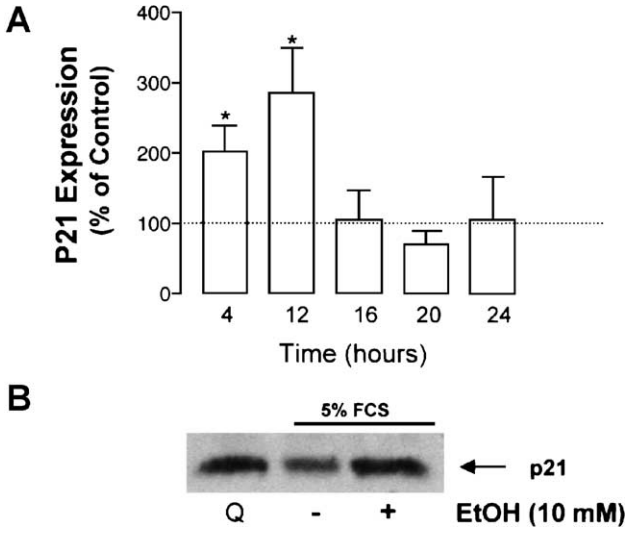


Fig. 4. Effect of ethanol on $p21^{waf1/cip1}$ expression. (A) Quiesced VSMC were stimulated with 5% fetal calf serum (FCS), for various times, in the presence of 160 mmol/l ethanol. Results (mean ± S.E.M, $n = 4$) are expressed as percent control (in the absence of ethanol). * $P < 0.05$ vs. control (FCS alone). (B) Representative western blot demonstrating p21 expression in quiesced (Q) and 5% FCS-stimulated VSMC ± ethanol (10 mmol/l) (6 h).

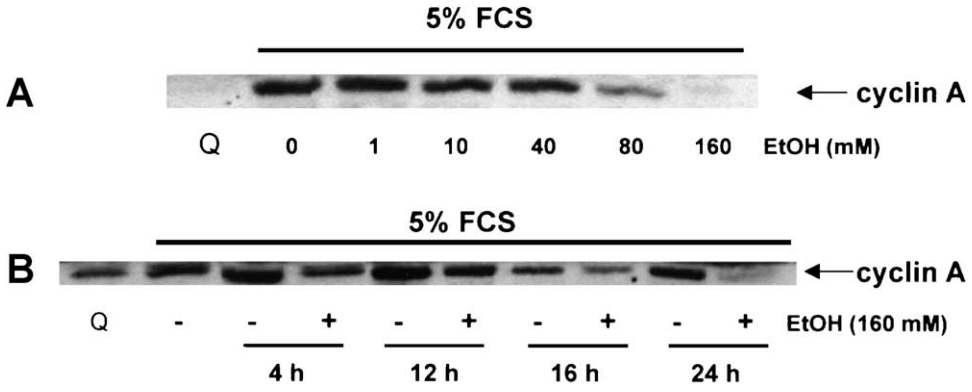


Fig. 5. Ethanol inhibits cyclin A expression. Representative Western blots of (A) quiescent VSMC stimulated with 5% FCS (16 h) in the absence or presence of various doses of ethanol (1–160 mmol/l) and (B) quiescent VSMC stimulated with 5% FCS in the absence or presence of ethanol (160 mmol/l) for various times (4–24 h). Experiments were repeated with similar results.

160 mmol/l) significantly induced $p21^{waf1/cip1}$ (Fig. 4). The expression of $p21^{waf1/cip1}$ in ethanol-treated cells was 222 ± 30% compared with corresponding controls (fetal calf serum alone) (10 mmol/l, 6 h, $P < 0.01$, and $n = 5$).

### 3.6. Effect of ethanol on cyclin A

Cyclin A is essential for cell cycle progression in S phase, and its expression is regulated through the E2F transcription factor, whose activity is, in turn, regulated by the phosphorylation of pRb. Quiescent VSMC expressed detectable levels of cyclin A which increased as early as 4 h after serum stimulation (Fig. 5). Ethanol inhibited the induction of cyclin A in a dose-dependent manner (Fig. 5A). Significant inhibition of cyclin A expression by ethanol was seen as early as 4 h and by 24 h, almost complete inhibition was observed (Fig. 5B).

## 4. Discussion

The major finding of our study is that in cultured vascular smooth muscle cells, passages 11–18, ethanol differentially modulated the expression/activity of key cell cycle regulatory molecules and inhibited the progression from $G_1$ to the S phase of the cell cycle. In particular, ethanol induced the expression of the important cell cycle inhibitor $p21^{waf1/cip1}$ and inhibited Cdk2 activity, resulting in a corresponding decrease of hyperphosphorylated Rb. In addition, ethanol inhibited the induction of cyclin A. These findings provide a possible molecular mechanism mediating the inhibitory effect of ethanol on vascular smooth muscle cell proliferation.

The proliferation of vascular smooth muscle cells plays a crucial role in the formation of vascular lesions, such as fibrous plaques in atherosclerosis and intimal thickening after balloon angioplasty (Ross, 1993; Schwartz et al., 1995). Accelerated smooth muscle cell proliferation is also a characteristic feature in arteries of hypertensive patients and animals (Dzau and Gibbons, 1993; Cho et al., 1997). Consequently, inhibition of vascular smooth muscle cell proliferation represents a potentially important therapeutic strategy for the treatment of diseases such as atherosclerosis and restenosis. The eukaryotic cell cycle is regulated by cyclins, Cdks, and their inhibitors. In mammalian cells, progression in $G_1$ phase and entry into S phase is believed to require the activity of Cdk2 in complex with cyclin A (Li and Brooks, 1999). As the name suggests, cyclins are a family of proteins which are synthesized and destroyed during each cell cycle. All cyclins currently described share an ~150 amino acid region of homology (the 'cyclin box') that binds to the N-terminal end of specific Cdks (McGill and Brooks, 1995). Cyclin A expression late in $G_1$ is important for $G_1$-to-S transition, because the inhibition of cyclin A kinase prevents S phase entry (Pagano et al., 1992). Ethanol inhibited both Cdk2 activity and cyclin A expression.

Several signals that control the cell cycle converge onto Rb (Li and Brooks, 1999). Hypophosphorylated Rb (pRB) binds to and inhibits several proteins involved in initiating DNA replication, such as E2F transcription factors. The pRb hyperphosphorylated by Cdks during late $G_1$ loses this ability and allows the cell to advance into S phase. Quiescent VSMC expressed high levels of pRb consistent with the fact that these cells are not in cycle. Ethanol inhibited serum-stimulated pRB phosphorylation. Because Cdk2 phosphorylates pRb in vitro (Akiyama et al., 1992) and may be a mediator of $G_1$/S transition (Pagano et al., 1993), the inhibition of Cdk2 activity may explain the ethanol-induced diminution of pRB phosphorylation.

Cdk activities are negatively regulated by Cdk inhibitor proteins. $p21^{waf1/cip1}$, also known as senescent cell-derived inhibitor 1 (Sdi 1) (Noda et al., 1994), has broad specificity and binds to various $G_1$ cyclin/Cdk complexes, including cyclin A–Cdk2 complexes to inhibit their activities (Xiong et al., 1993). In addition to its ability to inhibit Cdks,

$p21^{waf1/cip1}$ can bind directly to and inhibit the function of proliferating cell nuclear antigen which is a cofactor of DNA polymerase δ and is required during DNA replication and repair (Waga et al., 1994). These two distinct inhibitory activities of $p21^{waf1/cip1}$ reside in separate domains of the protein (Luo et al., 1995). The amino-terminal domain of $p21^{waf1/cip1}$ interacts with and inhibits Cdk2, whereas the carboxyl-terminal domain inhibits proliferating cell nuclear antigen. Ethanol induced the expression of $p21^{waf1/cip1}$ in VSMC. Other agents known to induce $p21^{waf1/cip1}$ include transforming growth factor-β (Datto et al., 1995), prostaglandin A2 (Gorospe et al., 1996), and nitric oxide (Ishida et al., 1997). Whether ethanol affected $p21^{waf1/cip1}$'s Cdk2- and/or proliferating cell nuclear antigen-inhibitory activity requires further investigation. Regardless, the fact that ethanol induced $p21^{waf1/cip1}$ expression predicts that ethanol prevents cell cycle progression. While our studies were performed on VSMC in vitro, the relevance of $p21^{waf1/cip1}$ in the context of vascular disease has been previously documented in vivo. For example, $p21^{waf1/cip1}$ is upregulated in arteries after vascular injury (Yang et al., 1996) and in the rat carotid artery balloon angioplasty model adenovirus-mediated overexpression of $p21^{waf1/cip1}$ in VSMC results in $G_1$ arrest and inhibition of cell growth (Chang et al., 1995). Tanner et al. (1998) reported that while $p21^{waf1/cip1}$ levels were undetectable in normal human arteries, they were elevated in atherosclerotic arteries and functioned to inhibit cell proliferation during arterial repair.

There have been previous reports of an effect of ethanol on cell cycle regulatory molecules in other tissues. Ethanol enhanced transcription of the $p21^{waf1/cip}$ gene and inhibited Cdk2 activity in human epithelial cells (Guo et al., 1997), while ethanol infusion increased p21 gene expression in the isolated perfused heart (Jankala et al., 2001). However, an ethanol-mediated inhibition of $p21^{waf1/cip}$ expression and increased pRb phosphorylation was reported in squamous cell carcinoma cell lines of the head and neck (Hager et al., 2001). These data, taken together with our results in VSMC suggest a tissue-specific, cell-dependent effect of ethanol on the regulatory molecules of the cell cycle. Vascular smooth muscle cells in normal arteries are abundant in contractile proteins and cannot proliferate (contractile phenotype) (Schwartz et al., 1995; Cappadona et al., 1999). However, the vascular smooth muscle cells in the neointima formed during atherosclerosis and restenosis after angioplasty have dedifferentiated properties (synthetic phenotype) (Bochaton-Piallat et al., 1996), similar to the cells used in this study. It may be of interest, therefore, to compare the effect of ethanol on vascular smooth muscle cells of different phenotype. Interestingly, a stimulatory effect of ethanol on the production of nitric oxide (NO) by endothelial cells has previously been reported (Davda et al., 1993; Hendrickson et al., 1999). NO itself is a potent inhibitor of vascular smooth muscle cell proliferation (Garg and Hassid, 1989; Van der Leyen et al., 1995). Taken together with our data, this suggests that ethanol may affect vascular smooth muscle cell growth directly by modulating cell cycle molecule expression and activity, and indirectly via its effect on endothelial cell NO production.

$Ca^{2+}$ and $Mg^{2+}$ are both believed to play an important role in cell proliferation (Bornfeldt et al., 1994; Wolf and Cittadini, 1999). Ethanol has been previously reported to cause a decrease in cytosolic-free $Ca^{2+}$ (Zhang et al., 1992), and a rapid depletion of VSMC intracellular $Mg^{2+}$ (Altura et al., 1995). In addition, ethanol has been reported to adversely affect mitochondrial cytochrome oxidase (Kennedy, 1998) and ATP synthesis (Devi et al., 1994). Therefore, the possibility that these effects of ethanol on ion distribution and/or cellular bioenergetic metabolism are responsible for modulation of cell cycle regulatory molecules and inhibition of VSMC proliferation warrants further investigation.

'Moderate' alcohol consumption is generally considered to be in the range 1–3 drinks/day (Tunstall-Pedoe et al., 1999; Klatsky et al., 1992; Thun et al., 1997; Friedman and Kimball, 1986; Goldberg et al., 2001), giving rise to blood alcohol levels of approximately 5–25 mmol/l. A blood alcohol level of 0.1 g%, the legal limit in many states, is approximately equivalent to 25 mmol/l ethanol. In alcoholics, blood alcohol level can reach in excess of 100 mmol/l. In this study, significant effects of ethanol on the activity and expression of key cell cycle regulatory proteins were demonstrated at concentrations less than 20 mmol/l. While a large number of observational studies have consistently demonstrated a J-shaped relation between alcohol consumption and total mortality, it is also clear that a consistent, strong, dose–response relation exists between increasing alcohol consumption and decreasing incidence of coronary heart disease (Pearson, 1996). The precise mechanism(s) responsible for the protective effect of ethanol against cardiovascular disease are not yet fully understood. Because coronary heart disease accounts for more than one-third of total deaths, abstainers have higher total mortality than those drinking 1–2 drinks/day. The J-shaped distribution for total mortality is thus considered to be the sum of the protective effect on coronary heart disease mortality and the detrimental effect of high levels of consumption on other causes of death such as stroke, cancers, cirrhosis, accidents, and suicide (Pearson, 1996).

The importance of our data and their relevance to the in vivo efficacy of ethanol remain speculative. However, our data demonstrate that ethanol inhibits key cellular events during the $G_1$ and S phases of the cell cycle in vascular smooth muscle cells. These effects of ethanol may play a role in the likely multifactorial process of ethanol-mediated protection against cardiovascular disease.

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