Ethanol Inhibits Fibroblast Growth Factor–Induced Proliferation of Aortic Smooth Muscle Cells

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Objective—Epidemiological studies have demonstrated that moderate alcohol consumption reduces mortality associated with coronary artery disease. The protective effect is correlated with the amount of ethanol consumed but is unrelated to the form of alcoholic beverage. Adoption of a favorable lipoprotein profile accounts for about half of the protective action of alcohol, but the remaining causative factors remain conjectural. Fibroblast growth factors (FGFs) play important roles in mediating smooth muscle cell (SMC) proliferation and migration, which are key factors in the atherosclerotic process. In the present study, we examined the effect of ethanol on FGF-mediated SMC growth and signaling.

Methods and Results— Pharmacologically relevant concentrations of ethanol inhibited the proliferation of a rat aortic SMC line (SV40LT-SMCs) in response to FGF1 and FGF2. Human aortic SMC growth was similarly inhibited by ethanol. Transition into the G_2/M phase was specifically affected. FGF-mediated phosphorylation of p42/p44 mitogen-activated protein kinase (MAPK) c-Raf, MAP kinase kinase kinase, MEK1/2 MAP kinase, kinase, stress-activated protein kinase/c-Jun–NH_2-terminal kinase, and p38 MAPK were variably reduced by ethanol. The inhibition of intracellular signaling by ethanol was correlated with inhibition of FGF receptor autophosphorylation. By contrast, neither epidermal growth factor receptor autophosphorylation nor epidermal growth factor–mediated p42/p44 MAPK activation was affected by ethanol.

Conclusions—The findings identify the FGF receptor as an inhibitory target for ethanol, which could account in part for the inhibitory actions of ethanol on SMC proliferation observed in vivo. (*Arterioscler Thromb Vasc Biol*. 2003;23: 1808-1813.)

Key Words: atherosclerosis ■ ethanol ■ fibroblast growth factor ■ cell cycle ■ aortic smooth muscle cells

Epidemiological studies have demonstrated that moderate alcohol consumption decreases the risk for complications of coronary artery disease (CAD) by as much as 50%, including myocardial infarction and cardiac death.[1] The lower risks for CAD-related mortality among the French (the "French paradox") were originally proposed to be due to red wine consumption.[2] However, the phenomenon is noted worldwide, and the protection against CAD is correlated with the amount of ethanol consumed rather than the type of alcoholic beverage.[3] The mechanism by which alcohol consumption reduces the risks for CAD appears to be multifactorial.[4] At least half of the benefit has been related to an increase in HDL levels in moderate drinkers.[5] Furthermore, long-term ethanol feeding reduced the severity of early atherosclerotic lesions in hyperlipidemic mice.[6] Ethanol inhibits human platelet activation[7] and enhances endothelial fibrinolytic activity,[8] both of which are important for thrombogenesis. However, the role of these effects of ethanol on protection against CAD, as well as other factors, remains conjectural.[9]

Intimal smooth muscle cells (SMCs) play a key role in the development of atherosclerotic lesions.[10] SMCs proliferate minimally in the intact artery but are stimulated to divide after arterial deendothelialization owing, in large measure, to the local accumulation of growth factors and cytokines at the injury site.[11] The mitogenic actions of fibroblast growth factors (FGFs) are keys to SMC proliferation. Notably, experimental reduction in FGF expression inhibits SMC proliferation after intimal injury in humans and laboratory animals.[12,13] A role for FGF2 and the FGF receptor 1 (FGFR-1) on the migration of SMCs has also been documented in vitro.[14]

In common with several other cell types,[15–18] the growth of SMCs in vitro is inhibited by ethanol. Hendrickson et al[19] reported that ethanol inhibits mitogen-activated protein kinase (MAPK) activity and the growth of SMCs in vitro. Ethanol also inhibits intimal hyperplasia in the arteries of pigs[20] and rabbits[21] after angioplasty.

In this study, we investigated the mechanism by which ethanol interferes with SMC proliferation. Pharmacologically relevant concentrations of ethanol inhibited the induction of SMC growth by FGF1 and FGF2. This was associated with a profound reduction in intracellular signaling mediators down-

stream of the FGFR and inhibition of FGFR-2 tyrosine autophosphorylation. The findings identify FGFR-2 as an inhibitory target of ethanol action.

Methods

Cell Cultures
SV40-immortalized rat aortic SMCs (SV40LT-SMCs) and human aortic SMCs (T/G HA-VSMCs) were obtained from American Type Culture Collection. Human cells were grown in dishes coated with rat tail type I collagen (BD-Biotech). SV40LT-SMCs were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, penicillin (100 IU/mL), streptomycin (100 μg/mL), and G418 (100 μg/mL, Mediatech). T/G HA-VSMCs were maintained in MDCB-131 SMC proliferation medium (BD-Biotech) supplemented with 5% fetal calf serum and human recombinant FGF2 (2 ng/mL), epidermal growth factor EGF (0.5 ng/mL), and insulin (5 μg/mL, Sigma).

Cell Growth Assays
Cells were seeded at a density of 10 000 cells/cm^2 in 12-well plates and cultured overnight. The cells were incubated for 24 hours in serum-free medium 199 (BD-Biotech) supplemented with 0.2% bovine serum albumin (SV40LT-SMCs) or MDCB131 (T/G HA-VSMCs) medium containing 5 μg/mL insulin. Growth factors were then added with or without ethanol. To compensate for the evaporation of ethanol, the medium was replaced daily. At selected times, cells were harvested by mild trypsinization and counted with a hematocytometer. In some experiments, cell growth was assessed with the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfenyl)-2H-tetrazolium)-based assay (Promega). For this procedure, cells were seeded in 96-well plates and incubated as described earlier. Assays were performed by adding MTS directly to the culture wells, incubating them for 30 minutes, and then recording the absorbance at 490 nm with a 96-well plate spectrophotometric reader (Dynatech MR600).

Cell Cycle Analysis
Subconfluent cells were incubated for 24 hours with 50 ng/mL FGF2 in the presence or absence of 85 mmol/L ethanol. After trypsinization, cells were fixed in 70% ethanol and incubated in 200 μg/mL RNAse I and 10 μg/mL propidium iodine for 30 minutes. Flow cytometry was performed with a commercially available instrument (Coulter Epics).

Western Immunoblotting
Cells were lysed in 50 mmol/L Tris-HCl–150 mmol/L NaCl, pH 7.5, buffer containing 1% Nonidet P40, 0.5% sodium deoxycholate, 100 mmol/L NaF, 2 mmol/L Na$_3$VO$_4$, 10 mmol/L phenylmethylsulfonylfluoride, 500 μmol/L 4-(2-aminoethyl)-benzenesulfonylfluoride, 150 nmol/L aprotinin, and 1 μmol/L leupeptin. Five micrograms of protein was electrophoresed in 12% sodium dodecyl sulfate denaturing polyacrylamide slab gels. After transfer to nitrocellulose membranes, bands were visualized by reaction with specific antiphosphoprotein antibodies directed against phospho–c-Raf, phospho–mitogen-activated protein kinase/extracellular signal–regulated kinase 1/2 (MEK1/2), phospho–p42/p44 mitogen-activated protein kinase (MAPK), phospho–stress-activated protein kinase/c-Jun–NH$_2$-terminal kinase (SAPK/JNK), and phospho–p38 MAPK (Cell Signaling). In brief, membranes were blocked in 5% bovine serum albumin and probed with anti-phosphoprotein antibody (1:1000) for 1 hour at room temperature. After secondary incubation in horseradish peroxidase–conjugated goat anti-mouse or goat-anti-rabbit IgG antibody (1:10 000) (Sigma), the immunocomplexes were visualized with an enhanced chemiluminescence kit (ECL) from Pierce. Band intensity was quantified by volume densitometric scanning of nonsaturated autoradiograms by using a software package (IMAGE, Scion Scientific).

FGFR-1, FGFR-2, or EGFR was immunopurified from cell lysates by addition of 4 μg/mL specific monoclonal antibody (Santa Cruz
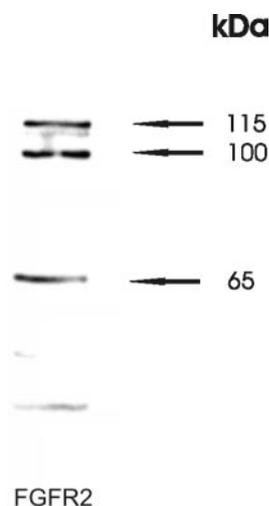
Figure 1. FGFR expression in rat SV40LT-SMCs. Cell lysates were probed for FGFR-2 by Western blot analysis, as described in Methods.

Biotech) to 100 μL of cell lysate. After a 16-hour incubation at 4°C, the immuncomplexes were captured on protein G–conjugated agarose (Roche Diagnostics) for 3 hours at 4°C. After recovery by centrifugation, the agarose pellets were washed twice with buffer containing 50 mmol/L Tris-HCl, pH 7.5, 500 mmol/L NaCl, 0.1% Nonidet P40, and 0.05% sodium deoxycholate, followed by 2 washes in 10 mmol/L Tris-HCl, pH 7.5, 0.1% Nonidet P40, and 0.05% sodium deoxycholate. Samples were analyzed on a 7% sodium dodecyl sulfate polyacrylamide gel, and the isolated proteins were transferred to nitrocellulose by electroblotting. Membranes were blocked by overnight incubation at 4°C in phosphate-buffered saline, pH 7.5, containing 0.1% Tween 20 (TBS) and 5% dried low-fat milk. Receptor phosphorylation was detected by incubating the membranes with anti-phosphotyrosine monoclonal antibody PY99 (1:1000, Santa Cruz) in TBS buffer containing 1% dried low-fat milk followed by incubation with secondary horseradish peroxidase–conjugated goat anti-mouse IgG antibody at 1:10 000 for 1 hour. Membranes were developed by an enhanced chemiluminescence reaction and exposed for autoradiography.

Statistics
Groups of data were analyzed by a 2-tailed Student's *t* test.

Results

FGFR Expression in Rat SV40LT-SMCs
The rat aortic SMC (SV40LT-SMC) cell line maintains defined SMC characteristics for >200 population doublings, including muscle-specific actin expression and growth inhibition by heparin.[22,23] It displays a low proliferative rate in serum-free medium while maintaining its phenotype. By contrast, cultures of SMCs from rat and human aortic explants can be maintained in culture for only a few passages and do not survive serum-free conditions for more than 24 to 48 hours. Thus, SV40-LT SMCs were used for studies of ethanol effects on SMC proliferation.

The FGFR expression pattern of SV40LT-SMCs is shown Figure 1. Blotting for FGFR-2 revealed 2 major bands at ≈115 and 100 kDa and another of lower intensity at 65 kDa. The 115-kDa band has the same size as the phosphorylated receptor and likely corresponds to the intact receptor or to the largest of the splicing variants expressed by this cell line.[24]
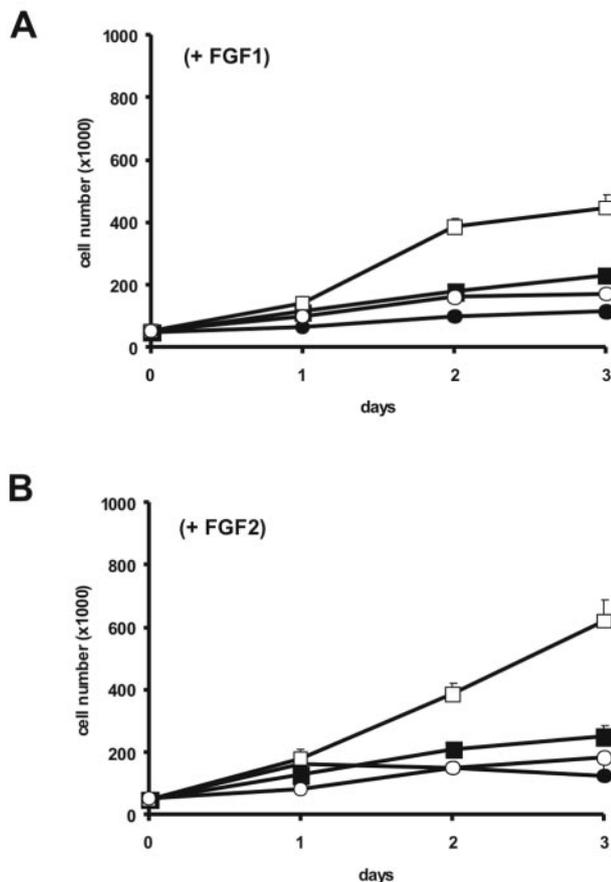
Figure 2. Ethanol-mediated inhibition of SMC proliferation. Serum-starved SV40LT-SMCs were treated with either FGF1 (A) or FGF2 (B) (50 ng/mL each) in the presence or absence of ethanol (85 mmol/L). Cell counts were determined at the indicated times. ○ Indicates control with no addition; ●, ethanol alone; □, FGF; and ■, FGF plus ethanol. Each data point is the mean±SD of quadruplicate determinations.

The 100- and 65-kDa bands might represent proteolytic products of FGFR-2. Their intensities were not affected by ethanol, nor were they phosphorylated. FGFR-1 was undetectable in SV40LT-SMCs (not shown). Positive controls for the anti–FGFR-1 antibody were demonstrated in colon carcinoma cell lines HCT116 and WiDr (not shown).

Ethanol Inhibits FGF-Dependent SMC Proliferation

FGF1 and FGF2 are both ligands for FGFR-2,[24] and an autocrine loop of FGF has been identified in SMCs, which is necessary for the survival of these cells in culture.[25] SV40LT-SMCs proliferated minimally in serum-free medium. Both FGF1 and FGF2 at 50 ng/mL induced SMC proliferation at a rate equivalent to that observed in the presence of 10% fetal calf serum (not shown). Ethanol (85 mmol/L) had minimal effect on the basal proliferative rate in serum-free medium alone but significantly decreased FGF1- and FGF2-induced proliferation (Figure 2) without causing cell detachment or death. The effect of ethanol on the FGF1- and FGF2-mediated proliferation was, however, different. Whereas 20 mmol/L ethanol was sufficient to maximally affect cell

Effect of Ethanol on Cell Cycle Progression

Treatment	Percent of Cells		
	G1	S	G2/M
None	69.4±1.5	18.1±1.7	12.4±1.5
Ethanol (85 mmol/L)	75.7±2.3*	14.9±2.4*	9.5±1.2*
FGF2 (50 ng/mL)	64.2±1.0*	22.1±0.6*	13.6±0.8
FGF2 (50 ng/mL)/Ethanol (85 mmol/L)	69.3±1.4†	19.6±1.3†	11.1±0.8*†

Values are mean±SD; n=5.
*$P<0.05$ vs untreated group; †$P<0.05$ vs FGF2 alone group

growth in response to FGF2 (77% inhibition), FGF1-induced proliferation required much higher ethanol concentrations (45% inhibition with 85 mmol/L ethanol; online Figure I at http://atvb.ahajournals.org). Maximal cell growth was observed at 50 ng/mL FGF1 and FGF2. Higher concentrations of FGF1 partially overcame the effect of ethanol (57% inhibition at 150 ng/mL FGF1), whereas inhibition by ethanol was not counteracted by even 150 ng/mL FGF2 (online Figure II). Ethanol had a similar effect on the FGF-mediated proliferation of human aortic SMCs (T/G HA-V SMCs). At 85 mmol/L, ethanol completely inhibited FGF1- and FGF2-dependent growth stimulation and reduced basal cell growth in the absence of FGF by ≈20% (online Figure III).

Accelerated G_1 arrest and a prolonged S-phase transition have been reported in cells after exposure to ethanol.[26,27] Cell cycle parameters for SV40LT-SMC growth were determined by flow cytometry (the Table). Cells were treated with FGF2 (50 ng/mL) for 24 hours. Ethanol by itself (85 mmol/L) decreased the number of mitotic cells (in the G_2/M phase) by 23% ($P<0.05$). FGF-dependent acceleration of cell entry into the S and G_2/M phases and the concomitant reduction in G_1 phase cells were completely inhibited by ethanol.

Ethanol Inhibits FGF-Mediated MAPK Activation

FGF2 induced the tyrosine phosphorylation of c-Raf, MEK1/2, SAPK/JNK, and p42/p44 and p38 MAPKs (Figure 3). Reductions in the phosphorylation of MEK1/2 and of p42/p44 and p38 MAPKs were observed at 85 mmol/L ethanol, and there was complete inhibition at 170 mmol/L ethanol. The phosphorylation of SAPK/JNK and c-Raf was also affected by ethanol but only at the higher ethanol concentration. The low basal levels of p42/p44 MAPK and SAPK/JNK phosphorylation were reduced by 85 mmol/L ethanol. In contrast with the inhibition of p42/p44 MAPK phosphorylation by FGF, ethanol had no effect on EGF-induced p42/p44 MAPK phosphorylation (Figure 4). The phosphorylation of MAPK in response to EGF was maintained even after 24 hours of incubation with ethanol.

Ethanol Inhibits FGFR-2 Phosphorylation

Given the multiple sites of ethanol inhibition in FGF signaling, we next considered whether ethanol inhibits the tyrosine autophosphorylation of FGFR-2 (Figure 5). Ethanol had little or no effect on the constitutive level of FGFR-2 tyrosine autophosphorylation but strongly inhibited its phosphoryla-
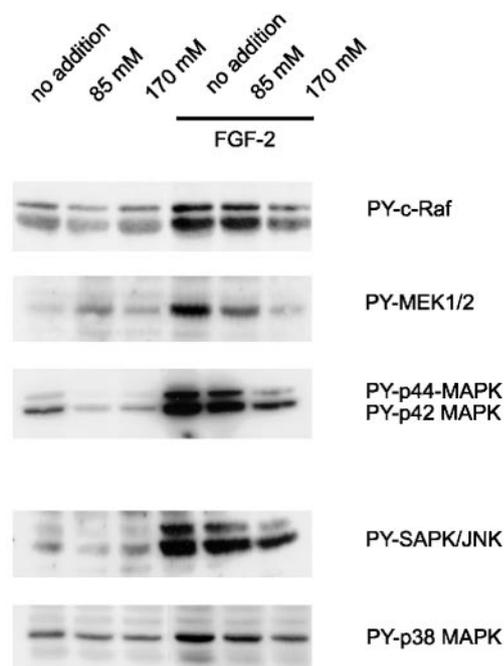
Figure 3. Ethanol-mediated inhibition of FGF-mediated intracellular signaling. SV40LT-SMCs were serum starved for 36 hours and treated with FGF2 (50 ng/mL) for 15 minutes in the presence or absence of the indicated concentrations of ethanol. Ethanol was added 30 minutes before FGF. Where indicated, cells were treated with the indicated concentrations of ethanol alone. Tyrosine phosphorylation of c-Raf, MEK1/2, SAPK/JNK, and p42/p44 MAPK and p38 MAPKs was determined by Western blotting with antibodies against the respective tyrosine-phosphorylated proteins (PY), as described in Methods.

tion by FGF2. By contrast, ethanol had no effect on EGF-induced EGFR autophosphorylation.

Discussion

Recent animal studies have demonstrated that ethanol inhibits arterial intimal hyperplasia. Long-term alcohol feeding inhib-
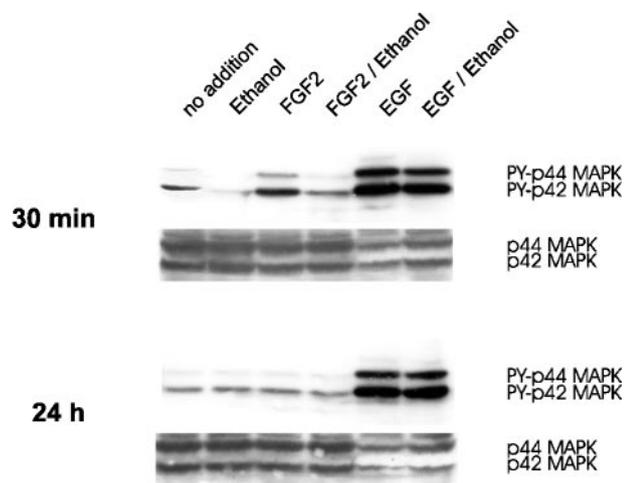
Figure 4. Specificity of inhibition of MAPK phosphorylation by ethanol. SMCs were stimulated with either FGF2 (50 ng/mL) or EGF (50 ng/mL) in the presence or absence of ethanol (85 mmol/L). Lysates were probed for tyrosine-phosphorylated (PY) and total p42/p44 MAPK by Western blot analysis, as described in Methods.
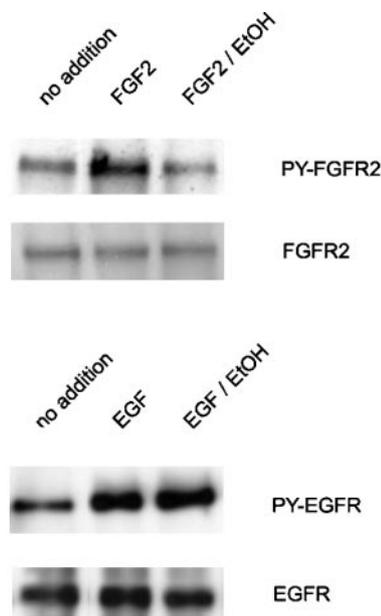
Figure 5. Ethanol (EtOH)-mediated inhibition of FGFR-2 and EGFR autophosphorylation. SV40LT-SMCs were serum-starved for 36 hours before addition of 85 mmol/L ethanol. Fifteen minutes later, FGF2 or EGF (each at 50 ng/mL) was added, and incubation was terminated after an additional 15 minutes. FGFR-2 was immunoprecipitated by addition of anti–FGFR-2 or anti-EGFR antibodies to the cell lysates. Complexes were protein G–purified and electrophoresed on denaturing 7% sodium dodecyl sulfate polyacrylamide gels, followed by Western blotting with either anti–phosphotyrosine–specific (PY) FGFR-2 or EGFR antibodies. Nitrocellulose membranes were developed by an enhanced chemiluminescence reaction, stripped, and immunoblotted with anti-receptor antibodies.

its the progression of atherosclerosis in C57BL/6 hyperlipidemic mice[28] and reduces vascular cell proliferation in hypercholesterolemic rabbits after balloon angioplasty.[21] Considerable reductions in neointimal hyperplasia, including SMC proliferation, were also observed in pigs after angioplasty.[20] Interestingly, in the last model, local delivery of 15% ethanol in the immediate postinjury phase was effective in reducing the size of neointimal lesions, which suggests that early signaling events might be specifically targeted by ethanol. Ethanol-induced inhibition of rat vascular SMC proliferation and activation of p42/p44 MAPK in vitro was subsequently demonstrated.[19] Taken together, ethanol appears to reduce SMC growth, an effect that has been invoked to participate in the protective action of moderate alcohol consumption on the complications of coronary heart disease and ischemic stroke.[29]

In the current study, we have identified the FGF signaling pathway in SMCs as an inhibitory target for ethanol. Pharmacologically relevant concentrations of ethanol potently inhibited FGF-induced SMC proliferation. FGF1 and FGF2 mitogenic activities were differently affected by ethanol. Interestingly, whereas FGF2 stimulates its own biosynthesis through an autocrine loop, FGF1 is unable to do so.[30] Conceivably, ethanol inhibition of the FGF2 effect is amplified through a blockade of the autocrine loop. Because of the higher mitogenic potency and susceptibility to ethanol dis-

played by FGF2, signaling studies were carried out with this growth factor.

Ethanol inhibited the proximal step in FGF-mediated signaling, FGFR-2 tyrosine autophosphorylation, and variably inhibited the phosphorylation of several intracellular signaling molecules downstream of FGFR-2, including c-Raf, MEK 1/2, SAPK/JNK, and p38 and p42/p44 MAPKs. By contrast, EGF signaling was unaffected by ethanol in SMCs, in terms of both EGFR autophosphorylation and activation of p42/p44 MAPK. EGF has been reported to be a mitogen for SMCs in culture.[31] However, to date, no role for EGF as an autocrine stimulus in these cells has been identified. Pastore et al[32] reported that an EGFR-targeted cytotoxin inhibits neointimal neoplasia in vivo. In another study, Trieu et al[33] found that selective inhibition of the EGFR decreased neointimal hyperplasia after vascular injury. Epiregulin and heparin-binding EGF (HBEGF), whose production by SMCs is under autocrine control, also bind to EGFR, and their mitogenic activity might have been the primary target of EGFR blockade. At least part of the mitogenic effect of HBEGF is mediated through the induction of expression of FGF2.[34] Therefore, ethanol might act indirectly of the mitogenic cascade initiated by EGFR activation.

As for receptor tyrosine kinases (RTKs) in general, signaling downstream of the FGFR is complex. However, p42/p44 MAPK has been invoked as a gatekeeper for SMC proliferation.[35,36] Moreover, specific inhibitors of MAPK phosphorylation markedly inhibit SMC growth.[36] This dependence of SMC growth on MAPK activation has been documented in vivo, in which the marked increase in p42/p44 MAPK phosphorylation after balloon catheter injury of the rat carotid artery can be effectively prevented by administering specific MEK inhibitors.[37] In the current study, the inhibition of SMC growth was correlated with a reduction in FGF-dependent MAPK activation. Ethanol also reduced the basal level of MAPK phosphorylation. For several reasons, however, it is unlikely that MAPK is directly targeted by ethanol. Importantly, in the current study, ethanol had no effect on EGF-induced MAPK activation. Ethanol also did not inhibit MAPK activation in response to phorbol ester, which bypasses receptor activation (data not shown). Given the basal phosphorylation of MAPK, even after serum starvation, it is likely that ethanol impairs the autocrine stimulation of MAPK, perhaps via inhibition of autocrine FGF-dependent stimulation. By extension, we suggest that ethanol-induced inhibition of the FGF signaling axis could account for the impairment of SMC growth in the presence of serum.[19]

In several cell types (eg, glia, neuronal cells), ethanol inhibits growth stimulation in response to other growth factors that signal via RTKs, including insulin,[38] insulin growth factor (IGF)-I,[39] and platelet-derived growth factor.[40] Given the multiplicity of growth factors contained within serum, in conjunction with regulation of growth by autocrine and paracrine stimulation by growth factors, it is possible that ethanol inhibits other receptor-mediated signaling pathways in SMCs. Nevertheless, the finding that abrogation of FGF and FGFR signaling specifically impairs SMC differentiation and growth[41–43] implies a strict dependence on FGF of SMC growth behavior. Thus, the linkage between ethanol-induced inhibition of SMC growth in this study and the inhibition by ethanol of SMC proliferation in vivo and in vitro is compelling.

The inhibitory action of ethanol on several growth factors has been localized to their respective RTKs. These interactions have been best characterized for the insulin receptor (IR) and IGF-IRs.[38,39] These receptors exhibit close homology, particularly within their catalytic domains.[39] Ethanol inhibits tyrosine autophosphorylation of IR and IGF-IR in several cell types and variably inhibits the tyrosine phosphorylation of key downstream signaling mediators, including IRS-1, IRS-2, and Shc and the activation of phosphatidylinositol 3-kinase.[16] The locus of ethanol action on these receptors likely resides within the tyrosine kinase domain itself. Notably, ethanol inhibits the kinase activity of the minimally defined 35-kDa IR kinase domain.[38] Similar inhibition of IGF-IR kinase activity has been observed (R. Rubin, unpublished data). Luo and Miller[17] demonstrated that ethanol inhibits platelet-derived growth factor autophosphorylation. However, other RTKs appear to be insensitive to ethanol. Notably, the EGFR is not directly inhibited by ethanol in neuronal cells and hepatocytes (R. Rubin, unpublished data), as well as SMCs in the current study. Given the structural similarities among the ethanol-sensitive RTKs, it is possible that ethanol interferes with the basic mechanism of kinase activation, perhaps at the level of activation-loop engagement.

Despite the proximal inhibition of RTKs by ethanol, there appears to be considerable diversity in the sensitivity of signaling elements within the tyrosine kinase signaling cascade to ethanol. For example, in a series of neuronal cells, there was considerable variation in the sensitivities of IRS-1, IRS-2, and Shc to ethanol.[16] In the current study, mediators downstream of FGFR-2 displayed differential sensitivity to ethanol (see Figure 3). These differences likely reflect a myriad cellular contextual issues, including receptor expression, the localization and binding affinities of signaling mediators to RTKs, phosphorylation of nontyrosine residues, and tyrosine phosphatase activity.

In conclusion, we have demonstrated that the FGFR signaling pathway is a target for ethanol. Several growth factors promote SMC proliferation. Among them, angiotensin II,[44] thrombin,[45] endothelin-1,[46] and HBEGF[34] act as mitogens either directly or through activation of FGF expression. It will be of interest to assess whether the effect of ethanol on FGFR signaling is specific and how other mitogenic pathways that appear refractory to the alcohol-inhibitory activity, such as the EGFR, are affected by perturbation of the FGF autocrine growth mechanism. Our results are resonant with the impairment of SMC proliferation after inhibition of FGF signaling in vivo by FGF-specific antibodies and antisense DNA directed against the FGFR.[12,41,42,47] These results serve as a basis for interpretation of the antimitogenic effect of ethanol on SMCs and support the idea that FGF2 plays a crucial role in initiating unrestrained SMC proliferation leading to atherosclerosis.

Acknowledgment

References

1. Klatsky AL. Alcohol and cardiovascular diseases: a historical overview. *Ann N Y Acad Sci*. 2002;957:7–15.

2. Belleville J. The French paradox: possible involvement of ethanol in the protective effect against cardiovascular diseases. *Nutrition*. 2002;18: 173–177.

3. Rimm EB, Klatsky A, Grobbee D, Stampfer MJ. Review of moderate alcohol consumption and reduced risk of coronary heart disease: is the effect due to beer, wine, or spirits. *BMJ*. 1996;312:731–736.

4. Agarwal DP. Cardioprotective effects of light-moderate consumption of alcohol: a review of putative mechanisms. *Alcohol Alcohol*. 2002;37:409–415.

5. Gaziano JM, Ruring MPH, Breslow JL, Goldhaber SZG, Rosner B, vanDenburgh M, Willett W, Hennekens CH. Moderate alcohol intake, increased levels of high-density lipoprotein and its subfractions, and decreased risk of myocardial infarction. *N Engl J Med*. 1993;329:1829–1834.

6. Dai J, Miller B, Lin RC. Alcohol feeding impedes early atherosclerosis in low-density lipoprotein receptor knockout mice: factors in addition to high density lipoprotein-apolipoprotein A1 are involved. *Alcohol Clin Exp Res*. 2002;21:11–18.

7. Rubin R. Effects of ethanol on platelets. *Lab Invest*. 1990;63:729–732.

8. Tabengwa EM, Wheeler CG, Yancey DA, Grenett HE, Booyse FM. Alcohol-induced up-regulation of fibrinolytic activity and plasminogen activators in human monocytes. *Alcohol Clin Exp Res*. 2002;26:1121–1127.

9. Rimm EB, Williams P, Fosher K, Criqui M, Stampfer MJ. Moderate alcohol intake and lower risk of coronary heart disease: meta-analysis of effects on lipids and hemostatic factors. *Br Med J*. 1999;319:1523–1528.

10. Ross R, Masuda J, Raines EW. Cellular interactions, growth factors, and smooth muscle proliferation in atherogenesis. *Ann N Y Acad Sci*. 1990; 598:102–112.

11. Libby P. Inflammation in atherosclerosis. *Nature*. 2002;420:868–874.

12. Lindner V, Reidy MA. Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against fibroblast growth factor. *Proc Natl Acad Sci U S A*. 1991;88:3739–3743.

13. Hanna AK, Fox J, Neschis DG, Safford SD, Swain JL. Antisense basic fibroblast growth factor gene transfer reduces neointimal thickening after arterial injury. *J Vasc Surg*. 1996;25:320–325.

14. Bilato C, Pauly RR, Melillo G, Monticone R, Gorelick-Feldman D, Gluzband YA, Sollott SJ, Ziman B, Lakatta EG, Crow MT. Intracellular signaling pathways required for rat vascular smooth muscle cell migration. *J Clin Invest*. 1995;96:1905–1915.

15. Seiler AEM, Ross BN, Rubin R. Inhibition of insulin-like growth factor-I and IRS-2 signaling by ethanol in SH-SY5Y neuroblastoma cells. *J Neurochem*. 2001;76:573–581.

16. Hallak H, Seiler AEM, Green JS, Henderson A, Ross BN, Rubin R. Inhibition of insulin-like growth factor-I signaling by ethanol in neuronal cells. *Alcohol Clin Exp Res*. 2001;25:1058–1064.

17. Luo J, Miller B. Basic fibroblast growth factor- and platelet-derived growth factor-mediated cell proliferation in B104 neuroblastoma cells: effect of ethanol on cell cycle kinetics. *Brain Res*. 1997;770:139–150.

18. Imperia PS, Chikkappa G, Philips PG. Mechanism of inhibition of granulopoiesis by ethanol. *Proc Soc Exp Biol Med*. 1984;175:219–225.

19. Hendrickson RJ, Cahill PA, McKillop IH, Sitzmann JV, Redmond EM. Ethanol inhibits mitogen activated kinase activity and growth of vascular smooth muscle cells in vitro. *Eur J Pharmacol*. 1998;362:251–259.

20. Liu MW, Anderson PG, Luo JF, Roubin GS. Local delivery of ethanol inhibits intimal hyperplasia in pig coronary arteries after balloon injury. *Circulation*. 1997;96:2296–2301.

21. Merritt R, Guruge BL, Miller DD, Chaitman BR, Bora PS. Moderate alcohol feeding attenuates postinjury vascular cell proliferation in rabbit angioplasty model. *J Cardiovasc Pharmacol*. 1997;30:19–25.

22. Reilly CF. Rat vascular smooth muscle cells immortalized with SV40 large T antigen possess defined smooth muscle cell characteristics including growth inhibition by heparin. *J Cell Physiol*. 1990;142: 342–351.

23. Simons M, Rosenberg RD. Antisense nonmuscle myosin heavy chain and c-myb oligonucleotides suppress smooth muscle cell proliferation in vitro. *Circ Res*. 1992;70:835–843.

24. Powers CJ, McLeskey SW, Wellstein A. Fibroblast growth factors, their receptors and signaling. *Endocr Relat Cancer*. 2000;7:165–197.

25. Miyamoto T, Leconte I, Swain JL, Fox JC. Autocrine FGF signaling is required for vascular smooth muscle cell survival in vitro. *J Cell Physiol*. 1998;177:58–67.

26. Sayeed S, Cullen JP, Coppage M, Sitzmann JV, Redmond EM. Ethanol differentially modulates the expression and activity of cell cycle regulatory proteins in rat aortic smooth muscle cells. *Eur J Pharmacol*. 2002;445:163–170.

27. Cook RT, Keiner JA, Yen A. Ethanol causes accelerated G_1 arrest in differentiating HL-60 cells. *Alcohol Clin Exp Res*. 1990;14:695–703.

28. Emeson EE, Manaves V, Singer T, Tabesh M. Chronic alcohol feeding inhibits atherosclerosis in C57BL/6 hyperlipidemic mice. *Am J Pathol*. 1995;147:1749–1758.

29. Djousse L, Ellison RC, Beiser A, Scaramucci A, D'Agostino RB, Wolf PA. Alcohol consumption and risk of ischemic stroke: the Framingham Study. *Stroke*. 2002;33:907–912.

30. Alberts GF, Hsu DK, Peifley KA, Winkles JA. Differential regulation of acidic and basic fibroblast growth factor gene expression in fibroblast growth factor-treated rat aortic smooth muscle cells. *Circ Res*. 1994;75:261–267.

31. Berk BC, Brock TA, Webb RC, Taubman MB, Atkinson WJ, Gimbrone MA Jr, Alexander RW. Epidermal growth factor, a vascular smooth muscle mitogen, induces rat aortic contraction. *J Clin Invest*. 1985;75:1083–1086.

32. Pastore CJ, Isner JM, Bacha PA, Kearney M, Pickering JG. Epidermal growth factor receptor-targeted cytotoxin inhibits neointimal hyperplasia in vivo: results of local versus systemic administration. *Circ Res*. 1995; 77:519–529.

33. Trieu VN, Narla RK, Myers DE, Uckun FM. EGF-genistein inhibits neointimal hyperplasia after vascular injury in an experimental restenosis model. *J Cardiovasc Pharmacol*. 2000;35:595–605.

34. Peifley KA, Alberts GF, Hsu DK, Feng SL, Winkles JA. Heparin-binding epidermal growth factor-like growth factor regulates fibroblast growth factor-2 expression in aortic smooth muscle cells. *Circ Res*. 1996;79:263–270.

35. Koyama H, Olson NE, Dastvan F, Reidy MA. Cell replication in the arterial wall: activation of signaling pathway following in vivo injury. *Circ Res*. 1998;82:713–721.

36. Plevin R, Scott PH, Robinson CJ, Gould GW. Efficacy of agonist stimulated MEK activation determines the susceptibility of mitogen activated protein kinase to inhibition in rat aortic smooth muscle cells. *Hypertension*. 1996;318:657–663.

37. Mii S, Khalil RA, Morgan KG, Ware JA, Kent KC. Mitogen-activated protein kinase and proliferation of human vascular smooth muscle cells. *Am J Physiol*. 1996;270:H142–H150.

38. Seiler AEM, Henderson A, Rubin R. Ethanol inhibits insulin receptor tyrosine kinase. *Alcohol Clin Exp Res*. 2000;24:1869–1872.

39. Resnicoff M, Sell C, Ambrose D, Baserga R, Rubin R. Ethanol inhibits the autophosphorylation of the insulin-like growth factor I (IGF-1) receptor and IGF-1 mediated proliferation of 3T3 cells. *J Biol Chem*. 1993;268:21777–21782.

40. Luo J, Miller M. Platelet-derived growth factor-mediated signal transduction underlying astrocyte proliferation: site of ethanol action. *J Neurosci*. 1999;19:10014–10025.

41. Fox J, Shanley JR. Antisense inhibition of basic fibroblast growth factor induces apoptosis in vascular smooth muscle cells. *J Biol Chem*. 1996; 271:12578–12584.

42. Casscells WLDA, Olwin BB, Wai C, Siegman M, Speir E. Elimination of smooth muscle cells in experimental restenosis: targeting of fibroblast growth factor receptors. *Proc Natl Acad Sci U S A*. 1992;89:7159–7163.

43. Liu MW, Li SJ, Chen YL. Local alcohol delivery may reduce phenotype conversion of smooth muscle cells and neointimal formation in rabbit iliac arteries after balloon injury. *Atherosclerosis*. 1996;127:221–227.

44. Itoh H, Mukoyama M, Pratt RE, Gibbons GH, Dzau VJ. Multiple autocrine growth factors modulate vascular smooth muscle cell growth response to angiotensin II. *J Clin Invest*. 1993;91:2268–2274.

45. Stouffer GA, Runge MS. The role of secondary growth factor production in thrombin-induced proliferation of vascular smooth muscle cells. *Semin Thromb Hemost*. 1998;24:145–150.

46. Peifley KA, Winkles JA. Angiotensin II and endothelin-1 increase fibroblast growth factor-2 mRNA expression in vascular smooth muscle cells. *Biochem Biophys Res Commun*. 1998;242:202–208.

47. Yukawa H, Miyatake S-I, Saiki M, Takahashi JC, Mima T, Ueno H, Nagata I, Kikuchi H, Hashimoto N. In vitro growth suppression of vascular smooth muscle cells using adenovirus-mediated gene transfer of a truncated form of fibroblast growth factor receptor. *Atherosclerosis*. 1998;141:125–132.

Arteriosclerosis, Thrombosis, and Vascular Biology

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