

Dose-dependent Activation of Antiapoptotic and Proapoptotic Pathways by Ethanol Treatment in Human Vascular Endothelial Cells

DIFFERENTIAL INVOLVEMENT OF ADENOSINE*

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Jie Liu, Zhigang Tian‡, Bin Gao, and George Kunos§

From the Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, Bethesda, Maryland 20892

Moderate but not heavy drinking has been found to have a protective effect against cardiovascular morbidity. We investigated the effects of ethanol (EtOH) treatment on the cell survival-promoting phosphatidylinositol 3-kinase (PI3K)/Akt pathway in cultured human umbilical vein endothelial cells (HUVEC). Exposure of cells to 2–20 mM EtOH resulted in rapid (<10 min) induction of Akt phosphorylation that could be prevented by pertussis toxin or the PI3K inhibitors wortmannin and LY294002. Among the downstream effectors of PI3K/Akt, p70S6 kinase, glycogen synthase kinase 3 α/β , and I κ B- α were phosphorylated, the latter resulting in 3-fold activation of NF- κ B. EtOH also activated p44/42 mitogen-activated protein kinase in a PI3K-dependent manner. Low concentrations of EtOH increased endothelial nitric-oxide synthase activity, which could be blocked by transfection of HUVEC with dominant-negative Akt, implicating the PI3K/Akt pathway in this effect. The adenosine A1 receptor antagonist 1,3-dipopylcyclopentylxanthine prevented the phosphorylation of Akt observed in the presence of EtOH, adenosine, or the A1 agonist N⁶-cyclopentyladenosine. Incubation of HUVEC with 50–100 mM EtOH resulted in mitochondrial permeability transition and caspase-3 activation followed by apoptosis, as documented by DNA fragmentation and TUNEL assays. EtOH-induced apoptosis was unaffected by DPCPX and was potentiated by wortmannin or LY294002. We conclude that treatment with low concentrations of EtOH activates the cell survival promoting PI3K/Akt pathway in endothelial cells by an adenosine receptor-dependent mechanism and activation of the proapoptotic caspase pathway by higher concentrations of EtOH via an adenosine-independent mechanism can mask or counteract such effects.

Alcohol has been the most widely used and misused drug throughout human history. Aside from its well known neurobehavioral effects, alcohol also influences cardiovascular variables. The ingestion of one or two alcohol-containing drinks has acute effects on heart rate, blood pressure, cardiac output,

myocardial contractility, and regional blood flow (1), actions that are generally not clinically important. During the last few decades, moderate alcohol consumption has been associated with a reduced risk for ischemic cardiovascular disease (2, 3), whereas chronic heavy drinking was found to increase cardiovascular morbidity and mortality (3). Although the cardioprotective effect of moderate drinking is generally attributed to an elevation of high density lipoproteins and reduced platelet aggregation (4), alcohol has been shown to alter endothelial function (5); such changes may also contribute to its cardioprotective effects. Vascular endothelial cells, because of their location at the interface of blood and the vessel wall, are susceptible to the influence of various blood-borne agents. The endothelium is now recognized as an important regulator of vascular tone, and the controlled proliferation of vascular endothelial cells is a key step in angiogenesis. In the present study we examined the hypothesis that ethanol may influence both pro- and antiapoptotic pathways in the vascular endothelium in ways that could contribute to its dose-dependent effects on cardiovascular morbidity.

The serine/threonine protein kinase Akt was originally discovered as the cellular homolog (c-Akt) of the transforming retrovirus AKT8 (6). It is a novel kinase with similarities to both protein kinase C and protein kinase A, so it is also referred to as protein kinase B (7). Akt is a downstream mediator of phosphatidylinositol 3-kinase (PI3K),¹ recruited to the plasma membrane by binding the lipid products of PI3K, phosphatidylinositol 3,4-bisphosphate and 3,4,5-trisphosphate, which bind to the pleckstrin homology domain present in a number of cytosolic signaling proteins including Akt (8). Akt is a multifunctional mediator of PI3K-dependent signaling and functions to promote cell survival as well as the transcription and translation of proteins involved in cell cycle progression (9). Receptor-induced activation of Akt is blocked by PI3K inhibitors and by the expression of dominant-negative forms of PI3K (10–17).

Phosphorylated Akt activates different downstream effectors, including endothelial nitric-oxide synthase (eNOS), an enzyme involved in vascular remodeling and angiogenesis and also responsible for maintaining systemic blood pressure (18,

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‡ On leave from the Institute of Immunology, University of Science and Technology of China, Hefei 230027, China.

§ To whom correspondence should be addressed: National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, 12420 Parklawn Dr., Rm. 120, MSC-8115, Bethesda, MD 20892-8115. Tel.: 301-443-2069; Fax: 301-480-0257; E-mail: gkunos@mail.nih.gov.

¹ The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; MAP, mitogen-activated protein; GSK, glycogen synthase kinase; eNOS, endothelial nitric-oxide synthase; PTX, pertussis toxin; CPA, N⁶-cyclopentyladenosine; DPCPX, 1,3-dipopylcyclopentylxanthine; GTP- γ S, guanosine 5'-3-O-(thio)triphosphate; MPMT, mitochondrial permeability transition; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; PARP, poly(ADP-ribose) polymerase; HUVEC, human umbilical vein endothelial cells; PBS, phosphate-buffered saline; ROS, reactive oxygen species; DiOC₆, 3,3'-dihexyloxycarbocyanine.

19). Another major downstream effector is p70S6 kinase, which phosphorylates the ribosomal S6 protein in response to stimulation with mitogens and preferentially increases the translation of mRNAs containing 5'-terminal oligopyrimidine tracts (14, 20). Glycogen synthase kinase 3 (GSK-3) is also a target of Akt (13, 21–25). Inhibition of GSK-3 by Akt inhibits apoptosis and promotes cell survival (22, 26). Akt is well established as a mediator of cell survival, as its activation protects against apoptosis induced by withdrawal of growth factor or serum (27). NF- κ B is a downstream mediator of Akt in anti-apoptotic signaling (28). When bound to its cytosolic inhibitor, I κ B, NF- κ B is sequestered in the cytoplasm. Upon its phosphorylation by I κ B kinases, I κ B is degraded, which allows NF- κ B to move to the nucleus and activate the transcription of anti-apoptotic proteins (29).

Here we report that in the presence of low concentrations (2–20 mM) of ethanol, the cell survival-promoting PI3K/Akt pathway and several of its downstream effectors, including eNOS, GSK-3, p70S6 kinase, and NF- κ B, are activated via an adenosine-dependent mechanism, whereas at concentrations of 50–100 mM, ethanol promotes serum deprivation-induced endothelial cell apoptosis independently of adenosine by activating caspase-3 and its downstream target, PARP-1. These dose-dependent, opposing effects on endothelial cell survival pathways may contribute to the bimodal effects of ethanol consumption on cardiovascular morbidity.

EXPERIMENTAL PROCEDURES

Materials—Ethanol, pertussis toxin (PTX), GF 109203X, wortmannin, and LY294002, *N*⁶-cyclopentyladenosine (CPA), 1,3-dipropylcyclopentylxanthine (DPCPX), and adenosine were purchased from Sigma. Rabbit anti-human antibodies to native and phosphorylated forms of Akt (Ser-473; catalog No. 9272 and 9271), I κ B- α (catalog No. 9242 and 9246), GSK-3 α/β (Ser-219; catalog No. 9331), p70S6 kinase (catalog No. 9205), p44/42 MAP kinase (catalog No. 9102 and 9101), and caspase-3 (catalog No. 9662) were purchased from New England Biolabs (Beverly, MA). Anti-poly(ADP-ribose) polymerase (PARP; catalog no. SA-253) and caspase-3 cellular activity assay kits were purchased from Biomol (Plymouth Meeting, PA). Anti-rabbit IgG (horseradish peroxidase-linked) and enhanced chemiluminescence reagent (ECL) detection system were obtained from PerkinElmer Life Sciences. [³⁵S]GTP γ S was purchased from PerkinElmer Life Sciences, and [α -³²P]dATP was obtained from Amersham Biosciences. TdT recombinant was purchased from Invitrogen.

Cell Culture—Primary cultured human umbilical vein endothelial cells (HUVEC) were purchased from ATCC. The plastic culture flask was precoated with 0.2% gelatin for at least 1 h prior to seeding the cells. HUVEC were maintained in EBM-2 medium (Clonetics) supplemented with 2% fetal bovine serum and growth factors such as hydrocortisone, hFGF-B, VEGF, R³-IGF-1, ascorbic acid, hEGF, GA-1000, and heparin plus 100 units/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. All experiments were performed using HUVEC at 2–4 passages, with no cells being used beyond the fourth passage. For serum starvation, the regular medium was removed and replaced with medium 199.

Western Blot Analyses—Western immunoblotting was done as previously described (30). HUVEC cells were cultured in serum-free medium overnight, prior to the addition of ethanol, to reduce the basal levels of Akt phosphorylation. Cell lysate protein (60 μ g) from an equal number of cells was size-fractionated by 10% SDS-PAGE and then transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked by incubation for 2 h in TPBS buffer (1 \times PBS, 0.1% Tween 20) containing 5% (w/v) nonfat dry milk at room temperature and then immunoblotted with primary antibody (at 1:1000 dilution in TPBS plus 3% bovine serum albumin) overnight at 4 °C and horseradish peroxidase-conjugated secondary antibody (1:2000 in blocking buffer) for 2 h at room temperature. Immunoreactive bands were visualized using an ECL detection system.

[³⁵S]GTP γ S Binding—Confluent HUVEC were rinsed twice in PBS and harvested by scraping. The cells were then homogenized in TME-Na buffer (50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM EGTA, 100 mM NaCl, pH 7.4), and the homogenate was centrifuged at 48,000 \times *g* for 20 min at 4 °C. The pellet was resuspended in TME-Na, homogenized, and

adjusted to a protein concentration of 1 μ g/ μ l with TME-Na. The membrane homogenate was then used for measuring agonist-stimulated [³⁵S]GTP γ S binding according to Griffin *et al.* (31). HUVEC membranes (100 μ g) were incubated in TME-Na buffer containing 0.1 mg/ml bovine serum albumin with 30 μ M GDP, 0.1 nM [³⁵S]GTP γ S, and different concentrations of ethanol in silanized glass tubes with or without 100 ng/ml PTX or 100 nM DPCPX. The total assay volume was 0.5 ml, and triplicate aliquots were incubated at 30 °C for 1 h. The reaction was terminated by the addition of 2 ml of ice-cold wash buffer (50 mM Tris-HCl, pH 7.4) followed by vacuum filtration through glass fiber (type B) filters. Filters were rinsed three more times with wash buffer, and the retained radioactivity was determined by liquid scintillation spectrometry. Basal binding was assayed in the absence of ethanol and in the presence of GDP.

Transfections and Fluorescent Cell Sorting—Plasmids pEGFP-N1 (CLONTECH) and dominant-negative Akt were co-transfected into HUVEC P2 cells. Using an initial seeding density of 1 \times 10⁴ cells/cm², HUVEC were ready for transfection at 18–24 h post-seeding. For each 75-cm² flask of cells to be transfected, 30 μ l of Lipofectin and 5 μ g of each DNA were diluted separately in 500 μ l of Opti-MEM I reduced serum medium. Solutions were combined, gently mixed, and incubated for 45 min at room temperature to allow formation of DNA-lipid complexes. The normal medium was replaced with 8 ml of Opti-MEM I reduced medium, and the DNA-lipid complexes were added subsequently to each flask and mixed gently by rocking the plate back and forth. Cells were incubated for 3 to 4 h at 37 °C in humidified air in 5% CO₂. The transfection mixture was replaced with basal media containing supplements. Transfected cells were identified by cell sorting at 18–24 h post-transfection, and only the positive cells were used for the eNOS activity assay.

eNOS Activity—eNOS activity was measured by monitoring the conversion of L-[³H]arginine to L-[³H]citrulline (eNOSdetect Assay Kit, Stratagene). Cells were trypsinized and washed with PBS and lysed in an adequate volume of homogenization buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA). The supernatant was separated from the homogenate, and protein concentration was adjusted to 5 μ g/ μ l. The reaction mixture was prepared on ice by adding the following components: 25 μ l of 2 reaction buffer (50 mM Tris-HCl, pH 7.4, 6 μ M BH₄, 2 μ M flavin adenine dinucleotide, 2 μ M flavin adenine mononucleotide), 5 μ l of 10 mM NADPH (freshly prepared in 10 mM Tris-HCl, pH 7.4), 1 μ l of [³H]arginine (50 μ Ci/ml), 5 μ l of 6 mM CaCl₂, and 4 μ l of H₂O. The reaction was initiated by the addition of 10 μ l of protein extract (total volume of 50 μ l) and carried out for 30 min at 37 °C. Rat cerebellum extract and *N* ω -nitro-L-arginine methyl ester HCl were used as positive or inhibitory control. The reaction was terminated by the addition of 400 μ l of stop buffer containing 50 mM HEPES, pH 5.5, and 5 mM EDTA. The reaction mixture was then passed through a provided resin. The [³H]citrulline generated was quantified by liquid scintillation spectrometry.

Luciferase Reporter Assay—The NF- κ B luciferase promoter construct was purchased from Stratagene. Transient transfection was performed as described above. The transfection mixture was replaced with medium M199 overnight. Cells were stimulated for another 8 h with ethanol and then washed twice with PBS and lysed in 200 μ l (for 12-well culture plate) of 1 \times passive lysis buffer. Cell lysates were centrifuged for 30 s at top speed in a refrigerated microcentrifuge, the cleared lysates were transferred to a fresh tube, and protein content was determined. Equal amounts of protein were used for reporter enzyme analyses using the Luciferase Reporter Assay System (Promega) and a luminometer.

Flow Cytometric Analysis of Apoptosis by Measurement of Mitochondrial Transmembrane Potential ($\Delta\Psi_m$) and the Generation of Reactive Oxygen Species (ROS)— $\Delta\Psi_m$ and ROS generation were measured according to (32). HUVEC were treated with or without ethanol for 6 h under standard conditions and harvested by trypsinization. $\Delta\Psi_m$ was measured directly by using 40 nM 3,3'-dihexyloxycarbocyanine (DiOC₆) (3), Molecular Probes, Eugene, OR). Fluorescence was measured after staining the cells for 15 min at 37 °C. To assess ROS generation by flow cytometry, cells were treated with 2 μ M hydroethidine (Molecular Probes) for 15 min at 37 °C. The probes were excited with a laser at 488 nm (250 milliwatts), and emission was measured through a 530/30 nm (DiOC₆) (3) or a 575/26 nm (ethidium) bandpass filter. Logarithmic amplification was used to detect the fluorescence of the probes.

DNA Fragmentation Assay—Following treatment, DNA was isolated using a DNA Isolation Kit (Gentra, Minneapolis, MN). Briefly, 300 μ l of cell lysates were incubated with 1.5 μ l of RNase A solution at 37 °C for 5 min. 100 μ l of protein precipitation solution was added to each sample, and after centrifugation at 13,000 \times *g* for 1 min, the superna-

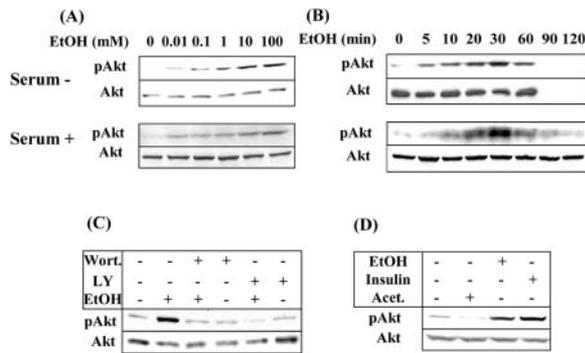


FIG. 1. Ethanol treatment increases Akt phosphorylation in HUVEC. HUVEC were incubated with various concentrations of EtOH for 30 min (A) or with 20 mM ethanol for the indicated time periods (B) under serum-starved condition (*upper panels*) or in serum-containing medium (*lower panels*). C, serum-starved HUVEC were preincubated for 30 min with 100 nM wortmannin (*Wort.*) or 25 μ M LY294002 (*LY*) prior to the addition of 10 mM ethanol for another 30 min. D, serum-starved cells were treated with 10 mM EtOH, 10 μ g/ml insulin (as positive controls), or 200 μ M acetaldehyde (*Acet.*) for 30 min. Cell extracts from *panels A–D* were subjected to Western blot analysis by using anti-phospho-Akt and anti-Akt antibodies as described under “Experimental Procedures.”

tant containing the DNA was precipitated by 300 μ l of isopropanol and washed by 70% ethanol. DNA pellets were rehydrated using DNA hydration solution and incubated at 65 $^{\circ}$ C for 1 h. DNA was end-labeled with 0.5 μ Ci of [α - 32 P]dATP using 25 units of terminal transferase enzyme at 37 $^{\circ}$ C for 1 h. The reaction was terminated by the addition of EDTA (pH 8.0) to a final concentration of 25 mM. Labeled DNA was separated from unincorporated radionucleotide by chroma-spin columns (CLONTECH) by agarose gel electrophoresis. The gel was transferred onto Whatman No. 3MM chromatography paper, dried, and analyzed by phosphorimaging (Typhoon, Molecular Dynamics).

TUNEL Assay—A TUNEL apoptosis detection kit (Upstate Biotechnology Inc.) was used for DNA fragmentation fluorescence staining according to the manufacturer’s protocol. In brief, following treatment the cells were fixed with 4% paraformaldehyde in 0.1 M NaH₂PO₄, pH 7.4, and incubated with a reaction mix containing biotin-dUTP and terminal deoxynucleotidyl transferase for 60 min. Fluorescein-conjugated avidin was applied to the sample, which was then incubated in the dark for 30 min. Positively stained fluorescein-labeled cells were visualized and photographed by fluorescence microscopy.

Measurement of Caspase-3 Activity—Caspase-3 activity was measured using a colorimetric assay kit (Biomol, Plymouth Meeting, PA) according to the manufacturer’s protocol. Cell lysates were centrifuged at 10,000 \times *g* for 10 min at 4 $^{\circ}$ C and their protein content determined (Bio-Rad). Equal amounts of cell lysates were incubated with Ac-DEVD-p nitroanilide substrate at 37 $^{\circ}$ C, and the absorbance at 405 nm was measured in a microtiter plate reader. The kinetic curve was obtained by recording data at 10-min intervals for 4 h.

Data Analysis—Data are reported as means \pm S.E. of at least three separate experiments, each performed in triplicate. Statistical significance was determined using Student’s *t* test.

RESULTS

Ethanol Induces Phosphorylation of Akt in HUVEC—A blood alcohol level of 0.1%, the legal limit for driving in many states, is equivalent to \sim 22 mM ethanol. The ethanol concentrations used in this study were 10 μ M–100 mM, which spans the range of behaviorally sub-threshold to severe intoxicating levels. Ethanol treatment caused strong, concentration-dependent activation of Akt in HUVEC (Fig. 1A), activation being evident in the presence of as little as 10 μ M ethanol. The time course is illustrated in Fig. 1B, with activation beginning at 5 min after the addition of ethanol and peaking at 30 min. Furthermore, the time- and concentration-dependent activation of Akt was evident both in the absence (*upper panels*) and in the presence of serum and growth factors (*lower panels*). Additionally, activation of Akt following ethanol treatment was completely antagonized by two structurally dissimilar inhibitors of PI3K, wortmannin and LY294002 (Fig. 1C); the ethanol metabolite

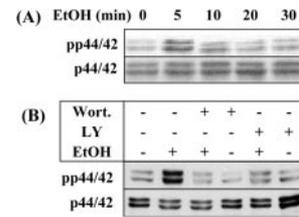


FIG. 2. Ethanol activates MAP kinase through a PI3K-dependent mechanism. A, phosphorylated p44/42 MAP kinase was visualized by Western blotting as described under “Experimental Procedures.” Serum-starved HUVEC were incubated with or without 20 mM ethanol for the indicated times. B, cells were preincubated for 30 min with 100 nM wortmannin (*Wort.*) or 25 μ M LY294002 (*LY*) prior to the addition of 20 mM ethanol for another 5 min.

acetaldehyde was inactive in this pathway (Fig. 1D).

Ethanol Induces MAP Kinase Activation in a PI3K-dependent Manner—Since the p44/42 MAP kinase can be activated by PI3K (33), we tested whether ethanol treatment can activate MAP kinase in HUVEC. As illustrated in Fig. 2A, treatment with 20 mM ethanol results in rapid phosphorylation of MAP kinase within 5 min, an effect that is abrogated in the presence of the PI3K inhibitors wortmannin or LY294002 (Fig. 2B).

Ethanol Induces Akt Activation by a PTX-sensitive G Protein-dependent Mechanism—To explore the mechanism by which ethanol treatment induces PI3K/Akt activation, we examined the effect of ethanol on GTP γ S binding in HUVEC membranes. As shown in Fig. 3A, in the presence of 2–100 mM ethanol GTP γ S binding in HUVEC membranes was increased by up to 25%. This effect was largely inhibited by PTX, a G_{i/o} protein inhibitor, or by DPCPX, an A1 adenosine receptor inhibitor. This indicates the possible involvement of G_{i/o} protein(s) and A1 adenosine receptors in the activation of Akt phosphorylation by ethanol treatment. The mechanisms involved in Akt phosphorylation were further examined by Western blot analyses. As shown in Fig. 3B, Akt phosphorylation in response to ethanol treatment was largely inhibited by PTX but not by GF 109203X, a protein kinase C inhibitor.

Activation of Downstream Effectors of Akt by Ethanol Treatment—We next investigated ethanol activation of pathways downstream of Akt. As shown in Fig. 4A, p70S6 kinase, GSK-3 α/β , and I κ B- α can all be phosphorylated by treatment with 10 mM ethanol. Further experiments with transfected NF- κ B promoter/luciferase constructs indicated NF- κ B promoter activity was increased by up to 3-fold in the presence of 2–20 mM ethanol, but with higher concentration of ethanol (50 mM) the activity declined (Fig. 4B). Similar results have been obtained for eNOS activity (Fig. 5). The increase in eNOS activity was dependent on ethanol concentration and on the duration of ethanol exposure. A time course study showed that eNOS activity peaked at 10 min following ethanol exposure (Fig. 5A). eNOS activity in HUVEC was increased by treatment with 20 mM ethanol, but activity declined toward basal levels with increasing ethanol concentrations (Fig. 5B). This ethanol-induced increase in eNOS activity could be blocked by transfection of HUVEC cells with dominant-negative Akt, implicating the PI3K/Akt pathway in eNOS activation by ethanol (Fig. 5C).

Ethanol Treatment Activates Akt by an A1 Adenosine Receptor-dependent Mechanism—It has been shown that some effects of ethanol are mediated by endogenous adenosine (34–37) and that adenosine activates Akt (38, 39). We wondered whether ethanol activated Akt in HUVEC by an adenosine receptor-dependent mechanism. As shown in Fig. 6, incubation of HUVEC with adenosine or the selective A1 adenosine receptor agonist CPA results in increased phosphorylation of Akt. Furthermore, pretreatment of HUVEC with the selective A1 receptor antagonist DPCPX almost completely prevented the increased Akt

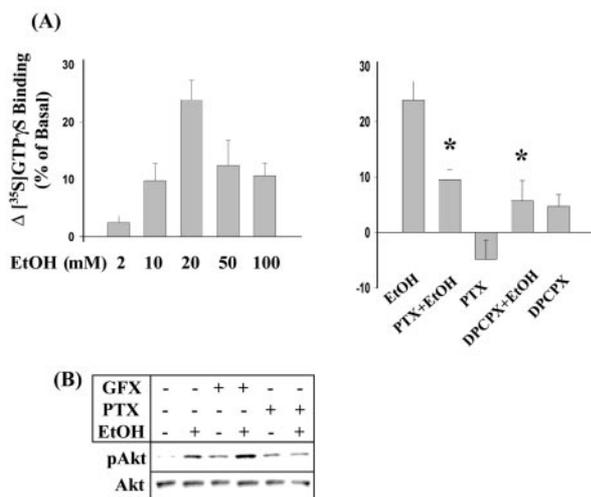


FIG. 3. Ethanol activates phosphorylation of Akt in HUVEC by a $G_{\beta\gamma}$ protein-dependent mechanism. A, [^{35}S]GTP γ S binding was measured in membrane preparations from HUVEC as described under "Experimental Procedures." * in the right panel, indicates significant difference ($p < 0.05$) from values with EtOH alone. B, serum-starved cells were preincubated for 50 min with 100 ng/ml PTX or 2 μM GF 109203X prior to the addition of 10 mM ethanol for another 30 min. Cell extracts were then prepared and subjected to Western blot analysis using anti-phospho-Akt and anti-Akt antibodies.

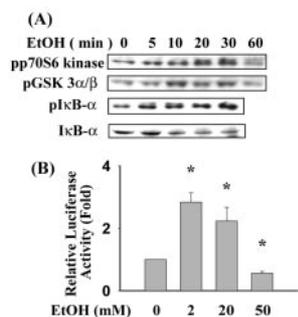


FIG. 4. Downstream effectors of Akt phosphorylated by ethanol treatment. A, HUVEC were treated with 10 mM of ethanol for various time periods. Cell extracts were then subjected to Western blotting analysis using anti-phospho-p70S6 kinase, anti-phospho-glycogen synthase kinase 3 α/β (pGSK 3 α/β), anti-phospho-I κ B- α , and anti-I κ B- α antibodies. The anti-phosphoprotein antibodies used are directed against epitopes known to be involved in the activation of the proteins. B, HUVEC cells were transiently transfected with NF- κ B promoter/luciferase construct. After 18 h, 2–50 mM ethanol was added for another 8 h. Cell extracts were prepared and luciferase activities were measured. Values are means \pm S.E. from three independent experiments, expressed as fold changes over control. *, indicates significant difference ($p < 0.05$) from ethanol-free control.

phosphorylation caused by ethanol, adenosine, or CPA, which implicates adenosine in this effect of ethanol.

High Concentrations of Ethanol Induce Apoptosis Independently of Adenosine—Fetal alcohol syndrome induced by maternal drinking has been linked to apoptotic cell death in the central nervous system. Therefore, we tested whether treatment with high concentrations of alcohol can also lead to apoptosis in vascular endothelial cells. Because there is increasing evidence that altered mitochondrial function is linked to apoptosis (40, 41), we first checked the ability of ethanol treatment to induce a mitochondrial permeability transition (MPT) state as an apoptotic early event, by measuring $\Delta\psi_m$ and ROS generation. We measured $\Delta\psi_m$ by monitoring the incorporation of the fluorescent probe DiOC $_6$ (3) into mitochondria, which is driven by the $\Delta\psi_m$ (42–44). Control cells exposed to DiOC $_6$ (3) exhibited bright DiOC $_6$ (3) fluorescence, and exposing them to 50 mM ethanol resulted in a decrease in DiOC $_6$ (3) fluorescence

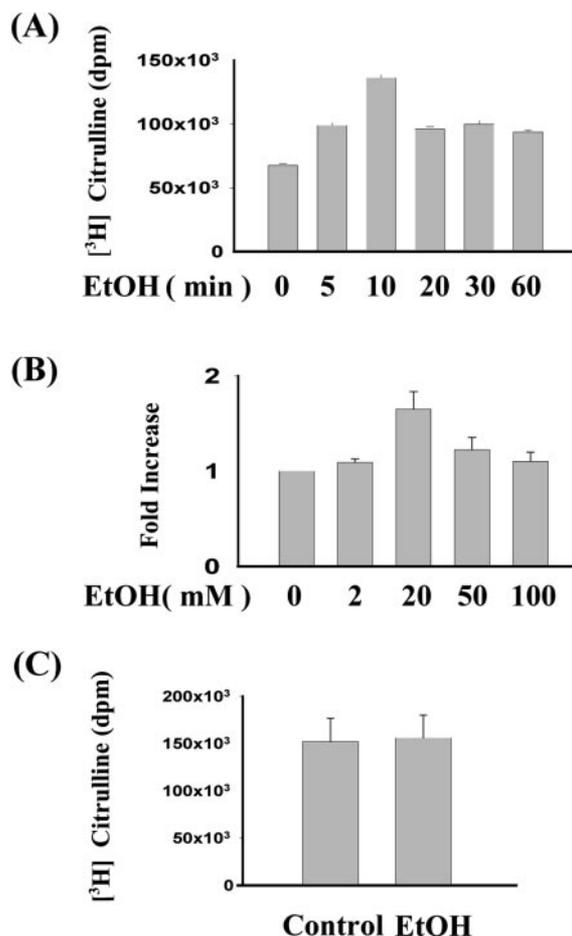


FIG. 5. Ethanol treatment activates eNOS by an Akt-dependent mechanism. HUVEC were treated with 20 mM ethanol for various time points (A) or with various concentrations of ethanol for 10 min (B). eNOS activity was measured in cell extracts as described under "Experimental Procedures." C, plasmids pEGFP-N1 and dominant-negative Akt were cotransfected into HUVEC as described under "Experimental Procedures." Transfected cells were identified and sorted by flow cytometry and were then treated with buffer (Control) or 20 mM ethanol for 10 min before measuring eNOS activity. Values are the means \pm S.E. from three independent experiments.

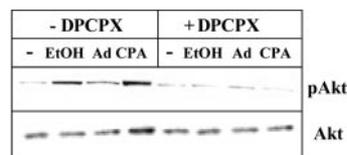


FIG. 6. Ethanol treatment stimulates Akt phosphorylation by an A1 adenosine receptor-dependent mechanism. HUVEC were pretreated with or without 100 nM DPCPX for 30 min, followed by 30-min incubation with 20 mM EtOH, 10 μM adenosine (Ad), or 1 μM CPA as indicated. Cell extracts were then subjected to Western blotting using anti-phospho-Akt and anti-Akt antibodies.

in 76% of the cells. Since a low ψ_m is associated with altered mitochondrial function, we also determined whether exposure to 50 mM ethanol potentiated mitochondrial O_2^- generation by the oxidative conversion of hydroethidine to ethidium. We noted that 5% of the cells exhibited an increase in ethidium fluorescence. As illustrated in Fig. 7, discrete cell populations shifted from the right lower quadrant to the left lower and left upper quadrants. Taken together, treatment with 50 mM ethanol not only resulted in a loss of ψ_m but also led to uncoupling of oxidative metabolism and to the generation of ROS in HUVEC cells as early as only 6 h of incubation. No significant changes in fluorescence have been noted in the presence of

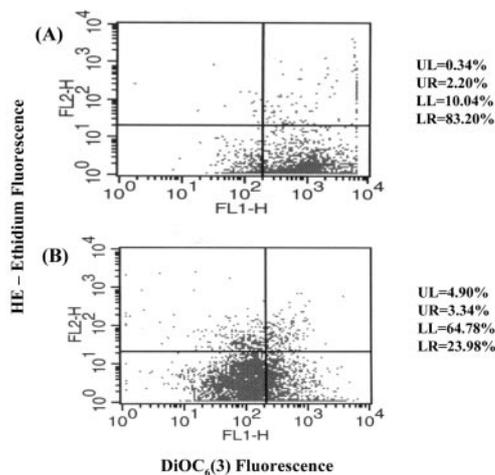


FIG. 7. Ethanol-induced alterations in $\Delta\psi_m$ and ROS generation in HUVEC. Confluent HUVEC were incubated without (A) or with 50 mM ethanol (B) for 6 h under normal conditions. Cells were then stained with DiOC₆ (3) and hydroethidine (HE) to measure $\Delta\psi_m$ and ROS generation, respectively. Control cells are shown as a discrete population exhibiting bright DiOC₆ (3) fluorescence and dim ethidium fluorescence (*lower right quadrant*). Ethanol (50 mM) decreased $\Delta\psi_m$, as demonstrated by a reduction in DiOC₆ (3) fluorescence (*lower left quadrant*), and increased ROS generation, as indicated by an increase in ethidium fluorescence (*upper left quadrant*).

lower concentration (2–20 mM) of ethanol (data not shown).

We next analyzed DNA fragmentation following exposure of HUVEC to ethanol and wortmannin (Fig. 8A). Incubation of HUVEC under conditions of serum and growth factor deprivation for 24 h caused a minority of the cells to undergo apoptosis as verified by a slight increase in DNA fragmentation compared with control. Exposure of serum-deprived HUVEC to 2–10 mM of ethanol did not significantly alter the levels of DNA fragmentation (Fig. 8A) and cell apoptosis (Fig. 8B). However, cells exposed to 50 mM ethanol displayed a dramatic increase in DNA fragmentation (Fig. 8A) and cell apoptosis (Fig. 8B). Moreover, incubation of serum-deprived HUVEC with 100 nM wortmannin, a PI3K inhibitor, also caused an increase in DNA fragmentation without influencing the ethanol-induced increase (Fig. 8A) and also increased cell apoptosis (Fig. 8B). This indicates that the PI3K/Akt pathway has a protective effect on cell survival. Finally, the selective adenosine A1 receptor antagonist DPCPX did not block 50 mM ethanol-induced apoptosis. Similar results were obtained by measuring the activity of caspase-3, an executioner of apoptosis that has been shown to mediate apoptosis in mammalian cells. As shown in Fig. 9A, serum starvation caused an increase in caspase-3 activity, which was not affected by 2 mM ethanol but was markedly potentiated by 20–50 mM ethanol treatment. Consistent evidence was obtained by immunoblotting, showing a significantly increased level of the active 17-kDa subunit of caspase-3 and increased cleavage of the 85-kDa PARP (Fig. 9B) in serum-deprived HUVEC exposed to 20–50 mM ethanol.

DISCUSSION

The present findings document a bimodal action of ethanol on pathways involved in endothelial cell survival. At concentrations up to 20 mM that do not cause significant intoxication *in vivo*, ethanol was found to activate the PI3K/Akt cell survival pathway in cultured human vascular endothelial cells. Consistent with this activation, several key downstream effectors of Akt known to be linked to anti-apoptotic, cell survival-promoting effects were also activated. In contrast, at concentrations of 50–100 mM, which cause severe intoxication *in vivo*, ethanol activated the pro-apoptotic caspase-3/PARP-1 pathway

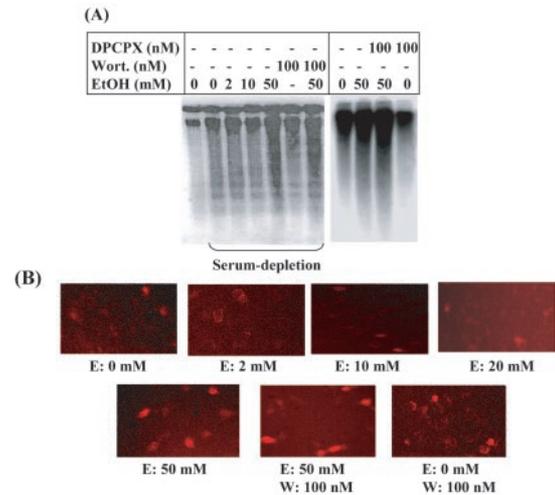


FIG. 8. High concentrations of ethanol induce apoptosis in HUVEC. Serum-starved HUVEC were treated with or without wortmannin (Wort.) or DPCPX for 30 min followed by incubation with various concentrations of EtOH for 30 min as indicated. Cells were then harvested for DNA fragmentation analysis (A) or TUNEL assay (B) as described under "Experimental Procedures."

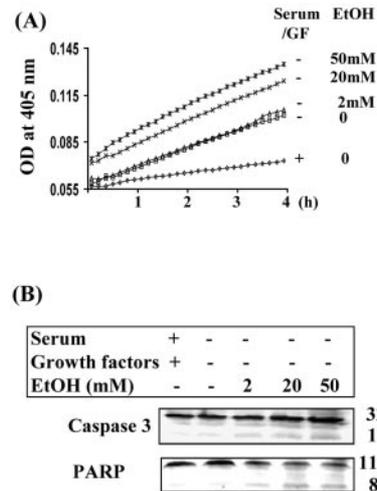


FIG. 9. High but not low concentrations of ethanol induce caspase-3 and PARP activation. Serum-starved HUVEC were incubated with various concentrations of ethanol for 24 h; cell extracts were then prepared and subjected to caspase-3 activity assay (A) or Western blotting using anti-caspase-3 and anti-PARP antibodies (B) as described under "Experimental Procedures." GF, growth factors.

and apoptosis, which is analogous to its pro-apoptotic effect in the central nervous system. To best interpret these findings, we propose a model in Fig. 10 that may partly explain the bimodal action of ethanol in the cardiovascular system.

In this model, treatment with low concentrations (<20 mM) of ethanol elicit activation of Akt and its downstream effectors including NF- κ B and eNOS. Some of these effectors, such as the p70S6 kinase, can also be activated by MAP kinase (33), and we found that ethanol rapidly activates MAP kinase in a PI3K-dependent manner (Fig. 2). It has been clearly shown that activation of Akt, eNOS, and NF- κ B play an important role in promoting angiogenesis and inhibiting apoptosis in HUVEC (27–29, 45). Importantly, the effects of ethanol treatment on Akt phosphorylation were evident not only in serum-starved cells but also in the presence of physiological levels of serum and growth factors, which suggests that similar changes are likely present in the intact organism. Thus, activation of Akt by low concentrations of ethanol likely promotes endothelial cell survival and thus may contribute to the protective

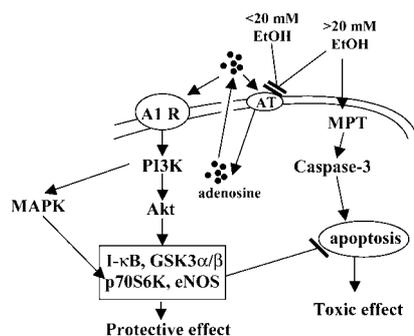


FIG. 10. **Bimodal action of ethanol on HUVEC.** See text for further explanation. AT, adenosine transporter; A1R, adenosine A1 receptor.

effect of moderate alcohol drinking against cardiovascular morbidity.

Furthermore, we provided evidence suggesting that ethanol activates Akt by an adenosine receptor-dependent mechanism. Adenosine is a signaling molecule that is released from tissue, including endothelial cells, in response to hypoxia, ischemia, or inflammation (46). The released adenosine can then act in an autocrine or paracrine fashion on G protein-coupled adenosine receptors to protect tissue from injury (47). Of the different adenosine receptor subtypes, A1 and A3 receptors are coupled to G_i/G_o and A1 receptors (which are present in vascular endothelial cells (47)) and have been linked to activation of the PI3K/Akt pathway (38). The receptor-mediated effects of adenosine are terminated by uptake into cells by facilitated diffusion via the nucleoside transporter (48). The finding that a selective A1 receptor antagonist, DPCPX, was able to inhibit the increase in Akt phosphorylation induced by ethanol treatment, adenosine, or the selective A1 agonist CPA strongly suggests that ethanol activates Akt in HUVEC by an adenosine receptor-dependent mechanism. However, the mechanism by which exposure to ethanol leads to adenosine receptor activation is not clear. It has been shown that acute ethanol treatment increases the extracellular level of adenosine by inhibiting adenosine uptake via the transporter in NG108-15 cells (49), S49 lymphoma cells (50), and hippocampal brain slices (51). A similar mechanism in HUVEC may account for the observed inhibition by DPCPX of EtOH-induced Akt phosphorylation. Further studies will be required to confirm this possibility.

In addition to activation of Akt, high concentrations (>20 mM) of ethanol also induce MPT, followed by activation of caspase-3 and apoptosis in HUVEC, which may counteract the anti-apoptotic effects of Akt activation. These opposing effects are distinguished not only by their differential sensitivity to ethanol but also by the differential involvement of adenosine. Unlike the adenosine-mediated activation of Akt, the pro-apoptotic effect of high concentrations of ethanol is independent of adenosine as well as Akt, as indicated by its resistance to inhibition by DPCPX or wortmannin. Mitochondria play a key role in the pathways of cell death (52). Mitochondrial damage not only causes "loss of function" resulting in a bioenergetic defect but also regulates effector mechanisms involving cytochrome *c* release into the cytosol (53). Cytosolic cytochrome *c* binds to Apaf-1 (the mammalian CED-4 homolog) and dATP, resulting in the recruitment and activation of procaspase-9. Activated caspase-9 then proteolytically activates caspase-3 (54). Thus, exposure of HUVEC to high concentrations of ethanol triggers the cascade of MPT followed by activation of caspase-3 and apoptosis. Mitochondria are known targets of ethanol in various types of cells, including hepatocytes (40) and neuronal cells (41), but the molecular mechanisms by which ethanol initiates the MPT are currently unknown.

Several mechanisms have been implicated in the cardioprotective effect of moderate alcohol drinking. These include modulation of blood lipoproteins (55), reduced platelet activation and thrombosis (55), and activation of ϵ protein kinase C (56). Inhibition of the synthesis of the vasoconstrictor peptide endothelin-1 by components of red wine may account for the added benefits accrued from drinking red wine (57).

The results of the present study suggest that, in addition to these mechanisms, ethanol induction of Akt activation may also contribute to the cardiovascular protective effect of moderate alcohol drinking. Akt phosphorylation results in the activation of a number of downstream effectors implicated in cell survival and also in the inhibition of apoptotic pathways, as suggested by the finding that the PI3K inhibitor wortmannin potentiates apoptosis in HUVEC. Together, these effects could promote angiogenesis induced by moderate hypoxia and would thus contribute to the protective effects of moderate drinking in ischemic heart disease.

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