Acute ethanol exposure disrupts VEGF receptor cell signaling in endothelial cells

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1Division of Molecular and Cellular Biochemistry, 2Alcohol Research Program, and 3Burn and Shock Trauma Institute, Department of Surgery, Loyola University Medical Center, Maywood, Illinois; 4Department of Medicine, University of California, San Diego; and 5Center for Wound Healing and Tissue Regeneration, University of Illinois at Chicago, Chicago, Illinois

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Radek KA, Kovacs EJ, Gallo RL, DiPietro LA. Acute ethanol exposure disrupts VEGF receptor cell signaling in endothelial cells. Am J Physiol Heart Circ Physiol 295: H174–H184, 2008. First published May 9, 2008; doi:10.1152/ajpheart.00699.2007.—Physiological angiogenesis is regulated by various factors, including signaling through vascular endothelial growth factor (VEGF) receptors. We previously reported that a single dose of ethanol (1.4 g/kg), yielding a blood alcohol concentration of 100 mg/dl, significantly impairs angiogenesis in murine wounds, despite adequate levels of VEGF, suggesting direct effects of ethanol on endothelial cell signaling (40). To examine the mechanism by which ethanol influences angiogenesis in wounds, we employed two different in vitro angiogenesis assays to determine whether acute ethanol exposure (100 mg/dl) would have long-lasting effects on VEGF-induced capillary network formation. Ethanol exposure resulted in reduced VEGF-induced cord formation on collagen and reduced capillary network structure on Matrigel in vitro. In addition, ethanol exposure decreased expression of endothelial VEGF receptor-2, as well as VEGF receptor-2 phosphorylation in vitro. Inhibition of ethanol metabolism by 4-methylpyrazole partially abrogated the effect of ethanol on endothelial cell cord formation. However, mice treated with r-butanol, an alcohol not metabolized by alcohol dehydrogenase, exhibited no change in wound vascularity. These results suggest that products of ethanol metabolism are important factors in the development of ethanol-induced changes in endothelial cell responsiveness to VEGF. In vivo, ethanol exposure caused both decreased angiogenesis and increased hypoxia in wounds. Moreover, in vitro experiments demonstrated a direct effect of ethanol on the response to hypoxia in endothelial cells, as ethanol diminished nuclear hypoxia-inducible factor-1α protein levels. Together, the data establish that acute ethanol exposure significantly impairs angiogenesis and suggest that this effect is mediated by changes in endothelial cell responsiveness to both VEGF and hypoxia.

hypoxia; angiogenesis; alcohol; wound healing

ANGIOGENESIS IS A TIGHTLY regulated process involving the migration, proliferation, and differentiation of endothelial cells into new vessels (10, 11, 26). Vascular endothelial growth factor (VEGF) is a heparin binding protein that is considered the most potent proangiogenic growth factor involved in vascular permeability, vascular dilation, endothelial proliferation, and angiogenesis (11, 46, 49). Three high-affinity receptors exist for VEGF: VEGFR-1 (fms-like tyrosine kinase-1), VEGFR-2 (fetal liver kinase-1/kinase domain region), and VEGFR-3 (fms-like tyrosine kinase-4), all of which are expressed almost exclusively on endothelial cells (15, 36). All three receptors are tyrosine kinase receptors that become autophosphorylated upon VEGF binding. VEGFR-1 has a higher affinity for VEGF, yet a decreased signaling capacity. Hence, VEGFR-1 is believed to be a negative regulator or a modulator of angiogenesis. VEGFR-2 has a lower affinity for VEGF, but has a direct involvement in endothelial cell signaling, resulting in endothelial cell mitogenesis. VEGFR-3 is primarily expressed on lymphatic endothelial cells and is involved in hypoxia-driven vascular development (37). Activation of VEGFRs results in the recruitment of adapter signaling molecules and other nonreceptor kinases, initiating signal transduction cascades to promote the upregulation of gene transcription and protein production of those mediators required for angiogenesis.

Moderate alcohol consumption, which is approximately one to two liquor drinks per day, is associated with various effects on the vasculature, including a reduction in atherosclerotic plaques in patients with cardiovascular disease (12, 27). Recent studies in our laboratory demonstrated that mice with a blood alcohol concentration (BAC) of 0.1% (100 mg/dl) at the time of injury exhibited a 50% reduction in wound vascularity (40). The reduction in vascularity occurred even though the level of VEGF in the wounds from ethanol-exposed mice was greater than that of control. These results suggested that ethanol has a direct effect on endothelial cell function that might be mediated through alterations in VEGF cell signaling.

In this study, the mechanism by which ethanol affects endothelial cell function and angiogenic responsiveness was further investigated. The data establish that acute ethanol exposure significantly perturbs VEGF signaling and hypoxia-inducible factor-1α (HIF-1α) translocation in endothelial cells and demonstrates the in vivo consequences of this impairment.

MATERIALS AND METHODS

Materials. DMEM, ITS+ premix, penicillin-streptomyccin, nonessential amino acids, and l-glutamine were purchased from Invitrogen. Fetal bovine serum, ethanol, and sodium bicarbonate were purchased from Sigma (St. Louis, MO).

Cell culture. Murine endothelial cells [small-vessel murine endothelial cells 4-10 (SVEC4-10)] were cultured in DMEM supplemented with fetal bovine serum (1 mg/ml), 1% nonessential amino acids, sodium bicarbonate (3.7 g/l), penicillin-streptomyccin (1:100), and ITS premix (1:500). For all experiments, cells were incubated overnight in serum-free DMEM and stimulated with appropriate conditions in

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serum-free DMEM to avoid any stimuli induced by factors in the bovine serum.

Ethanol recovery in vitro angiogenesis assay. Murine SV40 transformed SVEC4-10 (American Type Culture Collection, Rockville, MD) at 70–80% confluency were incubated for 8 h in 175 mm² flasks with either DMEM/10% FBS or 100 mg/dl of ethanol and DMEM/10% FBS at 37°C/5% CO₂. After 8 h, the ethanol media was replaced with fresh ethanol-free media, and the cells incubated for another 8 h. The cells were then trypsinized and incubated on an elliptical rotator at a concentration of 5 x 10⁴ for 30 min at room temperature with media in the presence or absence of 100 ng/ml of murine VEGF₁₆₄ (mVEGF₁₆₄) (R&D Systems, Minneapolis, MN). Following incubation, cord formation was assessed as described previously (30, 40). For each independent experiment, the number of endothelial cordlike structures formed in the presence of control mVEGF₁₆₄ was considered maximal (100%), and experimental values were determined as a percentage of maximal cord formation. This experiment was performed in quadruplicate. No difference in cell viability using Trypan blue was observed with ethanol treatment. The mean cord formation percentage of maximal cord formation. This experiment was performed TaqMan assay probes (Applied Biosystems) were used for the analyses in duplicate from three independent experiments in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Fold induction relative to the vehicle-treated control in vitro experiments was calculated using the comparative threshold cycle (Ct) method, where ΔCt is ΔCtstimulant – ΔCtvehicle. ΔCt is Ctgene – CtGAPDH, and Ct is the cycle at which an arbitrary detection threshold is crossed. Target gene expression was normalized to GAPDH. Relative RNA expression was subjected to statistical analysis by two-way ANOVA and Bonferroni’s post hoc tests.

Immunoblot for total and phosphorylated VEGFR-2. Seventy to eighty percent confluent SVEC4-10 cells were incubated in 100-cm² dishes (Falcon) with either serum-free DMEM alone, DMEM and 100 mg/dl of ethanol, DMEM and 100 mg/ml of mVEGF₁₆₄, or DMEM with 100 mg/ml of ethanol and 100 ng/ml of mVEGF₁₆₄ for 5 min. Cells were then washed twice with 1× PBS, tryspinized, and resuspended in serum-free DMEM. From each treatment group, 2 x 10⁵ cells/ml were added in duplicate to each well of a BD Biocoat angiogenesis plate (BD Biosciences, Bedford, MA), and the assay was performed according to the manufacturer’s protocol. After 18 h of incubation at 37°C, the cells were washed with Hanks’ buffered salt solution and stained with Calcein AM (Molecular Probes, Carlsbad, CA). The capillary cord structure within each well was viewed with an Olympus MVX10 microscope equipped with a DC71 camera. Both the cord junctions, defined as intersections of two or more tubes, and the tube length were counted within four random fields from each sample. Tubular length was calculated using Image J software (NIH Image). For each group, the number of cord junctions and tubular length formed in the presence of control mVEGF₁₆₄ were considered maximal (100%), and experimental values were determined as a percentage of the VEGF control. The mean number of cord junctions and tubular length for each group were subjected to statistical analysis using one-way ANOVA followed by Bonferroni’s posttest.

Endothelial cell tube formation assay. Murine SV40 transformed SVEC4-10 (American Type Culture Collection) were grown in six-well plates to 70–80% confluency. Cells were stimulated in quadruplicate with either serum-free DMEM alone, DMEM and 100 mg/dl of ethanol, DMEM and 100 mg/ml of mVEGF₁₆₄ (mVEGF₁₆₄) (R&D Systems, Minneapolis, MN). Following incubation, cord formation was assessed as described previously (30, 40). For each independent experiment, the number of endothelial cordlike structures formed in the presence of control mVEGF₁₆₄ was considered maximal (100%), and experimental values were determined as a percentage of maximal cord formation. This experiment was performed TaqMan assay probes (Applied Biosystems) were used for the analyses in duplicate from three independent experiments in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Fold induction relative to the vehicle-treated control in vitro experiments was calculated using the comparative threshold cycle (Ct) method, where ΔCt is ΔCtstimulant – ΔCtvehicle. ΔCt is Ctgene – CtGAPDH, and Ct is the cycle at which an arbitrary detection threshold is crossed. Target gene expression was normalized to GAPDH. Relative RNA expression was subjected to statistical analysis by two-way ANOVA and Bonferroni’s post hoc tests.

Immunofluorescence of endothelial cells for phosphor-VEGFR. SVEC4-10 cells were trypsinized following an overnight incubation with DMEM/10% FBS. The cells were then incubated on an elliptical rotator using the same treatment groups, as described previously (30, 40). Cells were then incubated in an appropriate horseradish peroxidase-conjugated secondary antibody [goat anti-rabbit for VEGFRs at 1:5,000 (ab6721, Abcam) and goat anti-mouse for GAPDH at 1:5,000 (Molecular Probes, Carlsbad, CA)]. After 1 h at room temperature, followed by enhanced chemiluminescence (ECL) detection with the ECL detection kit (Amersham Biosciences). Blots were stripped and reprobed with anti-VEGFR-2 (ab23734, Abcam, Cambridge, MA) and run at 150 V for 1 h. IgG beads and supernatants from VEGFR-2 beads were included and used as negative controls to assess for specificity of the immunoprecipitation.

Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad). Total protein was observed on the blot using Ponceau stain (Sigma). The blocked membrane was subsequently incubated for 2 h in blocking buffer (Tris-buffered saline, 0.1% Tween with 5% bovine serum albumin) at room temperature. Incubation in primary antibody against phosphorylated VEGFR-2 at 1:1,000 (ab5472, Abcam) or GAPDH at 1:5,000 (Fitzgerald Industries, Concord, MA) was performed in 5% bovine serum albumin overnight at 4°C. Membranes were then incubated in an appropriate horseradish peroxidase-conjugated secondary antibody [goat anti-rabbit for VEGFRs at 1:5,000 (ab6721, Abcam) and goat anti-mouse for GAPDH at 1:5,000 (Molecular Probes, Carlsbad, CA)] for 1 h at room temperature, followed by enhanced chemiluminescence (ECL) detection with the ECL detection kit (Amersham Biosciences). Blots were stripped and reprobed with anti-VEGFR-2 (ab23734, Abcam). The relative intensity of the bands corresponding to phosphorylated VEGFR-2 were normalized to total VEGFR-2 and are represented as percentage of the VEGF control. Values were subjected to statistical analysis by two-way ANOVA and Bonferroni’s post hoc tests.
scratched with a P100 pipette tip down the center of the chamber to create an in vitro wound. Cells were then stimulated with either serum-free DMEM alone, DMEM and 100 mg/dl of ethanol, DMEM and 100 ng/ml of mVEGF_{164} or DMEM with 100 mg/dl of ethanol and 100 ng/ml of mVEGF_{164} for either 3 or 5 min. Following incubation, the cells were washed twice with 1× PBS and fixed in 100% acetone for 30 min. After washing with 1× PBS, cells were blocked with 3% bovine serum albumin in PBS for 30 min. Primary antibody for phosphorylated VEGFR-2 at 1:500 (ab5472, Abcam) or control rabbit IgG at the same concentration as primary antibody were diluted with PBS, and cells were incubated in the primary antibody solution for 2 h at room temperature. Cells were washed and incubated with appropriate secondary antibody at 1:400 (goat anti-rabbit TRITC, Molecular Probes) for 1 h at room temperature. Cells were then washed and mounted with Prolong Antifade with 4,6-diamidino-2-phenylindole (Molecular Probes). Fluorescent images were captured on an Olympus BX51 microscope equipped with a DC71 camera using ×10 or ×40 objectives.

**Western blot for HIF-1α.** Seventy to eighty percent confluent SVEC4-10 cells were incubated in 100-cm dishes (Falcon) with either serum-free DMEM alone or DMEM and 100 mg/dl of ethanol for 24 h under normoxic (20% oxygen) or hypoxic (<1% oxygen) conditions. For hypoxic conditions, plates were placed in GasPak EZ Gas generating pouches (BD Biosciences), according to the manufacturer’s protocol and incubated at 37°C. For normoxic conditions, plates were placed in incubator without GasPak pouches and incubated at 37°C. Following incubation, cells were washed twice with 1× PBS and protein isolated, as previously described (5). Briefly, 1 ml of ice-cold PBS containing protease inhibitor mixture (complete EDTA-free, 1 tablet/50 ml; Roche) was added to the plates, and cells were scraped and collected into 1.5-ml centrifuge tubes. Cytoplasmic and nuclear extracts were obtained according to the manufacturer’s instructions using the NE-PER Nuclear and Cytoplasmic Extract Reagent Kit (Pierce). Total protein content was determined using the BCA Protein Assay (Pierce). Cell nuclear or cytoplasmic extracts were boiled for 3 min and loaded into a 4 –20% gradient SDS-PAGE gel along with 5 μl of biotinylated protein ladder (Cell Signaling Technology) and run at 150 V for 1 h. Normoxic and hypoxic PC-12 lysates were used as controls (Novus Biologicals, Littleton, CO).

Proteins were transferred to a polyvinylidene difluoride membrane (BioRad). Total protein was observed on the blot using Ponceau stain (Sigma). The blocked membrane was subsequently incubated for 2 h...
in blocking buffer (Tris-buffered saline, 0.1% Tween with 5% milk) at room temperature. Primary antibody for HIF-1α at 1:1,000 (Novus Biologicals) was incubated in 5% milk overnight at 4°C. An appropriate goat anti-rabbit horseradish peroxidase conjugated secondary antibody at 1:5,000 (ab6721, Abcam) was then incubated with the membrane for 1 h at room temperature, followed by ECL detection with the ECL detection kit (Amersham Biosciences). Membranes were stripped and reprobed for GAPDH using primary antibody at 1:5,000 (Molecular Probes) and developed with ECL as above. The relative intensity of the bands from normoxic nuclear extracts corresponding to HIF-1α were normalized to GAPDH and are presented as percentage of the hypoxic untreated (media) control. Values were subjected to statistical analysis by two-way ANOVA and Bonferroni’s post hoc tests.

RESULTS

Acute ethanol exposure induces a stable inhibition of endothelial cell cord formation in vitro. Previously, our laboratory determined that endothelial cells exposed to acute ethanol (100 mg/dl) for 4 h exhibited a significant reduction in cord formation (40). In the present experiments, we investigated whether acute ethanol exposure would cause a transient or stable inhibition of endothelial cell cord formation in vitro. The cord formation assay represents an in vitro system by which one can assess endothelial differentiation into capillary tubes, which is reminiscent of what occurs in vivo (24, 30, 43). Initially, we treated endothelial cells with increasing doses of ethanol, ranging from 50 to 300 mg/dl to identify the minimal inhibitory concentration for cord formation in the presence of VEGF. At doses of 100 mg/dl or greater, we could detect a significant reduction in cord formation in the presence of VEGF compared with controls. However, the level of inhibition did not increase at doses >100 mg/dl (data not shown).

After confirming the minimal inhibitory concentration for cord formation, we next sought to determine whether ethanol exposure induced a long-lasting or transient inhibition of endothelial differentiation. Endothelial cells were incubated with

Fig. 2. Acute ethanol exposure decreases VEGF receptor (VEGFR)-2 RNA expression in vitro. Murine endothelial cells (SVEC4-10) were incubated with increasing doses of ethanol, ranging from 50 to 300 mg/dl to identify the minimal inhibitory concentration for cord formation in the presence of VEGF. At doses of 100 mg/dl or greater, we could detect a significant reduction in cord formation in the presence of VEGF compared with controls. However, the level of inhibition did not increase at doses >100 mg/dl (data not shown).

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ethanol (100 mg/dl) or media alone for 8 h and allowed to recover for 8 h without ethanol. Following recovery period, cells were incubated with or without mVEGFR-164 for 4 h, which represents the time point of early differentiation of endothelial cells before the maximal response. Quantitation of cordlike structures at 4 h was used to assess the effect of ethanol on endothelial cell differentiation. Exposure of endothelial cells to ethanol resulted in a significant impairment in their ability to form cords. Even after a recovery period of 8 h with ethanol-free media, the ability of endothelial cells to form cordlike structures on the collagen was significantly decreased compared with control (Fig. 1A). Endothelial cells incubated with ethanol and VEGF exhibited a 60% reduction in percent cord formation compared with cells incubated with VEGF alone (Fig. 1B). The percent cord formation in cells treated with media alone was similar to that of cells treated with ethanol and VEGF, suggesting that the inhibition primarily involves VEGF signaling. No significant difference in cell viability was observed with ethanol treatment using Trypan blue exclusion (saline: 90% vs. ethanol: 93% viability).

We next assessed the ability of ethanol to inhibit capillary tube structure formation in Matrigel (Fig. 1C). Endothelial cells treated with ethanol in the presence of VEGF exhibited a significant reduction in both endothelial cord junction formation (~50% reduction) (Fig. 1D) and tubular length (~25% reduction) (Fig. 1E) compared with VEGF controls, further supporting a direct effect of ethanol on VEGF-induced capillary tube formation.

**Acute ethanol exposure induces a decrease in the expression of VEGFR-2 in vitro.** Because we had observed a reduction in VEGF-induced endothelial cell differentiation and capillary tube formation in the presence of ethanol, the effect of ethanol on the level of VEGFR-1 and -2 mRNA was examined. For VEGFR-1, incubation of endothelial cells with ethanol (100 mg/dl) for either 4, 8, or 24 h did not cause any significant change in the expression of VEGFR-1 mRNA under either hypoxic or normoxic conditions (Fig. 2A). For VEGFR-2, ethanol exposure had no effect on mRNA levels under normoxic conditions. However, under hypoxic conditions, ethanol caused a significant reduction in the expression of VEGFR-2, the receptor directly involved in endothelial cell mitogenesis and differentiation (Fig. 2B) (14, 15). Interestingly, the ethanol-mediated reduction occurred only when both ethanol and VEGF were present, as no reduction was seen with ethanol alone (Fig. 2B). The lack of an ethanol effect on VEGFR-2 under normoxic conditions suggests that the effects of ethanol on endothelial cells may be most profound when VEGFR-2 is upregulated, such as in a hypoxic environment.

**Acute ethanol exposure decreases VEGFR-2 phosphorylation.** One possible mechanism by which ethanol might influence the response to angiogenesis is via interference with receptor phosphorylation. To investigate the effect of ethanol on endothelial cell responsiveness to VEGF, we assessed phosphorylation of VEGFR-2 in vitro.

**Fig. 3.** Acute ethanol exposure decreases phosphorylation of VEGFR-2 in vitro. Murine endothelial cells (SVEC4-10) were incubated without or with VEGF (100 ng/ml) in the presence or absence of ethanol (100 mg/dl) for 5 min. Total protein (150 μg) from cell lysates was immunoprecipitated (IP) with an antibody specific to VEGFR-2. A: immunoprecipitates were run on a 4–20% gradient SDS-PAGE gel. The protein was transferred to polyvinylidene difluoride membrane and probed for phosphor-VEGFR-2 using a tyrosine 1054-specific antibody (top) and total VEGFR-2 (bottom). A portion of cell lysates from each group was treated with lambda phosphatase to assess specificity of phosphor-antibody (middle). A: representative blot for phosphorylated VEGFR-2 and total VEGF at 5 min. Similar results were observed from three independent experiments. IB, immunoblot. B: densitometric analysis of phosphor-VEGFR-2 normalized to total VEGFR-2. *P < 0.05 vs. VEGF. Statistical analysis was performed by one-way ANOVA and a Bonferroni posttest. C: immunofluorescence of endothelial cells for phosphor-VEGFR-2. Endothelial monolayers were either left alone (unstimulated) or scratched down the midline using a pipette tip. Green staining represents phosphor-VEGFR-2. Blue (4,6-diamidino-2-phenylindole) staining represents nuclei. Unstimulated, uninjured cells show little to no immunofluorescence at ×20 magnification (top right). Rabbit IgG was used as a negative control on a scratched endothelial monolayer and is shown at ×20 magnification with green filter only (top left). Media- and ethanol-treated endothelial cells show little to no immunofluorescence along “wounded” edge (middle, ×40 magnification). VEGF-stimulated endothelial cells show enhanced staining for phosphor-VEGFR-2 along “wounded edge” compared with VEGF-stimulated cells in the presence of ethanol (bottom, ×40 magnification). Representative photos are from three separate experiments (n = 3). Magnification bar = 20 μM.
on VEGFR function, we examined the levels of total and phosphorylated VEGFR-2 in endothelial cells exposed to ethanol in vitro. Endothelial cells were treated with media alone, ethanol alone, VEGF alone, or ethanol and VEGF for 5 min, as we determined this to be a time point of maximal phosphorylation of VEGFR-2 following VEGF stimulation. Total VEGFR-2 was immunoprecipitated from cell lysates, and phosphorylation of VEGFR-2 following VEGF stimulation. Total VEGFR-2 was detected using a tyrosine 1054-specific antibody. As expected, VEGF treatment caused an increase in the levels of phosphorylated VEGFR-2 (Fig. 3, A and B). Exposure of endothelial cells to ethanol significantly impaired VEGF-stimulated VEGFR-2 phosphorylation by ~50% (Fig. 3, A and B). Cell lysates treated with lambda phosphatase to dephosphorylate VEGFR-2 resulted in complete loss of detectable phosphorylated VEGFR-2, thus demonstrating the specificity of the phospho-antibody. The effect of ethanol on VEGFR-2 signaling was further examined using immunofluorescence of in vitro scratch wounds made within an endothelial cell monolayer. Endothelial cells exposed to ethanol and VEGF displayed a reduction in VEGFR-2 phosphorylation along the “wound” edge compared with VEGF alone (Fig. 3C). Unstimulated cells and cells exposed to either media or ethanol alone exhibited little to no VEGFR-2 phosphorylation. Thus the likely mechanisms for the ethanol-mediated reduction in endothelial cell cord formation in vitro and vessel formation in vivo include both a VEGFR-2 signaling defect at the level of receptor phosphorylation and diminished VEGFR-2 transcription (Fig. 2B).

Alcohol metabolism is involved in the inhibition of endothelial cell cord formation. To determine whether ethanol metabolism might be involved in the inhibition of endothelial cell differentiation into capillary tubes, SVEC4-10 were exposed to both ethanol and 4-MP, an inhibitor of alcohol dehydrogenase, a key enzyme intermediate within the ethanol metabolic pathway (50). Inhibition of ethanol metabolism by 4-MP yielded a partial reversal of the ethanol-mediated impairment of cord formation (Fig. 4), suggesting that ethanol metabolism is partially responsible for the observed effect. Interestingly, a similar pattern was seen in the absence of VEGF, that is, in the effect of ethanol and 4-MP on baseline cord formation without added stimulus. Unstimulated baseline cord formation was impaired by ethanol, and the effect of ethanol was partially reversed by inhibitor on ethanol metabolism with 4-MP (Fig. 2). Importantly, treatment of cells with 4-MP alone, without the addition of ethanol, had no significant effect on either baseline or VEGF-induced cord formation (Fig. 4).

Acute t-butanol exposure does not decrease wound vascularity in vivo. The by-products of ethanol metabolism by alcohol dehydrogenase, particularly NADH, acetaldehyde, and acetate, induce tissue damage through the production of protein adducts, reactive oxygen species (ROS), lipid peroxidation, and alterations in signal transduction (28, 32, 45). To determine the contribution of ethanol metabolism to the decrease in wound vascularity in vivo, we injected mice intraperitoneally with 1.4 g/kg of t-butanol, an alcohol not metabolized by alcohol dehydrogenase into toxic by-products, such as acetaldehyde (25). Wounds were harvested 7 and 10 days later for analysis of vascularity. In contrast to the response to ethanol, acute exposure of mice to acute t-butanol did not result in a decrease in vascularity at days 7 or 10 postwounding, time points that represent the peak of vessel density in healing wounds (Fig. 5) (30, 47). Together, the data suggest that ethanol metabolism is an important component of the ethanol-mediated impairment of wound angiogenesis.

Acute ethanol exposure increases wound hypoxia in vivo. The Hypoxyprobe-1 is a 2-nitroiminidazole hypoxia marker that is reductively activated at low oxygen concentrations, allowing it to form immunogenic covalent protein adducts with thiol groups in proteins, peptides, and amino acids (3, 4). Similar to our previous studies, wounds from ethanol-treated mice contained fewer vessels at day 7 (Fig. 6A) and day 10 (Fig. 6B) (40). Use of the Hypoxyprobe revealed that the wounds of mice exposed to ethanol also exhibited considerably more hypoxic cells compared with wounds from saline-treated animals. When these images were merged, we were able to determine that the hypoxic cells were not endothelial cells, as there is no evidence of colocalization of endothelial cell marker PECAM and the Hypoxyprob- positive cells. Because the fibroblast is the predominant cell type in day 7 and day 10 wounds, the hypoxic cells may represent, at least in part, the myofibroblast and fibroblast population. These data suggest that the decreased vascularity that occurs in wounds of mice exposed to ethanol is accompanied by an increase in the level of hypoxia.

Acute ethanol exposure reduces nuclear expression of HIF-1α in endothelial cells. The endothelial response to hypoxia is one of the most critical responses during wound healing. HIF-1α null mice are known to exhibit, in part, defects in VEGFR-2 expression and vessel formation and thus are deficient in their response to hypoxia and exhibit delays in wound healing (48). Since we observed a reduction in VEGF-2 expression in endothelial cells and identified that wounds from ethanol-treated mice were considerably more hypoxic, despite adequate levels of VEGF (40), we hypothe-
sized that ethanol exposure may impair HIF-1α expression, rendering the endothelial cell unresponsive to VEGF by interfering with the autocrine response to hypoxia. Endothelial cells were treated with or without ethanol under normoxic or hypoxic conditions for 24 h. Nuclear and cytoplasmic extracts were isolated and probed for HIF-1α protein expression by Western blot. Endothelial cells exposed to ethanol showed a significant decrease in nuclear protein expression of HIF-1α compared with media alone (Fig. 7, A and C). Extracts from PC-12 cells exposed to normoxic or hypoxic conditions served as negative and positive controls (Fig. 7B). No significant differences were observed in cytoplasmic expression of HIF-1α expression under hypoxic conditions, although there was a trend toward an increase in cytoplasmic HIF-1α expression from ethanol-treated cells (Fig. 7B). To ensure that this defect was solely at the protein level, we assessed the gene expression of HIF-1α under normoxic and hypoxic conditions. Endothelial cells exposed to hypoxia for 4, 8, or 24 h exhibited a significant increase in HIF-1α expression compared with cells exposed to normoxic conditions, although no significant differences were observed between treatment groups (Fig. 7D). Overall, this suggests a defect in the regulation of HIF-1α degradation or nuclear translocation in the presence of ethanol and VEGF during hypoxic conditions, rather than a change in gene expression.

**DISCUSSION**

Signaling via the VEGFRs, particularly VEGFR-2, mediates the molecular changes required for endothelial cell function during angiogenesis. The importance of these receptors in the formation of normal vasculature during embryonic development has been shown in mice, as mutants with inactivated VEGFR-1 or VEGFR-2 fail to develop normal vasculature and die in utero (44). In adult skin, a precise balance between pro- and anti-angiogenic factors maintains the quiescent state of adult vasculature (6, 7, 11, 19). Following injury, proangiogenic stimuli are released into the wound site and shift the balance toward promoting capillary growth.

The angiogenic process in wounds relies on the initial release of proangiogenic factors, and the regulation of endothelial differentiation into capillary tubes is primarily dependent on VEGF signaling and VEGFR phosphorylation. VEGFR levels in endothelial cells are known to modulate in response to environmental cues. Hypoxia can cause an increase in VEGFR expression (37, 38), and a significant increase in VEGFR-2 transcripts in microvascular endothelial cells has been seen in response to hypoxia (38). However, hypoxic cells also exhibit a reduction in VEGFR-2 phosphorylation (38). In addition to hypoxia, the level of VEGF in the surrounding environment can modulate the expression of VEGFRs. For instance, exogenous addition of VEGF downregulated cell surface receptor expression and reduced the cellular response to VEGF, while upregulating the mRNA expression as a means to replenish the...
endothelial surface with VEGFRs (51). This regulation in the presence of excess proangiogenic stimuli allows for the paracrine regulation of VEGFRs to control stimulation of endothelial cells.

Our laboratory’s previous studies demonstrated that the level of VEGF was significantly higher in wounds from ethanol-treated mice compared with saline controls (40). Thus there does not appear to be an ethanol-mediated defect in secretion or production of VEGF within the wound milieu, but rather an impaired response of the endothelial cell to the proangiogenic stimuli. Our results, demonstrating a long-term negative effect of ethanol on endothelial cord formation, as well as a negative effect on the formation of microvascular networks in Matrigel, suggest a direct effect of ethanol on endothelial cell function. Downregulation of VEGFRs by excess VEGF, as described above, may play a role in reducing the angiogenic response in wounds following ethanol exposure. Moreover, as shown above, ethanol exposure may lead to a reduction, in both the expression of VEGFR-2 transcript and the phosphorylation of VEGFR-2. In the context of the wound healing, endothelial cells may be rendered unresponsive to VEGF via ethanol-derived metabolic intermediates, resulting in reduced signaling capacity at both the protein and mRNA level. Extreme hypoxia due to the lack of vessel formation may lead to further production of ROS and prolonged endothelial cell dysfunction.

The ability of endothelial cells to adhere to the extracellular matrix, alter morphology, migrate, and differentiate is vital for physiological events, including wound healing. The effects of ethanol on cellular activity may be due directly to changes in membrane fluidity, or to the products of its metabolism via oxidation (28). Three enzyme systems are involved in ethanol oxidation and include alcohol dehydrogenase, the microsomal ethanol oxidizing system, and catalase. Ethanol is predominantly oxidized by ADH into acetaldehyde, which is further metabolized into acetate. Acetaldehyde is believed to induce numerous cellular alterations via increases in ROS, RNA and protein stability, and receptor levels (1, 9, 16, 28, 41). In vivo experiments using t-butanol, a tertiary long-chain alcohol known to increase membrane fluidity, did not induce changes in vascular density nor hydroxyproline content, suggesting that increasing membrane fluidity in and of itself would not inhibit wound angiogenesis. However, in vitro experiments to examine the effect of t-butanol on endothelial cells revealed a significant inhibition of endothelial cell cord formation, presumably through changes in membrane fluidity, even in the presence of VEGF (data not shown). This apparent paradox suggests that t-butanol exerts additional effects in vitro that may cause impaired cord formation, and that the actions of alcohols differ, depending on the system.

Our studies indicate that the metabolism of ethanol appears to play a role in the effect on endothelial cells, as in vitro incubation of endothelial cells with ethanol and an inhibitor of alcohol dehydrogenase partially restored the ability of the endothelial cells to differentiate into capillary tubes. These data correlate with the results of the in vitro recovery cord assay, which shows that the effects of ethanol are long lasting.

In these studies, many assessments of the effect of ethanol on endothelial cell function and phenotype were conducted
in vitro. The pharmacological response of cells in vitro may not completely mimic the intricate in vivo cell milieu in a live animal. Nevertheless, the in vitro investigations have allowed us to assess the direct effects of ethanol exposure on endothelial cell function, structure formation, and signaling in ways that are not readily accomplished in vivo. The current data support the in vivo analysis shown here and in our laboratory’s previous studies (40). However, wounds from mice exposed to ethanol would contribute additional important variables beyond what is included in in vitro experiments, such as extracellular matrix interactions, inflammation, and the presence of growth factors and cytokines derived from cell types beyond endothelium. While our studies generally support the concept that ethanol impairs endothelial cell function, our results differ from a few previous studies that suggest that ethanol exposure enhances angiogenesis. In one previous study, 0.4% (400 mg/dl) ethanol increased endothelial cell migration and induced in vitro angiogenesis in endothelial cells. Other studies suggest that, following gastric mucosa injury, excessively high levels (>600 mg/dl) of ethanol increased expression of bFGF and VEGF and stimulated mitogen-activated protein kinase in a gastric endothelial-derived cell line (20, 21). However, these doses are much higher than might be expected in most intoxicated patients (39). Ethanol at 0.25 g/kg has been shown to increase VEGF mRNA expression by 1.48-fold over control and increase angiogenesis in the chick chorioallantoic membrane (CAM) (17). These studies suggest that the effect of ethanol may be system dependent. Another important consideration might be the level of inflammation created by direct ethanol exposure to tissues such as mucosa and the CAM, as inflammatory cells themselves can promote an angiogenic response. An alternative explanation may be that, at very high concentrations, ethanol metabolism itself produces metabolic by-products that are angiogenic. A study conducted by Murray and Wilson (33) demonstrated that metabolites of glycolytic and oxidative metabolic pathways have angiogenic properties. In these studies, some of these metabolites induced a robust angiogenic response in the CAM assay in vivo and in chick embryonic capillary endothelial cells in vitro, whereas solely oxidative metabolites did not (33).

Increased wound hypoxia in response to ethanol was observed in the present studies. While hypoxia has been proposed to increase the expression of VEGFRs, prolonged hypoxia cannot sustain angiogenesis. Under initial hypoxic conditions, the endothelial cells express angiogenic receptors and allow for binding of angiogenic growth factors (15, 35). In wounds, a robust release of proangiogenic growth factors, particularly VEGF, occurs from platelets, epithelial cells, and immune cells. This binding of VEGF to VEGFRs and the resultant signal transduction cascade promotes the release of proteolytic enzymes to degrade the basement membrane. This process is critical for endothelial cell proliferation and migration (14, 18, 22). Once the endothelial cells differentiate into sprouting vessels, the new vasculature must be stabilized by reconstitution of the basement membrane, as well as recruitment of associated pericytes and smooth muscle cells. If hypoxia is prolonged, endothelial cells lose the ability to promote survival of adjacent cells and will continue to secrete matrix-degrading proteins.

Fig. 8. Proposed model for the effects of acute ethanol exposure on endothelial cell signaling during early wound healing. In normal wounds, a drop in oxygen tension induces the expression of hypoxia-inducible genes, such as VEGF in endothelial cells. This process involves the active translocation of HIF-1α to the nucleus. The resulting VEGF stimulates an autocrine loop that acts on the endothelial cell to initiate endothelial cell migration and proliferation. VEGF stimulates its cognate receptor, VEGF-R2, resulting in autophosphorylation, signal transduction, and further transcription of proangiogenic factors to allow for endothelial cell differentiation into capillaries. In the presence of ethanol, the hypoxia-induced translocation of HIF-1α is diminished in endothelial cells at the wound site (A). The decrease in HIF-1α activity would disrupt endothelial cell production of VEGF and impair its autocrine effect. Ethanol also causes a decreased production and responsiveness of VEGF-R2, including impaired receptor phosphorylation, which may also be a result of suppressed HIF-1α nuclear protein expression (B). Together, the effects of ethanol render endothelial cells less responsive to VEGF, leading to an impaired angiogenic response.
The finding of increased hypoxia in wounds of ethanol-treated mice correlates well with our laboratory’s previous results demonstrating significantly higher levels of VEGF at day 10 postwounding in mice exposed to ethanol (40). Hypoxia is known to stimulate VEGF production (2, 8, 14); hence, persistence of hypoxia may be at least partially responsible for the ethanol-induced increase in wound VEGF. The failure of endothelial cells to appropriately respond to VEGF in ethanol-treated mice would create a cycle in which low oxygen levels do not abate, despite adequate angiogenic stimuli. Our studies do not prove that the increase in tissue hypoxia exists because there is a defect in neovascularization into the wound. On the contrary, a defect in neovascularization may exist if the tissue is incessantly hypoxic. Numerous studies have demonstrated that ethanol exposure results in a dose-dependent increase in metabolic acidosis via disruption of the respiratory chain (23, 34, 42). If so, then the decrease in vascularity may be mediated, in part, by prolonged tissue hypoxia within the wound. Thus the ultimate consequence of ethanol exposure on angiogenesis may be multifactorial, if the ethanol exposure further disrupts VEGFR signaling.

Our combined in vitro and in vivo data suggest that ethanol-mediated defects in endothelial cell function might be initially attributed to ethanol and its metabolites, but may also derive from the resulting hypoxia. One molecule that we hypothesized might be affected by ethanol is HIF-1α, a hypoxia-inducible transcription factor involved in the expression of most hypoxia-inducible genes. Multiple studies suggest that the hypoxic-induced mitogenic response is critical for endothelial-driven angiogenesis during hypoxic stress (48). Our data demonstrate that, in the hypoxic environment, ethanol exposure impairs either the translocation of HIF-1α to the nucleus, or its stabilization, both of which are essential to its function as a regulator of hypoxia-inducible genes. Further studies are required to determine the specific mechanism behind ethanol-induced suppression of nuclear HIF-1α protein abundance. Interestingly, we also noted that HIF-1α expression increases in both the nuclear and cytoplasmic fractions in ethanol-treated cells under normoxic conditions. One possible explanation is that ethanol itself may shift the redox equilibrium, exacerbate oxidative stress within the wound milieu, resulting in prolonged hypoxia and endothelial cell dysfunction.

The present study demonstrates that the inhibitory effects of acute ethanol exposure are mediated by ethanol metabolization and result in a synergistic reduction in endothelial cell function, as summarized in Fig. 8. When exposed to ethanol, endothelial cells exhibit decreased VEGFR-2 function, including impaired receptor phosphorylation. Ethanol also perturbs the hypoxia-driven stabilization and translocation of HIF-1α to the nucleus within endothelial cells, further contributing to decreased responsiveness. The decrease in HIF-1α activity might delay or impair the production of VEGF by endothelial cells, disrupting the autocrine loop. Alternatively, decreased nuclear HIF-1α protein may induce changes in the transcription of adaptor molecules required for maximal VEGFR-2 signaling. Ultimately, the ethanol-mediated decrease in wound vascularity would lead to increased wound hypoxia and oxidative stress. Further studies are needed to understand the complete mechanism that leads to the impairment of physiological angiogenesis following acute ethanol exposure.

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