Am J Physiol Regulatory Integrative Comp Physiol
281: R365–R372, 2001.

rapid communication

Moderate levels of ethanol induce expression of vascular endothelial growth factor and stimulate angiogenesis

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Received 12 December 2000; accepted in final form 12 April 2001

Gu, Jian-Wei, Jesse Elam, Amanda Sartin, Wen Li, Raymond Roach, and Thomas H. Adair. Moderate levels of ethanol induce expression of vascular endothelial growth factor and stimulate angiogenesis. *Am J Physiol Regulatory Integrative Comp Physiol* 281: R365–R372, 2001.—Alcohol abuse has a negative impact on human health; however, epidemiological studies show that moderate consumption of ethanol (EtOH) reduces the risk of coronary heart disease, sudden cardiac death, and ischemic stroke. The mechanisms for these reductions in cardiovascular disease are not well established. Using cultured coronary artery vascular smooth muscle cells, we found that moderate levels of EtOH (10 and 20 mM) caused dose-related increases in both vascular endothelial growth factor (VEGF) mRNA (Northern blot) expression (1.9- and 2.6-fold) and VEGF protein (ELISA) expression (19 and 68%) compared with control ($P < 0.05$). EtOH at 0.25 $g \cdot kg^{-1} \cdot day^{-1}$ (7 days) increased VEGF mRNA expression by 1.48-fold over control, and increased vessel length density from 3.9 ± 0.7 (control) to 6.0 ± 0.3 mm/mm^2 ($P < 0.05$) in chick chorioallantoic membrane (CAM). We conclude that moderate levels of ethanol can induce VEGF expression and stimulate angiogenesis in chick CAM. Therefore, the results provide a theoretical basis for speculating that the cardiovascular-protective effects of moderate alcohol consumption may be partly mediated through VEGF-induced angiogenesis.

alcohol; vascular smooth muscle cells; chorioallantoic membrane

IT HAS LONG BEEN KNOWN that alcohol abuse can have damaging effects on the liver, central and peripheral nervous system, pancreas, skeletal muscle, myocardium, and fetus. Excessive ethanol intake may also promote certain forms of human cancer (17). However, during the past two decades, a number of epidemiological studies (2, 24, 30, 34) have suggested that moderate consumption of alcohol may protect against cardiovascular disease. Moderate ethanol intake is a negative risk factor for atherosclerosis and its clinical consequences, i.e., coronary heart disease, ischemic stroke, and peripheral vascular disease. It is especially significant that mortality and morbidity attributable to coronary heart disease is 40–60% lower in moderate drinkers compared with abstainers (10).

The mechanisms accounting for cardiovascular-protective effects of moderate alcohol consumption are not well established. Alterations in plasma lipoproteins, particularly increases in high-density lipoprotein (HDL) cholesterol, are thought to contribute to the protective effects of alcohol (9, 11). It is estimated that ~50% of the protective effect of moderate alcohol consumption is mediated through increased levels of HDL cholesterol (9). Other studies suggest that the beneficial effects of alcohol may also be mediated through prevention of vascular thrombosis and occlusion (15, 28). However, it is likely that additional actions of ethanol could contribute to its cardiovascular-protective effects, in which the possible mechanism could be ethanol-induced angiogenesis.

Angiogenesis is often observed to be a compensatory response to prolonged imbalances between the metabolic requirements of the tissues and the perfusion capabilities of the vasculature (1). Angiogenesis is thus a compensatory response to ischemia/hypoxia. The angiogenic process is initiated by degradation of the extracellular matrix and requires the migration and proliferation of endothelial cells. Many growth factors have been postulated to stimulate one or more steps of the angiogenic process; however, a growing belief is that vascular endothelial growth factor (VEGF) plays a key regulatory role in angiogenesis in both physiolog-

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ical and pathological conditions (5, 7). Special features of VEGF that make it a popular candidate for angiogenesis regulation are its relatively low expression in endothelial cells compared with nearly all other cells (4, 18), the restriction of its receptors to endothelial cells, and, perhaps most important, the fact that its expression is markedly enhanced by hypoxia both in vivo and in vitro (12, 13, 19, 22). Furthermore, VEGF-induced angiogenesis has a therapeutic effect in animal models of coronary ischemia (3, 14).

Ethanol is often used to solubilize other compounds for both in vitro and in vivo experiments. During the course of our studies on angiogenesis, we found an unexpected increase in VEGF expression in cell cultures that could not be attributed to the compound under study. Further experimentation suggested it was the ethanol we used to solubilize the compound that stimulated the expression of VEGF.

The purpose of the present study is to determine whether moderate levels of ethanol, i.e., concentrations that can be achieved in human subjects with moderate consumption, can stimulate VEGF expression and induce angiogenesis. The results show that moderate levels of ethanol can induce VEGF expression in coronary artery vascular smooth muscle cells (CAVSMC) and induce angiogenesis (as well as VEGF expression) in the chick chorioallantoic membrane (CAM).

METHODS

Cell cultures. CAVSMC were isolated from adult male mongrel dogs using a method previously described (12). The purity of the culture was confirmed by immunohistochemical staining using mouse anti-α-smooth muscle actin-fluorescein isothiocyanate conjugate (Sigma). The cells were seeded into sterile culture flasks at $\sim 5 \times 10^4$ cells/cm^2 and incubated at 37°C in a humidified atmosphere of 5% CO_2-20% O_2-75% N_2. Cell lines were used between *passages 4* and *8* in the experiments. The mouse hepatocytes were obtained from ATCC (H2.35). When the monolayers of cells reached ~80% confluence, standard medium (50% DMEM plus 50% M199) containing 10% FBS was replaced with media having 4% heat-inactivated FBS to reduce the mitogenic influence of growth factors and hormones. The cells were then treated with ethanol (10 or 20 mmol/l) for different periods, such as 2, 6, and 18 h.

VEGF protein. VEGF protein levels were measured in the media of cultured CAVSMC using sandwich ELISA (R&D Systems) as previously described (12). VEGF protein levels were normalized to the total amount of cellular protein and expressed as picograms per milligram of total cell protein. Cell protein content was determined in duplicate with BSA as the standard (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories).

Chick embryo CAM assay. Chick embryo CAM assays were performed by modification of previous methods (26, 32). Fresh fertile eggs from White Leghorn hens were incubated in forced-draft incubators at 37.8°C and 53% relative humidity. From *day 9* to *day 16*, normal saline (as control) or ethanol (Sigma Chemical, St. Louis, MO) at 0.125 or 0.25 $g \cdot kg^{-1} \cdot day^{-1}$ was administered into the air space through a 2- to 3-mm-diameter hole made in the center of the large end of the egg. Ethanol was diluted to 15% with normal saline. The control and test embryos always came from the same batch of eggs and were studied at the same time. In one series of experiments, total RNA was extracted from CAM for VEGF mRNA expression analysis after embryos were treated with ethanol or normal saline. On *day 16*, at 4 h after administration of saline or ethanol, the shell was removed from the large end of each egg, and the embryo and yolk were gently removed from the shell through the hole. CAM tissue was dissected from the shell and frozen immediately in liquid nitrogen. In a second series of experiments, digital images of the vasculature were acquired from CAMs still attached to the shell after embryos had been treated with ethanol or normal saline. On *day 16*, at 4 h after administration of test substances, the shell was removed from the large end of each egg, and the embryo and yolk were gently removed from the shell through the hole. The egg shell was cut longitudinally into three equal parts. After being gently rinsed with PBS, digital images of CAM vasculature were acquired in situ using a computerized image analysis system with a ×25 objective (Leitz). Blood vessels could be visualized because red blood cells were retained within the vessels (see Fig. 3). Vessel length density (mm/mm^2) was determined by analysis of randomly acquired skeletonized images of CAM vasculature using Optimas software (Seattle, WA). Thirty randomly acquired images each having an area of 25 mm^2 were analyzed on each CAM. The threshold for binarization of each image was performed by an investigator who did not have knowledge of the experimental group. The binary images were subsequently skeletonized (i.e., the vessels were reduced to a width of a single pixel).

Northern blot analysis. Total RNA isolation and Northern blot analyses were performed as previously described (12). The VEGF cDNA probe for CAVSMC was a 580-bp *Eco*R I-*Bam*H I fragment of the murine VEGF cDNA cloned into pBluescript plasmid [kindly provided by Dr. Werner Risau (23)]. The VEGF cDNA probe for chick embryo CAM was a 350-bp *Kpn* I-*Sac* I fragment of quail VEGF cDNA cloned into pBluescript plasmid [kindly provided by Dr. Ingo Flamme (6)]. The hypoxia-inducible factor (HIF)-1α cDNA probe for CAVSMC was a 310-bp *Kpn* I-*Sac* I fragment of human HIF-1α cDNA cloned into pBluescript plasmid (Novus-Biologicals). To verify the relative amounts of total RNA, filters were hybridized with a ^{32}P-labeled 28S rRNA antisense oligonucleotide probe (Ambion). The VEGF or HIF-1α mRNA was normalized against 28S rRNA in each sample.

Cell proliferation. Cell proliferation was determined by [^{3}H]thymidine incorporation. The uptake of [^{3}H]thymidine by human umbilical vein endothelial cells (HUVEC) or CAVSMC was used as an indicator of DNA synthesis, as described previously (12). Cells were cultured in standard media supplemented with 4% FBS in the absence (control) and presence of 20 mmol/l ethanol for 8 h. In a second series of experiments, we tested the effects of CAVSMC-conditioned media and ethanol-CAVSMC-conditioned media on proliferation of HUVEC or CAVSMC. We harvested the conditioned media from cultured CAVSMC 18 h after incubation in standard media in both the absence and presence of 20 mmol/l ethanol. The cells were cultured in the conditioned-media (that had or had not been exposed to ethanol) for 8 h. During the last 6 h of incubation, the cells were pulsed with [^{3}H]thymidine by adding 1 μCi per well. The cells were then washed, harvested, and processed for counting in a scintillation counter.

Metabolic consequences of ethanol. The metabolic consequences of ethanol in cultured MVSMC were studied by determining lactate production in the absence or presence of ethanol and the amount of ethanol that was metabolized by cultured CAVSMC. Lactate in media was measured using an enzymatic method (Sigma Lactate Kit) after 18 h of incuba-

tion in the absence or presence of 20 or 40 mmol/l ethanol. Metabolism of ethanol by CAVSMCs and mouse hepatocytes cultured in media having 26 mmol/l ethanol was determined by measuring changes in ethanol levels in the media in 6-h intervals using NAD-ADH kits (Sigma) and was corrected for evaporation of ethanol. The total amount of ethanol that was metabolized was normalized by total cell protein mass and expressed as micromoles per milligram.

Statistical analyses. All determinations were performed in duplicate, and each experiment was repeated at least three times. When indicated, data are presented as means ± SD or means ± SE . Differences were considered statistically significant when $P < 0.05$ by paired or unpaired *t*-test. All statistical calculations were performed with StatView software (BrainPower, Calabasa, CA).

RESULTS

Ethanol induces the expression of VEGF protein and mRNA in vitro. Exposing CAVSMC to ethanol caused a dose-dependent increase in the expression of VEGF protein and mRNA (Fig. 1*A*). Exposing CAVSMC to 10 and 20 mmol/l ethanol for 18 h caused VEGF protein levels in media to increase by 19% (2.64 ± 0.13 ng/mg, mean ± SD) and 68% (3.71 ± 0.25 ng/mg), respectively, compared with control (2.21 ± 0.13 ng/mg, $n = 6$; $P < 0.05$). Northern blot analyses indicated that VEGF mRNA expression was increased by 1.91- and 2.60-fold, respectively, over the control ($n = 6$; $P < 0.01$), when CAVSMC had been exposed to 10 and 20 mmol/l ethanol for 18 h. In addition, exposing CAVSMC to 20 mmol/l ethanol for 6 h increased VEGF protein levels in media by 27% (1.67 ± 0.05 ng/mg) compared with control (1.31 ± 0.12 ng/mg, $n = 4$; $P < 0.01$). Cells exposed to 10 or 20 mmol/l ethanol excluded trypan blue dye (>95%) and did not show changes in cell shape or attachment detectable by light microscopy.

Effect of ethanol on cell proliferation. As indicated in Fig. 2, adding 20 mmol/l ethanol directly into media had no effect on cell proliferation of either HUVEC or CAVSMC after 8-h incubation in standard media ($P > 0.05$, $n = 6$). Basal levels (control) of [^{3}H]thymidine uptake in HUVEC and CAVSMC were 9.6 ± 2.2 $\times 10^3$ cpm/well (mean ± SD) and 9.2 ± 1.8 $\times 10^3$ cpm/well, respectively. When the cells were cultured in CAVSMC-conditioned media, the levels of [^{3}H]thymidine uptake in HUVEC and CAVSMC were 12.2 ± 1.5 and 10.5 ± 1.6 $\times 10^3$ cpm/well, respectively. Figure 2 also indicates that exposure of HUVEC to ethanol-CAVSMC-conditioned media increased cell proliferation by 20% ($n = 6$, $P < 0.05$) compared with CAVSMC-conditioned media (control). In contrast, the ethanol-conditioned media had no effect on proliferation of cultured CAVSMC. Because CAVSMC produce VEGF but lack VEGF receptors and because HUVEC have VEGF receptors but express minimal amounts of VEGF, the results shown in Fig. 2 suggest that VEGF may mediate the proliferative effect of ethanol-CAVSMC-conditioned media on

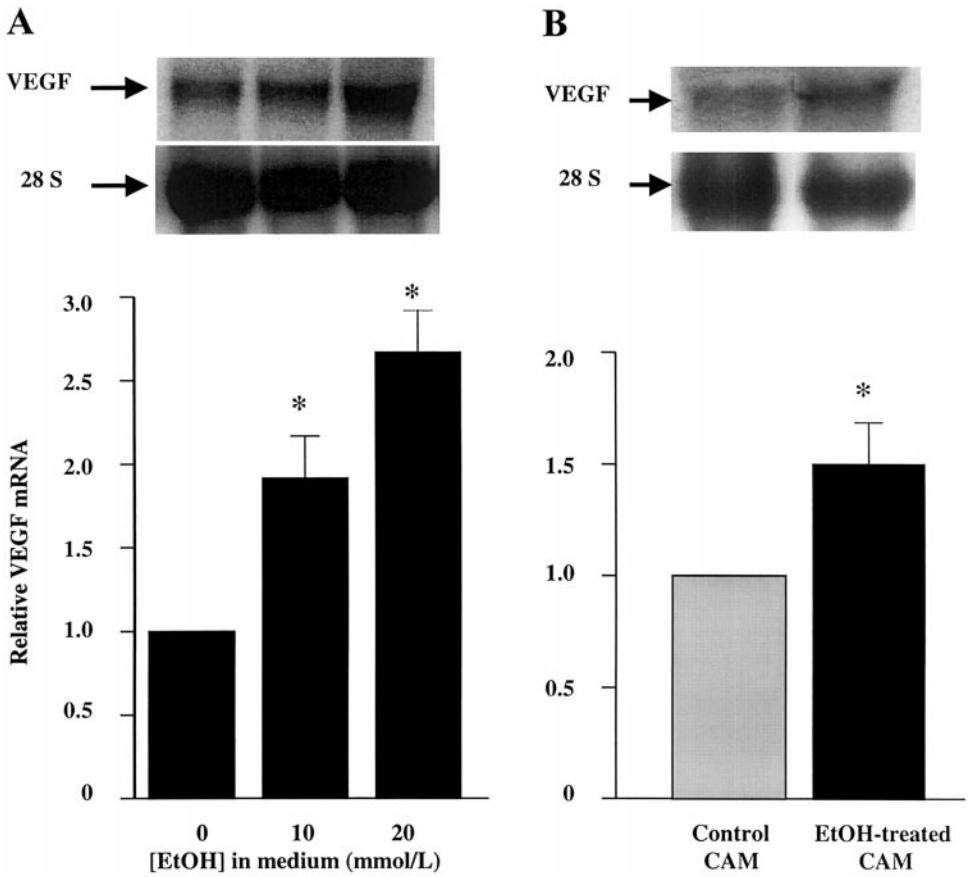

Fig. 1. Exposure to moderate levels of ethanol induces the expression of vascular endothelial growth factor (VEGF) mRNA in cultured coronary artery vascular smooth muscle cells (CAVSMC; *A*) and in chick embryo chorioallantoic membranes (CAM; *B*). *A*: CAVSMC were cultured in the absence and the presence of ethanol (EtOH; 10 or 20 mM) for 18 h. VEGF mRNA expression was increased by 1.91- and 2.60-fold, respectively, compared with control ($n = 6$; $^*P < 0.01$) when CAVSMC had been exposed to 10 and 20 mM EtOH for 18 h. *B*: moderate amounts of EtOH increased VEGF mRNA expression in chick embryo CAM. Embryos were treated with EtOH at 0.25 $g \cdot kg^{-1} \cdot day^{-1}$ for 7 days after having been incubated for 9 days. Northern blot analysis indicated that EtOH increased VEGF mRNA expression in chick embryo CAM by 1.48-fold over saline control ($n = 6$; $^*P < 0.05$).

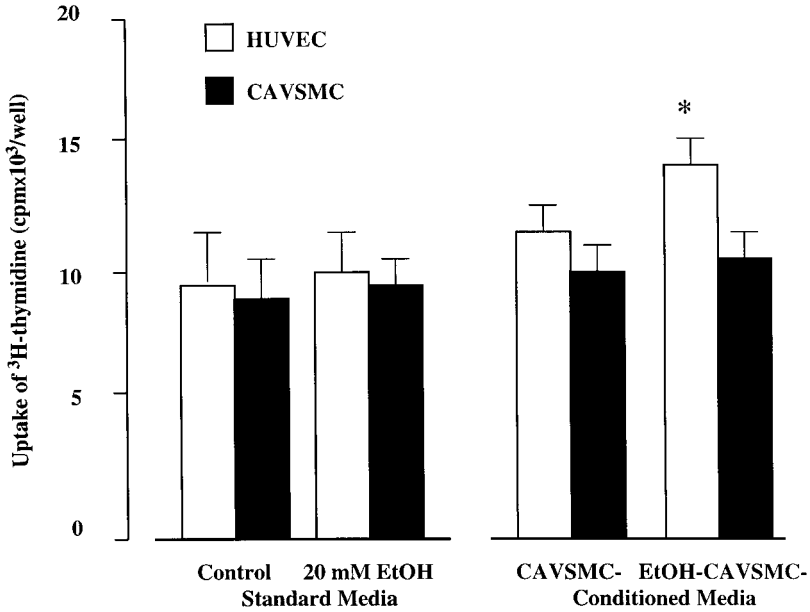

Fig. 2. The effects of ethanol on proliferation of human umbilical vein endothelial cells (HUVEC) and CAVSMC were determined by [^{3}H]thymidine incorporation. *A*: adding 20 mmol/l directly into media had no effect on proliferation of either HUVEC or CAVSMC after 8 h incubation in standard media. *B*: exposure of HUVEC to ethanol-CAVSMC-conditioned media increased cell proliferation by 20% ($n = 6$, $^*P < 0.05$) compared with CAVSMC-conditioned media (the level of [^{3}H]thymidine uptake in HUVEC was 12.2 ± 1.5 $\times 10^3$ cpm/well). Cell proliferation was unchanged in CAVSMC exposed to ethanol-CAVSMC-conditioned media, compared with CAVSMC-conditioned media.

HUVEC. However, further studies are necessary to confirm or refute this possibility.

VEGF mRNA expression in chick embryo CAM. We tested whether moderate levels of ethanol can increase VEGF mRNA expression in vivo using a chick embryo model. As shown in Fig. 1*B*, Northern blot analyses indicated that administration of ethanol at 0.25 $g \cdot kg^{-1} \cdot day^{-1}$ for 7 days increased VEGF mRNA expression in chick embryo CAM by 1.48 ± 0.16-fold (mean ± SD) compared with the saline-treated control group ($P < 0.05$, $n = 6$).

Effect of ethanol on CAM vascular growth. Figure 3 shows representative digital images of the CAM vasculature on *day 16* after embryos were treated with normal saline (control) or ethanol at 0.25 $g \cdot kg^{-1} \cdot day^{-1}$ for 7 days (from *days 9* to *16*). Note that administration of ethanol (Fig. 3, *C* and *D*) increased the formation of blood vessels as illustrated by greater branching of vessels and higher vascular density compared with saline-treated CAM (Fig. 3, *A* and *B*). Vessel length density (mm/mm^2) was determined by analysis of randomly acquired skeletonized images of CAM vasculature using Optimas software. We found that CAM vessel length density (VLD) was 3.94 ± 0.71 (mean ± SD) mm/mm^2 in the control group and 6.01 ± 0.33 mm/mm^2 in the ethanol-treated group ($n = 6$; $P < 0.05$), which represents a 53% increase in VLD. Regression analysis indicates that ethanol caused a dose-related increase in VLD ($r = 0.95$). Administration of ethanol at 0.125 and 0.25 $g \cdot kg^{-1} \cdot day^{-1}$ for 7 days caused VLD to increase by 39 ($n = 6$; $P < 0.05$) and 53% ($n = 6$; $P < 0.05$), respectively, compared with the control group.

Metabolic response to ethanol in cultured CAVSMC. We found that ethanol increased lactate production in a dose-dependent manner in cultured CAVSMC after 18 h incubation. Lactate levels in media were 3.5 ± 0.1 (mean ± SD), 4.5 ± 0.5, 5.2 ± 1.0, and 6.3 ± 0.8 mmol/l in the absence (control) and presence of ethanol at 10, 20, and 40 mmol/l, which represented an increase of 30, 48, and 81%, respectively, compared with the control ($n = 6$; $P < 0.05$). Figure 4*A* shows that there is a very strong linear decrease ($R = 0.999$, $P < 0.01$) in ethanol concentrations of the media having no cells during 12 h incubation. We consider that this decrease is due to the evaporation of ethanol during incubation. Ethanol concentrations in the media with CAVSMC decreased more significantly ($P < 0.01$) compared with the control (media having no cells). The ethanol levels were 26.76 ± 0.28 (mean ± SE), 24.33 ± 0.62, and 22.64 ± 0.47 mmol/l, respectively, at 0, 6, and 12 h incubation after adding 0.15% (vol/vol) ethanol into the media. The ethanol levels in the media cultured with mouse hepatocytes were 22.87 ± 0.87 and 21.12 ± 0.71 mmol/l, respectively, at 6 and 12 h after adding 0.15% (vol/vol) ethanol into the media. Changes of ethanol levels in the media-cultured cells were adjusted by the evaporation of ethanol, and the total amount of ethanol that was metabolized was normalized by total cell protein mass and expressed as micromoles per milligram. Figure 4*B* indicates that CAVSMC metabolized significant amounts of ethanol, which amounted to 1.9 ± 0.2 (mean ± SD) and 4.1 ± 0.3 μmol/mg total cell protein, respectively, after 6 and 12 h incubation in media having 0.15% (vol/vol) ethanol. The rate of ethanol metabolism in cultured mouse hepatocytes after 6 and 12 h incubation was 2-fold higher compared with CAVSMC ($n = 6$; $P < 0.01$).

Effect of ethanol on HIF-1α mRNA expression. We tested whether moderate levels of ethanol can induce mRNA expression of HIF-1α. Figure 5 shows that exposing CAVSMC to 20 mmol/l ethanol in media for 2 h incubation significantly increased HIF-1α mRNA expression by 1.4 ± 0.17-fold (mean ± SD) over the control ($n = 4$; $P < 0.05$).

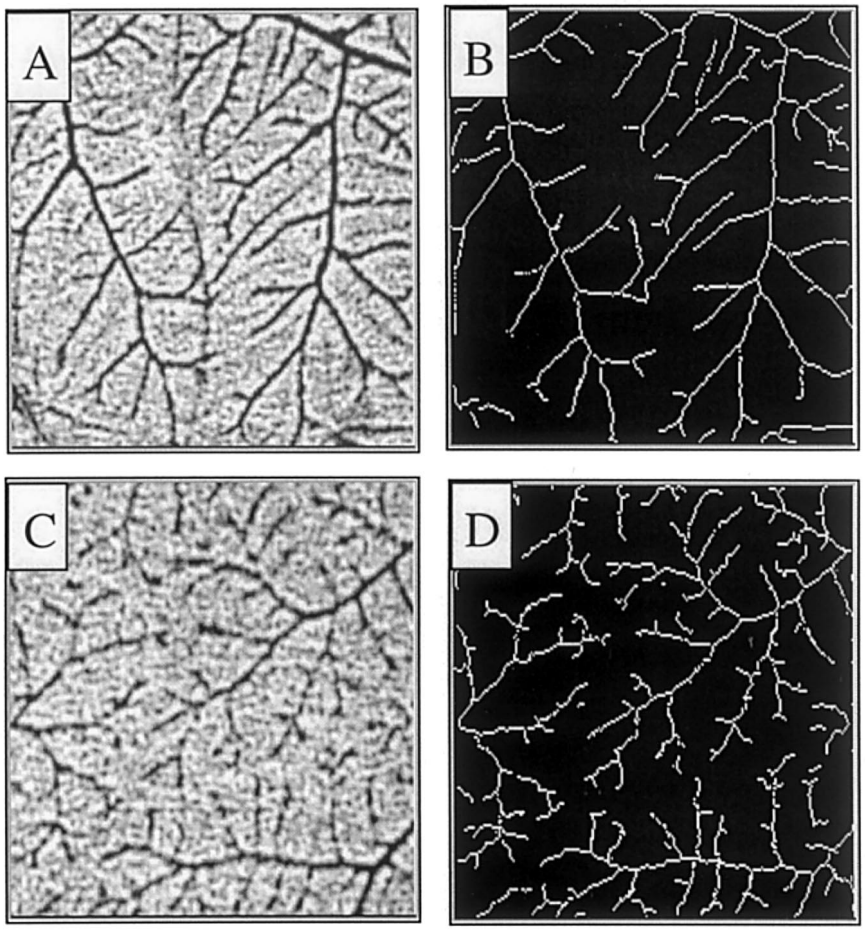

Fig. 3. Moderate amounts of EtOH-induced angiogenesis in chick embryo CAM. The embryos were treated with EtOH at 0.25 $g \cdot kg^{-1} \cdot day^{-1}$ for 7 days after having been incubated for 9 days. Digital images of the CAM vasculature were acquired with a computerized imaging system at a magnification of ×25. *A*: saline control; *B*: skeletonized image of saline control; *C*: EtOH-treated embryos; *D*: skeletonized image of EtOH-treated embryos.

DISCUSSION

In most human studies, moderate alcohol consumption is considered to be approximately one to two drinks per day. One standard drink is defined as 360 ml beer, 150 ml wine, or 45 ml liquor, each containing ~15 g of ethanol. The legal limit of blood alcohol concentration for operating a vehicle in the United States is usually 22 mmol/l or 100 mg/dl (0.1%). Therefore, the concentrations of ethanol used in the present study, i.e., 10 and 20 mmol/l for in vitro experiments and 0.125 or 0.25 $g \cdot kg^{-1} \cdot day^{-1}$ for in vivo experiments, are well within the levels that can be achieved in humans with moderate alcohol consumption (20, 29).

The results of the present study indicate that administration of moderate amounts of ethanol can increase VEGF mRNA expression and the vessel length density in chick embryo CAM (Figs. 1 and 3). Our data do not directly prove that VEGF causes these morphometric changes, i.e., angiogenesis. However, we have tested the proliferative effect of ethanol and ethanol-conditioned media on endothelial cells and vascular smooth muscle cells. Figure 2 indicates that adding 20 mmol/l ethanol into the media had no effect on the cell proliferation of either HUVEC or CAVSMC. The ethanol-CAVSMC-conditioned media, in which the VEGF levels were relatively higher, increased the proliferation of HUVEC by 20% ($n = 6$, $P < 0.05$). In contrast, cell proliferation was unchanged in cultured CAVSMC stimulated with ethanol-CAVSMC-conditioned media. It is well known that CAVSMC produce VEGF but lack VEGF receptors, and that HUVEC have VEGF receptors but produce only small amounts of VEGF. Together, these results are consistent with the speculation that ethanol-induced VEGF may play a role in the induction of angiogenesis. However, the ethanol-CAVSMC-conditioned media may also contain other factors that stimulate angiogenesis. Therefore, further studies, such as to investigate the time course of VEGF upregulation and vascular morphometric changes in the tissue during exposure to moderate levels of ethanol, and to apply anti-VEGF antibody, are necessary to confirm or refute this possibility.

Jones and associates (16) recently reported that intragastric administration of 1.5 ml of 25, 50, or 100% ethanol in rats induced VEGF expression and angiogenesis in the gastric mucosa. The induction of VEGF expression by ethanol in the Jones et al. (16) study was thought to result from ethanol-induced injury of the gastric mucosa with subsequent microvascular damage leading to tissue ischemia. The results of the present study indicate that much lower levels of ethanol can induce VEGF expression and stimulate angiogenesis in the absence of tissue injury or ischemia. The low levels

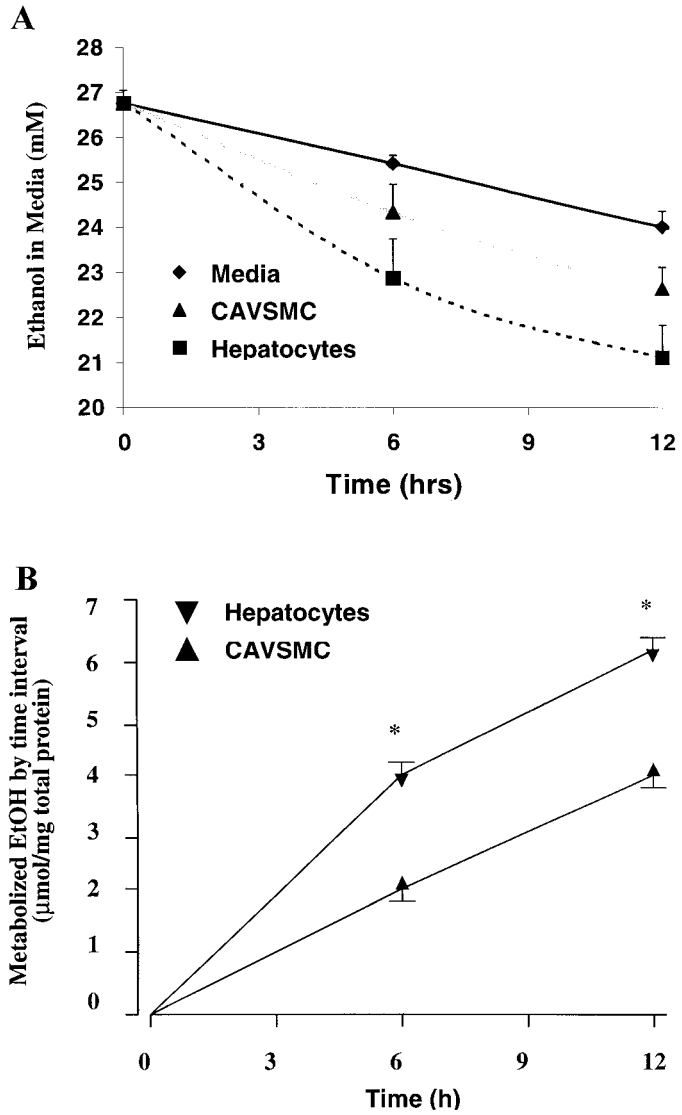

Fig. 4. EtOH metabolism in CAVSMC and hepatocytes was determined by changes of ethanol levels in media after adding 0.15% (vol/vol) ethanol. *A*: changes of ethanol concentrations in media in the absence and presence of CAVSMC or mouse hepatocytes during 12 h incubation. *B*: CAVSMC metabolized ethanol amounted to 1.9 ± 0.2 and 4.1 ± 0.3 μmol/mg total cell protein, respectively, after 6 and 12 h incubation. Mouse hepatocytes metabolized EtOH above twice as rapidly compared with CAVSMC ($n = 6$; $^*P < 0.01$).

of ethanol (10 or 20 mmol/l) used in the present study did not damage CAVSMC after 18 h of exposure, because the cells excluded trypan blue dye (>95%) and had normal morphology, as discussed above. Therefore, the induction of VEGF by moderate levels of ethanol in the present study cannot be attributed to ethanol-induced cellular injury.

We have concerns that exposing CAVSMC to 20 mmol/l ethanol for 18 h could be too long compared with moderate alcohol consumption in human settings. However, Fig. 4*A* shows that ethanol levels in the media cultured with CAVSMC significantly decreased >15% after 12 h incubation. In addition, exposing CAVSMC to 20 mmol/l ethanol for 6 h significantly increased VEGF protein levels in media by 27%, compared with control ($P < 0.01$), demonstrating that even a comparatively short exposure can induce VEGF expression. We could not find measurable ethanol concentrations in chick embryo blood 24 h after a 0.25 g/kg dose. Although there are considerable differences between cell culture or chick embryo models and humans, these findings suggest that our models, in terms of concentrations, doses, and exposure length for ethanol, mimic the relevant physiological conditions compared with moderate alcohol consumption in humans.

Many of the pathophysiological effects of alcohol ingestion relate to the metabolic consequences of ethanol in tissues or cells. In the present study, we found evidence of a metabolic response to ethanol in cultured CAVSMC. Figure 4*A* indicates that ethanol increased lactate production in a dose-dependent manner in cultured CAVSMC after 18 h incubation in the presence of ethanol at 10, 20, and 40 mmol/l. Figure 4*B* indicates that CAVSMC metabolized significant amounts of ethanol after 6 and 12 h incubation in media having 0.15% (vol/vol) ethanol. The rate of ethanol metabolism in cultured mouse hepatocytes was twofold higher compared with CAVSMC. These results are consistent with previous findings that ethanol can be metabolized in cardiovascular tissues. Soffia and Penna (32) found that incubation of rat heart homogenates with 116 mg/dl ethanol led to production of acetaldehyde, which is a metabolite of ethanol. Forsyth and associates (8) reported that acetaldehyde is oxidized by the heart in a rat model. The classical pathway for ethanol metabo-

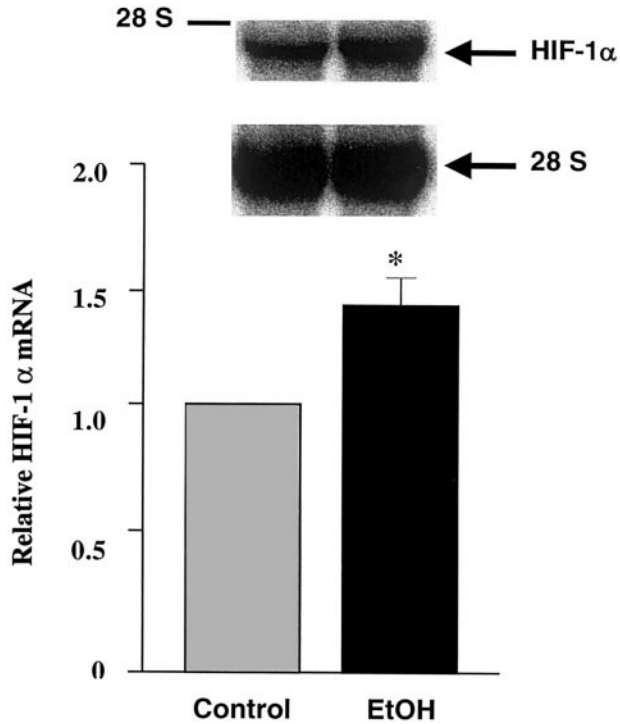

Fig. 5. Northern blot analysis indicated that exposing CAVSMC to 20 mmol/l EtOH in media for 2 h incubation significantly increased hypoxia-inducible factor (HIF)-1α mRNA expression by 1.4-fold over the control ($n = 4$; $^*P < 0.05$).

lism is oxidation of ethanol to form acetaldehyde, a process catalyzed by alcohol dehydrogenase (25). An important consequence of ethanol and acetaldehyde oxidation is an increase in both cytosolic and mitochondrial NADH/NAD ratios. The increase in NADH/NAD ratio can increase the activity of xanthine oxidase, a free radical-generating enzyme. Many recent studies support the hypothesis that the pathophysiological effects of ethanol ingestion are mediated primarily by the generation of free radicals (18).

Many studies (5) have demonstrated that HIF-1 is necessary for hypoxia-induced increase in VEGF expression. HIF-1 is a heterodimeric basic-helix-loop-helix-PAS domain transcription factor that binds to hypoxia-sensitive elements in the promoters/enhancers of O_2-sensing genes, such as VEGF, erythropoietin, glucose transporters, tyrosine hydroxylase, nitric oxide synthases, and heme oxygenase-1 (31). Genes that are transcriptionally activated by HIF-1 encode proteins that increase oxygen delivery or allow metabolic adaptation to limited oxygen availability. Previous studies demonstrated that ethanol caused hypoxia in the liver and the cardiovascular tissues by increasing oxygen consumption (21, 27). Recent data (31) suggested that some free radical molecules, such as hydroxyl radical, may be responsible for the hypoxia signal leading to HIF-1 expression. The current study shows that moderate level of ethanol can induce HIF-1α mRNA expression in cultured CAVSMC. All together, the evidence supports the hypothesis that ethanol-induced hypoxia signals may play an important role in VEGF induction by exposure to ethanol. Additional studies will be required to determine whether free radical molecules related to ethanol metabolism may be associated with induction of HIF-1 and VEGF expression.

In summary, this study demonstrates that moderate levels of ethanol upregulate the expression of VEGF mRNA and protein in cultured CAVSMC and induce VEGF mRNA expression as well as angiogenesis in chick embryo CAM. By using cell culture, we have found that CAVSMC can metabolize significant amounts of ethanol and that moderate levels of ethanol increase HIF-1α mRNA expression in CAVSMC. These findings will hopefully lead to tests of the hypothesis that induction of VEGF and angiogenesis by moderate levels of ethanol may represent an important mechanism of the cardiovascular-protective effect of moderate alcohol consumption in adult human settings.

Perspectives

Although the concentrations and doses of ethanol used in the present study are physiologically relevant in humans, there are considerable differences between the cell culture or chick embryo models and humans. We found that moderate levels of ethanol induce VEGF expression in cultured CAVSMC and increase VEGF mRNA expression as well as vessel length density in chick embryo CAM. The present study does not determine whether the cardiovascular-protective effects of moderate alcohol consumption are mediated by its ability to induce VEGF expression and angiogenesis. The significance of this study is that the results provide a theoretical basis for such speculation and may, therefore, encourage further experimentation. In our next steps, we will study whether moderate administration of ethanol can induce angiogenesis and VEGF expression in adult rat tissue and will test whether moderate alcohol consumption can increase VEGF expression in humans.

This study was supported by the American Heart Association Mississippi Affiliate (9810183MS), by an American Cancer Society Institutional Research Grant (IRG-98–275–01), and by National Heart, Lung, and Blood Institute Grant HL-51971. J.-W. Gu was a recipient of a Grant-in-Aid award of the American Heart Association Mississippi Affiliate and an American Cancer Society Institutional Research Grant award.

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