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Yong Qian

Jia Luo

Stephen S. Leonard

Gabriel K. Harris

Lyndell Millecchia

See next page for additional authors

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Hydrogen Peroxide Formation and Actin Filament Reorganization by Cdc42 Are Essential for Ethanol-induced *in Vitro* Angiogenesis*

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Yong Qian‡§, Jia Luo¶, Stephen S. Leonard‡, Gabriel K. Harris‡, Lyndell Millecchia‡, Daniel C. Flynn¶‖, and Xianglin Shi‡

From the ‡Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505 and ‖The Mary Babb Randolph Cancer Center and the ¶Department of Microbiology, Immunology, and Cell Biology, West Virginia University, Morgantown, West Virginia 26506-9300

This report focuses on the identification of the molecular mechanisms of ethanol-induced *in vitro* angiogenesis. The manipulation of angiogenesis is an important therapeutic approach for the treatment of cancer, cardiovascular diseases, and chronic inflammation. Our results showed that ethanol stimulation altered the integrity of actin filaments and increased the formation of lamellipodia and filopodia in SVEC4–10 cells. Further experiments demonstrated that ethanol stimulation increased cell migration and invasion and induced *in vitro* angiogenesis in SVEC4–10 cells. Mechanistically, ethanol stimulation activated Cdc42 and produced H_2O_2 a reactive oxygen species intermediate in SVEC4–10 cells. Measuring the time course of Cdc42 activation and H_2O_2 production upon ethanol stimulation revealed that the Cdc42 activation and the increase of H_2O_2 lasted more than 3 h, which indicates the mechanisms of the long duration effects of ethanol on the cells. Furthermore, either overexpression of a constitutive dominant negative Cdc42 or inhibition of H_2O_2 production abrogated the effects of ethanol on SVEC4–10 cells, indicating that both the activation of Cdc42 and the production of H_2O_2 are essential for the actions of ethanol. Interestingly, we also found that overexpression of a constitutive dominant positive Cdc42 itself was sufficient to produce H_2O_2 and to induce *in vitro* angiogenesis. Taken together, our results suggest that ethanol stimulation can induce H_2O_2 production through the activation of Cdc42, which results in reorganizing actin filaments and increasing cell motility and *in vitro* angiogenesis.

Both epidemiological and clinical studies demonstrate that alcohol abuse is directly associated with the increasing incidence of multiple organ diseases, such as liver injury, cardiovascular diseases, neurological disorders, and tumor promotion. High consumption of alcohol has been shown to enhance the invasion and metastasis of breast cancer, colon cancer, and liver cancer (1–5). Conversely, moderate alcohol consumption has also been shown to protect against the incidence of cardiovascular diseases (6). The molecular mechanisms of its pathogenic and protective effects are not fully established. Previous studies indicate that ethanol can induce angiogenesis (7, 8). Angiogenesis, the process of forming new blood vessels out of pre-existing capillaries (9), is a sequential event that involves (*a*) the disintegration of the basement membrane, (*b*) the migration of endothelial cells, (*c*) endothelial cell proliferation in cords with formation of new vascular channels, and (*d*) the formation of a new basement membrane (10). These processes of angiogenesis are associated with cancer, cardiovascular diseases, and chronic inflammation. The manipulation of angiogenesis has great potential as an intervention for a number of diseases. The stimulation of angiogenesis could prevent and improve ischemic heart failure, whereas the inhibition of angiogenesis could potentially cure cancers and chronic inflammation (9).

One of the major steps of angiogenesis is cell migration, which is pivotally governed by changes in the actin cytoskeleton. Actin is one of the most abundant proteins in eukaryotic cells. There are two different forms of actin, mono-actin (G-actin) and actin filaments (F-actin). Actin filaments are involved in a wide variety of cellular processes, including cell motility, cell cycle control, cellular structure, and cell signaling (11). They function in cellular processes by undergoing dynamic structural reorganization or remodeling, leading to the formation of discrete structures at the cell periphery for attachment to the substratum in response to extracellular signals. These structures include focal adhesions, stress fibers, lamellipodia, filopodia, and membrane ruffles, which are involved in cell attachment, cell migration, and signal transduction (12).

Because actin filaments play a critical role in cell migration, the remodeling of actin filaments may be essential for the process of angiogenesis. It has been found that insulin-stimulated glucose transport, gene expression, and alterations of cell morphology are dependent on the remodeling of actin filaments and that inhibition of the remodeling of actin filaments abrogates the induction of angiogenesis by insulin (13). The remodeling of actin filaments is essential for vascular endothelial growth factor-induced formation of tube-like structures and angiogenesis (14). Actin filaments assemble to form bundle structures that are oriented along the axis of the tubes at the periphery of the cells during the formation of vessel tubes. Inhibition of the actin filament assembly blocks tube formation (13). The remodeling of actin filaments results in the increases in cell spreading, nuclear extension, and DNA synthesis (15). The reorganization of actin filaments is also required for cultured rat hepatocytes to form three-dimensional structures or spheroids (16).

In this study we demonstrated that ethanol directly remodeled or reorganized the structure of actin filaments, increased cell migration and cell invasion, and induced *in vitro* angiogenesis in endothelial cells. We further showed that activation of Cdc42 was important for ethanol-induced *in vitro* angiogenesis.

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§ To whom correspondence should be addressed. Fax: 304-285-5938; Tel.: 304-285-6286; E-mail: yaq2@cdc.gov.

Inhibition of Cdc42 activation in endothelial cells abrogated the effects of ethanol. In addition, we found that ethanol stimulation of endothelial cells induced the production of H_2O_2 through activation of Cdc42, and elimination of H_2O_2 production abolishes the effects of ethanol on these cells. Measuring the time course of Cdc42 activation and H_2O_2 production revealed that the Cdc42 activation and the increase of H_2O_2 production lasted more than 3 h, which indicates the mechanisms of the long duration effect of ethanol on the changes in cell morphology, cell motility, and *in vitro* angiogenesis.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium, catalase, FITC[1]-phalloidin, TRITC-anti-Rabbit antibody were purchased from Sigma. Transwell cell migration chambers were purchased from Corning Costar (Corning, NY). Matrigel-coated invasion chambers and Matrigel-coated *in vitro* angiogenesis plates were purchased from BD Biosciences. Carboxymethyl dichlorofluorescein diacetate was purchased from Molecular Probes (Eugene, OR).

Plasmid Construct and Cell Preparation—Both dominant positive Cdc42 (phEFdpCdc42V12) and dominant negative Cdc42 (phEFdnCdc42N14) were the gifts from Dr. Alan Hall (University College London, UK) and Dr. Wang Lu-Hai (Mount Sinai School of Medicine). pcDNA3.1/Neo(+)/GPX1 was a gift from Dr. Larry Oberley (University of Iowa). pGST-WASP-CRIB was a gift from Dr. Pontous Aspenstrom (Ludwig Institute for Cancer Research, Uppsala, Sweden). SVEC4–10 cells, an immortal mouse endothelial cell line, were purchased from ATCC (Manassas, VA). Both phEFdpCdc42V12 and phEFdnCdc42N14 were transfected into SVEC4–10 cell with calphosphate kit (BD Biosciences), and the transfected cells were selected by G418 to make stable transfected cell lines. pcDNA3.1/Neo(+)/GPX1 was transiently transfected into SVEC4–10 cells with calphosphate kit (BD Biosciences).

Ethanol Exposure Method—Because of the volatility of ethanol, a method utilizing sealed containers (17) was used to maintain constant ethanol levels in the culture medium. 0.4% of ethanol was added directly to the culture medium in either tissue culture trays or dishes. The trays or dishes were then placed in sealed containers equipped with an ethanol-containing water bath in the bottom. The concentration of ethanol in the bath was the same as that in the culture medium. Ethanol from the bath evaporated into the air of the sealed container and maintained the ethanol concentration in the culture medium. A small volume of CO_2 (60 cc) was injected into the container before sealing. The ethanol bath was changed daily to maintain the appropriated ethanol concentration. In control cultures, the water bath contained no ethanol. All containers were incubated at 37 °C. Previous studies show that this sealed container method accurately maintains ethanol concentrations in the culture medium (17).

Immunofluorescence Assay—SVEC4–10 cells were grown on cover slides. After treatment, cells were fixed and permeabilized as previously described (18) followed by labeling with FITC-phalloidin for 20 min and mounting to the slides with Fluoromount (Fisher). A Zeiss LSM 510 microscope was used to obtain images. Scale bars were generated and inserted by LSM software.

Transwell Migration Assay—Transwell migration assays were conducted using modifications of the method described by manufacturer (BD Biosciences). Briefly, the cells were stimulated with 0.4% ethanol in serum-free media overnight. The Transwells were coated with E-C-L cell attachment matrix (Upstate Biotechnology) at 20 μg/ml and incubated for 1 h at 37 °C. The top chambers of the Transwells were loaded with 4×10^5 cells/ml in 0.4% ethanol in serum-free media, and the bottom chambers were filled with 5% fetal calf serum, Dulbecco's modified Eagle's medium media containing 0.4% ethanol. The 5% fetal calf serum served as an attractant for the cells. The Transwells were incubated in sealed chambers with 0.4% ethanol and 0.5% CO_2 at 37 °C for 18 h. After the incubation, the cells that had migrated were fixed with 10% formalin, stained with Harris modified Fisher hematoxylin (Fisher), and mounted on slides. These cells were counted using phase contrast microscopy. Data given for migrating cells represent the average of five typical fields per each sample.

Wound Healing Assay—The wound healing assays were performed according to the methods described by Meng *et al.* (4). SVEC4–10 cells were grown on coverslips to 100% confluent monolayers and then scratched to form a 100-μm "wound" using sterile pipette tips. The cells were then cultured with 0.4% ethanol in serum-free media in sealed chambers for 18 h, fixed on coverslips with formalin, and stained with FITC-phalloidin. A Zeiss LSM 510 microscope was used to obtain images. Scale bars were generated and inserted by LSM software.

Invasion Assay—Invasion assays were performed according to manufacturer's protocol (BD Biosciences). The cells were stimulated with 0.4% ethanol in serum-free media overnight. Cells (0.5 ml of 1.0×10^5 cells/ml) were loaded on pre-coated Matrigel 24-well invasion chambers (BD Biosciences). A 0.5-ml aliquot of 5% fetal calf serum medium containing 0.4% ethanol was added to the wells of the BD Falcon TC Companion plate to serve as the chemoattractant for the cells. The Matrigel invasion chambers were incubated in a sealed chamber with 0.4% ethanol and 0.5% CO_2 at 37 °C for 22 h. After the incubation, the invading cells were fixed with 10% formalin, stained with Harris modified hematoxylin (Fisher), and mounted onto slides. The invading cells were counted and analyzed according to manufacturer's instructions.

Cdc42 Activation Assay—Cdc42 activation assays were performed according to the method described by Edlund *et al.* (19). Briefly, the cells were washed with 1× phosphate-buffered saline supplemented with 1 mM $MgCl_2$. After the washing, the cells were lysed immediately with lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The lysates were centrifuged at 14,000 rpm for 15 min. A GST-WASP-CRIB fusion protein in glutathione *S*-transferase beads was added into the supernatants to pull-down active Cdc42 proteins, followed by incubation at 4 °C for 20 min. After the incubation, the beads were washed 2 times with cold wash buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride). The Cdc42 protein was eluted with sample buffer and subjected to 15% SDS-PAGE. The Western blot analysis was performed using anti-Cdc42 polyclonal antibody. Protein bands were visualized with an enhanced chemiluminescence reagent (Amersham Biosciences).

In Vitro Angiogenesis Assay—An *in vitro* angiogenesis assay was performed with slight modification according to the methods described by Tang *et al.* (20). In brief, the cells were incubated in 0.4% ethanol in serum-free media overnight. Cells (2 ml of $1–2 \times 10^5$ cell/ml) were loaded on pre-coated Matrigel basement membrane matrix dishes (BD Biosciences) and cultured in serum-free medium containing 0.4% ethanol at 37 °C for 5 h followed by staining with Diff-Qick (Fisher) for 20 min. Images were taken using an Olympus inverted microscope.

Cellular Hydrogen Peroxide (H_2O_2) Assay—H_2O_2 was monitored using a Biotech H_2O_2-560 quantitative H_2O_2 assay kit (Oxis International, Inc., Portland, OR). The assay is based on the oxidation of ferrous ions (Fe^{2+}) to ferric (Fe^{3+}) by H_2O_2 under acidic conditions. The ferric ion binds with an indicator dye, xylenol orange, to form a stable colored complex that can be measured at 560 nm using a PerSeptive Biosystems Cytofluor multiwell plate reader series 4000 (PerSeptive Biosystems Inc., Framingham, MA). Measurements were made at 37 °C using 1×10^6 cells suspended in 1 ml of phosphate-buffered saline. Student's *t* tests were performed using Sigma Stat programs.

H_2O_2 Assay in Adherent Cells—H_2O_2 assays in adherent cells were performed according to the methods described by Moldovan *et al.* (21). Briefly, cells were serum-starved overnight and then stimulated with 0.4% ethanol for the different periods of time. At the end of the stimulation, carboxymethyl dichlorofluorescein diacetate was added at a final concentration of 5 μM for 30 min. After the incubation, the cells were washed two times with phosphate-buffered saline and mounted on coverslips. A Zeiss LSM 510 microscope was used to obtain images. Scale bars were generated and inserted by LSM software.

RESULTS

Ethanol Stimulation Remodels the Integrity of Actin Filaments in SVEC4–10 Cells—Actin filaments mediate signal transduction in cells and undergo dynamic reorganization or remodeling in response to stimulation. Several activated forms of kinases remodel the structure of actin filaments to form the cell motile structures and change the structures of actin stress fibers. Here we sought to determine whether stimulation by ethanol has the capability to reorganize the structure of actin filaments in endothelial cells. SVEC4–10 cells, an immortal mouse endothelial cell line, were serum-starved overnight and stimulated with 0.4% ethanol for the different periods of time. After stimulation, the cells were fixed immediately and stained

[1] The abbreviations used are: FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; LSM, laser scanning microscope.

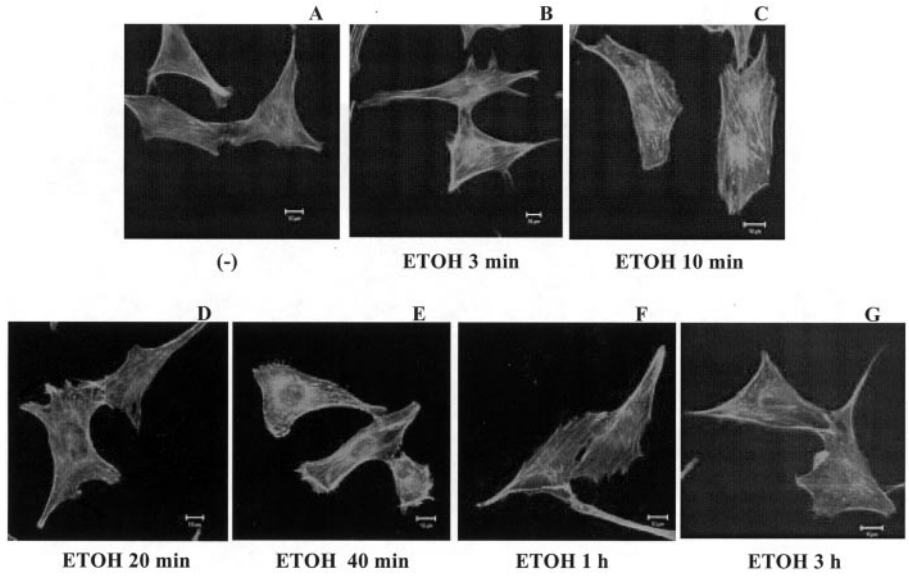

FIG. 1. **Ethanol stimulation induces the reorganization of actin filaments in endothelial cells.** SVEC4–10 cells were grown on coverslips and serum-starved overnight. Ethanol (0.4%) was used to stimulate the cells for the different periods of time as indicated. After stimulation, cells were fixed on coverslips and stained with FITC-phalloidin. Confocal microscopy was used to analyze the integrity of actin filaments and the changes in cell morphology.

with FITC-phalloidin followed by analysis using confocal microscopy. It was found that the actin stress fibers were well organized in the unstimulated SVEC4–10 cells, *i.e.* cells were evenly spread out, and few cell motile structures and rosette-like dots were found at the cell leading edges and within cell bodies (Fig. 1*A*). Upon stimulation with 0.4% ethanol for 3 min, actin filaments were remodeled and protruded the cell membrane at the leading edging to form cell motile structures such as lamellipodia and filopodia (Fig. 1*B*). After stimulation with ethanol for 10 min, the actin stress fibers dissociated to form the rosette-like structures around the cell body, and a substantial number of cell motile structures were found at the cell leading edge, indicating that the integrity of actin filaments was reorganized upon the stimulation with ethanol (Fig. 1*C*). The changes in the structure of actin filaments lasted for about 1 h and then began to recover (Fig. 1, *D–G*, and data not shown). Our data indicate that stimulation with ethanol altered the structure of actin filaments, which induced the dissociation of actin stress fiber structures to form actin rosette-like structures in the cell body and cell motile structures at the leading edges.

Ethanol Stimulation Increases Cell Migration and Cell Invasion through the Activation of Cdc42—The increase of lamellipodia and filopodia formation by ethanol stimulation suggests that small Rho GTPases may be activated in endothelial cells (Fig. 1). Cdc42 is involved in the formation of filopodia, and Rac is involved in the formation of lamellipodia (22). We sought to test whether Cdc42 and Rac were activated. SVEC4–10 cells were treated with 0.4% ethanol for various periods of time ranging from 3 min to 3 h. Glutathione *S*-transferase fusion protein pull-down assays for Cdc42 and Rac were then performed. The results demonstrate that Cdc42 was activated upon stimulation with 0.4% ethanol for 3 min, the peak of Cdc42 activation was detected around 40 min after the stimulation, and the activation lasted for more than 3 h (Fig. 2*A*). In contrast, no activation of Rac was observed (data not shown).

The increases in cell motile structure at the leading edge of cells indicate that ethanol may have the capability to increase cell migration and invasion. To test this possibility, wound healing assays were performed. SVEC4–10 cell were grown to 100% confluent monolayers on cover slips and were scratched to form a 100-μm wound with the sterile pipette tips. The cells were then incubated for 18 h with or without 0.4% ethanol stimulation. Ethanol-treated SVEC4–10 cells at the leading edges of the wound migrated and spread to cover the wound significantly faster than the unstimulated cells (Fig. 2*B*). We also utilized Transwell migration assays to test the ability of ethanol to increase the cell migration. The results are consistent with that of the wound healing assays (data not shown). Ethanol has been indicated to enhance the metastasis and invasiveness of several kinds of cancers. We used the Matrigel invasion assays to examine whether ethanol stimulation can increase the invasion of SVEC4–10 cells. The results demonstrated that ethanol stimulation significantly increases the invasiveness of SVEC4–10 cells (Fig. 2*C*). In the Matrigel assays, 22% of unstimulated SVEC4–10 cells crossed the gel barrier compared with 46% of the ethanol-stimulated cells ($p < 0.01$, $n = 3$).

We then investigated whether the activation of Cdc42 was required for ethanol-induced remodeling of actin filaments and increases in cell migration and cell invasion. Dominant negative Cdc42 was stably expressed into SVEC4–10 cells to establish a stable cell line (SVEC-Cdc42DN) to inhibit the activities of Cdc42. We then treated SVEC-Cdc42DN cells with 0.4% ethanol to examine changes in the integrity of actin filaments, cell migration, and cell invasion. Fig. 2*D* shows that the integrity of actin filaments of SVEC-Cdc42DN cells exhibited only a minor change upon the stimulation of ethanol. The actin stress fibers were well organized, and there was no increase in the formation of actin rosette-like structures or cell motile structures, indicating that the activation of Cdc42 is required for ethanol-stimulated reorganization of actin filaments. Furthermore, the expression of SVEC-Cdc42DN decreased the ability of ethanol to increase the cell migration and cell invasion (Fig. 2, *B*, *right panels*, and *C*). These data demonstrate that the activation of small Rho GTPase Cdc42 is essential for ethanol-stimulated reorganization of actin filaments as well as increases in cell motility and cell invasion.

Ethanol Induces in Vitro Angiogenesis through the Activation of Cdc42—The ability of ethanol to induce *in vitro* angiogenesis was tested. Fig. 3*A* shows the control without ethanol treatment. Untreated SVEC4–10 cells failed to form the tube-like structures and remained as individual cells on Matrigel up to 5 h of incubation (Fig. 3*A*). In contrast, ethanol-treated SVEC4–10 cells formed extensive tube-like structures on Matrigel within the same incubation time (Fig. 3*B*). Development of tube-like structures is characteristic of *in vitro* angiogenesis. The results indicate that ethanol stimulation induced *in vitro* angiogenesis in SVEC4–10 cells. Next, we tested whether the overexpression of dominant negative Cdc42 would affect the

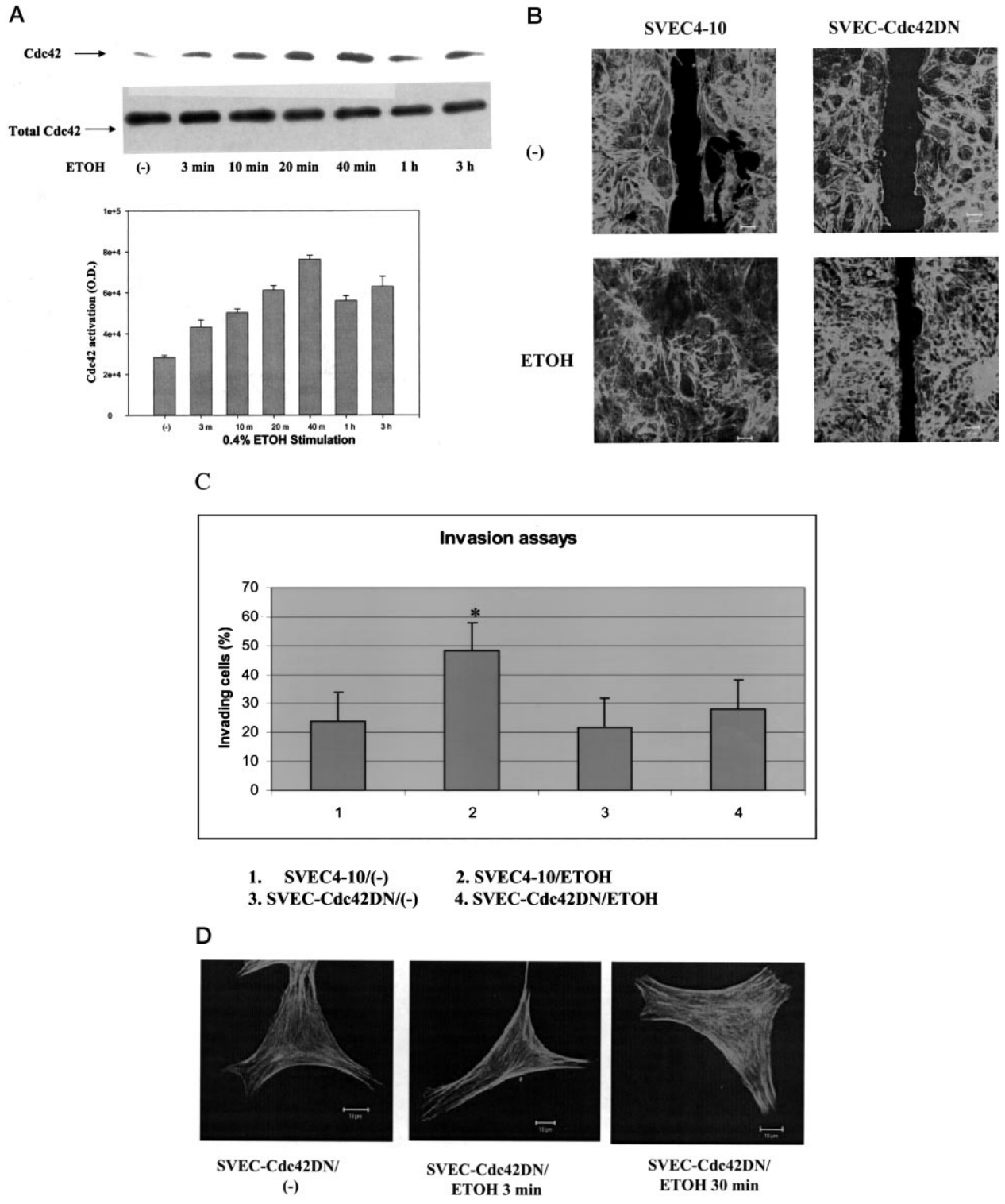

FIG. 2. **Ethanol stimulation remodels the structure of actin filaments and increases cell migration and invasion through the activation of Cdc42 in endothelial cells.** *A*, ethanol stimulation activates Cdc42. SVEC4–10 cells were serum-starved overnight and then stimulated with 0.4% ethanol for different periods of time as indicated. The cells were lysed after stimulation. Lysates were analyzed for Cdc42 activity. The proteins were resolved on SDS-PAGE gel, and anti-Cdc42 antibody was used to detect Cdc42 protein. The densitometries of Cdc42 protein bands were scanned using EglII software, and the results were plotted by SigmaPlot. *B*, ethanol stimulation increases cell migration through the activation of Cdc42. Both SVEC4–10 cells and SVEC-Cdc42DN cells were grown on coverslips to 100% confluent monolayers. Sterile pipette tips were used to scratch the confluent monolayer cells to form a 100-μm wound, and then the cells were cultured for 18 h with or without 0.4% ethanol. After the incubation, the cells were fixed on coverslips and stained with FITC-phalloidin followed by the analysis by confocal microscopy. *C*, ethanol stimulation increases the cell invasion through the activation of Cdc42. Both SVEC4–10 cells and SVEC-Cdc42DN were cultured on Matrigel-coated Transwells with or without 0.4% ethanol stimulation for 22 h. The invading cells were fixed and counted. Five fields were counted randomly. The uncoated Transwells were used as the controls. The percentage of invading cells was calculated according to manufacturer's institution. An *asterisk* indicates a significant increase. *D*, ethanol stimulation reorganizes the structure of actin filaments through the activation of Cdc42. SVEC-Cdc42DN cells were stimulated with 0.4% ethanol for different periods of time as indicated. After stimulation, cells were fixed on coverslips and stained with FITC-phalloidin followed by analysis with confocal microscopy.

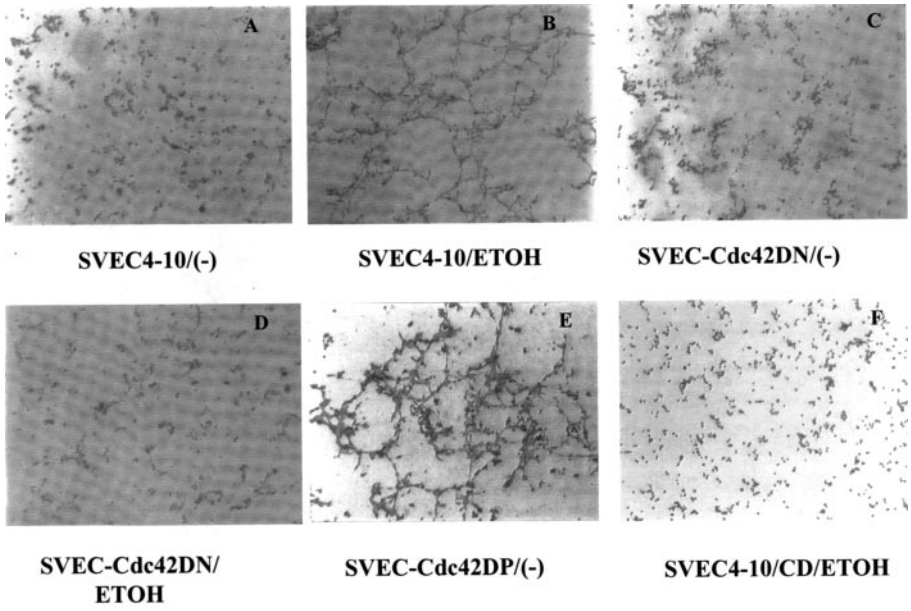

FIG. 3. **Ethanol stimulation induced *in vitro* angiogenesis through the activation of Cdc42 in SVEC4–10 cells.** SVEC4–10 cells, SVEC-Cdc42, and SVEC-Cdc42DP were grown on Matrigel with the different treatments as indicated for 5 h followed by staining with Diff-Qick. Images are representative of typical fields seen in three experiments. The magnification is 5×.

ability of ethanol to induce *in vitro* angiogenesis in SVEC4–10 cells. The results show that the expression of Cdc42 dominant negative protein in SVEC4–10 cells was sufficient to inhibit *in vitro* angiogenesis stimulated by ethanol (Fig. 3*D*), indicating that the activation of Cdc42 is involved in ethanol-induced *in vitro* angiogenesis. These results lead us to test whether the overexpression of dominant positive Cdc42 alone would induce *in vitro* angiogenesis in endothelial cells. Dominant positive Cdc42 was stably expressed into SVEC4–10 cells to create a stable cell line (SVEC-Cdc42DP). The results show that the expression of dominant positive Cdc42 was sufficient to induce *in vitro* angiogenesis in SVEC4–10 cells within 5 h of incubation (Fig. 3*E*), whereas neither SVEC4–10 cells nor SVEC-Cdc42DN cells were able to induce *in vitro* angiogenesis within the same period of time (Fig. 3, *A* and *C*). To the best of our knowledge, this is the first time that activation of Cdc42 alone has been demonstrated to be sufficient to induce *in vitro* angiogenesis. To investigate whether actin filaments are involved in ethanol-induced *in vitro* angiogenesis, we treated SVEC4–10 cells with an actin filament inhibitor, cytochalasin D, and examined its effects on ethanol-induced *in vitro* angiogenesis. Fig. 3*F* shows that cytochalasin D preincubation completely blocked the process of ethanol-stimulated *in vitro* angiogenesis, implying that the remodeling of actin filaments is essential for ethanol-stimulated *in vitro* angiogenesis. Taken together, the data demonstrate that the activation of Cdc42 is not only necessary to induce ethanol-stimulated *in vitro* angiogenesis but is sufficient to induce *in vitro* angiogenesis.

Ethanol Stimulation Induces the Formation of H_2O_2 through the Activation of Cdc42 in SVEC4–10 Cells—It has been shown that reactive oxygen species play a major role in ethanol-induced tissue damage (23). H_2O_2 is generated via dismuation of the superoxide radical (O_2^-). It can be converted to the hydroxyl radical (·OH) via Fenton-type reactions in the presence of metal ions. Thus, H_2O_2 is a key intermediate of reactive oxygen species. To investigate the role of H_2O_2 in ethanol-induced angiogenesis, we detected H_2O_2 formation from SVEC4–10 cells stimulated by ethanol. The results demonstrated that stimulation of the cells by ethanol increased H_2O_2 production (Fig. 4*A*). Treatment with catalase, a specific scavenger of H_2O_2, reduced the H_2O_2 production to the control level (Fig. 4*A*). To further confirm the functional roles of ethanol in H_2O_2 production, glutathione peroxidase was transiently overexpressed in SVEC4–10 cells to determine whether it could inhibit ethanol-induced H_2O_2 production. Glutathione peroxidase is an important antioxidant enzyme that removes H_2O_2 from cells and protects against oxidant damage (24). Our results showed that overexpression of glutathione peroxidase substantial decreased the production of H_2O_2 upon stimulation with ethanol (Fig. 4*A*). These results demonstrate that stimulation with ethanol induces the production of H_2O_2 in endothelial cells.

To determine whether the production of H_2O_2 upon stimulation with ethanol is regulated by Cdc42, SVEC4–10 cells expressing a dominant negative Cdc42 protein were stimulated with ethanol. The results showed that expression of dominant negative Cdc42 inhibited ethanol-induced H_2O_2 production (Fig. 4*B*). Interestingly, expression of dominant positive Cdc42 itself is sufficient to enhance H_2O_2 production (Fig. 4*C*). These results indicate that Cdc42 is important in ethanol-induced H_2O_2 production.

The Production of H_2O_2 Is Essential for Ethanol-induced Effects on SVEC4–10 Cells—To determine whether H_2O_2 mediated the effects of ethanol on SVEC4–10 cells, cells were pretreated with the H_2O_2 scavenger catalase for 2 h and subsequently stimulated with 0.4% ethanol. Immunofluorescence assays show that pretreatment with catalase blocked the ability of ethanol to induce remodeling of the structure of actin filaments in SVEC4–10 cells (Fig. 5*A*). To further confirm the role of H_2O_2 in mediating ethanol-induced actin filament remodeling, SVEC4–10 cells were incubated with 1 mM peroxide for 5 min followed by the immunofluorescence analysis. The results showed that the addition of peroxide partially mimicked the effects of ethanol on the changes of actin filament integrity (Fig. 5*A*, *4*), indicating that H_2O_2 production is important in ethanol-induced actin filament remodeling.

Both the cell invasion and the cell migration assays demonstrated that pretreatment with catalase inhibited the ability of ethanol to increase the migration and invasiveness in SVEC4–10 cell (Figs. 5, *B* and *C*). *In vitro* angiogenesis assays demonstrated that pretreatment with catalase abrogated the process of angiogenesis induced by ethanol (Fig. 5*D*). Fig. 5*D* also shows that overexpression of glutathione peroxidase had the same inhibitory effect as catalase did in ethanol-induced *in vitro* angiogenesis. These results indicate that the production of H_2O_2 mediates the ethanol-induced reorganization of actin filaments, the increase in cell migration and cell invasion, and *in vitro* angiogenesis in SVEC4–10 cells.

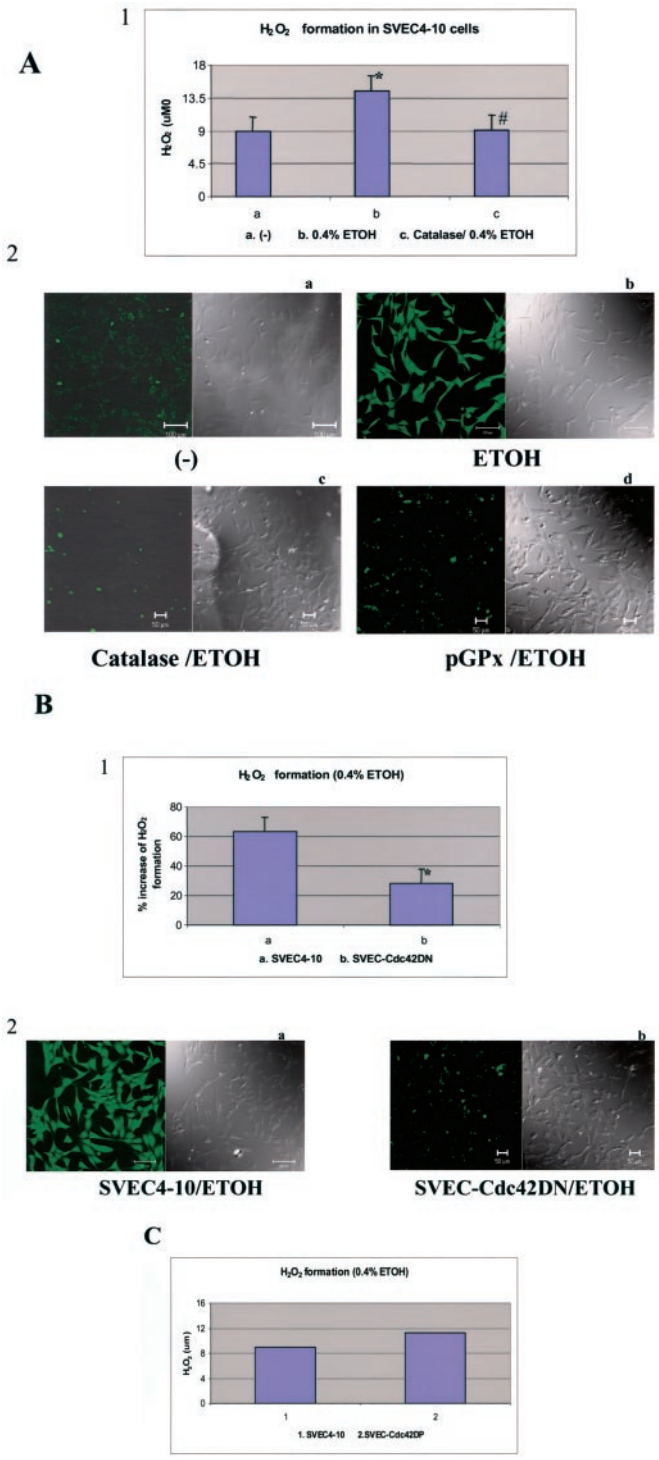

FIG. 4. **Ethanol stimulation produces the reactive oxygen species, H_2O_2, through the activation of Cdc42.** *A*, ethanol stimulation induces the production of H_2O_2 in SVEC4–10 cells. SVEC4–10 cells (1×10^5/ml) were stimulated with 0.4% ethanol for 3 min and then monitored for the production of H_2O_2 using a Biotch H_2O_2 quantitative H_2O_2 assay kit (*1*) and confocal microcopy (*2*). *a*, without stimulation; *b*, with stimulation by 0.4% ethanol; *c*, same as *b* but with 5000 units/ml catalase added; *d*, same as *b* but with overexpression of glutathione peroxidase. The *asterisk* indicates a significant increase, and the *pound sign* (#) indicates a significant decrease ($p < 0.05$, $n = 3$). A square region of interest, 500×500 μm, was centered on each fluorescent image, and the average gray value of the entire field (range 0–255, black = 0, white = 255) was extracted using Optimas image analysis software. The values of average gray level: *2a*, 8.09; *2b*, 59.81; *2c*, 1.30; *2d*, 5.05. *B*, ethanol-induced production of H_2O_2 is through the activation of Cdc42. SVEC4–10 cells (*a*) and SVEC-Cdc42DN (*b*) cells were monitored for the production of H_2O_2 before and after stimulation by 0.4% ethanol using a Biotech H_2O_2 quanitative H_2O_2 assay kit (*top panel*) and confocal microscopy (*bottom panels*). An *asterisk* indicates a significant decrease for that of SVEC4–10 cells ($p < 0.01$, $n = 3$). A square region of interest, 500×500 μm, was centered on each fluorescent image, and the average gray value of the entire field (range 0–255, black = 0, white = 255) was extracted using Optimas image analysis software. The values of average gray level: *2a*, 54.78; *2b*, 6.09. *C*, the overexpression of a constitutively dominant positive Cdc42 is sufficient to induce the production of H_2O_2. Both SVEC4–10 cells and SVEC-Cdc42DP cells were measured for the production of H_2O_2.

Ethanol Stimulation Induces a Long Duration of H_2O_2 Production—To further explore how the transient activation of Cdc42 can induce the reorganization of actin filaments in a relatively long period of time, the time course of H_2O_2 production upon ethanol stimulation were measured. SVEC4–10 cells were stimulated with ethanol for different periods of time ranging from 3 min to 3 h followed by the detection of H_2O_2 production using a confocal microscope. It was found that the increase of H_2O_2 production began in 3 min after ethanol stimulation and lasted for 3 h (Fig. 6). Further experiments revealed that the pattern of increase of H_2O_2 production lasted about 24 h (data not shown). The results indicate that the activation of Cdc42 upon ethanol stimulation induces a profound effect on the production of H_2O_2, which keeps actin filament remodeling to lead to the increases in cell motility and *in vitro* angiogenesis.

DISCUSSION

The ethanol concentration (400 mg/dl) applied here is relatively high but is physiologically relevant to the blood alcohol concentration of heavy alcoholics (25). In general, the concentrations of ethanol used for *in vitro* studies are higher than those required to produce similar effects *in vivo*. One example relates to the effects of ethanol exposure on cell proliferation. The duration of the cell cycle is increased by 29% in the telencephalic ventricular zone of rats with a blood alcohol concentration of ~180 mg/dl (26). Similarly, the length of the cell cycle of cultured neuroblastoma cells (used as a model for proliferating neuronal cells) is prolonged (+37%) by treatment with 400 mg/dl of ethanol (27).

In this report, we demonstrate that ethanol stimulation in endothelial cells could induce the remodeling of actin filaments, disrupt actin stress fibers, and induce the formation of cell motile structures. More importantly, the remodeling of the structure of actin filaments is concomitant with the increases in the cell motility and cell invasion. The reorganization of actin filaments is essential for cell signal transduction. It is a highly coordinated process that is regulated by cell signaling cascades. It has been shown that the reorganization of the structure of actin filaments is one of driving forces that cause the cell membrane to protrude forward to form the cell motile structures, which are involved in the increases in both actin filament polymerization and cross-linking (28–30). The reorganization of actin filaments plays a major role in the cell migration and invasion. It is essential in cell extension, attachment, contraction, and release during cell migration and cell invasion (31). We demonstrate here that ethanol stimulation could promptly reorganize the structure of actin filaments to induce the formation of cell motile structures and the dissociation of actin stress fibers within several minutes. Interestingly, similar results are found in insulin-mediated signal transduction, in which the reorganization of actin filaments occurs within several minutes upon the stimulation with insulin (13, 32). It is likely that the fast response of actin filaments to stimulation with ethanol enables the cells to adapt to changes in the environment properly and to transfer and propagate the signals.

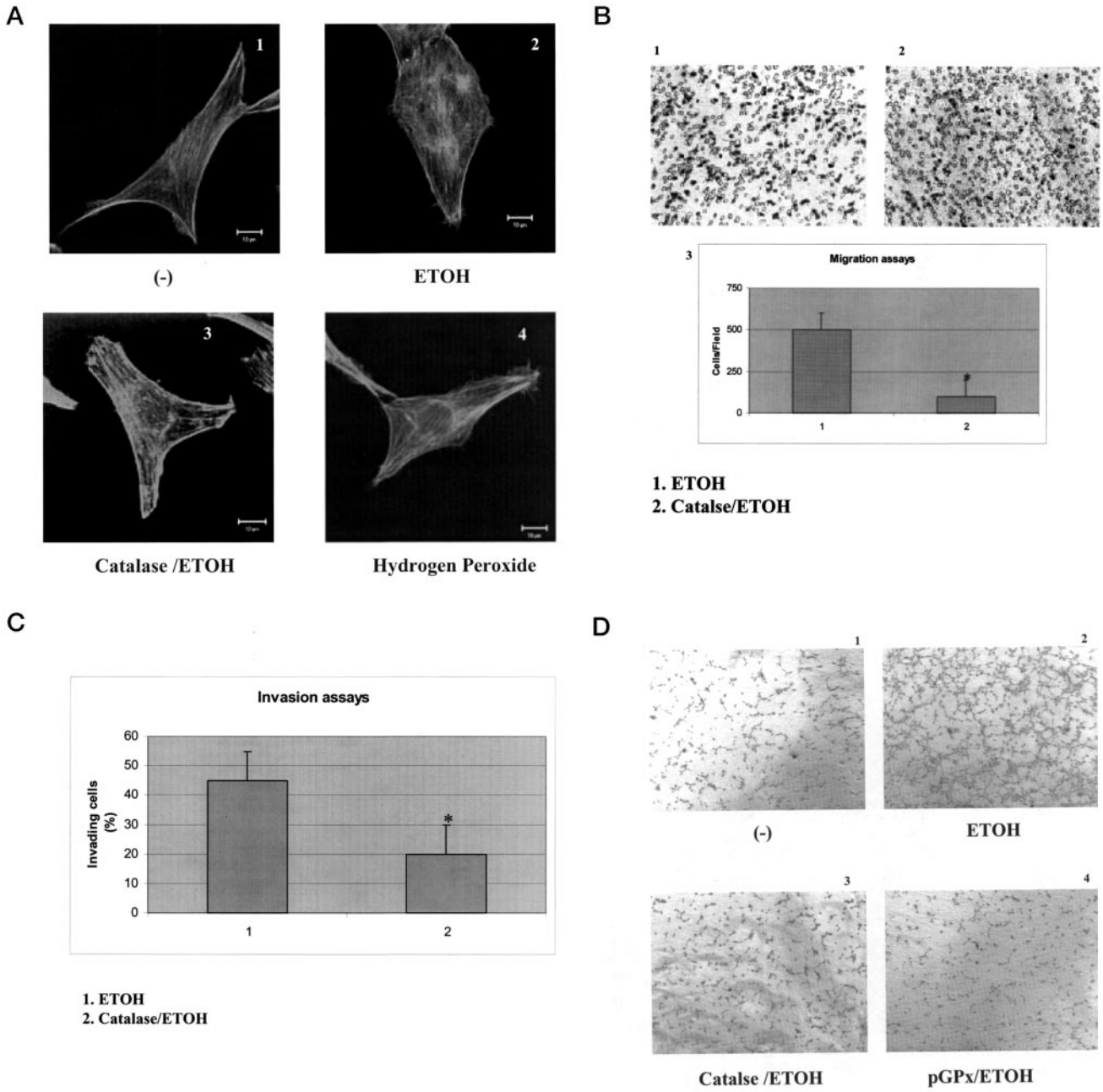

FIG. 5. **Elimination of H_2O_2 abrogates the effects of ethanol on SVEC4–10 cells.** *A*, the free radical scavenger, catalase, inhibits ethanol-induced reorganization of actin filaments. SVEC4–10 cells were grown on coverslips and serum-starved overnight. The cells in *panel 1* had no stimulation, and the cells in *panel 2* were stimulated with 0.4% ethanol for 3 min. The cells in *panel 3* were preincubated with 5000 units/ml catalase for 2 h followed by stimulation with 0.4% ethanol for 3 min. The cells in *panel 4* were stimulated with 1 mM exogenous hydrogen peroxide for 5 min. After stimulation, cells were fixed and stained with FITC-phalloidin followed by analysis with confocal microscopy. *B*, the free radical scavenger catalase inhibits ethanol-stimulated cell migration. SVEC4–10 cells were stimulated with 0.4% ethanol in serum-free media overnight followed by incubation in the Transwells for 18 h. The cells in *panel 2* were preincubated with 5000 units/ml catalase for 2 h. After incubation, the cells were fixed, and the images were taken using an Olympus inverted microscope (*panel 1* and *panel 2*). Five fields of the migrating cells were also counted using phase contrast microscopy. Cell migration data were analyzed and plotted using Microsoft Excel (*panel 3*). The *asterisk* indicates a significant decrease from the positive control ($p < 0.01$, $n = 3$). *C*, the free radical scavenger, catalase, inhibits ethanol-stimulated cell invasiveness. SVEC4–10 cells were preincubated with 5000 units/ml catalase for 2 h and then cultured on a Matrigel-coated Transwell with 0.4% ethanol stimulation for 22 h. The cells without preincubation of catalase served as controls. The invading cells were fixed, and five fields were counted randomly. The uncoated Transwells were used as controls. The percentage of invading cells was calculated according to manufacture's instruction. The data were analyzed and plotted by Microsoft Excel. The *asterisk* indicates a significant decrease from the positive control ($p < 0.01$, $n = 3$). *D*, inhibition of free radical formation inhibits *in vitro* angiogenesis-induced by ethanol. SVEC4–10 cells were either preincubated with 5,000 units/ml catalase for 2 h (*panel 3*) or were transiently overexpressed with glutathione peroxidase (*panel 4*). SVEC4–10 cells without the pretreatment were used as a positive control (*panel 2*). Cells were incubated on Matrigel with 0.4% ethanol for 5 h. *Panel 1* is a negative control in the absence of 0.4% ethanol. Images are representative of typical fields seen in three experiments. The magnification is 5×.

Rho GTPases Cdc42, Rac, and Rho play a central role in the reorganization of the structure of actin filaments. It has been demonstrated that Cdc42 regulates the formation of filopodia, Rac regulates the formation of lamellipodia, and Rho regulates the formation of stress fibers (22). The morphological changes in the structure of actin filaments, primarily the increase in the formation of filopodia and lamellipodia, upon stimulation by ethanol implies that Cdc42 and Rac could be activated. Indeed,

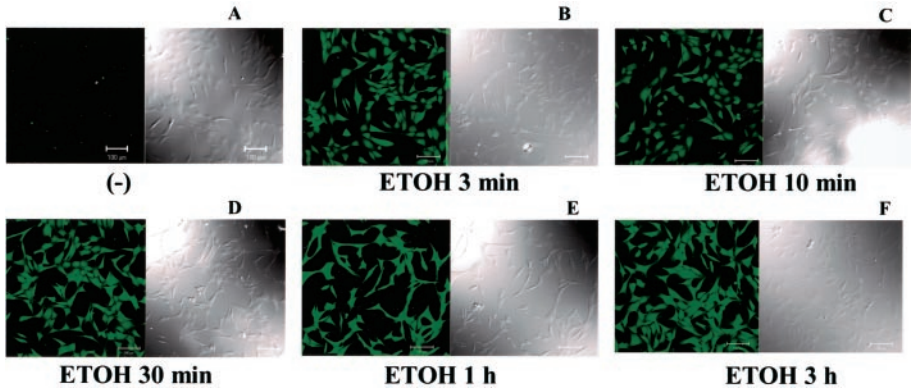

FIG. 6. **Time course of H_2O_2 production upon ethanol stimulation.** SVEC4–10 cells were stimulated with 0.4% ethanol for different periods of time ranging from 3 min to 3 h as indicated. After the stimulation, the cells were stained with carboxymethyl dichlorofluorescein diacetate. A Zeiss LSM 510 microscope was used to detect the production of H_2O_2. Images are representative of typical fields seen in three experiments. A square region of interest, 500 × 500 μm, was centered on each fluorescent image, and the average gray value of the entire field (range 0–255, black = 0, white = 255) was extracted using Optimas image analysis software. The values of average gray level: *A*, 2.3; *B*, 58; *C*, 38; *D*, 71; *E*, 72; *F*, 93.

our results demonstrate that ethanol stimulation activated Cdc42 in SVEC4–10 cells. However, no activation of Rac was detected upon stimulation with ethanol in SVEC4–10 cells (data not shown). A literature search found that it was not unprecedented that Cdc42 remodeled actin filaments to form both filopodia and lamellipodia structures independent of the activation of Rac (19, 33). The observation that expression of dominant negative Cdc42 blocked the effects of ethanol indicates that the activation of Cdc42 is necessary to reorganize the structure of actin filaments and to increase the cell migration and cell invasion upon stimulation with ethanol. Cdc42 has been found to control signaling pathways, such as apoptosis, cell cycle progression, and cell transformation (34). Recently, Cdc42 was found to be essential in $\alpha_V\beta_3$ integrin-mediated angiogenesis. Inhibition of $\alpha_V\beta_3$ integrin-dependent activation of Cdc42 results in the suppression of angiogenesis *in vivo* (35). Cdc42 was also found to mediate factor VIIa/tissue factor-induced angiogenesis (36). Mutation of its downstream kinase p21-activated kinase is sufficient to block angiogenesis (37). In this study, we demonstrated that Cdc42 is essential for ethanol-induced *in vitro* angiogenesis in SVEC4–10 cells. More importantly, we found that the overexpression of dominant positive Cdc42 is sufficient to induce *in vitro* angiogenesis in mouse endothelial cells. This is the first time that the overexpression of a constitutively dominant active Cdc42 alone has been demonstrated to induce *in vitro* angiogenesis. Our results also indicate that the *in vitro* angiogenesis observed in this study may be mediated through the reorganization of actin filaments. Cytochalasin D is an actin filaments inhibitor (38), and preincubation of SVEC4–10 cells with cytochalasin D blocked both the dominant positive Cdc42 (data not shown) and ethanol-induced *in vitro* angiogenesis.

Ethanol has been shown to induce *in vitro* angiogenesis through the activation of protein kinase C and mitogen-activated protein kinase signaling pathways (39). Recently, ethanol has been found to activate the angiogenic stimulator vascular endothelial growth factor through HIF-1α and Ras (7, 40). It is unclear at this time whether the activation of Cdc42 upon stimulation by ethanol is related to those pathways. Further studies are needed to identify the connections among these signaling pathways in ethanol-induced *in vitro* angiogenesis.

The results obtained from the present study show that H_2O_2 generated by ethanol-stimulated cells may play a key role in ethanol-induced angiogenesis and that Cdc42 is responsible for the H_2O_2 generation. The following experimental observations support these conclusions. (*a*) The generation of H_2O_2 upon stimulation by ethanol was visualized using confocal microscopy and detected by the change in fluorescence of scopoletin in the presence of horseradish peroxidase; (*b*) the increase of H_2O_2 production lasts a relatively long period of time; (*c*) catalase, a scavenger of H_2O_2, decreased its generation; (*d*) dominant negative Cdc42 cells generated a low level of H_2O_2 compared with wild type cells; (*e*) catalase prevented ethanol-induced remodeling of actin filaments, decreased in cell migration and cell invasion, and reduced angiogenesis; (*f*) the addition of peroxide extracellularly induced actin filament remodeling; and (*g*) overexpression of glutathione peroxidase decreased the H_2O_2 formation inside the cells and inhibited ethanol-induced angiogenesis. It should be noted that H_2O_2, a key member of the reactive oxygen species family, plays a pivoted role in various cellular processes. Recently, it has been reported that chronic alcohol toxicity is mediated primarily through oxidative stress in liver, central nervous system, and other tissues (23). Electron spin resonance and the analysis of indirect makers of oxidative stress, such as lipid peroxidation protein modification, and alternation in the levels of endogenous tissue antioxidants, lead to the hypothesis that oxidative stress is an important mediator of alcohol-induced cellular injury (23). Based on this hypothesis, therapeutic strategies have been developed for the treatment of alcohol toxicity by administration of antioxidants (23). The present study shows that H_2O_2 is an important mediator in ethanol-induced remodeling of actin filament integrity and *in vitro* angiogenesis, and Cdc42 is an upstream mediator of H_2O_2 generation. This study provides a molecular basis for the role of H_2O_2 in the ethanol-induced cell responses. Currently, we are actively identifying the downstream signals of H_2O_2 in mediating the effects of ethanol on SVEC4–10 cells.

Taken together, our data demonstrate that ethanol stimulation of endothelial cells has the ability to produce H_2O_2 through the activation of Cdc42, which induces the reorganization of actin filaments to lead to the increases in cell migration, cell invasion, and *in vitro* angiogenesis. Data also demonstrate that the overexpression of a constitutively dominant active mutant Cdc42 alone is sufficient to induce the formation of H_2O_2 and to cause *in vitro* angiogenesis. Our study identifies a novel signaling pathway that links the ethanol-induced changes in Cdc42, H_2O_2, actin filaments, and cell motility to *in vitro* angiogenesis. These observations may have implications for understanding the molecular mechanisms of angiogenesis in general.

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