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Induction of *in Vitro* Angiogenesis in the Endothelial-Derived Cell Line, EA hy926, by Ethanol Is Mediated through PKC and MAPK[1]

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We have previously shown that ethanol-induced injury to the gastric mucosa triggers increased expression of the angiogenic factors, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) and angiogenesis. To further investigate ethanol-induced angiogenesis, we used an *in vitro* angiogenesis model which employs the ability of an endothelial-derived cell line (EA hy926) to form tubelike structures resembling capillaries when plated on the matrix material, Matrigel. We report that serum-starved EA hy926 cells, incubated for as little as 5 minutes with ethanol concentrations of 1.0–2.5%, formed tubelike structures reflecting *in vitro* angiogenesis. Control cells, not incubated with ethanol, did not form tubelike structures. Incubation for 5 minutes with 2.5% ethanol resulted in increased activities of PKC and MAP kinase (ERK2) by 1.6-fold ($p < 0.05$) and 2.3-fold ($P < 0.001$), respectively. Futhermore, inhibitors of the MAPK kinase, MEK (PD98059) and PKC (GF 109203X) prevented the induction of *in vitro* angiogenesis by ethanol.

Key Words: **protein kinase C; mitogen activated protein kinase; ERK; MEK; signaling; kinase inhibitors; kinase activation.**

Angiogenesis, the development of new capillary vessels, is an important process in tissue development and wound healing (1). Angiogenesis occurs via a series of steps beginning with the localized degradation of the basement membrane of an existing microvessel through endothelial-derived proteases (2). This is followed by the rapid proliferation of endotheial cells (which normally have a very slow turnover time of years) and their migration in response to specific growth factors (e.g. basic fibroblast growth factor, bFGF, and vascular endothelial growth factor, VEGF). The migrating cells then form tubular structures which eventually join through anastomosis resulting in new capillaries (3).

We have previously demonstrated that ethanol-induced injury to the gastric mucosa *in vivo* results in the increased expression of the angiogenic growth factors, bFGF and VEGF, as well as rapid angiogenesis in the mucosa bordering the injury (4-7). Ethanol, itself, has been shown to trigger the activation of protein kinase C (PKC) and to augment the activation of the mitogen activated protein kinase (MAPK) by nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) in the neural cell line PC12 (8, 9). PKC has been shown to play an essential role not only in the proliferation of endothelial cells but also in their attachment, migration and spreading (10, 11). MAPK has also been implicated as being essential for the proliferative actions of bFGF and VEGF on endothelial cells (12). Thus, angiogenesis may be mediated, in part, through the activation of the PKC and/or MAPK signaling pathway(s).

An *in vitro* model for angiogenesis has been established using the cell line, EA hy926 (13). This cell line, obtained by hybridizing human umbilical vein endothelial cells (HUVECs) with the A549/8 human lung carcinoma cell line, has been demonstrated to possess a wide range of differentiated endothelial cell properties (14). These cells also undergo morphological rearrangements similar to the endothelial-derived HUVEC parental cells when seeded on the extracellular matrix material, Matrigel. These rearrangements, culminating in the formation of tubelike structures resembling capillaries—and thus *in vitro* angiogenesis—do not occur with the other parental line, A549/8 (13).

The aims of the present study were to investigate both the ability of ethanol to induce EA hy926 cells to undergo *in vitro* angiogenesis and the possible signaling mechanism(s) involved. This study demonstrated that ethanol stimulates serum-starved EA hy926 cells to undergo *in vitro* angiogenesis; that ethanol stimu-

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lates the activities of both MAPK and PKC in these cells; and that the specific inhibitor (PD98059) of the MAPK kinase, MEK, partially prevented ethanol-induced *in vitro* angiogenesis, while the specific inhibitor (GF 109203X) of PKC completely prevented ethanol-induced angiogenesis *in vitro* in these cells.

MATERIALS AND METHODS

Dulbecco's minimal essential medium (DMEM), antibiotic:antimycotic supplement and other tissue culture reagents were obtained from Fisher Scientific (Springfield, NJ). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Norcross, GA). Phorbol 12-myristate 13-acetate, GF 109203X and protein A sepharose were obtained from Sigma Chemical Co. (St. Louis, MO). PD98059 was obtained from Biomol (Plymouth Meeting, PA). Growth factor-reduced Matrigel was obtained from Becton Dickinson Labware (Bedford, MA). Rabbit polyclonal anti-ERK2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). LIVE/DEAD Viability/Cytotoxicity Kit was obtained from Molecular Probes, Inc. (Eugene, OR). Protein Kinase C Assay System was obtained from GibcoBRL (Gaithersburg, MD). [γ-^{32}P] ATP (6000 Ci/mmol) was obtained from Dupont NEN Research Products (Boston, MA).

Cell culture. EA hy926 cells were kindly provided by Dr. Cora-Jean Edgell, University of North Carolina School of Medicine, Chapel Hill, North Carolina. Cells were routinely grown in DMEM supplemented with 5% FBS and antibiotics in an atmosphere of 5% CO_2 and 95% air at 37°C in a humidified incubator. Subcultures were made from confluent stock cultures by trypsinization in PBS containing 0.5 mM EDTA and 0.25% trypsin.

In vitro angiogenesis assay. EA hy926 cells were grown in 60 mm tissue culture dishes until ~ 80% confluent. The growth medium was replaced with serum-free DMEM containing antibiotics:antimycotics and the cells were incubated for 36 additional hours. Inhibitors and/or ethanol were then added at the specified concentrations and the cells were further incubated for the indicated time period(s). The cells were then trypsinized, counted, and resuspended in DMEM containing 0.5% FBS (at 4×10^4 cells/ml). The wells of 24-well tissue culture plates were evenly coated with growth factor-reduced Matrigel (0.1 ml/well) which was allowed to solidify at 37°C for 30 minutes, according to the manufacturer's instructions, prior to plating cells. The indicated cell suspension was then plated at 1 ml/well onto the surface of the Matrigel and incubated at 37°C. Sixteen hours later, the cells were photographed using a Nikon inverted phase contrast photomicroscope (Nikon USA, Garden City, NY) with a video image analysis system (Image-1/FL, Universal Imaging Corp., Westchester, PA). Tube formation was quantified by counting the number of connected cells in randomly selected fields, under 200× magnification, and dividing that number by the total number of cells in the same field.

Determination of mitogen activated protein kinase (ERK2) activity. ERK activity was determined as previously described (15, 16). EA hy926 cells were grown in 60 mm tissue culture dishes and treated with the indicated inhibitor and/or ethanol as described under "In vitro angiogenesis assay." The cells were washed with ice-cold PBS and lysed on ice in 0.3 ml MAPK lysis buffer: 50 mM HEPES, pH 7.5; 100 mM NaCl; 1% NP-40; 40 mM phosphonitryl phenyl phosphate; 1 μM pepstatin; 0.2 mM PMSF; 2 μg/ml aprotinin; 1 μg/ml leupeptin; and 0.2 mM $NaVO_4$. Following centrifugation at 14,000 × g to remove the insoluble particulate, the protein concentration of the lysate was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). For each experimental sample, 30 μg of total protein was added to a conjugate of protein A sepharose (25 μl) and anti-ERK2 antibody (0.1 μg) in a total of 300 μl MAPK lysis buffer and mixed at 4°C for 2 hours. The conjugates were then pelleted by centrifugation at 14,000 × g for 1 minute and washed twice with 500 μl MAPK lysis buffer and twice with 500 μl wash buffer: 10 mM HEPES, pH 7.5 and 10 mM $MgCl_2$. Following the final wash, all buffer was removed and 40 μl of MAPK assay cocktail (10 mM HEPES, pH 7.5; 10 mM $MgCl_2$; 50 μM ATP; 30 μg myelin basic protein; and 4 μCi [γ-^{32}P] ATP) was added to each sample. The samples were then incubated at 30°C for 20 minutes and the reaction was terminated by the addition of 10 μl 5 × SDS-PAGE sample buffer (62.5 mM Tris-HCL, pH 6.8; 10% glycerol; 2% SDS; 5% β-mercaptoethanol; 0.65 mM dithiothreitol; 0.06% bromophenol blue). Then the samples were boiled for 2 minutes and loaded onto 15% acrylamide gels and electrophoresed. Following electrophoresis, the gels were fixed and stained with Coomassie brilliant blue in 10% acetic acid : 40% methanol and dried. The gels were autoradiographed and the myelin basic protein bands were cut out and counted in a scintilation counter.

Determination of protein kinase C activity. Protein kinase C activity was determined using an assay system based on the method previously described (17). EA hy926 cells were grown in 60 mm tissue culture dishes and treated with the indicated inhibitor and/or ethanol as described under "In-vitro angiogenesis assay." The cells were washed with ice-cold PBS and lysed on ice in 0.5 ml PKC homogenization buffer: 20 mM Tris-HCl, pH 7.4; 2 mM EGTA; 2 mM EDTA; 1% NP-40, 0.33 M sucrose; 0.2 mM $NaVO_4$; 100 mM NaF; 10 mM sodium pyrophosphate; 10 μg/ml aprotinin; 10 μg/ml leupeptin; 1 mM PMSF. Following centrifugation at 14,000 × g to remove the insoluble particulate, the protein concentration of the lysate was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). The lysate was diluted to 0.04 mg protein/ml in ice-cold dilution buffer (20 mM Tris-HCl, pH 7.4; 0.2 m NaCl; 0.5 mM EGTA; 0.5 mM EDTA; 10 mM β-mercaptoethanol) and 25 μl (1 μg of protein) was assayed using the PKC assay system according to the manufacturer's instructions.

Determination of cell viability in response to ethanol and kinase inhibitors. EA hy926 cells were grown on sterile glass coverslips in 12 well tissue culture plates and treated with the indicated inhibitor and/or ethanol as described under "In-vitro angiogenesis assay." The cells were then washed twice with sterile PBS and cell viability was determined using the LIVE/DEAD viability/cytotoxicity kit (Molecular Probes, Inc.; Eugene, OR) according to the manufacturer's instructions.

Statistical analysis. Results are expressed as the mean ± standard deviation (SD). Student's *t* test was used to determine statistical significance between control and treated cells. A p value of <0.05 was considered statistically significant. Comparisons of data between multiple groups were performed with analysis of variance (ANOVA).

RESULTS

Ethanol Induces Angiogenesis in Vitro

Serum-starved EA hy926 cells, incubated for 3 hours in DMEM containing ethanol at concentrations of 1.0%-2.5%, formed tubelike structures, characteristic of angiogenesis, when plated on growth factor-reduced Matrigel in the presence of 0.5% fetal bovine serum (Figures 1B and 1C; Table 1). These tubelike structures were usually formed within 16 hours after plating on the Matrigel. Serum-starved EA hy926 cells which were not incubated with ethanol failed to form tubelike structures when plated on growth factor-reduced Matrigel and remained as single cells (Figure 1A; Table 1). Formation of the tubelike structures was greastest when the EA hy926 cells were incubated for 3 hours in DMEM containing 2.5% ethanol. However, it is important to note that at this concentration, incubation for as little as 5 minutes was found to be sufficient to induce tube

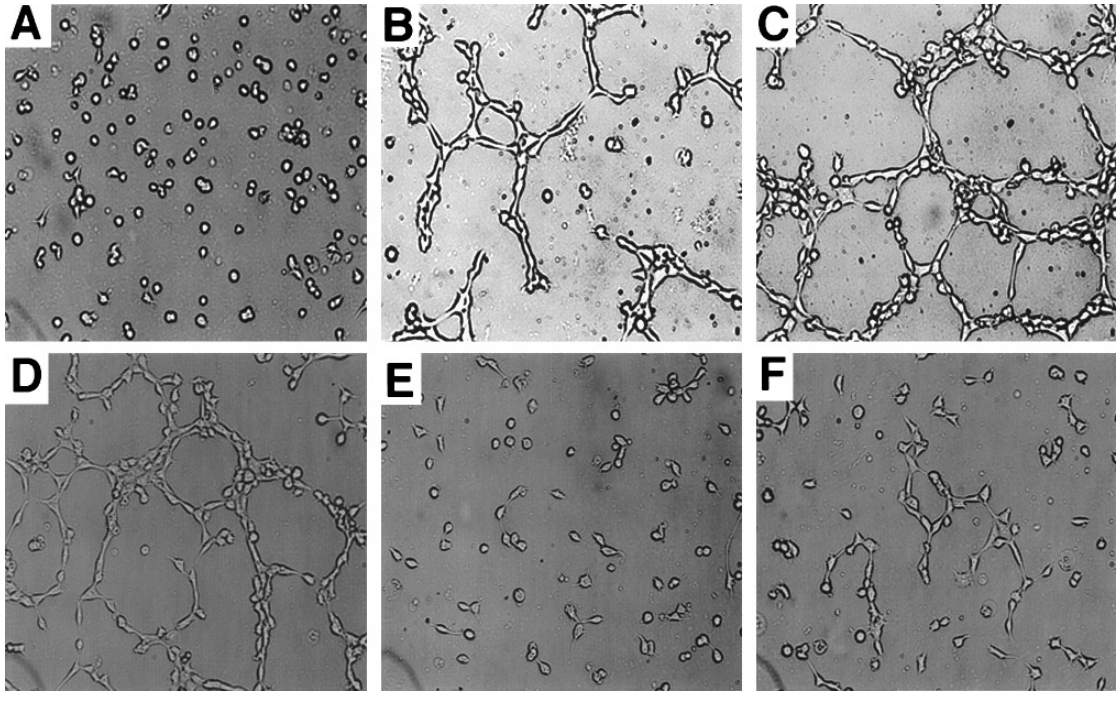

FIG. 1. Ethanol-induced *in vitro* angiogenesis. EA hy926 cells were treated as described in Materials and Methods under "In vitro angiogenesis assay." (**A**) EA hy926 cells which were serum-starved for 36 hours, failed to form tubelike structures when trypsinized and plated on growth factor-reduced Matrigel at a density of 4×10^4 cells/well in the presence of 0.5% FBS. (**B**) Serum-starved EA hy926 cells were incubated with 1.0% ethanol for 3 hours, trypsinized and plated on growth factor-reduced Matrigel at a density of 4×10^4 cells/well in the presence of 0.5% FBS. Extensive tubelike structures reflecting angiogenesis are clearly present where cells have migrated and joined together through anastomosis. (**C**) Serum-starved EA hy926 cells were incubated with 2.5% ethanol for 3 hours, trypsinized and plated on growth factor-reduced Matrigel at a density of 4×10^4 cells/well in the presence of 0.5% FBS. Again, tubelike structures reflecting angiogenesis are clearly present. (**D**) Serum-starved EA hy926 cells were incubated with 2.5% ethanol for 5 minutes, trypsinized and plated on growth factor-reduced Matrigel at a density of 4×10^4 cells/well in the presence of 0.5% FBS. The formation of tubelike structures demonstrates that incubation with this concentration of ethanol for 5 minutes is sufficient to induce *in vitro* angiogenesis. (**E**) Serum-starved EA hy926 cells were incubated with GF 109203X (5 μM) for 30 minutes. Ethanol was then added at a final concentration of 2.5% and the cells were incubated an additional 5 minutes and plated on growth factor-reduced Matrigel in the presence of 0.5% fetal calf serum. Cells failed to migrate or form tubelike structures demonstrating the requirement for PKC activity in the *in vitro* angiogenesis induced by ethanol. (**F**) Serum-starved EA hy926 cells were incubated with PD98059 (10 μM) for 30 minutes. Ethanol was then added at a final concentration of 2.5% and the cells were incubated an additional 5 minutes and plated on growth factor-reduced Matrigel in the presence of 0.5% fetal calf serum. The ability of the cells to form tubelike structures was impaired by $\sim$ 50% demonstrating the involvement of MAP (ERK) kinase in the *in vitro* angiogenesis induced by ethanol. Cells were photographed 16 hours after plating on growth factor-reduced Matrigel at a magnification of 200×. Photographs are representative of five standardized fields from three separate experiments. Quantitative data are given in Table 1.

formation (Figure 1D). Incubation in the presence of 5.0% ethanol resulted in the EA hy926 cells remaining as single cells on the matrigel (data not shown).

Ethanol Stimulates PKC Activity in EA hy926 Cells

Because ethanol has been shown to stimulate PKC activity in the neural cell line, PC12 (8), and because PKC has been shown to play a role in endothelial cell proliferation and migration (10, 11), the effect of ethanol on the PKC activity of EA hy926 cells was investigated. As shown in Figure 2, incubation of serum-starved EA hy926 cells in DMEM containing 2.5% ethanol for 5 minutes resulted in a 1.6-fold ($p < 0.04$) increase in PKC activity compared to control cells not treated with ethanol. This increase in PKC activity remained significantly ($p < 0.005$) elevated above control levels when the serum-starved EA hy926 cells were incubated in DMEM containing 2.5% ethanol for up to 1 hour; however, a decrease in PKC activity was observed after incubation in the presence of 2.5% ethanol for 3 hours (Figure 2). The ability of ethanol to stimulate the PKC activity of EA hy926 cells was also completely blocked by the specific inhibitor of PKC, GF 109203X (Figure 2). Incubation of serum-starved EA hy926 cells with 50 nM PMA for 5 minutes was found to significantly ($p < 0.004$) stimulate PKC activity by 2.2-fold (Figure 2).

Inhibition of PKC Prevents the Formation of Tubelike Structures

To ascertain whether the ethanol-induced in vitro angiogenesis of EA hy926 cells is mediated through

TABLE 1

Ethanol Induction of *in Vitro* Angiogenesis (Reflected by Tube Formation) in Serum-Starved EA hy926 Cells Plated on Growth Factor-Reduced Matrigel and the Effects of Inhibitors of PKC and MAPK

Treatment[a]	Tube formation (% of cells)[b]
Control	5.85 ± 2.09
1% ETOH	80.84 ± 11.04[c]
2.5% ETOH	97.74 ± 1.40[c]
5 μM GF 109203X + 2.5% ETOH	8.38 ± 2.40[d]
10 μM PD98059 + 2.5% ETOH	48.92 ± 19.28[c]

[a] EA hy926 cells were serum-starved for 36 hours before the growth medium was replaced with serum-free DMEM containing the indicated final concentration of ethanol. The cells were then incubated for an additional 3 hours and plated on growth factor reduced Matrigel in the presence of 0.5% fetal calf serum. For kinase inhibition, serum-starved EA hy926 cells were incubated for 30 minutes in serum-free DMEM containing either vehicle (controls) or the indicated inhibitor. Ethanol was then added at a final concentration of 2.5% and the cells were incubated an additional 5 minutes and plated on growth factor reduced Matrigel in the presence of 0.5% fetal calf serum. Tube formation was determined 16 hours after plating on Matrigel.

[b] Tube formation was determined by counting the number of connected cells in randomly selected fields, under 200× magnification, and dividing that number by the total number of cells in the same field. Five standardized fields were counted for each treatment and experiment. Each value is the mean ± SEM of data from three separate experiments.

[c] $P < 0.001$.

[d] Value is not statistically significant from control.

PKC, the effect of inhibiting PKC on this process was investigated. Serum-starved EA hy926 cells were incubated for 30 minutes in DMEM containing the selective PKC inhibitor, GF 109203X, at a final concentration of 5 μM. Ethanol was then added, at a final concentration of 2.5%, and the cells were further incubated for 5 minutes. When plated on growth factor-reduced Matrigel in the presence of 0.5% fetal bovine serum, these cells failed to form tubelike structures as assessed at 16 hours (figure 1E; Table 1). This result was not due to a general cytotoxicity of the inhibitor since the cells remained completely viable, compared to vehicle treated control cells, as determined by a viability/cytotoxicity assay (97 ± 1% viability).

Ethanol Stimulates MAPK (ERK2) Activity in EA hy926 Cells

MAPK has been shown to be involved in cell proliferation (18, 19), to be activated by PKC (20, 21), and has been implicated as a mediator of bFGF and VEGF-induced angiogenesis (12). Therefore, we examined the effect of ethanol on the ERK2 activity of EA hy926 cells. As shown in Figure 3A, incubation of serum-starved EA hy926 cells in DMEM containing 2.5% ethanol for 5 minutes resulted in a 2.3-fold ($p < 0.001$) increase in ERK2 activity compared to control cells not treated with ethanol. This stimulation of MAPK activity by ethanol was blocked completely by preincubation for 30 minutes with a specific inhibitor (PD98059) of the MAPK kinase, MEK (Figure 3A). The ERK2 activity returned to control levels when the serum-starved EA hy926 cells were incubated in DMEM containing 2.5% ethanol for 3 hours (Figure 3A). The ERK2 activity of EA hy926 cells was also determined to be stimulated by PKC as demonstrated by a 2.7-fold ($p < 0.0001$) increase in ERK2 activity in response to incubation for 5 minutes with 50 nM PMA (Figure 3A). Furthermore, preincubation for 30 minutes with GF 109203X completely blocked the ethanol-induced stimulation of MAPK activity (Figure 3A) Quantitative data for the MAPK activities are given in Figure 3B.

Inhibition of MAPK Only Partially Prevents the Formation of Tubelike Structures

To determine the involvement of MAPK in the ethanol-induced in vitro angiogenesis of EA hy926 cells, the effect of inhibiting the MAPK kinase, MEK, was investigated. Serum-starved EA hy926 cells were incubated for 30 minutes in DMEM containing the selective MEK inhibitor, PD98059, at a final concentration of 10 μM. Ethanol was then added, at a final concentration of 2.5%, and the cells were further incubated for 5 minutes. When plated on growth factor-reduced Matrigel in the presence of 0.5% fetal bovine serum, these cells did form tubelike structures, although tube formation was reduced by ~50% (Figure 1F; Table 1). Again, this result was not due to a general cytotoxicity of the inhib-

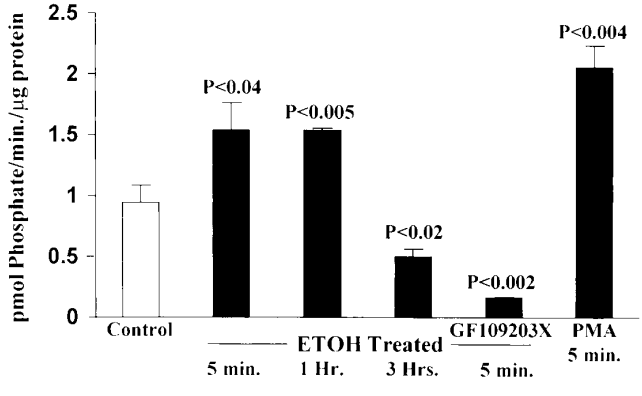

FIG. 2. Ethanol stimulation of PKC activity in EA hy926 cells. PKC activity was determined as described in Materials and Methods. Serum-starved EA hy926 cells were incubated for 30 minutes with either vehicle or GF 109203X (5 μM) followed by incubation with 2.5% ethanol for the indicated times. PMA was used at a final concentration of 50 nM. Data are from three separate experiments each performed in triplicate. Statistical significance is indicated by p values less than 0.05 as compared to vehicle treated control cells not incubated with ethanol.

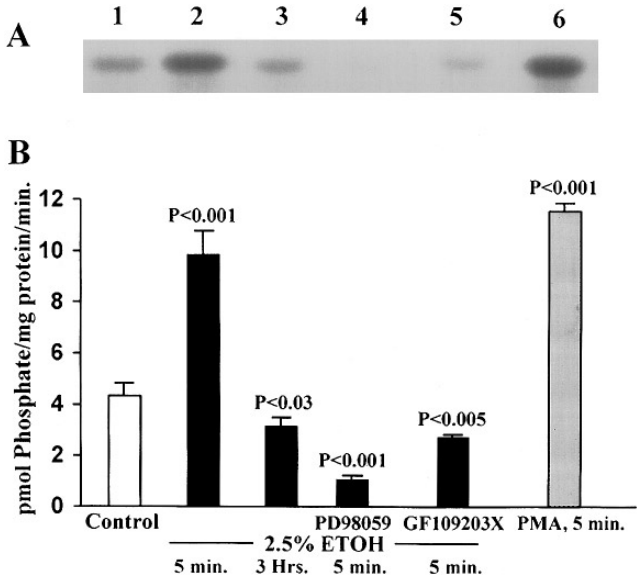

FIG. 3. Ethanol stimulation of ERK2 kinase in EA hy926 cells. ERK2 kinase activity was determined as described in Materials and Methods. (**A**) Representative autoradiograph of myelin basic protein phosphorylated by immunoprecipitated ERK2. Lane 1: EA hy926 cells treated with vehicle alone; Lane 2: EA hy926 cells incubated for 30 minutes with vehicle followed by incubation with 2.5% ethanol for 5 minutes; Lane 3: EA hy926 cells incubated for 30 minutes with vehicle followed by incubation with 2.5% ethanol for 3 hours; Lane 4: EA hy926 cells incubated for 30 minutes with PD98059 (10μM), followed by incubation with 2.5% ethanol for 5 minutes; Lane 5: EA hy926 cells incubated for 30 minutes with GF 109203X (5μM), followed by incubation with 2.5% ethanol for 5 minutes; Lane 6: EA hy926 cells incubated for 30 minutes with vehicle, followed by incubation with PMA (50 nM) for 5 minutes. (**B**) Quantitative analysis of three separate experiments each performed in triplicate. Statistical significance is indicated by *p* values less than 0.05 as compared to vehicle treated control cells not incubated with ethanol.

itor since the cells remained viable as determined by a viability/cytotoxicity assay (96 ± 1% viability).

DISCUSSION

Angiogenesis is a complex process involving endothelial cell proliferation, migration and tube formation (22) which are mediated through signaling mechanisms that remain incompletely defined (23). The development of in vitro models of angiogenesis, however, has greatly accelerated the experimental dissection of this process into a series of individual steps. This has led to the establishment that the extracellular matrix surrounding the endothelial cells serves not only as a structural framework but also as the source of the factors that signal the endothelial cells to proliferate, differentiate, migrate and eventually form new capillaries (2, 24). Important in the first step of this process is the increased production of collagenase and plasminogen activators by the endothelial cells which act in the local breakdown of the microvascular basement membrane. In this regard, it has been shown that phorbol esters, basic fibroblast growth factor, and vanadate all increase plasminogen activator production in vascular endothelial cells as well as induce angiogenesis (25-28). Phorbol esters strongly induce activation of PKC and vanadate nonselectively increases the level of tyrosine phosphorylation thereby activating signaling proteins such as MAPK (29, 30).

Our interest in the use of an in vitro angiogenesis model was to further understand the mechanisms involved in the angiogenesis which results from ethanol-induced injury to the gastric mucosa (5-7). We have previously demonstrated that ethanol-induced injury *in vivo* results in the increased expression of bFGF and its receptors and the increased expression of VEGF and ras (a mediator of cell proliferation and a putative regulator of VEGF expression) in the area bordering the gastric mucosal injury (4, 5). The finding in the present report is the first demonstration that ethanol, itself, can induce endothelial cells to undergo *in vitro* angiogenesis. The finding that the same concentration of ethanol that induces *in vitro* angiogenesis also increases PKC activity suggested that the ethanol-induced angiogenesis was mediated through the PKC pathway. This was confirmed by the demonstration that ethanol-induced angiogenesis could be completely blocked by inhibition of PKC activity. This is in agreement with published results which indicate that PKC is both necessary and sufficient for attachment, spreading, and migration of human endothelial cells (11) and that phorbol esters can induce in vitro angiogenesis (25).

It is well established that PKC can activate MAPK which, in turn, activates the gene transcription known to be involved in cellular proliferation and differentiation (19-21, 31, 32). We have demonstrated that ethanol stimulates ERK2 activity in EA hy926 cells and that this stimulation is blocked an inhibitor of PKC suggesting that it is mediated through PKC. The findings that an inhibitor of the MAPK kinase, MEK, blocks the ethanol-induced stimulation of ERK2 but only partially blocked the ethanol-induced in vitro angiogenesis suggests the involvement of a parallel, MAPK-independent, pathway. The nature of this pathway remains to be clarified; however, the finding that inhibition of PKC activity completely blocked ethanol-induced in vitro angiogenesis suggests that PKC is required for both the MAPK-dependent and MAPK-independent signaling leading to *in vitro* angiogenesis.

How ethanol stimulates PKC activity is also uncertain. Ethanol has been shown to activate PKC in human lymphocytes and human epidermal keratinocytes, both within 5 minutes of exposure (33, 34). In the case of human epidermal keratinocytes, ethanol exposure also induced the PKC-dependent mRNA expression of the transcriptional regulator, Jun/AP-1 (34). Ethanol has also been shown to stimulate PKC activity in the neural cell line, PC12, but this was only following

chronic exposure for 2 - 6 days and appears to result from increased expression of the isoforms, PKC_{δ} and PKC_{ϵ} (8). We did not detect increased expression in PKC protein levels in EA hy926 cells following exposure to ethanol (data not shown). Moreover, although ethanol stimulated the activities of both PKC and ERK2 in EA hy926 cells within 5 minutes, one might not expect protein levels to change in such a short time period. While ethanol has been shown to stimulate phospholipase C activity in liver cells leading to PKC activation (35), and to increased intracellular calcium in endothelial cells (36), the effects of ethanol on phospholipase C activity, calcium flux, and lipid phosphorylation appear to vary depending on cell type and membrane lipid content (36-38). Further investigation is required to fully understand the mechanism whereby ethanol stimulates PKC activity and increased PKC activity stimulates MAPK-dependent and MAPK-independent pathways leading to *in vitro* angiogenesis.

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