

2-Methoxyestradiol, an Endogenous Estrogen Metabolite, Induces Apoptosis in Endothelial Cells and Inhibits Angiogenesis: Possible Role for Stress-Activated Protein Kinase Signaling Pathway and Fas Expression

TIAN-LI YUE, XINKANG WANG, CALVERT S. LOUDEN, SHALLEY GUPTA, KODANDARAM PILLARISETTI, JUAN-LI GU, TIMOTHY K. HART, PAUL G. LYSKO, and GIORA Z. FEUERSTEIN

Departments of Cardiovascular Pharmacology (T.-L.Y., X.W., S.G., K.P., J.-L.G., P.G.L., G.Z.F.) and Toxicology (C.S.L., T.K.H.), SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406

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SUMMARY

2-Methoxyestradiol (2-ME) is an endogenous metabolite of estradiol-17 β and the oral contraceptive agent 17-ethylestradiol. 2-ME was recently reported to inhibit endothelial cell proliferation. The current study was undertaken to explore the mechanism of 2-ME effects on endothelial cells, especially whether 2-ME induces apoptosis, a prime mechanism in tissue remodeling and angiogenesis. Cultured bovine pulmonary artery endothelial cells (BPAEC) exposed to 2-ME showed morphological (including ultrastructural) features characteristic of apoptosis: cell shrinkage, cytoplasmic and nuclear condensation, and cell blebbing. 2-ME-induced apoptosis in BPAEC was a time- and concentration-dependent process ($EC_{50} = 0.45 \pm 0.09 \mu M$, $n = 8$). Nucleosomal DNA fragmentation in BPAEC treated with 2-ME was identified by agarose gel electrophoresis (DNA ladder) as well as *in situ* nick end labeling. Under the same experimental conditions, estradiol-17 β and two of its other metabolites, estriol and 2-methoxyestriol ($\leq 10 \mu M$), did not have an apoptotic effect on BPAEC. 2-ME activated stress-

activated protein kinase (SAPK)/c-Jun amino-terminal protein kinase in BPAEC in a concentration-dependent manner. The activity of SAPK was increased by $170 \pm 27\%$ and $314 \pm 22\%$ over the basal level in the presence of 0.4 and $2 \mu M$ 2-ME ($n = 3-6$), respectively. The activation of SAPK was detected at 10 min, peaked at 20 min, and returned to basal levels at 60 min after exposure to 2-ME. Inhibition of SAPK/c-Jun amino-terminal protein kinase activation by basic fibroblast growth factor, insulin-like growth factor, or forskolin reduced 2-ME-induced apoptosis. Immunohistochemical analysis of BPAEC indicated that 2-ME up-regulated expression of both Fas and Bcl-2. In addition, 2-ME inhibited BPAEC migration ($IC_{50} = 0.71 \pm 0.11 \mu M$, $n = 4$) and basic fibroblast growth factor-induced angiogenesis in the chick chorioallantoic membrane model. Taken together, these results suggest that promotion of endothelial cell apoptosis, thereby inhibiting endothelial cell proliferation and migration, may be a major mechanism by which 2-ME inhibits angiogenesis.

2-ME (Fig. 1) is a metabolite of the endogenous estrogen hormone estradiol-17 β and the oral contraceptive agent 17-ethylestradiol that is produced by sequential hepatic hydroxylation and methylation from the parent compounds (1). 2-ME is present in human blood and urine (1-3). It has been reported that 2-ME causes disturbances of mitosis in a cultured Chinese hamster cell line (4), produces abnormal metaphase in MCF-7 and HeLa cells (5), and inhibits tubulin polymerization (6). More recently, 2-ME was found to inhibit endothelial cell proliferation and angiogenesis in an *in vitro* capillary tube formation model. Therefore, it has been suggested that 2-ME may be a novel antiangiogenic therapeutic

agent (3). However, the mechanism of the antiangiogenic activity of 2-ME is not fully understood.

Angiogenesis, the generation of new capillaries from pre-existing vessels, is a critical process during development, wound healing, and various diseases such as cancer, adult blindness, and inflammatory disorders (7, 8). Angiogenesis involves several steps, commencing in enzymatic degradation of basement membrane, followed by vascular endothelial cells migration into the perivascular space, proliferation and alignment to form tubular structures, and, finally, new vessel formation (7, 9). Although the mechanisms leading to pathological angiogenesis are still unclear, recent evidence indi-

ABBREVIATIONS: BPAEC, bovine pulmonary artery endothelial cells; 2-ME, 2-methoxyestradiol; bFGF, basic fibroblast growth factor; IGF, insulin-like growth factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; CAM, chick chorioallantoic membrane; SAPK, stress-activated protein kinase; JNK, c-Jun amino-terminal protein kinase; GST, glutathione-S-transferase; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(α -aminoethyl ether)- N,N,N',N' -tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline.

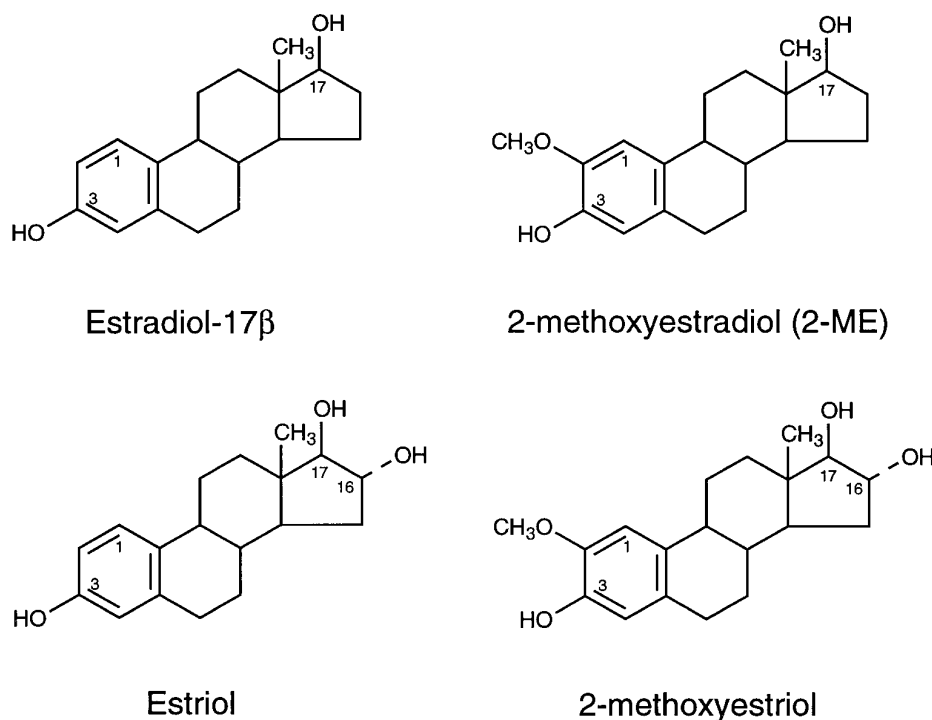


Fig. 1. Chemical structures of estradiol-17β, 2-ME, estriol, and 2-methoxyestriol.

cates that it is the result of an imbalance between antiangiogenic and proangiogenic factors, leading to vascular endothelial cell migration and proliferation. This pathological imbalance can be modulated through different mechanisms, of which apoptosis has been suggested to be an important pathway (10, 11).

Apoptosis, or programmed cell death, is an active, gene-directed form of cell death that is different from cell necrosis with respect to its morphology, biochemistry, pharmacology, and biological significance (12). It has been widely accepted that apoptotic cell death is an important mechanism that contributes to the reduction in cell growth (13). Recent studies by Brooks *et al.* (10) and Stromblad *et al.* (11) demonstrated that perturbation of angiogenesis on a CAM model by integrin $\alpha\beta 3$ antibody was mediated by selective promotion of apoptosis of angiogenic blood vessels. In this latter study, ~25–30% of the treated CAM cells, mainly blood vessel cells, showed evidence of nuclear condensation and DNA fragmentation, a hallmark of apoptotic cells. These findings demonstrated that induction of vessel cell apoptosis was a major mechanism for inhibition of angiogenesis.

The current study was undertaken to explore whether 2-ME induces endothelial cell apoptosis. To examine this possibility, we used BPAEC. Apoptosis was detected on the basis of morphological characteristics and DNA fragmentation. In addition, we studied the effect of 2-ME on the activity of SAPK in BPAEC. SAPK is a family of novel kinases that bind to the c-Jun transactivation domain and phosphorylate Ser63 and Ser73 and so is also called JNK (14, 15). SAPK/JNK was recently defined as being involved in the signaling pathways that lead to apoptosis (16, 17). The expression of Fas and Bcl-2 in 2-ME-stimulated BPAEC was determined in view of the death-promoting effect of Fas (18) and the antiapoptotic effect of Bcl-2 (19). Furthermore, to obtain more complete insight into the other components of angiogenesis, we explored the effects of 2-ME on vitronectin-induced

BPAEC migration *in vitro* (20) and bFGF-induced neovascularization in a CAM model *in vivo* (10); the former has been suggested to be a key cell behavior involved in angiogenesis.

Experimental Procedures

Materials. 2-ME, estradiol-17β, 2-methoxyestriol, estriol (Fig. 1), and forskolin were purchased from Sigma Chemical (St. Louis, MO). bFGF and tumor necrosis factor- α were obtained from Genzyme (Cambridge, MA). IGF (I and II) was provided by Boehringer-Mannheim Biochemicals (Indianapolis, IN). ApoptTag *in situ* apoptosis detection kit was purchased from Oncor (Gaithersburg, MD). Mouse anti-human JNK1 monoclonal antibody and protein G/Sepharose were obtained from PharMingen (San Diego, CA) and Pharmacia (Piscataway, NJ), respectively. [γ - 32 P]ATP (5000 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). DMEM was prepared by the Media Preparation Laboratory of SmithKline Beecham using materials from GIBCO (Grand Island, NY).

Cell cultures. BPAEC were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in DMEM supplemented with 10% heat-inactivated FCS in a humidified environment of 5% CO₂/95% air at 37° as previously described (21). Cells were initially cultured in T75 flasks and then subcultured onto 24-well tissue culture plates or T150 flasks at a density of 1×10^4 cells/ml. Cells at a subconfluent density were used. Before experiments, the medium was changed to FCS-free DMEM. BPAEC from passages 17–20 were used in all studies.

Morphological assessment and quantification of apoptosis. To quantify cells undergoing apoptosis, cell monolayers were fixed with 70% ethanol and stained with acridine orange as previously described (22). The morphological features of apoptosis (cell shrinkage, chromatin condensation, blebbing, and fragmentation) were monitored by fluorescence microscopy. At least 500 cells from randomly selected fields were counted. Data represent the mean \pm standard error of at least three independent experiments performed in duplicate.

Transmission electron microscopy. Endothelial cells were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, overnight at 4°. The cells were washed with 0.1 M cacodylate buffer

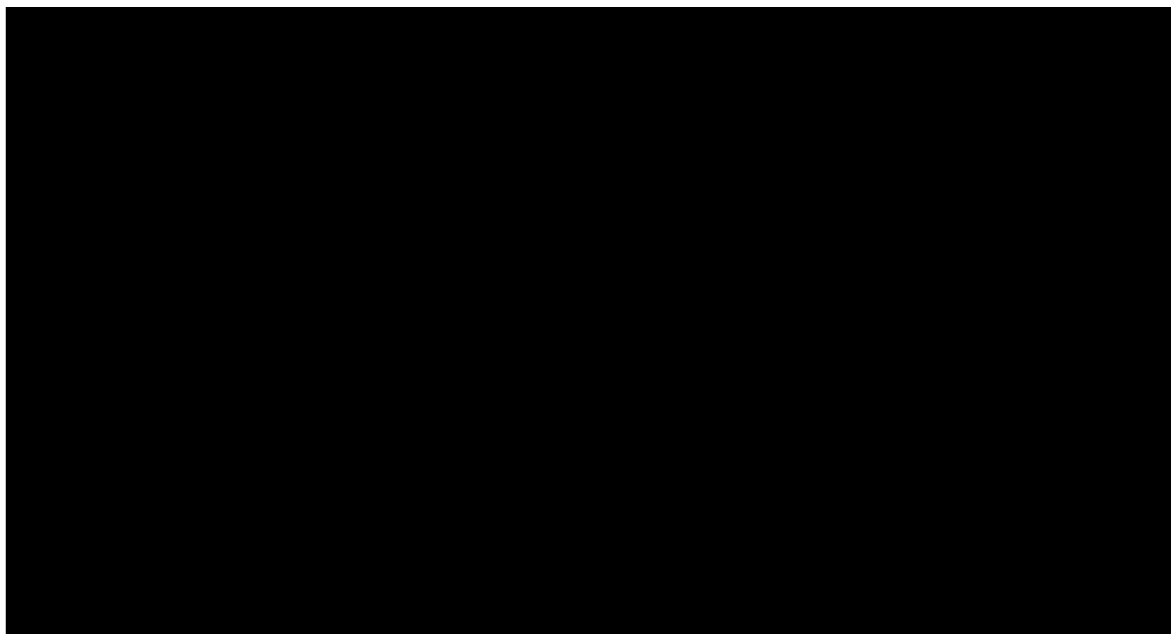


Fig. 2. *Top*, phase-contrast photomicrographs of BPAEC treated with vehicle (A), estradiol-17 β (10 μ M) (B), or 2-ME (2 μ M) (C) for 20 hr (original magnification, 100 \times). *Bottom*, fluorescence photomicrographs showing nuclear morphology of BPAEC treated with vehicle (A), estradiol-17 β (10 μ M) (B), or 2-ME (2 μ M) (C) for 20 hr and stained with acridine orange (original magnification, 400 \times).

and postfixed with 2% OsO₄, buffered with 0.1 M cacodylate buffer, pH 7.4, for 1 hr at 4°. The cells were dehydrated with graded alcohols to 2 \times 100% alcohol and 2 \times propylene oxide as previously described (23). Cells were removed from tissue culture wells with propylene oxide and embedded in pure Epon resin. One-micron-thick sections were cut, stained with toluidine blue, and examined at the light microscopic level to identify areas of interest. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and observed by transmission electron microscopy.

DNA fragmentation analysis. Cells (5–10 \times 10⁶) were lysed in lysis buffer containing 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, and 100 μ g/ml proteinase K. The lysates were incubated at 55° for 16 hr. After incubation, the lysates were gently extracted three times with phenol/chloroform/isoamyl alcohol. After centrifugation, the upper layer containing DNA was transferred to a

new tube, and 0.5 volume of 7.5 M ammonium acetate and 2.5 volumes of ethanol were added. The tube was kept at –20° overnight. After centrifugation, the resulting DNA pellet was dissolved in 0.1 ml of 10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA, and 10 μ g of DNase-free RNase was added and incubated at 37° for 3 hr. DNA was extracted with phenol/chloroform/isoamyl alcohol and precipitated again as described above. DNA electrophoresis was carried out in 1.8% agarose gels containing ethidium bromide, and DNA fragments were visualized under ultraviolet light.

In situ detection of apoptotic cells. *In situ* detection of apoptotic cells was performed by using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling with an ApopTag *in situ* apoptosis detection kit (Oncor). Briefly, BPAEC were cultured in four-chamber slides and treated with or without 2-ME for 24 hr. After treatment, cells were washed in PBS, fixed, incubated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice, and then labeled with fluorescent dUTP at strand breaks by deoxynucleotidyl transferase. The labeled cells were analyzed by fluorescence microscopy.

Fusion protein GST-c-Jun_(1–81) was made according to the method of Hibi *et al.* (14). The cDNA clone with a sequence encoding human c-Jun amino acids 1–81 was provided by Human Genome Sciences (Gaithersburg, MD) and subcloned into pGEX 4T-3, which contains a DNA sequence encoding GST. The GST-c-Jun expression vector, pGEX4T-3/c-Jun, was transformed into *Escherichia coli*. Expression of GST-c-Jun_(1–81) fusion protein was induced by isopropyl- β -thiogalactoside. *E. coli* were lysed and centrifuged, and the fusion protein GST-c-Jun_(1–81) was purified by glutathione-Sepharose chromatography.

SAPK/JNK assay. SAPK/JNK activity was measured using GST-c-Jun_(1–81) bound to glutathione-Sepharose 4B as described by Verheij *et al.* (24). Subconfluent monolayers of BPAEC were stimulated with 2-ME or vehicle for 20 min unless otherwise indicated. The cells were washed twice with cold phosphate buffer, pH 7.4, and then lysed in lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β -glycerophosphate, 1 mM dithiothreitol, 1 mM Na₃VO₄, 2 μ M leupeptin, 0.4 mM phenylmethylsulfonyl fluoride, 10% glycerol, 10 μ g/ml soybean trypsin inhibitor, 1% Triton X-100) on ice for 5–10 min. The nuclear-free supernatant was normalized for protein con-

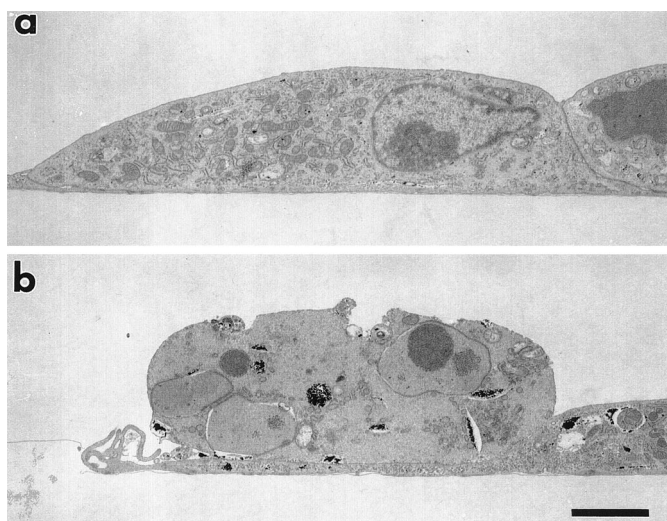


Fig. 3. Ultrastructural features of BPAEC cultures treated with 2-ME for 20 hr. *a*, Center, cell unaffected by the treatment. *Right*, cell with evident nuclear condensation. *b*, Apoptotic cell containing numerous DNA fragments. Bar, 2 μ m.

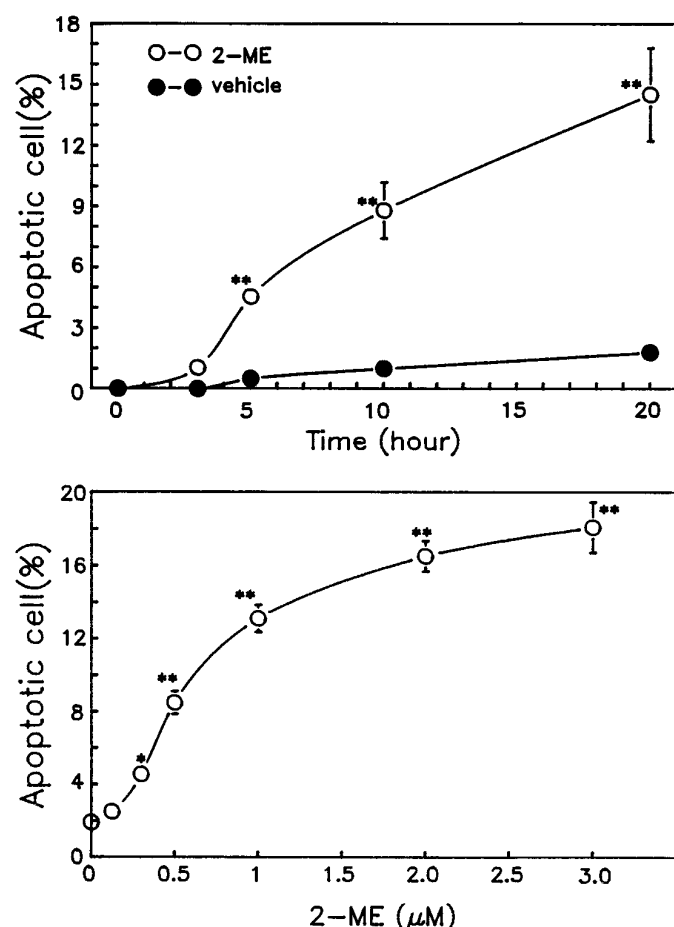


Fig. 4. Induction of apoptosis in BPAEC by 2-ME. A, Time course. Cells were cultured with 2-ME (2 μ M) for the indicated time intervals. Values are mean \pm standard error of four determinations. B, Concentration-dependency. Cells were cultured with the indicated concentration of 2-ME for 20 hr. The production of cells undergoing apoptosis was quantified by fluorescence microscopy after acridine orange staining. Values are mean \pm standard error of eight separate experiments performed in duplicate. *, $p < 0.05$; **, $p < 0.01$ versus basal.

TABLE 1

Induction of apoptosis in BPAEC by 2-ME and comparison with estradiol-17 β , 2-methoxyestriol, and estriol

BPAEC were treated with the test agents at the indicated concentrations for 20 hr, fixed with 70% ethanol, and stained with acridine orange. The morphological features of apoptosis (cell shrinkage, chromatin condensation, blebbing, and fragmentation) were monitored by fluorescence microscopy. At least 500 cells from randomly selected fields were counted. Data reported represent the mean \pm standard error of at least three independent experiments performed in duplicate or triplicate.

Compound	Concentration	Apoptosis
	μ M	%
Vehicle		1.91 \pm 0.31
2-ME	0.5	8.48 \pm 0.64 ^a
	2.0	16.51 \pm 0.85 ^a
Estradiol-17 β	2.0	2.14 \pm 0.40
	10.0	2.52 \pm 0.54
2-Methoxyestriol	2.0	0.83 \pm 0.13
	10.0	1.38 \pm 0.12
Estriol	2.0	0.88 \pm 0.21
	10.0	0.83 \pm 0.15

^a $p < .01$ versus vehicle.

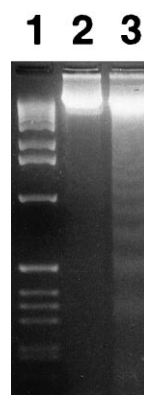


Fig. 5. Electrophoretic analysis of internucleosomal DNA fragmentation in 2-ME-treated BPAEC. BPAEC were treated with vehicle (lane 2) and 2 μ M 2-ME (lane 3) for 20 hr. Genomic DNA was isolated by phenol/chloroform extraction from BPAEC. DNA (10 μ g/lane) was loaded onto 1.8% agarose gels containing ethidium bromide. After electrophoresis, DNA bands were visualized under ultraviolet light. Lane 1, DNA size markers.

tent and immunoprecipitated with anti-SAPK antibody-conjugated Sepharose beads. The mixture was rotated at 4° for 3 hr. The phosphorylation buffer containing 4 μ g of GST-c-Jun₍₁₋₈₁₎, 10 μ Ci of [³²P]ATP, 125 μ M ATP, and 100 mM MgCl₂ was added to the SAPK-bound beads in assay buffer. The reaction was terminated after 20 min at 30° by the addition of protein loading buffer and heating at 95° for 3 min. Phosphorylated proteins were resolved on 10% SDS-polyacrylamide gel electrophoresis followed by autoradiography. A PhosphorImager (Molecular Dynamics, Sunnyvale, CA) was used to quantify the band intensities, and ImageQuant Version 3.0 software (Molecular Dynamics) was used to analyze the results as previously described (25).

Immunohistochemical studies. BPAEC were cultured in four-chamber slides and treated with vehicle or 2-ME for the indicated durations. The cells were fixed in 4% formaldehyde (EM-grade, EMS, Fort Washington, PA) at 4° for 2 hr and then prepared for immunoperoxidase staining using the Chem-mate Detection System (Bio Tek Instruments, Winooski, VT) according to the manufacturer's instructions. Briefly, endogenous peroxidase was quenched with 3% H₂O₂ in methanol for 30 min. Nonspecific immunoglobulin binding sites were blocked with normal goat serum for 20 min and then incubated with primary antibody, mouse anti-human Fas (Upstate Biotechnology, Lake Placid, NY) or mouse anti-human Bcl-2 (DAKO, Carpinteria, CA) antibody for 1 hr at room temperature. Cells were then incubated for 30 min with a biotinylated goat anti-mouse IgM (Fas) secondary antibody (1:200, Vector Laboratories, Burlingame, CA) or a biotinylated goat anti-mouse IgG (Bcl-2) secondary antibody (1:200, Biotek) followed by 30 min of incubation with the avidin-biotin complex (Biotek). Immunoglobulin complexes were visualized on incubation with 3,3'-diaminobenzidine, washed, counterstained with Harris' hematoxylin, air-dried, mounted with Crystal mount aqueous mounting media (Biomed, Foster City, CA), and examined by light microscopy.

Cell migration assay. BPAEC migration was monitored in a Transwell cell culture chamber by using a polycarbonate membrane with pores of 8 μ m (Costar, Cambridge, MA) as previously described (20, 25). Briefly, BPAEC were suspended in serum-free medium containing 0.2% bovine serum albumin at a concentration of 2×10^6 cells/ml. In the standard assay, 0.2 ml of cell suspension with a test agent or vehicle was placed in the upper compartment of the chamber. The lower compartment contained 0.5 ml of DMEM supplemented with 0.2% bovine serum albumin and the test agent or vehicle. Cell migration was induced with vitronectin (10 μ g/ml) (20). Incubation was at 37° in an atmosphere of 95% air/5% CO₂ for 20 hr.

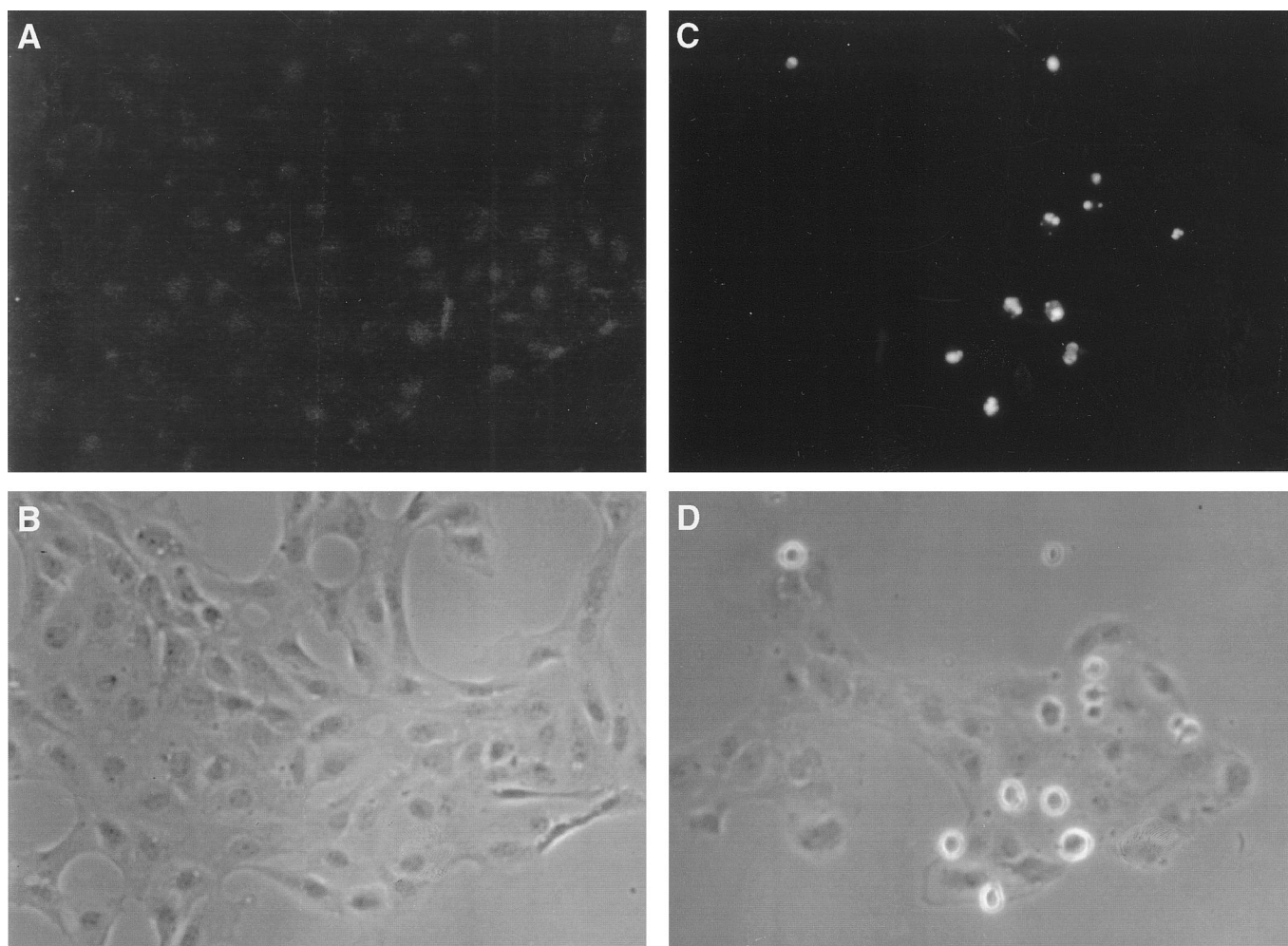


Fig. 6. *In situ* detection of DNA fragments. BPAEC were cultured in four-chamber slides and treated with 2-ME ($2 \mu\text{M}$) or vehicle for 20 hr. Cells were washed in PBS, fixed, incubated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice, and then labeled with fluorescent dUTP according to the manufacturer's instructions. The labeled cells were analyzed under fluorescence microscopy. *Top*, fluorescence photomicrographs. A, BPAEC treated with vehicle. C, BPAEC treated with 2-ME. *Bottom*, B and D phase contrast photomicrographs correspond to A and C.

After incubation, nonmigrated cells on the upper surface were scraped gently, and the filters were fixed in methanol and stained with 10% Giemsa stain. Stained cells were subsequently extracted with 10% acetic acid, and absorbance was determined at 600 nm.

CAM assay. Inhibition of angiogenesis was studied by CAM assay as previously described (26, 10). Angiogenesis was induced by b-FGF on the CAMs of 10-day-old chick embryos purchased from Truslow Farms (Chestertown, MD). Briefly, small holes were drilled into the blunt end over the air sac and broad side of day 10 fertilized chicken eggs. Using a Pasteur bulb, suction was applied to the hole over the air sac until the CAM dropped from the shell. A 1-cm^2 window was cut, through which a filter disk (Whatman Inc., Clifton, NJ) could be placed. The filters were saturated with $1 \mu\text{g/ml}$ b-FGF or PBS buffer (for measurement of the basal angiogenesis), and then the test agent ($10 \mu\text{l}$) or vehicle ($10 \mu\text{l}$) was added to the filter disks. After 72-hr incubation at 37° , CAMs with filters were fixed overnight at 4° with 4% paraformaldehyde. Angiogenesis was quantified by counting the number of vessels under the filter using NIH Image software. The angiogenesis was determined for 10–14 CAMs/treatment.

Statistical analysis. Results are expressed as mean \pm standard error. Statistical evaluation was performed by using one-way analysis of variance with subsequent *post hoc* paired comparisons. Differences with a value of $p < 0.05$ were considered significant.

Results

Morphological characterization of endothelial cell apoptosis. Phase-contrast microscopy showed morphological changes of apoptosis in 2-ME-treated endothelial cells (Fig. 2, *top*, C). When exposed to 2-ME for 20 hr, endothelial cells shrank and retracted from their neighbor cells, and the cytoplasm became condensed. When the endothelial cells were stained with acridine orange and assessed by fluorescence microscopy, cells with condensed chromatin or fragmented nuclei and blebbing of the plasma membrane were more clearly visualized (Fig. 2, *bottom*, C). Similar morphological changes were observed in endothelial cells treated with tumor necrosis factor- α , a known apoptotic agent on BPAEC (22) (data not shown). The study with transmission electron microscopy showed clear nuclear changes in 2-ME-treated BPAEC, including shrinkage, condensation of chromatin, and apoptotic bodies (Fig. 3).

2-ME-induced endothelial cell apoptosis was a time- and concentration-dependent process (Fig. 4). When exposed to 2-ME for 20 hr, a significant increase in the number of cells with apoptotic morphological changes was apparent at $0.3 \mu\text{M}$

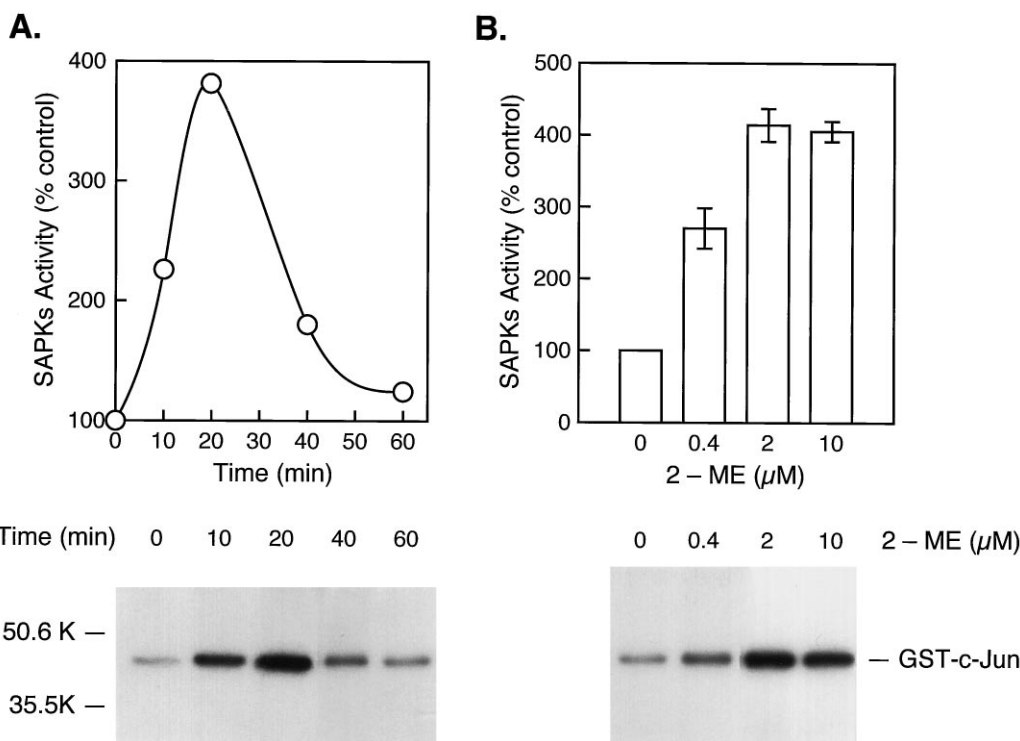


Fig. 7. Time course (A) and concentration dependence (B) of activation of SAPK by 2-ME. Subconfluent BPAEC were stimulated with 2 μ M 2-ME for the indicated periods of time (A) or at the concentrations indicated for 20 min (B) and lysed with ice-cold lysis buffer. The nuclear-free supernatant was normalized for protein content and immunoprecipitated with anti-SAPK monoclonal antibody (PharMingen)-conjugated Sepharose beads. The activity of SAPK was assayed using GST-c-Jun₍₁₋₈₁₎ as the substrate as described in the text. Phosphorylated proteins were resolved on SDS-polyacrylamide gel electrophoresis followed by autoradiography. The intensity of each band on the autoradiogram was quantified with a PhosphorImager using ImageQuant software (Molecular Dynamics). A and B, *bottom*, representative autoradiograms. A, *top*, quantitative result from a representative study performed in duplicate. B, *top*, quantitative results of three to six independent experiments.

2-ME, reaching a maximum value at 2–3 μ M 2-ME ($EC_{50} = 0.45 \pm 0.09 \mu$ M, $n = 8$). Under the identical conditions, estradiol-17 β , 2-methoxyestriol, and estriol ($\leq 10 \mu$ M) did not induce endothelial cell apoptosis, as shown in Fig. 2 (B) and Table 1. The concentration of FCS in medium below 2% had no effect on 2-ME-induced apoptosis in BPAEC. Quiescent confluent cultures of endothelial cells were less affected by 2-ME.

Accumulation of oligonucleosomes in endothelial cells undergoing apoptosis. 2-ME-induced DNA fragmentation was examined by agarose gel electrophoresis (Fig. 5). The characteristic degradation of DNA into oligonucleosomal-length fragmentations was observed when the endothelial cells were exposed to 2 μ M 2-ME for 20 hr. We further visualized DNA fragments *in situ* by using the terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling technique. A considerable fraction of endothelial cells treated with 2-ME showed positive staining (Fig. 6C) compared with the correspondent phase contrast photomicrograph (Fig. 6D); no positively stained cells were found in the vehicle- (Fig. 6, A compared with B) or estradiol-17 β - (data not shown) treated cultures.

Activation of SAPK/JNK in endothelial cells. The effects of 2-ME on SAPK/JNK activity in BPAEC are shown in Fig. 7. Exposure of endothelial cells to 2-ME induced a rapid activation of SAPK/JNK. A significant increase in SAPK/JNK activity was detected 10 min after stimulation, peaked at 20 min, and then returned to the basal levels at 60 min after stimulation. As shown in Fig. 7B, 2-ME-induced SAPK/

JNK activation in BPAEC was a concentration-dependent process. Some basal activities of SAPK/JNK were observed in unstimulated BPAEC. The SAPK/JNK activity was increased by $170 \pm 27\%$ ($n = 3$) and $314 \pm 22\%$ ($n = 6$) over the basal level in the presence of 0.4 and 2 μ M 2-ME, respectively. Under the same conditions, 1 mM of H₂O₂, a known activator of SAPK/JNK (15), increased the SAPK/JNK activity in BPAEC by $445 \pm 110\%$ ($n = 3$).

Effect of bFGF, forskolin, and IGF on the activation of SAPK/JNK and induction of apoptosis in BPAEC by 2-ME. In the presence of bFGF (100 ng/ml), forskolin (10 μ M), or IGF (100 ng/ml), 2-ME-induced activation of SAPK/JNK in BPAEC was prevented ($p > 0.05$ versus control, $n = 3-5$) (Fig. 8, *top*). These agents also reduced 2-ME-induced apoptosis in BPAEC significantly but not completely ($p < 0.05$ versus control) (Fig. 8, *bottom*). bFGF at low concentration (2 ng/ml) did not prevent the activation of SAPK/JNK by 2-ME and also had no effect on 2-ME-induced apoptosis in BPAEC.

Up-regulation of Fas and Bcl-2 expression in endothelial cells by 2-ME. Immunocytochemical analysis of Fas and Bcl-2 proteins in BPAEC was determined at 4 and 20 hr after treatment with 2-ME. These two time intervals were selected on the basis of the corresponding onset and peak of apoptotic cell death induced by 2-ME. The basal levels of both Fas and Bcl-2 in BPAEC were below the detectable level, as shown in Figs. 9, A and C, and 10C. However, a significant number of BPAEC expressing Fas protein were detected at 4 and 20 hr after stimulation (Fig. 9, B and D). Bcl-2 expression was observed 20 hr after 2-ME treatment (Fig. 10A) and

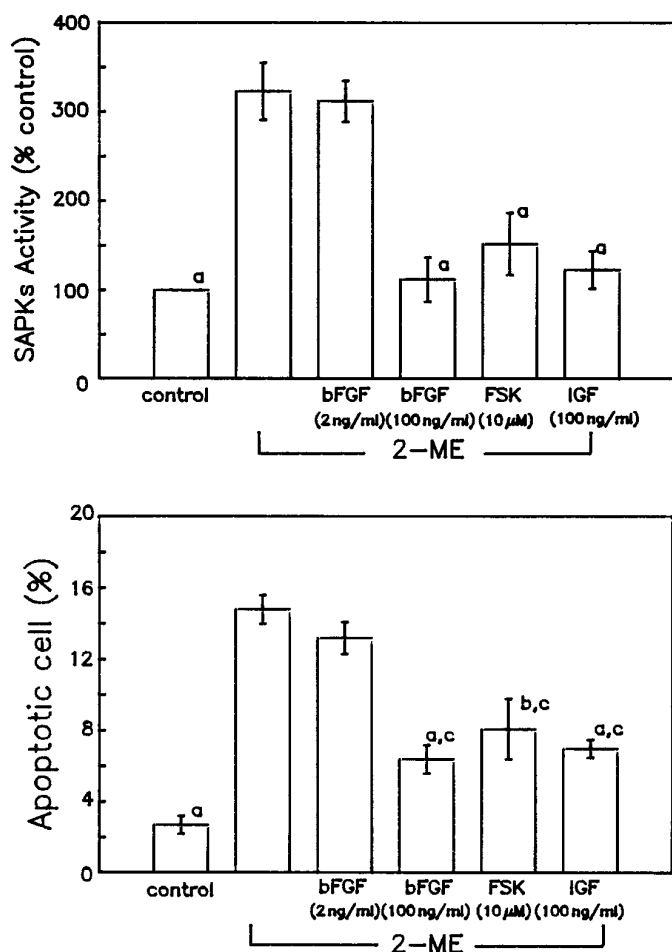


Fig. 8. Inhibition of 2-ME-induced SAPK/JNK activation (top) and 2-ME-induced apoptosis (bottom) by bFGF, forskolin (FSK), and IGF. Subconfluent BPAEC were pretreated with bFGF, forskolin, or IGF at the concentrations indicated for 1 hr and then stimulated with 2-ME for 20 min (SAPK assay) or 16 hr (apoptosis). The activities of SAPK and apoptosis of BPAEC were determined as described in the legends to Figs. 7 and 2, respectively. a, $p < 0.01$; b, $p < 0.05$ versus 2-ME alone; c, $p < 0.05$ versus control ($n = 3-5$).

showed a clear intracellular location (Fig. 10B), in accordance with previous reports (34). In 2-ME-treated BPAEC, Fas expression was detected in cells with normal morphology (Fig. 9, B and D), but the staining in these cells was not as strong as that in the apoptotic cells.

Inhibition of endothelial cell migration by 2-ME. Vitronectin induced a time- and concentration-dependent BPAEC migration that plateaued at 10 $\mu\text{g/ml}$ and 24 hr (Fig. 11, A and B). Therefore, 10 $\mu\text{g/ml}$ vitronectin and 24-hr incubation were selected for the chemotactic protocol. Treatment of BPAEC with 2-ME resulted in a dose-dependent inhibition of cell migration with an IC_{50} value of 0.71 ± 0.11 μM ($n = 4$) (Fig. 11C). Under the same conditions, estradiol-17 β (≤ 30 μM) had no significant effect on cell migration.

Inhibition of bFGF-induced angiogenesis on CAM model. bFGF induced a significant neovascularization on 10-day embryos after 72 hr incubation as shown by a representative photograph (Fig. 12B) compared with the vehicle (A). bFGF-induced angiogenesis was inhibited by 2-ME (Fig. 12C). Bar graph (Fig. 12D) shows the quantitative result. Angiogenesis, quantified by counting the increase in the

number of vessels, was increased by $38.3 \pm 9.8\%$ over the basal in the presence of bFGF ($p < 0.05$, $n = 14$). In the presence of 2-ME, however, the bFGF-induced angiogenesis on the CAM was inhibited and the level of angiogenesis was below the basal by $5.3 \pm 8.9\%$ ($p < 0.05$ versus bFGF alone, $n = 10$).

Discussion

In this study, we used morphological and biochemical techniques to demonstrate that 2-ME induces apoptosis in BPAEC. Under our experimental conditions, spontaneous BPAEC death was very low (1–2%), which is in accord with a previous observation (22). The effect of 2-ME was concentration dependent, with an EC_{50} value of 0.45 ± 0.09 μM . Significant numbers of apoptotic cells were detected 5 hr after treatment with 2-ME and reached 16.5% of total attached cells at 20 hr. Because the number of detached BPAEC increased during the period of treatment and only attached cells were fixed and counted, the actual number of apoptotic cell was higher than that reported here. We determined the number of floating cells at 20 hr after treatment with 2-ME (2 μM) in some samples. The floating cells were deposited onto microscope slides by using a Cytospin (Shandon Scientific, Cheshire, England, UK), fixed, and stained with acridine orange. They accounted for $5.6 \pm 1.2\%$ of total cells ($n = 6$).

Estradiol-17 β has been reported to have a ≥ 1000 -fold higher binding affinity to the cytosolic estrogen receptor than that of 2-ME (27). Conversely, 2-ME showed much more potent activity for induction of apoptosis in BPAEC compared with estradiol-17 β and its two other metabolites. These data strongly suggest that 2-ME-induced endothelial cell apoptosis is unlikely to be mediated through an estrogen receptor. Our results are also coincident with those of Fotsis *et al.* (3), who reported that the inhibitory effect of 2-ME on endothelial cell proliferation was 160–240-fold more potent than that of estradiol-17 β and its other metabolites that were tested in this study. Moreover, 2-ME was found in our study to have less apoptotic activity on confluent quiescent endothelial cells, indicating that endothelial cells in an active growth stage were more sensitive to 2-ME, which is also in accord with that observed in the cell proliferation study (3). The EC_{50} value of 2-ME for induction of apoptosis was 0.45 μM in the current study, which is close to the IC_{50} value of 0.14 μM for inhibition of endothelial cell proliferation by 2-ME (3). These results suggest an association between the induction of cell apoptosis and inhibition of cell proliferation by 2-ME and further suggest that 2-ME inhibited endothelial cell proliferation through an apoptotic mechanism.

The signaling pathways that lead to apoptosis have been the subject of intense investigation. Increasing evidence has suggested that the induction of programmed cell death involves activation of a signaling system, many elements of which, however, remain unknown (28). Recently, a family of novel kinases that bind to the c-Jun transactivation domain and phosphorylate Ser63 and Ser73 has been identified and termed SAPK or JNK (14, 15). Unlike the mitogen-activated protein kinases, the SAPK/JNK are weakly activated by growth factors but strongly activated by cellular stresses, such as ultraviolet light (17, 29), heat shock (29), and protein synthesis inhibitors (15). It has been demonstrated that over-

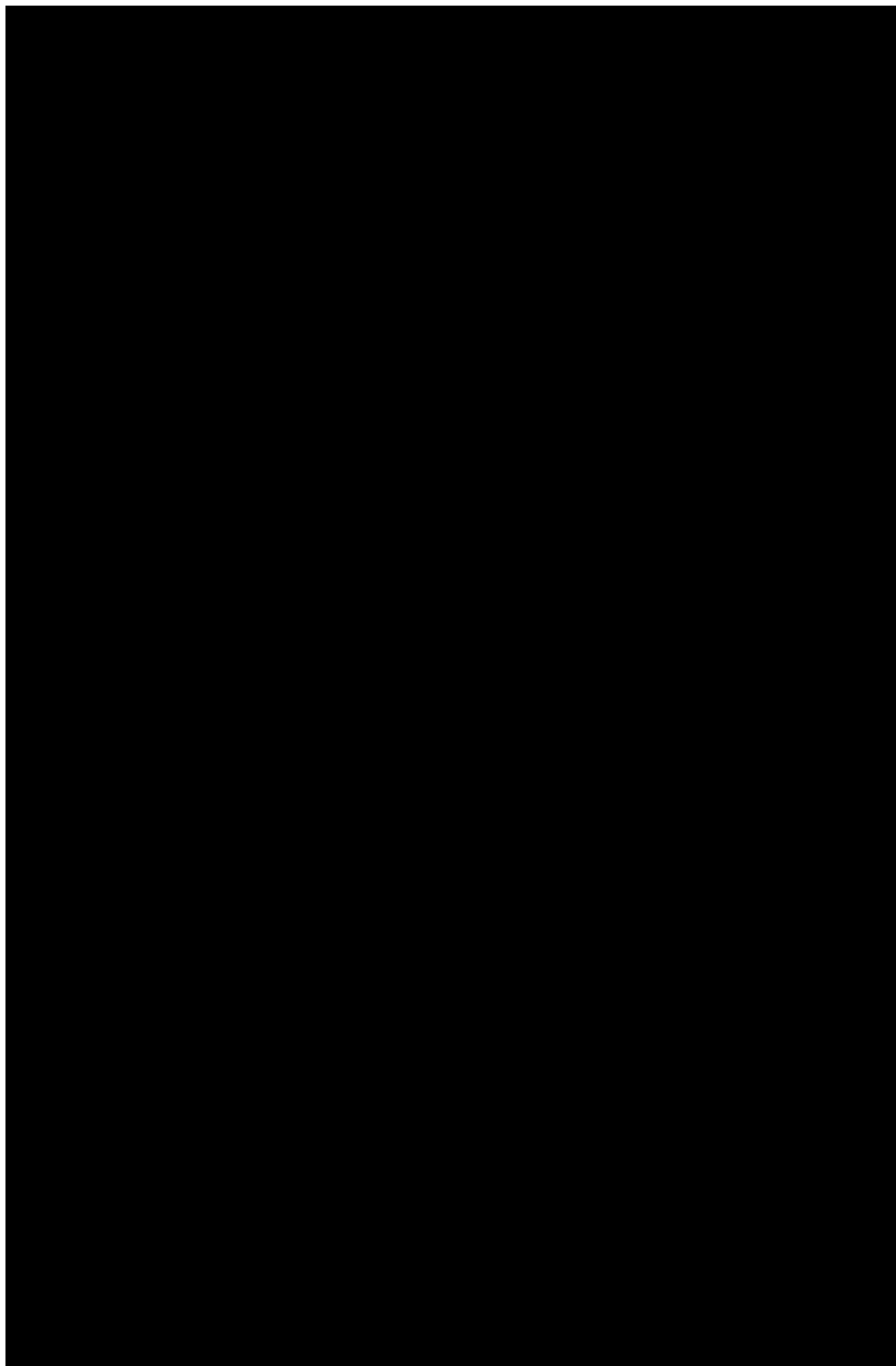


Fig. 9. Immunocytochemical detection of Fas protein in vehicle- (A and C) and 2-ME- (B and D) treated BPAEC for 4 hr (A and B) and 20 hr (C and D) (original magnification, 200 \times).

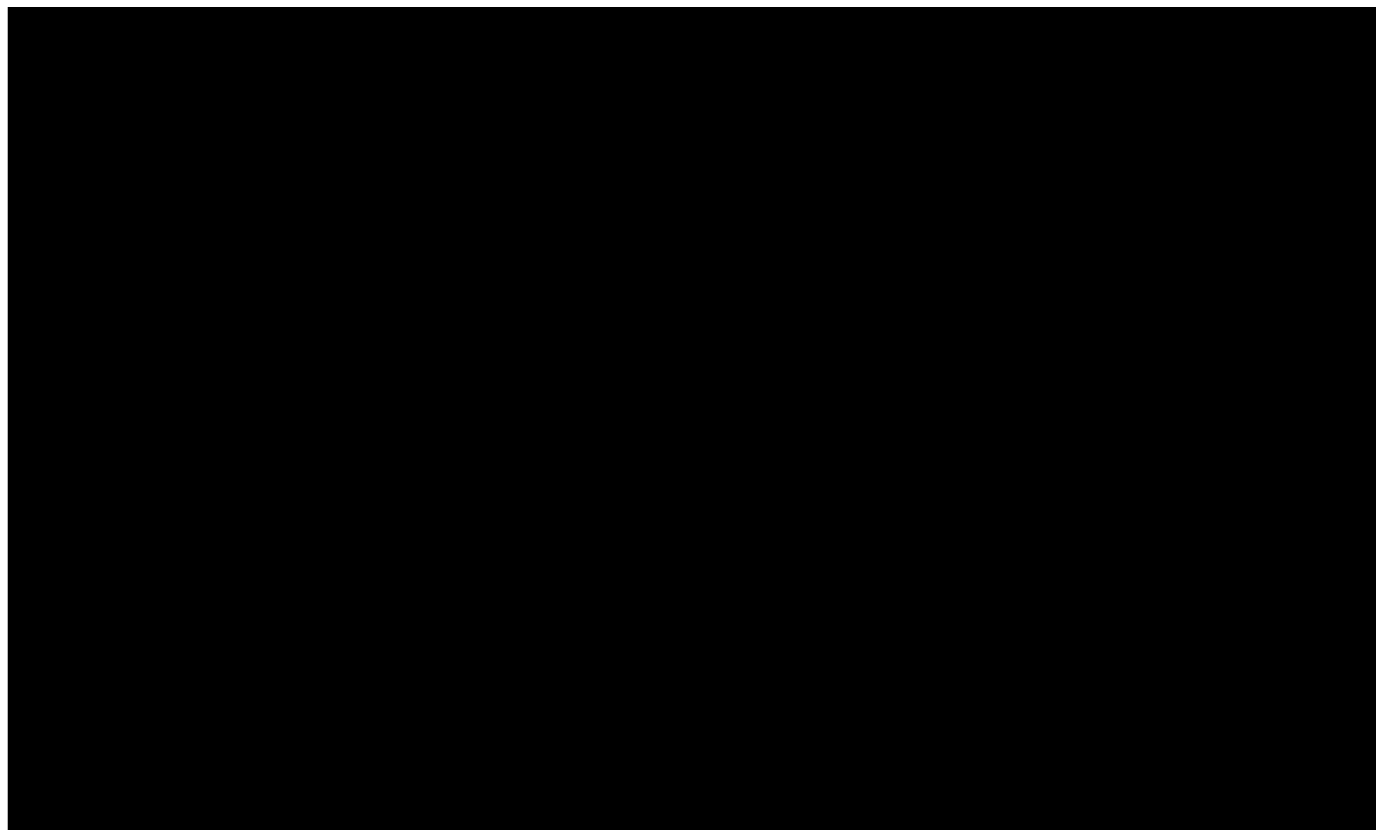


Fig. 10. Immunocytochemical detection of Bcl-2 protein in 2-ME- (A and B) and vehicle- (C) treated BPAEC for 20 hr [original magnification: A and C, 200 \times ; B, 400 \times (high magnification of apoptotic cells expressing Bcl-2)].

expression of SAPK (16) or activation of its upstream kinases (30) induces apoptosis and that interference with activation of SAPK protects against apoptosis (16). It was also reported that experimentally induced stable blockade of SAPK activation in cells with normal thermosensitivity was sufficient to confer resistance to cell death induced by diverse stimuli, including heat and the chemotherapeutic agents. The apparent relationship between the absence of SAPK activation after stress stimuli and resistance to cell death has suggested that SAPK may be a mediator of cell death (29). Moreover, recent studies have demonstrated that the sphingomyelin and SAPK/JNK signaling systems may be coordinated in the induction of apoptosis and that ceramide initiates apoptosis through the SAPK cascade (31, 24).

To investigate the possible involvement of SAPK/JNK in regulating 2-ME-induced apoptosis in BPAEC, we examined the effect of 2-ME on SAPK/JNK. The rapid activation of SAPK/JNK after 2-ME treatment (Fig. 7) is consistent with a role for the kinases in activation of transcription factors and stress-activated signaling cascades after cellular stress such as DNA damage. The range of concentrations of 2-ME for induction of SAPK/JNK coincided with the concentrations of 2-ME to elicit apoptosis. 2-ME ($<0.1 \mu\text{M}$) did not have an effect on SAPK/JNK activity and did not induce BPAEC apoptosis (data not shown). To further determine the involvement of SAPK/JNK activation in 2-ME-induced apoptosis in BPAEC, we studied the effects of bFGF, forskolin, and IGF on the activation of SAPK/JNK and induction of apoptosis by 2-ME. These agents have been reported to inhibit the activation of SAPK/JNK in other cell lines (16). In the presence

of bFGF, forskolin, or IGF, 2-ME-induced activation of SAPK/JNK in BPAEC was inhibited (Fig. 8, *top*). 2-ME-induced apoptosis in BPAEC was also significantly reduced (Fig. 8, *bottom*). In addition, bFGF at a concentration of 2 ng/ml, which did not inhibit the activation of SAPK/JNK, had no effect on 2-ME-induced apoptosis in BPAEC. The results further suggest that activation of SAPK/JNK may contribute to the induction of apoptosis in BPAEC by 2-ME. Activation of SAPK/JNK by 2-ME may trigger early genomic responses that ultimately lead to apoptosis. However, 2-ME-induced apoptosis in BPAEC was not completely prevented by bFGF, forskolin, or IGF at the concentrations that completely inhibited the activation of SAPK/JNK ($p > 0.05$ versus control), suggesting that the SAPK/JNK pathway is not the only system mediating 2-ME-induced apoptosis and that other mechanisms probably exist. The activation of SAPK/JNK in BPAEC by 2-ME was rapid and transient and much earlier and shorter than the time course of apoptosis. The different time courses between the activation of SAPK/JNK and the cell apoptosis were also observed in a variety of other cell types under different kinds of stresses (24, 30–32). The reason for this apparent discrepancy was unclear. One possibility is that activated c-Jun may sequester and regulate unknown proteins required in the apoptotic response (24). Although recent studies have demonstrated an important role of SAPK/JNK in the induction of apoptosis, the mechanism by which c-Jun mediates apoptosis is still not clear. The precise role of the SAPK/JNK pathway in 2-ME-induced apoptosis in BPAEC needs further exploration.

Apoptosis is an active gene-directed process of cellular

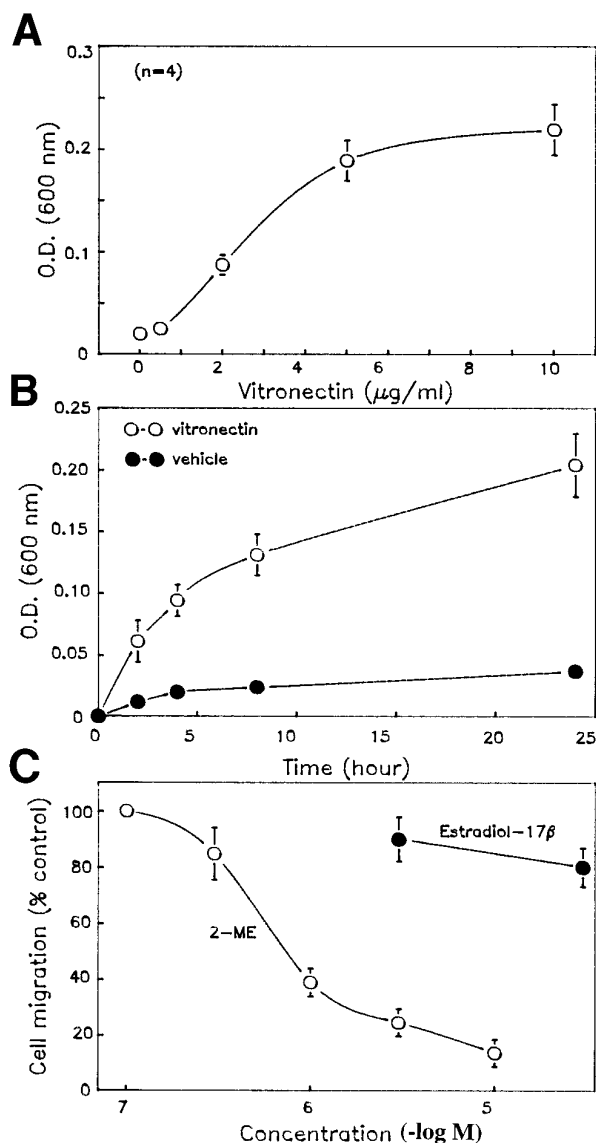


Fig. 11. Inhibition of BPAEC migration by 2-ME. BPAEC migration was monitored in a Transwell cell culture chamber and induced by vitronectin as described in the text. A and B, Concentration- and time-dependency of vitronectin- (10 $\mu\text{g/ml}$) induced BPAEC migration. C, Inhibition of vitronectin- (10 $\mu\text{g/ml}$) induced BPAEC migration by 2-ME. BPAEC treated with vehicle (basal) or 2-ME at the indicated concentrations were placed in the upper chamber and incubated for 20 hr. The migrated cells were fixed, stained, and determined. Values were mean \pm standard error of four independent experiments performed in triplicate.

suicide. The regulation of cell death seems to involve a balance between proapoptotic and antiapoptotic mediator genes. The primary function of Fas is to trigger programmed cell death (18, 34), and the activation of JNK has been found in Fas-associated signaling and cell death (35, 36). In contrast, Bcl-2 functions as an antiapoptotic factor, and overexpression of Bcl-2 markedly reduces cell killing induced by a wide variety of stimuli (19, 37). Two recent studies have demonstrated that estrogen protects the human breast cancer cell line MCF-7 from apoptosis due to up-regulation of Bcl-2 expression (38, 39). We were interested in determining whether Fas and Bcl-2 are involved in 2-ME-induced apoptosis in BPAEC. The data in the current study show that both Fas and Bcl-2 were expressed in BPAEC treated with 2-ME,

and more intense staining was exhibited in apoptotic cells than in the cells with normal morphology. Our results suggest that both Fas and Bcl-2 may be implicated in the modulation of endothelial cell death and survival in the presence of 2-ME. Although the interaction between Fas and Bcl-2 in regulating cell death has attracted great interest, it remains controversial whether Fas-mediated cytotoxicity is inhibited by Bcl-2. The reports dealing with this issue range from complete inhibition (40) to no effect (41). It has been confirmed that expression of Fas and Bcl-2 occurs via distinct pathways (41). In Bcl-2-deficient mice, the expression of Fas mRNA in a variety of tissues is the same as in normal mice, indicating that lack of Bcl-2 is not necessary for Fas expression (42). Our observations in the current study suggest that the increase in Bcl-2 in 2-ME-treated BPAEC may represent a reaction to oppose Fas-triggered programmed cell death. It is possible that 2-ME induced Bcl-2 expression is related to its weak estrogen effect (38, 39); however, the increased expression of Bcl-2 may not be sufficient to maintain survival of all cells. A similar observation was recently reported in ischemia-injured rat cardiomyocytes in which coexpression of Bcl-2 and Fas was found. The enhanced expression of Fas triggered a significant number of apoptotic cardiomyocytes despite the increase in Bcl-2, which was argued to preserve cell survival (43).

Endothelial cell migration has been believed to be an important step in the formation of new blood vessels. After enzymatic degradation of the associated basement membrane, endothelial cells adhere to matrix protein, migrate, proliferate, and then form capillary tubes. We were interested in determining whether 2-ME-induced apoptosis in BPAEC also modifies cell migration and hence further inhibits neovascularization. Vitronectin, a matrix protein known to induce endothelial cell adhesion and migration (20), induced a time- and concentration-dependent BPAEC migration that plateaued at 10 $\mu\text{g/ml}$ and 24 hr, in accordance with a previous report (20). 2-ME inhibited vitronectin-induced BPAEC migration in a concentration-dependent manner at a similar IC_{50} value (0.71 μM) that induced apoptosis. In a pilot receptor binding test using a human placenta solubilized $\alpha\text{v}\beta_3$ preparation, 2-ME did not affect vitronectin binding to the $\alpha\text{v}\beta_3$ receptor (data not shown). Therefore, inhibition of endothelial cell migration by 2-ME was most probably due to a reduction in cell motility of the apoptotic BPAEC.

The antiangiogenic activity of 2-ME was recently demonstrated in a capillary-like structure-forming model (3). D'Amato *et al.* (6) reported that 2-ME (100 μg) inhibited angiogenesis in the CAM assay in the presence of heparin. However, the details of their study was not published and the concentration of 2-ME was unusually high. Because the apoptotic effect of 2-ME and its inhibition of cell migration were observed under heparin-free conditions, we further studied the effect of 2-ME on neovascularization in the CAM model. The result of our study demonstrated that 2-ME, at concentrations similar to those inducing apoptosis and blocking cell migration, inhibited neovascularization. Moreover, heparin was not required for the antiangiogenic activity of 2-ME, indicating a different mechanism of action from that of corticosteroids, for which heparin or heparin fragments were required for the antiangiogenic activity (26).

The serum concentration of 2-ME during pregnancy has been reported to be 30 nM (2). The effective concentrations of

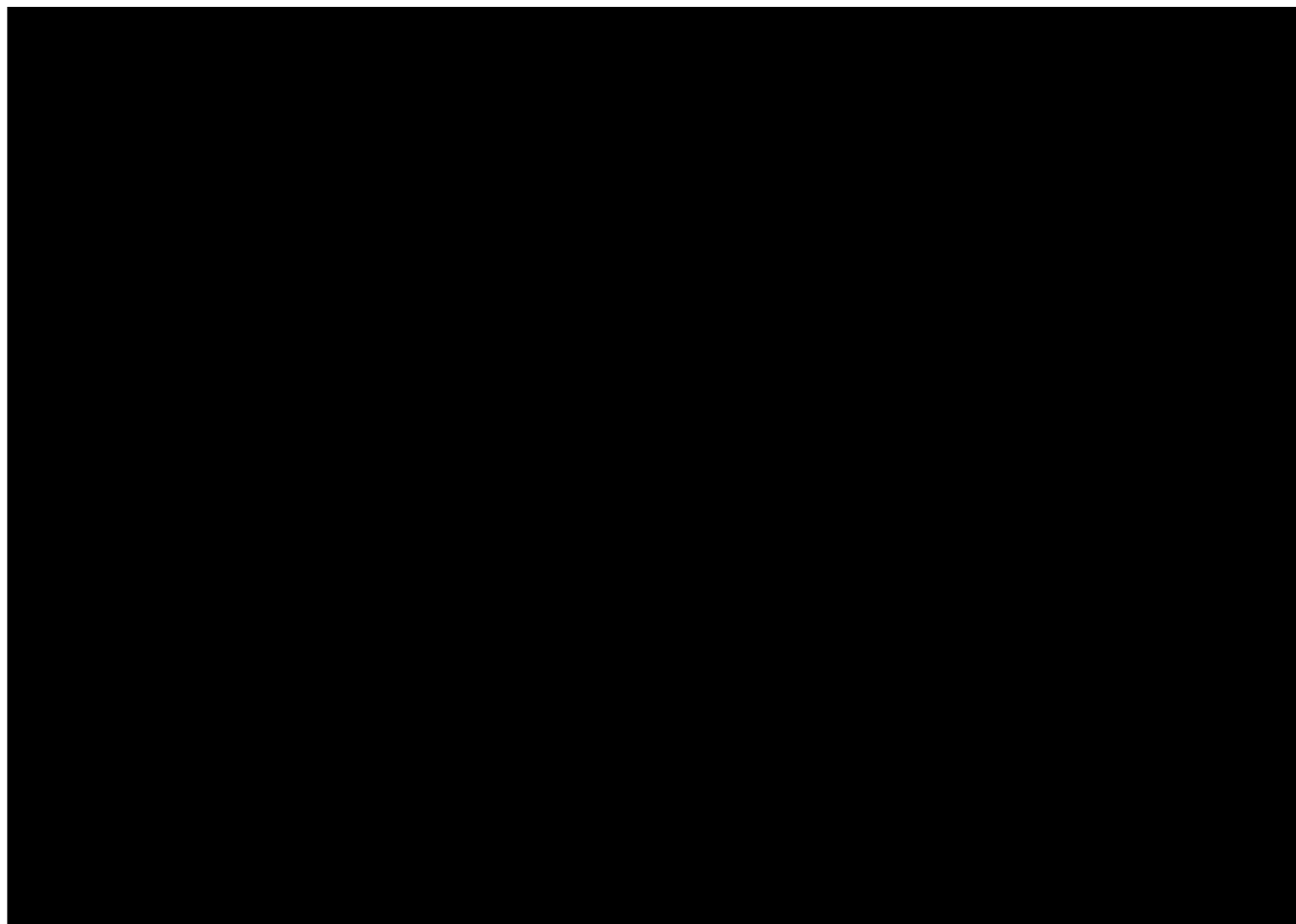


Fig. 12. Inhibition of bFGF-induced angiogenesis in CAM by 2-ME. CAM tissue from 10-day-old chick embryos was stimulated by filter disks saturated with 1 μ g/ml bFGF or PBS buffer (basal) as described in the text. 2-ME (2 μ M) or vehicle was added to the bFGF-treated disks. After 72-hr incubation at 37°, CAMs were fixed, and angiogenesis was quantified by counting the number of vessels under the filter using NIH Image software. A, Vehicle. B, bFGF. C, bFGF with 2-ME. D, Quantitative results of inhibition by 2-ME of bFGF-induced angiogenesis on CAM model. Values are mean \pm standard error of 10–14 CAMs/condition. *, $p < 0.05$ versus basal.

2-ME for inducing endothelial cell apoptosis in this study seemed to be 200–300 nM, which is much higher than the concentration in human serum. Therefore, it is questionable whether the effective antiangiogenic concentrations of 2-ME are of biological significance *in vivo*. However, 2-ME is a lipophilic compound that may accumulate in cells and cell membranes. It has been reported that the concentrations of lipophilic drugs such as calcium blockers (44) and propranolol (45) in membranes were much higher than those in plasma. The difference in concentration between lipid and aqueous phase was 2 orders of magnitude for the former and 30–40-fold for the latter. Whether 2-ME can reach an effective antiangiogenic level inside cells remains to be determined.

In summary, 2-ME causes endothelial cell apoptosis, possibly via activation of the SAPK/JNK signaling pathway and up-regulation of FAS expression in BPAEC. 2-ME inhibits angiogenesis in the CAM model, and this inhibition may occur via its apoptotic activity, therefore inhibiting endothelial proliferation and disturbing endothelial cell migration. The antiangiogenic activity of 2-ME could provide some therapeutic indications for this compound.

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References

1. Gelbke, H. P., and R. Knuppen. The excretion of five different 2-hydroxyoestrogen monomethyl ethers in human pregnancy urine. *J. Steroid Biochem.* **7**:457–463 (1976).
2. Berg, D., R. Sonsalla, and E. Kuss. Concentrations of 2-methoxyoestrogens in human serum measured by a heterologous immunoassay with an 125 I-labelled ligand. *Acta Endocrinol.* **103**:282–288 (1983).
3. Fotsis, T., Y. Zhang, M. S. Pepper, H. Adlercreutz, R. Montesano, P. P. Nawroth, and L. Schwelger. The endogenous oestrogen metabolite 2-methoxyestradiol inhibits angiogenesis and suppresses tumour growth. *Nature (Lond.)* **368**:237–239 (1994).
4. Wheeler, W. J., T. C. Hsu, A. Tousson, and B. R. Brinkley. Mitotic inhibition and chromosome displacement induced by estradiol in Chinese hamster cells. *Cytoskeleton* **7**:235–247 (1987).
5. Seegers, J. C., M. L. Aveling, C. H. van Aswegen, M. Cross, F. Koch, and W. S. Joubert. The cytotoxic effects of estradiol-17 β , catecholestradiols and methoxyestradiols on dividing MCF-7 and Hella cells. *J. Steroid Biochem.* **32**:797–809 (1989).
6. D'Amato, R. J., C. M. Lin, E. Flynn, J. Folkman, and E. Hamel. 2-Methoxyestradiol, an endogenous mammalian metabolite, inhibits tubulin polymerization by interacting at the colchicine site. *Proc. Natl. Acad. Sci. USA* **91**:3964–3968 (1994).
7. Folkman, J., and Y. Shing. Angiogenesis. *J. Biol. Chem.* **267**:10931–10934 (1992).
8. Fan, T.-P.D., R. Jagger, and R. Bicknell. Controlling the vasculature:

- angiogenesis, anti-angiogenesis and vascular targeting of gene therapy. *Trends Pharmacol. Sci.* **16**:57–66 (1995).
9. Bischoff, J. Approaches to studying cell adhesion molecules in angiogenesis. *Trends Cell Biol.* **5**:69–73 (1995).
 10. Brooks, P. C., A. M. P. Montgomery, M. Rosenfeld, R. A. Reisfeld, T. Hu, G. Kiler, and D. A. Cheresh. Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* **79**:1157–1164 (1994).
 11. Stromblad, S., J. C. Becker, M. Yebra, P. C. Brooks, and D. A. Cheresh. Suppression of p53 activity and p21^{WAF1/CIP1} expression by vascular cell integrin $\alpha_v\beta_3$ during angiogenesis. *J. Clin. Invest.* **98**:426–433 (1996).
 12. Gerchenson, L. E., and R. J. Rotello. Apoptosis: a different type of cell death. *FASEB J.* **6**:2450–2455 (1992).
 13. Thompson, C. B. Apoptosis is the pathogenesis and treatment of disease. *Science (Washington D. C.)* **267**:1456–1462 (1995).
 14. Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.* **7**:2135–2148 (1993).
 15. Kyriakis, J. M., P. Banerjee, E. Nikolakaki, T. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch, and J. R. Woodgett. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature (Lond.)* **369**:156–160 (1994).
 16. Xia, Z., M. Dickens, J. Raingeaud, R. J. Davis, and M. E. Greenberg. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science (Washington D. C.)* **270**:1326–1331 (1995).
 17. Derijard, B., M. Hibi, I. H. Wu, T. Barrett, B. Su, T. Deng, M. Karin, and R. J. Davis. JNK-1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76**:1025–1037 (1994).
 18. Nagata, S., and P. Golstein. The Fas death factor. *Science (Washington D. C.)* **267**:1449–1456 (1995).
 19. Reed, J. C., T. Miyashita, S. Takayama, H. G. Wang, T. Sato, S. Krajewski, C. Aime-Sempe, S. Bodrug, S. Kitada, and M. Hanada. Bcl-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. *J. Cell. Biochem.* **60**:23–32 (1996).
 20. Leavesley, D. I., M. A. Schwartz, M. Rosenfeld, and D. A. Cheresh. Integrin β_1 - and β_3 -mediated endothelial cell migration is triggered through distinct signaling mechanisms. *J. Cell Biol.* **121**:163–170 (1993).
 21. Yue, T. L., P. J. McKenna, J. L. Gu, H. Y. Cheng, R. E. Ruffolo, and G. Z. Feuerstein. Carvedilol, a new vasodilating β adrenoceptor blocker antihypertensive drug, protects endothelial cells from damage initiated by xanthine-xanthine oxidase and neutrophils. *Cardiovasc. Res.* **28**:400–406 (1994).
 22. Polunovsky, V. A., C. H. Wendt, D. H. Ingbar, M. S. Peterson, and P. B. Bitterman. Induction of endothelial cell apoptosis by TNF α : modulation by inhibitors of protein synthesis. *Exp. Cell Res.* **214**:584–594 (1994).
 23. Varani, J., M. K. Dame, C. G. Taylor, V. Sarma, R. Merino, R. G. Kunkel, G. Nunez, and V. M. Dixit. Age-dependent injury in human umbilical vein endothelial cells: relationship to apoptosis and correlation with a lack of A20 expression. *Lab. Invest.* **73**:851–858 (1995).
 24. Verheij, M., R. Bose, X. H. Lin, B. Yao, W. D. Jarvis, S. Grant, M. J. Birrer, E. Szabo, L. I. Zon, J. M. Kyriakis, A. Haimovitz-Friedman, Z. Fuks, and R. N. Kolesnick. Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature (Lond.)* **380**:75–79 (1996).
 25. Yue, T. L., X. K. Wang, C. P. Sung, B. Olson, P. J. McKenna, J. L. Gu, and G. Z. Feuerstein. Interleukin-8: a mitogen and chemoattractant for vascular smooth muscle cells. *Circ. Res.* **75**:1–7 (1994).
 26. Crum, R., S. Szabo, and J. Folkman. A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. *Science (Washington D. C.)* **230**:1375–1378 (1985).
 27. Merriam, G. R., N. J. MacLusky, M. K. Picard, and F. Naftolin. Comparative properties of the catechol, estrogens: I. Methylation by catechol-O-methyltransferase and binding to cytosol, estrogen receptors. *Steroids* **36**:1–11 (1980).
 28. McConkey, D. J., and S. Orrenius. Signal transduction pathways in apoptosis. *Stem Cells* **14**:619–631 (1996).
 29. Zanke, B. W., K. Boudreau, E. Rubie, E. Winnett, L. A. Tibbles, L. Zon, J. Kyriakis, F. F. Liu, and J. R. Woodgett. The stress-activated protein kinase pathway mediates cell-death following injury-induced by cisplatin, UV irradiation or heat. *Curr. Biol.* **6**:606–613 (1996).
 30. Johnson, N. L., A. M. Gardner, K. M. Diener, C. A. Lange-Carter, J. Gleavy, M. B. Jarpe, A. Minden, M. Karin, L. I. Zon, and G. L. Johnson. Signal transduction pathways regulated by mitogen-activated/extracellular response kinase kinase induce cell death. *J. Biol. Chem.* **271**:3229–3237 (1996).
 31. Westwick, J. K., A. E. Bielawska, G. Dbaibo, Y. A. Hannun, and D. A. Brenner. Ceramide activates the stress-activated protein kinases. *J. Biol. Chem.* **270**:22689–22692 (1995).
 32. Sawai, H., T. Okazaki, H. Yamamoto, H. Okano, Y. Takeda, M. Tashima, H. Sawada, M. Okuma, H. Ishikura, H. Umehara, and N. Domae. Requirement of AP-1 for ceramide-induced apoptosis in human leukemia HL-60 cells. *J. Biol. Chem.* **270**:27326–27331 (1995).
 33. Latinis, K. M., and G. A. Koretzky. Fas ligation induces apoptosis and Jun kinase activation independently of CD45 and Lck in human T cells. *Blood* **87**:871–875 (1996).
 34. Peter, M. E., F. C. Kischkel, S. Hellbardt, A. M. Chinnaiyan, P. H. Krammer, and V. M. Dixit. CD95 (APO-1/Fas)-associating signalling proteins. *Cell Death. Diff.* **3**:161–170 (1996).
 35. Wilson, D. J., K. A. Fortner, D. H. Lynch, R. R. Mattingly, I. G. Macara, J. A. Posada, and R. C. Budd. JNK, but not MAPK, activation is associated with Fas-mediated apoptosis in human T cells. *Eur. J. Immunol.* **26**:989–994 (1996).
 36. Tian, Q., J. C. Taupin, S. Elledge, M. Roberson, and P. Anderson. Fas-activated serine/threonine kinase (FAST) phosphorylates TIA-1 during Fas-mediated apoptosis. *J. Exp. Med.* **182**:865–874 (1995).
 37. Reed, J. C. Bcl-2 and the regulation of programmed cell death. *J. Cell. Biol.* **124**:1–5 (1994).
 38. Wang, T. T. Y., and J. M. Phang. Effects of estrogen on apoptotic pathways in human breast cancer cell. *Cancer Res.* **55**:2487–2489 (1995).
 39. Teixeira, C., J. C. Reed, and M. A. C. Pratt. Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. *Cancer Res.* **55**:3902–3907 (1995).
 40. Jaattela, M., C. M. Benedic, M. Tewari, J. A. Shayman, and V. M. Dixit. Bcl-x and Bcl-2 inhibit TNF and Fas-induced apoptosis and activation of phospholipase A2 in breast carcinoma cells. *Oncogene* **10**:2297–2305 (1995).
 41. Strasser, A., A. W. Harris, D. C. Huang, P. H. Krammer, and S. Cory. Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis. *EMBO J.* **14**:6136–6147 (1995).
 42. Suzuki, A., A. Matsuzawa, and T. Iguchi. Down regulation of Bcl-2 is the first step on Fas-mediated apoptosis of male reproductive tract. *Oncogene* **13**:31–37 (1996).
 43. Kajstura, J., W. Cheng, K. Reiss, W. A. Clark, E. H. Sonneck, S. Krajewski, J. C. Reed, G. Olivetti, and P. Anversa. Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. *Lab. Invest.* **74**:86–107 (1996).
 44. Herbette, L. G., Y. M. H. Van Erve, and D. G. Rhodes. Interaction of 1,4-dihydropyridine calcium channel antagonists with biological membranes: lipid bilayer partitioning could occur before drug binding to receptors. *J. Mol. Cell. Cardiol.* **21**:187–201 (1989).
 45. Pruett, J. K., T. Walle, and U. Walle. Propranolol effects on membrane repolarization time in isolated canine Purkinje fibers: threshold tissue content and the influence of exposure time. *J. Pharmacol. Exp. Ther.* **215**:539–543 (1980).

Send reprint requests to: Tian-Li Yue, Ph.D., Department of Cardiovascular Pharmacology, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, UW2510, King of Prussia, PA 19406-0939. E-mail: tian-li.yue@sbphrd.com