Effects of 2-Methoxyestradiol on Human Microvascular Endothelial Cells (HMEC-1)

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ABSTRACT

The formation of new blood vessels (angiogenesis) is critical for the growth of many tumors. Altered angiogenesis is involved in various diseases such as diabetic retinopathy, arthritis, and psoriasis. Potential benefits of developing an avenue for the therapeutic control of angiogenesis are, therefore, enormous. A search for angiogenesis inhibitors has been the focus of many investigations. One such inhibitor, which caught the attention of many researchers, is 2-methoxyestradiol (2-ME). 2-ME is an endogenous metabolite of estradiol-17β. The current study was undertaken to characterize the angiostatic effects of 2-ME in human microvascular endothelial cells. Transforming Growth Factor β (TGF-β) has been shown to play a vital role in regulating endothelial cell growth and differentiation, as well as apoptosis. We, therefore, investigated whether TGF-β is modulated by 2-ME using the human microvascular endothelial cell line, HMEC-1. It was found that 2-ME induced increase in TGF-β1 levels in HMEC-1, suggesting that 2-ME may mediate some of its effects in endothelial cells via regulating TGF-β1 production. When exposed to 2-ME (1000 nM), HMEC-1 showed morphological features characteristic of apoptosis: cell shrinkage, cytoplasmic and nuclear condensation, and exposure of phosphatidylserine on cell surface. 2-ME-induced apoptosis in HMEC-1 was a time and concentration-dependent process. Under the same experimental conditions, estradiol-17β, the parent compound, did not have an apoptotic effect on HMEC-1. 2-ME transiently activated SAPK/JNK (stress-activated protein kinase/c-Jun amino-terminal protein kinase). Also, 2-ME induced activation of p38 signaling pathway, which appears to be a survival pathway since inhibition of p38 resulted in cell death in HMEC-1. 2-ME, however, did not suppress or activate the ERK1/2 MAP Kinase, which suggest that modulation of ERK may not be involved in 2-ME mediated apoptosis. In addition, 2-ME up-regulated the expression of cleaved caspase-3, the executioner of apoptosis, confirming the morphological data. Interestingly, the p38 inhibitor (SB203580) also increased caspase-3 activity, supporting the notion that p38 is a survival pathway in HMEC-1. These findings suggest that 2-ME mediated apoptosis in human microvascular endothelial cells involve SAPK/JNK pathway and caspase-3 while the activation of p38 kinase pathway may represent a survival signal to counteract the 2-ME induced apoptotic events.

RÉSUMÉ

La formation de nouveaux vaisseaux sanguins (angiogenèse) revêt une importance critique pour la croissance de nombreux tumeurs. L'altération de l'angiogenèse entre en jeu dans diverses maladies comme la rétinopathie diabétique, l'arthrite et le psoriasis. Les avantages potentiels d'un contrôle thérapeutique de l'angiogenèse sont par conséquent considérables. La recherche des inhibiteurs de l'angiogenèse a été au coeur de nombreuses études. Un des inhibiteurs, qui a retenu l'attention de nombreux chercheurs, est le 2-méthoxyestradiol (2-ME). Le 2-ME est un métabolite endogène de l'estradiol-17β. La présente étude a été entreprise pour caractériser les effets antiangiogéniques du 2-ME sur les cellules endothéliales microvasculaires de l'être humain. L'angiogenèse est un processus complexe qui fait entrer en jeu diverses hormones, des cytokines et des facteurs de croissance. Le facteur de croissance transformant β (TGF-β) joue un rôle crucial dans la régulation de la croissance et de la différenciation des cellules endothéliales, et de l'apoptose. Nous avons donc cherché à déterminer si le TGF-β est modulé par le 2-ME au moyen de la lignée de cellules endothéliales microvasculaires de l'être humain, HMEC-1. Exposée au 2-ME (1000 nM), la HMEC-1 a affiché des caractères morphologiques caractéristiques de l'apoptose : rétrécissement des cellules, condensation cytoplasmique et nucléaire, et exposition de la phosphatidylsérine à la surface des cellules. L'apoptose provoquée par le 2-ME dans la HMEC-1 est un processus qui dépend du temps et des concentrations. Dans les mêmes conditions expérimentales, l'estradiol-17β, le composé d'origine, n'avait pas d'effet apoptosique sur la HMEC-1. Le 2-ME activait provisoirement la SAPK/JNK (protéine-kinase activée par le stress/protéine-kinase N-terminale c-Jun). Par ailleurs, le 2-ME provoquait l'activation de la voie de signalisation p38, qui semble être une voie de survie étant donné que l'inhibition de p38 entraînait la mort des cellules dans la HMEC-1. Cependant, le 2-ME ne supprimait ni n'activait la kinase ERK1/2 MAP, ce qui incite à croire que la modulation de l'ERK n'entre peut-être pas en jeu dans l'apoptose dont la médiation est assurée par le 2-ME. En outre, le 2-ME a entraîné la régulation positive de l'expression de la caspase-3 segmentée, le responsable de l'apoptose, ce qui confirme les données morphologiques. Il est intéressant de constater que l'inhibiteur p38 (SB203580) entraîne également une activité accrue de la caspase-3, ce qui semble confirmer le fait que p38 est une voie de survie dans la HMEC-1. Le 2-ME provoquait également la production du TGF-β1. Ces résultats incitent à croire que l'apoptose à médiation 2-ME dans les cellules endothéliales microvasculaires de l'être humain fait intervenir la voie SAPK/JNK et la caspase-3. Il se peut que les cellules activent provisoirement la kinase p38 pour qu'elle émette des signaux de survie afin d'équilibrer l'apoptose provoquée par le 2-ME. Enfin, le 2-ME a entraîné une hausse des concentrations du TGF-β1 dans la HMEC-1, ce qui incite à croire que le 2-ME peut assurer la médiation de certains de ses effets sur les cellules endothéliales en régulant la production du TGF-β1.

LIST OF ABBREVIATIONS

2-ME	2-methoxyestradiol
ATF2	activating transcription factor-2
BPAEC	bovine pulmonary aortic endothelial cells
BSA	bovine serum albumin
COMT	catechol-O-methyltransferase
CRE	cyclic AMP response element
DAPI	4,6-diamidine-2-phenylindole dihydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
E2	estradio-17β
ECL	enhanced chemiluminescent system
EGF	epidermal growth factor
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal regulated kinase
EtOH	ethanol
FBS	fetal bovine serum
HMEC-1	human microvascular endothelial cell line, CDC/EU, HMEC-1
JNK	c-Jun N-terminal kinase
MAPK	mitogen activated protein kinase
nM	nano molar
PBS	phosphate buffer saline
PARP	poly(ADP-ribose) polymerase

PI	protease inhibitor
pM	pico molar
PS	phosphatidylserine
SAPK	stress activated protein kinase
SDS	sodium dodecyl sulfate
SEK1	SAPK/ERK kinase 1
SHBG	sex hormone binding globulin
TBS	tris buffer saline
TBS/T	tris buffer saline/ tween
TCF	ternary complex factor
TGF-β	transforming growth factor-beta
TMLC	transfected mink lung cells
TREs	TPA response elements
μCi	micro currie

INTRODUCTION

2-Methoxyestradiol, once considered an inactive by product of estradiol-17ß, has recently emerged as a promising agent in cancer treatment. 2-ME targets both the tumor and the endothelial cells by inducing apoptosis. It inhibits rapidly proliferating cells and suppresses blood vessel formation at several stages in the angiogenic cascade. Several potential targets and pathways of action have been suggested for 2-ME. However, the meachanism of angiostatic activity of 2-ME is not fully understood.

Biosynthesis and Metabolism of 2-Methoxyestradiol:

Estrogens undergo a series of hydroxylation, methylation, and conjugation reactions after their synthesis. Although these modifications were initially thought to be part of a metabolic process that enhanced their subsequent elimination from the body, it is now evident that at least some of the products of these reactions may possess unique activities mediated independently of the estrogen receptor (MacCarthy-Morogh et al, 2000). 2-ME is an estradiol-17ß metabolite. Endogenous 2-ME is synthesized in vivo by the hydroxylation at the 2-position of estradiol and subsequent O-methylation of

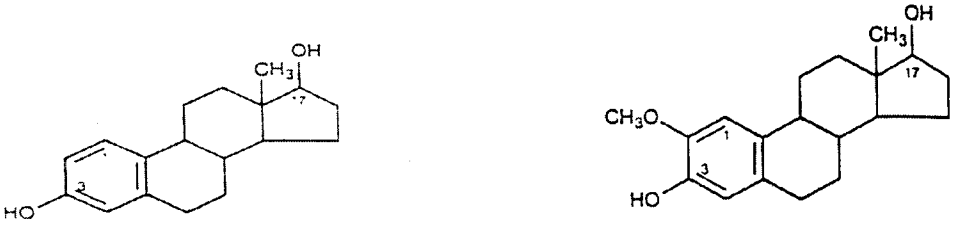

Figure 1: Structural representation of estradiol-17ß and 2-methoxyestradiol.

that hydroxyl group by catechol-O-methyltransferase (COMT) (Martucci, 1983, Ball et al, 1983). Despite being an estradiol metabolite, the relative affinity of 2-ME for estrogen receptors α and β is less than 0.5% and .008% that of estradiol (Pribluda et al, 2000). 2-ME is present in human blood and urine (Fotsis et al, 1994). Human serum levels of 2-ME range from 30 pM (adult men) to 30 nM (pregnant women) (Berg et al, 1992). 2-ME in circulation preferentially binds to the Sex Hormone Binding Globulin (SHBG), for which it has higher affinity than testosterone or estradiol-17ß (Dunn et al, 1980).

Effects of 2-ME on Cell Proliferation:

2-ME inhibits the growth of many cell types, including human breast cancer lines, *in vitro* (Seegers et al, 1989), and oral administration of 2-ME inhibits the growth of transplanted Meth-A sarcoma and B16 melanoma in C3H mice (Fotsis et al, 1994) and human MDA-MB-453 breast carcinoma cells in immunodeficient mice (Klauber et al, 1997). 2-ME, at low micromolar concentration, induces a reversible mitotic arrest (Attala et al, 1996) and apoptosis in retinoic acid-differentiated neuroblastoma SH-SY5Y cells (Nakagawa-Yagi et al, 1996) and in human lung and pancreatic cancer cells (Schumacher et al, 1999). 2-ME also inhibits tubulin polymerization by interacting at the colchicine site (D'Amato et al, 1994). Moreover, 2-ME was found to inhibit endothelial cell proliferation and angiogenesis and it has been suggested that 2-ME may be a novel antiangiogenic therapeutic agent (Yue et al, 1997).

Angiogenesis:

Angiogenesis, the generation of new capillaries from preexisting vessels, is a critical process during development, wound healing, and various diseases such as cancer, adult blindness, and inflammatory disorders (Folkman, 1992, Fan et al, 1995). Angiogenesis involves several steps, commencing in enzymatic degradation of basement membrane, followed by vascular endothelial cell migration into the perivascular space, proliferation and alignment to form tubular structures, and, finally, new vessel formation (Folkman, 1992, Bischoff, 1995). Although the mechanisms leading to pathological angiogenesis are still unclear, recent evidence indicates 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 (Brooks et al, 1994; Stomblad et al, 1996).

Apoptosis:

Animal cells can self-destruct via an intrinsic program of cell death. This phenomenon is called apoptosis and is a form of programmed cell death that is characterized by specific morphologic and biochemical properties. Morphologically, apoptosis is characterized by a series of structural changes in dying cells: blebbing of the plasma membrane, condensation of the cytoplasm and nucleus, and cellular fragmentation into membrane apoptotic bodies (Steller, 1995). Biochemically, apoptosis is characterized by the degradation of chromatin, initially into large fragments of 50-300 kilobases and subsequently into smaller fragments that are monomers and multimers of

200 bases (Oberhammer et al., 1993). The execution of apoptosis minimizes the leakage of cellular constituents from dying cells. As a result, there is no inflammatory response during apoptosis. This feature of apoptosis distinguishes it from necrosis. Necrosis results from trauma that causes injured cells to swell and lyse, releasing the cytoplasmic material, stimulating an inflammatory response. Although apoptosis is important for the normal development and health of an animal, its abnormal activation may contribute to a number of diseases, for example, AIDS, neurogenerative disorders, and ischemic injury. On the other hand, impaired apoptosis may be a significant factor in the etiology of such diseases as cancer, autoimmune disorders, and viral infections (Thompson, 1995).

Cell death is as essential as cell proliferation for the regulation of mammalian development. Hormones, cytokines, and growth factors play important roles in modulating such processes by transmitting their signals through specific signaling pathways, thereby affecting the decision of cell survival or death. However, the signaling pathways leading to apoptosis after 2-ME treatment, as well as the mechanism(s) whereby specific growth factors, such as TGF-β, are modulated by 2-ME are poorly understood.

2-ME's Mechanism of Action:

The process by which 2-ME affects cell growth remains unclear. However, a number of studies have implicated various mechanisms of action and cellular targets. 2-ME has been shown to induce changes in the levels and activities of various proteins involved in the progression of cell cycle. These include cofactors of DNA replication and

repair (Klauber et al, 1997, Lottering et al, 1996); cell cycle kinases and regulators, such as $p34^{cdc2}$ and cyclin B (Lottering et al, 1996; Attala et al, 1996; Zoubine et al, 1999); regulators of cell arrest and apoptosis, such as tubulin (D'Amato et al, 1994; Hamel et al, 1996), BCL-2 (Yue et al, 1997), and p53 (Kataoka et al, 1998; Mukhopadhyay and Roth, 1997; Seegers et al, 1997); and transcription factor modulators, such as SAPK/JNK, one of the mitogen activated protein kinases (Yue et al, 1997; Attala et al, 1998).

Tubulin:

2-ME interacts with the colchicine binding site on ß-tubulin and seems to induce a metaphase arrest by functioning as an antimicrotubule agent (D'Amato et al, 1994). However, the precise effects of 2-ME on tubulin have not been resolved entirely. Whereas several studies have demonstrated that 2-ME inhibits tubulin assembly *in vitro*, Attalla et al showed that, similar to paclitaxel, 2-ME promotes tubulin polymerization in intact cells. Although treatment of cells with 2-ME at concentrations that are relevant for biological effects does not cause gross disturbances of microtubule structures in cells, the same is probably true of other antimicrotubule agents, such as colchicine or paclitaxel. The underlying effect of these drugs at biologically relevant concentrations may be on the kinetics of mitotic spindle microtubule dynamics, rather than in the alteration of microtubule polymerization (Jordan and Wilson, 1998). Moreover, in a panel of tumor cell lines, the IC50's for the cell proliferation were one to two orders of magnitude lower than those for inhibition of tubulin polymerization and interaction with the colchicine binding site (apparent *K*i: 22 μM) (D'Amato et al, 1994). There is no clear correlation between the ability of several analogs to inhibit tubulin polymerization and cell

proliferation (Pribluda et al, 2000). Hence it is not clear how 2-ME's interaction with tubulin could account for inhibition of proliferation.

BCL-2 and p53:

The induction of cell death by 2-ME may be mediated via effects on known apoptosis regulators such as BCL-2 or p53 (Mukhopadhyay and Roth, 1997; Attala et al, 1998). BCL-2 suppresses apoptosis. BCL-2 is phosphorylated and thus rendered inactive in K562 leukemic cells treated with 2-ME (Attala et al, 1998). This is likely to be a general response to antimicrotubule agents because BCL-2 is also phosphorylated in cells treated with paclitaxel or colchicine (Jordan and Wilson, 1998). In paclitaxel-treated cells, BCL-2 is phosphorylated (on Ser-70, Ser-87, and Thr-69), which leads to its inactivation (Yamamoto et al, 1999.). Phosphorylated BCL-2 is thought to be unable to bind and inactivate the proapoptotic BAX protein. Relatively high concentrations of 2-ME (5 μM) have also been reported to increase endogenous wild-type p53 protein post-transcriptionally in cancer cells resulting in programmed cell death (Mukhopadhyay and Roth, 1997). Using different lung cancer cell lines or temperature-sensitive p53 lymphoblast cell lines in which p53 was mutated, 2-ME was found to be ineffective at inhibitng proliferation. Antisense p53 oligonucleotides rendered cells refractory to 2-ME. On the other hand, a recent study reports apoptosis induced by 2-ME to be independent of p53 in certain pancreatic cell lines. Therefore, while p53 may play a role in the mechanism of action of 2-ME in certain cell lines, it can also induce cell cycle arrest and apoptosis independent of p53 (Pribluda et al, 2000).

Mitogen Activated Protein Kinase Pathways:

Yue et al have shown that 2-ME mediated apoptosis in bovine pulmonary aortic endothelial cells involve the SAPK/JNK pathway, one of the MAP kinase signaling pathways (1997). In mammalian systems, at least six independent signaling units appear to function; among them, the biochemical properties of three MAPKs, the stress activated protein kinases (SAPKs), the extracellular signal regulated kinases (ERKs), and the p38 MAPKs (p38s), have been characterized in some detail (Ichijo, 1999).

SAPK/JNK Pathway:

Part of the cellular response to toxins, physical stresses and inflammatory cytokines occurs by signalling via the SAPK pathways. SAPK, also known as c-Jun amino terminal kinases (JNK), is a member of the mitogen-activated protein kinase (MAPK) family of proteins. Among the processes modulated by SAPK pathway are apoptosis, transformation, development, immune activation, inflammation, and adaptation to environmental changes (Tibbles and Woodgett, 1999).

SAPK/JNK

SAPK/JNKs are activated by a highly diverse group of extracellular signals. Activation occurs via phosphorylation at Thr183 and Tyr185 by the dual specificity enzyme SEK1/MKK4 and also by yet to be unidentified kinases (Davis, 1994, Sanchez et al., 1994, Yan et al., 1994). In the JNK subgroup, three genes (jnk1, jnk2, jnk3) and ten

different splicing have been described (Ichijo, 1999). JNKs are activated by two distinct MAPKKs, MKK4/SEK1 and MKK7 (Ichijo, 1999).

SEK1/MKK4 and MKK7

SAPK/ERK kinase (SEK1), also called MKK4 and Jun kinase kinase (JNKK), is a protein kinase that activates the MAP kinase homologue SAPK (or JNK) and functions

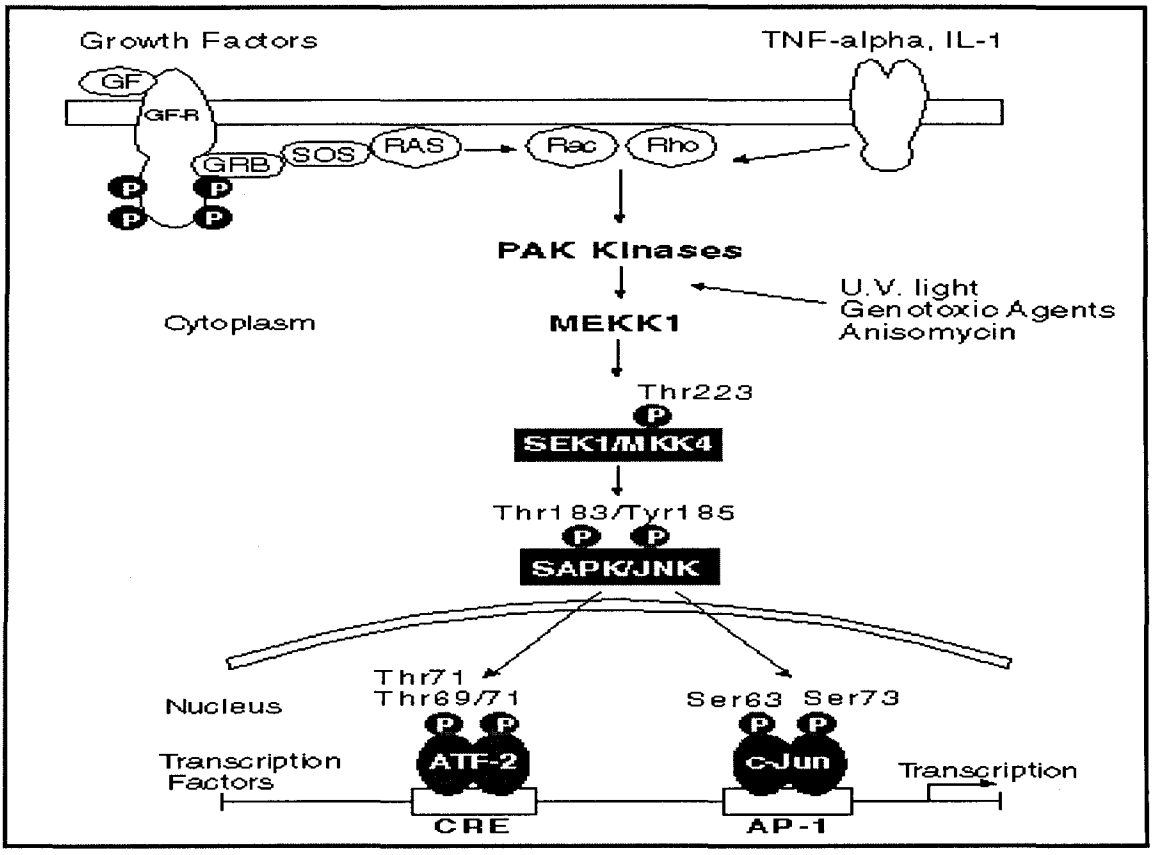

Figure 2: *SAPK/JNK Pathway*

in a stress activated protein kinase cascade. SEK1, as well as MKK7, is regulated by phosphorylation on a serine and a threonine residue. SEK1 and MKK7 are structurally

quite similar (49% identity) and do not appear to display any preference for the different JNK isoforms (Tibbles and Woodgett, 1999).

MEKK1

In contrast to MAPKK's, i.e. MKKs, MAPKKKs are highly divergent in structure and gene number. Eleven different MAPKKKs have been identified as upstream activators of JNK pathway (Widmann et al, 1999). MEEK1, one of the MAPKKKs, was first shown, when overexpressed, to activate the ERKs via phosphorylation of MEK (hence the name MEKK Kinase). However, lower level expression revealed a distinct preference for activation of SEK1 (Yan et al, 1994).

MAPK/ERK Pathway:

MAP Kinase (ERK1/2) Signaling Pathway plays a critical role in the regulation of cell growth and differentiation. ERKs are thought to act in multiple biochemical signals because they are activated by a wide variety of extracellular signals. Upon growth factor stimulation, protein kinase cascades are sequentially activated from Raf, MEK, ERK1/2 and then to Elk-1 and p90RSK. Phosphorylation of Raf at Ser259 and Ser621 is required for Raf-1 activity. Activation of MEK1 and MEK2 by Raf occurs through phosphorylation at Ser217 and Ser221 (Derijard et al., 1994).

ERK1/2:

Mitogen-activated protein kinases were first identified as insulin-induced protein kinases. It was subsequently demonstrated that these proteins were regulated by tyrosine

and threonine phosphorylation via phosphorylation by MEKs. In mammalian cells there are two well characterized and highly related classical MAPKs termed Extracellular Signal Regulated Kinases (ERK1/2). A number of less related protein kinases have been identified including ERK3, ERK5 and ERK6. The ERKs are activated by a variety of mitogenic stimuli as well as differentiation signals. MAPK activation largely requires Ras, which activates Raf. However, protein kinase C has been shown to phosphorylate Raf, leading to MAPK activation (Marais et al, 1998).

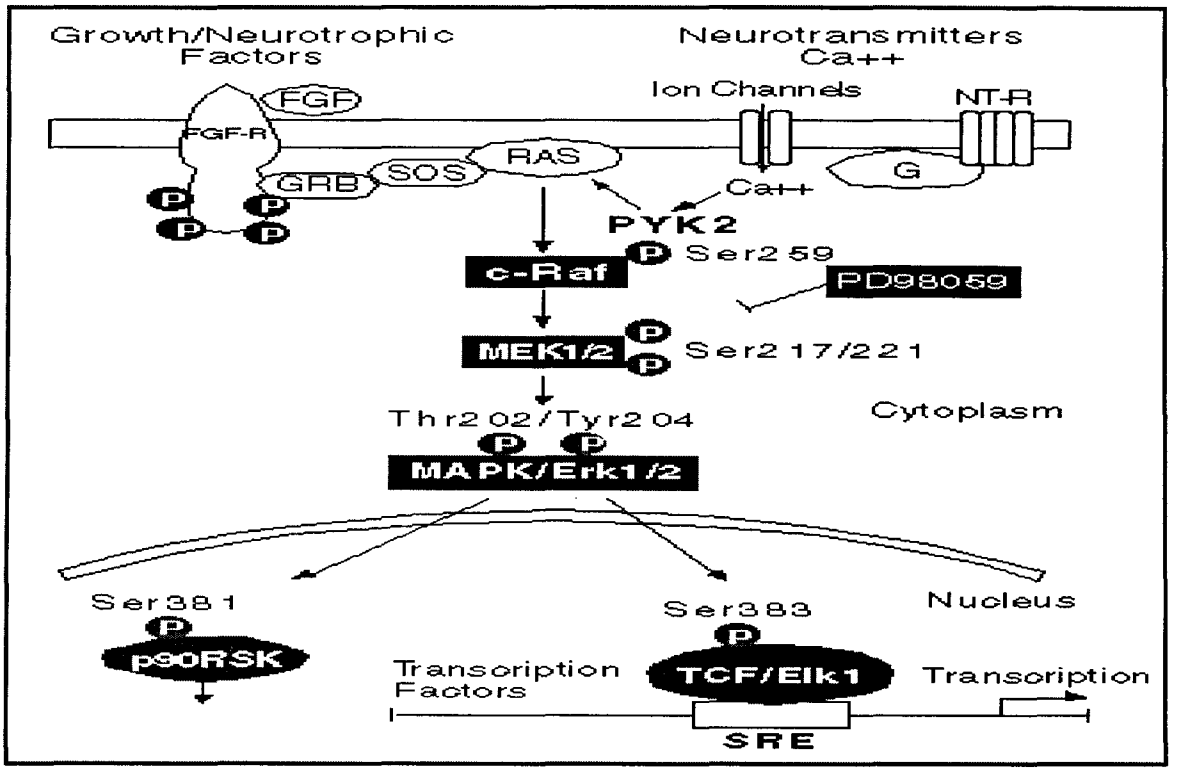

Figure 3: *MAPK/ERK Pathway*

ERKs are inactivated by dephosphorylation by specific protein phosphatases such as MKP1 (CL100) and PAC1. Downstream substrates include Elk1, phospholipase A2 and p90Rsk1 (Zheng and Guan, 1993).

MEK: MAP Kinase Kinase

A critical protein kinase lies upstream of ERK1/2 kinases and stimulates their enzymatic activity. The structure of this protein kinase, denoted MEK1, for MAP kinase or ERK kinase, was elucidated from a complementary DNA sequence and shown to be a protein of 393 amino acids (43,500 daltons) (Crews et al, 1992). MEK is a dual-specificity kinase that phosphorylates the tyrosine and threonine residues on ERKs 1 and 2 required for activation. Two related genes encode MEK1 and MEK2 which differ in their binding to ERKs and, possibly, in their activation profiles. MEKs do not phosphorylate either SAPK or p38 MAPK. MEKs are substrates for several protein kinases including the Rafs (c-, A- and B-), Mos, Tpl-2, and MEKK1. MEKs are phosphorylated by these kinases at two serine residues. MEK1/2 is strongly inhibited by PD98059. ERK1/2 are activated by MEK1/2 through phosphorylation of Thr202 and Tyr204. The phosphorylation of transcription factor Elk-1 is critical for transcriptional activation (Brott et al, 1993).

P38 MAPK Pathway:

The p38 signalling transduction pathway plays an essential role in regulating many cellular processes including inflammation, cell differentiation, cell growth, and death (Ono and Han, 2000).

p38

Although many p38-activating stimuli are pro-apoptotic, the biological outcome of p38 is highly divergent and appears to be largely dependent on cell type or cellular

context. p38 MAP kinases are composed of four genes including p38α, p38β, p38γ, and p38δ (Ichijo, 1999).

MKK3 and MKK6

MKK3 and MKK6 are closely related dual specificity protein kinases that activate p38 MAP kinase by phosphorylation at its activation site, Thr-Gly-Tyr. Phosphorylation of p38 MAP kinase dramatically stimulates its ability to phosphorylate protein substrates such as ATF-2 and Elk-1. MKK3 and MKK6 are activated by different forms of cellular stress and inflammatory cytokines through phosphorylation at Ser189 and Thr193 for

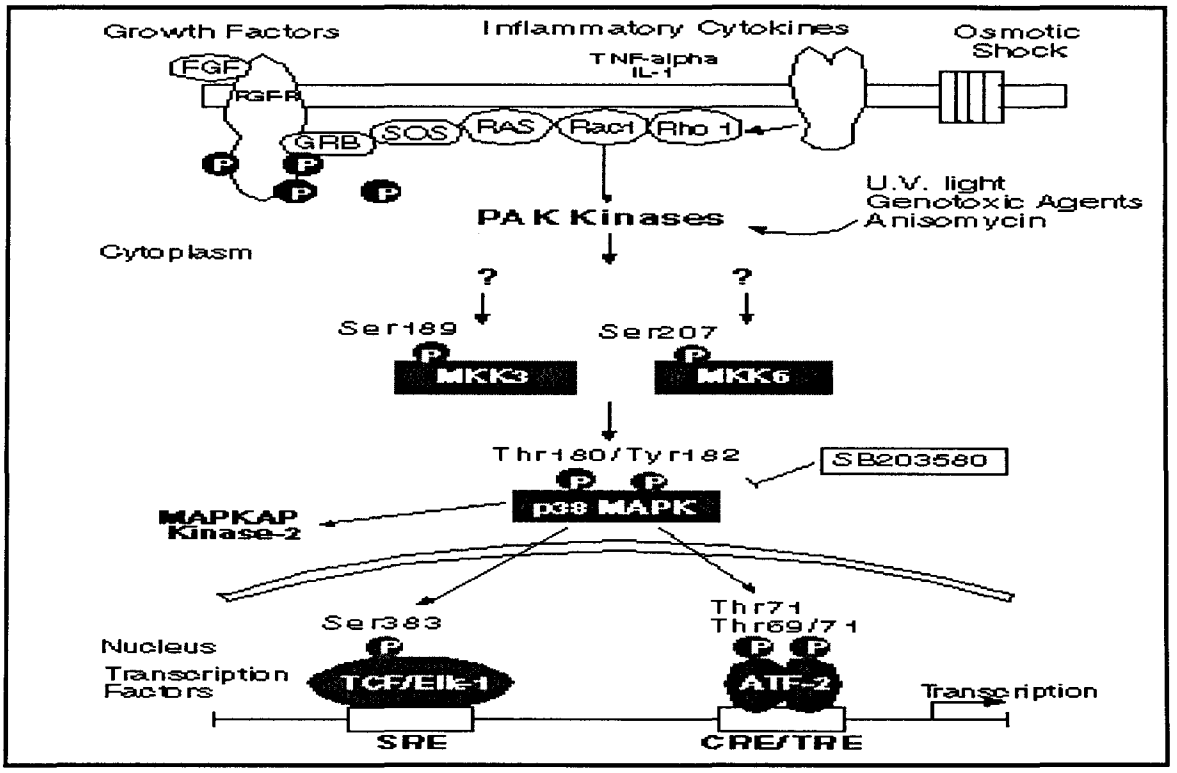

Figure 4: *P38 MAPK Pathway*

MKK3 and Ser207 and Thr211 for MKK6 (Raingeaud et al., 1996, Han et al., 1996).

A number of p38 relatives have been identified which all contain the signature Thr-Gly-Tyr activation domain sequence. These incude: ERK6, SAP kinase-3, and p38beta. SAPK3 and ERK6 appear to be homologous and are regulated by MKK6 phosphorylation. The physiological role of the different p38 isoforms is still unclear but there is some suggestion that they may differentially couple to MKK3 or MKK6 and have distinct substrate specificities. p38 is inhibited by SB203580 but this drug has no effect on ERK6 (Jiang et al., 1996).

Major Targets of the MAPK Pathways:

Elk1

Elk1 (also known as p62 ternary complex factor, TCF) is a transcription factor that mediates growth factor stimulation of the c-fos promoter. Elk1 binds to DNA in part via interaction with Serum Response Factor. While Elk1 is a bona fide ERK substrate, it remains unclear whether Elk1 is regulated by the SAPK pathway since certain residues important for transcriptional activation are poorly phosphorylated by SAPKs compared with ERK2. However, SAPK phosphorylation of Elk1 may mediate transcriptional activation of the fos promoter in response to a variety of stresses (Whitmarsh et al., 1995).

c-Jun

c-Jun transcription factor, a major target of JNK, participates in various biological responses. Its expression is induced in response to both proliferative and stressful stimuli. Via a leucine zipper, c-Jun forms homodimers. It also forms heterodimers with Fos and

other jun-related proteins which, together, comprise the AP-1 transcription factors that bind TPA response elements (TREs). c-Jun mediates transcriptional regulation in response to a variety of stimulants. It is tightly regulated posttranslationally and is phosphorylated in two distinct regions. C-terminal phosphorylation sites lie proximal to the DNA binding domain and prevent DNA binding. These sites are dephosphorylated in response to growth stimulation and likely function to repress the activity of the factor under resting conditions (Pulverer et al, 1991). c-Jun is also phosphorylated at two residues proximal to the major transactivation domain. These residues (Ser 63 and 73) are

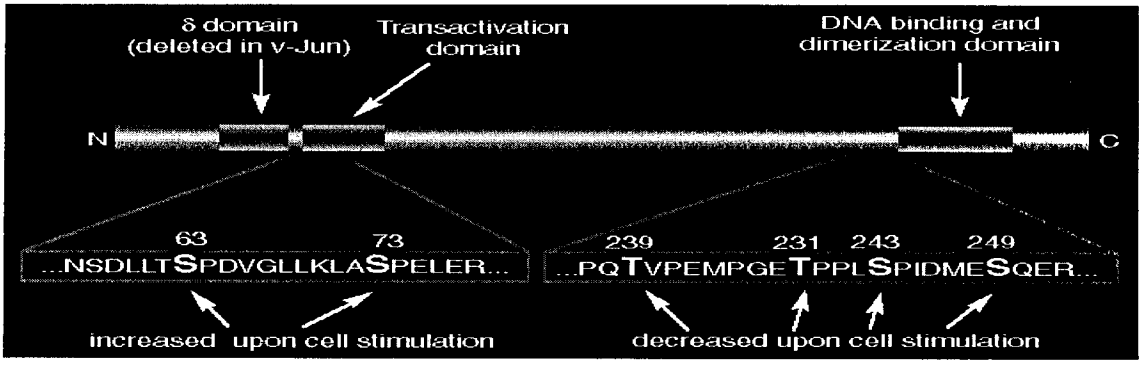

Figure 5: *c-Jun*

required to be phosphorylated for efficient transactivation function. The kinases responsible for this modification in vivo are the SAPK/JNKs. These kinases bind with very high affinity to a region in c-Jun termed the delta domain. This region is deleted in v-Jun. v-Jun is not phosphorylated but is transcriptionally active. Thus, binding of inactive SAPK to Jun may represent a mechanism for inhibiting the function of the transcription factor. Once activated, the SAPK will phosphorylate c-Jun and dissociate. Mutation of the phosphorylation sites prevents dissociation thus resulting in inactive c-Jun. The activation of c-Jun has been implicated in a variety of processes including

embryonic development, cellular proliferation, and transformation, and the initiation of apoptosis in response to various stresses (Black et al., 1994, Huang et al., 2000).

ATF-2

ATF2 contains a docking domain for SAPKs analogous to the delta domain of c-Jun. This region has very limited amino acid identity with c-Jun but acts as an independent interaction site. ATF-2 (also called CRE-BP1) binds to both AP-1 and CRE response elements and is a member of the ATF/CREB family of leucine zipper proteins. ATF-2 is known to interact with a variety of viral oncoproteins and cellular tumor suppressors, and has been shown to be a target of the SAPK/JNK and p38 MAP kinase signaling pathways. Various forms of cellular stress including genotoxic agents, inflammatory cytokines and UV irradiation stimulate the transcriptional activity of ATF-2. Activation of ATF-2 by cellular stress requires phosphorylation of Thr69 and Thr71 (Livingstone et al., 1995).

2-ME and Endothelial cells:

Endothelial cells are not necessarily more sensitive than other cell types to the antiproliferative activity of 2-ME (Pribluda et al, 2000). The first evidence of angiostatic effect of 2-ME came from the study by Fotsis et al on different types of endothelial cells, namely bovine aortic, bovine adrenal cortex microvascular, and human umbilical vein endothelial cells (1994). The half-maximal inhibitory concentration (IC50) of 2-ME on the bovine microvascular endothelial cell was observed to be ~0.15 μM. 2-ME has been shown to effectively disrupt angiogenesis at different stages in the formation of new blood vessels (Pribluda et al, 2000). The inhibitory effects of 2-ME have been observed

in the chick chorioallantonic membrane and the corneal micropocket vascularization assays in mice (Yue et al, 1997; Klauber et al, 1997). In the former, 2-ME at low micromolar concentrations (2μM), similar to those inhibiting proliferation and inducing apoptosis, inhibited angiogenesis induced by bFGF. In the corneal micropocket assay, oral administration of 2-ME (150 mg/kg p.o.) inhibited angiogenesis induced either by bFGF or VEGF, at doses efficacious for antitumor activity.

Transforming Growth Factor β:

Transforming growth factor (TGF) β, a pleiotropic cytokine involved in regulating growth and differentiation, can exert both pro-apoptotic and anti-apoptotic effects depending on the cell type or circumstances (Huang et al, 2000). Although named originally for its ability to induce a transformed phenotype in cultured cells, TGF-β and its signalling pathway are frequently involved in suppressing the growth of human tumors. However, depending on the cell type and the environment, it may stimulate or inhibit growth, regulate developmental fate in an instructive or a selective manner and contribute to both the initiation and the resolution of processes involved in inflammation and tissue repair (Moses and Serra, 1996). TGF-β is known to be a potent mediator of angiogenesis. The microendothelium has been shown to be a major target tissue for endocrine TGF-β (Dickson et al, 1995). Studies of human atherosclerotic plaque have indicated TGF-β1 expression by vascular cells within lesions (Nikol et al, 1992). Similarly, TGF-β1 expression within the vasculature is upregulated in the context of diabetes and hypertension in animal models of vascular disease (Pollman et al, 1999). The study by Schulick et al suggested that alterations in the local abundance of TGF-β1

in the arterial wall promotes vascular cell transdifferentiation, vascular wall remodeling, arterial lesion growth, and lesion regression associated with apoptosis (1998). Furthermore, several studies have indicated that TGF-β1 induces endothelial cell apoptosis in cultured bovine pulmonary aortic endothelial cells (BPAEC) and in human umbilical vein endothelial cells (HUVEC) (Pollman et al, 1999, Tsukada et al,1995).

Rationale:

Angiogenesis is critical in several physiologic and pathologic states. It enables the normal physiologic process of the menstrual cycle, the inflammatory response for host protection, and wound healing. On the other hand, pathologic states such as diabetic retinopathy, rheumatoid arthritis, and psoriasis also involve extensive angiogenesis (Reiser et al, 1998). A search for angiogenesis inhibitors has been the focus of many investigations. 2-ME is one such inhibitor. The study based on bovine pulmonary aortic endothelial cells suggested apoptosis to be an important pathway through which 2-ME inhibits angiogenesis (Yue et al, 1997).

The current study was undertaken to explore the effects of 2-ME in microvascular endothelial cells. To examine the possible effects, we used a human dermal microvascular endothelial cell line (HMEC-1). We analyzed whether 2-ME inhibited the cell growth and proliferation and if apoptosis was involved. In addition, we explored some of the possible MAP Kinase signaling pathways that could be taken by 2-ME in its role as an angiostatic agent in HMEC-1. We also examined whether or not 2-ME may mediate some of its effects in HMEC-1 by regulating the protein levels of TGF-β, a potent mediator of angiogenesis as well as apoptosis.

Materials and Methods

2-ME and estradiol-17β were bought from Stereloids Inc. The primary antibodies, SAPK-p, ATF-2p, c-Jun-p, p38-p, ERK1/2-p and cleaved Caspase-3, as well as the secondary antibody (Anti-Rabbit IgG, HRP linked antibody), were obtained from Cell Signaling Technology. STAT-3 primary antibody was purchased from Sanra Cruz Biotechnology. The p38 pathway inhibitor and ERK-1/2 pathway inhibitor, SB203580 and PD98059 respectively, were obtained from Calbiochem. TGF-β1 was purchased from Austral Biologicals.

Cell Culture

Human microvascular endothelial cell line, CDC/EU.HMEC-I (Ades et al, 1992) was a gift from Drs. F. W. Ades (Centers for Disease Control. Atlanta, GA) and T.J. Lawley (Emery University, Atlanta, GA). HMEC-1 was obtained by immortalizing neonatal foreskin endothelial cells with the SV40 large T antigen. These cells have been shown to display the same morphologic, phenotypic and functional characteristics as the normal human microvascular endothelial cells (Ades et al, 1992). HMEC-1 were cultured in MCDB 131 medium (Gibco BRL) supplemented with 10 % FBS, 2 mM glutamine, 10 ng/mL epidermal growth factor (Collaborative Biomedical Products), 1 μg/mL hydrocortisone (Steraloid Inc.), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin (Gibco BRL). During serum starvation and short 2-ME treatments, HMEC-1 were cultured in phenol red free Endothelial Base Medium (Clonetics-BioWhittaker), supplemented with 2 mM glutamine and 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin (Gibco BRL).

Transfected Mink Lung Cells (TMLC) were a generous gift from Dr. Rifkin. TMLC were grown in DMEM supplemented 10% FBS, 200 μg/mL geniticin, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin.

Cell Proliferation Assay

Cells were plated at a density of 1.5 x 10^4 cells/ml in 100mm dishes. On the next day, when the cells were 50-60 % confluent, they were serum starved for 2-3 hours in phenol free Endothelial Base Medium. Cells were then treated with either vehicle (<.001% ethanol, EtOH) or 100 and 1000nM of 2-ME. Cells were fed every two days and treated cells were replenished with fresh drug. Cells were harvested at 48 hours and at 120 hours. Cell growth was monitored first by trypsinizing the cells and then by staining them with trypan blue (Sigma). The viable cells were counted using the hemocytometer. All experiments were done in triplicates.

Cell Growth Analysis: [^{3}H] Thymidine Incorporation Assay

DNA synthesis was determined using [^{3}H]-thymidine incorporation assay. Subconfluent cell monolayers were serum-starved for 3 hours and treated with 0-1000 nM of 2-ME for 24 or 48 hours in a 24 well plate. [^{3}H]-thymidine (1 μCi/mL, Amersham Pharmacia Biotech) was added with 2-ME for the 24-hour incubation. However, when the cells were treated with 2-ME for 48 hours, [^{3}H]-thymidine was added in the last 24 hour. The cells were then washed three times with ice cold PBS and once with 5% trichloroacetic acid and then solubilized with 1% SDS. Liquid scintillation counting was used to measure the amount of incorporated radioactivity.

For the experiments with the MAP Kinase signaling pathway inhibitors, SB203580 and PD98059, and 2-ME, the cells were pretreated with the corresponding

inhibitors (50 μM). After 2-3 hours, the cells were treated with 2-ME for 24 hours. Estradiol-17β was used at 100 and 1000 nM and also in combination with 2-ME. The thymidine incorporation assay was performed as described.

Annexin-V-FLUOS Staining: Visualization of Phosphatidylserine Exposure on Cell Surface

Cells were plated at a density of 1.5 x 10^4 cells/ml in chamberslides (Nunc Inc). On the next day, when the cells were 50-60 % confluent, they were serum starved for 2-3 hours in phenol free Endothelial Base Medium. Cells were treated either with vehicle (<.001% ethanol, EtOH) or 100 and 1000nM of 2-ME, prepared in Endothelial Base Medium. After 24 hours, the treatment medium was aspirated and 100 μL of staining solution (2 % propidium iodide and Annexin-V-fluorescein in 100 μL of Hepes Buffer, obtained from Roche) was added to each chamber. The cells were incubated with the staining solution for 15 minutes at room temperature. After the incubation, the chambers were removed and coverslips were placed on the slide. The cells were analyzed with the fluorescence microscopy with an excitation wavelength at the range of 450-500 nm and detection in the range of 515-565 nm (green).

DAPI Staining: Visual Analysis of Nuclear Condensation

HMEC-1 cells were plated and treated according to Annexin-V-FLUOS Staining procedure. After treating the cells with 100 and 1000nM of 2-ME, treatment medium was aspirated and 100 μL of 1 μg/mL DAPI (Roche) was added, prepared in phenol free Endothelial Base Medium. After removing the chambers and placing coverslips on the slides, the cells were analyzed with fluorescence microscopy.

Western Blot Analysis: Analysis of Enzymatic Activity

To determine the activity of Mitogen Activated Protein Kinases and their transcription factors due to 2-ME treatment, HMEC-1 cell monolayers were grown overnight in 60mm dishes in MCDB-131 medium with the supplements. They were then washed with sterile PBS once and incubated in phenol free Endothelial Base Medium for 2-3 hours to remove any endogenous growth factors. The cells were then treated with the desired concentrations of 2-ME, prepared in Endothelial Base Medium, for 10, 30, and 60 minutes. To determine the activity of cleaved Caspase-3 at the end of apoptotic cascade, the cells were treated with 2-ME for at least 24 hours.

After 2-ME treatments, HMEC-1 cell monolayers were washed with 1X Phosphate Buffered Saline (PBS) and lysed immediately with 500 μL of the Western lysis buffer (150 mM sodium chloride, 50 mM Tris-Hcl pH 7.5, 50 mM sodium fluoride, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, 5 mM EDTA pH 8.0, 10% glycerol, 1% NP-40). Protease inhibitor cocktails were added to each well (10x PMSF: .35 g PMSF diluted in 20 mL isopropanol, 20x PI: 10mg/mL BSA, 50 μg/mL leupeptin, 500 μg/mL benzamide, 500 μg/mL STI, 100 μg/mL pepstatin, 1 mM PMSF). The cells were then scraped and the extracts were transferred to microcentrifuge tubes. The tubes were gently vortexed for 10–15 seconds to shear the DNA and reduce sample viscosity. Afterwards, they were kept on ice. Before preparing the samples for loading, the Bio-Rad protein assay was performed on the samples. Using the standard curve, drawn with the SIGMA-Plot, the protein concentration was determined in each sample. 45 μg of the protein samples were transferred to a new set of tubes. To each sample, 10 μL of 5X sample buffer was added along with 3 μL of β-mercaptoethanol (Sigma). After

a gentle vortex, the prepared samples, along with 10 μL of Prestained Protein Marker (New England Biolabs), were heated at 100 °C for 5 minutes. After a brief centrifuge, the samples and the marker were loaded onto SDS-PAGE mini gel. The samples were subsequently electrotransferred onto nitrocellulose membrane (Schleicher and Schuell Inc.). After transfer, the nitrocellulose membrane was washed with 25 mL of 1X TBS (1L of 10X TBS: 24.2 g Tris base, 80 g NaCl; pH adjusted to 7.6 with HCl) for 5 minutes at room temperature. The membrane was then incubated in 25 mL of blocking buffer (1X TBS, 0.1% Tween-20 with 5% nonfat dry milk) for 3 hours at 4 °C. After the incubation, the membrane was washed three times for 5 minutes each with 15 mL of TBS/T (1X TBS, 0.1% Tween-20). It was then incubated in primary antibody (Cell Signalling) at the appropriate dilution in 10 mL primary antibody dilution buffer (1X TBS, 0.1% Tween-20 with 5% BSA) with gentle agitation overnight at 4°C. The membrane was washed three times for 5 minutes each with 15 mL of TBS/T. After the wash, the membrane was incubated with HRP-conjugated secondary antibody (Cell Signaling Technology) at 1:2000 in 10 mL of blocking buffer with gentle agitation for 3 hours at 4°C. After three washes for 5 minutes each with 15 mL of TBS/T, the protein was detected with the enhanced chemiluminescent (ECL) system (Pharmacia Biotech Inc.). To confirm equal protein loading in each well, the membrane was washed once with TBS/T and reincubated in primary STAT-3 antibody overnight. STAT-3 protein levels were detected as previously described.

Flow Cytometry: Cell Cycle Analysis

HMEC-1 cells were plated at 3.0 x 10^4 cells/mL in 100mm dishes. When cells reached 50-60% confluency, they were serum starved and pretreated with either vehicle

(DMSO, <.001%) or p38 inhibitor SB203580 (10 μM) for 2-3 hours. Cells were then treated with vehicle or 2-ME at 100 and 1000nM in duplicates. 24 hours after the treatment, the cells were scraped and the duplicates were combined into 50 mL tubes. They were then centrifuged at 2000 rpm for 5 minutes. After decanting the supernatant, the cells were resuspended in 500 μL of Krishan's Buffer, which consisted of 75 μM Propidium Iodide, 0.1 M Sodium Citrate, 0.02 mg/mL RnaseA and 0.3% Triton-X-100 (Sigma). After a brief vortex, 50-mL tubes containing the cells were incubated on ice for 30 minutes in the dark. They were centrifuged again at 2000 rpm for 5 minutes. After decanting the supernatant, the cells were resuspended in another 500 μL of Krishan's Buffer. The tubes were briefly vortexed and were transferred to special fluorocytometry tubes. They were then analysed with flow cytometry.

PAI/L Assay: Measurement of TGF-β Levels in Conditioned Medium

HMEC were treated with 2-ME (100-200 nM) up to 48 hours and conditioned medium was collected in siliconized eppendorf tubes. Protease inhibitor cocktails were added to conditioned medium (10x PMSF, 20x PI).

In order to measure TGF-β in the conditioned medium, Transfected Mink Lung Cells (TMLC) were plated at $1.6x10^5$ cells/mL in a 96 well plate in DMEM medium containing geniticin. Cells were incubated for 3 hours at 37^0 C and in 5% CO_2/ air atmosphere. TGF-β1 standards (0-40 pM) were prepared in a medium consisting half of HMEC medium and half of 0.1% BSA-DMEM medium. In order to measure active levels of TGF-β1, no preparation was needed. However, to measure total TGF-β1 (active + latent), samples were heated at 70^0C for 10 min. After the incubation, wells were aspirated and TGF-β1 standards were added 100 μL per well in triplicates. Samples were

added 50 μL per well with 50 μL of 0.1% BSA-DMEM medium. The plate was incubated overnight. Next day, wells were aspirated and washed once with cold PBS. After the wash, 70 μL of 1X PharMingen Lysis Buffer (BD PharMingen) was added to each well. After 30-45 min incubation at room temperature, the lysates (45 μL) were transferred to luminometer plates. Before reading the plate, 10 μL of ATP cocktail (dH_2O: 4 μL; 0.1M ATP (A and C): 3 μL; 0.5M KH_2PO_4 (pH 7.8): 2 μL; 1M $MgCl_2$: 0.5 μL) was added to each well. Each well received 50 μL of 0.25 mM Luciferin (Roche Diagnostics) and the plate was read with a Berthold Luminometer. The concentration of TGF-β1 in the samples were read from the standard curve, taking into consideration the appropriate dilution factors. The concentration of TGF-β1 obtained from PAI/L Assay was corrected by the conditioned medium protein, measured by the Bradford method (Bio-Rad).

Statistics

Data are expressed as mean ± S.D. and compared by Student's t-test. A p-value of 0.05 or less was considered significant.

RESULTS

2-ME induced HMEC-1 apoptosis: Apoptosis has characteristic morphological features including membrane blebbing, rounding up and detachment of cells, and a condensation of the cytoplasm and nucleus. To determine if estrogen or its metabolite 2-methoxyestradiol (2-ME) can affect the endothelial cell viability, we treated cultures with estradiol-17β, 2-ME or vehicle as a control. Phase-contrast microscopy was used to visualize the endothelial cell morphologic changes induced by these treatments. Phase-contrast microscopy showed morphological changes of apoptosis in 2-ME-treated endothelial cells **(Fig. 1B)**. When exposed to 2-ME (1000 nM) for 48 hrs, endothelial cells shrank and retracted from their neighbouring cells, and the cytoplasm became condensed. A significant portion of the cells became rounded and non-adherent, suggestive of cells undergoing apoptosis. However, estradiol-17β did not change the cell viability in HMEC-1 (data not shown). The fluorescent dye DAPI binds selectively to DNA and forms strongly fluorescent DNA-DAPI complexes with high specificity (Russel et al, 1975). When the endothelial cells were stained with DAPI and assessed by fluorescence microscopy, cells with condensed chromatin were observed **(Fig. 2B)**. When cells undergo apoptosis, phosphatidylserine, a phospholipid, is exposed on the extracellular surface of the cell membrane (Franklin and McCubrey, 2000). When HMEC-1 were treated with 2-ME for two days and stained with Annexin-V, which has high affinity for phosphatidylserine, treated cells showed intense binding of fluorecently tagged Annexin-V on the cell surface **(Fig. 3B)**, indicating apoptosis has taken place. Moreover, the rounded feature of the apoptotic cells was also visible.

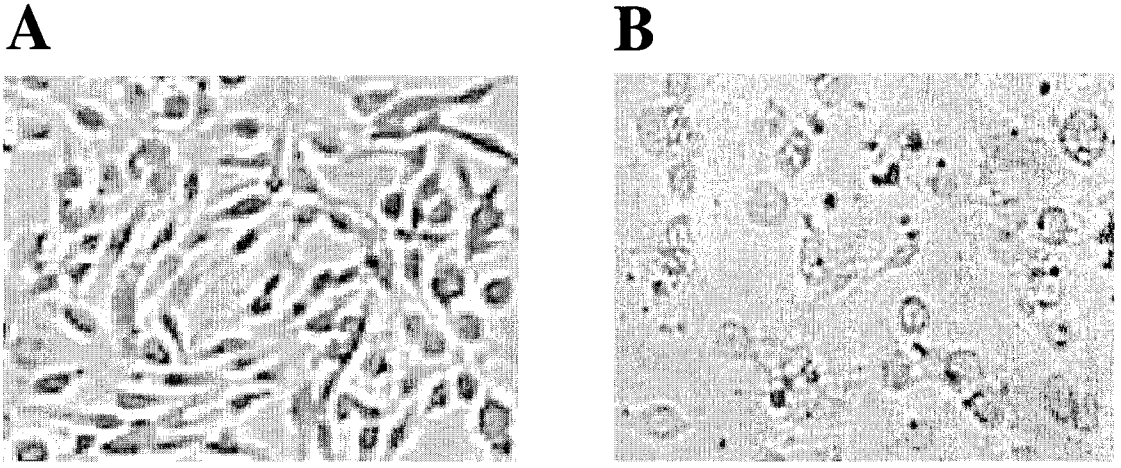

Figure 1: 2-ME induced apoptosis in Human Microvascular Endothelial Cells (HMEC-1). Subconfluent monolayers of HMEC-1 were treated with either vehicle (<.001% ethanol, EtOH) or 1000 nM of 2-ME in phenol red free Endothelial Base Medium (EBM) supplemented with glutamine. After 2 days, EtOH treated (A) and 2-ME treated (B) cells were photographed.

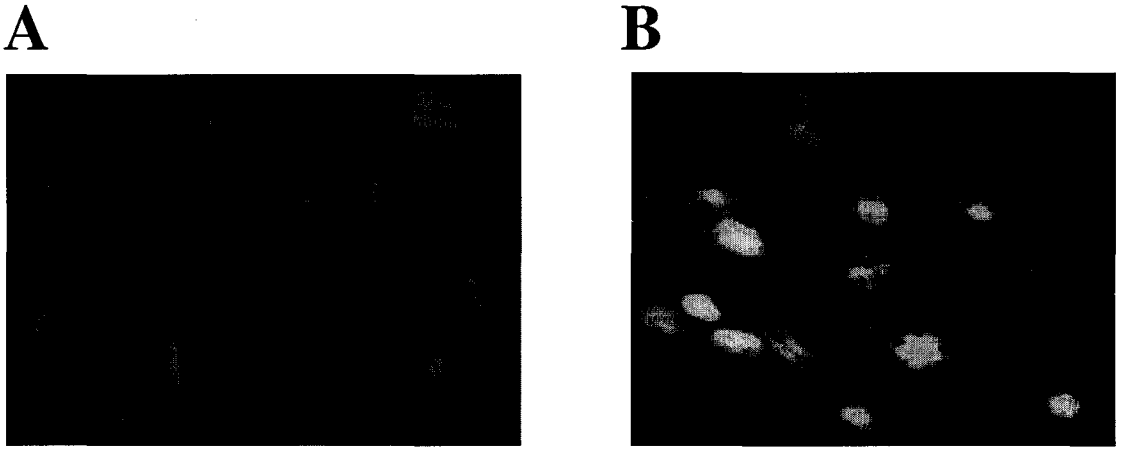

Figure 2: 2-ME induced Nuclear Condensation in Human Microvascular Endothelial Cells (HMEC-1). Subconfluent monolayers of HMEC-1 were treated with either vehicle (<.001% ethanol, EtOH) or 1000 nM 2-ME in phenol red free Endothelial Base Medium (EBM) supplemented with glutamine. After 2 days, EtOH treated (A) and 2-ME treated (B) cells were stained with 1 ug/mL DAPI and visualized under fluorescence microscopy.

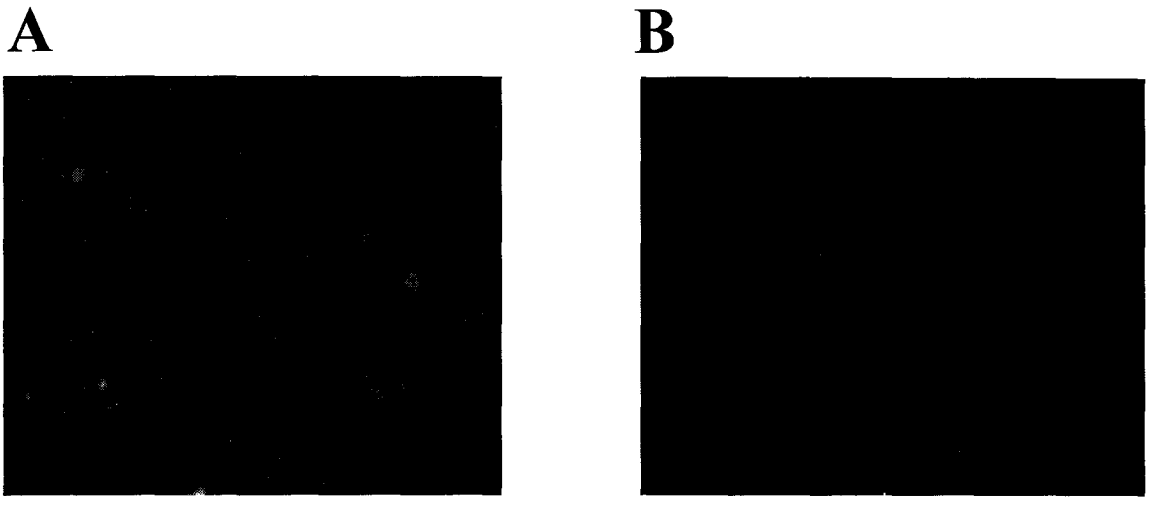

Figure 3: 2-ME induced apoptosis in Human Microvascular Endothelial Cells (HMEC-1). Subconfluent monolayers of HMEC-1 were treated with either vehicle (<.001% ethanol, EtOH) or 1000 nM 2-ME in phenol red free Endothelial Base Medium (EBM) supplemented with glutamine. After 2 days, EtOH treated (A) and 2-ME treated (B) cells were stained with Annexin-V-fluorescein and photographed using the fluorescence microscope.

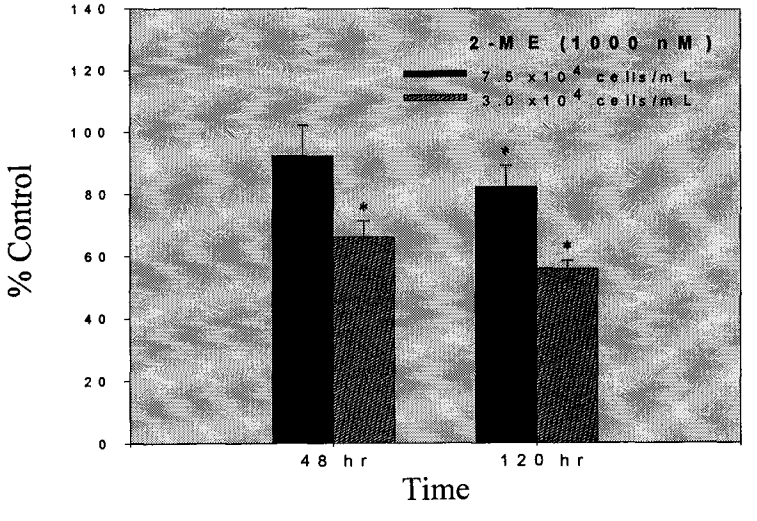

Figure 4: Effects of 2-ME on Cell Proliferation in Human Microvascular Endothelial Cells (HMEC-1). Confluent (plating density: $7.5x10^4$ cell/ml) and subconfluent (plating density: $3.0x10^4$ cell/ml) monolayers of early passage HMEC-1 were treated with either vehicle (<.001% ethanol, EtOH) or 1000 nM of 2-ME for 48 and 120 hours. After trypsinising and adding trypan blue, viable cells were counted using the hemocytometer (*$p<.05$).

2-ME was anti-proliferative in HMEC-1: Actively proliferating human microvascular endothelial cells were treated with different concentrations of 2-ME and cell proliferation was observed. 2-ME induced endothelial cell apoptosis was time-dependent. When subconfluent monolayers of HMEC-1 (plating density 3.0 x 10^4 cells/ml) were treated with 1000 nM of 2-ME only 70-75% of the cells remained viable after two days **(Fig. 4)**. On the fifth day, only 55-60% remained viable compared to the control. Moreover, quiescent confluent cultures of endothelial cells were less affected by 2-ME since more than 90% of confluent monolayers of HMEC (plating density 7.5 x 10^4 cells/ml) remained viable after the second day of 2-ME treatment and approximately 80% remained viable on the fifth day.

2-ME inhibited DNA synthesis in HMEC-1: The effects of 2-ME on DNA synthesis in endothelial cells, measured by thymidine incorporation assay, are shown in **Fig. 5**. Confirming our previous observation on cell proliferation, the effect of 2-ME on HMEC-1 was found to be time and cell density dependent. At 24 hours, cells plated at low density and treated with 1000 nM of 2-ME showed significant growth inhibition. When the cells were plated at a high density, even 48 hours of 1000 nM 2-ME treatment showed no significant growth inhibition. At 48 hours, cells plated at low density and treated with 200, 500 and 1000 nM of 2-ME showed significant growth inhibition.

2-ME effect was independent of estrogen receptor: Since 2-ME induced significant changes in endothelial cell morphology, viability, and proliferation, the mechanism of 2-ME action in our cell type needed to be explored. Since 2-ME is an estrogen derivative,

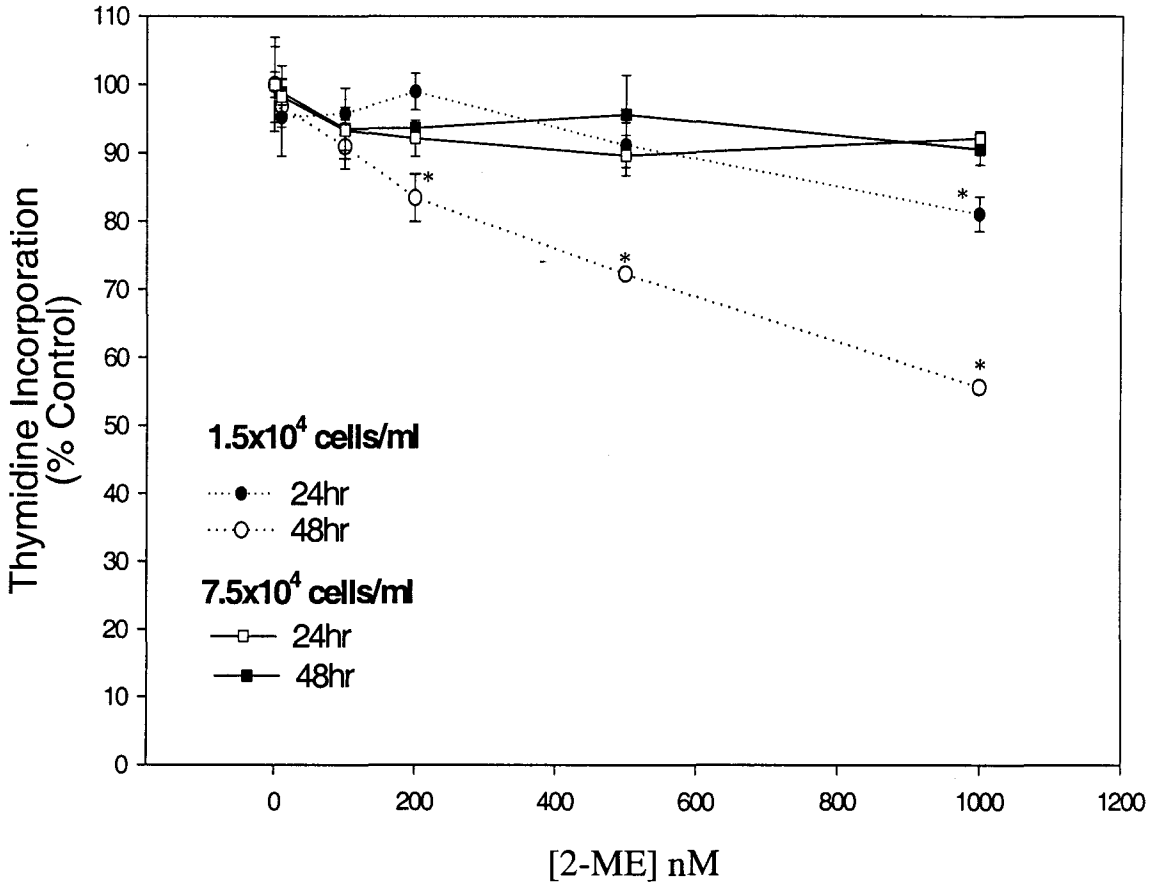

Figure 5: Effects of 2-ME on DNA Synthesis in Human Microvascular Endothelial Cells (HMEC-1). Confluent (plating density: $7.5x10^4$ cell/ml) and subconfluent (plating density: $1.5x10^4$ cell/ml) monolayers of early passage HMEC-1 were treated with either vehicle (<.001% ethanol, EtOH) or 10, 100, 200, 500, and 1000 nM of 2-ME for 24 and 48 hours. DNA synthesis was determined using [^{3}H]-thymidine incorporation assay (* $p<.05$).

we analyzed whether 2-ME could be mediating its anti-proliferative effect on HMEC-1 via the estrogen receptor. Estradiol-17β on its own at 1000 nM had no significant effect in HMEC-1 growth as measured by [^{3}H] thymidine incorporation assay **(Fig. 6)**. 2-ME alone at 1000 nM caused significant growth inhibition in HMEC-1, as observed previously. When cells were cultured with estradiol-17β and 2-ME at 1000 nM, similar result was obtained as with 2-ME alone. 100 nM of either estradiol-17β or 2-ME alone or the combination of both estradiol-17β and 2-ME at 100 nM had no significant effect on HMEC-1 DNA synthesis.

Possible pathways of 2-ME action: In order to study possible mechanisms of 2-ME action in HMEC-1, we explored whether some of the classical Mitogen Activated Protein Kinases were active in our microvascular endothelial cell. We treated HMEC-1 with SB203580 and PD98059, the inhibitors of p38 and ERK signaling pathways respectively. Both inhibitors caused a significant growth inhibition in HMEC-1 **(Fig. 7)**. SB203580 had a stronger growth inhibitory effect in HMEC-1 than PD98059. However, high concentrations of inhibitors used (50 μM) in the experiment raised the question of cytotoxicity of these inhibitors. We, therefore, analyzed the dose effects of these inhibitors in HMEC-1. SB203580 caused a significant decrease in DNA synthesis even at 5 μm **(Fig. 8)**. PD98059 at lower concentrations had little effect on HMEC-1 growth inhibition (data not shown).

2-ME activated SAPK/JNK pathway: The effects of 2-ME on SAPK/JNK activity in HMEC-1 are shown in **Fig. 9A**. Exposure of endothelial cells to 100, 500, and 1000 nM

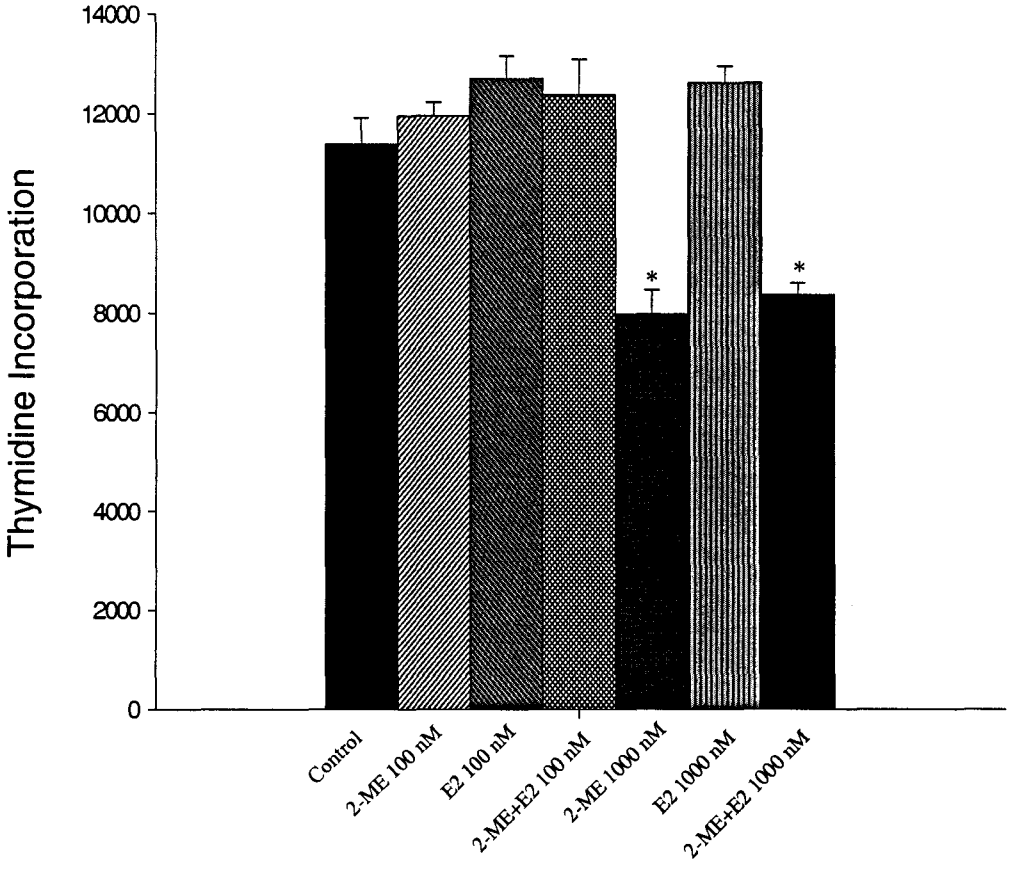

Figure 6: Effects of Estradiol-17 ß (E2) on DNA Synthesis in Human Microvascular Endothelial Cells (HMEC-1). Subconfluent monolayers of early passage HMEC-1 were treated with either vehicle (<.001% ethanol, EtOH) or 100 and 1000 nM of 2-ME and Estradiol-17 ß either alone or together for 24 hours. DNA synthesis was determined using [^{3}H]-thymidine incorporation assay (* $p<.05$).

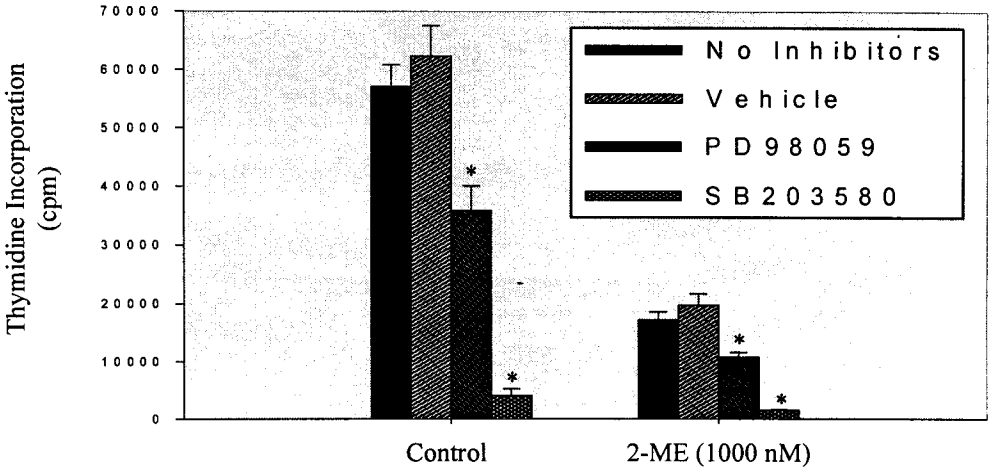

Figure 7: Effects of p38 Pathway Inhibitor (SB203580) and ERK1/2 Inhibitor (PD98059) on DNA Synthesis in Human Microvascular Endothelial Cells (HMEC-1). Subconfluent monolayers of early passage HMEC-1 were treated with either vehicle (<.001% ethanol, EtOH) or 1000 nM of 2-ME with or without 50 μM of SB203580 or PD98059 for 24 hours. DNA synthesis was determined using [^{3}H]-thymidine incorporation assay (* $p<.05$).

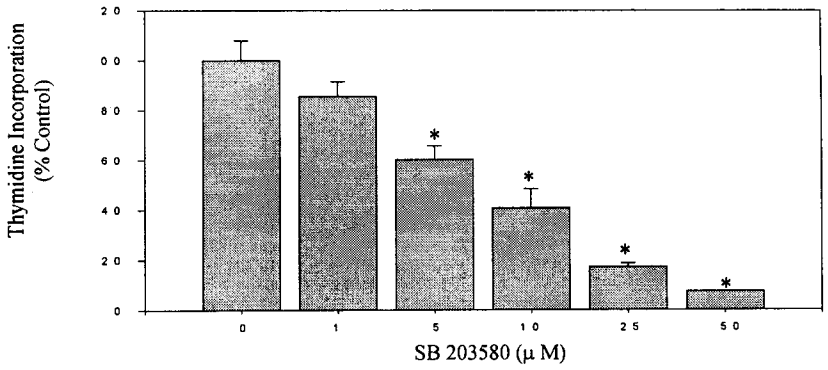

Figure 8: Effects of Increasing Concentration of p38 Pathway Inhibitor (SB203580) on DNA Synthesis in Human Microvascular Endothelial Cells (HMEC-1). Subconfluent monolayers of early passage HMEC-1 were treated with either vehicle (<.001% DMSO) or 1, 5, 10, 25, and 50 μM of SB203580 for 24 hours. DNA synthesis was determined using [^{3}H]-thymidine incorporation assay (* $p<.05$).

of 2-ME induced a rapid activation of SAPK/JNK 1 and 2 and/or 3. A significant increase in SAPK/JNK activity was detected 10 minutes after stimulation and the activation declined after 30 minutes. No activation was observed 60 minutes after the 2-ME stimulation. As shown in **Fig. 9A**, 2-ME-induced SAPK/JNK activation in HMEC-1 was not concentration-dependent. Some basal activities of SAPK/JNK were observed in unstimulated HMEC-1. Equal loading of protein in each well can be observed, as indicated by Stat3 **(Fig. 9B)**. The effects of 2-ME on the transcription factor c-Jun, the downstream target of SAPK/JNK, showed a similar response **(Fig. 10B)**. Activated c-Jun paralleled the activity of SAPK and declined to basal levels after 60 minutes. When the cells were treated with SB203580, which causes growth inhibition in HMEC-1, c-Jun was also phosphorylated **(Fig. 13)**.

2-ME did not modulate ERK Pathway: The effects of 2-ME on other classical MAP Kinases, namely p38 and ERK1/2, were analyzed in a similar fashion as SAPK/JNK Kinase. Similar to SAPK/JNK activation, p38 Mitogen Activated Potein Kinase was also activated due to 100, 500, and 1000 nM doses of 2-ME **(Fig. 9B)**. p38 activation was maximum at 10 minutes. However, unlike the SAPK/JNK activation, which diminished at 60 minutes, some p38 activation was still observed even after 60 minutes of 2-ME treatment. The transcription factor ATF-2, downstream target of p38, also showed similar activities as its upstream kinase **(Fig. 10A)**. However, unlike c-Jun, the untreated cells showed considerable ATF-2 activity at 10 minutes. This could be explained by the fact that ATF-2 is a common target for both SAPK/JNK and p38. Since both kinases showed some basal activities at 10 minutes in unstimulated cells, they could be additively

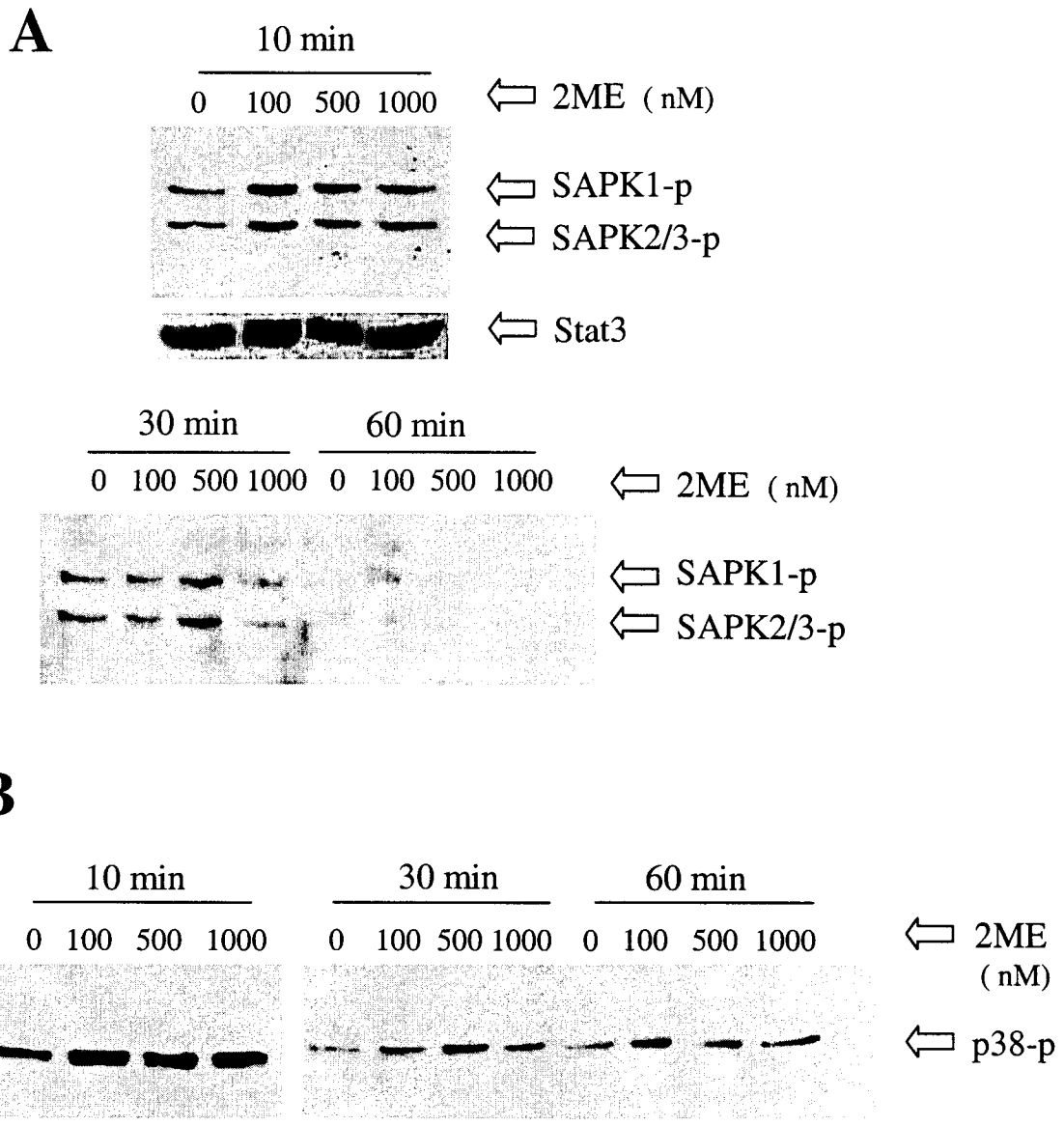

Figure 9: Effects of 2-ME on Stress Activated Protein Kinase (SAPK) and p38 Activity in Human Microvascular Endothelial Cells (HMEC-1): Subconfluent monolayers of early passage HMEC-1 were treated with either vehicle (<.001% ethanol, EtOH) or 100, 500, and 1000 nM of 2-ME for 10, 30, and 60 minutes. The cells were then processed for western immunoblotting to assess the enzymatic activity of the three isoforms of SAPK (A) and the enzymatic activity of p38-p (B). Equal loading of protein is observed, as indicated by Stat3 (A).

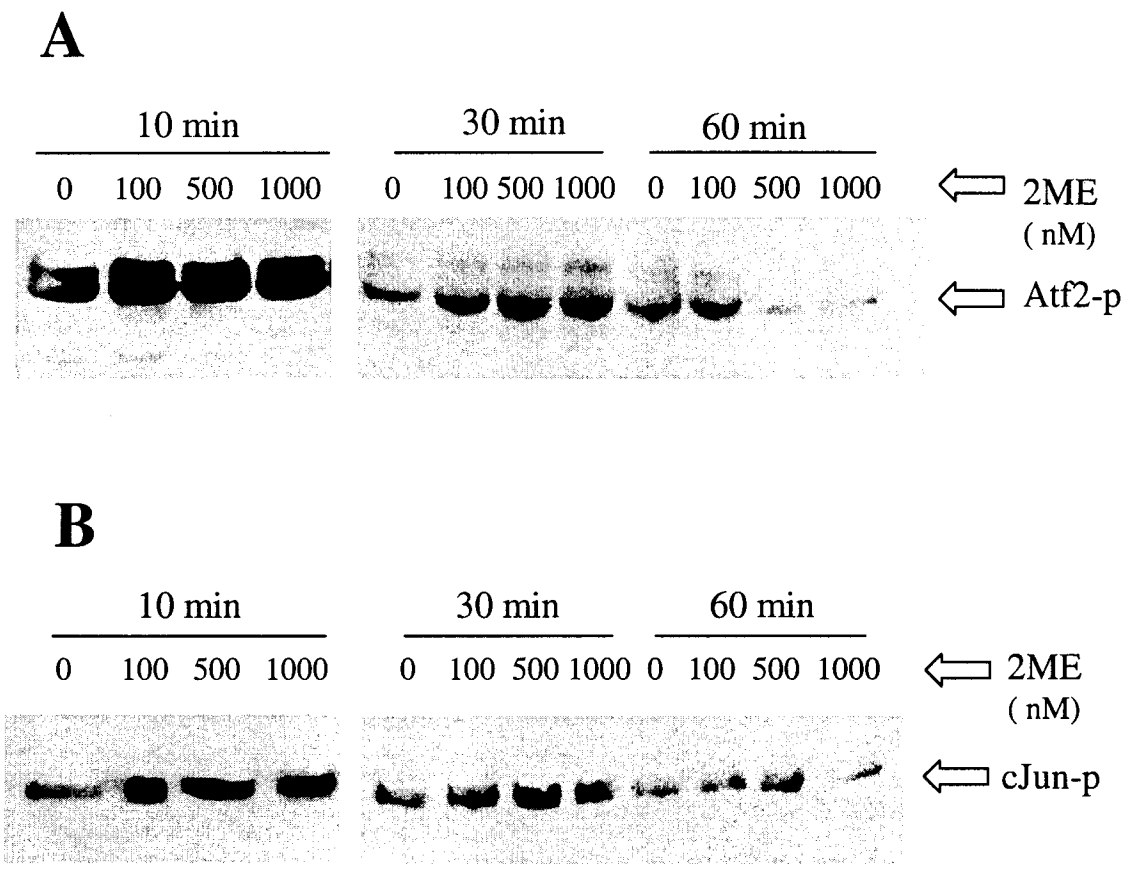

Figure 10: Effects of 2-ME on the Enzymatic Activity of Transcription Factors ATF-2-p and cJun-p in Human Microvascular Endothelial Cells (HMEC-1): Subconfluent monolayers of early passage HMEC-1 were treated with either vehicle (<.001% ethanol, EtOH) or 100, 500, and 1000 nM of 2-ME for 10, 30, and 60 minutes. The cells were then processed for western immunoblotting to assess the enzymatic activity of ATF-2-p (A) and cJun-p (B).

phosphorylating ATF-2. The other MAP Kinase, ERK1/2, did not respond to 2-ME treatment. Both in 2-ME treated and in untreated cells, ERK1/2 was found to be phosphorylated **(Fig. 11)**. The ERK pathway could be constitutively active in HMEC-1 since even at 0 minute, unstimulated cells showed high ERK activity.

2-ME caused increase in caspase-3 activity: The effect of 2-ME on caspase-3, the executioner of apoptosis, is shown in **Fig. 12** and **14**. 100 nM of 2-ME caused lower expression of cleaved, activated caspase-3 whereas 1000 nM of 2-ME induced increased expression of activated caspase-3. As can be seen from **Fig. 14**, the greatest increase in caspase expression was observed when the cells were treated with both SB203580 and 2-ME (1000 nM). SB203580 alone caused caspase-3 expression in control cells.

2-ME arrested HMEC-1 in G2/M phase: The role of 2-ME in HMEC-1 cell cycle was investigated using flow cytometry. Subconfluent monolayers of HMEC-1 were pretreated with SB203580 or vehicle for 2-3 hours. Control and 2-ME treated cells were then stained with PI and the cell cycle distribution was determined **(Table 1)**. The DNA histogram showed that the majority of cells were in G0-G1 phase with only 9.5% of cells in the G2/M phase without the SB203580 treatment and only 11.4% of cells with the inhibitor treatment. 100 nM of 2-ME had no drastic effect on the overall cell cycle. The synthetic analogue of 2-ME, 16-epiestriol, had no effect on the overall cell cycle distribution either (data not shown). In contrast, 1000 nM of 2-ME treatment dramatically enhanced the percentage of cells in the G2/M phase. About one third of cells were in the G2/M compartment of cell cycle. There seems to be little difference between the cells

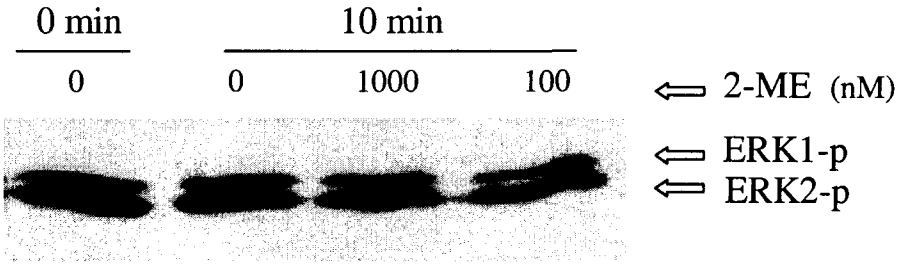

Figure 11: Effects of 2-ME on the Enzymatic Activity of ERK1/2 in Human Microvascular Endothelial Cells (HMEC-1): Subconfluent monolayers of early passage HMEC-1 were treated with either vehicle (<.001% ethanol, EtOH) or 1000 and 100 nM of 2-ME for 10 minutes. The cells were then processed for western immunoblotting to assess the enzymatic activity of both isoforms of ERK1 and ERK2.

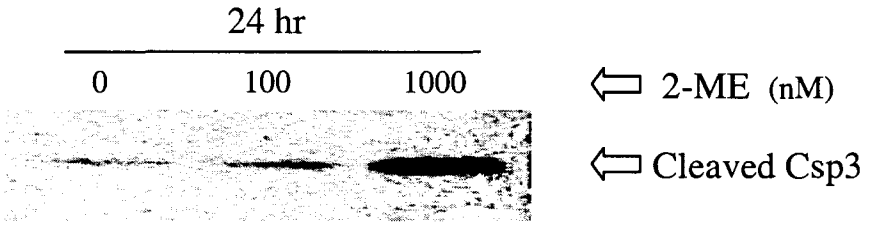

Figure 12: Effects of 2-ME on Caspase-3 in Human Microvascular Endothelial Cells (HMEC-1): Subconfluent monolayers of early passage HMEC-1 were treated with either vehicle (<.001% ethanol, EtOH) or 1000 and 100 nM of 2-ME for 24 hours. The cells were then processed for western immunoblotting to assess the activity of cleaved caspase-3.

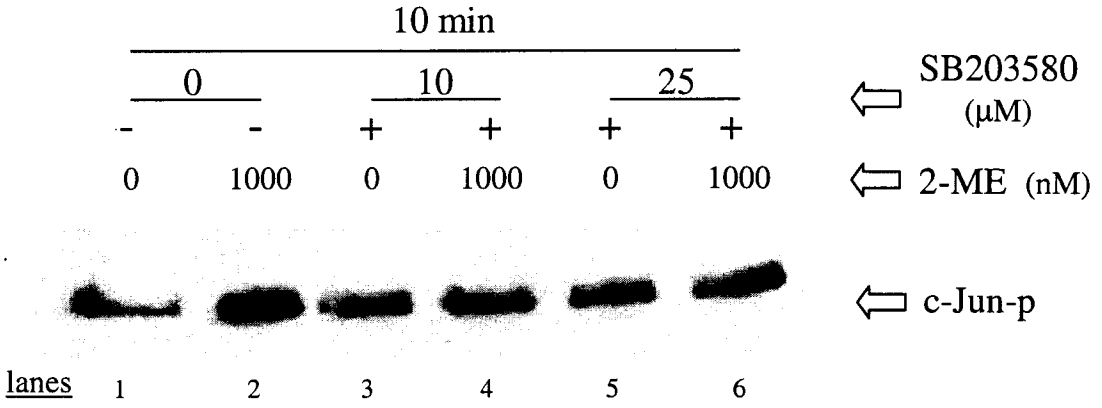

Figure 13: Effects of 2-ME on the Enzymatic Activity of cJun in the Presence or Absence of p38 Pathway Inhibitor (SB203580) in Human Microvascular Endothelial Cells (HMEC-1): Subconfluent monolayers of early passage HMEC-1 were pretreated with or without 10 or 25 μM of SB203580 for 2-3 hours and then treated either with vehicle (<.001% ethanol, EtOH) or 1000 nM of 2-ME for 10 minutes. The cells were then processed for western immunoblotting to assess the activity of cJun-p.

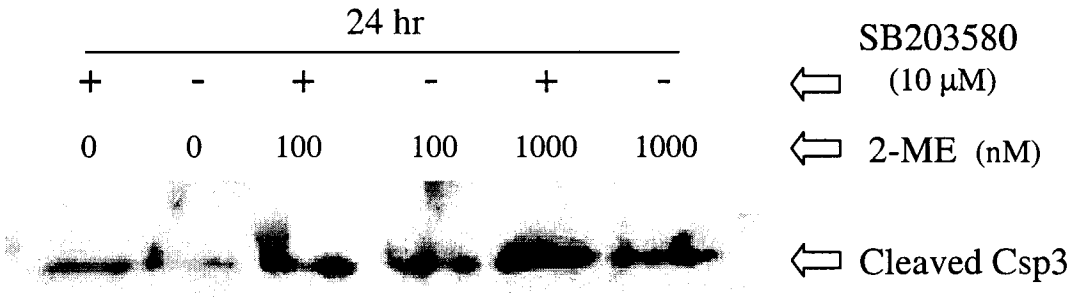

Figure 14: Effects of 2-ME on the Activity of Cleaved Caspase-3 in the Presence or Absence of SB203580 in Human Microvascular Endothelial Cells (HMEC-1): Subconfluent monolayers of early passage HMEC-1 were pretreated with or without 10 μM of SB203580 for 2-3 hours and then treated with either vehicle (<.001% ethanol, EtOH) or 1000 nM of 2-ME for 24 hours. The cells were then processed for western immunoblotting to assess the activity of cleaved caspase-3.

PHASES	Control		2ME-100 nM		2ME-1000 nM	
	(- SB)	(+ SB)	(- SB)	(+ SB)	(- SB)	(+ SB)
% G0-G1	85.5	85.3	91.3	92.9	43.1	67.3
% S	5.0	3.3	5.6	0.0	29.0	1.2
% G2-M	9.5	11.4	3.1	7.1	27.9	31.5

Table 1: Effects of 2-ME on Cell Cycle Distribution in HMEC-1: Subconfluent monolayers of early passage HMEC-1 were pretreated with or without 10 μM of SB203580 for 2-3 hours and then treated with either vehicle (<.001% ethanol, EtOH) or 100 and 1000 nM of 2-ME for 24 hours. The cells were stained with Propidium Iodide cocktail and the distribution of cell cycle was determined by flow cytometry.

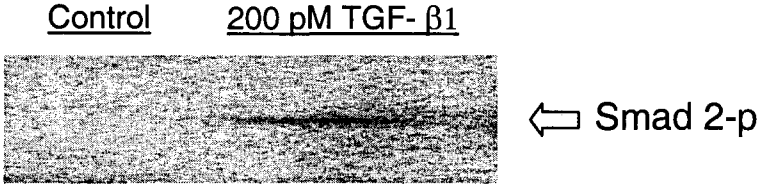

Figure 15: TGF-β_1 induces Smad2 Phosphorylation in Human Microvascular Endothelial Cells (HMEC-1): Subconfluent monolayers of early passage HMEC-1 were treated with 200 pM of TGFβ_1 for 30 minutes. The cells were then processed for western immunoblotting to assess the activity of Smad2.

that were left untreated with the p38 inhibitor (G2/M: 27.9%) and those that were pretreated with the inhibitor (G2/M: 31.5%).

2-ME induced TGF-β1 production in HMEC-1: Transforming Growth Factor β (TGF-β) has been shown to be a mediator of endothelial cell apoptosis (Pollman et al, 1999; Tsukada et al, 1995). We investigated whether TGF-β signaling mechanism is active in our microvascular endothelial cell. We, therefore, explored the role of TGF-β1 in the activation of Smad2, one of the key intracellular mediators of TGF-β signaling.

As it can be seen from **Fig. 15**, TGF-β1 caused an increase in Smad2 phosphorylation in HMEC-1. Since 2-ME exerted dramatic angiostatic effect on HMEC-1, we further investigated whether the levels of TGF-β1 was modulated by 2-ME. It was observed that 2-ME caused an increase in TGF-β1 levels in HMEC-1 as measured by the Plasminogen Activator Inhibitor-Luciferase Assay **(Fig. 16)**. 2-ME (200 nM) caused a significant increase in the levels of total TGF-β1 after 48 hours of treatment (~55 pM compared to ~40 pM in control, $p<.05$).

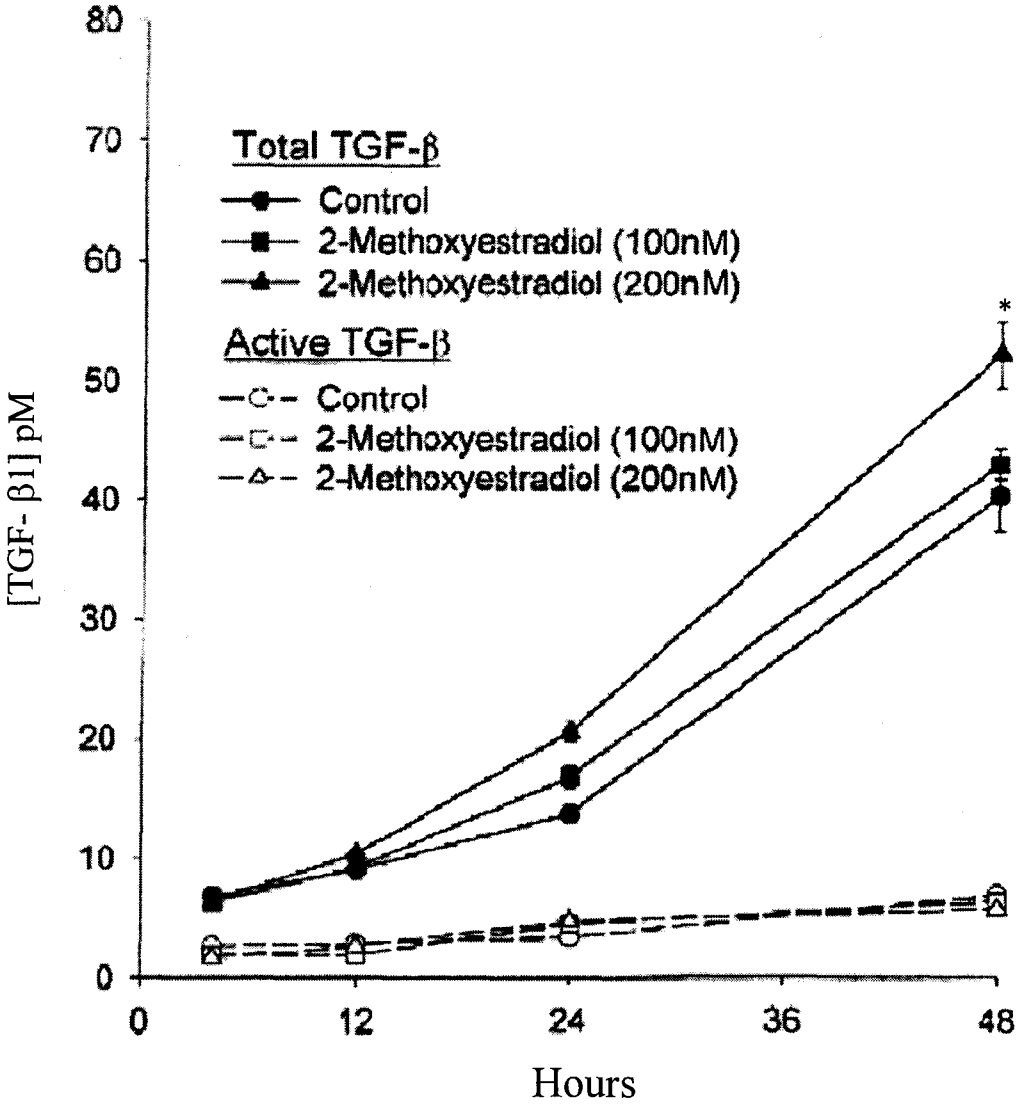

Figure 16: 2-ME induces TGF-β1 production in Human Microvascular Endothelial Cells (HMEC-1): Subconfluent monolayers of early passage HMEC-1 were treated with 100 and 200 nM of 2-ME for 12, 24 and 48 hours. Conditioned mediums were collected in siliconized tubes after the corresponding time intervals. The concentration of TGF-β_1 in the conditioned medium was measured using the PAI/L Assay. Concentration of TGFβ_1 was standardized by the conditioned medium protein (* $p<.05$: total TGFβ_1 : Control vs 2-ME-200 nM).

DISCUSSION

In this study we used morphological and biochemcal techniques to demonstrate that 2-ME induces anti-proliferative and apoptotic effects in Human Microvascular Endothelial Cells (HMEC-1). 2-ME circulates in the blood at a range of 30 pM in men to 30 nM in pregnant women, much lower than that at which its anti-proliferative, antiangiogenic effects occur (Berg et al, 1992, D'Amato at al, 1994). The half-maximal inhibitory concentration of 2-ME ranges from 300 nM in Jurkat lymphoblast tumour cells to 5 μm in Lung carcinoma cells (A549) (Pribluda et al, 2000). Our results demonstrate that 2-ME caused anti-proliferative and apoptotic effect in HMEC-1 at 1μM. After 2-ME treatment, as detected by the Phase-contrast microscopy, cells looked shrunk and rounded **(Fig. 1B).** In the cells not undergoing apoptosis, phosphatidylserine is contained in the inner leaflet of the cell membrane. However, when the cells are induced to undergo apoptosis, phosphatidylserine rearranges itself in the membrane and is exposed on the extracellular surfaces of the cell membrane (Franklin and McCubrey, 2000). When the cells are exposed to a phospholipid binding protein, a complex forms. Thus, 2-ME treated cells undergoing apoptosis showed intense binding of such a phospholipid binding protein, Annexin V, which is fluorescent labeled **(Fig. 3B).** Moreover, our data on DAPI staining indicated that 2-ME treated cells had condensed chromatin, a hallmark of apoptosis **(Fig. 2B).**

Our results on cell proliferation assay indicated that 2-ME had less apoptotic activity on confluent quiescent endothelial cells, suggesting that endothelial cells in an

active growth stage were more sensitive to 2-ME, which is in accord with the observation made by Fotsis et al (1994). The effect of 2-ME was also concentration dependent as indicated by the thymidine incorporation assay. 1000 nM of 2-ME caused nearly 45-50% inhibition, but 2-ME at less than 200 nM caused no significant changes in HMEC-1 DNA synthesis **(Figure 5)**.

The signaling pathways that lead to apoptosis have been the subject of intense investigation. Since 2-ME is derived from estradiol-17β it was possible that 2-ME mediated apoptosis involved the estrogen receptor. Estradiol-17β has been reported to have a 1000-fold higher binding affinity to the cytosolic estrogen receptor than that of 2-ME (Yue et al, 1997). When cells were cultured with estradiol-17β and 2-ME, HMEC-1 only responded to 2-ME alone suggesting that HMEC-1 was refractory to estradiol-17β. Given that estradiol-17β has high affinity to the estrogen receptor compared to its metabolite, it also suggests that the inhibitory effect of 2-ME observed is unlikely to be mediated through the estrogen receptor. Our results are also consistent with those of Fotsis et al. (1994), who reported that the inhibitory effect of 2-ME on endothelial cell proliferation was much more potent than that of estradiol-17β.

Increasing evidence suggest that the induction of programmed cell death involves activation of one or several signaling systems, many elements of which, however, remain unknown. Unlike the ERK MAP kinases, the SAPK/JNK are weakly activated by growth factors but strongly activated by cellular stresses, such as ultraviolet light, heat shock, and protein synthesis inhibitors (Yue at al, 1997). It has been demonstrated that

overexpression of SAPK or activation of its upstream kinases induces apoptosis and that interference with activation of SAPK protects against apoptosis (Xia et al, 1995). 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 (Zanke et al, 1996).

To investigate the possible involvement of SAPK/JNK in mediating 2-ME-induced apoptosis in HMEC-1, we examined the effect of 2-ME on SAPK/JNK. 2-ME treatment caused potent activation of JNK/SAPK in a transient fashion. The rapid activation of SAPK/JNK after 2-ME treatment **(Fig. 9A)** has been shown to be associated with apoptosis in Bovine Pulmonary Aortic Endothelial Cells (BPAEC) (Yue at al, 1997). The range of concentrations of 2-ME for induction of SAPK/JNK activation, however, did not coincide with the concentrations of 2-ME to elicit apoptosis. 100 nM of 2-ME had a stimulatory effect on SAPK/JNK activity **(Fig. 9A)** but did not induce substantial HMEC-1 apoptosis. This could be explained by the fact that transient SAPK/JNK activation may not be the only mechanism through which 2-ME causes apoptosis (Yue at al, 1997). Yamamoto et al have shown that BCL-2, the anti-apoptotic protein, was rendered inactive by SAPK/JNK at the G2/M phase of the cell cycle (1999). One of the postulated mechanisms for 2-ME's anti-tumor effect is the disruption of microtubules and the subsequent growth arrest of proliferating cells in the G2/M phase of the cell cycle. In HMEC-1, 1000 nM of 2-ME, but not 100 nM, caused significant growth arrest at the

G2/M phase, which is consistent with the notion that only micromolar (1-5 μm) doses of 2-ME causes G2/M cell cycle arrest (Attala et al, 1996, Qadan et al, 2001). Taken together, these reports suggest that 2-ME mediated apoptosis involves transient SAPK/JNK activation as well as G2/M arrest leading to BCL-2 inactivation and subsequent apoptosis. To further delineate the involvement of SAPK/JNK activation in 2-ME-induced apoptosis in HMEC-1, we studied the effects of 2-ME on the activity of one of the transcription factors downstream of SAPK. c-Jun, one of the prime targets of SAPK, showed a similar response as its upstream kinase. It is important to note that the control cells also showed some SAPK as well as c-Jun activity **(Fig. 9A and 10A)**, but it was minimal compared to the treated cells. Moreover, the SAPK cascade is susceptible to a small variation in temperature or osmolarity (Franklin and McCubrey, 2000), which can happen during the addition of fresh treatment media to the cells.

Activation of SAPK/JNK by 2-ME may trigger early genomic responses that ultimately lead to apoptosis. The activation of SAPK/JNK in HMEC 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 (Verheij et al, 1996; Johnson et al, 1996) 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 (Verheij et al, 1996). Although several 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 (Yue et al, 1997). When

HMEC-1 were treated with the inhibitor of p38 MAP kinase (SB203580), which had caused growth inhibition in HMEC-1 **(Fig. 7, 8)**, it seem to have caused an increase in the phosphorylation state of cJun **(Fig. 13:** lane 1 vs lane 3 and 5**).** This suggests that growth inhibition in HMEC-1 may involve early activation of c-Jun.

Since SAPK pathway may not be the only pathway that leads to apoptosis, we examined the effects of 2-ME on other classical MAP Kinase pathways, namely ERK and p38. Several studies have indicated that the cell growth promotion or inhibition is a balance between several MAP Kinase pathways. Gupta et al have showed that VEGF promotes the growth of human dermal microvascular endothelial cells by inhibiting SAPK/JNK but upregulating ERK pathway (1999). When HMEC-1 was treated with 2-ME, no changes in the phosphorylation state of ERK were observed **(Fig. 11)**. In fact, both isoforms of ERK (1 and 2) were found to be constitutively active in HMEC-1. Therefore, the growth inhibitory effect of 2-ME was not likely to be via the ERK pathway. This result was consistent with the result obtained using the ERK pathway inhibitor, PD98059, where at 10 μm, the inhibitor had no significant effect on cell growth (data not shown). PD98059 at 50 μm significantly inhibited HMEC-1 growth **(Fig. 7)**. However, the growth inhibition observed may be due to the inhibitor's cytotoxic effect and may not be due solely to its blocking the ERK pathway in HMEC-1.

We investigated the effects of 2-ME on the activity of p38 MAP Kinase pathway. The p38 signalling transduction pathway plays an essential role in regulating many cellular processes, including cell differentiation, cell growth and death (Ono and Han,

2000). SB203580, the p38 pathway blocker, decreased HMEC growth even at 1 and 5 μm **(Fig. 8).** Although SB203580 has been shown to block pathways other than p38 at concentrations above 10 μm (Whitmarsh et al, 1997), the significant decrease in growth observed at 5 μm suggests that p38 is a survival pathway in HMEC-1. Western analysis of p38 MAP Kinase showed an increase in phosphorylation after 2-ME treatment at concentrations ranging from 100 to 1000 nM **(Fig. 9B)**. This increase in activity was transient and decreased to basal levels after 60 minutes of 2-ME treatment. Furthermore, similar results were obtained for ATF2, the transcription factor downstream of p38 **(Fig. 10A)**. Cells could be triggering this survival pathway in order to balance the apoptotic SAPK/JNK signaling pathway. Several studies have indicated that early p38 kinase activity was necessary for cell survival in the presence of apoptosis causing cytokines, such as tumour necrosis factor α (Roulston et al, 1998; Varghese et al, 2001). One of the possible upstream kinases of p38, SEK1 (SAPK and ERK kinase 1) or MKK4, the only kinase that can activate both SAPK and p38 in vitro (Moriguchi et al, 1995), had shown no activity in response to 2-ME treatment (data not shown). In fact, activation of SEK1 is not essential for either p38 or SAPK activation in some cell types. It has been shown that sorbitol and UV radiation could activate SAPK in the complete absence of SEK1 (Tibbles and Woodgett, 1999). Therefore, it is possible that different isoforms of MKKs are activating SAPK and p38. MKK3 or 6 could be responsible for p38 activation and MKK7 could be phosphorylating SAPK in HMEC-1.

Apoptosis is an active gene-directed process of cellular 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, and the activation of JNK has been found in Fas-associated signaling and cell death. 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 (Yue et al, 1997). Studies have demonstrated that estrogen protects the human breast cancer cell line MCF-7 from apoptosis due to up-regulation of BCL-2 expression (Yue at al, 1997). Moreover, Attala et al have shown that JNK/SAPK indirectly phosphorylates and inactivates BCL-2, the antiapoptotic factor (1998). Inactivation of BCL-2 leads to the formation of apoptosome, consisting of activated procaspase-9, Apaf-1, and cytochrome c. This complex cleaves and activates procaspase 3 (Franklin and McCubrey, 2000). We, therefore, decided to observe the effect of 2-ME on the downstream effector of this apoptotic cascade, namely caspase-3, which once activated can cleave cellular proteins. These proteins include PARP, PAK, and certain isoforms of ICAD (Franklin and McCubrey, 2000).

We examined the cleaved, active form of procaspase-3 after 2-ME treatment. Our results showed an increase in caspase activity due to 2-ME treatment. As we had expected, 1000 nM of 2-ME caused greater activity of caspase-3 as opposed to 100 nM **(Fig. 12 and 14)**. This increase in caspase-3 activity confirms the morphological observations of apoptosis seen in HMEC-1 after 2-ME treatment **(Fig. 1-3).** Moreover, pretreating HMEC with SB203580, the blocker of survival (p38 MAP Kinase) pathway in our cellular model, caused increased caspase activity as well. Treatment of 2-ME with SB203580 caused the greatest increase in caspase-3 activity when compared to control cells. This is consistent with p38 being an anti-apoptotic pathway in HMEC-1.

Micromolar concentrations (1-5 μM) of 2-ME has been shown to cause G2/M cell cycle arrest and has been postulated to be one of the possible mechanisms of 2-ME's antiproliferative effects on various cell types (Attala et al, 1996, Qadan et al, 2001). Our results on HMEC-1 suggested a similar phenomenon. A significant number of cells were pushed to the G2/M compartment after 1000 nM of 2-ME treatment compared to the control **(Table 1).** The therapeutic low dose of 2-ME (100 nM), however, did not cause G2/M cell cycle arrest in HMEC-1. Thus, the findings with the therapeutic high dose of 2-ME (1000 nM) suggest a correlation may exist between 2-ME induced G2/M arrest and apoptosis in HMEC-1 **(Table 1, Fig. 14)**.

Steroids have profound effects on the endothelium. The recent discovery of potent naturally occurring and synthetic angiostatic steroids uncovered novel aspect of endothelial cell physiology. A growing number of studies have implicated TGF-ß1 as a potent mediator of apoptosis in endothelial cells (Pollman et al, 1999; Tsukada et al, 1995). Our study on the interaction between 2-ME, the anti-angiogenic steroid, and TGF-ß1 indicated that 2-ME causes an increase in TGF-ß1 levels in HMEC-1. 2-ME induced phopshorylation of ATF2 may provide a possible mechanism whereby 2-ME caused an increase in TGF-ß1 levels. ATF-2 binds to CRE-like elements [T(G/T)ACGTCA] in the promoter of many genes. Indeed, a specific role for ATF2 in the expression of transforming growth factor-ß2 has been established (Gupta et al, 1995). Moreover, TGF-ß1 promoter has been shown to have AP-1 binding sites (Kim et al, 1991). Since ATF2 binds to CRE as well as AP-1 binding sites, the increase in activity of ATF2 after 2-ME

treatment **(Fig. 10A)** could translate into the increase in the levels of TGF-ß1 observed in HMEC-1 **(Fig. 16)**.

In summary, 2-ME causes endothelial cell apoptosis, possibly via activation of the SAPK/JNK signaling pathway and the up-regulation of caspase-3 in HMEC-1. These cells seem to trigger the p38 MAP Kinase signaling pathway as a protective mechanism in response to 2-ME treatment. The decrease in HMEC-1 DNA synthesis and induction of apoptosis by 2-ME do not appear to involve the ERK MAP Kinase signaling pathway. One of the mechanisms of 2-ME induced apoptosis in HMEC-1 seem to involve cell cycle arrest at the G2/M phase. Finally, 2-ME induces the production of TGF-ß1, which may play a role in 2-ME mediated apoptosis in HMEC-1.

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