2-Methoxyestradiol Induces Cell Cycle Arrest and Mitotic Cell Apoptosis in Human Vascular Smooth Muscle Cells

Yu Gui, Xi-Long Zheng

Abstract—It has been shown that 2-methoxyestradiol (2-ME) inhibits cell proliferation and DNA synthesis in human aortic smooth muscle cells. However, the cellular mechanisms underlying the antiproliferative activity of 2-ME are unclear. The present study was performed to explore the cellular mechanisms whereby 2-ME leads to growth inhibition and apoptosis of human smooth muscle cells. Our results demonstrate that at 1 hour of treatment, 1 μmol/L 2-ME induces multiple spindles, overamplified centrosomes, and multipolar cytokinesis, whereas 10 μmol/L 2-ME causes completely damaged spindle, disoriented centrosomes, and missegregated chromosomes. At 6 hours of treatment, the mitotic index was increased and reached a maximal level, and cells with 4N DNA content (4N cells) began to accumulate. The increased mitotic cells induced by 2-ME were apoptotic as detected by both annexin V and TUNEL staining. Blockage of cells in G1, phase by thymidine prevented 2-ME–induced apoptosis. In addition, the increased mitotic index declined concurrently when even more 4N cells accumulated at 12 to 48 hours of treatment with 10 μmol/L 2-ME. Furthermore, in response to 2-ME, cells delayed entry into the next cell cycle and exhibited aneuploidy or micronuclei. Some aneuploidy cells continued to synthesize DNA. We conclude that 2-ME treatment not only arrests cells in mitosis and promotes mitotic cell apoptosis, but also causes cells to undergo “mitotic slippage” and endoreduplication. The induction of mitotic cell arrest and apoptosis may be a major cellular mechanism by which 2-ME inhibits proliferation of human smooth muscle cells. (Hypertension. 2006;47:271-280.)

Key Words: apoptosis ■ estrogen ■ muscle, smooth vascular

Smooth muscle cell (SMC) proliferation and apoptosis play important roles in the pathogenesis of vascular proliferative diseases, such as atherosclerosis and restenosis. Recent human and animal experimental studies indicate that cell cycle inhibition holds great potential as a therapeutic strategy for vascular proliferative diseases. Regulation of the cell cycle is considered a key mechanism for controlling SMC proliferation.

Cell cycle progression into G1, S, G2, and M phases is controlled by cell cycle checkpoints that ensure the correct order and timing of transitions. During mitosis, the spindle assembly checkpoint monitors segregation of sister chromatids, inhibiting the onset of anaphase until all of the chromosomes are properly attached to the mitotic spindle apparatus. It has been shown that treatment with microtubule-interfering agents, such as nocodazole and taxol, induces preanaphase arrest in early mitosis resulting from effects on the mitotic spindle. This can be followed by tetraploidy arrest because of aberrant exit from mitosis without sister chromatid segregation and cytokinesis, a process known as “mitotic slippage.” As a result of mitotic failure, cells undergo apoptosis. In addition, after mitotic slippage induced by prolonged exposure to microtubule-interfering agents, cells may inappropriate continue the next cell cycle and enter S phase with 4N DNA content, a process known as endoreduplication. Endoreduplication can be prevented by inhibition of cyclin E/cyclin-dependent kinase (CDK) 2 activity through induction of p21, a CDK inhibitor.

2-methoxyestradiol (2-ME), a metabolite of the endogenous estrogen, is present in human blood at concentrations ranging from picomolar to tens of nanomolars. 2-ME exhibits antiproliferative and antiangiogenic activity in a variety of cells and an ex vivo model, suggesting that 2-ME could be a novel antiangiogenic and antiangiogenic therapeutic agent. Additionally, it appears that the antiproliferative activity of 2-ME results mainly from triggering of apoptosis. Recently, 2-ME was found to mediate estradiol-induced inhibition of DNA synthesis and cell proliferation in human aortic SMCs in an estrogen receptor–independent manner. However, the mechanism underlying the antiproliferative activity of 2-ME is not clearly understood. Given that 2-ME can interact with tubulin directly at the colchicine-binding site and inhibit tubulin polymerization, we hypothesized that the antiproliferative action of 2-ME in human SMCs may result from mitotic cell arrest and apoptosis through disruption of the mitotic spindle apparatus.

Our present studies were designed to investigate the cellular mechanisms by which 2-ME inhibits proliferation of...
human vascular SMCs. In response to treatment with 2-ME, cell cycle profiles were first examined by laser scanning cytometry (LSC). Mitotic spindles, centrosomes, and chromosomes were inspected by immunofluorescent staining and confocal microscopy. Apoptosis was evaluated by annexin V apoptotic assay and TUNEL assay.

Methods

Materials
Human vascular SMCs derived from aorta were purchased from the American Type Culture Collection. Kaighn’s modification of Ham’s F12 (F12K) medium, FCS, trypsin-EDTA, insulin, transferrin, and selenium were purchased from Invitrogen. Antibodies to cyclins A, B, and D3, and CDKs 1 and 2 were from BD Biosciences. 2-ME, anti-p27, anti-phospho-histone H3 (ser10), and anti-α-actin were from Sigma-Aldrich.

Cell Culture
Human vascular SMCs were cultured in Kaighn’s modification of Ham’s F12 (F12K) medium containing 10% FCS and supplemented with insulin–selenium–transferrin. The identity of SMCs was confirmed by immunostaining with antibody against smooth muscle α-actin. After seeding in the F12K medium with 10% serum for 48 hours, cells were treated with or without 2-ME for time periods indicated in the relevant figures. For cell proliferation experiments, the culture medium was changed every 2 days for up to 6 days; cell numbers were counted with a hemocytometer.

Cell Cycle Analysis by LSC
Cell cycle analysis and 5-bromodeoxyuridine (BrdUrd) incorporation were performed using laser scanning cytometer (CompuCyte Corp), as described previously. In addition, BrdUrd pulse-chase assay was performed to track the position changes of the BrdUrd-positive cells in cell cycle phases.

Immunofluorescence Studies
For most immunocytochemical experiments, cells grown on coverslips were fixed with 4% paraformaldehyde for 20 minutes at room temperature and permeabilized with 100% methanol overnight at −20°C. Cells were then washed with PBS and blocked with 2% skim milk for 30 minutes. For studies of mitotic index, cells were stained with an antibody (1:300 dilution) against p27, CDKs 1 and 2, and cyclins D3, A, B, and E were used to detect the respective proteins.

Assays for Apoptosis
DNA fragmentation analysis of SMCs treated with or without 2-ME at the concentrations indicated for 48 hours was performed as described previously. Live cell apoptosis was performed using Annexin V-FITC apoptosis detection kit (Sigma) according to the manufacturer’s recommendations. To determine whether mitotic cells are apoptotic, cells were subjected to TUNEL assay and phosphohistone H3 staining. The TUNEL assay was modified from the APO-BrdUrd TUNEL assay kit (Molecular Probes).

Western Blot Detection
SMCs grown in 100 mm Petri dishes after treatment as described, were lysed, and equal amounts of protein from each sample were separated by 11% SDS-PAGE as described previously. Antibodies against p27, CDKs 1 and 2, and cyclins D3, A, B, and E were used to detect the respective proteins.

Statistical Analysis
Results represent the mean value (mean±SEM) of experiments as indicated in the relevant figures. Statistical comparisons were performed with the Student t test for unpaired observations or with ANOVA followed by Bonferroni’s correction for comparisons of ≥3 groups. P value <0.05/n (where n is the number of comparisons) was considered to indicate a statistically significant difference.

Results
2-ME Inhibits Proliferation and Induces Apoptosis of Human Vascular SMCs
To determine the effects of 2-ME on SMC proliferation, human SMCs were cultured for ≤6 days in 10% serum in the absence and presence of 2-ME (0.1, 1, and 10 μmol/L). We were inspected by fluorescence microscopy and photographed with a CCD camera. Mitotic cells were identified by the presence of condensed DNA and by phosphohistone H3 (ser10)–positive staining. The mitotic index was calculated as the percentage of mitotic cells versus the total cell count.

For experiments regarding the microtubule network, mitotic spindle, and centrosome, anti-α-tubulin and anti-γ-tubulin were used as primary antibodies and anti-mouse IgG-Alexa Fluor 488 and anti-rabbit IgG-Texas Red as secondary antibodies, respectively. Confocal images were acquired with a laser scanning confocal microscope (Leica Microsystems) under a ×100 oil immersion lens and photographed with Cooled Scientific CCD camera (Princeton Instruments). Stacked images collected at 0.8-mm planes were analyzed and processed for deconvolution by Imaris software.

Figure 1. 2-ME inhibits proliferation and induces apoptosis of human vascular SMCs. Human SMCs were seeded at a density of 10 000 cells/well in 12-well plates and cultured in F12K medium containing 10% FBS for 24 hours. After changing to fresh medium (day 0), cells were treated without or with different concentrations of 2-ME for up to 6 days. During this period, the culture medium was changed at 48-hour intervals. Viable cells were counted every day using a hemocytometer. Values are expressed as mean±SEM (n=5; A). *P<0.05 and **P<0.001. Cells, after treatment without or with 2-ME for 48 hours, were lysed for DNA extraction. DNA (10 μg/lane) was loaded onto 1.8% agarose gels (B). M indicates 100-bp DNA ladder marker.
found that viable cell numbers were reduced in response to 0.1 μmol/L 2-ME compared with the control cells without the treatment of 2-ME ($P<0.05$). Viable cell numbers were even more significantly decreased in the presence of 1 μmol/L and 10 μmol/L 2-ME compared with the control cells ($P<0.001$; $n=5$; Figure 1A). After 6 days of treatment with 10 μmol/L 2-ME, the viable cells decreased to $0.5 \pm 0.2 \times 10^4$ versus the seeding level of $1.0 \times 10^4$ ($P<0.01$; $n=5$), suggesting a cytotoxic effect.

To investigate whether the cytotoxic effect induced by 2-ME involves induction of apoptosis, DNA fragmentation, a hallmark of apoptosis, was assayed. Human SMCs treated with 1 μmol/L 2-ME displayed a typical DNA laddering pattern, which was enhanced in the cells treated with 10 μmol/L 2-ME (Figure 1B). These results indicate that 2-ME causes apoptosis in human SMCs.

### 2-ME Inhibits DNA Synthesis and Induces Accumulation of Cells With 4N DNA Content

To explore whether the antiproliferative effect of 2-ME on human SMCs is through a mechanism regulating the cell cycle, cell cycle profiles in response to 2-ME were examined by LSC. Human SMCs were stimulated with 2-ME (0.1, 1, and 10 μmol/L) for up to 48 hours. Cells in S phase, which

![Figure 2. Effects of 2-ME on BrdUrd incorporation and cell cycle profile. After treatment with 2-ME (0.1, 1, and 10 μmol/L) for 24 hours, cells were labeled with 10 μmol/L BrdUrd for 1 hour and then fixed for detection with anti-BrdUrd antibody and PI staining using LSC. Representative scattergrams show BrdUrd staining intensity (y axis, FITC integral) vs DNA content (x axis, PI integral). BrdUrd-positive cells are distributed in the regions of upper quadrants (A). Histograms represent the number of cells vs DNA content (B). 2N and 4N indicate cells with 2N and 4N DNA contents.](http://hyper.ahajournals.org/DownloadedFrom/.../byguestonApril19,2017)
Effects of 2-ME on Cell Cycle Profile

<table>
<thead>
<tr>
<th>Groups</th>
<th>6 Hours</th>
<th>12 Hours</th>
<th>24 Hours</th>
<th>48 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2N</td>
<td>S</td>
<td>4N</td>
<td>2N</td>
</tr>
<tr>
<td>Control</td>
<td>68±4</td>
<td>28±3</td>
<td>5±3</td>
<td>68±4</td>
</tr>
<tr>
<td>0.1 μmol/L</td>
<td>66±5</td>
<td>27±4</td>
<td>5±3</td>
<td>67±4</td>
</tr>
<tr>
<td>1 μmol/L</td>
<td>60±3</td>
<td>25±3</td>
<td>15±2</td>
<td>64±4</td>
</tr>
<tr>
<td>10 μmol/L</td>
<td>57±5</td>
<td>21±3</td>
<td>20±4</td>
<td>50±5</td>
</tr>
</tbody>
</table>

Summarized data (n=5) of cells with 2N and 4N DNA contents (2N and 4N cells) and cells in S phase (the BrdUrd-positive cells) in response to different time treatments with 2-ME (0.1 to 10 μmol/L). Numbers indicate percentage values±SEM.

are synthesizing DNA actively, were detected by incorporation of BrdUrd. The scattergram shows that BrdUrd-positive cells (high FITC integral) were reduced from 12 to 48 hours in the presence of 1 and 10 μmol/L 2-ME (Figure 2A and Table). Cell distributions in cell cycle phases according to their DNA contents are displayed as histograms in Figure 2B. The histogram shows that treatment with 0.1 μmol/L 2-ME did not cause a significant change in the cell cycle distribution compared with the control. In contrast, treatment with 10 μmol/L 2-ME resulted in accumulation of cells with ≥4N DNA content (4N cells) as early as 6 to 12 hours, which was more significant at 24 to 48 hours (Figure 2B and Table 1). The increase in 4N cells coincided with a decrease in 2N cells. However, treatment with 1 μmol/L 2-ME only caused a temporary (at 6 to 12 hours) increase in cells with ≥4N DNA content. Cell cycle profiles in response to 2-ME are summarized in the Table. Note that the decrease in the S phase (BrdUrd-positive) cells in response to 2-ME treatment was concentration and time-dependent. The accumulation of cells with ≥4N DNA content in the presence of 10 μmol/L 2-ME was also time-dependent. These results suggest that 2-ME arrests cells at cell cycle stages with ≥4N DNA content.

2-ME Induces Cell Cycle Arrest in the Mitotic Phase

Cells with ≥4N DNA content could be in the G2 phase, mitotic phase, or postmitotic phases (tetrploidy). To evaluate whether 2-ME induces cell cycle arrest at mitosis, we stained cells with phosphohistone H3 (serine 10), a mitotic marker, and DAPI, a DNA-specific dye. Mitotic cells, containing condensed DNA and positive for phosphohistone H3, were significantly increased in the presence of 1 to 10 μmol/L 2-ME up to 24 hours (Figure 3). In response to 2-ME treatment (1 to 10 μmol/L), mitotic cells began to increase at 3 hours of treatment, reached a maximum at 6 hours, and then slowly declined but remained above the untreated control (Figure 3). However, treatment with 0.1 μmol/L 2-ME only temporarily (at 3 to 6 hours) induced a small increase in mitotic cells (Table). Summarized results showed that in the presence of 10 μmol/L 2-ME, the mitotic index was 22±3% after 6 hours of treatment, then decreased to 12±2% and 8±2% at 24 hours and 48 hours, respectively. Note that in the presence of 10 μmol/L 2-ME for 24 hours and 48 hours, cells with ≥4N DNA content were 47±4% and 60±5% (Table); therefore, not all 4N cells are mitotic cells. The decline in mitotic index coincident with the accumulation of 4N cells after 24 hours and 48 hours of treatment indicates that a fraction of the mitotic cells might have undergone mitotic slippage.

2-ME Induces Mitotic Slippage and Endoreduplication, Which Is Associated With an Increase in CDK2 and a Reduction in p27 Expression

To evaluate whether accumulation of 4N cells was because of mitotic slippage, BrdUrd pulse-chase assays were carried out to trace changes of the cell cycle position of BrdUrd-positive cells after S phase as described in the Methods section. As shown by the representative results (Figure 4A), in the control groups, the majority of BrdUrd-positive cells started to arrive at the 2N region after 6 hours and significantly accumulated at the 2N region after 12 hours, indicating that those cells had already finished cell division and become daughter cells in G1 phases. At 24 hours, some of the daughter cells had entered into the G2/M phase of the next cell cycle as they appeared at the 4N region. In contrast, in the groups treated...
with 1 and 10 μmol/L 2-ME, most of the BrdUrd-positive cells had arrived at the 4N region by 6 hours, and stayed there until 24 hours. After 24 hours of treatments, the majority of BrdUrd-positive cells left for the 2N region in the group treated with 1 μmol/L 2-ME but not in the group treated with 10 μmol/L 2-ME. These results suggest that, in response to 2-ME, cells underwent mitotic slippage after the exit from mitotic arrest.

In order to determine whether mitotic cells pursue endoreduplication, we next searched for aneuploid cells that continually synthesized DNA in response to 2-ME. In the cells treated with 1 to 10 μmol/L 2-ME for 24 hours, BrdUrd was added for 1 hour to label S-phase cells. We observed that some of the aneuploid cells were BrdUrd positive (Figure 4B, arrows), indicating that 2-ME treatment induces endoreduplication in human SMCs. Because not every 4N cell could be distinguished by microscopy, we were unable to quantify how many 4N cells were in the S phase. In searching for other supportive evidence, we examined the expressions of the cell cycle regulatory proteins cyclins D3, B, A, and E; CDKs 1 and 2; and the CDK inhibitor p27. Representative results from 4 sets of independent experiments showed that CDK2 was increased significantly in response to 1 and 10 μmol/L 2-ME. The increase in CDK2 protein level was concurrent with a decrease in p27 expression (Figure 4C). The expression levels of CDK1 and cyclins A, B, E, and D3 were not changed by the presence of 2-ME.
Figure 5. Concentration-dependent effects of 2-ME on the microtubule network, mitotic spindle, and centrosome integrity. Human SMCs were treated with 2-ME at the concentrations indicated for 24 hours, fixed with 100% methanol, and then stained with anti-α-tubulin and PI (A). The microtubule network was examined by fluorescence microscopy. After exposure to 2-ME for 1 hour, cells were fixed and stained with anti-α-tubulin, anti-γ-tubulin, and DAPI. The images for the mitotic spindle, centrosome, and chromosome of cells in mitosis and cytokinesis were acquired by confocal microscopy (B). Cells in interphase depicted (C) were examined after treatment with 2-ME for 12 hours. Microtubule network and mitotic spindles are in green; centrosomes are red; chromosomes are blue.
2-ME Induces Damage of Mitotic Spindle, Impact of Centrosome and Chromosome Integrity, and Aberrant of Cytokinesis

To explore the cellular mechanism underlying the mitotic arrest induced by 2-ME, given that 2-ME can inhibit tubulin polymerization and disrupt microtubule function, we examined the effects of 2-ME on the microtubule network, mitotic spindle, and centrosome integrity by detection of immunofluorescent signals of α-tubulin and γ-tubulin, a centrosomal component. Representative results showing the effects of 2-ME on the microtubule network in response to 2-ME are illustrated in Figure 5A. The microtubule network remained intact in the presence of 0.1 and 1 μmol/L 2-ME. However, after treatment with 10 μmol/L 2-ME, the microtubule network exhibited a disorganized structure, distributing at the membrane region and perinuclear cytoplasmic regions. The gap between those 2 regions indicates tubulin disrupted by 2-ME. In addition, note that mitotic microtubule bundles in the presence of 0.1 μmol/L 2-ME displayed bipolar spindle morphology. In contrast, in the presence of 1 μmol/L 2-ME, the mitotic microtubule appeared as irregular multipolar bundles. The effects of 2-ME on the mitotic spindle at the early stage were additionally examined by confocal microscopy. Representative confocal images of cells incubated in the absence and presence of 2-ME for 30 minutes to 1 hour are shown in Figure 5B. Control mitotic cells have normal bipolar spindles, 2 pairs of centrosomes each of which attach to a spindle pole, and chromosomes aligned to the metaphase plate. Treatment with 0.1 μmol/L 2-ME did not cause an obvious change in mitotic cells. However, after treatment with 1 μmol/L 2-ME, mitotic cells displayed irregular multipolar spindles, metaphase plate, and disoriented chromosome, concurrent with the appearance of multipolar centrosomes, which are still attached to the poles. In cells treated with 10 μmol/L 2-ME, the mitotic spindle and metaphase plate were disrupted completely; centrosomes appeared in the

Figure 6. Mitotic cells undergo apoptosis in response to 2-ME. Live human SMCs, incubated with different concentrations of 2-ME for 6 hours, were stained with Annexin V, PI, and Hoechst for 10 minutes and inspected by fluorescence microscopy. For the bottom 3 panels, cells were pretreated with thymidine for 12 hours before addition of 2-ME. Images represent 3 sets of independent experiments (A). White arrows indicate Annexin V positive/PI negative (early apoptotic cells). Cells, treated with and without 2-ME for 6 hours, were fixed with 4% paraformaldehyde and detected with antiphosphohistone H3 and TUNEL assay (B). DNA was counterstained with DAPI. Note the excellent agreement of the signals for antiphosphohistone H3 with TUNEL staining. Percentages of mitotic cells and corresponding TUNEL-positive cells were displayed (C). For each coverslip, ≥500 cells were counted. **Increases in mitotic index are significant when compared with the control (P<0.001; n=4). *Differences in TUNEL-positive cells between the 2-ME–treated groups and control were significant (P<0.001, n=4). #, ##P<0.001 when comparing groups treated with 10 μmol/L 2-ME with groups pretreated with thymidine (n=4).
middle of the chromosomes, which seemed improperly packed. In the cytokinesis process, the control cells divided into 2 equal daughter cells, each of which contains a centrosome, a chromosome, and a bundle of mitotic spindles connecting to the midbody. In the presence of 0.1 μmol/L 2-ME, cells were still able to divide into 2 equal daughter cells, but 1 of them has 2 centrosomes, whereas the other has only 1. In response to 1 μmol/L 2-ME, cells divided multipolarly with multiple spindles, overamplified centrosomes, and improperly segregated chromosomes. Damaged spindle bundles were linked to the midbody. One daughter cell has 2 chromosomes and 3 centrosomes, whereas the other has 3 centrosomes without a chromosome. In the cells treated with 10 μmol/L 2-ME, spindles were completely disrupted, chromosomes asymmetrically segregated or failed to segregate, and the midbody was missing. In this case, if cytokinesis occurred, 1 daughter cell would have double the number of chromosomes, whereas the other would have none. Indeed, we observed the appearance of cleaved cells with a centrosome but without a chromosome (data not shown). Furthermore, after treatment with 2-ME for 1 hour, the interphase cells did not exhibit abnormal microtubule spindle, centrosomal, and chromosomal structure (data not shown). Studies were carried out to inspect the interphase cells in the groups exposed to 2-ME for 12 hours, when 4N cells were accumulated and mitotic cells declined. As shown in Figure 5B (right), the interphase cells in the 0.1 μmol/L 2-ME–treated group seemed to have normal microtubules, centrosomes, and chromosomes. In contrast, in the 1 to 10 μmol/L 2-ME–treated groups, the interphase cells were multinuclear, containing multiple centrosomes.

**Mitotic Arrest Cells in Response to the Treatment of 2-ME Undergo Apoptosis**

Based on the finding that human SMCs displayed a DNA laddering pattern in response to 2-ME treatment (Figure 1B) to further characterize the apoptotic effect induced by 2-ME, we evaluated apoptosis in live cells (Figure 6A) with Annexin V and propidium iodide (PI) staining. To visualize all of the cells, Hoechst dye, a cell-permeable DNA dye, was also applied during the staining. The results show that in the presence of 2-ME (1 and 10 μmol/L) for 6 hours, cells that had condensed DNA as detected by Hoechst dye were Annexin V-positive and/ or PI positive, suggesting that these mitotic cells were apoptotic. In addition, to see whether blockage of entry into mitosis would affect apoptosis, we pretreated cells with thymidine for 12 hours to synchronize the midbody at the boundary of G1 to S phase and then added 10 μmol/L 2-ME in the presence of thymidine for another 6 hours. Preincubation of thymidine for 12 hours, which arrested 93% of cells in the G10 phase (data not shown), significantly reduced the Annexin V– and PI–positive cells induced by 10 μmol/L 2-ME (Figure 6A). Interestingly, the cells with multiple nuclei were Annexin V and PI negative when treated with 2-ME for 6 hours (data not shown), implying that the aneuaplloid cells are nonapoptotic at this stage. Next, in order to confirm that the mitotic cells were apoptotic, we stained the cells with both phosphohistone H3 (a mitosis marker) and TUNEL (an apoptosis marker). The nuclei were counterstained with DAPI. The increased mitotic cells identified by both DAPI and phosphohistone H3 stainings were in excellent agreement with those positive for TUNEL staining in the presence of 2-ME (Figure 6B). Our accumulated data showed that 0.1 μmol/L 2-ME increased TUNEL-positive cells, although there was no significant DNA fragmentation detected (Figure 1B). The TUNEL-positive cells after treatment of 10 μmol/L 2-ME were significantly reduced in the group synchronized in G10 phases by thymidine (Figure 6C). In addition, the presence of thymidine prevented the reduction of p27 and increase of CDK2 in response to 2-ME treatment (data not shown). Quantitative data of the percentage of TUNEL-positive cells with concurrent mitotic index in response to 6 hours of 2-ME treatment at different concentrations are shown in Figure 6C. Note that in response to 2-ME treatment, the increase in mitotic cells coincided with the induction of apoptosis. The presence of thymidine completely inhibited apoptosis induced by 2-ME.

**Discussion**

Antiproliferative effects of 2-ME have been found in a variety of cell types, including human breast cancer, myeloma, and endothelial cells. The process by which 2-ME affects cell growth may involve arresting cells in G2/M and inducing apoptosis. In human aortic SMCs, it has been shown that 2-ME mediated the inhibitory effects of estradiol on cell proliferation and DNA synthesis independent of estrogen receptor. It has also been reported in an abstract that 2-ME induced both G2/M and G10 arrest based on flow cytometry data. However, the precise cell cycle phase at which 2-ME arrests human SMCs has not been documented. Our data demonstrate the sequential events that occur in human SMCs in response to 2-ME. In the presence of 2-ME (1 to 10 μmol/L) for as little as 30 minutes to 1 hour, the mitotic spindle was damaged, and centrosomal and chromosomal integrity were affected. Exposure of cells to 2-ME for 1 to 3 hours led to aberrant cytokinesis. Subsequently, increases in mitotic cells were detected after 3 to 6 hours of treatment, which coincided with apoptosis in mitotic cells detected with the TUNEL assay. Additionally, mitotic cell apoptosis induced by 2-ME was prevented by synchronization of cells in G10 with thymidine. These results suggest that the antiproliferative effect of 2-ME is through induction of cell cycle arrest in mitosis and promotion of mitotic cell apoptosis, and the cellular mechanisms underlying 2-ME–induced effects involve rapid disruption of the mitotic spindle and centrosomal integrity. Along with evidence that 2-ME can interact with tubulin directly to inhibit tubulin polymerization, it is possible that 2-ME activates the spindle checkpoint and induces the onset of apoptosis in mitotic cells. However, how activation of the spindle checkpoint promotes apoptosis and whether phosphorylation of histone H3 (ser 10) during mitosis plays a role in mitotic cell apoptosis are unknown, although phosphorylation of histone H2B at serine 14 by mammalian Mst1/Mst4 kinase has been linked to mitotic cell apoptosis. Given that aurora kinases can phosphorylate histone H3 at both serine 10 and serine 28, it will be interesting to see whether aurora kinases play a role in mitotic cell apoptosis induced by 2-ME.

It has been shown that most mammalian cells undergo adaptation of the mitotic spindle checkpoint in the presence of microtubule-disrupting agents. Once cells adapt to the
spindle checkpoint, they can aberrantly exit from mitosis and enter interphase in a tetraploidy state. In our studies, the induction of mitotic arrest and aberrant cytokinesis by 2-ME may contribute to the accumulation of 4N cells observed after 6 to 12 hours of treatment. The significant accumulation of 4N cells after 24 to 48 hours of treatment with 10 μmol/L 2-ME may result from the processes of mitotic slippage and endoreduplication. This is supported by the following findings: (1) declined mitotic index occurs concurrent with the accumulation of 4N cells; (2) BrdUrd-positive cells delayed entry into the next cell cycle and turned into aneuploid or micronuclear cells; and (3) some aneuploid cells continued to synthesize DNA. In addition, the increase in CDK2 expression and the reduction of p27 in response to 2-ME may also support the occurrence of endoreduplication. This observation is consistent with the finding that loss of CDK inhibitors p21 and p27 results in upregulation of CDK2 activity during endoreduplication induced by mitotic spindle disruption.7 Another possibility is that CDK2 plays a role in apoptosis independent of its roles in the normal cell cycle, as observed in cardiomyocyte apoptosis.18 Furthermore, our studies suggest that polyploidy or multiple nuclei could arise from more than two different mechanisms: multipolar cleavages with uneven amounts of chromosomes as seen in cells treated with 1 μmol/L 2-ME or failure of chromosome segregation because of complete disruption of the mitotic spindle as seen in cells treated with 10 μmol/L 2-ME. Therefore, after mitotic slippage, 4N cells induced by 1 μmol/L 2-ME only temporarily increased. Because of uneven chromosomal segregation, the daughter cells could have <4N DNA content. In contrast, the chromosome was not segregated in response to 10 μmol/L 2-ME, and 4N cells could propagate as tetraploid/polyploidy cells.

In response to 2-ME, mitotic slippage and endoreduplications leading to the formation of polyploidy and aneuploidy could potentially increase the genomic instability of vascular SMCs. Aneuploidy has been documented to be a characteristic of a variety of human tumors,17 and genetic instability of aneuploid cells has been proposed to contribute to the development of cancer.19 In addition, polyploidization has been reported to be associated with hypertrophic vascular SMCs in hypertensive animals and patients.20,21 An increase in apoptosis of vascular SMCs is also documented in the arterial wall of hypertension.22 Therefore, it will be important to determine whether 2-ME effects on vascular SMCs could contribute to the pathogenesis of vascular hypertensive disease.

The effective concentration of 2-ME for inhibiting human SMC proliferation and inducing apoptosis in the present study was between 0.1 and 1 μmol/L, which is similar to the concentration used to inhibit endothelial cell growth and angiogenesis in vitro by others.23 These effective concentrations of 2-ME seem much higher than that in normal human plasma,8 which is in the range of picomolars to tens of nanomolars. However, it has been found that vascular SMCs from one origin can more effectively metabolize estradiol to generate 2-ME than those from another.24 Note that 2-ME has been used at the concentrations >1000 mg/day in participants in clinical trials.25 It is possible, therefore, that lipophilic 2-ME might reach higher concentrations in some cellular regions than in the plasma. In addition, the finding that the rapid effect of 2-ME is to induce mitotic cell arrest and apoptosis through impact on the mitotic spindle, whereas SMCs usually remain in a quiescent cell cycle stage in intact vessels, raises an important question of whether 2-ME affects vascular SMCs in vivo. As we know, under certain pathophysiological conditions, such as atherosclerosis and restenosis, vascular SMCs are highly proliferative. Inhibition of cell cycle progression has been found to prevent the development of these diseases.1 Therefore, it will be important to examine the effects of 2-ME in models of postangioplasty restenosis and atherosclerosis in vivo. Under in vivo condition, the concentration of 2-ME could be much lower, and it is also possible that the mechanisms of antiproliferative actions of 2-ME, if any, may be through other mechanisms independent of apoptosis. Our in vitro study indicates that 0.1 μmol/L 2-ME could induce an abnormal centrosome number in cells after cytokinesis (Figure 5). As documented, aberrant centrosome itself can cause genomic instability and tumor progression.26 However, we cannot determine whether the lower concentrations of 2-ME have nonantiproliferative actions, because it has been reported recently that 2-ME stimulated the growth of breast cancer cells through activation of the estrogen receptor.27 Taken together, our data have demonstrated the antiproliferative effect of 2-ME on human vascular SMCs in vitro, which certainly allows us to better understand the pathological roles of 2-ME.

Acknowledgments

This work was supported by a grant from the Heart and Stroke Foundation of Alberta (to X.-L. Z.), X.-L. Z. and Y.G. are recipients of a New Investigator Award and a Postdoctoral Fellowship Award, respectively, from the Heart and Stroke Foundation of Canada. We are very grateful to Dr Michael P. Walsh for advice during the course of this work and in preparation of the article and for providing the LSC through his Tier I Canada Research Chair and associated equipment grant from the Canada Foundation for Innovation with matching funds from Alberta Innovation and Science Research Infrastructure, the Alberta Heritage Foundation for Medical Research, and the University of Calgary.

References


2-Methoxyestradiol Induces Cell Cycle Arrest and Mitotic Cell Apoptosis in Human Vascular Smooth Muscle Cells
Yu Gui and Xi-Long Zheng

Hypertension. 2006;47:271-280; originally published online December 27, 2005;
doi: 10.1161/01.HYP.0000199656.99448.dc
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/47/2/271

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2006/01/30/01.HYP.0000199656.99448.dc.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/