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# 2-Methoxyestradiol, an Endogenous Metabolite of Estrogen, Enhances Apoptosis and $\beta$-Galactosidase Expression in Vascular Endothelial Cells

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**2-Methoxyestradiol (2ME) is an endogenous metabolite of estradiol (E2) and is known to inhibit tumor angiogenesis. In the present study, the direct effects of 2ME on the vascular endothelial cells were examined. 2ME enhanced apoptosis and $\beta$-galactosidase expression in bovine vascular endothelial cells. A nitric oxide (NO) donor S-nitroso-N-acetyl penicillamin (SNAP) also enhanced $\beta$-galactosidase expression, suggesting a possible role of NO in mediating the action of 2ME. 2ME increased the cellular content of nitric oxide synthase (NOS) and the production of NO. In addition, 2ME altered the membrane localization pattern of NOS. These suggest that the effects of 2ME on apoptosis and senescence of vascular endothelial cells were mediated, at least partly, by NOS and NO.** 

It has been reported recently that 2-methoxyestradiol (2ME), an endogenous metabolite of estrogen, inhibits angiogenesis and thereby suppresses the growth of tumors (1, 2). 2ME is known to be an inhibitor of tubulin polymerization, and inhibits tumor angiogenesis probably by causing cell cycle arrest in vascular endothelial (VE) cells. However, it is not known whether this compound induces apoptosis and/or cellular senescence in addition to the cell cycle arrest. Apoptosis is a cellular mechanism by which those cells suffered from severe DNA damage are eliminated (3). Cellular senescence reflects a gradual process in which after a particular number of cell doubling, a cell eventually loses replicative capacity (4, 5). Both of them are thought to be tumor-suppressive mechanisms. In the present study, therefore, we examined the effects of 2ME on the apoptosis and senescence of bovine VE cells.

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## MATERIALS AND METHODS

*Materials.* Estradiol (E2) and 2ME were obtained from Sigma Chemical Co. (St. Louis, MO). Anti-endothelial nitric oxide synthase (NOS) antibody, S-nitroso-N-acetyl penicillamin (SNAP) and 5-bromo-4-chloro-3-indoyl-$\beta$-D-galactopyranoside (X-gal) were purchased from Wako Pure Chemicals, Tokyo.

*Cell culture.* VE cells were prepared from bovine carotid arteries as reported (6). $5\times10^5$ of bovine VE cells were cultured for different periods of time in 5 ml of Dulbecco' s modified Eagle' s medium (DMEM) + 5% fetal bovine serum (FBS) with or without reagents. Since DMEM contains a estrogenic compound phenol red as a pH indicator, some of the experiments were carried out using phenol red-free DMEM (7).

*Apoptosis.* The cells cultured as described above were assayed for apoptosis by fluorescent microscopy following staining with acridine orange and ethidium bromide, as described by Piazza et al. (8). In some experiments, apoptosis was analyzed by the level of fragmented DNA contained in cell lysates following treatment with 1 $\mu$g/ml of 2ME. After 3 to 4 days of culture with 1 $\mu$g/ml of 2ME, the DNA was extracted by phenol method and subjected for electrophoresis using 2% agarose gel. Molecular size marker was $\phi$X174 Hae III fragment.

*The expression of $\beta$-galactosidase.* Cells were washed in phosphate buffered saline, fixed for 5 min at room temperature in 2% formaldehyde/0.2% glutaraldehyde, washed, and incubated at 37°C with fresh senescence-associated $\beta$-galactosidase staining solution: 1 mg/ml of X-gal/40 mM citric acid/sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/ 2 mM $MgCl_2$. Staining was evident in 2 - 4 hr and maximal in 12 - 16 hr (4).

*NO production.* Cells were cultured in phenol red-free culture medium with 1 $\mu$g/ml of 2ME or E2 for 48 h. The supernatants were collected and stored at −20°C until the nitrite content was analyzed. The culture medium contained 0.4 mM L-arginine, but to all cell cultures 1 mM L-arginine (Sigma) was added 24 hours before the harvest. Nitrite was detected by mixing 0.1 ml of Griess reagent (9). After incubation for 30 minutes in the dark at room temperature, the absorbance was measured at 540 nm.

*Cellular content and localization of NOS.* The cells were cultured for 4 days with or without 1 $\mu$g/ml of E2 or 2ME and were fixed with glutaraldehyde and formalin. NOS was quantitated using rabbit anti-NOS antibody, biotinylated anti-rabbit IgG, avidin-biotinylated horse radish peroxidase and $H_2O_2$ and 2,2′-azino-di-[3-ethyl-benzthizoline sulfonate] (ABTS). The light absorbance was measured at 402 nm. To analyze the subcellular localization pattern of NOS, it was



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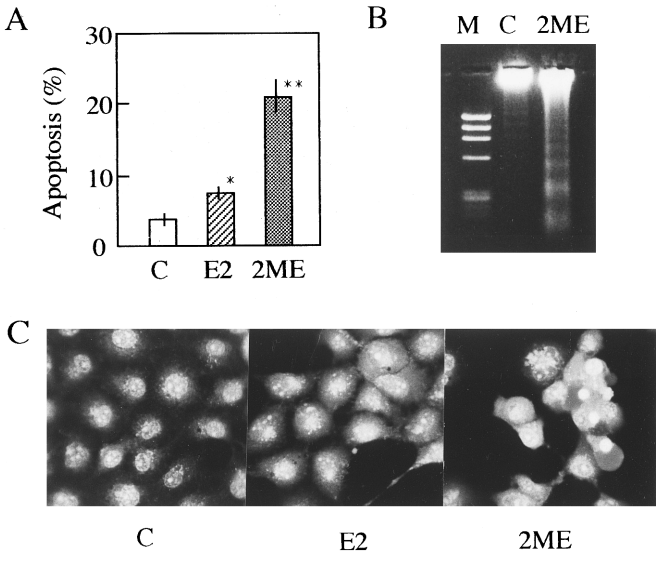


**FIG. 1.** Effects of 2ME on the apoptosis of VE cells. (A) Apoptosis of VE cells cultured for 4 days with or without 1 $\mu$g/ml of E2 or 2ME. (B) Fragmentation of DNA of VE cells cultured with or without 1 $\mu$g/ml of 2ME. (C) Nuclear morphology of VE cells cultured for 4 days with 1 $\mu$g/ml of 2ME. (200×) C, control; E2, estradiol; 2ME, 2-methoxyestradiol. *$p<0.05$; **$p<0.01$. (n=3).

visualized by immunocytochemical method using rabbit anti-NOS antibody, biotinylated anti-rabbit IgG antibody, avidin-biotinylated alkaline phosphatase, nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (10). Nuclei were stained with Mayer's hematoxylin solution.

## RESULTS

The growth of VE cells was inhibited by 20% and 80% after four days of culture with 1 $\mu$g/ml of E2 and 2ME, respectively. E2 had no remarkable effect on the morphology of VE cells. In contrast, 2ME-treated cells became enlarged and flattened, some of which were multinucleated. In addition, fragmented and/or micronuclei typical to apoptotic cells appeared. DNA ladder typical to apoptosis was also recognized on agarose gel electrophoresis. These indicate that apoptosis accounts for 2ME-inhibition of cell proliferation, at least partly (Fig. 1).

However, the remarkable anti-proliferative effect of 2ME on VE cells might not be explained solely by apoptotic cell death, and a possible involvement of other mechanisms such as senescence in the 2ME action was suggested. To clarify the latter possibility, $\beta$-galactosidase, a biomarker of senescence, was stained with X-gal (4). The $\beta$-galactosidase-positive VE cells increased gradually beyond the 5th passage in *in vitro* culture. 2ME significantly increased the ratio of $\beta$-galactosidase positive VE cells during the 5th to 7th passage (Fig. 2A). In the culture with SNAP, the VE cells became elongated and fibroblast-like, some of which were

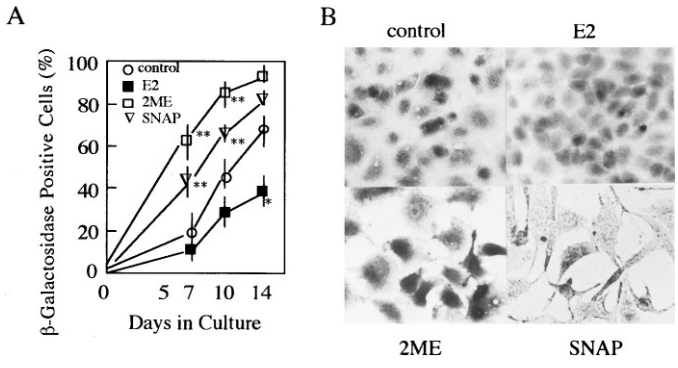


**FIG. 2.** Effect of E2 and 2ME on the expression of $\beta$-galactosidase. VE cells were cultured for up to 14 days with or without E2 (1 $\mu$g/ml), 2ME (1 $\mu$g/ml) or SNAP (10 $\mu$g/ml ), and were stained with X-gal. (A) Ratio of $\beta$-galactosidase positive cells. (B) Immunocytochemical staining of $\beta$-galactosidase of VE cells cultured with or without E2, 2ME and SNAP (200×).

positively stained with $\beta$-gal (Fig. 3C). In contrast, E2 reduced the ratio of $\beta$-galactosidase positive cells.

Tumor growth, angiogenesis, and cellular senescence, may be related with NO (11). Therefore, the effects of 2ME on NO production and cellular NOS content were investigated. 2ME increased both cellular NOS content and NO production (Fig. 3A and B). In addition, immunocytochemical study using the antibody disclosed that 2ME altered the membrane localization of NOS. As shown in Fig. 3C, NOS was seen at the cell boundary in control culture, while this localization pattern disappeared in 2ME-treated cells.

DMEM has been reported to be estrogenic, which appears to be due to phenol red used as a pH indicator (7). In fact, the number of VE cells cultured in a phenol red-free DMEM was approximately 40% less than that of cells in conventional DMEM. However, in either the conventional or phenol red-free medium, lower concen-

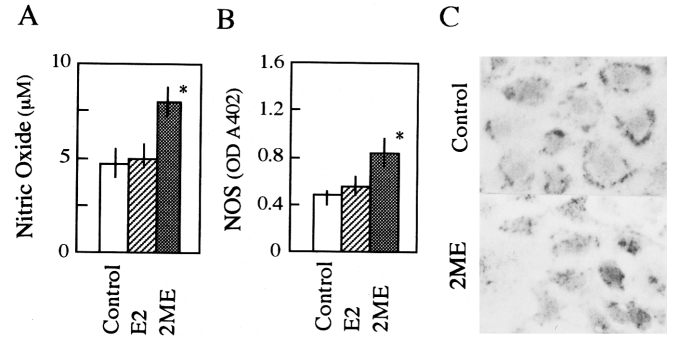


**FIG. 3.** Effects of 2ME on the production of NOS and NO and the subcellular localization of NOS. VE cells ($1 \times 10^6$) were cultured for 2 days with or without 1$\mu$g/ml of E2 or 2ME. (A) NO in the culture media was measured with Griess reagent. (B) NOS in the VE cells was quantitated with specific anti-NOS as described in Methods. *$p<0.05$ (n=3). (C) NOS was visualized by immunocytochemical method as described in Methods. (×200).

trations of E2 and 2ME (100 pg/ml and 100 ng/ml) had no significant effects on cell growth, apoptosis, $\beta$-galactosidase expression and NO production. These indicate that the 2ME effects became apparent only at pharmacological concentrations.

## DISCUSSION

Growth inhibition of VE cells by 2ME was associated with increases in apoptosis and $\beta$-galactosidase expression. Seegers et al. reported that 2ME (0.3 to 0.6 $\mu$g/ml) induced apoptosis in transformed cells but not in normal human fibroblast cell line (12). In contrast, Fotosis et al. reported that 2ME inhibited the growth of non-malignant VE cells (2). In our present experiment, 2ME induced apoptosis of normal VE cells. These indicate that rapidly growing normal and tumor cells are susceptible to 2ME. On the other hand, Syridopoulos et al. reported that E2 inhibited TNF$\alpha$-induced apoptosis. In their experiment, however, E2 itself did not inhibit apoptosis. Therefore, their results did not always contradict with our present data (13). The molecular mechanism of 2ME-induction of apoptosis remains to be studied. Although 2ME is known to be an inhibitor of tubulin polymerization like colchicin, it is not clear how this 2ME action connects with the activation of proteolytic enzymes such as caspases which activate nucleases and degrades cellular constituents (14–16).

It is likely that $\beta$-galactosidase expression reflects one of the changes in cell function that invariably accompanies senescence (17). p53 tumor suppressor triggers senescence program in tumor cells, suggesting that genes regulated by p53 may be playing important roles in 2ME-induction of $\beta$-galactosidase expression (17). The nature of p53-inducible genes is not clarified and the mechanism of the 2ME-stimulation of $\beta$-galactosidase expression remains to be studied.

NOS gene has binding sequence for estrogen receptor (18). However, since the affinity of 2ME to the receptor is almost negligible, the 2ME-enhancement of NOS expression is likely to be mediated by estrogen receptor-independent, post-transcriptional mechanisms (15, 16). This speculation may be supported by the recent report in which the release of bioactive NO was increased by inhibiting superoxide anion production (19). On the other hand, Hishikawa et al. and Hayashi et al. reported that NOS was upregulated by estrogen through estrogen receptor-mediated mechanisms (20, 21). In our study, E2 had no remarkable effect on NO and NOS production (Fig. 3). The exact reason for this discrepancy is not known. In their experiments, however, biologically active molecules such as ionomycin and fibroblast growth factor were used simultaneously with estrogen, and these differences in experimental conditions may be responsible for the discrepancy, at least partly (20, 21).

On the other hand, 2ME alters the membrane localization pattern of NOS (Fig. 3). This 2ME effect can be explained by its inhibitory effect on tubulin polymerization (15, 16). NOS, localized at the caveolae of the plasma membrane, produces NO which may have physiological effects on the biologically active constituents of caveolae (16, 22). In contrast, NOS localized at different part of membrane produces NO, which may be toxic to the cells and may trigger apoptosis (10, 23). It is also conceivable that the altered localization of NOS may cause changes in the functional proteins such as ras and src. These may facilitate the process of cellular senescence (24, 25).

As shown in this study, 2ME can induce apoptosis and senescence in VE cells. These 2ME effects are associated with the altered localization pattern of NOS and increased production of NOS and NO, suggesting that cellular NOS content and NO production may be involved in the induction of apoptosis and senescence (23). These also indicate that 2ME is an useful anti-angiogenic and anti-cancer compound which can be used for the chemotherapy and chemoprevention of solid tumors.

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