Journal of Steroid Biochemistry & Molecular Biology 117 (2009) 74–80

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Antioxidant effect of estrogen on bovine aortic endothelial cells

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ARTICLE INFO

Article history:
Received 2 January 2009
Received in revised form 15 July 2009
Accepted 17 July 2009

Keywords:
Oxidative stress
Estrogen
Bovine aortic endothelial cell
Hydrogen peroxide
Apoptosis

ABSTRACT

Objective: This study discussed the role of estrogen as an antioxidant in the damage of vascular endothelial cells.
Design: We treated bovine aortic endothelial cells (bAEC) either with 1 mM of H_2O_2 alone or with 1 μM of 17β-estradiol (E_2) for 24 h followed by 1 mM of H_2O_2 for 3 h. The cell survival was evaluated by MTT assay, cellular apoptosis by fluorescence activated cell sorter (FACS) and Hoechst 33342 staining, oxidative stress by intracellular reactive oxygen species (ROS) and apoptosis after oxidative stress by western blotting for phospho-p38, p38, and Bcl-2.
Results: MTT assay showed that bAEC viability was reduced to 55.7 ± 3.0% and 39.1 ± 3.7% after 30 and 60 min of H_2O_2 treatment, respectively. E_2 and H_2O_2 treated cells did not show significant decrease in the cell survival. Similarly the FACS analysis and Hoechst 33342 stain showed that the latter decreased cellular apoptosis induced by H_2O_2. Intracellular ROS increased by 181.6 ± 68.9% in the former and by 37.0 ± 3.9% in the latter ($P < 0.05$). The expression of phospho-p38 mitogen-activated protein kinase (MAPK) was higher in the latter.
Conclusions: E_2 mediates antioxidant effects on the oxidative stress induced by H_2O_2. This antioxidant effect on bAEC may elucidate the scientific basis of hormone therapy for maintaining cardiovascular integrity in postmenopausal women.

1. Introduction

Postmenopausal women spend 1/3 of their life under the condition of female hormone deficiency, and acute and chronic symptoms of hormone deficiency appear in 75–85% postmenopausal women. Because of the prolongation of the average life-span these days, the health management of such postmenopausal women is a very important social issue.

In numerous clinical studies, the risk of cardiovascular diseases and osteoporosis due to postmenopausal hormone deficiency has been reported, and based on such reports, hormone replacement therapy has been administered [1–3]. According to numerous retrospective studies in the past, administration of estrogen to postmenopausal women lowered the incidence of cardiovascular diseases by 30–50% and in the combination therapy with added progesterone, a similar prophylactic effect has been detected [4,5]. In addition, it has been reported that female hormones mediate potent effects on the maintenance of the integrity of the vascular wall [6]. It has been reported that intravenous injection of 17β-estradiol prevented the peroxidation of low density lipoprotein and estrogen patch decreased the peroxide production in postmenopausal women. Thus hormone therapy in postmenopausal women has been shown to have an antioxidant effect [7]. It has been explained by antioxidant effects, relaxation of blood vessels, reduction of the formation of thrombi, improvement of plasma lipids, and improvement of the vascular endothelial cell-dependent vascular reaction level [8–11]. But its molecular biological mechanism has not been elucidated yet.

However, the Women's Health Initiative (WHI) trials sponsored by the US National Institutes of Health and scheduled to be completed in 2007, were discontinued in July 2002 because it has been reported that in the cases treated with the combination of estrogen–progesterone, the risk of breast cancer was increased as well as the incidence of coronary artery diseases, stroke, and the development of thrombi, and thus the risk was higher than the benefit [12]. The result of the WHI study was reported immediately by the press without further detailed analysis, and it was a great shock to postmenopausal women under hormone therapy or clinicians prescribing hormone therapy. However, upon the analysis of the research result of WHI, it was discovered that in healthy

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0960-0760/$ – see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.jsbmb.2009.07.006

aged women who underwent menopause a long time ago, hormone therapy was not effective on the prevention of newly developed cardiovascular diseases.

Nonetheless, based on studies on the vascular aging, although estrogen was not effective on preventing the thickening of the coronary artery endothelium in postmenopausal women, it reduced the frequency of the formation of thrombi by arteriosclerosis [13], and thus hormone therapy may delay the onset of cardiovascular diseases in women at the menopausal transitional period. Large scale studies on hormone therapies with new groups of patients are ongoing [14].

Cardiovascular disease is one of the leading cause of morbidity and mortality not only in the male but also in the female [15]. According to the 2004 statistics of the causality of death reported by the National Statistical Office, in Korean women, the death caused by vascular diseases reached 24.6% [16]. In premenopausal women, the ratio of the incidence of myocardiac infarction to the male in the same age group is 1:10, which is noticeably lower. After menopause the risk begins to be increased, and in the age of 70–80s, it is 1:1.5 which is almost comparable to the male. This suggests the fact that estrogen may mediate beneficial actions on the cardiovascular system, and such research results have been reported [2,17]. Therefore, numerous studies to characterize the mechanism causing cardiovascular diseases and the effect of hormones directly on blood vessels have been conducted.

It is well known that hydrogen peroxide (H_2O_2) is the main functional group of oxygen species. It induces oxidative stress by causing DNA damage and lipid peroxidation [18], separates cytochrome C from mitochondria, and thus induces cell death [19]. The MAP kinase signal transduction system is diversely involved in cell proliferation and cell death in response to stimulation [20,21]. The p38 MAP kinase has been reported to be involved in the survival of cells in differentiated adipocytes [22], and there is a contradictory study that in the white blood cell line HL-60 cells, it is involved in the apoptosis induced by ROS [23].

This study was conducted to examine the role of estrogen as antioxidants in cell injury caused by oxidative stimulation induced by hydrogen peroxide (H_2O_2), and to examine whether the phospho-p38 MAPK signal transduction system is involved in the reaction.

2. Methods

2.1. Cell culture and pretreatments

Bovine aortic endothelial cells (bAECs) were cultured with Dulbecco's Modified Eagle Medium (DMEM, Gibco/BRL, MD, U.S.A.) in a 37 °C, 5% CO_2 incubator.

To stimulate with oxidative stress, the control group was cultured with 1 mM H_2O_2 (Sigma Chemicals, St. Louis, MO, U.S.A.) alone, and for the estrogen treatment group, 1 μM 17β-estradiol (E_2) (Sigma Chemicals, St. Louis, MO, U.S.A.) was added and precultured 24 h prior to the stimulation with oxidative stress by 1 mM H_2O_2.

2.2. Oxidative stress and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) analysis

In 96-well plates, 1×10^4/ml bAEC cells were cultured, and the oxidative stress group was stimulated with 1 mM H_2O_2 (Sigma Chemicals) diluted with phosphate buffered saline (PBS) for 15, 30, 60 min, and the estrogen-treated group was pretreated with 1 μM 17β-estradiol (E_2) for 24 h, and after the pre-incubation, stimulated with oxidative stress by 1 mM H_2O_2 for 15, 30, 60 min.

The evaluation of sensitivity of cells to oxidants was performed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Colorimetric assay kit, Chemicon Inc., CA, U.S.A.) assay method that is a modification of tetrazolium-based colorimetric assay, and the light absorbance was measured at 450 nm.

2.3. Measurement of intracellular reactive oxygen species (ROS)

In 6-well tissue culture dishes, 5×10^4/ml bAEC cells were cultured, and exposed to 1 mM H_2O_2 for 3 h. To the estrogen-treatment group, 1 μM 17β-estradiol was added 24 h before the oxidative stress stimulation by 1 mM H_2O_2, and after the preculture, they were stimulated with oxidative stress by the same method.

Subsequently, cells were washed with PBS buffer solution twice, added 30 μM 2,7-dichlorofluorescin diacetate (DCF-DA) (Sigma Chemicals, St. Louis, MO, U.S.A.), incubated for 1 h in a 37 °C incubator containing 5% CO_2, and analyzed by flow cytometer (FACScan, Becton-Dickinson, Mountain View, CA, U.S.A.).

2.4. Morphological assessment of cell apoptosis

On 4-well chamber slides, 1×10^4/ml bAEC cells were cultured and exposed to 1 mM H_2O_2 for 3 h. The estrogen-treatment group was also exposed to H_2O_2 for 3 h after the pretreatment, similarly to the method 2. They were washed 2 times with PBS, fixed with 3.7% formaldehyde, washed again with PBS, added 10 μg/ml Hoechst 33342 (Sigma Chemicals, St. Louis, MO, U.S.A.), reacted in a dark room at room temperature for 1 h, and after washing, examined by immunofluorescence microscope.

2.5. Western blotting

2×10^5/ml bAEC cells were cultured in 100 mm^2 dishes, and stimulated with 30% H_2O_2 were diluted to 1 mM with PBS as oxidative stress for 3 h. The estrogen-treatment group was stimulated with oxidative stress for 3 h after the pretreatment by the identical method described above.

To examine the effect of suppression of phospho-p38 activity, the control group as well as the estrogen-treatment group were pretreated with 20 μM SB203580 (Sigma Chemicals) for 1 h, and stimulated with oxidative stress. Cells cultured under each condition were scraped and collected, centrifuged at 4 °C, 12,000 rpm, for 5 min, and the separated cells were lysed by reacting with 100 μl lysis buffer at 4 °C for 30 min. The cell lysate was centrifuged at 4 °C, 12,000 rpm to extract proteins, and the concentration of protein was measured by the Bio-Rad Protein Assay (Bio-Rad, Philadelphia, PA, U.S.A.). 50 μg protein was boiled for 5 min, and electrophoresed on 10% sodium dodecyl sulfate (SDS)/polyacrylamide gel at 100 V for 2 h. Subsequently, proteins were transferred to a nitrocellulose paper at 360 mA for 1 h, and the membrane was blocked for 1 h with 5% nonfat dried milk and 0.05% Tween 20 solution. As primary antibodies, phospho-p38, p38 MAP kinase (Thr180/Tyr182) (Cell signaling Technology, Beverly, MA, U.S.A.), and Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) antibody were used, and reacted with the membrane at 4 °C overnight. The membrane was washed with Tris-buffered saline and Tween 20 (TBST) 3 times, and each membrane was incubated with secondary antibody (Calbiochem, San Diego, CA, U.S.A.) for 1 h at room temperature, and washed with TBST for 3 times. Specific protein bands were obtained by the ECL Western blotting system (Amersham, Piscataway, NJ, U.S.A.). By applying β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), blots were standardized.

2.6. The analysis of apoptosis using FACS

Cells treated as described above were collected, washed with PBS, and resuspended with 100 μl Annexin V binding buffer (140 mM NaCl, 10 mM Hepes, pH 7.4, 25 mM $CaCl_2$). 5 μl Annexin V-FITC conjugate and 5 μl propidium iodide (PI) were added and reacted in a dark room for 15 min. 400 μl Annexin V binding buffer was added again, and analyzed by the FACScan (Becton-Dickinson).

2.7. Statistical analysis

Results were obtained by repeating the experiments described above 3 times, and all data were presented as the mean ± standard deviation, and the comparison of each groups was performed by the SPSS (SPSS Inc, Chicago, U.S.A.), and analyzed by *t*-test. The statistically significant level was $P < 0.05$.

3. Results

3.1. The antioxidant effect of estrogen on H_2O_2 in bAECs

The bAECs were treated with 1 mM H_2O_2 and the cell survival was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Colorimetric assay kit, Chemi-con Inc., CA, U.S.A.). In the H_2O_2 treated group, the cell survival rate was decreased to 55.7 ± 3.0% after 30 min treatment, and 39.1 ± 3.7% after 60 min treatment. The cell survival was decreased statistically significant in the group treated for 30 min, and the cell death was increased significantly in proportion to the duration of H_2O_2 treatment ($P < 0.05$). However, in the estrogen-pretreatment group, despite of the stimulation with H_2O_2, the cell number was decreased only to 64.3 ± 3.9% after 30 min treatment and 66.0 ± 4.8% after 60 min treatment. Cell survival rate was decreased in proportion to the duration of H_2O_2 treatment, but it was not statistically significant. In the comparison of the cell survival rates of the H_2O_2-treated group and the estrogen-H_2O_2 treated group according to the duration of H_2O_2 treatment, the cell survival rate was not significantly different after the treatment for 15 min, but, the cell survival rate was statistically significantly different after 30 and 60 min between two groups ($P < 0.05$) (Fig. 1).

3.2. The measurement of intracellular reactive oxygen species

After the treatment with 1 mM H_2O_2 for 3 h, ROS was substantially increased statistically significantly to 181.6 ± 68.9% ($P < 0.05$).

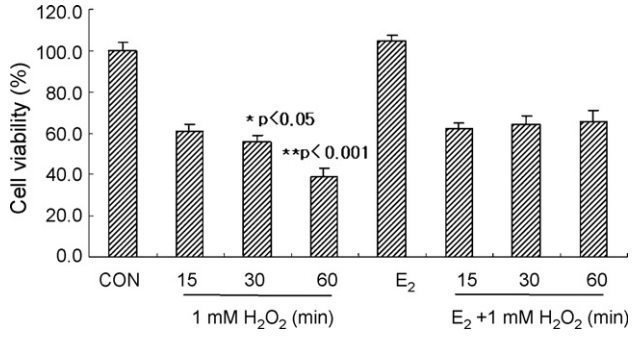

Fig. 1. 17β-estradiol inhibited H_2O_2 induced bAEC death. Cells were pretreated with 17β-estradiol (E_2) at the indicated concentrations for 24 h prior to incubation with 1 mM H_2O_2. Cell viability was measured as described in Section 2. The asterisks represent significant differences compared with control (*$P < 0.05$). When bAECs were treated with H_2O_2, cell viability was diminished time dependently. The asterisks also represent significant differences compared with 17β-estradiol pretreatment group (*$P < 0.05$). 17β-estradiol pretreatment effectively inhibited H_2O_2-induced cell death. Con, control.

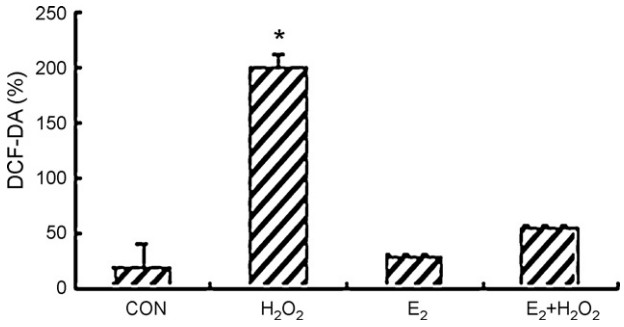

Fig. 2. ROS production in H_2O_2 treated bAECs. bAECs were incubated for 3 h with H_2O_2 in the absence or presence of 1 μM 17β-estradiol (E_2) for 24 h, and added DCF-DA to culture dish subsequently. After additional 1 h incubation, the fluorescence intensity of cells was measured by flow cytometric analysis. The experiment was repeated 3 times independently and the results were similar. The population of cells with DCF-DA fluorescence was elevated in the H_2O_2 treated group compared with that in the control group (*$P < 0.05$). Pretreatment of 17β-estradiol effectively inhibited intracellular ROS production. Con, control.

However, in the estrogen-pretreated group, it was detected to be 37.0 ± 3.9%. Thus, in the group pretreated with 1 μM 17β-estradiol (E_2) for 24 h and subsequently stimulated with 1 mM H_2O_2 oxidative stress, the result showed that it decreased ROS statistically significantly ($P < 0.05$) (Fig. 2).

3.3. The morphological change of bAECs by H_2O_2

In the control group, after the treatment with 1 mM H_2O_2 for 3 h, the number of bovine aortic endothelial cells was markedly decreased under microscope, and after the Hoechst 33342 staining, the typical apoptotic cell finding of the condensed nucleus was detected. In the estrogen-pretreatment group, smaller number of cells showed apoptotic findings under microscope in comparison to the control group (Fig. 3).

3.4. Western blot analysis

In the estrogen-treatment group, stimulated with the oxidative stress H_2O_2, the expression of phospho-p38 MAPK was increased more than in the control group stimulated with the oxidative stress H_2O_2, and it was decreased by the pretreatment with the phospho-p38 MAPK inhibitor SB203580 (Sb) (Fig. 4A). In addition, the Western blot showed the expression of Bcl-2 which is located on the downstream pathway of phospho-p38 MAPK. The Bcl-2 expression was decreased in the cases treated with H_2O_2 alone, but in the cases treated with H_2O_2 after the pretreatment with 17β-estradiol, the expression of Bcl-2 protein was not decreased (Fig. 4B).

3.5. The analysis of apoptosis after the treatment with SB203580

In response to H_2O_2 treatment, the apoptosis of bAECs was increased by 48.8 ± 2.4%, and when the cells were pretreated with 17β-estradiol, cell apoptosis was decreased by 3.5 ± 2.4%. Treated with 20 μM SB203580 for 1 h and subsequently treated with 17β-estradiol and H_2O_2, it was increased again to 23.6 ± 7.1% (Fig. 5).

4. Discussion

In recent studies, it has been reported that inflammatory diseases including cell injury caused by oxidative stress or cytokines, induced the death of endothelial cells by cellular apoptosis [24–26]. ROS such as peroxide negative ion, peroxide and hydroxyl radicals generated during the metabolic process and accumulated in the body, are converted to more potent oxidants such as hydroxyl sub-

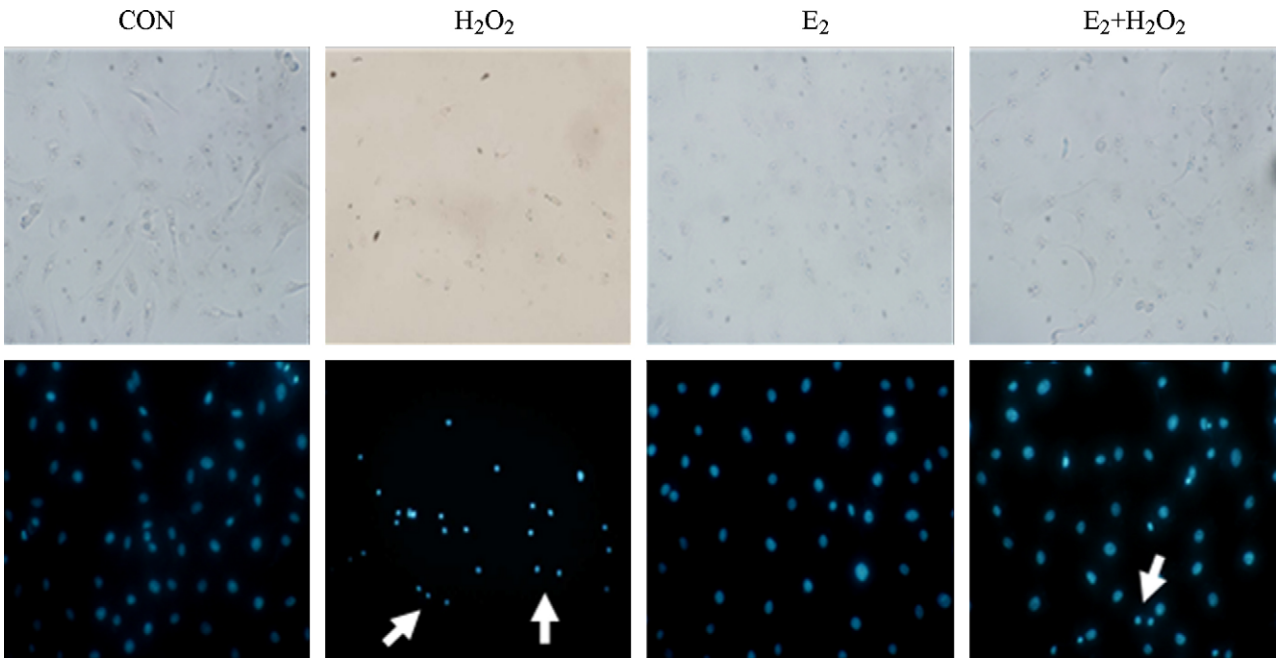

Fig. 3. Light microscopic finding and Hoechst 33342 staining of apoptosis induced by H_2O_2. Cells were exposed to 1 mM concentration of H_2O_2 for 3 h. (A) Light microscopic appearance of cells (200×). The cell density was markedly decreased due to detachment of dead cells after H_2O_2 treatment as compared with other groups. (B) Hoechst 33342 staining was used to detect the characteristic change of the apoptotic nuclear (arrow) morphology and was visualized under a fluorescence microscope (200×). When bAECs were treated with H_2O_2, cell apoptotic bodies and nuclear chromatin condensation (arrow) were observed. However, 17β-estradiol pretreatment reduced apoptotic bodies and nuclear chromatin condensation. Con, control.

stances, induce cell necrosis or apoptosis, DNA damages and cause substantial damages by oxidization of protein and lipid [27,28].

ROS also has been shown to be the substances that induced the abnormality in the nerve system such as Creutzfeldt-Jacob [29] and Alzheimer's disease [30]. Although it is not a disease, ROS induce DNA damages which are deeply involved in the aging process of the body [31]. Such ROS could be generated in mitochondria, leucocytes, peroxisomes, and the cytochrome p450 system. In normal individuals, the balance of ROS and antioxidants must be maintained, and the case with the increased active oxygen species by the disruption of such balance is referred to as the oxidative stress condition [32]. Recently, it has been considered that the elevation of such oxidative stress can cause heart diseases, hypertension, arteriosclerosis, malignant tumors, and degenerative diseases such as arthritis [33].

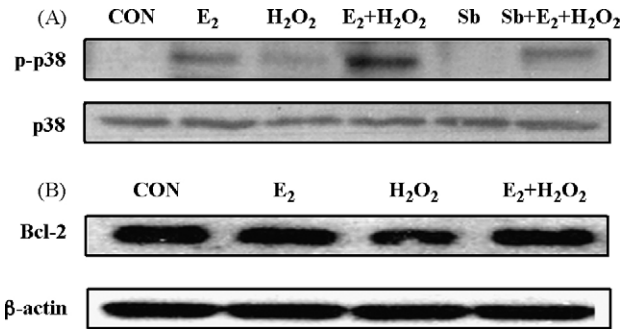

Fig. 4. Western blot analysis of p38 MAPK and Bcl-2 during H_2O_2 induced cell death with or without estradiol pretreatment. bAECs were incubated for 3 h with H_2O_2 in the absence or presence of 1 μM 17β-estradiol. Proteins were extracted for the western blots and probed with specific antibodies to ascertain the phosphorylation of p38 (A), Bcl-2(B). Cells were pre-incubated with or without 20 μM SB203580 (Sb, p38 inhibitor) for 1 h before treatment 1 μM 17β-estradiol. All experiments were repeated 3 times, and the data showed a typical representative result obtained from three independent experiments. Incubation of bAECs with pretreatment of 17β-estradiol demonstrated the increased expression of the phospho-p38 MAPK. The expression of anti-apoptotic Bcl-2 was decreased after H_2O_2 treatment. Con, control.

Until now, numerous studies have been conducted to prolong a life to the maximum life-span by increasing the antioxidant protective mechanism or decreasing ROS. Studies on antioxidants have been carried out actively, nonetheless, it is limited to the examination of its biological effects in many studies. Antioxidants that suppress the generation of ROS have been reported to suppress cell apoptosis [24,34,35]. In addition, it has been reported that in keratinocytes, apoptosis induced by the treatment with H_2O_2 was suppressed by 17β-estradiol, and the suppression of the chemokine, interferon-gamma-induced protein (IP-10), monocyte chemotactic protein-1 (MCP-1) and regulated upon activation, normal T-cell expressed, and secreted (RANTES) production were observed [36–38]. It has been reported that in mice performed bilateral oophorectomy, the concentration of lipid peroxides in the serum and the liver was elevated, and the increased concentration of lipid peroxides was decreased by hormone replacement therapy. Similarly, in women who underwent bilateral oophorectomy, the concentration of lipid peroxides was increased [39]. The antioxidant effects of estrogen on neurons and heart muscles have been reported, and, studies on its direct mechanism are ongoing [40–42]. Ejima et al. have reported the protective action of 17β-estrogen from oxidative stress on cultured bovine aortic endothelial cells, and estrogen showed antioxidant effects by increasing the antioxidant enzyme thiol/disulfide oxidoreductase. And estrogen which has structural similarity to tamoxifen or flavonoid, acts on antioxidative responsive element (ARE) resulting in the increase of protein disulfide isomerase (PDI) gene [43]. Dias-Flores et al. have reported that by significantly increasing the glutathion reductase level in the uterus, it mediates detoxification actions against oxidative stress [44]. In addition, in experiments using dogs, estrogen has been reported to have protective functions on myocardiac infarction [45].

In our study, efforts have been made to examine the antioxidant effect of 17β-estradiol on vascular endothelial cells. The effect of the pretreatment of 17β-estradiol on the oxidative stress of bovine aor-

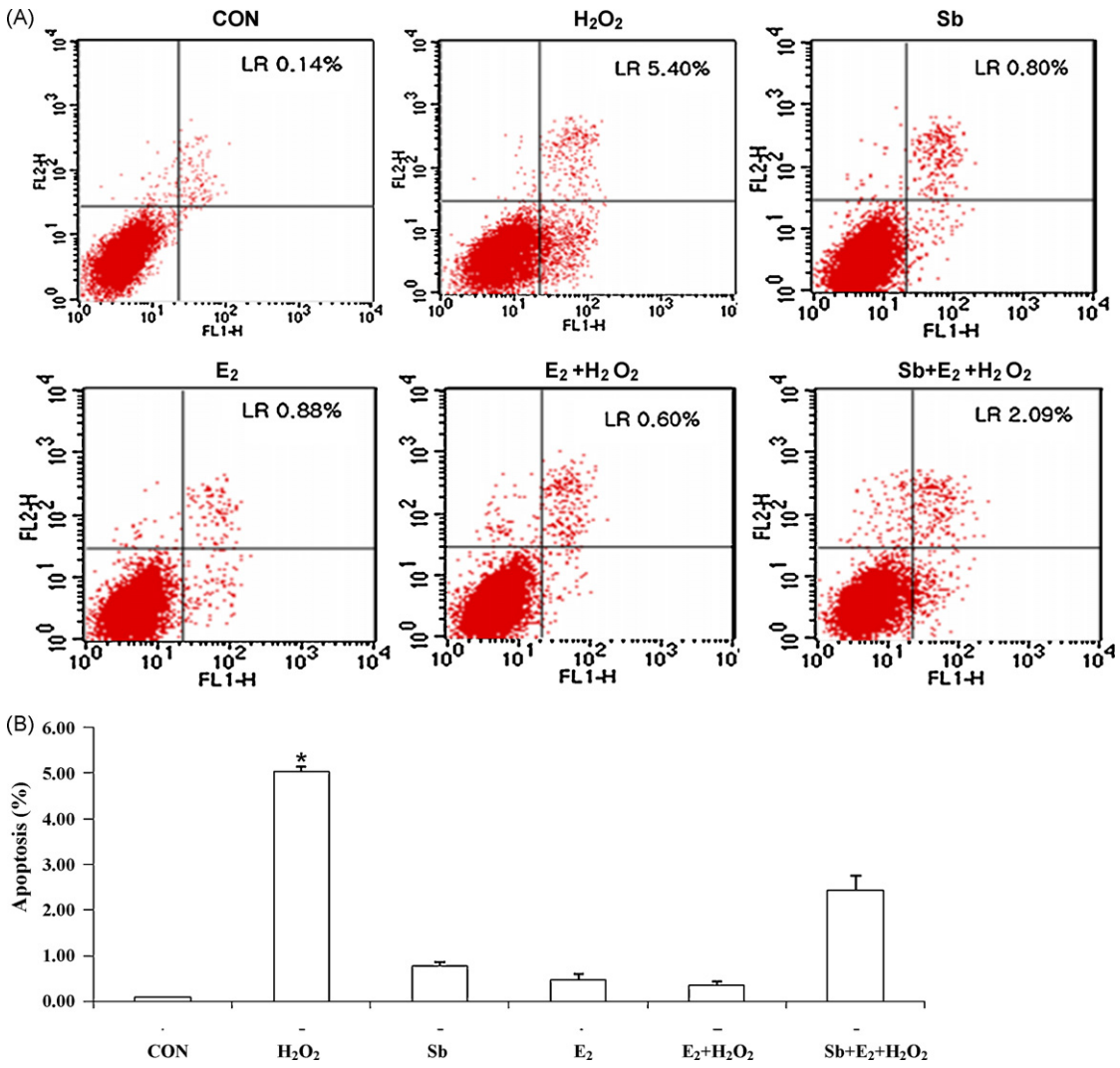

Fig. 5. FACS analysis of bAECs labeled with Annexin V and propidium iodide (PI). (A) bAECs were incubated in the absence of 1 mM H_2O_2 for 3 h or incubated for 1 h with 20 μM SB203580 (p38 inhibitor Sb) or incubated for 24 h with 1 μM of 17β-estradiol (E_2). Cells were stained with Annexin V and PI. The *X*-axis represents Annexin V related fluorescence and the *Y*-axis represents PI-related fluorescence. (B) The fluorescent cells in experiment (A) were quantified (*$P < 0.05$). The percentage of apoptotic cells was elevated in the H_2O_2-treated group compared with that in the control group. Pretreatment with 17β-estradiol effectively inhibited apoptotic cell percentage. But pretreatment with SB203580 percentage of apoptotic cells were increased.

tic endothelial cells was assessed by the MTT assay. The oxidative stress induced by the repeated H_2O_2 treatments showed the suppression of cell growth, and the antioxidant effect of 17β-estradiol was observed. By the measurement of intracellular ROS, it was confirmed that ROS increased by H_2O_2 treatment were decreased by the pretreatment of 17β-estradiol. In addition, Hoechst staining showed that the apoptosis of cells induced by oxidative stress was decreased by the pretreatment with 17β-estradiol.

The MAP kinase signal transduction system is involved in cellular metabolism from cell proliferation to cell death upon stimuli [20,21]. MAPK is classified largely to three groups, extracellular signal regulate kinase (ERK) 1/2, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAPK [46]. ERK1/2 has been shown to control mitogenesis and it is regulated by growth factors [47,48] and JNK/SAPK has been shown to be involved in apoptosis [49]. In HUVEC cells stimulated with H_2O_2, ebselen suppresses p38 MAPK and thus it mediates antioxidant effects [50].

In our study, to examine the antioxidant effect of 17β-estradiol on the cell signal transduction system, Western blot experiments were performed, and it was confirmed that the pretreatment with 17β-estradiol elevates the expression of phospho-p38 MAPK and Bcl-2 in comparison with the control group. In addition, it was found that by the pretreatment with the phospho-p38 MAPK suppressor SB203580, the expression of phospho-p38 MAPK and Bcl-2 was decreased. The FACS analysis showed the similar results.

Therefore, our study showed that in bAEC line, the pretreatment of 17β-estradiol mediates a protective effect on the oxidative stress induced by the treatment with H_2O_2, and in signal transduction systems, the phospho-p38 MAPK and Bcl-2 pathway were found to be associated. Exposure of oxidative stress was reported to go through intracellular pathway via MAP kinase kinase (MKK) 4/7 and MKK3/6, the upstream activators of JNK/SAPK and p38, respectively, also localize in both the cytoplasm and the nucleus [51]. And other reported that activation of the SAPK2/p38 pathway was required to trigger actin polymerization, make dysregulation against ERK pathway and consequently lead to membrane blebbing which was an early manifestation of oxidative toxicity that is associated with disruption of the endothelial layer integrity [52]. However, the cells pretreated with the phospho-p38 MAPK suppressor SB203580 and then treated with 17β-estradiol, did not show a noticeable reduction, and thus it could be inferred that other signal transduction

systems may be co-existing for 17β-estradiol mediating protective functions.

The possible molecular pathways of estrogen influencing on cardiovascular system has been reported, but little was known about antioxidant effect. Stice et al. asserted that estrogen played roles on protection cardiovascular system from ischemia by activating NFκB, inducing heat-shock protein (hsp)-72 and decreasing the expression of soluble epoxide hydrolase [53]. Estradiol also reported to promote the association of hsp-90 with endothelial nitric oxide synthase and reduce the Ca2+ requirement for its activation [54]. Another study asserted that cardioprotective effect of estradiol was due to increasing bioavailability of nitric oxide (NO) via estrogen receptor (ER) pathway and activating MAPK, phospholipase c-dependent pathway via autocrine loop involving fibroblast growth factor (FGF)-2 [55].

Estrogen stimulated endothelial cell was also thought to release anandamide and inhibit the secretion of serotonin from adenosine diphosphate (ADP)-stimulated platelets [56]. A study reported that reduction in plasma plasminogen activator inhibitor-1 (PAI-1) levels observed after treatment of postmenopausal women with hormone therapy reflected local ER-dependent mechanisms in bAEC [57]. Lui et al. made several statements on protective effect of estradiol and VEGF on tumor necrosis factor (TNF)-α induced apoptosis. They reported that activation of p44/42 Ca2+-calmodulin dependent protein kinase (CCDPK) signaling together with inhibition of p38 CCDPK signaling by estradiol 0.1 pmol/L to 100 nmol/L appeared to be an important mechanism for its survival effect on bAEC [58,59]. From the viewpoint of anti-apoptotic effect, 17β-estradiol prevented the apoptosis induced by TNF-α in bAEC with activating endothelial NO production by phosphatidylinositol-3 kinase (PI3K)-Akt pathway [60]. There has been several studies concerning about molecular mechanism, but not yet established. Further research on molecular mechanism of estradiol would clarify the association of estradiol and endothelial cell function.

In this study, it is considered that studies on the direct antioxidant effect of estrogen on vascular endothelial cells could provide a scientific basis for hormone replacement therapy in postmenopausal women, and it may contribute to not only the prevention of cardiovascular diseases but also the improvement of the health of the elderly.

Acknowledgement

The authors wishes to acknowledge the financial support of the Catholic Medical Center Research Foundation made in the program year of 2006.

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