

Tumor-suppressor Protein p53 Sensitizes Human Colorectal Carcinoma HCT116 Cells to 17 α -estradiol-induced Apoptosis via Augmentation of Bak/Bax Activation

Cho Rong Han¹, Ji Young Lee¹, Dongki Kim², Hyo Young Kim², Se Jin Kim², Seokjoon Jang², Yoon Hee Kim^{1,2}, Do Youn Jun¹ and Young Ho Kim^{1*}

¹Laboratory of Immunobiology, School of Life Science and Biotechnology, College of Natural Sciences, Kyungpook National University, Daegu 702-701, Korea

²Daegu Science High School, Daegu 706-852, Korea

Received September 23, 2013 / Revised October 23, 2013 / Accepted October 24, 2013

The regulatory effect of the tumor-suppressor protein p53 on the apoptogenic activity of 17 α -estradiol (17 α -E₂) was compared between HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells. When the HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells were treated with 2.5~10 μ M 17 α -E₂ for 48 h or with 10 μ M for various time periods, cytotoxicity and an apoptotic sub-G₁ peak were induced in the HCT116 (p53^{+/+}) cells in a dose- and time-dependent manner. However, the HCT116 (p53^{-/-}) cells were much less sensitive to the apoptotic effect of 17 α -E₂. Although 17 α -E₂ induced aberrant mitotic spindle organization and incomplete chromosome congregation at the equatorial plate, G₂/M arrest was induced to a similar extent in both cell types. In addition, 17 α -E₂-induced activation of Bak and Bax, $\Delta\psi$ m loss, and PARP degradation were more dominant in the HCT116 (p53^{+/+}) than in the HCT116 (p53^{-/-}) cells. In accordance with enhancement of p53 phosphorylation (Ser-15) and p53 levels, p21 and Bax levels were elevated in the HCT116 (p53^{+/+}) cells treated with 17 α -E₂. The HCT116 (p53^{-/-}) cells exhibited barely or undetectable levels of p21 and Bax, regardless of 17 α -E₂ treatment. On the other hand, although the level of Bcl-2 was slightly lower in the HCT116 (p53^{+/+}) than in the HCT116 (p53^{-/-}) cells, it remained relatively constant after the 17 α -E₂ treatment. Together, these results show that among the components of the 17 α -E₂-induced apoptotic-signaling pathway, which proceeds through mitotic spindle defects causing mitotic arrest, subsequent activation of Bak and Bax and the mitochondria-dependent caspase cascade, leading to PARP degradation, 17 α -E₂-induced activation of Bak and Bax is the upstream target of proapoptotic action of p53.

Key words : 17 α -estradiol, mitotic arrest, activation of Bak and Bax, p53 phosphorylation (Ser-15), mitochondrial apoptosis

Introduction

Estrogens can exert biological effect on target cells by the intracellular estrogen receptor (ER)-mediated genomic mechanism, the plasma membrane ER-mediated non-genomic mechanism associated with cell signaling pathways, or an ER-independent mechanism [5, 30]. The ER-mediated genomic action of estrogens is elicited by binding with the nuclear receptors estrogen receptor α (ER α) and β (ER β), and the subsequent transcriptional regulation of gene expression [13]. The plasma membrane ER-mediated action

of estrogens rapidly triggers second messenger signaling events, in which activated ERs do not directly alter the expression of target genes. The ER-independent action of estrogens is induced at pharmacological concentrations (in the micromolar range) and is not blocked by ER antagonists such as ICI 162,780 or tamoxifen [31].

As the predominant and most biologically active estrogen, 17 β -estradiol (17 β -E₂) is known to reduce neuronal apoptosis at physiological concentrations (in the nanomolar range) in various *in vivo* and *in vitro* neurodegenerative conditions [4, 31]. However, 17 β -E₂ induces apoptotic cell death in osteoclasts and thymocytes, suggesting that the apoptotic regulatory activity of 17 β -E₂ may differ depending upon the target cell types [21, 25]. On the other hand, although 17 α -estradiol (17 α -E₂), which is a stereoisomer of 17 β -E₂ and fails to interact effectively with the ER, has been considered hormonally inactive and little attention has been given to its roles, 17 α -E₂ turns out to be as potent as 17 β -E₂ in protecting neu-

*Corresponding author

Tel : +82-53-950-5378, Fax : +82-53-955-5522

E-mail : ykim@knu.ac.kr

This is an Open-Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

rons from toxic stress conditions [12]. This neuroprotective action of 17 α -E₂ appears to be mediated by ER-independent nongenomic mechanisms. In this regard, the clinical application of 17 α -E₂ as a neuroprotective therapeutic agent, is expected to be more beneficial than 17 β -E₂, in that 17 α -E₂ possesses low genomic effects and equipotent nongenomic effects compared with 17 β -E₂, thus avoiding the adverse effects of 17 β -E₂.

In relation to the cytoprotective or apoptogenic effect of 17 β -E₂ toward malignant tumor cells, a number of studies have indicated that 17 β -E₂ stimulates cancer cell survival and proliferation at a physiological dose through an ER-dependent mechanism, whereas 17 β -E₂ inhibits cancer cell proliferation at a pharmacological dose by inducing apoptosis or microtubule disruption independent of the ER. Recently, several studies have reported that an endogenous metabolite of 17 β -E₂, 2-methoxyestradiol (2-MeO-E₂) can be a promising anticancer drug candidate [24]. The anticancer actions of 2-MeO-E₂ at pharmacological concentrations are exerted by inducing apoptosis, arresting cell growth at the G₁/S boundary and/or G₂/M boundary, or inhibiting angiogenesis [3, 15, 28]. However, little information is known regarding the effect of 17 α -E₂ on tumor cell proliferation and apoptosis.

Recently, we have shown that a pharmacological dose (5~10 μ M) of 17 α -E₂, but not 17 β -E₂, can induce apoptosis in human acute leukemia Jurkat T cells, which are known to not express ERs [9], via a mitochondria-dependent caspase cascade activation [20]. The 17 α -E₂-induced apoptosis occurs mainly in G₂/M-arrested cells and is accompanied by an increase in Bcl-2 phosphorylation at Thr-56 [20], a known site that can be phosphorylated by the Cdk1/cyclin B1 kinase during G₂/M phase [14]. More recently, we have reported that the apoptogenic activity of 17 α -E₂ was due to the impaired mitotic spindle assembly causing prometaphase arrest and prolonged Cdk1 activation, phosphorylation of the Bcl-2 family members (Bcl-2, Mcl-1 and Bim), Bak activation, and mitochondria-dependent caspase cascade activation [18].

In the present study, to elucidate further how 17 α -E₂-induced apoptotic events are regulated by the tumor suppressor protein p53, we investigated the effect of 17 α -E₂ (2.5~10 μ M) on mitotic arrest, microtubule network organization, and mitochondria-dependent apoptotic signaling pathway using human colorectal carcinoma cell clones HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}).

Materials and Methods

Reagents, antibodies, and cells

An ECL Western blotting kit was purchased from Amersham (Arlington Heights, IL, USA), and Immobilon-P membrane was obtained from Millipore Corporation (Bedford, MA, USA). The anti-caspase-3 antibody was purchased from Pharmingen (San Diego, CA, USA), and anti-p53, anti-poly (ADP-ribose) polymerase (PARP), anti-Bak, anti-Bax, anti-Bcl-2, and anti- β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-caspase-9 was purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-Bak (Ab-1) and anti-Bax (6A7) were obtained from Calbiochem (San Diego, CA, USA). The anti-phospho-p53 (Ser-15) was obtained from Cell Signaling (Beverly, MA, USA). The monoclonal anti-p21 antibody was purchased from Neomarkers (Freemont, CA, USA). Human colorectal carcinoma cell lines HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) were provided by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD, USA). Both HCT116 cells were maintained in DMEM (Hyclone, (Gaithersburg, MD, USA) containing 10% FBS (Hyclone), and 100 μ g/ml gentamycin.

Cytotoxicity assay

The cytotoxic effect of 17 α -E₂ on HCT116 cells was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [20]. Briefly, cells (1 \times 10⁴/well) were incubated with a serial dilution of 17 α -E₂ in 96-well plates. At 44 h after incubation, 50 μ l of MTT solution (1.1 mg/ml) was added to each well and incubated for an additional 4 h. The colored formazan crystal produced from MTT was dissolved in DMSO and OD values of the solutions were measured at 540 nm by a plate reader.

Flow cytometric analysis

Flow cytometric analysis for the cell cycle of HCT116 cells exposed to 17 α -E₂ was done as described elsewhere [19]. Changes in the mitochondrial membrane potential ($\Delta\psi$ m) following treatment with 17 α -E₂ were measured after staining with 3,3'-dihexyloxycarbocyanine iodide (DiOC₆) [26]. Activation of Bak and Bax in HCT116 cells following treatment with 17 α -E₂ was measured by flow cytometry as previously described [22].

Immunofluorescence Microscopy

HCT116 cells adhered onto glass cover slips were fixed

with cold methanol for 3 min [22]. The cells were rinsed four times with cold PBS containing 0.5% Triton X-100, and blocked with 10% goat serum for 30 min. The cells were then incubated with monoclonal anti- α -tubulin (1:2,500) overnight at 4°C. For detection, the cells were treated with Alexa Fluor 488-labeled goat anti-mouse IgG (1:1,000) for 1 h at room temperature. Thereafter, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) to label the nuclei. Images were visualized and photographed using a Carl Zeiss MicroImaging Confocal Laser Scanning Microscope.

Preparation of cell lysate and western blot analysis

Cell lysates were prepared by suspending 3×10^6 HCT116 cells in 200 μ l of the lysis buffer (137 mM NaCl, 15 mM EGTA, 1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, 1 mM PMSF, and 2.5 μ g/ml proteinase inhibitor E-64, pH 7.2). Cells were disrupted by sonication and extracted for 30 min at 4°C. An equivalent amount of protein lysate (15~20 μ g) was electrophoresed on a 4~12% NuPAGE gradient gel and then electrotransferred to an Immobilon-P membrane. The detection of each protein was carried out with an ECL Western blotting kit, according to the manufacturer's instructions.

Statistical analysis

Unless otherwise indicated, each result in this paper is representative of at least three separate experiments.

Results and Discussion

Comparison of cytotoxic effect of 17 α -E₂ between HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells

To examine whether 17 α -E₂-induced apoptotic cell death is affected by tumor suppressor protein p53, the cytotoxicity of 17 α -E₂ (2.5~10 μ M), the cytotoxic effects of 17 α -E₂ on HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells were compared. As determined by MTT assay, the viabilities of HCT116 (p53^{+/+}) cells treated for 24 h with 17 α -E₂ at concentrations of 2.5 μ M, 5 μ M, and 10 μ M were 96%, 80%, and 61.6%, respectively (Fig. 1). Under the same conditions, the cell viabilities of HCT116 (p53^{-/-}) cells were not affected within the range of 2.5~5 μ M, and declined to the level of 78.9% in the presence of 10 μ M. When HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells were treated with 10 μ M 17 α -E₂ for various time periods, the apoptotic sub-G₁ peak was detected in HCT116 (p53^{+/+}) cells as early as 12 h after the treatment and then

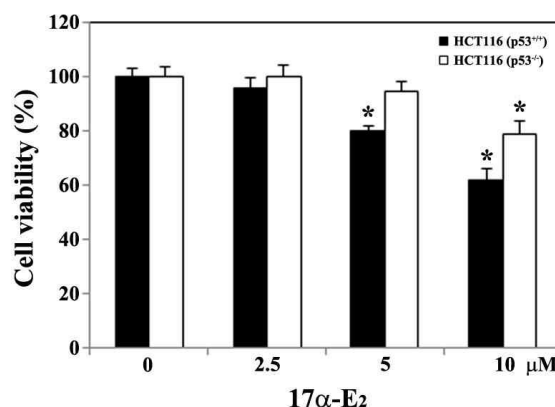


Fig. 1. Cytotoxic effect of 17 α -E₂ on human colorectal carcinoma cell clones HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells. For cell viability analysis, continuously growing cells (1×10^4 /well) were incubated at the indicated concentrations of 17 α -E₂ in a 96-well plate for 24 h and the final 4 h was incubated with MTT. The cells were sequentially processed to assess the colored formazan crystal produced from MTT as an index of cell viability. Each value is expressed as the mean \pm SD (n=3 with three replicates per independent experiment). * $p < 0.05$ compared with control. A representative study is shown and two additional experiments yielded similar results.

the ratio of apoptotic sub-G₁ cells was elevated up to the level of 74.1% at 48 h, whereas the apoptotic sub-G₁ peak in HCT116 (p53^{-/-}) cells was first detected at 12 h and the time-dependent elevation of apoptotic sub-G₁ peak was not so significant as compared with HCT116 (p53^{+/+}) cells (Fig. 2). In addition, an elevation in the ratio of G₂/M cells were observed in both HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells at 12 h following 10 μ M 17 α -E₂, and then the ratio of G₂/M cells appeared to decrease, possible due to elimination of G₂/M-arrested cells by inducing apoptotic cell death.

These results show that HCT116 (p53^{+/+}) cells were more sensitive to the cytotoxicity of 17 α -E₂ as compared with HCT116 (p53^{-/-}) cells, and suggest that the apoptotic effect of 17 α -E₂, which is responsible for the cytotoxicity, could be augmented in the presence of the tumor suppressor protein p53.

Effect of 17 α -E₂ on the cellular microtubule network in HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells

The 17 α -E₂-induced apoptotic signaling pathway in Jurkat T cells has previously been shown to proceed through direct inhibition of microtubule formation causing prometaphase arrest, prolonged activation of Cdk1, and subsequent mediation of mitochondria-dependent apoptotic events including

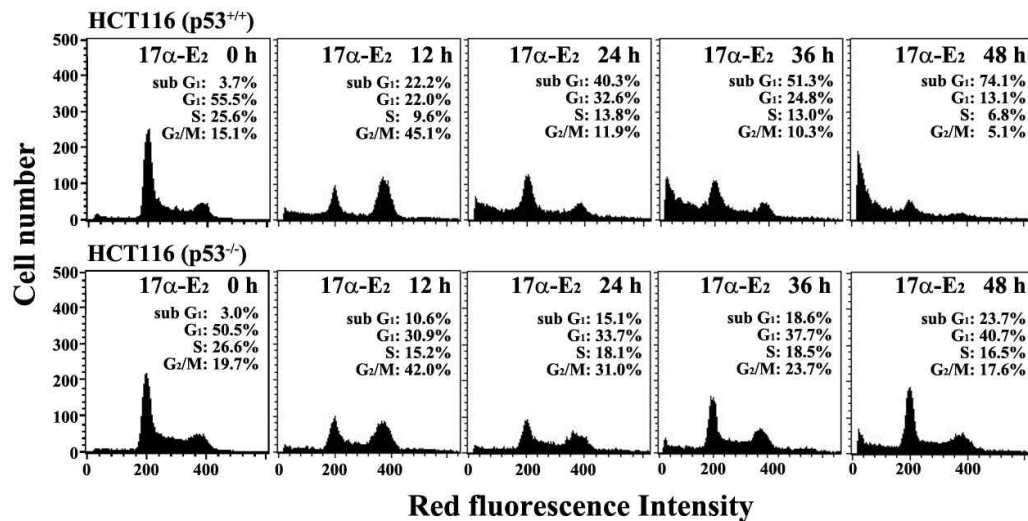


Fig. 2. Flow cytometric analysis of the cell cycle distribution in HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells following exposure to 17α-E₂ for various time periods. The cells were incubated at a density of 2.5×10^5 /ml with 10 μM 17α-E₂ for 12, 24, 36, or 48 h. The analysis of cell cycle distribution was performed on an equal number of cells (2×10^4) by flow cytometry after staining of DNA by PI as described in Materials and Methods. A representative study is shown and two additional experiments yielded similar results.

Bak activation, $\Delta\psi_m$ loss, and resultant caspase cascade activation [18]. Because 17α-E₂-induced apoptotic sub-G₁ peak was more apparent in HCT116 (p53^{+/+}) than in HCT116 (p53^{-/-}) cells, we decided to examine whether the impairment of mitotic spindle network, which can be caused by 17α-E₂, is modulated by the presence of p53. The effect of 17α-E₂ on the organization of the microtubule network in HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells was investigated by immunofluorescence microscopy using anti-α-tubulin antibody. Although the microtubule network in continuously growing HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells showed a normal arrangement with the majority of cells in interphase, both cell types exhibited, in common, an aberrant bipolar array of microtubules after treatment with 10 μM 17α-E₂ for 12 h (Fig. 3). In addition, DAPI staining revealed that while some chromosomes in the cells treated with 10 μM 17α-E₂ were aligned at the equator of the mitotic spindle, other chromosomes failed to localize at the equator, regardless of the presence of p53.

These results indicate that the 17α-E₂-induced defect in the organization of mitotic spindle network, which is known to be the primary target for the apoptotic action of 17α-E₂ [24], and subsequent mediation of incomplete alignment of the chromosomes at the equatorial plate, leading to prometaphase arrest of the cell cycle, were not influenced by the presence or absence of p53.

Effect of p53 on 17α-E₂-induced activation of Bak and Bax, and mitochondrial membrane potential ($\Delta\psi_m$) loss in HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells

To examine whether p53 contributes to modulation of the

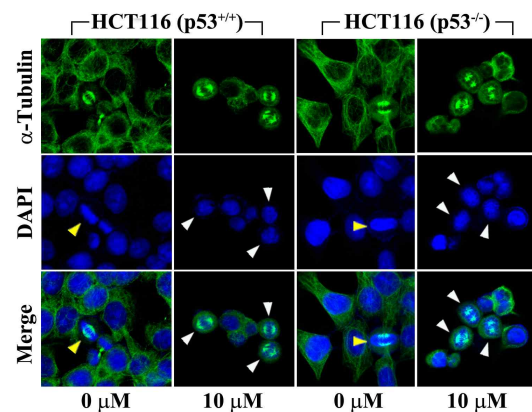


Fig. 3. Effect of 17α-E₂ on the organization of microtubule network in HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells. After treatment of cells with 10 μM 17α-E₂ for 12 h, the cells were fixed with cold methanol for 3 min, permeabilized, incubated with mouse monoclonal anti-α-tubulin, and then treated with Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin. The cells were then stained with 4',6-diamidino-2-phenylindole (DAPI) to label the nuclei. Images were obtained using a confocal laser scanning microscope. Symbols: white arrowhead, incomplete/complete chromosome congression at the equatorial plate. A representative study is shown; two additional experiments yielded similar results.

downstream events of the 17 α -E₂-induced mitotic arrest, resulting from the mitotic spindle defects, the 17 α -E₂-induced activation of Bak and Bax, and mitochondrial membrane potential ($\Delta\psi$ m) loss were compared between HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells. As shown in Fig. 4A, although continuously growing HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells untreated with 17 α -E₂ showed barely detectable levels of apoptotic-sub-G₁ cells, the apoptotic sub-G₁ cells following treatment with 10 μ M 17 α -E₂ for 24 h appeared to increase to a level of 45.0% in HCT116 (p53^{+/+}) cells and 18.0% in HCT116 (p53^{-/-}) cells. Under these conditions, when the $\Delta\psi$ m loss of cells treated with 17 α -E₂ was measured by DiOC₆ staining, the ratio of negative fluorescence in HCT116 (p53^{+/+}) cells treated with 10 μ M 17 α -E₂ were 39.8% (Fig. 4B). However, the ratio of negative fluorescence in HCT116 (p53^{-/-}) cells treated with 10 μ M 17 α -E₂ was only 11.2%. Since current results demonstrated that 17 α -E₂-induced $\Delta\psi$ m loss was more dominant in HCT116 (p53^{+/+}) cells than in HCT116 (p53^{-/-}) cells, it was likely that the 17 α -E₂-induced mitochondrial damage and subsequent mitochondria-dependent activation of caspase cascade might be positively modulated by p53.

To examine this prediction further, 17 α -E₂-induced activation of the pro-apoptotic multidomain Bcl-2 family members, Bak and Bax, were compared between HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells. As shown in Fig. 5, both Bak and Bax

activations, as evidenced by their N-terminal conformational changes detected using an active conformation-specific anti-Bak antibody (Ab-1) and an active conformation-specific anti-Bax antibody (6A7), were more significantly observed in HCT116 (p53^{+/+}) cells than in HCT116 (p53^{-/-}) cells. Previously, it has been reported that either Bak activation or Bax activation can mediate permeabilization of the mitochondrial outer membrane (MOM) to trigger mitochondrial cytochrome *c* release into cytoplasm, leading to the caspase cascade activation [6, 8]. Consequently, current and previous results indicated that p53-mediated enhancement in the 17 α -E₂-induced mitochondrial membrane potential ($\Delta\psi$ m) loss was associated with the elevation in the level of Bak and Bax activations in the presence of p53.

Numerous studies have reported that chemotherapeutic agents-induced apoptotic signaling pathways are frequently associated with mitochondria-dependent apoptotic events [2, 10, 11]. In addition, it has been shown that an alteration in the expression ratio of Bak to Bcl-2 and/or Bax to Bcl-2, resulting in an enhancement in the ratio of Bak to Bcl-2 and/or Bax to Bcl-2, is often prerequisite for provoking the activation of Bak and/or Bax during the mitochondria-dependent apoptosis induced by chemotherapeutic agents [1, 6, 7, 16]. To examine the upstream pro-apoptotic events that mediate 17 α -E₂-induced activation of Bak and Bax, the levels of Bcl-2 family proteins, such as the pro-apoptotic Bcl-2 family mem-

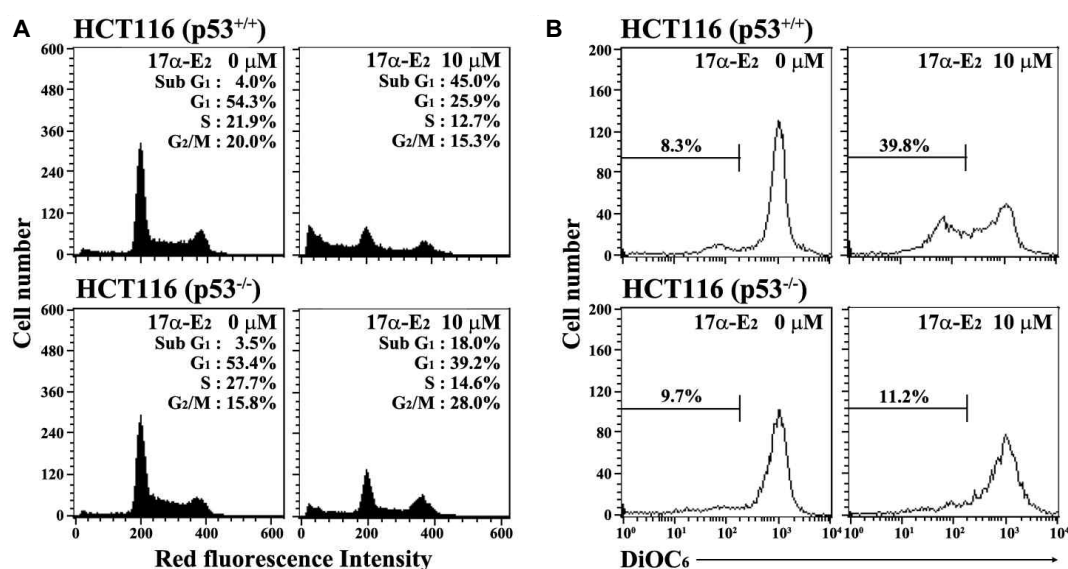


Fig. 4. Apoptotic changes in the cell cycle distribution (A), and mitochondrial membrane potential ($\Delta\psi$ m) loss (B) in HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells after exposure to 17 α -E₂. After the cells were incubated at a density of 2.5×10^5 /ml with 10 μ M 17 α -E₂ for 24 h, cell cycle distribution and $\Delta\psi$ m loss of the cells were determined through flow cytometric analysis of PI staining and DiOC₆ staining, respectively. A representative study is shown and two additional experiments yielded similar results.

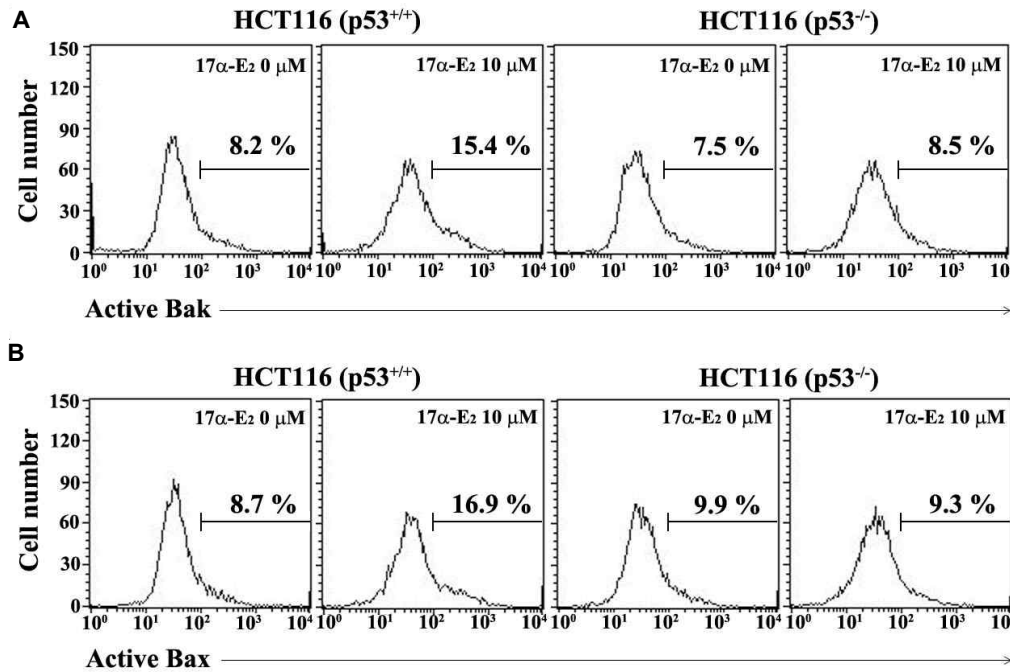


Fig. 5. Flow cytometric analysis of Bak activation (A) and Bax activation (B) in HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells after 17α-E₂ treatment. The cells were incubated at the concentration of 2.5×10^5 /ml with 10 μM 17α-E₂ for 24 h, and subjected to flow cytometric analysis of the activation of Bak and Bax as described in Materials and Methods. A representative study is shown and two additional experiments yielded similar results.

bers (Bak and Bax) and anti-apoptotic Bcl-2, and the activation of caspase-9, and -3, and PARP degradation were compared by western blot analysis between HCT115 (p53^{+/+}) and HCT116 (p53^{-/-}) cells treated with 17α-E₂.

The p53 expression was easily detected in HCT116 (p53^{+/+}), but was not detected in HCT116 (p53^{-/-}) cells (Fig. 6A). In HCT116 (p53^{+/+}) cells treated with 5~10 μM 17α-E₂, the level of phosphorylated p53 at Ser-15 and total p53 were enhanced dose-dependently. Although the up-regulation of p21, which act as a negative cell cycle regulator for G₁ arrest [23, 29], was remarkable in HCT116 (p53^{+/+}) after 17α-E₂ treatment, it was barely detected in HCT116 (p53^{-/-}) cells. Under these conditions, caspase-9 and -3 activations, and PARP degradation were more significantly observed in HCT116 (p53^{+/+}). As shown in Fig. 6B, a time-kinetic analysis revealed that the levels of anti-apoptotic Bcl-2 and pro-apoptotic Bak appeared to remain relatively constant in HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells following exposure to 10 μM 17α-E₂, whereas that of Bax appeared to increase in both cell types at 24~48 h, with more dominant increase in the presence of p53. In addition, the expression of p53 and phosphorylated p53 at Ser-15, both of which were detected only in HCT116 (p53^{+/+}) cells, were enhanced time-dependently after 17α-E₂ treatment. Under these conditions, the level of

p21 was enhanced in HCT116 (p53^{+/+}) cells, whereas the enhancement was barely or not detected in HCT116 (p53^{-/-}) cells. These results suggested that 17α-E₂-induced Bax activation might be due to up-regulation in its expression level, whereas 17α-E₂-induced Bak activation might not require the up-regulation of its expression level. Recently, it has been shown that p53 can exert a pro-apoptotic role at the mitochondria through direct interaction of p53 with anti-apoptotic Bcl-2, which results in induction of mitochondria-dependent apoptosis [17, 27]. It is noteworthy that the differential expression pattern of Bcl-2, which exhibited a relatively lower level in HCT116 (p53^{+/+}) than in HCT116 (p53^{-/-}) cells, might also contribute to render HCT116 cells more susceptible to the onset of Bak and Bax activations. Consequently, these previous and current results indicated that the 17α-E₂-induced activation of Bak and Bax, which occurred more dominantly in the presence of p53, was exerted by not only a transcription-dependent mechanism of p53 via up-regulation of Bax level, but also a transcription-independent mechanism of p53 via direct binding to Bcl-2.

In conclusion, these results show that HCT116 (p53^{+/+}) cells were more sensitive to the cytotoxicity of 17α-E₂, which is attributable to more potent induction of apoptosis, as compared with HCT116 (p53^{-/-}) cells. Although the 17α-E₂-induced

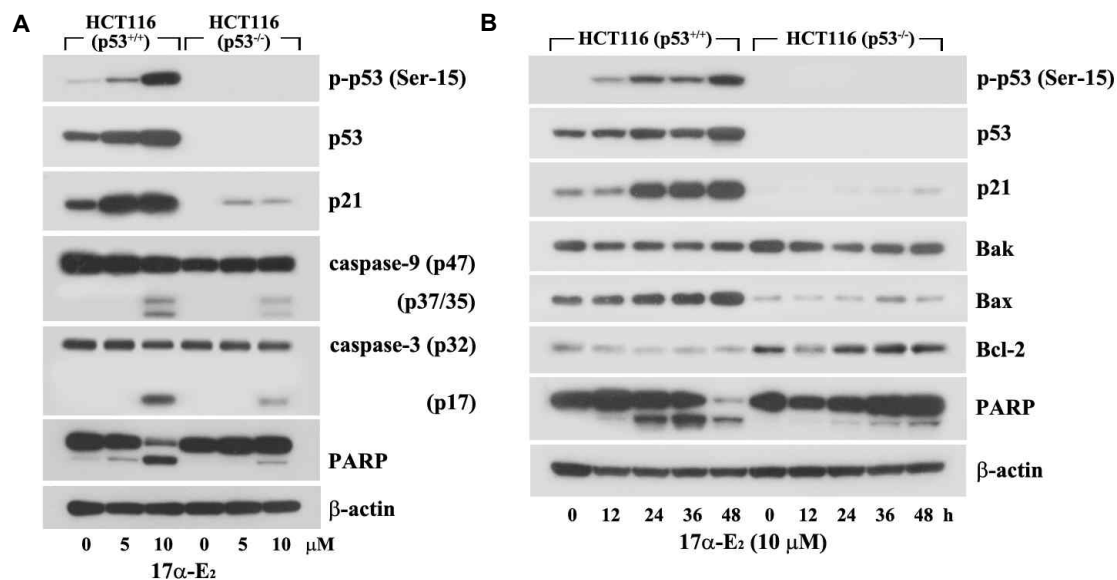


Fig. 6. Western blot analysis of dosage effect of 17α-E₂ on mitochondria-dependent caspase cascade (A), and kinetic analysis of 17α-E₂-induced apoptotic events (B) in HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells. HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells were incubated at a density of 2.5×10⁵/ml with 17α-E₂ for 48 h, or with 10 μM 17α-E₂ for various time periods before preparation of the cell lysates. Western blot analysis was performed, as described in Materials and Methods, in order to assess p53 phosphorylation at Ser-15, p53, p21, Bak, Bax, Bcl-2, activation of caspase-9 and -3, PARP cleavage, and β-actin in HCT116 (p53^{+/+}) and HCT116 (p53^{-/-}) cells treated with 17α-E₂. A representative study is shown and two additional experiments yielded similar results.

defect in the organization of mitotic spindle network and subsequent mediation of incomplete alignment of the chromosomes at the equatorial plate, causing G₂/M arrest, were not influenced by p53, the downstream events including both Bak and Bax activations and Δψ_m loss were observed more potently in the presence of p53. In HCT116 (p53^{+/+}) cells following 17α-E₂ treatment, the levels of phosphorylated p53 at Ser-15, total p53, and Bax were markedly enhanced in a dose- and time-dependent manner, suggesting that 17α-E₂-induced activation of Bak and Bax might be positively modulated by the pro-apoptotic action of p53. These results provide insight into the molecular and cellular mechanism underlying the pro-apoptotic role of p53 in the Bak and Bax activations, provoked by a microtubule-targeting drug, 17α-E₂.

Acknowledgement

This work was supported by the Kyungpook National University Research Fund, 2010, Republic of Korea.

References

- Adams, J. M. and Cory, S. 2007. Bcl-2-regulated apoptosis: mechanism and therapeutic potential. *Curr Opin Immunol* **19**, 488-496.
- Ashkenazi, A. and Dixit, V. M. 1999. Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol* **11**, 255-260.
- Batsi, C., Markopoulou, S., Kontargiris, E., Charalambous, C., Thomas, C., Christoforidis, S., Kanavaros, P., Constantinou, A. I., Marcu, K. B. and Kolettas, E. 2009. Bcl-2 blocks 2-methoxyestradiol induced leukemia cell apoptosis by a p27^{Kip1}-dependent G₁/S cell cycle arrest in conjunction with NF-κB activation. *Biochem Pharmacol* **78**, 33-44.
- Behl, C. 1998. Effects of glucocorticoids on oxidative stress-induced hippocampal cell death: implications for the pathogenesis of Alzheimer's disease. *Exp Gerontol* **33**, 689-696.
- Behl, C. and Holsboer, F. 1999. The female sex hormone estrogen as a neuroprotectant. *Trends Pharmacol Sci* **20**, 441-444.
- Chipuk, J. E. and Green, D. R. 2008. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol* **18**, 157-164.
- Chipuk, J. E., Moldoveanu, T., Llambi, F., Parsons, M. J. and Green, D. R. 2010. The BCL-2 family reunion. *Mol Cell* **37**, 299-310.
- Czabotar, P. E., Colman, P. M. and Huang, D. C. 2009. Bax activation by Bim? *Cell Death Differ* **16**, 1187-1191.
- Danel, L., Menouni, M., Cohen, J. H., Magaud, J. P., Lenoir, G., Revillard, J. P. and Saez, S. 1985. Distribution of androgen and estrogen receptors among lymphoid and haemopoietic cell lines. *Leuk Res* **9**, 1373-1378.
- Desagher, S. and Martinou, J. C. 2000. Mitochondria as the central control point of apoptosis. *Trends Cell Biol* **10**, 369-

- 377.
11. Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B. and Martinou, J. C. 1999. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol* **144**, 891-901.
 12. Dykens, J. A., Moos, W. H., Howell, N., Dykens, J. A., Moos, W. H. and Howell, N. 2005. Development of 17 α -estradiol as a neuroprotective therapeutic agent: rationale and results from a phase I clinical study. *Ann NY Acad Sci* **1052**, 116-135.
 13. Evans, R. M. 1988. The steroid and thyroid hormone receptor superfamily. *Science* **240**, 889-895.
 14. Furukawa, Y., Iwase, S., Kikuchi, J., Terui, Y., Nakamura, M., Yamada, H., Kano, Y. and Matsuda, M. 2000. Phosphorylation of Bcl-2 protein by CDC2 kinase during G₂/M phases and its role in cell cycle regulation. *J Biol Chem* **275**, 21661-21667.
 15. Gao, N., Rahmani, M., Dent, P. and Grant, S. 2005. 2-Methoxyestradiol-induced apoptosis in human leukemia cells proceeds through a reactive oxygen species and Akt-dependent process. *Oncogene* **24**, 3797-809.
 16. Gross, A., McDonnell, J. M. and Korsmeyer, S. J. 1999. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* **13**, 1899-1911.
 17. Ha, J. H., Shin, J. S., Yoon, M. K., Lee, M. S., He, F., Bae, K. H., Yoon, H. S., Lee, C. K., Park, S. G., Muto, Y. and Chi, S. W. 2013. Dual-site interactions of p53 protein transactivation domain with anti-apoptotic Bcl-2 family proteins reveal a highly convergent mechanism of divergent p53 pathways. *J Biol Chem* **288**, 7387-7398.
 18. Han, C. R., Jun, D. Y., Kim, Y. H., Lee, J. Y. and Kim, Y. H. 2013. Prometaphase arrest-dependent phosphorylation of Bcl-2 family proteins and activation of mitochondrial apoptotic pathway are associated with 17 α -estradiol-induced apoptosis in human Jurkat T cells. *Biochim Biophys Acta* **183**, 2220-2232.
 19. Jun, D. Y., Kim, J. S., Park, H. S., Han, C. R., Fang, Z., Woo, M. H., Rhee, I. K. and Kim, Y. H. 2007. Apoptogenic activity of auraptene of *Zanthoxylum schinifolium* toward human acute leukemia Jurkat T cells is associated with ER stress-mediated caspase-8 activation that stimulates mitochondria-dependent or -independent caspase cascade. *Carcinogenesis* **28**, 1303-1313.
 20. Jun, D. Y., Park, H. S., Kim, J. S., Kim, J. S., Park, W., Song, B. H., Kim, H. S., Taub, D. and Kim, Y. H. 2008. 17 α -estradiol arrests cell cycle progression at G₂/M and induces apoptotic cell death in human acute leukemia Jurkat T cells. *Toxicol Appl Pharmacol* **231**, 401-412.
 21. Kameda, T., Mano, H., Yuasa, T., Mori, Y., Miyazawa, K., Shiokawa, M., Nakamaru, Y., Hiroi, E., Hiura, K., Kameda, A., Yang, N. N., Hekeda, Y. and Kumegawa, M. 1997. Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. *J Exp Med* **186**, 489-495.
 22. Lee, J. W. and Kim, Y. H. 2011. Activation of pro-apoptotic multidomain Bcl-2 family member Bak and mitochondria-dependent caspase cascade are involved in *p*-coumaric acid-induced apoptosis of Jurkat T cells. *J Life Sci* **21**, 1678-1688.
 23. Lepley, D. M. and Pelling, J. C. 1997. Induction of p21/WAF1 and G₁ cell-cycle arrest by the chemopreventive agent apigenin. *Mol Carcinog* **19**, 74-82.
 24. Mueck, A. O. and Seeger, H. 2010. 2-Methoxyestradiol-Biology and mechanism of action. *Steroids* **75**, 625-631.
 25. Okasha, S. A., Ryu, S., Do, Y., McKallip, R. J., Nagarkatti, M. and Nagarkatti, P. S. 2001. Evidence for estradiol-induced apoptosis and dysregulated T cell maturation in the thymus. *Toxicology* **163**, 49-62.
 26. Park, H. S., Jun, D. Y., Han, C. R., Woo, H. J. and Kim, Y. H. 2011. Proteasome inhibitor MG132-induced apoptosis via ER stress-mediated apoptotic pathway and its potentiation by protein tyrosine kinase p56^{lck} in human Jurkat T cells. *Biochem Pharmacol* **82**, 1110-1125.
 27. Tomita, Y., Marchenko, N., Erster, S., Nemajerova, A., Dehner, A., Klein, C., Pan, H., Kessler, H., Pancoska, P. and Moll, U. M. 2006. WT p53, but not tumor-derived mutants, bind to Bcl2 via the DNA binding domain and induce mitochondrial permeabilization. *J Biol Chem* **281**, 8600-8606.
 28. Verenich, S. and Gerk, P. M. 2010. Therapeutic promises of 2-methoxyestradiol and its drug disposition challenges. *Mol Pharm* **7**, 2030-2039.
 29. Vermeulen, K., Van Bockstaele, D. R. and Berneman, Z. N. 2003. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif* **36**, 131-149.
 30. Wise, P. M. 2003. Estrogens: protective or risk factors in brain function? *Prog Neurobiol* **69**, 181-191.
 31. Wise, P. M., Dubal, D. B., Wilson, M. E., Rau, S. W. and Bottner, M. 2001. Minireview: neuroprotective effects of estrogen-new insights into mechanisms of action. *Endocrinology* **142**, 969-973.

초록 : 17 α -Estradiol에 의한 인체 대장암 세포주 HCT116의 에폭토시스에 수반되는 Bak/Bax의 활성화에 미치는 종양억제단백질 p53의 강화효과

한초롱¹ · 이지영¹ · 김동기² · 김효영² · 김세진² · 장석준² · 김윤희^{1,2} · 전도연¹ · 김영호^{1*}

(¹경북대학교 자연과학대학 생명과학부, ²대구과학고등학교)

17 α -estradiol (17 α -E₂)의 에폭토시스 유도활성에 미치는 종양억제단백질 p53의 조절효과를 조사하고자, 17 α -E₂에 의해 유도되는 에폭토시스 현상들을 인체 대장암 세포주 유래 클론인 HCT116 (p53^{+/+}) 및 HCT116 (p53^{-/-}) 세포에서 비교하였다. HCT116 (p53^{+/+}) 및 HCT116 (p53^{-/-}) 세포를 17 α -E₂ (2.5~10 μ M)로 처리하거나 혹은 HCT116 (p53^{+/+}) 및 HCT116 (p53^{-/-}) 세포를 10 μ M 17 α -E₂로 시간 별로 처리한 결과, HCT116 (p53^{+/+})에 있어서는 세포독성과 에폭토시스-관련 sub-G1 peak의 비율은 처리농도와 시간에 의존적으로 나타났다. 그러나 HCT116 (p53^{-/-}) 세포의 경우는 이러한 현상이 미약하게 나타났다. 17 α -E₂에 의해 유도되는 비정상적 유사분열방추사 형성, 중기판 염색체 배열의 미완성, 이에 따른 유사분열정지(G₂/M arrest) 등의 현상은 HCT116 (p53^{+/+}) 및 HCT116 (p53^{-/-}) 세포에서 유사한 수준으로 나타났다. 이에 반해, 17 α -E₂에 의해 유도되는 Bak과 Bax의 활성화, 미토콘드리아의 막전위 상실($\Delta\psi$ m loss), 그리고 PARP 분해 등의 현상은 HCT116 (p53^{-/-}) 세포에 비해 HCT116 (p53^{+/+}) 세포에서 훨씬 높은 수준으로 확인되었다. 아울러 17 α -E₂로 처리된 HCT116 (p53^{+/+}) 세포에서 확인되는 p53 (Ser-15)의 인산화 및 p53 수준의 증가와 일치하여, 세포 내의 p21 및 Bax 수준도 현저히 증가하였다. 이때 17 α -E₂로 처리된 HCT116 (p53^{-/-}) 세포에서는 p21 및 Bax의 발현수준이 매우 낮았다. 한편, 에폭토시스 억제단백질인 Bcl-2 단백질 수준은 HCT116 (p53^{-/-}) 세포에 비해 HCT116 (p53^{+/+}) 세포에서 다소 낮았으나, 이러한 Bcl-2 단백질 수준은 17 α -E₂ 처리 후에도 크게 변화하지 않는 것으로 나타났다. 이러한 결과들은 17 α -E₂ 처리에 의해 유도되는 에폭토시스 유도 경로의 구성원들의 변화, 즉 비정상적 유사분열방추사 형성 및 이에 따른 유사분열정지(G₂/M arrest), 뒤이은 Bak 및 Bax의 활성화, 미토콘드리아의 막전위 상실, 그리고 이에 수반되는 caspase cascade 활성화 및 PARP 분해로 진행되는 에폭토시스 현상들 중에서, Bak 및 Bax의 활성화 단계가 종양억제단백질 p53의 에폭토시스 증진 활성화에 의해 양성적으로 조절되는 작용 타겟임을 보여준다.