Mitochondrial Dynamics of Bcl-2 Family During E2-Induced Apoptosis Correlates with the Malignant of Endometrial Cancer Cells

Takahiro Yaguchi (✉ yaguchi@iuhw.ac.jp)

International University of Health and Welfare Department of Healthcare: Kokusai Iryo Fukushima Daigaku
Fukuoka Hoken Gakubu <https://orcid.org/0000-0002-4660-5123>

Hirofumi Taira

International University of Health and Welfare: Kokusai Iryo Fukushima Daigaku

Misaki Kamenno

Konan University: Konan Daigaku

Junji Kawakami

Konan University: Konan Daigaku

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Mitochondrial dynamics of Bcl-2 family during E₂-induced apoptosis correlates with the malignant of endometrial cancer cells

Takahiro Yaguchi ¹, Hirofumi Taira ¹, Misaki Kamenno ², Junji Kawakami ²

Running title: E₂ disturbs mitochondrial membrane potentials via a different mechanism in HEC1 and HEC50B cells

1 Department of Medical Laboratory Science, Graduate School of Health and Welfare Sciences, International University of Health and Welfare, 137-1 Enokizu, Okawa, Fukuoka 831-8501, Japan

2 Department of Nanobiochemistry, Frontiers of Innovative Research in Science and Technology (FIRST), Konan University, 7-1-20 Minatojima-Minamimachi, Chuo-ku, Kobe, 650-0047, Japan

Corresponding to Takahiro Yaguchi. Department of Medical Laboratory Science, Graduate School of Health and Welfare Sciences, International University of Health and Welfare, 137-1 Enokizu, Okawa, Fukuoka, 831-8501, Japan

Tel.: +81-944-89-2000; Fax. +81-944-89-2000

E-mail address: yaguchi@iuhw.ac.jp

Abstract

Endometrial adenocarcinoma has two type: Type I has an estrogen-derived etiology and low-grade endometrial cancer cells, on the other hand, Type II occurs estrogen-independent and is high-grade endometrial cancer cells. However, it is not well understood that the relationship between the exposure of estrogen and the grade of endometrial cancer cells. So, we investigated how estrogen affected the grade of endometrial cancer cells. In the present study, we focused on the localization and expression of Bcl-2 family that regulate the mitochondrial membrane potential in HEC1 and HEC50B cells. Cell viability was decreased by 17- β -estradiol (E_2) and E_2 disturbed the mitochondrial membrane potential followed by activating caspase-9 and caspase-3 in both cells. These results suggested that E_2 induced apoptosis in HEC1 and HEC50B cells. B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated death promoter (Bad) expression were decreased and B-cell lymphoma-extra large (Bcl-X_L) and Bcl-2-associated X protein (Bax) expression were increased at the mitochondrial outer membrane in HEC1 cells. On the other hand, Bcl-2 and Bcl-X_L expression were decreased and Bad and Bax expression were increased at the mitochondrial outer membrane in HEC50B cells. In conclusion, the dynamics of the Bcl-2 family during E_2 -induced apoptosis was found to be different depending on the grade of endometrial cancer cells. We hope that the dynamics of Bcl-2 family proteins such as Bcl-X_L and Bad will be used to diagnose the grade of malignant of endometrial cancer cells.

Key words: E_2 , mitochondrial membrane potential, Bcl-2 family, caspase, apoptosis

Introduction

The number of patients suffering from endometrial adenocarcinomas is increasing over the world, because the endometrial adenocarcinomas are difficult to discover more than cervical cancer. Endometrial adenocarcinomas have two types, such as Type I and Type II. It is well-known that Type I is dependent on the continuously exposure of estrogen, on the other hand, Type II is independent of estrogen [1-4]. In histological classify, it is divided into Grade I, Grade II and Grade III according to the percentage of

undifferentiated [5-7]. Type II and Grade III endometrial adenocarcinomas are more aggressive endometrial cancer cells [8-10].

B-cell lymphoma 2 (Bcl-2) family proteins, such as Bcl-2, B-cell lymphoma-extra large (Bcl-X_L), Bcl-2-associated death promoter (Bad) and Bcl-2-associated X protein (Bax), play an important role in the maintenance of mitochondrial membrane potentials with changing the affinities, localization and local relative abundance of these proteins [11]. The affinities between the Bcl-2 family proteins are the result of protein conformation changes, most of which occur on the mitochondrial outer membrane. Bcl-2 and Bcl-X_L belong to anti-apoptotic proteins and contain the Bcl-2 homolog (BH)1, BH2, BH3 and BH4 domains. Bad and Bax belong to pro-apoptotic proteins and Bad contain the BH3 domains, on the other hand, Bax contain the BH1, BH2 and BH3 domains [12-14]. Bcl-2, Bcl-X_L and Bax have the transmembrane domain, but not Bad. The affinity between Bcl-2 and Bax is higher than that between Bcl-2 and Bad. Moreover, the affinity between Bcl-X_L and Bad is higher than that between Bcl-X_L and Bax. Thus, Bcl-X_L recruits Bax on the mitochondrial outer membrane followed by Bax homo-oligomerization and pore formation within the mitochondrial outer membrane [15-17]. The mitochondrial membrane potentials are disturbed by Bax pore formation followed by releasing cytochrome c (Cyt c) and/or second mitochondria-derived activator of caspase (SMAC)/DIABLO from mitochondria to cytosol. The activation of caspase-9 by Cyt c and/or the neutralization of inhibitor of apoptosis (IAP) family of apoptosis of SMAC/DIABLO induce apoptosis [18-21].

The present study focused on the mitochondrial membrane potential and the dynamics of Bcl-2 family proteins in HEC1 and HEC50B cells. 17- β -estradiol (E₂) disturbed mitochondrial membrane potential depending on changing the localization of Bcl-2 family. Especially, there was a significant difference in the dynamics of Bcl-X_L (anti-apoptotic protein) and Bad (apoptotic protein) between HEC1 and HEC50B cells during E₂-disturbed mitochondrial membrane potential. In conclusion, the susceptibility to E₂ and the dynamics of Bcl-2 family proteins treated with E₂ have been changing as becoming the malignancy of endometrial cancer cells, interestingly.

Materials and Methods

Source and Cell culture

We used two cell lines: Grade II endometrial carcinoma HEC1 cells, and Grade III endometrial carcinoma HEC50B cells. These cells were obtained from the Japan Health Sciences Foundation (Tokyo, Japan). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated bovine serum and 20 mmol/L of L-Glutamine without phenol red at 37°C in a 5% CO₂ incubator.

Cell viability

Cell viability for 5,000 cells per 96-well plate was evaluated by MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay. Cells were cultured for 24 h and 48 h in serum-free DMEM in the presence or absence of E₂. After 24 h or 48 h, 20 µL of MTT solution (5 mg/mL) was added to each well, samples were incubated for 2 h at 37°C. After 100 µL of stop solution (20% SDS, 50% dimethyl formamide) was added to each cell, samples were incubated for overnight at room temperature and were measured by absorbance at 570 nm with Microplate reader (SH-9000) (CORONA ELECTRIC, Ibaragi, Japan). Data are expressed as percentage of independent basal levels.

Reverse transcription-polymerase chain reaction (RT-PCR)

Cells were scraped and total RNAs were purified using TRIzol reagent (Thermo Fisher Scientific, Kanagawa, Japan). Subsequently, 5 µg of total RNAs was used for cDNA synthesis. RT reaction was performed using Reverse transcriptase, M-MLV, RNase H⁺ (Wako, Osaka, Japan) according to manufacturer's instructions. Each cDNA (1 µL) was amplified with rTaq polymerase (TOYOBO, Osaka, Japan) and loaded in a thermal cycler (Thermo Fisher Scientific, Kanagawa, Japan) with the following steps: first step, 94°C for 2 min followed by 30 cycles, 94°C for 1 s, 62°C for 15 s and 72°C for 30 s. The reaction was carried out using primers (Table 1).

Assay of mitochondrial membrane potentials

We visualized mitochondrial membrane potentials using DePsipher™ kit (Trevigen, MD, USA). Cells were incubated in a presence and absence of E₂ for 6 h. After washing with phosphate buffered saline (PBS), cells were incubated in a DePsipher™ kit solution at 37°C for 30 min. After washing with PBS, the fluorescent signals were

observed with BZ-X810 (KEYENCE, Tokyo, Japan). The aggregate red form has absorption/emission of 585/590 nm and the green monomeric form has absorption/emission of 510/527 nm.

Mitochondria extraction

Cells were treated with E₂ or vehicle control for 6 h and collected cells. Cells were sonicated with 200 µL of mitochondria fraction solution [250 mM sucrose, 0.5 mM EGTA, 10 mM Tris-HCl (pH7.4)]. After centrifugation at 1,000 g for 10 min, supernatant (sup) was transferred into a new tube. After centrifugation at 8,000 g for 10 min, sup was transferred into a new tube and these samples were used as cytosol fraction. The pellet (ppt) was lysed with 50 µL of TBST [150 mM NaCl, 0.1% (v/v) Triton X-100 and 20 mM Tris-HCl (pH7.4)] and vortexed vigorously. After centrifugation at 5,000 rpm for 5 min, sup was used as mitochondria fraction. Subsequently, we carried out Western blotting.

Western blotting

The protein concentration of each sample was measured with a BCA protein assay kit (Takara Bio, Shiga, Japan). Lysates from mitochondria fraction (5 µg) and cytosol fraction (10 µg) were loaded onto 15% (v/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. PVDF membranes were blocked in TBST containing 1% BSA and incubated with antibodies against Bcl-2, Bcl-X_L, Bad, Bax, Cyt c (Abcam, Tokyo, Japan), and β-actin (Sigma-Aldrich, Tokyo, Japan) followed by Histofine Simple Stain MAX-PO (Nichirei Bioscience, Tokyo, Japan). Immunoreactivity was detected with an ECL plus (GE healthcare, NJ, USA) and visualized using MultiImager II chemBox (Ikeda Trading Corporation, Tokyo, Japan). Signal density was measured with an Image J software.

Enzymatic assay of caspase activity

Caspase activation was measured using a caspase fluorometric substrates; Ac-Leu-Glu-His-Asp-MCA for caspase-9 substrate peptide and Ac-Asp-Asn-Leu-Asp-MCA for caspase-3 substrate peptide (PEPTIDE institute, Osaka, Japan). Cells were incubated in a presence and absence of E₂ for 12 h. Protein (50 µg) was mixed with PBS and

substrate peptide (10 μ M) followed by incubating 37°C for 2 h. After that, the fluorescent signals were measured using a spectrofluorometer (RF-5300) (Shimadzu, Kyoto, Japan) with excitation at 380 nm and emission at 460 nm.

Statistical analysis

Data from 4 to 7 independent experiments are expressed as mean $\pm$ SEM. Statistical analysis was carried out using unpaired *t*-test. *P* values below 0.05 were considered statistically significant.

Results

E₂ decreases cell viability in HEC1 and HEC50B cells

We investigated the effect of E₂ on cell viability in endometrial cancer cells, such as HEC1 cells (Grade II) and HEC50B cells (Grade III). E₂ decreased cell viability in a concentration-dependent manner (100 nM-100 μ M) at 24-h and 48-h treatment, with reaching 38.9% $\pm$ 1.57 (*P*<0.01) and 43.7% $\pm$ 1.39 (*P*<0.01), respectively. On the other hand, E₂ attenuated cell viability at concentration above 50 μ M in HEC50B cells, with reaching 52.7% $\pm$ 0.92 (*P*<0.01) and 55.1% $\pm$ 1.53 (*P*<0.01) at 24-h and 48-h treatment, respectively. There was no critical difference between 24-h treatment and 48-h treatment in HEC1 and HEC50B cells. These results suggest that E₂ significantly downregulated the cell viability of HEC1 and HEC50B cells, especially at concentrations above 50 μ M.

E₂-induced estrogen receptor mRNA expression in HEC1 and HEC50B cells

Subsequently, we examined the expression of estrogen receptor (ER) and estrogen-related receptor (ERR) using RT-PCR in HEC1 cells (Fig. 2a: upper panel) and HEC50B cells (Fig. 2a: lower panel). ERR α mRNA only was expressed in HEC1 and HEC50B cells, however, ER α , ER β , ERR β and ERR γ mRNAs were not expressed. E₂ (50 μ M) did not change the expression of ER α , ER β , ERR α , ERR β and ERR γ mRNA in HEC1 cells (Figure 2b: upper panels) and HEC50B cells (Fig. 2b: lower panels). These results suggest that E₂ reduces cell viability via the ERR α pathway and that E₂-induced cell death may not be related to *de novo* protein synthesis.

E₂ disturbs mitochondrial membrane potentials in HEC1 and HEC50B cells

We investigated whether E₂ (50 μ M)-induced cell death is mediated by a disturbance in mitochondrial membrane potential in HEC1 and HEC50B cells. The mitochondrial membrane potentials were visualized using DePsipher™ kit. In both cells, the red fluorescent signals alone were found in control cells (Fig. 3a, 3b: upper panels). In contrast, E₂ (50 μ M)-treated cells were observed the green fluorescent signals (Fig. 3a, 3b: lower panels). These data imply that 6-h treatment with E₂ (50 μ M) disturbs mitochondrial membrane potentials in HEC1 and HEC50B cells. Thus, E₂ (50 μ M)-induced cell death was mitochondrial-dependent apoptosis in HEC1 and HEC50B cells.

E₂ changes the dynamics of Bcl-2 family proteins on mitochondria in HEC1 and HEC50B cells

To examine the mechanisms of disturbance of mitochondrial membrane potential induced by E₂ (50 μ M), we focused on the localization and expression of Bcl-2 family proteins at the mitochondrial outer membrane. In HEC1 cells, 6-h treatment with E₂ (50 μ M) attenuated Bcl-2 and Bad expression level at mitochondria ($P<0.01$ and $P<0.01$ as compared with non-treatment cells, respectively, *t*-test) (Fig. 4b, 4c). E₂ (50 μ M) enhanced the expression of Bcl-X_L and Bax at mitochondria ($P<0.01$ and $P=0.04$ as compared with non-treatment cells, respectively, *t*-test) (Fig. 4a, 4d). In HEC50B cells, on the other hand, E₂ (50 μ M) decreased Bcl-X_L and Bcl-2 expression at mitochondria ($P=0.04$ and $P<0.01$ as compared with non-treatment cells, respectively, *t*-test) (Fig. 5a, 5b). E₂ (50 μ M) increased the expression of Bad and Bax at mitochondria ($P=0.05$ and $P=0.05$ as compared with non-treatment cells, respectively, *t*-test) (Fig. 5c, 5d). These results suggest that the localization and expression of Bcl-2 family proteins induced by E₂ (50 μ M) had different regulation between HEC1 and HEC50B cells, notably Bcl-X_L and Bad. To obtain further evidence for the effect of E₂ (50 μ M) on mitochondria, we examined the expression of mitochondrial Cyt c. In both cells, E₂ significantly accelerated the release of Cyt c from mitochondria in HEC1 and HEC50B cells ($P<0.01$ and $P=0.02$ as compared with non-treatment cells, respectively, *t*-test) (Fig. 4e, Fig. 5e). These results suggest that 6-h treatment with E₂ (50 μ M) accelerates the disappearance of mitochondrial Cyt c in HEC1 and HEC50B cells.

E₂ enhances caspase-9 and caspase-3 activity through mitochondrial damage in HEC1 and HEC50B cells

Finally, we investigated whether the disturbance of the mitochondrial membrane potentials led to activate caspase-9. In HEC1 cells, E₂ (50 μ M) activated caspase-9 (Fig. 6a) and caspase-3 (Fig. 6b), reaching $117.0\% \pm 4.5$ ($P < 0.01$) and $112.0\% \pm 2.1$ ($P < 0.01$) at 12-h treatment, respectively. In HEC50B cells, E₂ (50 μ M) also accelerated caspase-9 (Fig. 7a) and caspase-3 (Fig. 7b) activity, reaching $112.3\% \pm 2.6$ ($P = 0.03$) and $117.0\% \pm 2.7$ ($P = 0.03$) at 12-h treatment, respectively. These results suggest that E₂ (50 μ M) disturbs mitochondrial membrane potential followed by facilitating the activity of caspase-9/-3.

Discussion

In the present study, E₂ (50 μ M) induced apoptosis in HEC1 cells (Grade II) and HEC50B cells (Grade III). In a previous report, we reported that E₂ (1 μ M) promoted cell viability by increasing the expression of ATP-binding cassette G2 (ABCG2) in Ishikawa 3H-12 cells (Grade I) [22]. Moreover, the effect of the endocrine disruptor bisphenol A (BPA) on cell viability in grade of endometrial cancer cells only enhanced cell proliferation in Ishikawa 3-H-12 cells (Grade I) and HEC265 cells (Grade I). However, HEC151 cells (Grade II), HEC251 cells (Grade II), HEC108 cells (Grade III) and HEC50B cells (Grade III) were not affected by BPA treatment [23]. These results implied that the susceptibility to drugs such as E₂ and BPA differed depending on the grade of endometrial cancer cells. As becoming high-grade endometrial cancer cells, major changes may have occurred at the epigenetic and proteomic levels in these cells. The results of the present study demonstrated that the dynamics of Bcl-2 family, such as Bcl-X_L, Bcl-2, Bad, and Bax, on the mitochondrial membrane after E₂ stimulation is different between HEC1 and HEC50B cells. Anti-apoptotic proteins, Bcl-2 and Bcl-X_L, etc., and apoptotic proteins, Bad and Bax, etc., are called as Bcl-2 family. Bcl-2 family is divided into three groups according to the difference in function, (A) anti-apoptotic proteins (Bcl-2, Bcl-X_L, MCL-1, etc.) including Bcl-2 homology (BH) 1, BH2, BH3 and BH4, (B) pro-apoptotic BH3-only proteins (Bad, Bid, Bik and Bim, etc.), and (C) pro-apoptotic pore-formers (Bax and Bak, etc.) including BH1, BH2 and BH3 [24-27]. These BH3-only proteins induce structural changes by binding to Bax, furthermore,

induce apoptosis with counteracting anti-apoptotic effect by binding to Bcl-2. The oligomerize of pro-apoptotic pore-forming proteins within the mitochondrial outer membrane induces the permeabilize mitochondrial membrane followed by releasing Cyt c and SMAC/DIABLO [28, 29]. E₂ (50 μ M) upregulated the expression of Bcl-X_L and Bax at the mitochondrial outer membrane, however, downregulated the expression of Bcl-2 and Bax at the mitochondrial outer membrane in HEC1 cells. On the other hand, E₂ (50 μ M) enhanced the expression of Bcl-X_L and Bcl-2 at the mitochondrial outer membrane, however, reduced the expression of Bad and Bax at the mitochondrial outer membrane in HEC50B cells. Interestingly, E₂ (50 μ M) stimulation induced apoptosis in HEC1 and HEC50B cells, however the expression of Bcl-X_L and Bad on mitochondria showed the opposite expression pattern between HEC1 and HEC50B cells (Fig. 4, 5, 8). In HEC50B cells, notably, the localization and expression of Bcl-X_L and Bad was a typical expression pattern during apoptosis. In contrast, the localization and expression of Bcl-X_L and Bad were consistent with those of anti-apoptosis in HEC1 cells. We hypothesized that as endometrial cancer cells become high grade, the affinities and localization of Bcl-2 family proteins induced by E₂ may be shifting from anti-apoptotic to apoptotic patterns. Generally, it is well-known that Bcl-X_L plays an important role in apoptosis, and the increase of Bcl-X_L expression at mitochondria indicates the inhibition of the disturbance of the mitochondrial membrane potential. According to a previously report, co-expression of Bcl-X_L and p53 is important for cell survival, thus, Bcl-X_L induced the survival of cancer cells by inhibiting the function of p53 [30, 31]. In RT-PCR analysis, p53 mRNA was expressed in HEC1 cells but not HEC50B cells (Supplementary Fig. 1a, b). Moreover, the expression of p53 mRNA was not induced by E₂ (50 μ M) (Supplementary Fig. 1a, b). The disruption of the mitochondrial membrane potential by E₂ treatment is thought to occur independently of p53 in HEC1 and HEC50B cells. On the other hand, Bad is a member of BH3-only family and does not have transmembrane domain. The BH3 domain of activator BH3-only proteins such as Bad and Bid binds to the BH3 domain-binding groove in Bax followed by eliciting a series of conformation changes such as Bax homo-oligomerization and pore formation [32, 33]. Therefore, these results suggest that the decrease of the expression of Bad on the mitochondrial outer membrane indicated resistance to E₂-induced apoptosis in HEC1 cells. However, it remains unknown what kind of dimers of Bcl-2 family proteins are formed on the mitochondrial outer membrane during E₂-induced apoptosis in HEC1 and

HEC50B cells. We are currently carrying out examining the partners of Bcl-2 family proteins at the mitochondrial outer membrane using immunoprecipitation during E₂-induced apoptosis in HEC1 and HEC50B cells.

Caspase is one of the apoptosis-induced factors and plays an important role in programmed cell death. Caspase-9/-3 pathway is downstream of the disturbance of the mitochondrial membrane potential. Cyt c and SMAC/DIABLO are released from mitochondria to cytosol through Bax pore during mitochondria-dependent apoptosis [34]. Cytosolic Cyt c binds apoptosis protease-activating factor 1 (APAF-1) and accelerates the formation of apoptosome. After that, apoptosome recruited and activated the initiator caspase procaspase-9. On the other hand, SMAC/DIABLO inhibits the inhibitor of apoptosis (IAP), a suppressor of caspase-9. In the present study, we examined on mitochondrial dynamics of Cyt c in HEC1 and HEC50B cells. Mitochondrial Cyt c was decreased as compared with non-treatment cells significantly. The disappearance of mitochondrial Cyt c by E₂ treatment caused to disturb the mitochondrial membrane potential in HEC1 and HEC50B cells. The disturbance of the mitochondrial membrane potential induced to activate caspase-9 followed by activating caspase-3 in HEC1 and HEC50B cells. We expected to observe the increase of cytosolic Cyt c treated with E₂, however cytosolic Cyt c was not significantly increased in HEC1 and HEC50B cells. These results suggest that E₂ may activate caspase via a cytosolic Cyt c-independent pathway in HEC1 and HEC50B cells. In future, it is necessary to investigate whether E₂ induces apoptosis through a SMAC/DIABLO pathway in both cells.

In conclusion, we obtained a new evidence that there was the relationship with the localization and expression of Bcl-2 family proteins and the malignance of endometrial cancer cells. Especially, as the cells became high grade of endometrial cancer cells, the expression of Bcl-X_L at mitochondrial was changed from increase to decrease, and the expression of Bax was changed from decrease to increase during E₂-induced apoptosis. These results may exhibit a useful marker of the malignance of endometrial cancer cells.

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Conflict of interests

The authors declare no conflicts of interest in association with the present study.

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Figure legends

Fig. 1

E₂ attenuates cell viability in HEC1 cells and HE50B cells. (a) HEC1 cells and (b) HEC50B cells were treated with E₂ at concentration as indicated for 24 h and 48 h. Cell viability for 5,000 cells per well (96 well) was measured using an MTT assay. In the graphs, each point represents the mean $\pm$ SEM ratio of basal level (n=5-7 independent experiments). ** P <0.01; * P <0.05, †† P <0.01; † P <0.01, P values, unpaired t -test.

Fig. 2

RT-PCR analysis is performed for ER α , ER β , ERR α , ERR β , ERR γ and GAPDH mRNA expressed in HEC1 cells (a: upper panel) and HEC50B cells (a: lower panel). HEC1 cells (b: upper panels) and HEC50B cells (b: lower panels) were treated with E₂ (50 μ M) for 0, 0.5, 1, 3 and 6 h and RNA was extracted followed by RT-PCR. Note that a similar result was obtained with 3 independent experiments.

Fig. 3

E₂ (50 μ M) disturbs mitochondrial membrane potentials in HEC1 cells and HEC50B cells. HEC1 cells (a) and HEC50B cells (b) were treated with E₂ (50 μ M) for 12 h in serum free culture medium, and mitochondrial membrane potentials were observed with BZ-X810. The aggregate red form (a: left 2 panels, b: left 2 panels) was detected at an absorption/emission of 585/590 nm and the green monomeric form (a: right 2 panels, b: right 2 panels) was detected at an absorption/emission of 510/527 nm. Scale bars, 50 μ m. Note that a similar result was obtained with 4-5 independent experiments.

Fig. 4

E₂ (50 μ M) changes the expression and localization of Bcl-2 family and Cyt c in HEC1 cells. After 6-h treatment with E₂ (50 μ M), Western blotting for Bcl-X_L (a), Bcl-2 (b), Bad (c), Bax (d), Cyt c (e) and β -actin (f) was performed in the mitochondrial and cytoplasmic fractions. In the graph, each column represents the mean $\pm$ SEM ratio against basal normalized immunoreactive intensities for mitochondrial and cytoplasmic fractions untreated with E₂ (50 μ M) (n=4-6 independent experiments). ** P <0.01; * P <0.05, P values, unpaired t -test.

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Fig. 6

E₂ (50 μ M) activates caspase-9 and caspase-3 in HEC1 cells. After 12-h treatment with E₂ (50 μ M), activities of caspase-9 (a) and -3 (b) were measured with spectrofluorometer (RF-5300). Note that a similar result was obtained with 5 independent experiments. ** P <0.01, P values, unpaired t -test.

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Fig. 8

Schematic diagram for E₂ signaling pathways.

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