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Reactive oxygen species-independent G1 arrest induced by evening primrose extract in Ehrlich ascites tumor cells

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Abstract

We previously demonstrated that evening primrose extract (EPE) induced apoptosis in Ehrlich ascites tumor cells (EATC), and this effect was specific on tumor cells. Furthermore, our results demonstrated that EPE exposure elicited a rapid increase in the activity of superoxide dismutase and intracellular peroxides levels. These changes caused translocation of Bax to mitochondria and a subsequent release of mitochondrial cytochrome *c*. However, no activation of caspase-3 was observed in EPE-treated EATC. On the other hand, apoptosis-inducing factor (AIF) was translocated from mitochondria to nuclei. The EPE-induced translocation of AIF was suppressed with the addition of catalase, suggesting that the rapid intracellular peroxide levels after addition of EPE triggers off induction of apoptosis, which is AIF-mediated and caspase-independent. In this study, we have shown that EPE elicited a dose-dependent accumulation of cells in the G1 phase and inhibited DNA synthesis. Our results also demonstrated that cell cycle arrest and inhibition of proliferation in EATC by EPE are associated with decreased Rb phosphorylation. Furthermore, inhibitions of Rb phosphorylation and DNA synthesis by EPE were not suppressed with the addition of catalase. The present study suggests that intracellular peroxides, which trigger off induction of apoptosis, are not the trigger of EPE-induced G1 arrest in cell cycle.
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Keywords: Evening primrose extract; Rb phosphorylation; G1 arrest; Reactive oxygen species; Ehrlich ascites tumor cells

1. Introduction

Oenothera biennis L., one species of evening primroses, is a herbal plant. The edible oil from the seeds of evening primrose oil has been shown to have several pharmacological effects such as anti-diabetic [1,2], anti-inflammatory [3,4], anti-premenstrual [5] as well as anti-tumoric [6–8].

We have demonstrated previously that evening primrose extract (EPE) from defatted seeds of *O. biennis* L. induced apoptosis in Ehrlich ascites tumor cells (EATC), while mouse embryo fibroblast cells (NIH3T3), used as a normal cell model, were completely resistant to the cytotoxic activity of EPE. Furthermore, we demonstrated that EPE exposure elicited a rapid increase in the activity of superoxide dismutase (SOD) and intracellular peroxides levels. These changes caused translocation of Bax to mitochondria, and a subsequent release of mitochondrial

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cytochrome *c*. One of the main consequences of mitochondrial cytochrome *c* release is the activation of caspase-3. However, no caspase-3 activation was observed. On the other hand, apoptosis-inducing factor (AIF) was translocated from mitochondria to nuclei [9, 10]. Hydrogen peroxide induces AIF translocation and apoptosis, which has been shown to be caspase-independent [11]. EPE-induced translocation of AIF was suppressed with the addition of catalase, suggesting that the rapid increase in intracellular peroxides levels after addition of EPE triggers off induction of apoptosis [10]. Hydrogen peroxide has been shown to possibly mediate the induction of apoptosis in response to external stimuli in several tumor cell lines [12,13].

The retinoblastoma gene (Rb) product is well known as a tumor suppressor and is either absent or mutated in many human tumors. This product is a phosphoprotein (110–116 kDa) that is expressed in most normal cells of vertebrates [14], and acts as a tumor suppressor by providing a cell cycle checkpoint between the G1 and S phases [15,16], and undergoes differential phosphorylation during the cell cycle. The active hypophosphorylated form of Rb is primarily associated with resting or fully differentiated cells and becomes increasingly phosphorylated throughout the cell cycle until late mitosis, when substantial dephosphorylation occurs. The hypophosphorylated Rb interacts with a number of cellular proteins including the E2F transcription factor, several cyclins, c-myc, and p46. The activity of Rb is negatively regulated by cyclin-dependent kinases (cdks), which phosphorylated Rb in late G1. Thus, the hyperphosphorylated form is primarily found in proliferating cells.

The present study, therefore, employed the same conditions of our previous studies, such as the same EATC cell line and culture conditions to elucidate the intracellular peroxides-dependent mechanisms governing cell cycle dynamics during growth arrest by EPE.

2. Materials and methods

2.1. Materials

EPE was prepared by Oryza Oil and Fat Chemical Co. Ltd, Japan. The seeds of evening primrose, *O. biennis* L. defatted with hexane were extracted with 70% ethanol and evaporated to dryness in vacuo. Fetal calf serum (FCS) was purchased from Thermo Trace Ltd. (Melbourne, Australia). Mouse anti-Rb antibody was obtained from Pharmingen (San Diego, CA). Biotinylated goat anti-mouse IgG and horse radish peroxidase-coupled streptavidin were obtained from DAKO (Kyoto, Japan). Other chemicals used in this study were special grade commercial products.

2.2. Cell culture

EATC were cultured in humidified atmosphere of 5% CO_2 in air at 37 °C for 3–4 days in Eagle's minimum essential medium containing 10% FCS. Then the cells were washed and cultured again at a concentration of 1×10^6/ml in fresh medium. EPE was dissolved in dimethyl sulfoxide (DMSO) and diluted in cultured medium immediately before use (final DMSO concentration $<0.5\%$). In all the experiments control cultures were made up of medium, DMSO and the cells.

2.3. Assay of cell viability

Cell viability of EATC was determined by the Trypan blue exclusion analysis. The tumor cells (1×10^6/ml) treated with EPE were incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C for 24 h in Eagle's minimum essential medium containing 10% FCS. To a cell suspension was added an equal volume of 0.4% Trypan blue reagent (Sigma) and percentages of viable cells were evaluated under the field microscope. Assays were performed in triplicate.

2.4. Measurement of DNA synthesis

Cells were labeled with [methyl-^{3}H]thymidine from 23 to 24 h after EPE addition. The labeled cells were washed twice in phosphate buffered saline (PBS) and radioactivity of the acid-insoluble fraction was measured as the amount of DNA synthesis [17].

2.5. Measurement of cell cycle changes

EPE-induced cell cycle changes were analyzed by Laser Scanning Cytometer (Olympus LSC 101) using

PI staining. Briefly, after designated treatments, cells were washed twice in PBS and incubated with freshly prepared PI-stained buffer (0.1% Triton X-100 in PBS, 20 μg/ml PI, 200 μg/ml RNase) for 1 h at 37 °C in the dark [18].

2.6. Preparation of protein for western blot analysis of Rb

EPE-treated cells were washed twice in PBS and resuspended in 100 μl of lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM ethylenediaminetetra-acetic acid (EDTA), 0.5% sodium deoxycholate, 1% Nonidet P-40, 10 μg/ml pepstatin, 1 mM sodium vanadate, 50 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonate, pH 7.5) for 20 min on ice followed by freeze-thawing three times. Lysates were centrifuged at 17 500*g* for 20 min at 4 °C and supernatant was collected for western blot [18]. Protein concentrations were determined by the Bradford method [19].

2.7. Western blotting analysis of Rb

Protein (20 μg) for Rb assay was loaded onto each lane of a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and the separated proteins were blotted to 0.45 μm polyvidyline fluoride membranes (Amarsham Pharmacia Biotech). After an overnight blocking with 5% non-fat milk, 0.1% Tween-20, in PBS, the membrane was stained with anti-Rb antibody for 1 h at room temperature. After washing, the membrane was reincubated with 1:1500 diluted biotinylated mouse IgG for 1 h at room temperature. The membrane was washed several times, and then incubated with 1:400 diluted horse radish peroxidase-coupled streptavidin for 1 h at room temperature. After several washing steps the color reaction was developed with 3-amino-9-ethylcalbazole [18].

2.8. Statistical analysis

Data are represented as mean ± SD (standard deviation from the mean) and statistical evaluations of cell viability and DNA synthesis were made using analysis of variance with Fisher's post hoc comparison test. $P < 0.05$ was used to indicate a statistically significant difference.

3. Results

3.1. Effects of EPE on DNA synthesis

The effect of EPE on [^{3}H]thymidine incorporation into acid-insoluble fraction was examined in EATC. As shown in Fig. 1, EPE inhibited DNA synthesis significantly as compared with non-treated cells.

3.2. EPE and cell cycle changes

EPE-induced cell death in EATC was also evaluated by cell cycle analysis with PI staining. The changes in cell cycle profile induced by EPE are shown in Fig. 2. Cells incubated with EPE resulted in an accumulation of a discrete sub-population of signals under the G1 cell cycle region and a decrease in S cell cycle region.

3.3. Effect of EPE on Rb phosphorylation

EPE decreased hyperphosphorylated Rb (pRb) levels and increased hypophosphorylated (Rb) levels in a dose- and time-dependent manner (Fig. 3(A) and (B)).

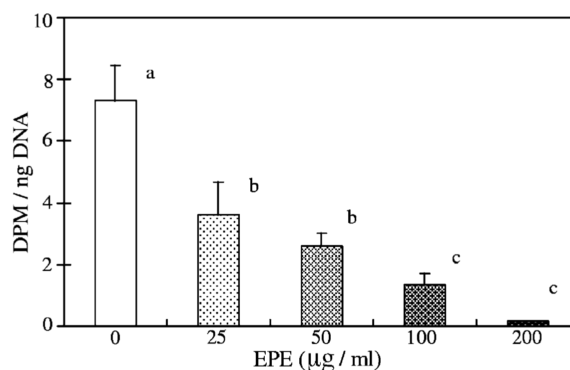

Fig. 1. Effect of EPE on DNA synthesis in Ehrlich ascites tumor cells. Cells were cultured in Eagle's minimum essential medium containing 10% FCS for 3–4 days, were diluted and incubated again in fresh medium with or without EPE. Cells were labeled with [^{3}H]thymidine from 23 to 24 h after EPE addition. Radioactivity of acid-insoluble fraction was measured as the amount of DNA synthesis. EPE extract was dissolved in dimethyl sulfoxide (DMSO) (final concentration of DMSO was <0.5%). Each point is the mean (± SD) of three experiments. Data not sharing common alphabet are significantly different ($P < 0.05$) using Fisher's test.

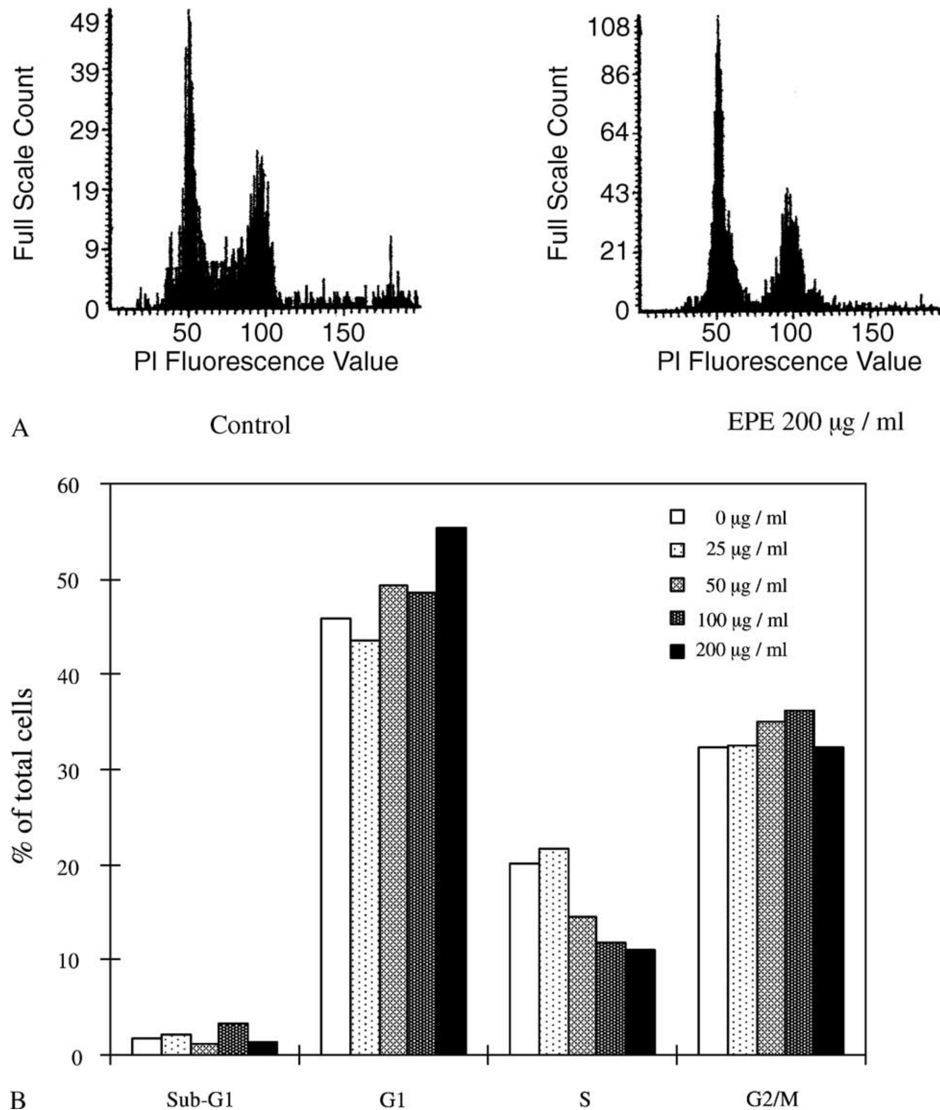

Fig. 2. Effect of EPE on cell cycle in Ehrlich ascites tumor cells. Cells were incubated with EPE for 24 h, washed twice in PBS, and incubated with freshly prepared PI-stained buffer for 1 h at 37 °C in the dark. The staining was analyzed by Laser Scanning Cytometer. Results are representative of three separate determinations.

3.4. Effects of catalase on cell viability and DNA synthesis

Catalase reacts with hydrogen peroxide to from water and molecular oxygen, which is one of the primary defense mechanisms against hydrogen peroxide. In a previous study, we demonstrated that the rapid increase in intracellular peroxides levels after addition of EPE triggers off induction of apoptosis. We examined the effect of catalase on cell viability by Trypan blue method. Furthermore, to determine whether the EPE-induced increase in intracellular peroxide was related to inhibition of DNA synthesis, we examined the effect of catalase on DNA synthesis. As shown in Table 1, EPE decreased cell viability and this effect was reversed with the addition of 100 units/ml catalase 5 min before the addition of 200 μg/ml EPE. However,

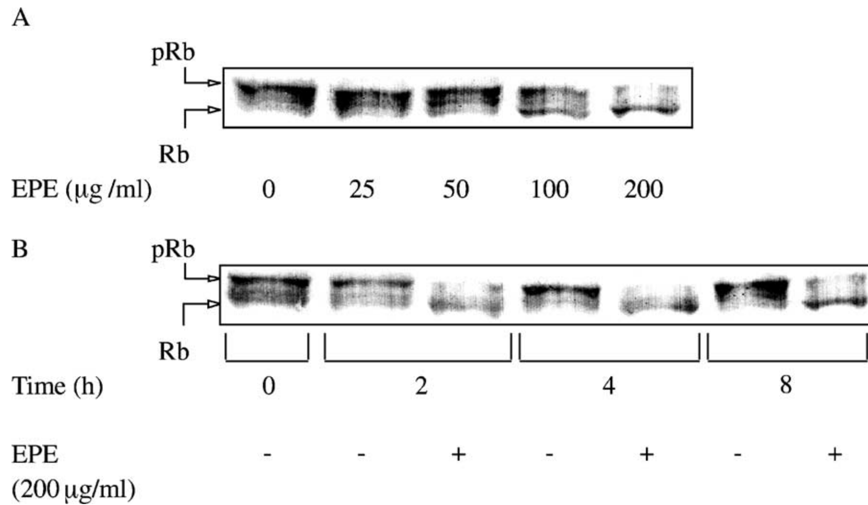

Fig. 3. Effect of EPE on changes in Rb phosphorylation. In dose-dependent study (A), cells were cultured in Eagle's minimum essential medium with various concentrations of EPE (0–200 μg/ml) and harvested at 8 h. In time-dependent study (B), cells were cultured with 200 μg/ml EPE and harvested at indicated times. Lysates were analyzed by Western blot. Results are representative of three separate determinations. pRb, hyperphosphorylated Rb; Rb, hypophosphorylated Rb.

the addition of catalase did not reverse the EPE-induced decrease in DNA synthesis.

3.5. *Effects of catalase on Rb phosphorylation*

To determine whether the EPE-induced increase in intracellular peroxide is associated with the phosphorylation of Rb, we examined the effect of catalase on Rb phosphorylation using western blot analysis. As shown in Fig. 4, EPE decreased hyperphosphorylated Rb (pRb) levels and increased hypophosphorylated (Rb) levels. However, these effects were not reversed with the addition of 100 units/ml catalase 5 min before the addition of 200 μg/ml EPE.

4. Discussion

The significant finding in this study is that EPE caused a decrease in DNA synthesis, an accumulation of cells in the G1 phase, and a decrease in the phosphorylation of Rb protein, in its growth inhibitory effect in EATC. We previously demonstrated that a rapid increase in intracellular peroxide levels after addition of EPE triggers off induction of apoptosis [9, 10]. Contrarily to this observation however, this rapid increase in intracellular peroxide levels did not trigger off inhibition of cell growth arrest in this study.

We showed in previous studies that EPE caused a statistically significant decrease in the viability of tumor cells, which was shown to be apoptotic. Furthermore, we demonstrated that EPE exposure elicited a rapid increase in the activity of SOD and intracellular peroxide levels. These changes caused translocation of Bax to mitochondria, and subsequent release of mitochondrial cytochrome *c*. One of the main consequences of mitochondrial cytochrome *c* release is the activation of caspase-3. However, no caspase-3 activation was observed. On the other hand,

Table 1
Effects of catalase (CAT) on cell viability and DNA synthesis in EPE-treated Ehrlich ascites tumor cells

	Cell viability (%)	DNA synthesis (DPM/μg DNA)
Control	95.2 ± 1.0^a	7311 ± 1139^a
EPE	34.2 ± 3.0^b	145 ± 29^b
EPE + CAT	89.8 ± 3.1^a	131 ± 24^b

Catalase (100 units/ml) was added 5 min before the addition of 200 μg/ml EPE. Cell viability was determined at 24 h after EPE addition by the Trypan blue exclusion analysis. Each point is the mean (± SD) of three experiments. Data not sharing common alphabet are significantly different ($P < 0.05$) using Fisher's test.

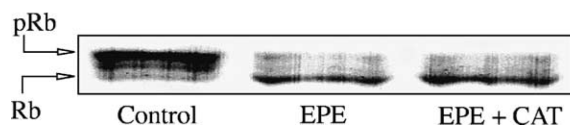

Fig. 4. Effect of catalase (CAT) on EPE-induced changes in Rb phosphorylation in Ehrlich ascites tumor cells. Catalase (100 units/ml) was added 5 min before the addition of 200 μg/ml EPE, and cells were harvested at 8 h. Lysates were analyzed by Western blot.

AIF was translocated from mitochondria to nuclei. Hydrogen peroxide induces AIF translocation and apoptosis, which has been shown to be caspase-independent [11]. EPE-induced translocation of AIF was suppressed with the addition of catalase, suggesting that the rapid increase in intracellular peroxides levels after addition of EPE triggers off induction of apoptosis [10].

We demonstrated that EPE elicited a dose-dependent accumulation of cells in the G1 phase and inhibited DNA synthesis. The notable components of EPE are polyphenols (60% w/w), and several studies have shown that polyphenols, such as epicallocatechin and epigallocatechin-3-gallate in tea [18,20], resveratrol in grape seeds [21], carnoaic acid in rosemary [22], and a polyphenolic fraction from grape seeds [23], cause growth arrest in tumor cells by eliciting G0–G1 phase of the cell cycle.

The mechanisms of cell cycle progression and arrest have been extensively studied, and the involvement of Rb proteins and also of several cdks in the G1 phase has been reported [24]. Rb proteins play important roles in the control of progression through the G1 stage of the cell cycle [24,25]. In early G1, hypophosphorylated Rb proteins are present as a complex with the transcription factor E2F, thereby inactivating E2F. The phosphorylation of Rb proteins in the mid-to-late G1 phase owing to the action of cdk–cyclin complexes results in dissociation of the Rb–E2F complex and allows E2F to activate transcription of several genes, such as *cyclin A*, thymidine kinase and c-*myc*, which are required for the progression through late G1 and into S phase of the cell cycle [26]. Our results demonstrate that treatment with EPE caused a dose- and time-dependent decrease in hyperphosphorylated Rb and an increased in hypophosphorylated Rb proteins in EATC. These findings imply that cell cycle arrest and growth inhibition in EATC by EPE are associated with decreased Rb phosphorylation.

Recent studies have suggested that reactive oxygen species (ROS), including peroxides, may mediate the induction of G1 arrest in cell cycle via the inhibition of Rb phosphorylation in response to external stimuli in various cell types, such as the human fibroblast cells [27], pulmonary epithelial cells [28] and HepG2 cells [29]. Tetrandrine increased intracellular ROS, activated p53, and increased expression level of $p21^{waf1}$ and Bax, and induced cell cycle arrest in G1 phase (via $p21^{waf1}$ and dephosphorylation of pRb) and apoptosis (via Bax) in Neuro 2a mouse neuroblastoma cells [30]. However, our results showed that inhibitions of phosphorylation of Rb and DNA synthesis by EPE were not hindered by addition of catalase, and therefore suggested that peroxides are not the trigger of EPE-induced G1 arrest.

In conclusion, we demonstrated previously that an EPE-induced rapid increase in intracellular peroxides level triggers off induction of apoptosis in EATC. However, the findings in the present study suggest that intracellular peroxides do not cause an EPE-induced G1 arrest. Hence, EPE-induced inhibitions of the growth of EATC are via at least two pathways differentially modulated by ROS, notably intracellular peroxides. These are (a) the EPE-induced apoptosis pathway which is dependent on increases in hydrogen peroxide and (b) the EPE-induced inhibition of cell proliferation which is hydrogen peroxide independent. The identification of factors that trigger the EPE-induced G1 arrest is currently under investigation.

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