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ORIGINAL ARTICLES

Exogenous phosphatidylethanolamine induces apoptosis of human hepatoma HepG2 cells *via* the bcl-2/bax pathway

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Abstract

AIM: To investigate the signaling pathways implicated in phosphatidylethanolamine (PE)-induced apoptosis of human hepatoma HepG2 cells.

METHODS: Inhibitory effects of PE on human hepatoma HepG2 cells were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell cycle, apoptosis and mitochondrial transmembrane potential ($\Delta\Psi m$) were analyzed by flow cytometry. Immunocytochemical assay and Western blotting were used to examine Bcl-2, Bax and caspase-3 protein levels in HepG2 cells treated with PE.

RESULTS: PE inhibited the growth of HepG2 cells in a dose- and time- dependent manner. It did not affect the cell cycle, but induced apoptosis. PE significantly decreased $\Delta\Psi m$ at 0.25, 0.5 and 1 mmol/L, respectively, suggesting that PE induces cell apoptosis by decreasing the mitochondrial transmembrane potential. The Bcl-2 expression level induced by different concentrations of PE was lower than that in control groups. However, the Bax expression level induced by PE was higher than that in the control group. Meanwhile, PE increased the caspase-3 expression in a dose- and time-dependent manner.

CONCLUSION: Exogenous PE induces apoptosis of human hepatoma HepG2 cells *via* the bcl-2/bax pathway.

Key words: Apoptosis; Bcl-2; Bax; Caspase-3; Phospha tidylethanolamine; Human hepatoma HepG2 cell

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INTRODUCTION

Phospholipids, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (SM), are the dominant lipid constituents of the membranes of animal cells, in which they are distributed in an asymmetrical fashion and act as the matrix for the support and organization of different membrane proteins[1]. In addition to this structural role, many individual phospholipid constituents are known to be involved in specific signaling functions necessary for cells to respond to external stimuli[2-4].

Some studies have provided evidence that membrane

phospholipid asymmetry is disturbed in one of the early stages of apoptosis[5-7]. Specifically, a translocation of PS and PE from the internal to external surface of the plasma membrane appears to be a fundamental mechanism through which apoptotic cells are recognized and eliminated by phagocytic macrophages[8-10]. PS and PE externalization could arise either through inactivation of aminophospholipid translocase (APTL), whose normal function maintains the asymmetric distribution of PS and PE in cells[11,12] or by accelerating reversal of movement of the phospholipid. Inhibition of APTL could induce apoptosis of central nervous system (CNS)-derived HN2-5 and HOG cells, activating caspase-3, indicating that abnormal distribution of cell membrane phospholipid can induce apoptosis[13]. PS externalization is a characteristic feature of the apoptotic cells. It has been shown that the externalized PS serves as a marker for detecting the apoptotic cells, and PE exposed to the cell surface forms lipid rafts with PS during apoptosis[14].

It was recently reported that overexpression of Raf kinase inhibitor protein (RKIP), a member of the phosphatidylethanolamine-binding protein (PEBP) family, can inhibit the Raf-ERK1/2 pathway[15]. However, the effects of PE on cell proliferation or apoptosis remain unclear. Our results in this study suggest that exogenous PE induces apoptosis of human hepatoma HepG2 cells *via* the bcl-2/bax pathway.

MATERIALS AND METHODS

Materials and agents

RPMI-1640 medium and fetal calf serum were purchased from GIBCO (Canada). PE, MTT and propidium iodide (PI) were purchased from Sigma. Annexin V-FITC apoptosis detection kit was from BD Biosciences (USA). Monoclonal antibodies were obtained from Cell Signaling Technology (USA).

Cell culture

Human hepatoma cell line HepG2 was obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). SMMC7721, HEK293 and HeLa cells were provided by Molecular Biology Center of the First Affiliated Hospital, Xi'an Jiaotong University. HepG2 cells (5.0×10^4 cells/mL) were cultured in RPMI-1640 supplemented with 100 mL/L fetal bovine serum, containing 2.0 mmol/L glutamine and 20 μg penicillin-streptomycin/mL in 50 mL/L CO_2 at 37℃, and allowed to adhere for 24 h. The experiments were divided into four groups: control group, 0.25 mmol/L PE, 0.5 mmol/L PE, and 1 mmol/L PE treatment groups.

MTT assay for cell viability

HepG2 cells (2×10^4 cells/well) were seeded onto 96-well plates and incubated with test substances for an indicated time at 37℃ in an atmosphere containing 50 mL/L CO_2. Then, 20 μL MTT solution (5 g/L) was added into each well and incubated for another 4 h. Supernatants were removed and formazan crystals were dissolved in 200 μL dimethylsulfoxide. Finally, optical density was determined at 490 nm by POLARstar + OPTIMA (BMG Labtechnologies, Germany).

Cell cycle analysis by flow cytometry

DNA content per duplicate was analyzed by flow cytometry (BD Biosciences). Adherent cells were harvested by brief trypsinization, and washed with PBS, fixed in 700 mL/L ethanol, stained with 20 μg/mL PI containing 20 μg/mL RNase (DNase free) for 30 min, and analyzed by flow cytometry. The number of cells at the G0/G1, S, and G2/M phases was calculated.

Detection of HepG2 cell apoptosis by annexin-V/PI staining

HepG2 cells were treated with PE at 37℃ in an atmosphere containing 50 mL/L CO_2 for 48 h, then harvested and washed twice with PBS. The cells were labeled by incubation with 5 μL FITC-annexin V and 10 μL PI at 250 μg/mL for 10 min in the dark at room temperature. The cells were washed with PBS again and examined by flow cytometry. Apoptosis was routinely quantified by counting the number of cells stained with FITC-labeled annexin V.

$\Delta\Psi m$ examination

HepG2 cells (1×10^6/mL) were washed twice with PBS, incubated with rhodamine 123 (10 μg/mL) at 37℃ for 30 min, then washed with PBS and analyzed by flow cytometry.

Immunocytochemical assay

HepG2 cells were grown on glass culture slides coated with poly-lysine in a 24-well plate and treated with PE. Slides with cells were fixed in 40 mg/L paraformaldehyde for 20 min, and then incubated with monoclonal antibody (anti-Bcl-2, anti-Bax, anti-caspase-3), which was labeled with FITC-conjugated goat anti-rabbit IgG. Positive-staining-area percentages were calculated under SP2 confocal microscope (Leica, Germany). Additionally, fluorescence intensity in 20 positive cells was evaluated.

Western blotting

HepG2 cells were plated at 5×10^4 cells/tissue culture dish in six-well plates with RPMI-1640. After exposure to the inhibitor at different concentrations, the cells were washed with PBS and subsequently lysed in 200 μL of a lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 0.1 mg/L sodium azide, 10 mL/L NP-40, 1 mmol/L phenylmethylsulfonyl fluoride. Insoluble material was removed by microcentrifugation at 13000 r/min for 15 min at 4℃. Cell lysates (80 μg of protein/lane) were subjected to electrophoresis on 100 mg/L SDS-PAGE. The proteins were transferred to a polyvinylidene difluoride membrane (NEN Life Science Products, Boston, MA, USA).

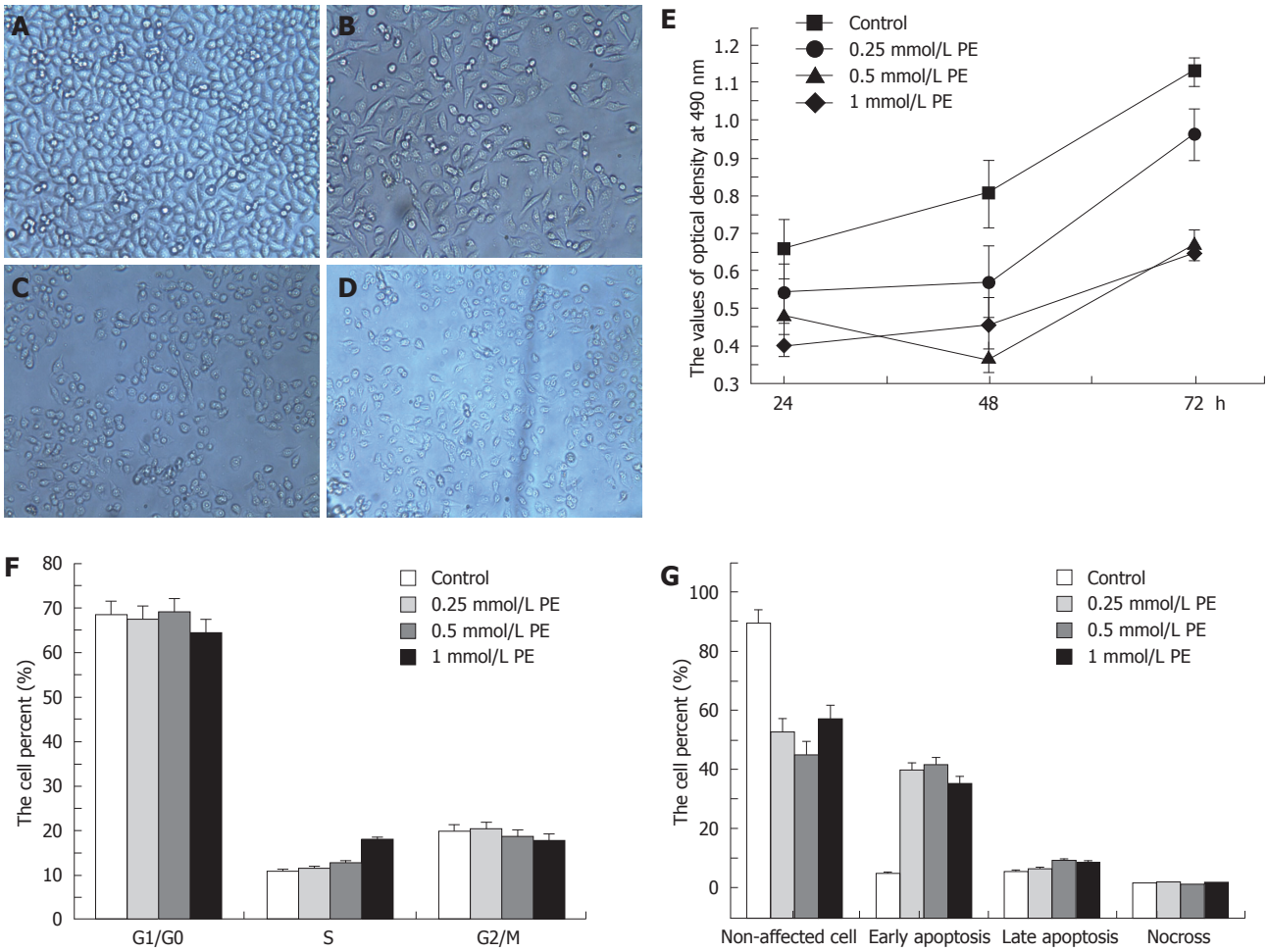

Figure 1 Effect of exogenous PE on the growth and apoptosis of human hepatoma HepG2 cells. A: Control group; B: 0.25 mmol/L PE; C: 0.5 mmol/L PE; D: 1 mmol/L PE; E: 48 h after treatment, inhibition of cell growth shown by MTT assay; F: Cell cycle in human hepatoma HepG2 cells shown by PI staining 24 h after PE treatment, with data showing the cell percentages at G1/G0, S and G2 phases; G: Apoptosis of human hepatoma HepG2 cells shown by annexin- V/PI staining 24 h after PE treatment, with data showing the percentages of non-affected, early and late apoptotic cells and necrosis. The results are given as mean ± SD from three experiments.

After blocked with Tris-buffered saline containing 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5 mL/L Tween 20, 10 mg/L bovine serum albumin, the membrane was incubated with different monoclonal antibodies (R&D Systems) and anti-glyceraldehyde-3-phosphate dehydrogease, respectively. For chemiluminescence detection, WB membranes were incubated in the dark with ECL (Amersham) which is a luminol-based enhanced chemiluminescence substrate for horseradish peroxidase. The luminescent signal was recorded and quantified with the Syngene G box (Syngene, UK) which consists of a high-performance CCD videocamera with focus stabilized optics, in a mini darkroom enclosure. The instrument is linked to a computer that controls the instrument and handles the data. The luminescent signal is detected by the CCD camera and transmitted to the controller unit and the data are sent to the computer for analysis and documentation.

Statistical analysis

All data were expressed as mean ± SD and analyzed by SPSS 11.0 software. Analysis of data was performed using t test. $P < 0.05$ was considered statistically significant.

RESULTS

PE inhibited growth of HepG2 cells

The growth of SMMC7721, HEK293, HeLa and HepG2 cells was detected by MTT assay at different time points after treatment with PE at different concentrations. The results showed that PE inhibited the growth of these cells as well as normal cells (HEK293), suggesting that the inhibition is non-specificic (Table 1). HepG2 cells were chosen in our study.

Polygon or fusiform HepG2 cells were observed in the control group with intact and distinct peripheria (Figure 1A), but round HepG2 cels were found in the PE treatment groups with ambiguous peripheria (Figure 1C and D). PE inhibited the growth of HepG2 cells in a dose-and-time dependent manner (Figure 1E). PE did not affect the cell cycle (Figure 1F), but induced apoptosis (Figure 1G).

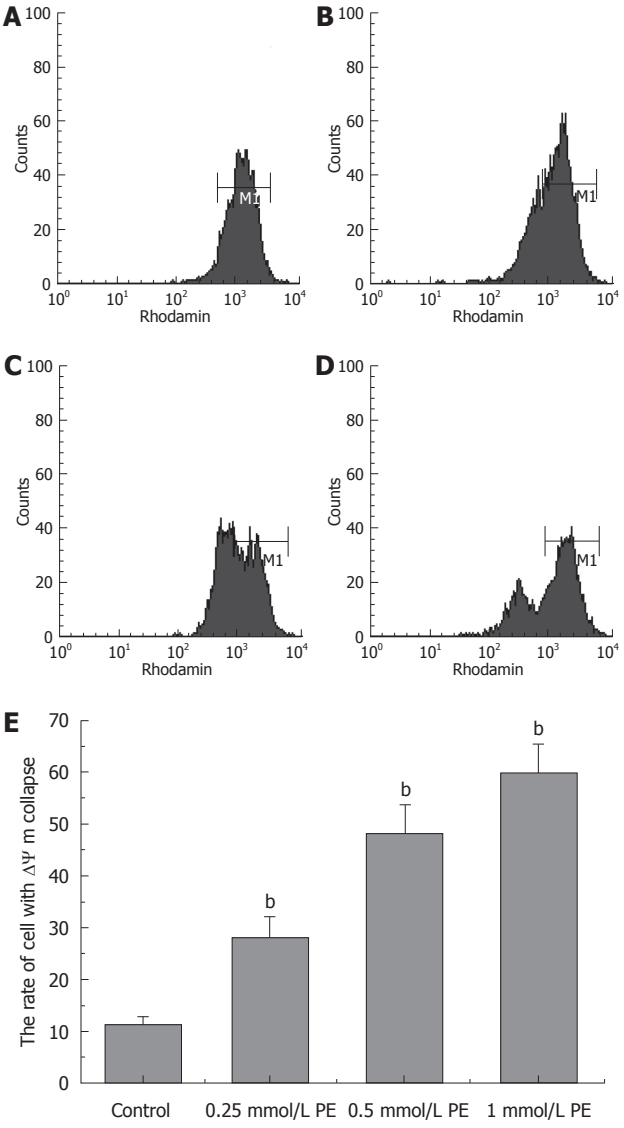

Figure 2 Effects of exogenous PE on the ΔΨm of human hepatoma HepG2 cells. A: Control group; B, C, D: Groups treated with 0.25, 0.5 and 1 mmol/L PE at 24 h; E: Flow cytometry analysis of cells with ΔΨm collapse in human hepatoma HepG2 cells shown by rhodamine staining at 24 h. The results are given as mean ± SD from three repeat experiments. $^bP < 0.01$ *vs* control group.

Effect of PE on ΔΨm of HepG2 cells

The uptake of rhodamine123, a lipophilic fluorescent dye absorbed by mitochondria, is positively correlated with ΔΨm. The descent of rhodamine can reflect the collapse of ΔΨm[16]. In order to determine the effect of PE on ΔΨm, the uptake of rhodamine was detected by flow cytometry. PE significantly decreased the ΔΨm at 0.5 mmol/L and 1 mmol/L (Figure 2C-E), suggesting that PE induces cell apoptosis by decreasing the ΔΨm.

PE induced apoptosis of Bcl-2/Bax in HepG2 cells

Bcl-2 and Bax are involved in the maintenance of mitochondria membrane stability[17,18]. Immunocytochemical assay showed that Bcl-2 expression in HepG2 cells was significantly suppressed 24 h after treatment with PE, showing a negative correlation with the PE dosage (Figure 3A-E), while Bax expression was significantly increased 24 h after treatment with PE, showing a positive correlation with the PE dosage (Figure 4A-E). The results of Western blotting and immunocytochemical assay were similar. The Bcl-2 expression level was lower in different PE treatment groups than in the control group at different time points (Figure 3F). However, the Bax expression level was higher in different PE treatment groups than in the control group (Figure 4F).

Involvement of caspase-3 in HepG2 cell apoptosis induced by PE

Caspase-3, a key regulatory protease from which many signaling pathways merge for the execution of apoptosis, participates in apoptosis induced by bcl-2/bax, p38 and JAK-STAT[19,20]. We detected the caspase-3 expression in HepG2 cells after treatment with PE. Immunocytochemical assay showed that caspase-3 expression was significantly increased, showing a positive correlation with the PE dosage 24 h after treatment (Figure 5A-E). The caspase-3 expression level was higher in PE treatment groups than in the control group (Figure 5F). However, PE increased the caspase-3 expression in a dose- and time-dependent manner (Figure 5F).

DISCUSSION

PE is an important phospholipid component, which is involved in the formation of membrane asymmetry. PE locates at the intracellular leaflet of normal cell membranes, and is exposed to the cell surface during apoptosis. It has been shown that externalization of PE is a signal of early apoptosis[21]. However, the effect of PE on cell apoptosis remains unclear.

In this study, PE inhibited the growth of HepG2 cells (Figure 1A-E) in a dose-dependent manner. Because the cell cycle and apoptosis are involved in the regulation of cell growth, they were detected with a flow cytometer 24 h after treatment with PE in our study. PE did not significantly affect the cell cycle (Figure 1F), but induced apoptosis of HepG2 cells (Figure 1G), suggesting that PE induces apoptosis by inhibiting the growth of HepG2 cells.

At the early stage of cell apoptosis, ΔΨm was decreased before chromatin condensation and DNA fragmentation. It was recently reported that mitochondrial dysfunction is essential to the apoptotic pathway, and loss of ΔΨm may be an early event in the apoptotic process[22]. Reduced ΔΨm induces cytochrome C release from the mitochondria, and causes apoptosis[23-25]. In this study, 0.5 and 1 mmol/L of PE significantly decreased the ΔΨm (Figure 2C-E), suggesting that PE induces apoptosis of HepG2 cells *via* the mitochondrial pathway. Loss of ΔΨm was found to be closely associated with the expression of Bcl-2 and Bax. It is generally thought that the expression of Bax increases following death stimulation, and then translocates at the mitochondria to induce cytochrome

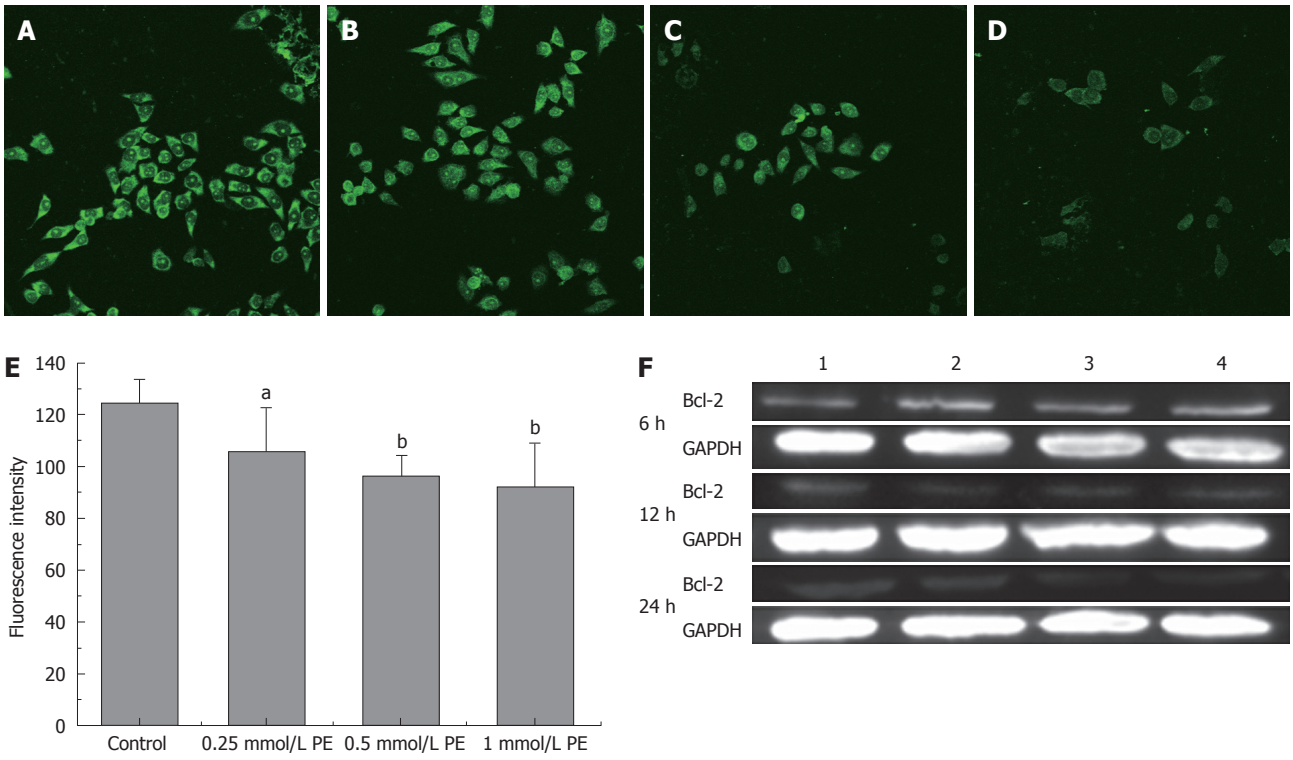

Figure 3 Inhibitory effect of exogenous PE on bcl-2 expression in human hepatoma HepG2. A: Cells in control group; B: 0.25 mmol/L PE; C: 0.5 mmol/L PE; D: 1 mmol/L PE; E: Fluorescence intensity; F: 48 h after treatment and Western blotting. The results are presented as mean ± SD. Lane 1: Control group; lanes 2-4: 0.25, 0.5 and 1 mmol/L PE treatment groups. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

Table 1 Inhibitory effect of PE on the growth of different types of cells (mean ± SD)

Time of treatment	24 h				48 h			
PE (mmol/L)	0	0.25	0.5	1	0	0.25	0.5	1
SMMC7721	0.75 ± 0.06	0.59 ± 0.03^{b}	0.42 ± 0.02^{b}	0.30 ± 0.02^{b}	0.96 ± 0.03	0.42 ± 0.02^{b}	0.36 ± 0.02^{b}	0.30 ± 0.02^{b}
HepG2	0.66 ± 0.08	0.54 ± 0.08^{b}	0.48 ± 0.07^{b}	0.40 ± 0.03^{b}	0.81 ± 0.09	0.57 ± 0.10^{b}	0.36 ± 0.03^{b}	0.45 ± 0.08^{b}
HEK293	0.63 ± 0.02	0.39 ± 0.10^{b}	0.40 ± 0.01^{b}	0.35 ± 0.02^{b}	0.80 ± 0.03	0.40 ± 0.02^{b}	0.31 ± 0.02^{b}	0.24 ± 0.03^{b}
HeLa	1.28 ± 0.09	1.16 ± 0.06^{a}	1.08 ± 0.04^{a}	0.95 ± 0.09^{b}	1.66 ± 0.11	1.29 ± 0.01^{b}	1.15 ± 0.05^{b}	1.01 ± 0.06^{b}

$^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* control group.

C release[26,27]. It is also known that Bax undergoes post-translational modification during apoptosis of HepG2 cells in response to various stimuli with interferon alpha and chemotherapeutic drugs, and the cleaved form of Bax is a potent inducer of apoptosis[28-30]. Anti-apoptotic Bcl-2 inhibits the pro-apoptotic function of Bax. In the present study, PE up-regulated the expression of Bax and down-regulated the expression of Bcl-2 in HepG2 cells in a dose-and time-dependent manner. Bax expression was observed 12 and 24 h after treatment with 0.5 and 1 mmol/L PE (Figure 4E). The expression of Bax was negatively corelated with decreased $\Delta\Psi$m, suggesting that increased Bax expression may be involved in the decreased $\Delta\Psi$m. Increased Bax/Bcl-2 proportion will lead to release of cytochrome C from the mitochondria, thus inducing cell apoptosis[31]. Experiments *in vitro* have proved that PE plays a key role in cytochrome C transmembrane transport in liposomes composed of acid-phospholipid and neutro-phospholipid[32]. When the amount of the PE in PA/PE/PC system is increased, cytochrome C transmembrane transport increases in liposome[32].

Caspases are cystein proteases that play a key role in cascade activation during apoptosis induced by many stimuli[33-36]. Activation of initiator of caspases (procaspases 8-10) leads to proteolytic activation of downstream effector caspases (caspase-3, -6, -7). The activation of caspase-3 is a common event in two major pathways, death receptor and mitochondrial pathways[37-40]. In the present study, we proved that PE up-regulated caspase-3 expression in a dose-dependent manner, suggesting that exogenous PE induces apoptosis of human hepatoma HepG2 cells *via* the bcl-2/bax pathway.

COMMENTS

Background

Phosphatidylethanolamine (PE) is one of the dominant lipid constituents in the membranes of animal cells in which it is distributed in an asymmetrical fashion. Many individual phospholipid constituents are known to be involved in specific signaling functions necessary for cells to respond to external stimuli, but the specific signaling function of PE is largely unknown.

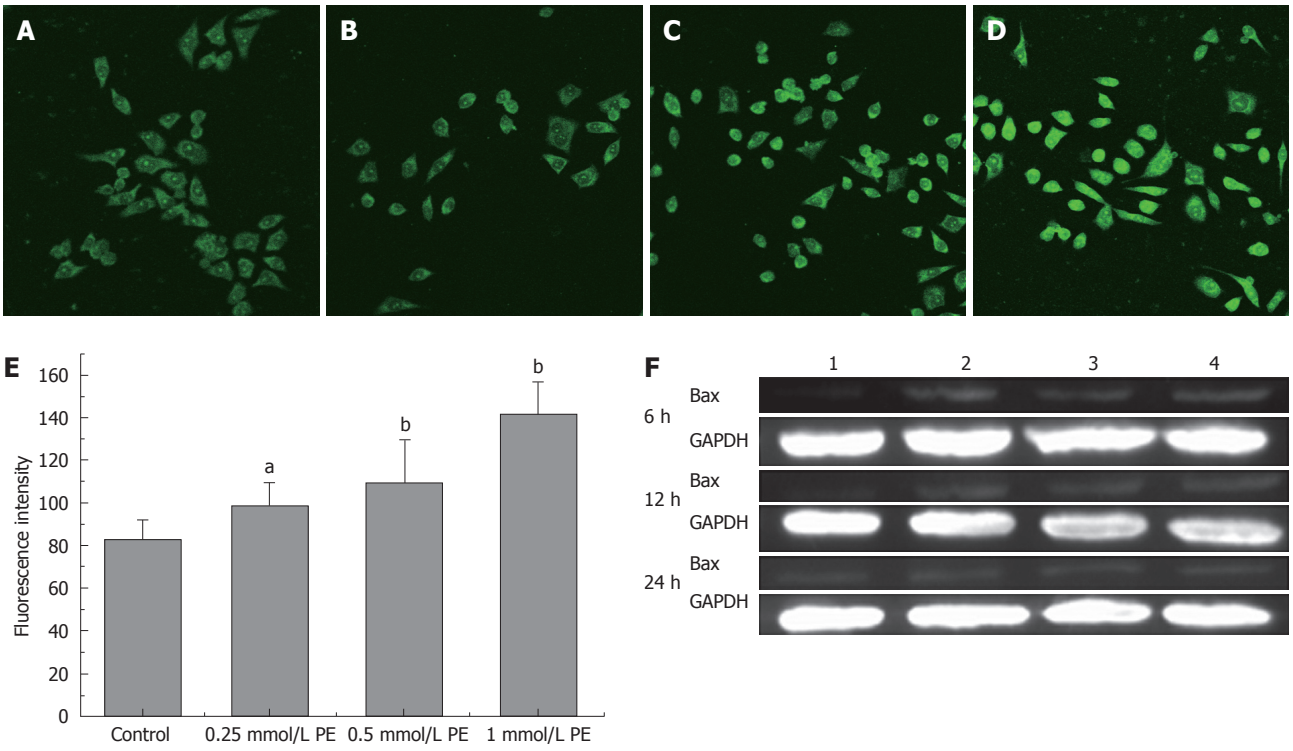

Figure 4 Inhibitory effects of exogenous PE on bax expression in human hepatoma HepG2 cells. A: Control group; B: 0.25 mmol/L PE; C: 0.5 mmol/L PE; D: 1 mmol/L PE; E: Fluorescence intensity; F: 48 h after treatment and Western blotting. The results are presented as mean ± SD. Lane 1: Control group; lanes 2-4: 0.25, 0.5 and 1 mmol/L PE treatment groups. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

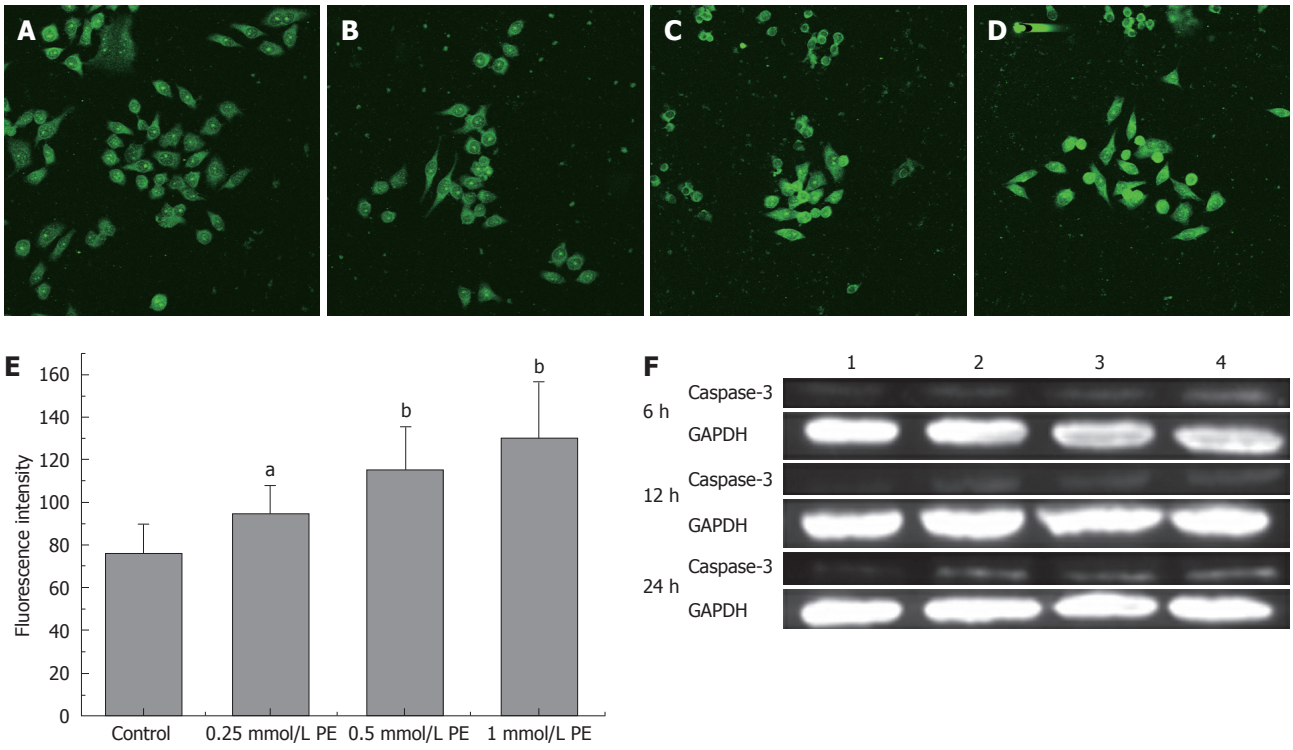

Figure 5 Inhibitory effect of exogenous PE on caspase-3 expression in human hepatoma HepG2 cells. A: control group; B: 0.25 mmol/L PE; C: 0.5 mmol/L PE; D: 1 mmol/L PE; E: Fluorescence intensity, F: 48 h after treatment and Western blotting. The results are presented as mean ± SD. Lane 1: Control group; lanes 2-4: 0.25, 0.5 and 1 mmol/L PE treatment groups. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

Research frontiers

Translocation of phosphatidylserine (PS) and PE from the internal to external surface of plasma membrane appears to be a fundamental mechanism underlying apoptotic cells. Aminophospholipid translocase involving the asymmetric distribution of PE in cells was inhibited to induce apoptosis in our study, implying that PE externalization leads to apoptosis. The authors of this paper demonstrated that exogenous PE could induce apoptosis of human hepatoma HepG2 cells.

Innovations and breakthroughs

PS externalization is a typical feature of apoptotic cells. It has been shown that externalized PS serves as a marker for detecting apoptotic cells. PE exposed to the cell surface forms lipid rafts with PS during apoptosis. This is the first study to report that the bcl-2/bax pathway is involved in PE-induced apoptosis of HepG2 cells.

Applications

PE is a dominant component of liposome. This study revealed the mechanism of liposome cytotoxicity to cells.

Peer review

In the present work, the authors showed that PE, one of the important phospholipid components in cell membrane, inhibited the growth of HepG2 cells by inducing apoptosis, but did not change the cell cycle. Furthermore, PE down-regulated Bcl-2 expression, and up-regulated Bax expression in HepG2 cells, induced $\Delta\Psi m$ collapse, and increased the caspase-3 expression level. Therefore, exogenous PE could induce apoptosis of human hepatoma HepG2 cells *via* the bcl-2/bax pathway. So it is an interesting work.

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