0026-895X/02/6103-524–532$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 61:524–532, 2002

Vol. 61, No. 3
1235/964811
Printed in U.S.A.

Endothelin-1 Protects Ovarian Carcinoma Cells against Paclitaxel-Induced Apoptosis: Requirement for Akt Activation

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Received July 25, 2001; accepted December 5, 2001

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Endothelin-1 (ET-1) is a powerful mitogenic peptide produced by different tumors. In ovarian carcinoma cells, ET-1 acts as an autocrine growth factor, selectively through ET_A receptor (ET_AR), which is predominantly expressed in tumor cells. The aim of this study was to examine whether ET-1 plays a role in the sensitivity of three ovarian carcinoma cell lines (OVCA 433, HEY, and SK-OV-3) to apoptosis induced by two different stimuli. Our results demonstrated that the addition of ET-1 markedly inhibited serum withdrawal and paclitaxel-induced apoptosis in a concentration-dependent manner, as demonstrated by Annexin-V assay, sub-G_1 peak in DNA content histograms, internucleosomal DNA fragmentation, and terminal deoxynucleotidyl transferase-mediated dUTP biotin nick-end labeling method. Pretreatment of the cells with an ET_AR antagonist, BQ 123, reversed the ET-1-induced protective effect. Paclitaxel-induced apoptosis resulted in the phosphorylation of Bcl-2 that was suppressed by the addition of ET-1. Further analysis of the signaling pathway demonstrated that ET-1 stimulated Akt activation. The phosphatidylinositol 3-kinase (PI3-K) inhibitor wortmannin blocked ET-1-induced Akt phosphorylation. Inhibition of ET-1-stimulated mitogen-activated protein kinase activity did not affect ET-1 protection from paclitaxel-mediated apoptosis. Moreover, BQ 123 blocked the Akt-mediated pathway activated by ET-1, sensitizing ovarian carcinoma cells to paclitaxel treatment. These results establish a novel role for ET-1 in determining protection of ovarian carcinoma cells against paclitaxel-induced apoptosis through Bcl-2–dependent and PI3-K–mediated Akt pathways and suggest that ET-1 and ET_AR could represent important targets for anticancer therapy.

Endothelin-1 (ET-1) is produced primarily in endothelial cells and in elevated amounts by several malignancies (Shichiri et al., 1991; Levin, 1995). ET-1 acts through two distinct subtypes of G protein-coupled receptors, ET_A and ET_B, with equal affinity. ET_A receptor (ET_AR) selectively binds ET-1, whereas ET_BR binds both ET-1 and ET-3 (Rubanyi and Polokoff, 1994). We previously demonstrated that ET-1 is present at high concentrations in ovarian cancer ascites and is overexpressed in primary and metastatic ovarian carcinomas compared with normal ovarian tissues (Bagnato et al., 1999; Salani et al., 2000). Binding studies demonstrated that the ET_AR subtype is the dominant functional receptor present in ET-1 producing ovarian carcinoma cells (Bagnato et al., 1995; Bagnato and Catt, 1998). In these tumor cells, ET-1 selectively acts as an autocrine growth factor through ET_AR, as demonstrated by the inhibitory proliferative effects induced by a specific ET_AR antagonist. Binding of ET-1 to the ET_AR results in the activation of a pertussis toxin-insensitive G protein that stimulates phospholipase C activity, Ca^{2+}/protein kinase C signaling, and phosphorylation and activation of p42 MAP kinase (MAPK) (Bagnato et al., 1997).

Recent evidence suggests that ET-1 modulates apoptosis induced by serum starvation and chemical treatment and acts as survival factors for endothelial cells (Shichiri et al., 1997), fibroblasts (Shichiri et al., 1998), smooth muscle cells (Wu-Wong et al., 1997; Diep et al., 2000; Shichiri et al., 2000), colon carcinoma (Eberl et al., 2000a,b) and glioblastoma cells (Egidy et al., 2000).

Apoptosis is the predominant mechanism of cytotoxicity induced by chemotherapeutic agents (Fisher, 1994). The failure of cancer cells to activate apoptosis may lead to multidrug resistance. Many intracellular signaling pathways involving growth factor receptors and their downstream signaling molecules may converge in a common apoptosis regulatory mechanism (Collins and Rivas, 1993). Growing evidence indicates that the serine/threonine protein kinase Akt/PKB is a key regulation of cell survival. Activation of Akt occurs through the direct binding of the phosphoinositide

This work was supported by grants from the Associazione Italiana Ricerca sul Cancro, Ministero della Sanità, and fellowships from the Fondazione Italiana Ricerca sul Cancro (to D.S., F.S., and L.R.).

ABBREVIATIONS: ET, endothelin; ET_AR, endothelin A receptor; SFM, serum-free medium; EGF, epidermal growth factor; TUNEL, TdT-mediated dUTP biotin nick-end labeling; mAb, monoclonal antibody; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase.

products of the PI3-kinase to its pleckstrin homology domain. Phosphoinositol lipids also activate a protein kinase cascade that phosphorylates Akt, resulting in further activation. Activated Akt phosphorylates survival-mediating targets including the Bcl-2 family proteins, inhibiting apoptosis and promoting cell survival (Datta et al., 1997; Cardone et al., 1998). Thus, a key in successful antitumor therapy is to enhance the susceptibility of the tumor to undergo apoptosis in response to several stimuli.

Paclitaxel is a potent antineoplastic agent for the treatment of advanced metastatic breast and ovarian carcinoma (Rowinsky and Donehower, 1995). At the cellular level, paclitaxel induces tubulin polymerization and microtubule formation, blocks the cell cycle in mitosis, and induces programmed cell death (Wahl et al., 1996). At the biochemical levels, paclitaxel increases tyrosine phosphorylation of the antiapoptotic protein Bcl-2 (Blagosklonny et al., 1996).

In an attempt to understand the molecular mechanisms underlying paclitaxel resistance in ovarian carcinoma, we conducted the current study on OVCA 433, HEY, and SK-OV-3 ovarian carcinoma cell lines, which produce elevated amounts of ET-1 and express abundant high-affinity ET_AR (Bagnato et al., 1995). We found that ET-1 rescues paclitaxel-induced apoptosis in ovarian cancer cells and that ET_A receptor blockade inhibits the ET-1–induced protective effect against paclitaxel-mediated apoptosis. We also provide experimental evidence for the role of Bcl-2 in the protection induced by ET-1 on paclitaxel-mediated apoptosis and that the ET_AR-mediated antiapoptotic effects require Akt-activation through the PI3-kinase pathway. This study has revealed a plausible molecular mechanism underlying the paclitaxel resistance phenomenon in ovarian cancers that overexpress ET-1/ET_AR and suggests that pharmacological ET_AR blockade using specific ET_AR antagonist may provide novel approach to the treatment of ovarian carcinoma in combination therapy

Materials and Methods

Cell Culture. OVCA 433 and HEY human ovarian carcinoma cell lines were a generous gift from Dr. Giovanni Scambia (Catholic University School of Medicine, Rome, Italy). The SK-OV-3 cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine, penicillin, and streptomycin sulfate at 37°C under 5% CO_2/95% air. All culture reagents were from Invitrogen (Paisley, Scotland).

Treatment. Approximately 2×10^6 cells were plated in supplemented medium in 100-mm tissue culture Petri dishes and incubated at 37°C for 24 or 72 h in serum-free medium (SFM). After 24 h in SFM, cells were exposed for 20 h to paclitaxel (Bristol-Myers Squibb, Latina, Italy) at doses of 10, 60, and 100 nM. ET-1 (Peninsula Laboratories, Belmont, CA) was used at the doses ranging from 0.1 to 100 nM. In combination experiments, cells were exposed simultaneously to ET-1 (100 nM) and paclitaxel (60 or 100 nM) for 20 h. When wortmannin (200 nM; Sigma, Milan, Italy), PD98059 (50 μM; Calbiochem, San Diego, CA), BQ 123 (1 μM; Peninsula) and BQ 778 (1 μM; Peninsula) were used, cells were preincubated with the inhibitors for 1 h (PD98059), 30 min (wortmannin), or 20 min (BQ 123, BQ 788) before paclitaxel plus ET-1 treatment. EGF treatment (Collaborative Biomedical Products, Bedford, MA) was also performed as a positive control of Akt activation. The HL60 promyelocytic cell line treated for 6 h with 0.15 μM camptothecin (Sigma) was used as positive control in DNA fragmentation analysis.

Apoptosis. Cells floating in the culture supernatants were collected by centrifugation and pooled with adherent cells recovered from the plates. Cells were washed, assayed for cell viability (trypan blue exclusion test), and counted (Coulter Counter ZM; Beckman Coulter, Fullerton, CA). Aliquots of treated or control cells were differentially processed according to the analyses to be performed. Analyses of DNA fragmentation and sub-G_1 peak in DNA content were performed as described previously (Del Bufalo et al., 1996). For Annexin-V assay, 1×10^6 cells were double-stained with fluorescein isothiocyanate-conjugated Annexin-V and propidium iodide using the Vybrant Apoptosis Kit according to the manufacturer's instructions (Molecular Probes, Eugene, OR) and were immediately analyzed by cytofluorometric analysis. Terminal deoxynucleotidyl transferase-mediated bromo-dUTP-biotin DNA breaks labeling assay was performed using APO-BRDU kit (BioSource International, Camarillo, CA). Cells were fixed in 80% ethanol for 1 h at 4°C, then processed according to the manufacturer's instruction and analyzed by cytofluorometric analysis. Apoptosis was also detected by enzyme-linked immunosorbent assay (ELISA^{PLUS}kit; Roche Molecular Biochemicals, Mannheim, Germany). Briefly, cells in 96-well plates were treated with test reagents for 20 h at 37°C. The cell supernatants were collected and centrifuged at 200 g for 10 min. The amount of histone-associated DNA fragments (mono- and oligonucleosomes) in the cell supernatants was determined at A_{405} in a spectrophotometer using monoclonal antibodies (mAb) directed against DNA and histones. Apoptotic cells were detected in situ by the TUNEL method using the ApopDETEK in situ apoptosis detection kit (Enzo Diagnostic, New York, NY) as reported previously (Leonetti et al., 1999). Briefly, 50 μl of TUNEL reaction mixture was applied to the cytospin preparation and the slides were incubated for 45 min at 37°C. The incorporated Bio-16-dUTP was then stained with streptavidin-biotinylated horseradish peroxidase complex. The entire complex was visualized by using diaminobenzidine as chromogen. The percentage of apoptotic cells was determined by microscopic examination of TUNEL-treated slide at 200× magnification. For each slide, five fields were examined and 100 cells in each field were evaluated.

Western Blot Analysis. Western blot analysis to evaluate the expression of Bax and the phosphorylation of Bcl-2 proteins was performed as reported previously (Del Bufalo et al., 1996). Anti-Bcl-2 mAb (clone 124, 1:200 dilution; DAKO A/S, Glostrup, Denmark), anti-Bax polyclonal antibody (N20, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-human HSP 72/73 mAb (Ab-1, clone W27, Calbiochem) and peroxidase labeled anti-mouse antibody NF-825 (Amersham Biosciences, Piscataway, NJ) were used. To evaluate the activation of Akt, cells were lysed in lysis buffer (62.5 nM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 nM dithiothreitol; 0.1% bromphenol blue). Proteins (100 μg) were electrophoresed on a 10% SDS-polyacrylamide gel. Membranes were immunoblotted with anti-Akt mAb, (1:1000; Cell Signaling Tech, Beverly, MA) or anti-phospho-Akt (Ser 473, 1:1000; Cell Signaling Tech). For immunoblot analysis of the mobility shift of MAP kinase, cells were stimulated, treated with lysis buffer, and separated on 12.5% polyacrylamide gels. The blots were then incubated with anti-p42 MAP kinase polyclonal antibody (1:1000, anti-ERK2; Santa Cruz Biotechnology, Inc.). Peroxidase labeled anti-rabbit antibody (Santa Cruz) was used according manufacturer's instructions. Blots were developed with enhanced chemiluminescence (ECL; Amersham).

Results

Endothelin-1 Reduces Serum-Deprivation-Induced Apoptosis in Ovarian Carcinoma Cells. Antiapoptotic effect of ET-1 was studied in three ovarian carcinoma cell lines, HEY, OVCA 433, and SK-OV-3, whose expression of the various components of the endothelin system has been described previously (Bagnato et al., 1995). The dissociation constants ranged from 0.02 nM for OVCA 433 cells to 0.15

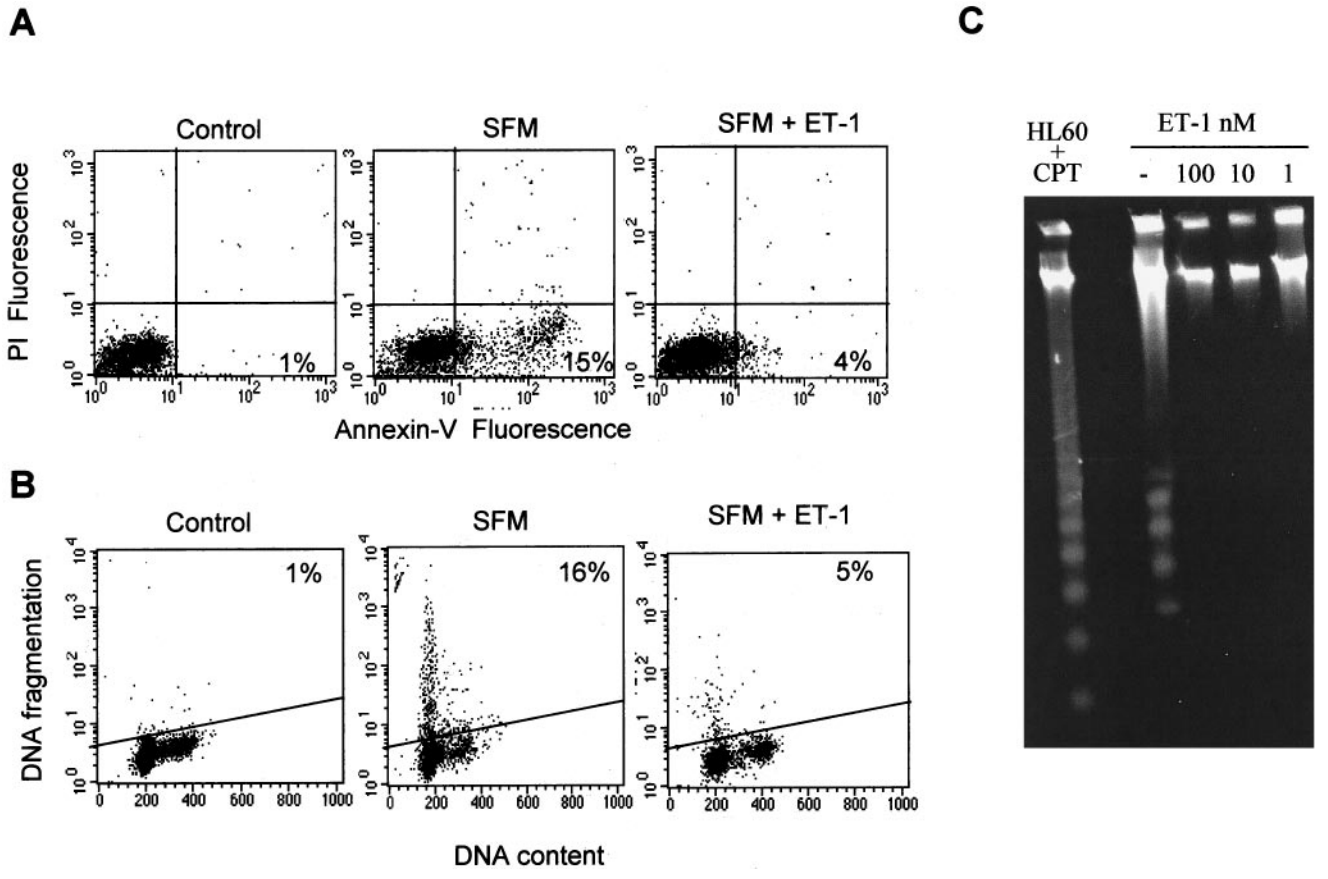


Fig. 1. ET-1 protects OVCA 433 cells from serum-deprivation-induced apoptosis. Annexin-V (A) and TUNEL (B) assays of OVCA 433 cells grown in SFM for 48 h in the absence or presence of 100 nM ET-1. The percentage of apoptotic cells calculated by flow cytometric analysis is reported. C, fragmented DNA was extracted from cells deprived of serum for 72 h in the absence or presence of different concentrations of ET-1 (1 nM up to 100 nM) and separated by electrophoresis. HL60 cells treated for 6 h with 0.15 μM camptothecin (CPT) were used as positive control.

nM for SK-OV-3 and for HEY cells, and receptor number ranged from 2,600 sites/cell for SK-OV-3 cells to 35,600 sites/cell for HEY cells and 43,600 for OVCA 433 cells. To evaluate the role of ET-1 on apoptosis induced by serum starvation, OVCA 433 cells, which expressed the largest number of high-affinity receptors for ET-1, were grown for 48 h in SFM in the presence or absence of 100 nM ET-1. A pool of detached and adherent cells was analyzed for the presence of early apoptotic events upon Annexin-V staining (Fig. 1A). Annexin-V positive cells were evident after deprivation of serum; apo-

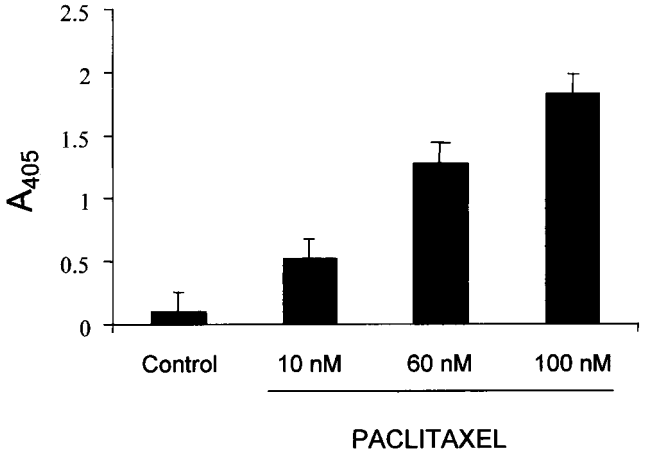


Fig. 2. Paclitaxel induces apoptosis in OVCA 433 cells in a dose-dependent manner. Cells were serum-starved for 24 h and treated with increasing concentrations (10, 60 and 100 nM) of paclitaxel for 20 h. Apoptosis was detected by enzyme-linked immunosorbent assay, which detects histone-associated DNA fragments. Each value represents the mean ± S.D. of three determinations. The results shown are representative of four experiments.

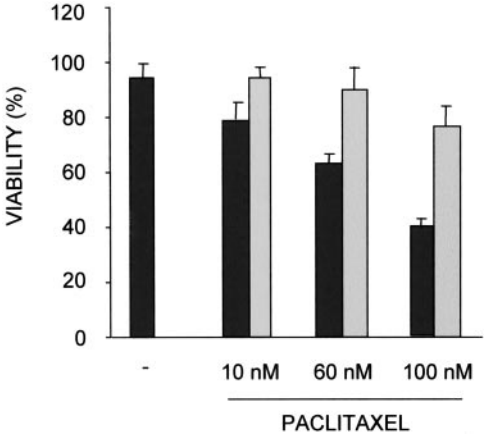


Fig. 3. ET-1 protects OVCA 433 cells from paclitaxel-induced cytotoxicity. OVCA 433 cells were serum-starved for 24 h and treated with increasing concentrations (10, 60, and 100 nM) of paclitaxel for 20 h in the absence (■) or presence (▨) of 100 nM ET-1. Cell viability at the end of treatments was evaluated by trypan blue exclusion test. Each value represents the mean ± S.D. of three determinations. The results shown are representative of three independent experiments.

ptotic cells were about 15% of total population. Moreover, TUNEL analysis (Fig. 1B) confirmed the percentage of apoptotic cells induced by serum-deprived OVCA 433 cells and showed that serum deprivation gave a positive signal in the G_1 phase region. When cells were grown in the presence of 100 nM ET-1, a reduced fraction (about 5%) of OVCA 433 cells undergoing apoptosis was observed. Analysis of DNA fragmentation after 72 h of serum starvation showed a marked enhancement of nucleosomal ladder formation (Fig. 1C). ET-1 dose dependently (1 nM up to 100 nM) inhibited the fragmented DNA induced by 72 h of serum starvation in OVCA 433 cells as demonstrated by inhibition of the DNA ladder formation.

Endothelin-1 Suppresses Paclitaxel-Induced Apoptosis in Ovarian Carcinoma Cell Lines. To investigate whether paclitaxel elicited cytotoxicity by induction of apoptosis, OVCA 433, SK-OV-3, and HEY cells were treated with paclitaxel and were examined for apoptosis-related parameters. As reported in Fig. 2, different concentrations of paclitaxel are able to induce apoptosis in a dose-dependent manner in serum-deprived OVCA 433 cells. The A_{405} value, a quantitative measurement of histone-associated DNA fragments, increases from about 0.5 to 1.85 when cells were treated for 20 h with increasing paclitaxel concentrations ranging from 10 to 100 nM.

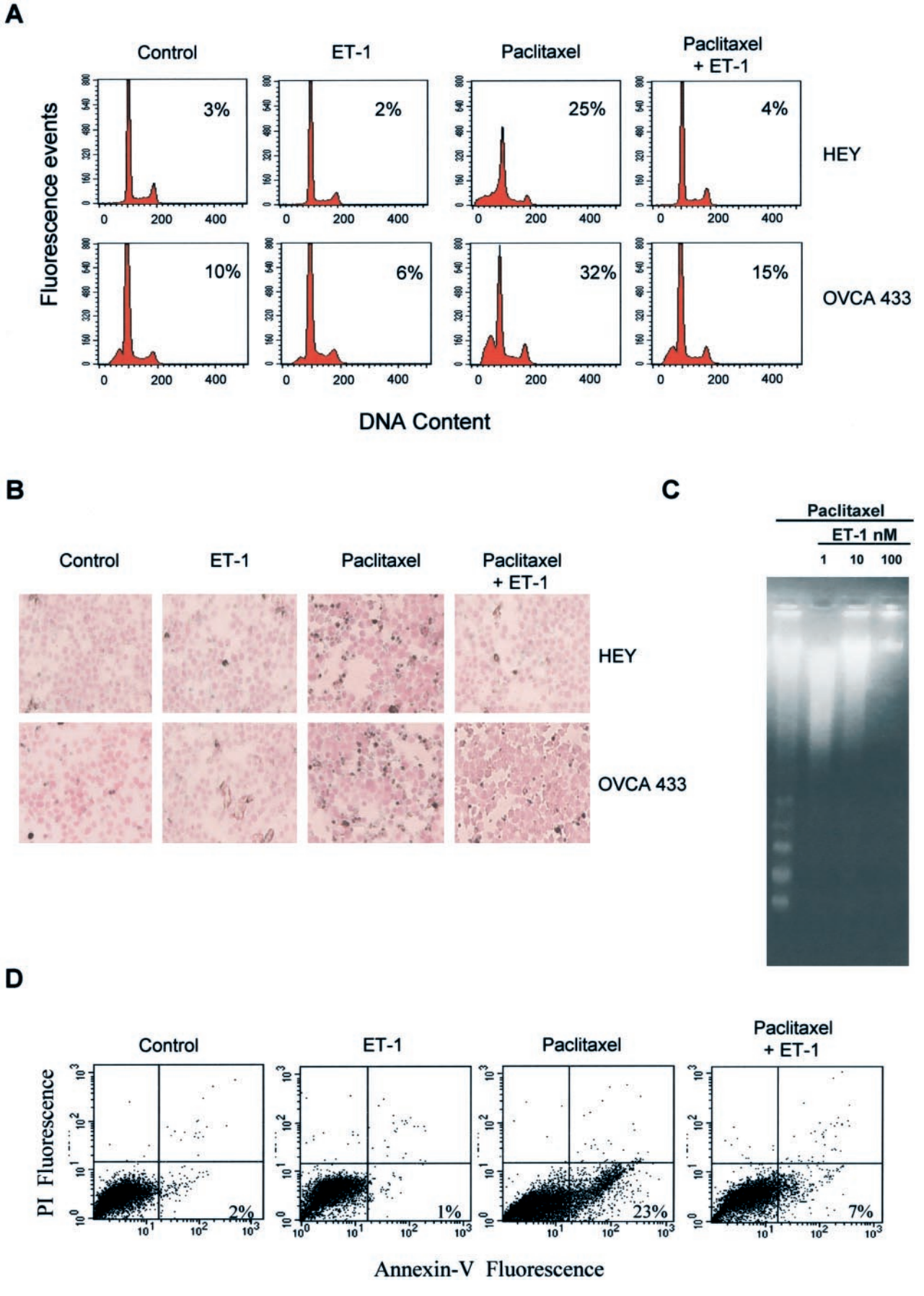
To analyze the role of ET-1 on paclitaxel-induced cytotoxicity, the viability of OVCA 433 cells was measured after treatment with different concentrations of the drug ranging from 10 to 100 nM in the absence or presence of 100 nM ET-1 (Fig. 3). A decreased viability (from 80 to 40%) was observed with increasing concentrations of paclitaxel and ET-1 protects ovarian carcinoma cells against paclitaxel-induced cytotoxicity at all concentrations used.

To further investigate the role of ET-1 on paclitaxel induced-apoptosis, OVCA 433 and HEY ovarian carcinoma cell lines were exposed for 20 h to 60 nM paclitaxel in combination with 100 nM ET-1. Cytofluorometric analysis of DNA content reveals the presence of sub-G_1 peaks after treatment with paclitaxel in both cell lines (Fig. 4A). OVCA 433 and HEY dead cells after paclitaxel treatment were about 32 and 25%, respectively. Addition of 100 nM ET-1 inhibited the paclitaxel-induced apoptosis in both cell lines (15 versus 32% for OVCA 433 cells and 4 versus 25% for HEY cells). These data are complemented by TUNEL staining shown in Fig. 4B. Apoptotic cells (brown staining) are well evident after treatment with 60 nM paclitaxel in both cell lines and addition of ET-1 (100 nM) reduced the number of TUNEL-positive cells. The percentage of apoptotic cells in sub-G_1 peak is similar to that measured by the TUNEL assay. Interestingly, in SK-OV-3 cells, which expressed the lowest number of ET-1 receptors, ET-1 was unable to rescue cells from paclitaxel-induced apoptosis, indicating that ET-1 receptor number represent a central role of this signaling pathway to mediate resistance. Thus, a similar percentage of apoptotic cells (about 20%), as evaluated by Annexin-V assay, was observed when treating cells with 60 nM paclitaxel for 20 h in the presence or absence of 100 nM ET-1. Electrophoresis of DNA samples extracted from OVCA 433 cells treated with 60 nM paclitaxel (Fig. 4C) showed a marked DNA fragmentation. Treatment with ET-1, ranging from 10 to 100 nM, reduced nucleosomal ladder induced by 60 nM paclitaxel in a dose-dependent manner. A double-staining method that determines the binding of fluorescein isothiocyanate-conjugated Annexin-V to phosphatidylserine on the cell surface, and the staining of the DNA by propidium iodide, was set out on OVCA 433 cells to confirm the above observations. Figure 4D shows that 23% of the early apoptotic cells is evident after 16 h of 60 nM paclitaxel treatment and it decreases to 7% in the presence of ET-1. Altogether, these data demonstrate that ET-1 antagonizes paclitaxel-induced apoptosis in ovarian cancer cells.

Endothelin-1 Suppresses Paclitaxel-Induced Apoptosis via the ET_A Receptor. To characterize the ET-1 receptor subtype mediating the survival effect, we examined whether ET-1 receptor antagonists can abrogate the protective effect of ET-1 in OVCA 433 cells. The number of floating cells induced by exposure for 20 h to 60 nM paclitaxel alone or in combination with different concentrations of ET-1 (0.1 to 100 nM) or with 100 nM ET-1 in the presence of ET-1 receptor inhibitors was evaluated (Fig. 5A). Values were calculated as the percentage of the number of floating cells found after treatment with paclitaxel, which was set to 100%. The number of floating dead cells undergoing apoptosis is reduced by ET-1 exposure (0.1 nM to 100 nM) in a dose-dependent manner. A significant ($p < 0.001$) apoptotic inhibition was induced with as low as 0.1 nM ET-1 and a maximal antiapoptotic effect (80.2 $\pm$ 4.0% survival of control apoptotic cells) was induced with 100 nM ET-1. Pretreatment of cells with BQ 123 (1 μM), the selective antagonist for ET_AR, completely blocked the protective effect induced by ET-1, whereas addition of the ET_BR antagonist, BQ 788 (1 μM), did not show significant effect on ET-1-induced apoptosis protection. Neither BQ 123 nor BQ 788 alone affected the apoptotic frequency of OVCA 433 cells (data not shown). These results were confirmed by in situ TUNEL assay. As reported in Fig. 5B, the addition of ET_AR antagonist, BQ 123, completely reverted the protective effect induced by ET-1, being the percentage of apoptotic cells similar to that observed after exposure to paclitaxel alone. These results suggest that the antiapoptotic effect induced by ET-1 is mediated by the ET_AR.

Endothelin-1 Reduces Bcl-2 Phosphorylation Induced by Paclitaxel. To define the molecular mechanism by which ET-1 protects from apoptosis induced by paclitaxel, the expression of some Bcl-2 family proteins involved in apoptosis was evaluated by Western blot analysis in OVCA 433 cells. Because paclitaxel was previously shown to induce phosphorylation of Bcl-2, thereby inactivating Bcl-2 and inducing apoptosis in several cancer types (Haldar et al., 1995), we first evaluated Bcl-2 expression after paclitaxel and ET-1 treatment alone or in combination. As reported in Fig. 6, the exposure to 100 nM ET-1 increased Bcl-2 protein expression of about 2 fold above control. Treatment with 60 and 100 nM paclitaxel induced phosphorylation of Bcl-2 protein that can be detected, as slower migrating band in Western blots. Addition of 100 nM ET-1 was able to reduce Bcl-2 phosphorylation induced by 100 nM paclitaxel. Therefore, inhibition of paclitaxel-induced apoptosis by ET-1 could be the result of increased Bcl-2 activity. On the contrary, the expression of Bax protein, which counteracts the survival function of Bcl-2, was not affected by treatment with paclitaxel in the presence or in the absence of ET-1.

Activation of Akt Is Required for ET-1-Mediated Protection from Paclitaxel-Induced Apoptosis. Akt activa-



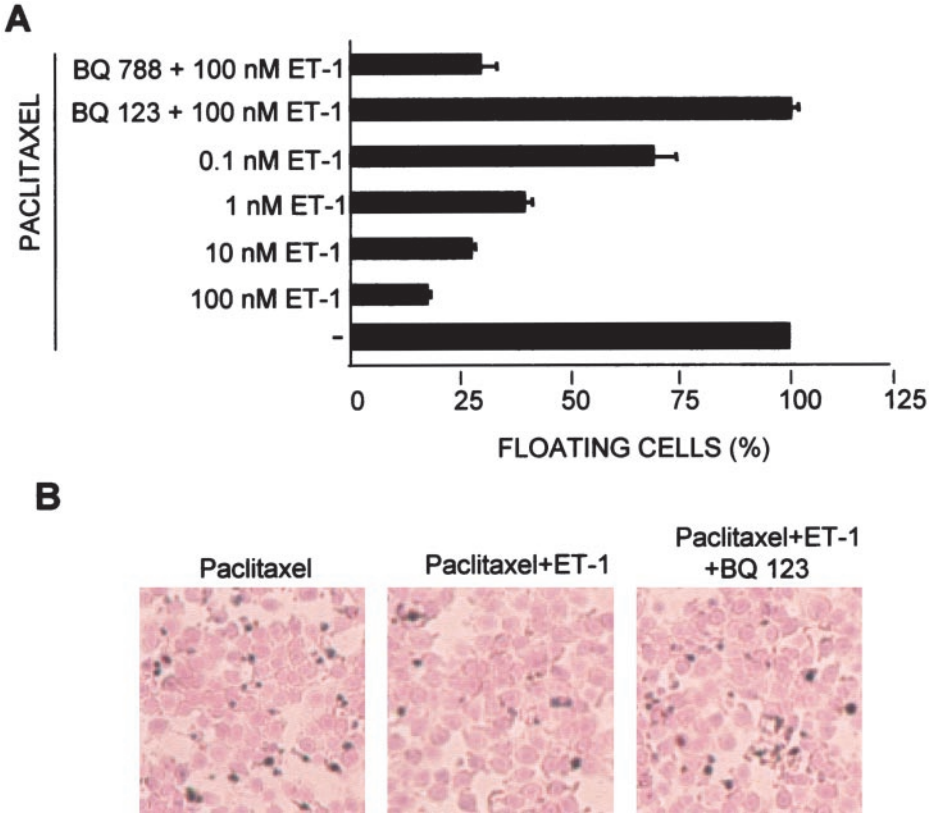


Fig. 5. Protection from paclitaxel-induced apoptosis by ET-1 is dose-dependent and is mediated via ET_AR. A, serum-starved OVCA 433 cells were treated with 60 nM paclitaxel in the absence or presence of different concentrations of ET-1 (0.1 nM up to 100 nM). Where reported, cells were preincubated with BQ 123 (1 μM) and BQ 788 (1 μM). Values were calculated as the percentage of the number of floating cells found after treatment with paclitaxel, which was set to 100%. Each column represents the mean ± S.D. of six determinations. B, detection of apoptosis by TUNEL staining in serum-starved OVCA 433 cells treated with 60 nM paclitaxel in the absence or the presence of 100 nM ET-1 and preincubated with or without 1 μM BQ 123. Original magnification, 200×.

tion has been shown to protect cells from apoptosis induced from different stimuli (Downward, 1998). Therefore, we examined whether ET-1 is able to activate Akt in OVCA 433 cells. Because serine 473 is located in the kinase domain of Akt, and its phosphorylation is required to Akt activation, the activation of Akt was determined with an antibody specific for the phosphorylated serine 473 of Akt. As shown in Fig. 7A, the increased phosphorylation of Akt was detected within 5 min after ET-1 exposure to serum-deprived cells. ET-1 was equipotent with EGF in stimulating Akt activation. Because Akt has been found to be regulated by PI3-K (Coffer et al., 1998), we investigated whether the PI3-K inhibitor, wortmannin, inhibited the ET-1–induced Akt activation. The treatment of serum-starved OVCA 433 cells with 150 nM wortmannin for 30 min before the addition of 100 nM ET-1 results in the decrease of Akt phosphorylation. Preincubation of OVCA 433 cells with BQ 123 before the addition of 100 nM ET-1 resulted in a marked decrease in Akt phosphorylation, suggesting that ET_AR activation is required for that ET-1-induced Akt activation. Both Akt and MAPK pathway have been shown to have anti-apoptotic regulatory properties in different cell types and in response to different stimuli. Figure 7A shows that ET-1 stimulation of OVCA 433 cells activates both Akt and MAPK activities.

Finally, we investigated whether Akt or MAPK activation was involved in the protective effect of ET-1 (Fig. 7B). Treatment with 60 nM paclitaxel in OVCA 433 cells that were not prestimulated with ET-1 resulted in the inhibition of Akt activation. Twenty hours after paclitaxel treatment, Akt and MAPK were still activated in ET-1–treated cells. Furthermore, a second challenge with ET-1 for 5 min increased the phosphorylated activated form of Akt and MAPK in cell exposed to paclitaxel. Pretreatment before the addition of ET-1 with 150 nM wortmannin or 1 μM BQ 123 significantly inhibited the ability of ET-1 to activate Akt signaling. To investigate whether the reduction of phospho-Akt induced by wortmannin is associated with reversion to paclitaxel-induced apoptosis, cytofluorometric analysis of DNA content was performed (Fig. 7C). The ET-1-induced antiapoptotic effect was completely blocked by pretreatment with the wortmannin. In contrast, inhibition of MAPK activation by PD98059 (50 μM for 1 h), a selective inhibitor of MAPK kinase, had no effect on the ability of ET-1 to protect against paclitaxel-induced apoptosis. Thus, indicating that the PI3-K–dependent Akt pathway but not MAPK activation plays a central role in ET_AR-mediated protection against paclitaxel-dependent apoptosis in ovarian carcinoma cells.

Discussion

Several growth factors and growth factor receptors have been shown to modulate apoptosis in ovarian carcinoma cells (Edwards and Bartlett, 1999; Coppola et al., 1999). Our previous studies demonstrated a novel mechanism in the growth

Fig. 4. ET-1 protects OVCA 433 and HEY cells from paclitaxel-induced apoptosis. OVCA 433 and HEY cells were serum-deprived for 24 h (Control) and then treated for 16 h (D) or 20 h (A, B, C) with 60 nM paclitaxel in the absence or in presence of 100 nM ET-1. A, flow cytometric analysis of DNA content. The percentage of cells with hypodiploid DNA content is reported. B, detection of apoptosis by TUNEL staining in OVCA 433 and HEY cells. Original magnification, 200×. C, fragmented DNA was extracted from serum-deprived OVCA 433 cells treated with paclitaxel (60 nM) in the absence or in the presence of different concentration of ET-1 (10 nM up to 100 nM) and separated by electrophoresis. D, detection of apoptosis by annexin-V assay in OVCA 433 cells. The percentage of annexin-V positive cells is reported.

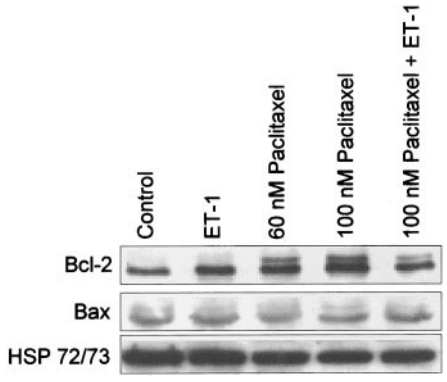


Fig. 6. ET-1 reduces Bcl-2 phosphorylation induced by paclitaxel. Western blot analysis of the antiapoptotic protein Bcl-2 and the proapoptotic protein Bax in OVCA 433 cells after paclitaxel treatment in presence and absence of ET-1 (100 nM). The relative amount of the transferred proteins were quantified and normalized to the corresponding HSP 72/73 protein amounts.

control of ovarian carcinoma mediated by the ET-1 autocrine loop that selectively occurs via ET_AR (Bagnato et al., 1999). Here, we have provided evidence that ET-1, acting as an antiapoptotic factor through activation of ET_AR, can confer resistance to paclitaxel-induced apoptosis.

ET-1 has been shown to inhibit apoptosis induced by different stimuli in normal (Shichiri et al., 1997, 1998, 2000; Wu-Wong et al., 1997) and transformed cell lines (Eberl et al., 2000b; Egidy et al., 2000). Apoptosis represents a fundamental intracellular program that is regulated at various levels within the signaling pathways (Williams and Smith, 1993). In the current study, we have demonstrated a relevant role for ET-1 as an antagonist of apoptosis induced by both serum-withdrawal and paclitaxel. The observation that ET-1 may function as an antiapoptotic factor in response to diverse agents suggests that it blocks a common late intracellular apoptosis pathway. The mechanism by which ET-1 can function to protect specific cell types from apoptosis is not currently defined. We investigated the molecular mechanism by which ET-1 induced antiapoptotic pathway in OVCA 433 cells and found that after paclitaxel treatment, Bax expression is unaffected by ET-1, whereas Bcl-2 expression is increased. Moreover, ET-1 is capable of inhibiting Bcl-2 phosphorylation induced by paclitaxel, thereby affecting its ability to inactivate its antiapoptotic activity. Suenobu et al. (1999) showed that Bcl-2 expression was unaffected by ET-1 in endothelial apoptosis and Diep et al. (2000) showed that Bax/Bcl-2 ratio did not change after ET-1 pretreatment in ω-3 fatty acid-induced apoptosis of vascular smooth muscle cells. Thus ET-1 is likely to block or reduce apoptosis by different mechanisms depending on the cell type.

Different cell types may use either Akt or MAPK signaling pathways for survival in response to different proapoptotic stimuli. Akt phosphorylates the Bcl-2 family member Bad and caspase-9, inhibiting their proapoptotic activity. The phosphorylation of these proteins seems to be a mechanism for the antiapoptotic function for Akt. (Page et al., 2000; Mitsuuchi et al. 2000). Recently, it has been shown that ET-1 induced the Akt cell survival pathway in serum-starved prostate cancer cells through the transactivation of the insulin-like growth factor receptor (Sumitomo et al., 2001). It should be noted that even though the MAPK pathway did not have an antiapoptotic function in the OVCA 433 cells, MAPK has been shown to have significant survival functions in other cell types. ET-1 exerts its antiapoptotic action by activation of the MAP kinase pathway for endothelial and vascular smooth muscle cells in serum deprivation-induced apoptosis and for rat fibroblasts in c-myc–induced apoptosis. In ovarian carcinoma cells ET-1 is able via autocrine mechanism to activate mitogenic signaling that leads to MAPK phosphorylation (Bagnato et al., 1997).

Our results indicate that ET-1 protects ovarian cancer cells from paclitaxel-induced apoptosis by a Bcl-2–dependent mechanism and involves the activation of Akt. We also showed that Akt cell survival pathway is inhibited by ET_AR antagonist, suggesting that ET_AR blockade in ovarian carcinoma cells could result in the suppression of resistance to paclitaxel-induced apoptosis.

Our findings are particularly relevant to understanding the function of ET-1 receptors in ovarian cancer. Controversial results were reported concerning the ET-1 receptor involved in mediating antiapoptotic effect. ET-1 functions as a survival factor through ET_AR in rat fibroblasts and human smooth cells, and through ET_BR in rat endothelial cells (Shichiri et al., 1997, 1998, 2000). In human prostate smooth muscle cells, ET-1 protects from paclitaxel-induced apoptosis acting through ET_AR (Wu-Wong et al., 1997). The ET_AR is also involved in ET-1 inhibition of apoptosis in colon carcinoma cells and the addition of bosentan, a mixed ET_AR and ET_BR antagonist, enhances FasL-induced apoptosis in colon carcinoma and glioblastoma cells (Eberl et al., 2000b; Egidy et al., 2000).

Interestingly, we found that the addition of a specific ET_AR antagonist, BQ 123, blocked the ET-1–induced resistance to paclitaxel-mediated apoptosis. Furthermore, BQ 123 blocked the ET-1–induced activation of Akt, indicating that ET-1 contributes to trigger resistance to paclitaxel via activation of antiapoptotic signaling pathways such as those for Akt through ET_AR binding. The present results add further information on the overall importance of ET-1/ET_AR in regulating ovarian carcinoma cell proliferation and survival. ET_A receptor blockade in ovarian tumor cells inhibits cell growth (Bagnato et al. 1995, 1997, 1999), cell migration and invasion (Rosanò et al., 2001), and vascular endothelial growth factor-dependent neovascularization (Salani et al., 2000). Furthermore, ET_AR antagonist inhibits transactivation of EGF, which is partly responsible for MAPK activation in ovarian carcinoma cells (Vacca et al., 2000).

New therapeutic strategies using specific ET_AR antagonists provide an additional approach to the treatment of ovarian carcinoma in which ET_AR blockade could result in the tumor growth inhibition by reducing tumor growth and inducing massive apoptosis. Furthermore, the therapeutic use of specific ET_AR antagonist combined with conventional chemotherapy would more effectively induce apoptosis by contributing to the paclitaxel treatment. Thus, expanding these studies in vivo, we can explore the potential cooperative antitumor effect of combination therapy in which ET_AR blockade increasing the commitment toward apoptosis could potentiate the antitumor activity of conventional chemotherapeutic, agents such as paclitaxel.

Acknowledgments

We are grateful to Drs. Gabriella Zupi and Pier Giorgio Natali for critical reading of the manuscript and insightful comments.

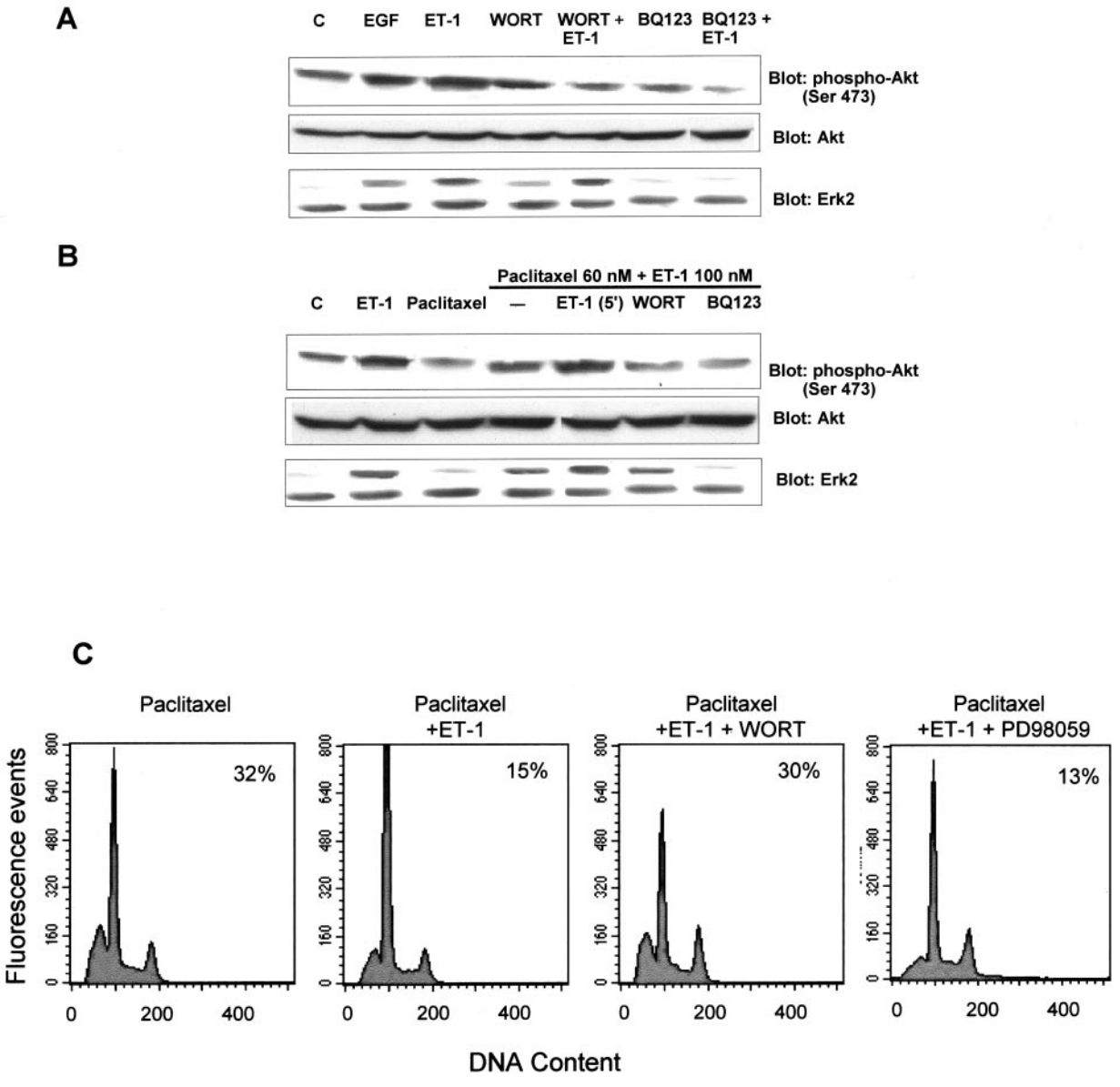


Fig. 7. ET-1 activates PI3-K-dependent Akt to protect cells from paclitaxel-induced apoptosis through ET_AR. A, Western blot analysis of serum-starved OVCA 433 cells was performed with anti-phospho-Akt, anti-Akt, or anti-Erk2 antibodies. Cells were treated with 100 nM ET-1 (24 h), or 10 ng/ml EGF, or 150 nM wortmannin (WORT, 30 min). Exposure to ET-1 was also performed after pretreatment with 1 μM BQ 123 (15 min) or 150 nM wortmannin (30 min). B, serum-starved OVCA 433 cells were treated with ET-1 (100 nM) in the presence or absence of 60 nM paclitaxel. As indicated, cells were restimulated an additional 5 min with 100 nM ET-1. Paclitaxel-treated cells were preincubated for 30 min with wortmannin (150 nM) or with BQ 123 (1 μM) and then treated with ET-1. The cells were lysed and immunoblotted with anti-phospho-Akt or anti-Erk2 antibodies. The results are representative of three independent experiments. C, flow cytometric analysis of DNA content of OVCA 433 cells treated with 60 nM paclitaxel in the absence or in the presence of 100 nM ET-1 preincubated with or without 150 nM wortmannin (WORT) or 50 μM PD98059. The percentage of apoptotic cells in the sub-G1 peak is reported.

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