Mechanisms for 2-methoxyestradiol-induced apoptosis of prostate cancer cells

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Abstract **Prostate and breast carcinomas are sex hormone-related carcinomas, which are known to be associated with an over-expression of the proto-oncogene Bcl-2. Here, we report that 2-methoxyestradiol (2-ME), an endogenous metabolite of estrogen that does not bind to nuclear estrogen receptors, effectively induces apoptosis in Bcl-2-expressing human prostate and breast carcinoma cells in vitro and in a rat prostate tumor model in vivo. In several cell lines derived from prostate, breast, liver and colorectal carcinomas, 2-ME treatment led to an activation of c-Jun N-terminal kinase (JNK) and phosphorylation of Bcl-2, which preceded the induction of apoptosis. In summary, our data suggest that 2-ME induces apoptosis in epithelial carcinomas by causing phosphorylation of JNK, which appears to be correlated with phosphorylation of Bcl-2.**

Key words: Apoptosis; Bcl-2; Breast cancer; 2-Methoxyestradiol; Prostate cancer; Stress-activated protein kinase/c-Jun N-terminal kinase

1. Introduction

Apoptosis has been recognized as an important regulator of tumor development and net tumor growth is dependent on the balance between proliferation and apoptosis [1]. Another crucial regulator of tumor progression and metastasis is angiogenesis, as described by Folkman and coworkers [2,3]. Hormone-related breast and prostate carcinomas are common in populations of the Western society and the incidences of both diseases are rising [4,5]. The proto-oncogene Bcl-2 protects cells from different forms of apoptotic stimuli by preventing caspase activation [6,7]. It has been shown that hormone therapy-resistant prostate tumor cells expresses Bcl-2, whereas normal prostate cells are Bcl-2-negative [8,9]. Furthermore, breast carcinomas commonly express Bcl-2 [10].

2-Methoxyestradiol (2-ME), an endogenous metabolite of the endogenous estrogen hormone estradiol-17β, has been shown to potently inhibit angiogenesis and tumor growth in vivo [11]. The growth-inhibitory properties of 2-ME have been associated with its effects on tubulin polymerization, causing an increased stability of microtubules, which probably results in cell cycle arrest [11–13]. Endothelial cells treated with 2-ME displayed morphological signs of apoptosis after as early as 5 h which was associated with an activation of stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) [14]. 2-ME has recently been shown to cause growth inhibition [15] and also apoptosis in human lung and pancreatic cancer cells [16,17]. Interestingly, the apoptotic effects of 2-ME appears to be most effective in rapidly growing cells, particularly tumor cells in vitro and in vivo, although the underlying molecular mechanism is not yet recognized [18–22]. The antiproliferative and apoptotic effects of 2-ME in human breast carcinoma cells and endothelial cells have recently been demonstrated to occur independently of the described estrogen receptors (α and β) [23]. The inhibitory effects on tumor growth and angiogenesis of 2-ME are increasingly well recognized, but the physiological function for 2-ME remains to be determined.

Mitogen-activated protein kinases (MAPKs) transduce signals from the cell membrane to the nucleus in response to different stimuli and participate in intracellular signaling pathways that control a wide spectrum of cellular processes, including cell growth, differentiation and stress responses [24–26]. In contrast to p42 extracellular signal-regulated kinase (ERK)2 and p44 ERK1, which are activated by mitogenic stimuli, the JNK/SAPK and p38 MAPKs are activated by pro-inflammatory cytokines and environmental stress such as UV light, γ-irradiation, heat shock, osmotic shock, shear stress, growth factor withdrawal, ceramides and inhibition of protein synthesis. Upon activation, SAPK/JNK can phosphorylate several transcription factors like c-Jun, ATF-2 and Elk-1 thereby regulating gene expression [27–29]. It has been shown that activation of JNK and p38 MAPKs promotes apoptosis, e.g. upon growth factor withdrawal in rat PC12 cells [30]. Similar to the case for ERKs, activation of SAPK/JNK requires phosphorylation at conserved threonine and tyrosine residues, by SEK1/MKK4/JNK kinase [29,31]. G protein-coupled receptors (GPCRs) are integral membrane proteins involved in the transmission of signals from the extracellular environment to the cytoplasm, which recently have been shown to regulate MAPK cascades, including ERK, SAPK/JNK and the p38 MAPK pathways [32].

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Abbreviations: ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; 2-ME, 2-methoxyestradiol; Ptx, pertussis toxin; SAPK, stress-activated protein kinase; TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick-end labeling

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We report here that 2-ME treatment induced apoptosis in rapidly growing cells, like prostate and breast carcinoma cells. Moreover, 2-ME treatment of rats transplanted with a spontaneous prostate tumor resulted in a reduction of the tumor volume compared to vehicle-treated rats. The potent apoptotic effects of 2-ME were found to be associated with activation of JNK, causing subsequent phosphorylation and inactivation of the proto-oncogene Bcl-2.

2. Materials and methods

2.1. Materials

2-ME, estradiol-17β, forskolin, curcumin and propidium iodide were obtained from Sigma Chemical (St. Louis, MO, USA). Pertussis toxin (Ptx) was obtained from Calbiochem (La Jolla, CA, USA) and z-VAD-fmk was obtained from Enzyme Systems Products (Livermore, CA, USA).

2.2. Cell culture and reagents

The human prostate cancer cell lines PC-3, LNCaP and DU-145, the breast cancer cell lines MCF-7 and MDA-MB-468, the human colon carcinoma cell line SW-480 and the human hepatocarcinoma cell line HepG2 were routinely grown in RPMI 1640 (PC-3, LNCaP and DU-145), minimal essential medium (MCF-7) or Dulbecco's minimal essential medium (DMEM; MDA-MB-468, SW-480, HepG2) containing 10% fetal bovine serum (FBS), L-glutamine and penicillin (Gibco BRL, Paisley, UK). At least 24 h before experimentation the medium for MCF-7 was changed to EX-MEM, which does not contain phenol red, supplemented with 10% FBS.

2.3. Primary cell culture

The mammary epithelial cell organoids were isolated from human normal breast and the method for culturing organoids was modified from an earlier publication [33,34]. Normal breast tissue was immediately placed in cold phosphate-buffered saline (PBS), minced into pieces of about 1 mm^3 and washed three times with PBS. The minced tissues were incubated at 37°C for 8–10 h in an enzymatic solution containing 75% M199, 25% DMEM, 10% FBS, 10 μg/ml insulin, 145 μg/ml hyaluronidase and 0.6 μg/ml collagenase (type I). Isolated cells were collected by centrifugation at 300×*g* for 10 min and resuspended in medium I (75% DMEM, 25% Ham's F12, 10% FBS, 5 μg/ml insulin, 5 μg/ml T3, 5 μg/ml adenine, 10 pg/ml epidermal growth factor (EGF), 20 μg/ml hydrocortisone and 50 μg/ml gentamicin). After 1 week of culturing at 37°C in 95% air, 5% CO_2, medium I was replaced by medium II (75% DMEM, 25% Ham's F12, 10% FBS, 5 μg/ml insulin, 10 pg/ml EGF, 20 μg/ml hydrocortisone, 141 μg/ml phosphoethanolamine and 50 μg/ml gentamicin). The viability of isolated cells was greater than 85% as assessed by trypan blue dye exclusion.

2.4. DNA fragmentation assay

DNA fragmentation analysis was performed on confluent cells harvested from tissue culture dishes as well as on cells collected by centrifugation of supernatants from confluent cell cultures (1×10^6/ml), treated with 10 μM 2-ME for different time periods as indicated in the figures. The cell pellet was resuspended in 20 μl of sterile 10 mM EDTA, 50 mM Tris–HCl, pH 8.0, 0.5% (w/v) sodium lauryl sarcosinate and 0.5 mg/ml proteinase K and incubated at 37°C for 1 h. RNase A (1 mg/ml) was added and the sample was incubated for 15 min at 65°C. DNA was extracted using phenol–chloroform and electrophoresed on a 1.2% agarose gel in the presence of 0.1 μg/ml ethidium bromide for 2–4 at 80 Vh. DNA was visualized by UV light and gels were photographed.

2.5. In situ detection of apoptosis

The TUNEL (terminal deoxynucleotide transferase-mediated dUTP nick-end labeling) method was performed according to the instructions supplied by the manufacturer (Roche Molecular Bioscience, Mannheim, Germany). Cells were cultured on collagen I-coated culture slides (Biocoat, Becton Dickinson, Bedford, MA, USA). Paraffin-embedded, formalin-fixed 4 μm thick tumor sections were used for the investigations of apoptotic cells by TUNEL, as described earlier [35].

2.6. FACS analysis

Cell cycle studies were done by flow cytometry according the method described by Vindelöv et al. [36]. Briefly, $1–2\times10^6$ cells were incubated with or without 2-ME; floating and attached cells were then collected and pooled, washed with cold PBS and lysed in a NP-40-containing buffer [36]. After treatments with trypsin and RNase A, nuclei were stained with propidium iodide and analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA, USA). Apoptotic cells were determined by their hypochromic, sub-G1, staining profiles.

2.7. In vivo experiments

Dunning R3327-PAP prostate tumors, originally provided by Dr. N. Altman (The Papanicolaou Cancer Research Institute, Miami, FL, USA), were transplanted bilaterally subcutaneously into the flanks of Copenhagen×Fisher F_1 male rats. This tumor sub-line has been well characterized and is androgen-sensitive [37]. Rats were housed under standard conditions with food and water ad libitum. Four to five months post-implantation, when the tumor volume was approximately 1200 mm^3, the rats were allocated consecutively to treatment with vehicle (five rats with eight tumors; NaCl 0.2 ml given intraperitoneally daily for 14 days) or 2-ME (six rats with 11 tumors, 12.5 mg/kg/day, total volume 0.2 ml). 2-ME was dissolved in sterile sesame oil at a concentration of 125 mg/ml. All animal experiments were done with permission of the local ethical committee. Diameters of tumors were measured by microcalipers and the tumor volume was calculated as described earlier [38].

2.8. Western blot analysis

Cells grown on 10 cm dishes were treated with 10 μM 2-ME for different time periods, washed with PBS and lysed in 100 μl lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% NP-40, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). After centrifugation, supernatants were collected and protein concentrations were measured by using the Bio-Rad Protein Assay Kit. Equal amounts of protein were subjected to SDS–gel electrophoresis in 10–12% polyacrylamide gels, and blotted onto polyvinylidene difluoride membranes. Membranes were probed with antibodies against the following proteins: phosphorylated and total SAPK/JNK, phosphorylated and total p38 (New England Biolab, Beverly, MA, USA), phosphorylated and total ERK1/2 (affinity-purified polyclonal antibodies, a kind gift of Dr. Lars Rönnstrand, Ludwig Institute for Cancer Research, Uppsala, Sweden), Bcl-2, caspase 1, 3 and 8 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Signals were detected by an enhanced chemoluminescent (ECL) system.

2.9. Statistics

Values are expressed as means ± S.E.M. For comparisons between groups the Mann–Whitney *U*-test was used. A P value less than 0.05 was considered to be statistically significant.

3. Results

3.1. 2-ME induces apoptosis in breast and prostate carcinoma cell lines but not in normal breast cells

To investigate the cellular effects of the estradiol metabolite 2-ME, we incubated different cell lines with 2-ME and followed cell survival. 2-ME treatment of two human breast carcinoma cell lines, MCF-7 (estrogen receptor-α-positive) and MDA-MB-468 (estrogen receptor-α-negative), as well as two human prostate carcinoma cell lines, PC-3 and LNCaP, potently induced apoptosis, as demonstrated by a DNA fragmentation assay (Fig. 1A). The kinetics of 2-ME-induced apoptosis were different in the investigated cell lines with a maximal effect observed using 10 μM (data not shown). The most rapid response was evident in MDA-MB-468 cells, in which DNA fragmentation was already seen after 6 h of incubation with 2-ME. DNA fragmentation occurred after 24 h of 2-ME treatment in PC-3 cells, and after 36 h and 48 h in LNCaP and MCF-7 cells, respectively. A TUNEL assay confirmed the apoptotic effect of 2-ME in all cell lines (Fig. 1B). FACS

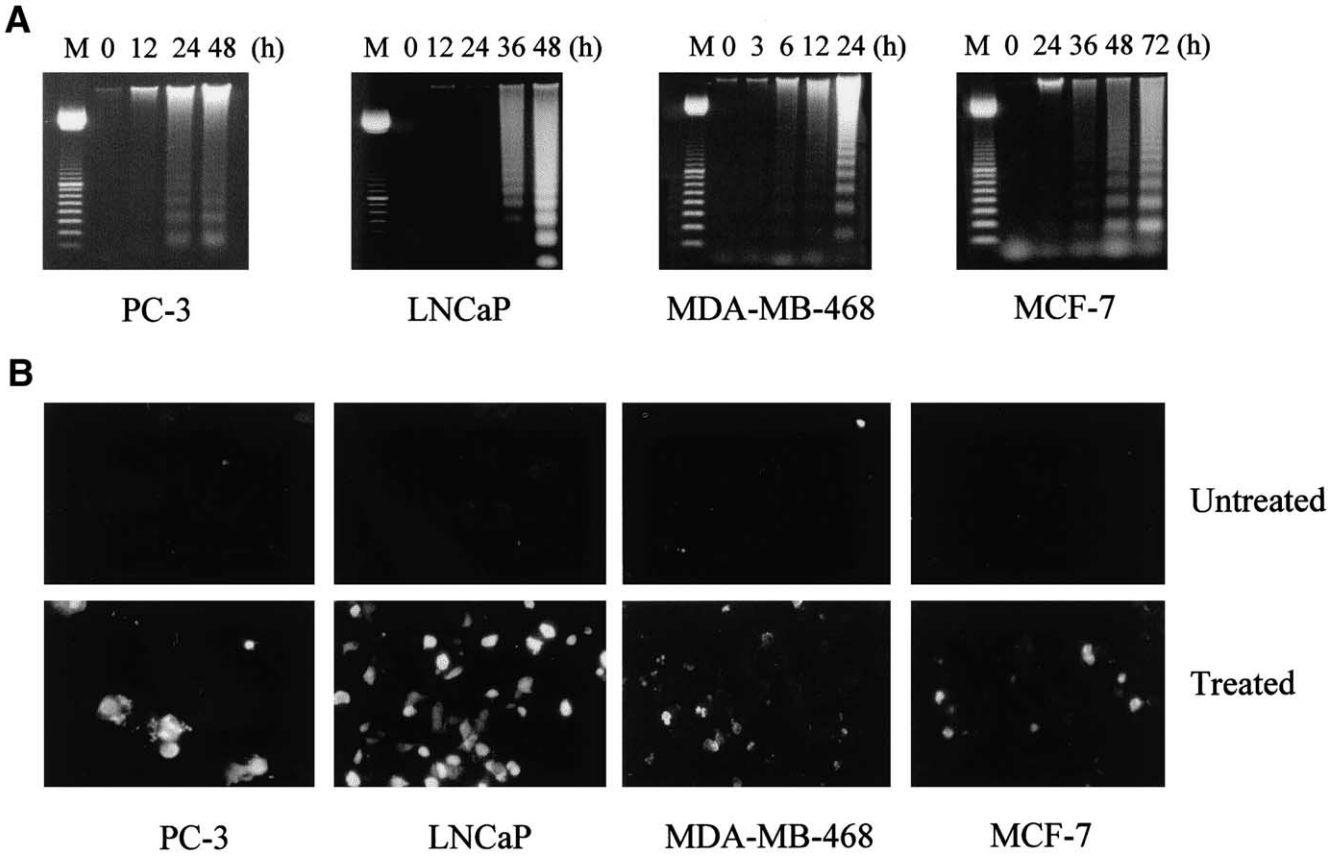


Fig. 1. 2-ME induces apoptosis in human prostate and breast carcinoma cell lines but not in normal primary breast cells. A: DNA was extracted from attached cells as well as from cells floating in the medium of the human prostate (PC-3, LNCaP) and breast (MDA-MB-468, MCF-7) carcinoma cell lines. The cells were treated with 10 μM 2-ME for indicated times in medium containing 10% FBS. DNA was electrophoresed in 1.2% agarose gel and visualized by UV light. B: The presence of apoptotic cells was identified by TUNEL assay according to the manufacturer's manual (Roche) in various cell lines treated or not with 10 μM 2-ME. PC-3 cells were treated for 24 h, LNCaP cells for 36 h, MDA-MB-468 cells for 12 h and MCF-7 cells for 36 h, before fixation and processed for TUNEL staining to visualize apoptotic cells. C: Cells were harvested at different time points after treatment with 10 μM 2-ME or estradiol-17β (E2), fixed, stained with propidium iodide and analyzed for DNA staining profiles by FACS analysis. The results from the FACS analysis in the different cell lines are shown in the figures to the left and were performed after 24 h, 72 h, 36 h and 48 h of treatment in MDA-MB-468, MCF-7, PC-3 and LNCaP cells, respectively. The sub-G1 fraction is determined as the fraction in M1 and the percentage of sub-G1 cells in the different cell lines are shown in the panels to the right. D: Normal primary breast cells and MDA-MB-468 cells were treated with 2-ME for indicated times in medium containing 10% FBS and subjected to DNA fragmentation assay.

analysis was used to quantify the sub-G1 fraction of cells, as a marker of the number of apoptotic cells. 2-ME increased the sub-G1 portion of cells by time, to approximately 30% of MDA-MB-468 (24 h), 40% of MCF-7 (72 h), and 35–40% of PC-3 and LNCaP cells (48 h). Moreover, in PC-3 and LNCaP cells, 2-ME induced apoptosis more effectively than treatment with estradiol (Fig. 1C). In contrast, 2-ME treatment of normal breast cells did not induce apoptosis (Fig. 1D). We therefore conclude that 2-ME potently induces apoptosis in prostate and breast carcinoma cells, although with different kinetics.

3.2. 2-ME inhibits prostate tumor growth in vivo by the induction of apoptosis of endothelial and tumor cells

To investigate the apoptotic effect of 2-ME in vivo, Copenhagen × Fisher F_1 rats transplanted with the spontaneous Dunning R3327-PAP rat prostate tumors received intraperitoneal injections of 2-ME (12.5 mg/kg/day) for 14 days. Under these conditions 2-ME significantly reduced the relative tumor growth to 67 ± 18% ($n = 11$), compared to 149 ± 50% in the control group ($n = 8$, $P < 0.05$, Fig. 2A). The mean tumor weight in the control group was 11.9 ± 1.3 g ($n = 8$) compared to 6.1 ± 0.6 g in the 2-ME-treated group ($n = 11$, $P < 0.02$). In tumors from rats treated with 2-ME macroscopically fibrotic tissue was observed in their centers (Fig. 2B), implying that 2-ME caused massive cell death in the tumor and/or endothelial cells. This was further confirmed by TUNEL, clearly identifying apoptotic tumor and endothelial cells (Fig. 2C). Rats tolerated at least 14 days of 2-ME treatment without visible signs of side effects (like hair loss, diarrhea, infection, lethargy or severe weight loss). The average body weight, including tumors, in the control group before treatment was 478 ± 10 g, and 476 ± 9 g in the 2-ME group. At the end of the treatment period the body weight, including tumors, was 448 ± 8 g in the control group and 407 ± 12 g in the 2-ME-treated group. In summary, 2-ME treatment inhibits growth of solid prostate tumors in vivo, caused by induction of apoptosis of endothelial and tumor cells. Importantly, no major toxic side effects were observed during 2-ME treatment of rats, except a moderate loss of weight.

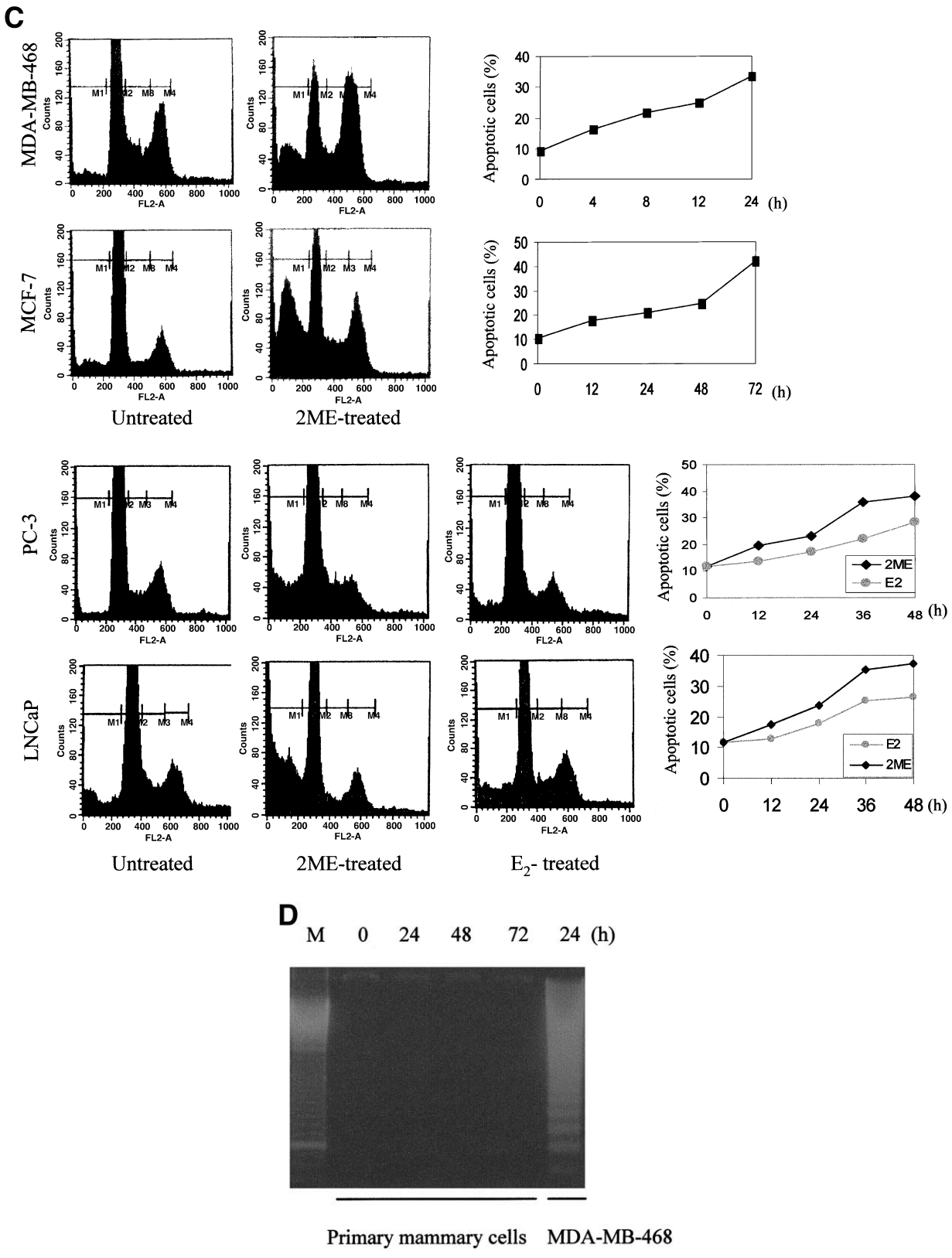


Fig. 1 (*Continued*).

3.3. 2-ME activation of SAPK/JNK results in caspase-dependent apoptosis

Next we studied the molecular details of the cellular effects of 2-ME. Addition of 2-ME activated SAPK/JNK in the investigated tumor cell lines after 20–60 min of stimulation (Fig. 3A). The strongest induction of SAPK/JNK phosphorylation was observed in MDA-MB-468 cells, in which also apoptosis occurred most rapidly. In contrast, challenge of MDA-MB-

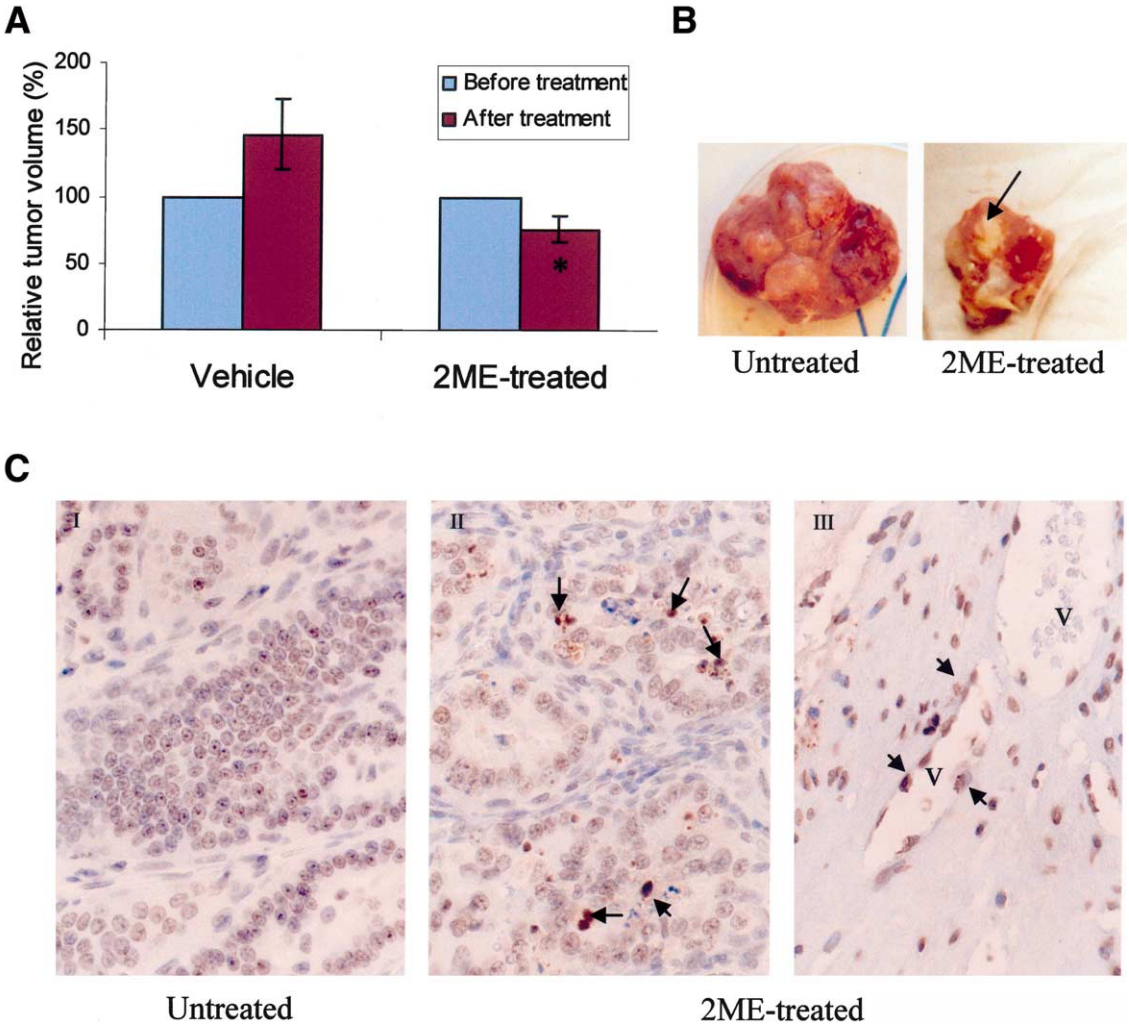


Fig. 2. 2-ME inhibits growth of a rat prostate tumor (Dunning R3327-PAP) in vivo, by induction of apoptosis in tumor and endothelial cells. A: Intraperitoneal injections of 2-ME (12.5 mg/kg/day) for 14 days significantly (*$P < 0.05$) reduced the relative tumor growth in comparison with rats treated with vehicle (0.2 ml NaCl, 9 mg/ml). B: Photographs of tumors grown in rats treated with vehicle or 2-ME. Macroscopically fibrotic tissue was observed in the center of 2-ME-treated tumors (arrow), while vehicle-treated tumors were composed of macroscopically viable cells. C: TUNEL assay performed on sections counterstained with hematoxylin from sections of tumors treated with vehicle or 2-ME. TUNEL-positive apoptotic tumor and endothelial cells were identified in 2-ME-treated tumors, indicated by arrows (tumor cells) and arrowheads (endothelial cells). Blood vessels are indicated with V. 40× magnification.

468 cells with 2-ME did not activate ERK1/2 (Fig. 3B) or p38 (data not shown). Curcumin has been reported to inhibit AP-1, NF-κB and SAPK/JNK activation, with the highest sensitivity against SAPK/JNK activation induced by UV-C or γ-irradiation [39]. Although curcumin is not a specific inhibitor of SAPK/JNK activation, we used curcumin in the present study since we found that curcumin inhibited in a dose-dependent manner the 2-ME-induced activation of SAPK/JNK in MDA-MB-468 cells (data not shown). Curcumin prevented the 2-ME-induced apoptosis in PC-3 (data not shown) and MDA-MB-468 cells (Fig. 3C). As shown in Fig. 3D, simultaneous treatment with the general caspase inhibitor z-VAD-fmk partially (MDA-MB-468 and LNCaP cells) or totally (MCF-7 and PC-3 cells) inhibited the 2-ME-induced apoptosis. Therefore, 2-ME activates SAPK/JNK in the investigated breast and prostate carcinoma cell lines, resulting in a caspase-dependent apoptosis.

3.4. *Inhibition of 2-ME-induced phosphorylation of SAPK/JNK by simultaneous treatment with Ptx*

Next we aimed to investigate which proteins could be upstream of SAPK/JNK in the 2-ME-induced apoptosis. Surprisingly, the 2-ME-induced activation of SAPK/JNK was inhibited in a dose-dependent manner by preincubation with Ptx indicating an involvement of heterotrimeric $G_{i/o}$ proteins (Fig. 4A). Furthermore, Ptx also inhibited the 2-ME-induced apoptosis in MDA-MB-468 cells (Fig. 4B). Activation of G_i is known to reduce cellular levels of cAMP [40]. Interestingly, application of forskolin, a G protein-independent activator of adenylate cyclase, to MDA-MB-468 cells 30 min before treatment with 2-ME rescued cells from undergoing apoptosis (data not shown). Therefore, our data suggest that 2-ME activates $G_{i/o}$ proteins, leading to a modulation of cAMP, activation of JNK and subsequent apoptosis in MDA-MB-468 cells.

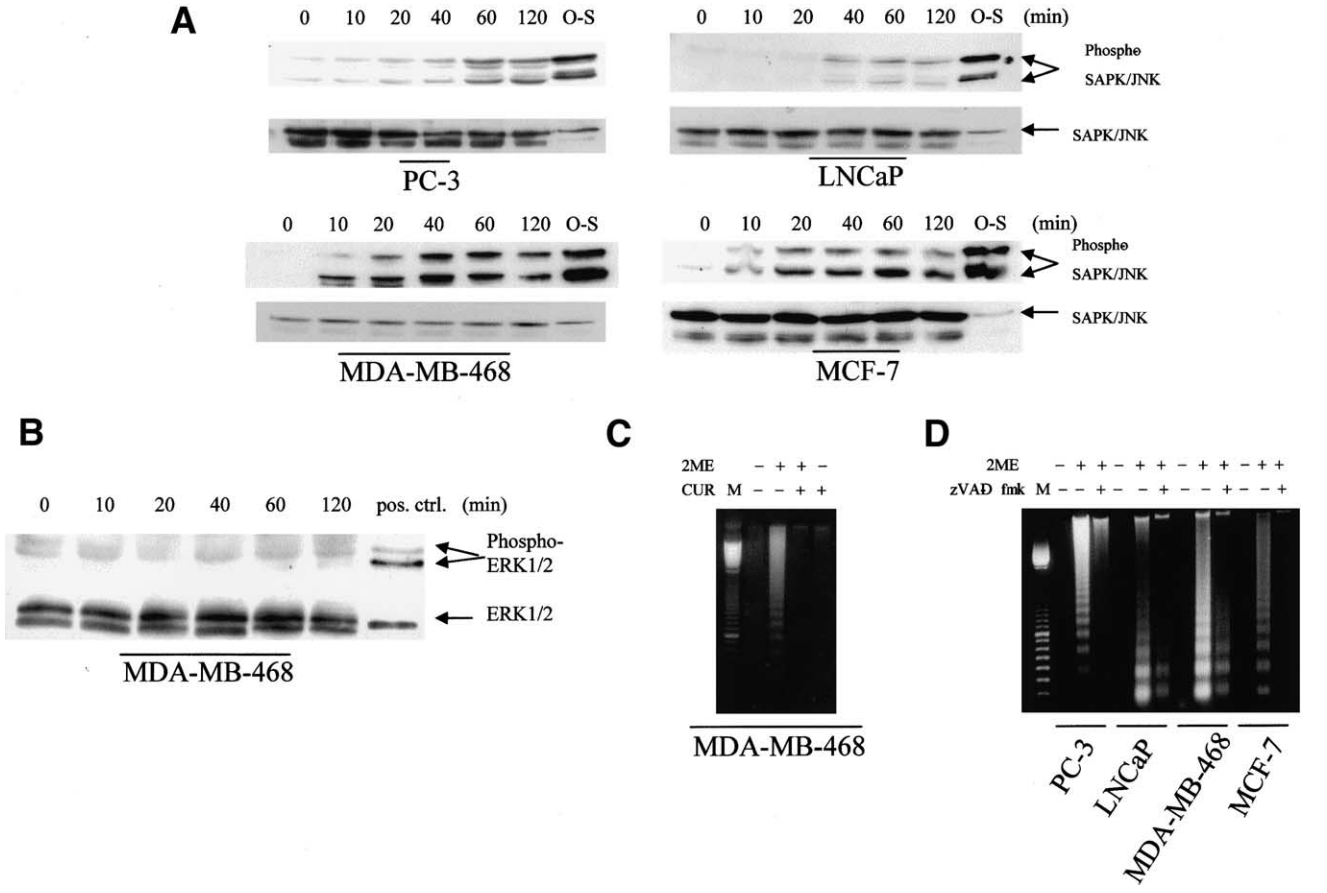


Fig. 3. 2-ME induces activation of SAPK/JNK, which results in caspase-dependent apoptosis. A: Human prostate (PC-3, LNCaP) and breast (MDA-MB-468, MCF-7) carcinoma cells were treated with 10 μM 2-ME. Cells were collected at the indicated time points, and the activated as well as total levels of endogenous SAPK/JNK were determined by Western blot analysis. Cells treated with osmotic shock (O-S; 0.5 M NaCl; 30 min) were used as a positive control. B: MDA-MB-468 breast carcinoma cells were treated with 10 μM 2-ME. Cells were collected at the indicated time points, and the activated as well as total levels of endogenous ERK1/2 were determined by Western blot analysis. Cells treated with 20% FBS for 30 min were used as a positive control (pos. ctrl.). C: DNA fragmentation analysis from MDA-MB-468 cells treated with 10 μM 2-ME for 24 h in the absence or presence of 10 μM curcumin. D: DNA was extracted from attached and floating cells in the medium of the PC-3, LNCaP, MDA-MB-468 and MCF-7 carcinoma cell lines, treated with 2-ME in the presence or absence of 10 μM of a pan-specific caspase inhibitor (z-VAD-fmk) for the time required to obtain maximal apoptosis, in medium containing 10% FBS. DNA was electrophoresed in 1.2% agarose gel and visualized by UV light.

3.5. *Phosphorylation of Bcl-2 in prostate and breast carcinoma cells is associated with 2-ME-induced apoptosis*

Since Bcl-2 is a key component in regulation of cell survival and apoptosis we wondered whether it is involved in the observed apoptotic effect of 2-ME. 2-ME treatment resulted in a phosphorylation of Bcl-2 in prostate (PC-3, LNCaP) and breast (MCF-7, MDA-MB-468) carcinoma cell lines (Fig. 5A). Kinetics of the 2-ME-induced phosphorylation of Bcl-2, differed in the investigated cell lines, but always preceded the onset of apoptosis (see Fig. 1). To investigate whether the phosphorylation of Bcl-2 is linked to the apoptotic effect of 2-ME, the human prostate carcinoma cell line DU-145 that lacks Bcl-2 was studied (Fig. 5B). 2-ME treatment activated SAPK/JNK, but did not induce apoptosis in DU-145 cells (Fig. 5C), while challenge with 10 ng/ml transforming growth factor-β1 (TGF-β1) was able to induce apoptosis, as shown by DNA fragmentation analysis (Fig. 5D). In MDA-MB-468 cells, Ptx and curcumin could inhibit the 2-ME-induced phosphorylation of Bcl-2. Furthermore, the caspase inhibitor z-VAD-fmk also prevented 2-ME-induced phosphorylation of Bcl-2 in MDA-MB-468 cells (Fig. 5E). Taken together, our data show that the 2-ME-induced phosphorylation of Bcl-2 is prevented by Ptx, curcumin and a caspase inhibitor, treatments which also inhibited 2-ME-induced apoptosis. Our results thus propose a link between 2-ME, JNK activation, phosphorylation of Bcl-2 and apoptosis.

3.6. *2-ME also induces apoptosis in hepatic and colon carcinoma cell lines*

To investigate whether the apoptotic effect of 2-ME is more general and not only limited to breast and prostate carcinoma cell lines, we included HepG2 (human hepatocarcinoma) and SW-480 (human colorectal carcinoma) cells in our study. 2-ME treatment of these cells for 48 h clearly resulted in apoptosis, as shown by DNA fragmentation assays (Fig. 6A). Again, pretreatment with Ptx inhibited the 2-ME-mediated effects (Fig. 6A). Furthermore, 2-ME activated SAPK/JNK in these cells (Fig. 6B) and also caused phosphorylation of Bcl-2 (Fig. 6C). Therefore, the identified molecular pathway for 2-ME-induced apoptosis, i.e. activation of SAPK/JNK preceding phosphorylation of Bcl-2, was found in several different epithelial carcinoma cell lines thus supporting the notion of a general mechanism of the cellular effects of 2-ME.

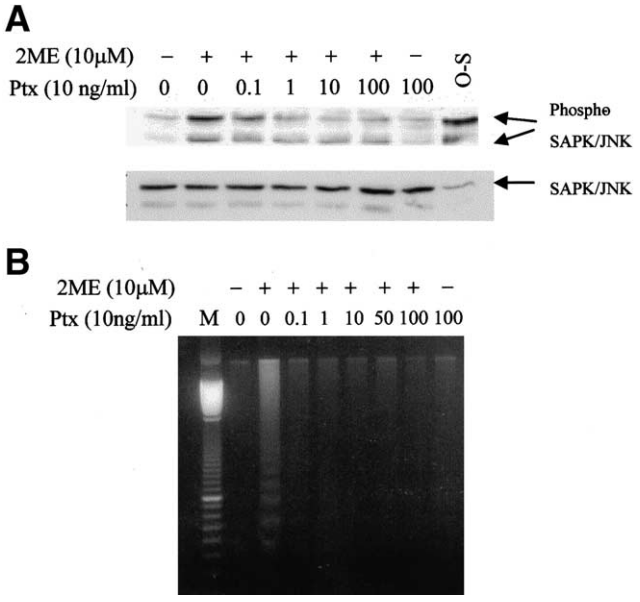


Fig. 4. 2-ME-mediated activation of SAPK/JNK and induction of apoptosis are mediated by GPCR activation. A: MDA-MB-468 cells were pretreated with increasing concentrations of Ptx for 12 h and then challenged with 10 μM 2-ME for 1 h. Cells were collected and the levels of phosphorylated endogenous SAPK/JNK were determined by Western blot analysis. Cells treated with osmotic shock (O-S; 0.5 M NaCl, 30 min) were used as a positive control. The levels of total SAPK/JNK were determined as a control for equal loading. B: DNA was extracted from attached cells and cells floating in the medium of the MDA-MB-468 cells. The cell lines had been treated with 10 μM 2-ME for 24 h in the absence or presence of Ptx at increasing concentrations in medium containing 10% FBS. DNA was electrophoresed in 1.2% agarose gel and visualized by UV light.

4. Discussion

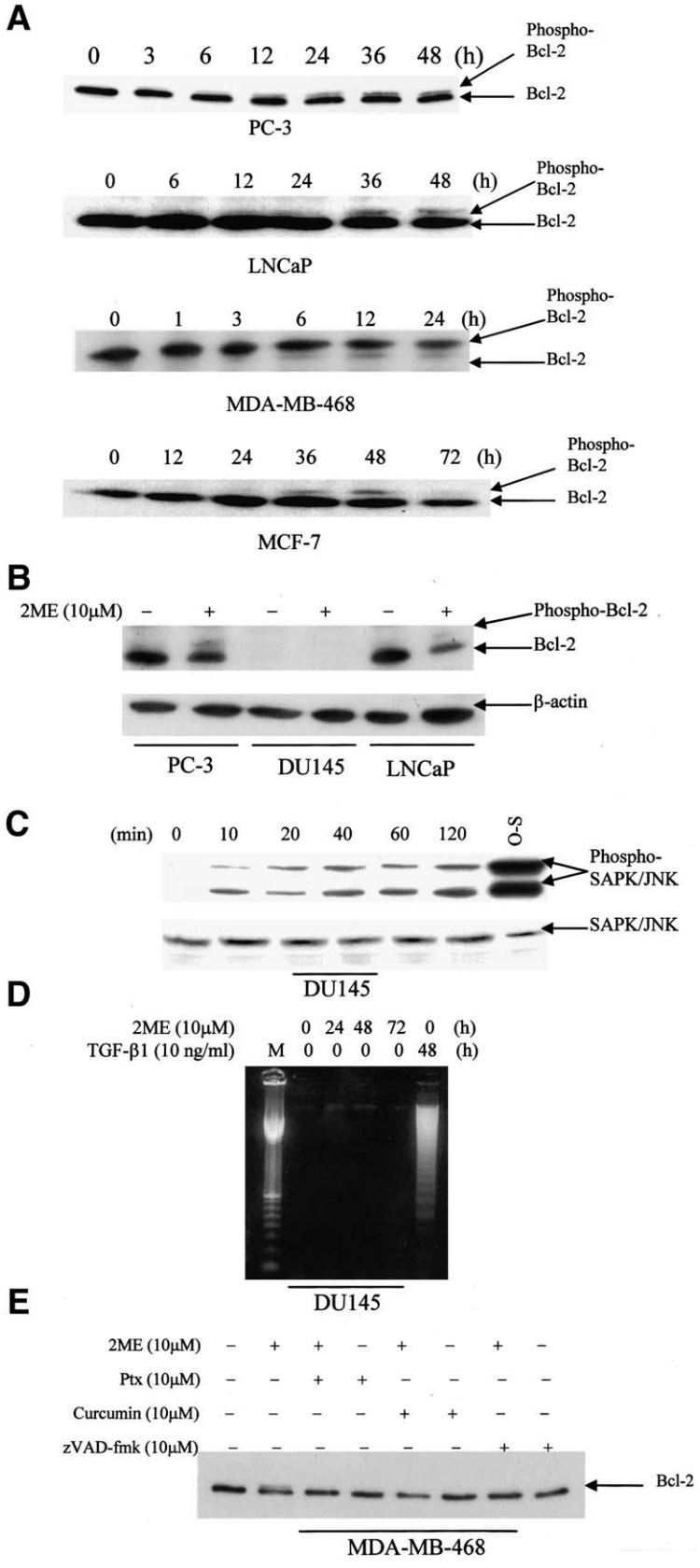
We report here that 2-ME potently induces apoptosis in several common carcinoma epithelial cell lines, derived from prostate, breast, liver and colorectal cancers. Moreover, 2-ME treatment of rats transplanted with Dunning R3327-PAP prostate tumors resulted in a reduction of tumor volume, associated with an increase of the number of apoptotic endothelial and tumor cells. No major toxic side effects of 2-ME treatment were observed in these in vivo studies. Investigations of the molecular mechanism by which 2-ME treatment activates an apoptotic pathway revealed that 2-ME treatment causes activation of SAPK/JNK and phosphorylation of Bcl-2 that precedes apoptosis.

2-ME potently induced apoptosis of the human prostate carcinoma cell lines PC-3 and LNCaP, and the human breast carcinoma cell lines MDA-MB-468 and MCF-7, but with variations in kinetics. The in vivo experiments performed in non-castrated rats, using the transplantable spontaneous rat prostate Dunning R3327-PAP as a model, showed that 2-ME treatment could in fact cause tumor regression to half the size compared to vehicle treatment, after 2 weeks of treatment. Since 2-ME treatment potently inhibits prostate tumor growth also in vivo, due to its capability to induce apoptosis of endothelial as well as malignant cells in the tumor without severe toxic side effects, it is an interesting candidate to improve the therapeutic arsenal in treatment of advanced prostate carcinoma.

2-ME given to the examined tumor cell lines caused a rapid activation of SAPK/JNK, which was most pronounced in MDA-MB-468 cells, in accordance with the earlier observed effect of 2-ME on endothelial cells [14]. 2-ME did not lead to any ERK1/2 phosphorylation, suggesting a specific activation of the SAPK/JNK MAPK pathway. Curcumin has been shown to inhibit SAPK/JNK activation, probably inhibiting the kinases upstream of SAPK/JNK kinase [39,41]. In the current study, this drug inhibited 2-ME-induced SAPK/JNK activation as well as 2-ME-induced apoptosis in MDA-MB-468 cells, suggesting an involvement of SAPK/JNK activation in these processes. Most likely caspases are activated after treatment with 2-ME, since a pan-specific caspase inhibitor could prevent 2-ME-induced apoptosis. However, we were not able to detect any expression of the active forms of caspase 1, 3 or 8, by Western blot analysis of lysates from 2-ME-treated cells (data not shown).

In our effort to further characterize the molecular mechanisms by which 2-ME could induce apoptosis in epithelial-derived tumor cells, we tested a possible role of GPCRs, by using Ptx, which specifically inhibits G_i and G_o proteins [40]. Ptx decreased the 2-ME-induced activation of SAPK/JNK, as well as the 2-ME-induced apoptosis of MDA-MB-468 cells in a dose-dependent manner. Furthermore, the cellular levels of cAMP have been reported to decrease when G_i proteins are activated [40] and we found that forskolin, which increases cAMP levels [42], prevented the 2-ME-induced apoptosis of MDA-MB-468 cells, suggesting that 2-ME activates a putative GPCR (data not shown). In fact, 2-ME treatment has earlier been reported to decrease the cAMP levels in MCF-7 cells [13]. There are also other reports of non-nuclear actions of steroids, including that estradiol-17β can potentiate kainate-induced current in neurons and attenuate the EGF-mediated Erk-1/2 activity in breast carcinoma cells, probably by activating a GPCR [43,44]. The apoptosis induced by 2-ME is not likely to be mediated by the classical estrogen receptor, since MDA-MB-468 cells, which do not express estrogen receptor-α, responded well to 2-ME. In addition, the observed rapid 2-ME-induced activation of SAPK/JNK that we observed in MDA-MB-468 cells is unlikely to be mediated by transcriptional activity of nuclear estrogen receptors. Moreover, previous studies have shown that 2-ME has little or no binding affinity for the classical estrogen receptor and 2-ME lacks estrogenic activity in the uterus [45,46]. In a recent report by La Vallee et al., 2-ME was shown to inhibit proliferation and to induce apoptosis clearly independently of the estrogen receptors α or β [23]. Recently, it has been reported that estrogens have rapid modulatory effects on activation of Erk-1/2, initiated by EGF and activation of JNK induced by taxol or UV [44,47,48]. These effects of estrogens have been suggested to occur via putative receptors in the plasma membrane. Moreover, activation of JNK by natural and synthetic estrogens has been demonstrated [49]. These reports suggest that estrogens could have effects on alternative membrane-bound receptors. Further studies are needed to determine the molecular mechanism for the observed activation of SAPK/JNK by 2-ME.

We report here that 2-ME does not induce apoptosis in normal mammary cells, suggesting a specific apoptotic effect of 2-ME on rapidly growing tumor cells, in line with the recent report from Huang et al., that 2-ME selectively kills human leukemia cells but not normal lymphocytes [22]. Inter-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Phospho-Bcl-2
Bcl-2
MDA-MB-468
0 12 24 36 48 72 (h)
Phospho-Bcl-2
Bcl-2
MCF-7
B
2ME (10μM) – + – + – +
Phospho-Bcl-2
Bcl-2
β-actin
PC-3 DU145 LNCaP
C
(min) 0 10 20 40 60 120 O-S
Phospho-SAPK/JNK
SAPK/JNK
DU145
D
2ME (10μM) 0 24 48 72 0 (h)
TGF-β1 (10 ng/ml) M 0 0 0 0 48 (h)
DU145
E
2ME (10μM) – + + – + – + –
Ptx (10μM) – – + + – – – –
Curcumin (10μM) – – – – + + – –
zVAD-fmk (10μM) – – – – – – + +
Bcl-2
MDA-MB-468

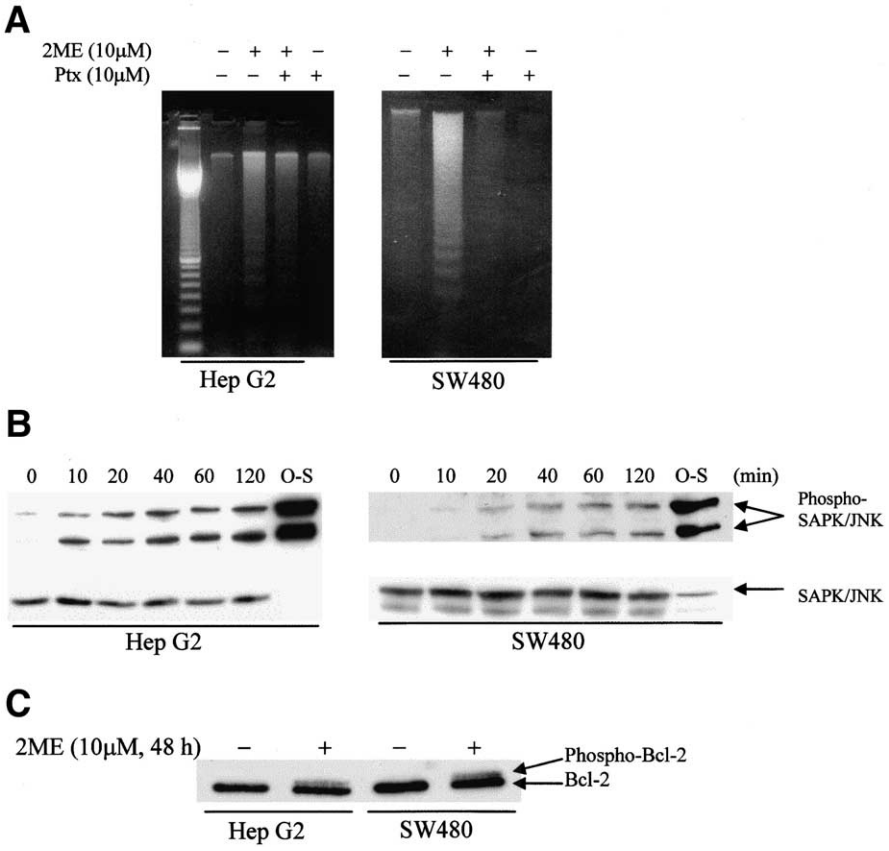

Fig. 6. The 2-ME-induced apoptosis in human liver and colorectal carcinoma cell lines is mediated by GPCR and SAPK/JNK activation, causing phosphorylation of Bcl-2. A: DNA was extracted from attached cells as well as from cells floating in the medium of the human liver (HepG2) and colorectal (SW-480) carcinoma cell lines. The cell lines were treated with 2-ME in the presence or absence of Ptx for 48 h in medium containing 10% FBS. DNA was electrophoresed in 1.2% agarose gel and visualized by UV light. B: HepG2 and SW-480 cells were treated with 10 μM 2-ME. The cells were collected at the indicated time points and the levels of phosphorylated endogenous SAPK/JNK were determined by Western blot analysis. Cells treated with osmotic shock (O-S; 0.5 M NaCl, 30 min) were used as a positive control. The level of total endogenous SAPK/JNK was determined as a control for equal loading. C: HepG2 and SW-480 cells were treated with 10 μM 2-ME for 48 h. Cells were collected and the expression of Bcl-2 was determined by Western blot analysis. Phosphorylated Bcl-2 was recognized as a slower-migrating band as indicated by an arrow.

estingly, 2-ME was shown to inhibit the transcription of the superoxide dismutase (SOD) enzymes, which protects cells from damage induced by superoxide radicals and inhibition of SOD activity, resulting in apoptosis. Tumor cells are more dependent on SOD than normal cells, since tumor cells have higher superoxide production but lower levels of SOD than normal cells [22]. The reported effect of 2-ME on regulation of SOD might be correlated with our finding of the selective induction of apoptosis by 2-ME only in tumor cells. It is important to note that all observed effects of 2-ME in the current study were caused by pharmacological concentrations. However, since no serious side effects of 2-ME treatment were observed in the animal experiments, a therapeutic use of 2-ME may be possible.

Bcl-2 belongs to a family of proteins, the members of which all regulate apoptosis and function as molecular determinators of cell survival [50–52]. Over-expression of Bcl-2 is associated with many types of cancers including breast and prostate carcinomas [53,54]. The role of Bcl-2 phosphorylation in regulation of apoptosis is unclear, since some studies show potentiation of its anti-apoptotic function, while other studies show inactivation of its anti-apoptotic function [55–57]. Bcl-2 has

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Fig. 5. 2-ME-induced Bcl-2 phosphorylation in human prostate carcinoma cell lines is necessary for induction of apoptosis. A: PC-3, LNCaP, MDA-MB-468 and MCF-7 cells were treated with 10 μM 2-ME. The cells were collected at the indicated time points and the expression of Bcl-2 was determined by Western blot analysis. Phosphorylated Bcl-2 was recognized as a slower-migrating band. B: PC-3, DU-145 and LNCaP cells were treated with 10 μM 2-ME. The cells were collected at the indicated time points and the expression of Bcl-2 was determined by Western blot analysis. Phosphorylated Bcl-2 was recognized as a slower-migrating band. The same filter was reprobed with actin antibodies to show that equal amounts of proteins were loaded. C: DU-145 cells were treated with 10 μM 2-ME for indicated times. The cells were collected at the indicated time points and the phosphorylated SAPK/JNK and total SAPK/JNK levels were determined by Western blot analysis. D: DNA was extracted from attached cells as well as from cells floating in the medium of the DU-145 prostate carcinoma cell lines, treated with 2-ME or TGF-β1 for indicated time periods. DNA was electrophoresed in 1.2% agarose gel and visualized by UV light. E: MDA-MB-468 carcinoma cells were treated with 10 μM 2-ME for 24 h in the absence or presence of Ptx (10 ng/ml), 10 μM curcumin and 10 μM z-VAD-fmk, then cells were collected and the expression of Bcl-2 was determined by Western blot analysis. Phosphorylated Bcl-2 was recognized as a slower-migrating band and is indicated by an arrow.

been shown to be phosphorylated during apoptosis induced by other microtubule-affecting drugs like paclitaxel, which has similar stabilizing effects on the microtubule system as reported for 2-ME. Treatment with paclitaxel can activate ERK and SAPK/JNK in MCF-7 breast carcinoma cells, and in both breast and prostate carcinoma cells, paclitaxel treatment causes phosphorylation and inactivation of Bcl-2, leading to apoptosis [58–60]. When the SAPK/JNK pathway was blocked, no phosphorylation of Bcl-2 occurred and cells were rescued from apoptosis [60], indicating that Bcl-2 is phosphorylated, directly or indirectly by SAPK/JNK [61,62]. Furthermore, 2-ME treatment of a erythromyeloid leukemia cell line (K562) has been reported to activate SAPK/JNK and cause phosphorylation of Bcl-2 [63]. Filippovich et al. [64] showed that radiation-induced apoptosis of human myeloma cells is preceded by Bcl-2 phosphorylation. The present study shows that 2-ME treatment also results in SAPK/JNK activation and phosphorylation of Bcl-2 in all investigated epithelial carcinoma cell lines that responded with apoptosis. We demonstrated that Ptx treatment prevented the 2-ME-induced phosphorylation of Bcl-2 and concomitant apoptosis of cells suggesting that 2-ME activates the SAPK/JNK pathway. When curcumin was used, the 2-ME-induced phosphorylation of Bcl-2 was prevented, supporting the notion that activation of SAPK/JNK is associated with the phosphorylation of Bcl-2. Interestingly, MacCarthy-Morrogh et al. [65] recently reported that a derivative of 2-ME resulted in increased phosphorylation of Bcl-2 and apoptosis of human mammalian cell lines in vitro, when compared to the effects of the parent 2-ME. Our finding that 2-ME treatment of DU-145 prostate carcinoma cells, which lack Bcl-2 expression, causes SAPK/JNK activation but not apoptosis suggests that Bcl-2 expression is important for 2-ME-induced apoptosis in carcinoma cells and the presence of Bcl-2 could therefore be necessary for efficient treatment of patients with 2-ME. Also in the human hepatocarcinoma and colorectal carcinoma cell lines (HepG2, SW-480), 2-ME treatment resulted in an activation of SAPK/JNK and in phosphorylation of Bcl-2, which preceded apoptosis. Our data suggest that 2-ME causes activation of the SAPK/JNK pathway, which leads to phosphorylation of Bcl-2 and apoptosis in several investigated cell lines derived from breast, prostate, liver and colorectal carcinomas. Furthermore, treatment with 2-ME inhibited prostate tumor growth in vivo by causing apoptosis in tumor and endothelial cells, without any apparent major side effects on the animals. Since 2-ME inhibits angiogenesis, lacks estrogenic activity and shows minimal toxicity in vivo, while it has potent cytotoxic effects on several common epithelial tumor cells, it is an attractive candidate for future clinical trials for prevention or treatment of carcinomas.

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