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Up-Regulation of Bcl-2 through ERK Phosphorylation Is Associated with Human Macrophage Survival in an Estrogen Microenvironment

Manikandan Subramanian and Chandrima Shaha

Estrogen is a known immunomodulator with pleiotropic effects on macrophage function that partly accounts for the gender bias observed in numerous autoimmune, cardiovascular, and neurodegenerative disorders. The effect of estrogen on the survival of human macrophages is largely unknown, and in this study we demonstrate that 17β-estradiol (E2) provokes a death response in human THP-1 macrophages by initiating Bax translocation from cytosol to the mitochondria; however, a concomitant up-regulation of Bcl-2 creates a Bax to Bcl-2 ratio favorable for Bcl-2, thus ensuring cell survival. Both Bcl-2 up-regulation and Bax translocation are estrogen receptor-dependent events; however, Bcl-2 augmentation but not Bax translocation is dependent on Ca2+ increase, activation of protein kinase C, and ERK phosphorylation. This estrogen-induced Bcl-2 increase is crucial for the survival of THP-1 macrophages as well as that of human peripheral blood monocyte-derived macrophages, which is evident from E2-induced cell death under small interfering RNA-mediated Bcl-2 knockdown conditions. Hence, this study demonstrates that E2-induced Bcl-2 up-regulation is a homeostatic survival mechanism necessary for the manifestation of immunomodulatory effect of estrogen on human macrophages. The Journal of Immunology, 2007, 179: 2330–2338.

Macrophages express estrogen receptor subtypes α and β (1–3) and are therefore capable of responding to increase in estrogen during the follicular phase of menstrual cycle (4), at the time of exposure from exogenous sources such as phytoestrogens (5), following administration for prophylactic and therapeutic purposes (6), or during accidental exposure to estrogenic chemicals (7, 8). Estrogen affects a variety of macrophage functions; for example, it can reduce accumulation of cholesteryl esters in macrophages (2), stimulate production of NO (9, 10), increase arachidonic acid release (11), regulate activation-related events (2, 12), decrease monocyte adhesion to vasculature (13), enhance macrophage phagocytosis (14), and facilitate Ca2+ influx (10). Some functions are implicated in mediating the gender bias observed in numerous autoimmune (15), cardiovascular (16), and neurodegenerative disorders (17). In addition, estrogen is able to modulate macrophage death, which is of great relevance because macrophage survival or death is crucial for disease pathogenesis (18). However, data available on the influence of the hormone on the macrophage cell death process are contradictory. Existing literature show that macrophage-like U937 cells undergo apoptosis when exposed to estrogen (19), but the same cell type is protected from TNF-α-induced apoptosis by the hormone (20). According to other reports, estrogen is able to induce apoptosis in undifferentiated U937 monocytes, but macrophages differentiated from these cells are refractory to such effects of estrogen (21). Further examples of cell types in which estrogen is reported to induce cell death include bone macrophages like murine osteoclasts, preosteoclastic FLG 29.1 cell line, and mouse peritoneal macrophages (22–25).

The apparently paradoxical effect of estrogen on apoptosis in cells of the monocytic lineage could be interpreted to be the result of its ability to differentially modulate antiapoptotic and proapoptotic proteins like the members of the Bcl-2 family that share sequence-homology domains within the group (26). The various pro-apoptotic and antiapoptotic Bcl-2 family members are able to heterodimerize (26), and their relative concentrations function as a rheostat for the apoptotic program (26). Certain apoptotic stimuli like exposure to NO (27), oysterol (28), and activation-inducing agents increase macrophage Bcl-2 or other members of the Bcl-2 family of proteins like BII-1 (29), but the involvement of Bcl-2 family members in regulating the macrophage death pathway is not completely understood. In the context of tumor development, mechanisms regulating macrophage death are important because these cells constitute a large proportion of the tumor cells and are evidently important for either progression or regression of tumors (30). Survival of macrophages in estrogen microenvironment is relevant especially in the cells populating estrogen target tissues like uteri, breast, brain, and cervix. Also, understanding of the mechanism of macrophage survival under altered Bcl-2 level becomes important in the backdrop of development of Bcl-2 small molecule inhibitors, antisense oligonucleotides, and RNA interference against Bcl-2, which were intended to be used for treatment of resistant carcinoma and some of which are currently in phase I and phase II clinical trials (31–35). This study was designed to identify the key players that are vital for modulating human macrophage survival under estrogen exposure.

We demonstrate that 17β-estradiol (E2) treatment not only provokes a death response via Bax translocation in macrophages derived from THP-1 human acute monocytic leukemia cells, but also...
initiates an antiapoptotic response through the up-regulation of Bcl-2 via a Ca\(^{2+}\)-dependent ERK-mediated pathway. The importance of E2-induced Bcl-2 up-regulation in macrophage physiology is demonstrated by increased cell death when Bcl-2 is down-regulated through interference with Ca\(^{2+}\) influx, ERK phosphorylation, or small interfering RNA (siRNA)-mediated Bcl-2 mRNA degradation. E2 also induces cell death in human peripheral blood monocyte-derived macrophages (MDM) when Bcl-2 is knocked down with anti-Bcl-2 siRNA.

**Materials and Methods**

**Materials**

E2 (cyclodestrin encapsulated) was obtained from Sigma-Aldrich. ICI 182780, PPT (4,4'-d(4-Propyl)-1H-pyrazole-1,3,5-triyl)trisphenol), and DPN (2,3-bis(4-hydroxyphenyl)-propionitrile) were procured from Tocris Cookson. Primary Ab against Bcl-2 was obtained from Santa Cruz Biotechnology. Abs against phospho-ERK, whole ERK, and phospho-CREB were purchased from StressGen Biotechnologies. Anti-Bax, anti-cytotochrome c, and anti-C1D14 FITC Abs were purchased from BD Biosciences. Secondary Abs raised in either mice or rabbits conjugated to HRP were obtained from Jackson ImmunoResearch Laboratories. Fluo-3-acetoxyethyl ester (fluo-3-AM) and secondary anti-mouse IgG conjugated to Alexa Fluor 488 are obtained from Molecular Probes. Anti-actin and anti-estrogen receptor C-terminal Abs were from Calbiochem. The Vybrant apoptosis detection system was purchased from Promega. All reagents for Western blotting and ECL development were obtained from Amersham Biosciences. The Bcl-2 siRNA was purchased from Upstate Biotechnology, whereas Cy3-labeled negative control siRNA was procured from Ambion. Transpass R2 transfection reagent was from New England Biolabs. Phenol-red free RPMI 1640 and dextran-coated charcol-stripped FCS was obtained from Biological Industries. Verapamil, PMA, Ca\(^{2+}\) ionophore A 23187, EGTA, P998059, bisindolylmaleimide (BIM), Histopaque 1077, and any other chemical used were obtained from Sigma-Aldrich, unless otherwise mentioned.

**Peripheral blood monocyte isolation, cell lines, and cell culture**

Peripheral blood was collected from healthy male volunteers after obtaining informed consent as per regulations of the Institutional Human Ethics Committee (National Institute of Immunology, New Delhi, India). The Peripheral blood was collected from healthy male volunteers after obtaining informed consent as per regulations of the Institutional Human Ethics Committee (National Institute of Immunology, New Delhi, India). The PBMC were isolated by density gradient centrifugation using Histopaque 1077, and any other chemical used were obtained from Sigma-Aldrich, unless otherwise mentioned.

**Intracellular free Ca\(^{2+}\) assay**

Changes in intracellular free Ca\(^{2+}\) concentration were monitored using the Ca\(^{2+}\) binding fluorescent probe fluo-3-AM as previously described (36). Briefly, 10\(^6\) cells/ml loaded with 0.5 \(\mu\)M fluo-3-AM containing 0.1% saponin at room temperature for 30 min and subsequently incubated with the formation of transfection complexes. The siRNA transfection complexes were added at a final concentration of 15 pmol to 10\(^5\) cells/well grown in 24-well plates and incubated for 6 h following which fresh complete medium was added. Transfection efficiency was estimated by observing Cy3 fluorescence of the negative control siRNA with a Nikon TE2000-E fluorescence microscope using a tetramethyl rhodamine filter (530-550 nm). Target protein knockdown was assessed 24 h posttransfection by probing extracts of transfected cells on Western blots with anti-Bcl-2 Ab.

**Subcellular fractionation**

THP-1 macrophages and human peripheral blood MDM were transfected with 15 pmol SMARTpool Bcl-2 siRNA or negative control siRNA using Transpass R2 transfection reagent as per the manufacturer’s instructions. Briefly, Bcl-2 siRNA or negative control siRNA were added to transfection reagent diluted in serum-free medium and incubated for 20 min to allow the formation of transfection complexes. The siRNA transfection complexes were added at a final concentration of 15 pmol to 10\(^5\) cells/well grown in 24-well plates and incubated for 6 h following which fresh complete medium was added. Transfection efficiency was estimated by observing Cy3 fluorescence of the negative control siRNA with a Nikon TE2000-E fluorescence microscope using a tetramethyl rhodamine filter (530-550 nm). Target protein knockdown was assessed 24 h posttransfection by probing extracts of transfected cells on Western blots with anti-Bcl-2 Ab.

**SDS-PAGE and Western blot**

Whole cell lysates were prepared by mixing cells with lysis buffer (0.5 M sucrose, 10 mM Tris, pH 7.4) containing 1 mM EDTA and protease inhibitor cocktail from Roche Diagnostics with a mixture of various protease inhibitors. Cell lysis was performed by sonication (Sonifier 450; Branson) on ice at 50% duty cycle for a total of 9 cycles. The sonicate was centrifuged at 4000 \(\times\) g for 10 min to obtain the nuclear pellet. From the resulting supernatant, the mitochondrial fraction was extracted by further centrifugation at 10,000 \(\times\) g for 10 min (38) in an ultracentrifuge (SW55Ti rotor, optima XL-110k; Beckman Coulter). The postmitochondrial supernatant was further centrifuged at 100,000 \(\times\) g for 30 min to isolate the microsomal fraction as a pellet and the supernatant obtained was the cytosolic fraction.

**RT-PCR**

Total RNA was isolated using Trizol reagent (Invitrogen Life Technologies), and cDNA was synthesized as previously described (36). The specific primers used were the following: Bcl-2 (sense) 5\'-GGGGAGAACCTTCCAGGGA-3\', (antisense) 5\'-AGGCCACCCAGGGTGATGCCA-3'; and actin (sense) 5\'-GGGGGGGCCTCCAAGCACA-3', (antisense) 5\'-CTCTTTAATGTCAACACAAGTTT-3'. PCR was performed after determining the cycle number in which a linear amplification of serially diluted template could be achieved. The PCR products were then resolved on 1.5% agarose gel and visualized by ethidium bromide staining and quantitated by densitometry.

**siRNA transfection**

THP-1 macrophages and human peripheral blood MDM were transfected with 15 pmol SMARTpool Bcl-2 siRNA or negative control siRNA using Transpass R2 transfection reagent as per the manufacturer’s instructions. Briefly, Bcl-2 siRNA or negative control siRNA were added to transfection reagent diluted in serum-free medium and incubated for 20 min to allow the formation of transfection complexes. The siRNA transfection complexes were added at a final concentration of 15 pmol to 10\(^5\) cells/well grown in 24-well plates and incubated for 6 h following which fresh complete medium was added. Transfection efficiency was estimated by observing Cy3 fluorescence of the negative control siRNA with a Nikon TE2000-E fluorescence microscope using a tetramethyl rhodamine filter (530-550 nm). Target protein knockdown was assessed 24 h posttransfection by probing extracts of transfected cells on Western blots with anti-Bcl-2 Ab.

**Immunocytochemistry**

Formaldehyde (4%) fixed cells were blocked with 3% normal goat serum containing 0.1% saponin at room temperature for 30 min and subsequently incubated with primary Ab at 1:50 dilution for 1 h at 37°C followed by secondary Ab conjugated to Alexa Fluor 488 at 1/100 dilution for 1 h at the same temperature. Subsequently, the cells were washed and resuspended in PBS and analyzed on a BD LSR flow cytometer equipped with an air-cooled 488 nm argon ion laser.

**Densitometry**

Densitometric measurements for quantification of signals on immunoblots or ethidium bromide stained agarose gels were performed using a UVP gel documentation instrument, and the acquired data were analyzed with the LabWorks image analysis and acquisition software (v4.0, UVP). At least three Western blots per experiment were quantitated to arrive at the average value of the signal. All measurements were normalized to internal loading controls.
**FIGURE 1.** Estrogen increases Bcl-2 expression in THP-1 macrophages through an estrogen receptor-dependent mechanism. **A,** Increase in Bcl-2 levels after 24 h treatment with various doses of E2 is shown on representative immunoblots of THP-1 macrophage lysates probed with anti-Bcl-2 Ab (80 ng/ml). Actin was used as a loading control. **B,** Time-kinetic analysis of Bcl-2 expression with THP-1 macrophage lysates collected at different time points after 10 nM E2 exposure (0–6 h) showing a time-dependent increase in Bcl-2 levels. Actin was used as a loading control. **C,** Flow cytometric analysis of Bcl-2 expression as determined by immunostaining of THP-1 macrophages with anti-Bcl-2 mAb (600 ng/ml) after 24 h of treatment of cells with E2 and ICI 182780. Note the distinct shift obtained with E2 as compared with vehicle treated control (VT) lanes 1 and 2, 10 nM E2 treatment with ICI (lane 3), and 100 nM PPT, an estrogen receptor antagonist, (lane 4) as compared with vehicle treated control (VT lane 5). **D,** Western blot showing a time-dependent augmentation in Bcl-2 protein levels occurring from 1 h onward doubling at 4 h after treatment with E2 as compared with constitutive Bcl-2 levels (vehicle treated). **E,** Relative Bcl-2 expression levels normalized to actin as compared with vehicle treated control, calculated by densitometric analysis of multiple immunoblots as detailed in Materials and Methods. Error bars in A, B, and E are ± SEM with n = 3 experiments in duplicate. *, p < 0.05 as compared with vehicle treated; §, p < 0.05 as compared with E2 treated. F. Estrogen receptor expression was analyzed by flow cytometry with THP-1 macrophages immunostained with anti-estrogen receptor Ab reactive to both estrogen receptors α and β. The marker represents the percentage population that stain positive for the receptors. CSA, control secondary Ab.

**Statistical analysis**

Data were analyzed by Student’s t test and values are expressed as mean ± SEM. The values were considered significantly different at p < 0.05.

**Results**

E2 does not affect THP-1 macrophage viability while it increases Bcl-2 levels

To examine whether E2 has any effect on cell survival, THP-1 macrophages were exposed to several doses of the hormone ranging from 1 nM to 1 μM and cell death was estimated at 24 h by propidium iodide (1 μg/ml) exclusion method. No significant cell death could be recorded with any of the doses as compared with vehicle treated (cyclodextrin dissolved in water) controls. Percentage of survival was as follows: vehicle treated, 94 ± 1; 1 nM E2 treatment, 95 ± 2; 10 nM E2 treatment, 96 ± 1; 100 nM E2 treatment, 96 ± 2. Contrary to our findings, some studies show that estrogen causes death in cells of monocytic lineage (19, 21–25). Arguably, if estrogen is able to induce death in cells of similar lineage, the failure of the hormone to do so in THP-1 macrophages could mean differential regulation of the proapoptotic and antiapoptotic proteins leading to maintenance of viability. A distinct increase in THP-1 macrophage Bcl-2 above the constitutive levels was observed with 10 nM, 100 nM, and 1 μM E2 treatment (Fig. 1A). Because beyond 10 nM there was no appreciable increase in Bcl-2 (Fig. 1A) and 100 nM being within the physiological range (39), all other studies used this dose of E2. With the same dose, a time-dependent augmentation in Bcl-2 protein levels occurred from 1 h onward doubling at 4 h from the constitutive levels at 0 h (Fig. 1B) and was sustained till 72 h (data not shown). Flow cytometric quantitation of Bcl-2 expression in cells of different treatment groups labeled sequentially with anti-Bcl-2 Ab followed by secondary Ab conjugated to Alexa Fluor 488 showed a distinct shift in the mean fluorescence intensity (MFI), which doubled after treatment with E2 as compared with constitutive Bcl-2 levels (vehicle treated) (Fig. 1C). Presence of estrogen receptor antagonist ICI 182780 prevented a shift in fluorescence labeling (Fig. 1C, E2+ICI), and the MFI was similar to vehicle treated controls (Fig. 1C). Therefore, both densitometric quantification of Western blots and flow cytometric analysis demonstrated a 1.5- to 1.9-fold increase in Bcl-2 expression. An increase in Bcl-2 mRNA transcription level after E2 treatment (Fig. 1D) suggested Bcl-2 up-regulation through the genomic route. E2 action on cells could possibly occur through either a receptor-mediated or a receptor-independent pathway (40), therefore to gain insights into which receptor involvement, estrogen receptor antagonists, and agonists were used. Presence of a specific estrogen receptor antagonist ICI 182780 during E2 exposure prevented Bcl-2 up-regulation (Fig. 1E, lane 3). Subtype-specific estrogen receptor agonists, namely DPN, a specific agonist for estrogen receptor-β (41) and PPT, a specific agonist for estrogen receptor-α (42) were able to up-regulate Bcl-2 (Fig. 1E, lanes 4 and 5) in the absence of E2. Therefore, data with both agonists and antagonists implicated estrogen receptor involvement. The presence of estrogen receptors was confirmed by flow cytometric analysis of cells stained with anti-estrogen receptor Ab recognizing both estrogen compared with vehicle treated; §, p < 0.05 as compared with E2 treated.
receptors α and β that demonstrated expression of estrogen receptors in >95% of THP-1 macrophages (Fig. 1F).

Taken together, these data established the capability of E2 to induce an estrogen receptor-dependent increase in Bcl-2 mRNA and protein in human THP-1 macrophages.

E2 induces Bax translocation to the mitochondria in THP-1 macrophage

In many cell systems, the Bax to Bcl-2 ratio serve as a control for the balance of pro-apoptotic and anti-apoptotic pathways. In THP-1 macrophages, the Bax to Bcl-2 ratio is elevated upon receipt of apoptotic stimuli (43, 44). In THP-1 macrophages, within 4 h of E2 exposure, translocation of Bax from cytosol to the mitochondria occurred (Fig. 2A, lanes 3 and 4). Presence of the estrogen receptor antagonist ICI 182780 during E2 treatment prevented this translocation of Bax (Fig. 2A, lanes 9 and 10). The estrogen receptors β and α agonists DPN and PPT, respectively, were able to induce Bax translocation in the absence of E2 (Fig. 2A, lanes 5–8). Over a period of 2 h after E2 treatment, there was a clear increase in the expression of mitochondria associated Bcl-2 and Bax (Fig. 2B), but the ratio of the two proteins remained in favor of Bcl-2 (Fig. 2C). Because of the concomitant increase of Bcl-2 levels the cell survival pathway was favored even after Bax translocation to mitochondria. Taken together, data showed that exposure to E2 induced a death response in the macrophages through a estrogen receptor mediated pathway.

Bcl-2 expression is dependent on intracellular Ca2+ concentration

There is a close relationship between Bcl-2 expression and intracellular Ca2+ changes because although Bcl-2 can regulate release of Ca2+ from the endoplasmic reticulum stores (45), Ca2+ is reported to be able to regulate Bcl-2 expression through the ERK signaling pathway (46). Intracellular Ca2+ levels doubled within a minute after addition of E2 (Fig. 3A) and a second peak of Ca2+ increase occurred without any further addition of E2 at around 90 min, and this level of ~90 nM was maintained till 140 min, the time point at which the last measurement was made (Fig. 3A). Presence of the ICI 182780 (Fig. 3A, ICI+E2) could attenuate the increase in Ca2+ (Fig. 3A), indicating that Ca2+ modulation was an estrogen receptor-dependent phenomenon. The source of Ca2+...
that contributed to the intracellular increase was of extracellular origin because presence of EGTA (Fig. 3A, lane 2) did not show any detectable increase in Bcl-2 levels. Therefore, a link between PKC phosphorylation (46) prompted us to check the effect of E2 on ERK. ERK phosphorylation regulates Bcl-2 expression

Prior knowledge on the effects of Ca\(^{2+}\) on ERK phosphorylation (46) prompted us to check the effect of E2 on ERK. ERK phosphorylation occurred within 5 min of E2 exposure (Fig. 4A, lane 2) and was dependent on intracellular Ca\(^{2+}\) levels because verapamil could prevent ERK phosphorylation (Fig. 4A, lane 3). To check upstream events to ERK phosphorylation, BIM, a protein kinase C (PKC) inhibitor, was used at 1 \(\mu\)M concentration (51) and it was able to inhibit ERK phosphorylation (Fig. 4B, lane 4). PD98059, a selective pharmacological antagonist that inhibits MEK-1, which phosphorylates and activates ERK, was able to partially inhibit estrogen-induced Bcl-2 increase (Fig. 4C, lanes 3 and 4) when used at a dose of 25 \(\mu\)M (52). Therefore, a link between PKC pathway, phosphorylation of ERK, and Bcl-2 increase could be established. Downstream to ERK phosphorylation, CREB phosphorylation occurred (Fig. 4D), suggesting that possibly CREB phosphorylation regulates Bcl-2 expression
could mediate ERK-induced effects and that ERK consequently acts as a prosurvival protein inducing Bcl-2 increase. Because along with Bcl-2 increase, there was a concomitant translocation of Bax, it was of interest to see whether the PKC-ERK pathway was involved in Bax translocation. E2-induced Bax translocation (Fig. 4E, lanes 3 and 4) could not be prevented by either verapamil (Fig. 4E, lanes 5 and 6) or PD98059 (Fig. 4E, lanes 7 and 8), showing that Bax translocation was not dependent on Ca$^{2+}$ or ERK phosphorylation. Because the primary function of Bax is to facilitate cytochrome c release into the cytosol from the mitochondria, both mitochondrial and cytosolic fractions of E2-treated and untreated cells in the presence of verapamil and PD98059 were checked. In both cases, at around 12 h, a distinct cytochrome c release into the cytosol was observed that was not visible at 2 h (Fig. 4E). Collectively, these data suggest that E2 mediates increase in Bcl-2 levels via the Ca$^{2+}$-PKC-ERK signaling pathway, but Bax translocation was independent of this signaling cascade.

**E2 does not affect human peripheral blood MDM viability while it up-regulates Bcl-2**

In view of the results obtained with THP-1 macrophages, we used human peripheral blood MDM as a cellular system to validate the effects of E2 in primary cells. Exposure of human peripheral blood MDM to E2 did not induce any loss of viability. The percentage of survival was as follows: vehicle treated, 95 ± 2; 1 nM E2 treatment, 93 ± 3; 10 nM E2 treatment, 95 ± 2; 100 nM E2 treatment, 96 ± 1; and 1 μM E2 treatment, 95 ± 1. Considering our results with THP-1 macrophage, we checked Bcl-2 levels and >1.5 fold up-regulation of Bcl-2 protein was observed after E2 treatment in human peripheral blood MDM (Fig. 5A, lane 2) as compared with constitutive Bcl-2 levels (Fig. 5A, lane 1) and presence of estrogen receptor antagonist ICI 182780 prevented Bcl-2 increase from constitutive levels by 75% (Fig. 5A, lane 3). To further confirm action through estrogen receptors and elucidate the receptor subtypes involved, DPN and PPT were used and both treatments increased Bcl-2 expression (Fig. 5A, lanes 4 and 5) in the absence of E2, suggesting involvement of both estrogen receptors β and α. Also, to directly demonstrate the presence of estrogen receptors in human peripheral blood MDM, immunocytochemistry was performed with an Ab that recognizes both estrogen receptors α and β and analyzed by flow cytometry, which showed that >95% of human peripheral blood MDM express estrogen receptors (Fig. 5B).

**E2 induces THP-1 macrophage cell death in Bcl-2 knockdown conditions**

Because E2 initiated a concomitant translocation of Bax along with Bcl-2 increase, the question was how the cells would behave under Bcl-2 knockdown conditions. For this experiment, several routes of Bcl-2 inhibition in the presence of E2 were exploited. L-type Ca$^{2+}$ channel blocker verapamil, PKC inhibitor BIM, and MEK inhibitor PD98059 were used to reduce Bcl-2 levels and cell fate was followed. Because the described treatments could have global effects affecting other pathways, Bcl-2 inhibition through the use of siRNA against Bcl-2 was used during E2 treatment. Fig. 6, A and B, demonstrates Bcl-2 decrease in the presence of Bcl-2 siRNA in THP-1 macrophages and human peripheral blood MDM, respectively. A 60% down-regulation of constitutive Bcl-2 in THP-1 macrophages (Fig. 6A, second lane) as compared with cells transfected with negative control siRNA (Fig. 6A, first lane) could be achieved. The transfection efficiency was ~95% estimated by fluorescence microscopic analysis of Cy3-labeled negative control siRNA. In E2-treated THP-1 macrophages, Bcl-2 up-regulation could be significantly knocked down with siRNA against Bcl-2 (Fig. 6A, last lane) as compared with E2 treatment in the presence of negative control siRNA (Fig. 6A, third lane). In human peripheral blood MDM, ~30% down-regulation of constitutive Bcl-2 was achieved with Bcl-2 siRNA (Fig. 6B, second lane) as compared with cells treated with negative control siRNA (Fig. 6B, first lane). The transfection efficiency of siRNA was ~95% as detected by observing fluorescence of Cy3-labeled negative control siRNA. As observed in THP-1 macrophages, siRNA against Bcl-2 was able to knockdown E2 induced Bcl-2 up-regulation significantly in human peripheral blood MDM (Fig. 6B, fourth lane) as well as compared with E2 treatment in the presence of negative control siRNA (Fig. 6B, third lane). Analysis of viability in the presence of agents that inhibited Bcl-2 increase, namely verapamil, BIM, or PD98059, during E2 treatment showed a significant increase in cell death as compared with only E2 group (Fig. 6C). When the number of THP-1 macrophages entering the death pathway after estrogen exposure was estimated in a siRNA-mediated Bcl-2 knockdown condition by calculating cells showing phosphatidylserine exposure, nuclear propidium iodide staining, or both, then ~53%, 63%, and 82% of the cells tested positive at 2, 4, and 6 h (Fig. 6D, iv–vi), respectively, as compared with 1% in the absence of estrogen exposure (Fig. 6D, iii). Human peripheral blood MDM behaved similarly,
Flow cytometric analysis of THP-1 viability by simultaneous Annexin V and propidium iodide staining of cells transfected with negative control siRNA treated with (ii) or without (i) E2, Bcl-2 siRNA treated without (iii) or with 10 nM E2 for 2 h (iv), 6 h (v), or in the presence of ICI 182780 (vi). The number of THP-1 viability by simultaneous Annexin V and propidium iodide staining of cells transfected with negative control siRNA treated with (ii) or without (i) E2, Bcl-2 siRNA treated without (iii) or with 10 nM E2 for 2 h (iv), 4 h (v), and 6 h (vi). Error bars are representative of three independent experiments. Error bars are ± SEM with n = 3 experiments. *p < 0.05, first lane vs second; #p < 0.05, first lane vs third; §p < 0.05, third lane vs fourth. C, THP-1 macrophages were preincubated with 20 μM verapamil or 25 μM PD98059 for 10 min or 1 μM BIM for 30 min before treatment with 10 nM E2 for 12 h. Cell death was analyzed by fluorescence microscopy using propidium iodide (1 μg/ml) staining. Data are representative of three independent experiments. Error bars are ± SEM with n = 3 experiments. *p < 0.05 for E2 vs Ver+E2, BIM+E2, and PD+E2. D, Flow cytometric analysis of THP-1 viability by simultaneous Annexin V and propidium iodide staining of cells transfected with negative control siRNA and treated with (ii) or without (i) E2, Bcl-2 siRNA treated without (iii) or with 10 nM E2 for 2 h (iv), 4 h (v), and 6 h (vi). E, Flow cytometric analysis of THP-1 viability by simultaneous Annexin V and propidium iodide staining of cells transfected with negative control siRNA treated with (ii) or without (i) E2, Bcl-2 siRNA treated without (iii) or with 10 nM E2 for 2 h (iv), 6 h (v), or in the presence of ICI 182780 (vi). The y-axis represents propidium iodide labeling and the x-axis represents Annexin V labeling. The percentage shown represents cells analyzed that lie within each quadrant.

FIGURE 6. Inhibition of E2-induced Bcl-2 up-regulation results in cell death. The Bcl-2 siRNA-mediated Bcl-2 knockdown efficiency is shown on Western blots of extracts of THP-1 macrophages (A) or human peripheral blood MDM (B) treated with 10 nM E2 or without E2 and probed with mouse monoclonal anti-Bcl-2 Ab (80 ng/ml). Negative control siRNA was used as a target gene knockdown specificity control. Data represent the relative Bcl-2 expression levels normalized to actin as compared with vehicle treated control, calculated by densitometric analyses of multiple immunoblots as detailed in Materials and Methods. Error bars are ± SEM with n = 3 experiments in duplicate. *, p < 0.05 first lane vs second; #, p < 0.05, first lane vs third; §, p < 0.05, third lane vs fourth. C, THP-1 macrophages were preincubated with 20 μM verapamil or 25 μM PD98059 for 10 min or 1 μM BIM for 30 min before treatment with 10 nM E2 for 12 h. Cell death was analyzed by fluorescence microscopy using propidium iodide (1 μg/ml) staining. Data are representative of three independent experiments. Error bars are ± SEM with n = 3 experiments. *p < 0.05 for E2 vs Ver+E2, BIM+E2, and PD+E2. D, Flow cytometric analysis of THP-1 viability by simultaneous Annexin V and propidium iodide staining of cells transfected with negative control siRNA and treated with (ii) or without (i) E2, Bcl-2 siRNA treated without (iii) or with 10 nM E2 for 2 h (iv), 4 h (v), and 6 h (vi). E, Flow cytometric analysis of THP-1 viability by simultaneous Annexin V and propidium iodide staining of cells transfected with negative control siRNA treated with (ii) or without (i) E2, Bcl-2 siRNA treated without (iii) or with 10 nM E2 for 2 h (iv), 6 h (v), or in the presence of ICI 182780 (vi). The y-axis represents propidium iodide labeling and the x-axis represents Annexin V labeling. The percentage shown represents cells analyzed that lie within each quadrant.

and the number of human peripheral blood MDM entering the death pathway after estrogen exposure was 40% and 57% at 2 and 6 h (Fig. 6E, iv and v), respectively, as compared with 10% in the absence of estrogen exposure (Fig. 6E, iii). ICI 182780 could prevent cell death after E2 exposure (Fig. 6E, vi). This result clearly showed that Bcl-2 knockdown makes the THP-1 macrophages and human peripheral blood MDM susceptible to the death-inducing effects of E2.

Discussion
The ability of macrophages to respond to estrogen plays an incisive role in macrophage function (9–14), and in this study we establish the mechanism by which estrogen regulates the macrophage mitochondrial death pathway. Our report illustrates the function of two Bcl-2 family members with disparate biological properties, namely Bax and Bcl-2 (26) in regulating estrogen-induced effects on macrophage survival. The ability of estrogen to influence macrophages would depend on the presence of functional receptors unless the hormone acts through a receptor independent pathway (40). Because both THP-1 macrophages and human peripheral blood MDM contained functional receptors proven by the ability of estrogen agonists to mimic E2 action on Bcl-2 and Bax and estrogen antagonists to prevent such action, the cells were therefore competent to respond to E2 via receptors. Clearly, the effect of E2 on Bcl-2 was a genomic effect mediated through a PKC-ERK signaling pathway because there was an actual increase in Bcl-2 transcript level. It was unlikely that estrogen responsive elements in bcl-2 gene (53) were directly involved in responding to E2 because interference with the events of Ca2+ influx, PKC activation, and ERK phosphorylation could prevent E2 induced Bcl-2 increase. Changes in cellular Ca2+ induced by any stimuli are an important event for a cell in terms of its survival (54). There is a close relationship between Bcl-2 and Ca2+ because although Ca2+ can mediate Bcl-2 increase, Bcl-2 can also regulate cellular Ca2+ through manipulation of endoplasmic reticulum Ca2+ stores (45). Bcl-2-induced modulation of Ca2+ was excluded by the inability of siRNA-mediated Bcl-2 knockdown to affect E2-induced Ca2+ elevation (data not shown), but experiments with L-type Ca2+ channel blocker that resulted in a reduction of Ca2+ and, consequently, inhibited Bcl-2 increase suggested a situation like hippocampal neurons in which estrogen activates rapid Ca2+ influx (55). Interestingly, there were two peaks of Ca2+ increase...
both of which could be inhibited by verapamil, but although inhibition of the first resulted in inhibition of Bcl-2, lowering of the second peak did not prevent Bcl-2 increase, suggesting that involvement of the second peak of Ca\(^{2+}\) in Bcl-2 expression is unlikely and increased Ca\(^{2+}\) at that point in time could be serving some other purpose.

The augmentation of Bcl-2 levels by E2 in both THP-1 macrophages and human peripheral blood MDM suggested the creation of a favorable condition for the cells to survive. Similar instances of estrogen-induced Bcl-2 up-regulation by \(\sim 1.3\) fold have been demonstrated to protect B cells from BCR-mediated apoptosis (56). Increase in Bcl-2 under estrogen exposure is also observed in neurons (57) and MCF-7 breast cancer cells (58). Although Bcl-2 must ensure cell viability as demanded by certain conditions of stress, cell death is facilitated by translocation of Bax from cytosol to the mitochondria (59). In the mitochondria, Bax could either be overwhelmed by the amount of Bcl-2 present and the equilibrium will shift to Bcl-2 ensuring survival or in low Bcl-2 conditions Bax will prevail and facilitate release of cytochrome c ensuring death (59). As Bax translocation was independent of Ca\(^{2+}\) increase and activation of the PKC pathway, this provided us with an opportunity to investigate Bcl-2 function without interfering with Bax translocation. The crucial role of estrogen induced Bcl-2 increase was obvious from experiments in which L-type Ca\(^{2+}\) channel blocker verapamil restricted Ca\(^{2+}\) influx, and consequently CREB phosphorylation, resulting in an increase in Bcl-2. Translocation of Bax from cytosol to mitochondria is Ca\(^{2+}\) signaling-independent but estrogen receptor-dependent. Interference with Bcl-2 increase through inhibition of Ca\(^{2+}\) (verapamil), PKC (BIM), phosphorylation of ERK (PD98059), and Bcl-2 mRNA degradation via Bcl-2 siRNA leads to cell death in presence of estrogen due to elevated levels of Bax.

The authors have no financial conflict of interest.

Disclosures

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