Chapter 8

Summary
The modulation of macrophage survival and function by estrogen (E2) is of immense importance in the context of regulation of all functions pertaining to immune responses. Previous studies have described the effect of E2 on survival and function of murine and rat macrophages and these studies have been used to extrapolate the findings to human macrophages. However, the effect of E2 is highly species and cell-type specific and therefore to understand E2 action on human cells it is essential to directly investigate the effect of the hormone on macrophages derived from human sources. This thesis is the first description of the effect of E2 on human macrophage survival and the signaling cascades involved in the macrophage apoptotic pathway. For this study, human THP-1 monocytic cells, an acute monocytic leukemia cell line was differentiated to macrophages and used as a model system to study estrogen action. In addition, ex-vivo human peripheral blood monocyte derived macrophages were used to verify findings derived from the model cell line.

Our data clearly demonstrates expression of the two sub-types of E2 receptors ER-α and ER-β in the THP-1 macrophage cell line as well as in human peripheral blood monocyte-derived macrophages. These receptors are differentially distributed in the various sub-cellular compartments, for e.g., ER-α sub-type is expressed on the plasma membrane and the cytoplasm, while the ER-β sub-type is expressed exclusively in the cytoplasm. This distribution pattern is unlike in several other cells where the E2 receptors are localized within the nucleus. The knowledge of the distribution pattern of receptors is important in the context of studying functional effects of E2 and this study demonstrates the importance of such differential distribution in executing E2 function as described below.

E2 induces both a pro-survival and a pro-apoptotic signal in the form of upregulation of Bcl-2 and mitochondrial translocation of Bax respectively in both THP-1 derived macrophages as well as in human peripheral blood monocyte derived macrophages. The E2-induced upregulation of Bcl-2 was mediated through membrane bound ER-α receptors as was evident from the ability of membrane impermeable E2 to stimulate Bcl-2 increase and downregulation of this increase through ER-α siRNA mediated intervention. This ER-α knockdown mediated Bcl-2 inhibition was specific because siRNA against ER-β could not inhibit Bcl-2 increase. The critical role of Bcl-2 upregulation was evident from the dramatic increase in cell death when Bcl-2 elevation was inhibited by using either Bcl-2 or ER-α siRNA thereby allowing the
translocated Bax to carry out the pro-apoptotic function. This demonstrated that though there was an increase in the level of pro-apoptotic Bax in the mitochondria upon exposure to E2, a concomitant upregulation of mitochondria localized Bcl-2 ensured that the net ratio of Bcl-2:Bax remained the same thereby favoring cell survival. The Bax translocation signals were mediated through ER-β because ER-β siRNA interfered with the translocation. The stimulus for change in Bax location coming from E2 was independent of surface receptors because membrane impermeable E2 and ER-α siRNA were ineffective in inducing or abrogating translocation respectively. Therefore, dichotomous effect of E2 mediated through the two ER subtypes, ER-α mediating Bcl-2 increase and ER-β arbitrating Bax translocation was evident. These data taken together demonstrate that there is a fine balance between the levels of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax upon exposure to E2, and the ratio of the two determines cell fate.

The signaling pathways for E2 receptor induced effects on the Bcl-2 and Bax were primarily worked out on THP-1 cells. The E2-induced upregulation of Bcl-2 was transcriptionally regulated and could be inhibited by (a) siRNA for Bcl-2 (b) the L-type Ca\(^{2+}\) channel inhibitor verapamil, (c) PKC inhibitor bisindoleylmaleimide VIII and (d) MEK inhibitor PD 98,059 demonstrating that the upregulation of Bcl-2 was signaled by a process which was dependent upon Ca\(^{2+}\) influx via the L-type Ca\(^{2+}\) channel, with subsequent activation of PKC, phosphorylation of ERK, and activation of CREB. On the contrary, E2-induced activation and mitochondrial translocation of Bax occurred via signaling through intracellular alkalinization mediated by activation of sodium-hydrogen exchangers as demonstrated by the inhibition of Bax translocation by specific sodium-hydrogen exchanger inhibitor amiloride that lowered Na\(^{+}\) levels and prevented alkalinization of the cytoplasm. Inhibition of Bax translocation by preventing intracellular alkalinization rescued the Bcl-2 knockdown cells from death upon exposure to E2. This confirmed that Bax acts as the major pro-apoptotic signal upon exposure to E2.

This thesis also establishes that E2 modulates a number of macrophage functions via an estrogen receptor dependent pathway, for e.g., it enhances the macrophage phagocytic ability, increases nitric oxide generation by activation of iNOS, and inhibits LPS induced secretion of inflammatory cytokines such as IL-1β,
IL-8 and IL-12. Despite the ability of E2 to modulate a number of factors that may affect the outcome of macrophage infection by intracellular pathogens, no significant effect of E2 was observed on the initial infection or clearance of *Leishmania major* parasites *in vitro* or the onset or progression of *L. major* infection *in vivo* in a mouse model of cutaneous leishmaniasis.

Taken together, this study highlights the importance of E2 signaling through distinct ER subtypes in modulating the mitochondrial death pathway of human monocyte derived macrophages and underscores the importance of integrative signaling modality from multiple estrogen receptor pools in modulating E2 responses in human macrophages. The observations raise interesting possibilities of exploring the use of selective estrogen receptor modulators specific for ER-α or ER-β or those which could signal exclusively through the membranous or cytoplasmic pool of receptors to manipulate death pathway in human macrophages. The development and use of such agonists and antagonists could be used to target specific receptor population in target cells to achieve desired therapeutic effects like manipulation of death pathways in favor or against cell survival.
Up-Regulation of Bcl-2 through ERK Phosphorylation Is Associated with Human Macrophage Survival in an Estrogen Microenvironment

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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.


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 prophyllactic 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 proapoptotic 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), oxysterol (28), and activation-inducing agents increase macrophage Bcl-2 or other members of the Bcl-2 family of proteins like BH-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\textsuperscript{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\textsuperscript{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 (cycloexdin encapsulated) was obtained from Sigma-Aldrich. IC\textsubscript{50} 182780, PPT (4,4'-4',4'-4)-Propyl-[1H]-pyrazole-1,3,5-trisphosphol, and DPN (2,3-bis[4-hydroxyphenyl]-propanitrile) 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-cytochrome c, and anti-CtD4 FITC Abs were purchased from BD Biosciences. Secondary Abs raised in either mice or rabbits conjugated to HRP were obtained from Jackson ImmunoResearch Laboratories. Fluor-3-ace	}

- 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.

- 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 PBMC were isolated by density gradient centrifugation using Histopaque 1077, and centrifuged at 400 × g for 35 min at 25°C. The mononuclear cell population was isolated from the plasma-Histopaque interface, and the monocytes were further purified by washing off the nonadherent cells after incubating the total PBMC for 1 h at 37°C. The homogeneity of the obtained population was determined by analyzing immunostaining of the cells with an Ab against monocyte-specific marker CD14-conjugated to FITC. The monocytes were further cultured for 7 days to allow macrophage differentiation. THP-1 cells, a human acute monocytic leukemia cell line obtained from American Type Culture Collection were maintained in RPMI 1640 medium supplemented with 10% FCS. Macrophage differentiation was induced by treatment of THP-1 cells with 10 ng/mL PMA for 36 h, following which cells were maintained in PMA-free medium for 12 h before experimentation. Forty-eight hours before experiment, both cell types were transferred to phenol red-free RPMI 1640 supplemented with 10% dextran-coated charcoal-stripped FCS to remove any extraneous source of estrogen.

- Intracellular free Ca\textsuperscript{2+} assay

Changes in intracellular free Ca\textsuperscript{2+} concentration were monitored using the Ca\textsuperscript{2+}-binding fluorescent probe fluo-3-AM as previously described (36). Briefly, 10\textsuperscript{6}cells/ml loaded with 0.5 \muM fluo-3-AM containing 0.5 \muM pluronic acid F-127 were used for different experiments. and free Ca\textsuperscript{2+} was monitored at an excitation of 480 nm and emission of 520 nm with a Fluorolog (BMG Technologies). Fluorescence values were converted into absolute intracellular free Ca\textsuperscript{2+} concentration, using procedures and calculations as previously described (36).

- Cell viability assay

For cell viability assays, propidium iodide exclusion by cells and identifying phosphatidylserine exposure by Annexin V-labeling was conducted as previously described (37) using Vybrant apoptosis assay kit. Labelling was analyzed by flow cytometry in which data acquisition was done with a HD LSR flow cytometer equipped with a 488 nm air-cooled argon ion laser. Analysis was done using WinMDI software (Microsoft v.2.8).

#### 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'-GTGGAGAGCCTCTCAGGGGA-3', (antisense) 5'-AGGCCACCAAGGTTAGTCAC-3', and actin (sense) 5'-GTGGGGCCCGAGGACCA-3', (antisense) 5'-CTCTTAATGTCAGCCAGATTC-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 Transferr 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\textsuperscript{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 (530–580 nm). Target protein knockdown was assessed 24 h posttransfection by probing extracts of transfected cells on Western biots with anti-Bcl-2 Ab.

- Subcellular fractionation

THP-1 macrophages were harvested and suspended in homogenization 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 (Sonifer 450; Branson) on ice at 30% duty cycle for a total of 9 cycles. The sonicate was centrifuged at 4000 × g for 10 min to obtain the nuclear pellet. From the resulting supernatant, the mitochondrial fraction was extracted by further centrifugation at 10,000 × g for 10 min (38) in an ultracentrifuge (SW20Ti rotor, optimus XL-110K; Beckman Coulter). The postmitochondrial supernatant was further centrifuged at 100,000 × g for 30 min to isolate the microsomal fraction as a pellet and the supernatant obtained was the cytosolic fraction.

- SDS-PAGE and Western blot

Whole cell lysates were prepared by mixing cells with lysis buffer (0.125 M Tris, 4% SDS, 20% glycerol, and 10% 2 ME), and the lysates were resolved on 12% SDS-PAGE gel following which they were transferred onto nitrocellulose membrane as previously described (36). The quantitation of protein in cell lysates was conducted with a CBX protein assay kit (Bio-Rad). Western blots were incubated with 5% ECL blocking reagent in 0.05% PBS-Tween 20 for 1 h to block nonspecific binding sites. Primary and secondary Abs were used at appropriate dilutions and reactivity was visualized by ECL using an ECL Western blotting detection system in which membranes were exposed to x-rays for appropriate time periods and subsequently developed before densitometry.

- 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

Densiometric 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 (v.4.0.0.8; 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.
Bcl-2 0.05 for E2 "E2 ICL estrogen transduction increased levels of estrogen receptors and Bcl-2 expression in THP-1 macrophages."  

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 (800 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 182870. Note the distinct shift obtained with E2 as compared with vehicle treated control (VT) and E2 with ICI 182870 (E2+ICI). CSA, control secondary Ab; US, unstained cells. Data shown at bottom are the MFI of the indicated treatment groups and the fold change in Bcl-2 expression (n = 3), r, p < 0.05 for vehicle treated vs E2 treated; #, p < 0.05 for E2 vs E2 + ICI. D. RT-PCR analysis for the expression of Bcl-2 at 12 h after treatment with E2 (10 nM) (E2) showing increase in Bcl-2 amplification (304 bp) with cDNA isolated from THP-1 macrophages (lane 2) as compared with vehicle treated (VT) control (lane 1). Actin RT-PCR served as loading control. E. Immunoblots for Bcl-2 on THP-1 extracts at 24 h after the following treatments: lane 1, vehicle treated (VT); lane 2, 10 nM E2 treatment; lane 3, pretreatment with 1 μM ICI 182870 before E2 treatment; lanes 4 and 5, treatment with 100 nM DPN, an estrogen receptor β-specific agonist; 100 nM PPT, an estrogen receptor α-specific agonist. Data shown below immunoblots represent the 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. r, 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 recognizing 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 at the following: vehicle treated, 94 ± 1; 1 nM E2 treatment, 95 ± 2; 10 nM E2 treatment, 96 ± 1; 100 nM E2 treatment, 96 ± 1; and 1 μM 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 10 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 182870 prevented a shift in fluorophore labeling (Fig. 1C) E2–ICI), and the MFI was similar to vehicle treated control (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 transcript 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 182870 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. F. Estrogen receptor expression was analyzed by flow cytometry with THP-1 macrophages immunostained with anti-estrogen receptor Ab recognizing both estrogen receptors α and β. The marker represents the percentage population that stain positive for the receptors. CSA, control secondary Ab.
FIGURE 2. Estrogen treatment induces translocation of Bax from cytosol to mitochondria in THP-1 macrophages. A, Immunoblot showing Bax translocation performed on cytosolic and mitochondrial fractions obtained from 4 h lysates of THP-1 macrophage exposed to different treatments as indicated. The purity of the mitochondrial fraction was determined by probing for cytochrome c (Cyt C) using an anti-cytochrome c Ab (500 ng/ml). Cyt c, cytosolic fraction; m, mitochondrial fraction. B, Time-kinetic analysis of Bcl-2 up-regulation and Bax translocation through immunoblotting of cytosolic and mitochondrial fractions of THP-1 macrophages with 20 nM E2 (80 ng/ml) and anti-Bax Abs (900 ng/ml). Silver-stained gel (top) shows profiles of mitochondrial and cytosolic fractions with equal amounts of protein loaded. Immunoblot analysis (bottom three panels) is shown of Bcl-2, Bax, and cytochrome c in cytosol and mitochondrial fraction of cells after 10 nM E2 treatment collected at indicated time points. C, Data represents absolute values of Bcl-2 and Bax and their ratio at various time intervals after E2 treatment as determined by densitometric analysis of specific immunoreactive bands on blots, representatives of which are shown in B. Data are representative of three independent experiments.

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 point upstream of irreversible damage to cellular constituents where Bax translocation to the mitochondria from cytosol occur 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 an 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-1-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, E2 + EGTA) during estradiol treatment prevented both Ca"²⁺ peaks (Fig. 3A). To determine the route of Ca"²⁺ influx, we used voltage gated Ca"²⁺ channel blockers like pimozide for T-type channels (47) and verapamil for L-type channels (48) and Na⁺/Ca²⁺ exchange blockers like benzamil (49) and bepridil (50). Pimozide, bepridil or benzamil could not prevent Ca"²⁺ influx (data not shown), but verapamil was able to reduce both Ca"²⁺ peaks when added at two different time points, one before addition of E2 (Fig. 3A, V1) and another just before the second increase of Ca"²⁺ (Fig. 3A, V2), suggesting the involvement of L-type Ca²⁺ channels. Microscopically, a clear increase in labeling with Ca²⁺ binding fluorescent dyes rhod-2-AM was evident in the E2 treatment group (Fig. 3B, iv) as compared with vehicle treated controls (Fig. 3B, ii). Cells treated with E2 in the presence of ICI 182780 (Fig. 3B, vi) and verapamil (Fig. 3B, viii) did not show any detectable increase in Ca²⁺ levels in the cells, the constitutive levels of Ca²⁺ being non-detectable by fluorescence microscopy. To establish whether this increase in Ca²⁺ was related to Bel-2 expression, verapamil was used to prevent Ca²⁺ entry after E2 treatment. Verapamil added before initiation of E2 treatment (Fig. 3C, V1 + E2) inhibited Bel-2 increase significantly as compared with that induced by E2 alone (Fig. 3C, E2). However, addition of verapamil just before the second peak of Ca²⁺ (Fig. 3C, V2 + E2) could not produce statistically significant inhibition of Bel-2 levels. Collectively, these experiment established that E2 was able to induce a biphasic Ca²⁺ increase through L-type Ca²⁺ channels and the first peak of Ca²⁺ was linked to Bel-2 increase.

**ERK phosphorylation regulates Bel-2 expression**

Prior knowledge on the effect of Ca²⁺ 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 Ca²⁺ 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 μM 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 Bel-2 increase (Fig. 4C, lane 3 and 4) when used at a dose of 25 μM (52). Therefore, a link between PKC pathway, phosphorylation of ERK, and Bel-2 increase could be established. Downstream to ERK phosphorylation, CREB phosphorylation occurred (Fig. 4D), suggesting that possibly CREB
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 Ca2+ 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 Ca2+-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 macrophages, 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 Ca2+ 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. 6A 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) 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, first 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, nuclearpropidium 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.
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.

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 an target gene knockdown specificity control. Data represent the relative Bcl-2 expression levels normalized to actin as compared with vehicle treated control, calculated by densiometric 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 analyses of THP-1 viability by simultaneous Annexin V and propidium iodide staining of cells transfected with negative control siRNA and treated with (i) or without (ii) 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 (i) or without (ii) E2. Bcl-2 siRNA treated without (iii) or with 10 nM E2 for 2 h (iv), 4 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 shows cells labeled that lie within each quadrant.
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 MDKM suggested the creation of a favorable condition for the cells to survive. Similar instances of estrogen-induced Bcl-2 up-regulation by −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 the consequent inhibition of the PKC pathway prevented ERK phosphorylation resulting in Bcl-2 decrease but did not inhibit Bax translocation as a result of which cell death ensued. Although the above data confirmed the importance of the altered Bax/Bcl-2 level for survival, both PKC and ERK activity could involve other pathways as well. Therefore, unequivocally prove the involvement of Bcl-2 in macrophage survival at the time of estrogen exposure, we used Bcl-2 siRNA to knockdown Bcl-2 levels during E2 treatment, and our studies establish that Bcl-2 up-regulation is an absolute requirement for macrophage survival in the presence of estrogen. The reduced number of late apoptotic cells observed in THP-1 macrophages as compared with human peripheral blood MDM could be attributed to higher expression of antiapoptotic proteins in THP-1 (60) due to its leukemic nature.

Clearly, E2 shows two distinct set of effects. Although increase in Ca^{2+} leads to ERK phosphorylation resulting in an increase in Bcl-2, which is an antiapoptotic signal, a distinct proapoptotic signal in the form of translocation of Bax from cytosol to mitochondria was generated by the Ca^{2+} signaling-independent but estrogen receptor-dependent pathway (Fig. 7). Therefore, in macrophages, estrogen shows a dichotomous effect and depending on other factors that could influence Bax or Bcl-2 proteins in a given circumstance, a survival or a death pathway would be chosen. How might our findings be integrated into a model of macrophage behavior in high estrogen microenvironments? From a pathological point of view, there are two possible scenarios in which this estrogen mediated cell survival could be of relevance. Optimal, macrophage survival at tumor or inflammatory sites during chemotherapy is essential during the orderly process of cell death to restrict harm to host tissues (61). Our report provides a new insight into macrophage function in response to estrogen where the hormone is able to generate a cytoprotective response in macrophages through manipulation of the Bcl-2 proteins. This function will be crucial under conditions of Bcl-2 down-regulation (31–35) when macrophage population will be depleted if Bcl-2 knockdown is attempted in tumors of estrogen targets. On the contrary, in many tumors, tumor-associated macrophages play a protumorigenic role by secreting growth factors and angiogenic factors and current antitumor strategy includes targeted destruction of these cells in which case macrophage killing through Bcl-2 down-regulation would be beneficial for tumor therapy. From a physiological point of view, estrogen-induced Bcl-2 up-regulation is obligatory for human macrophage survival and hence necessary for the manifestation of the immunomodulatory effects of estrogen on macrophages.

Disclosures
The authors have no financial conflict of interest.

References


Oestrogen modulates human macrophage apoptosis via differential signalling through oestrogen receptor-α and β

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Abstract

Human macrophages express oestrogen receptors and are therefore competent to respond to the hormone present in their microenvironment, which is implicated in sexual dimorphism observed in several immune and autoimmune phenomena. An earlier study from this laboratory demonstrated 17β-oestradiol (E2) induced apoptosis in macrophages derived from human peripheral blood monocytes and THP-1 acute monocytic leukaemia cell line, when Bcl-2 was down-regulated; however, the involvement of E2 receptor subtypes in the modulation of death pathways in these cells remain unknown. Using macrophages derived from THP-1 human acute monocytic leukaemia cells as a model, we demonstrate that plasma membrane associated oestrogen receptor (ER) α participate in E2 induced Bcl-2 increase, through activation of the mean arterial pressure-kinase (MAPK) pathway whereas cytosolic ER-β transmits signals for the pro-apoptotic event of Bax translocation. The mechanistic basis of Bax translocation comprised of ER-β mediated increase in intracellular pH, facilitated by activation of the Na+/H+ exchanger. Intracellular alkalinization accompanied by concomitant Bcl-2 increase and Bax migration does not cause cellular apoptosis; however, siRNA mediated down-regulation of ER-α during E2 exposure leads to inhibition of Bcl-2 increase and consequently apoptosis due to the unopposed action of mitochondrial Bax. In summary, this study underscores the importance of integrative signalling modality from multiple oestrogen receptor pools in modulating oestrogen effects on human monocyte-derived macrophage apoptotic signalling pathway, which opens new vistas to explore the use of selective oestrogen receptor modulators in apoptosis-based therapies.

Keywords: oestrogen • macrophage • oestrogen receptor • Bcl-2 • Bax • apoptosis • alkalinization

Introduction

Macrophages derived from the differentiation of monocytes express steroid hormone receptors and are therefore sensitive to the hormones present in their microenvironment. The study of steroid hormone action on modulation of human macrophage function is of significant interest because these versatile cells are involved in the regulation of immune response and consequently are relevant to pathogenesis of many diseases. The ovarian steroid oestrogen is able to exert pleiotropic effects on macrophages, including modulation of the death pathway, for example, it exerts paradoxical effects on human U937 macrophages where cell death is induced by oestrogen [1], but the same hormone accords protection to these cells from TNF-α induced apoptosis [2]. Similar effect is exerted on murine osteoclasts, where oestrogen exposure leads to caspase-dependent apoptosis [3, 4]. A previous study from this laboratory demonstrated 17β-oestradiol (E2) induced apoptosis in macrophages derived from human peripheral blood monocytes and THP-1 acute monocytic leukaemia cell line, when Bcl-2 was down-regulated [5]. It is well established that the survival of a cell in response to certain apoptotic stimuli depends on the critical ratio of mitochondrial Bcl-2 and Bax. Consequent to sensing of apoptotic stimuli, Bax, which exists as an inactive monomer in the cell cytoplasm, migrates to the mitochondria to interact with the existing mitochondrial Bcl-2 and the resulting interaction determines the fate of the cell, higher or lower ratio of Bcl-2/Bax being anti-apoptotic or pro-apoptotic [6], respectively.

For the change of location of Bax, a conformational alteration occurs in the Bax protein, leading to exposure of the mitochondrial targeting sequence resulting in its translocation to the outer mitochondrial membrane. Multiple mechanisms have been proposed as instrumental in initiating Bax translocation, which include neutralization of several pro-survival proteins by BH3 only members of the Bcl-2 family such as Noxa and Puma [7].
fluxes [22], Elicitation of a particular event in response to E2 could be dependent on the relative concentrations of the two receptors on effects elicited by oestrogen because signalling through separate subtypes could have diverse outcome [15]. This is evident from the distinct phenotypes obtained with ER-α and ER-β [16], and in addition, evidence for differential receptor activity come from studies showing overlapping but exclusive sets of downstream target genes for the two subtypes [17]. The classical model of ER action is where ligand bound ER interacts with oestrogen response elements in target genes and initiates transcription by modulating co-repressors and co-activators. Conversely, ERs can also interact with other transcription factors like activating protein-1 and stimulating protein-1 to initiate transcription [18, 19]. In addition to the above mode of actions, oestrogen may elicit effects through genomic or non-genomic mechanisms by binding to oestrogen receptors localized on the plasma membrane of target cells [20] and activate mean arterial pressure-kinase (MAPK) signalling [21] or induce intracellular Ca\(^{2+}\) fluxes [22]. Elicitation of a particular event in response to E2 could be dependent on the relative concentrations of the two ER subtypes, for example, U937 monocytes expressing mostly ER-β are sensitive to oestrogen induced apoptosis; however, after differentiation to macrophages, receptor population expressed is predominantly ER-α, as a result of which the apoptosis inducing effect of oestrogen becomes ineffective [23].

The purpose of this study was to investigate the involvement of the two ER subtypes in mediating apoptosis associated events in human macrophages using THP-1 monocyte derived macrophages as a model system. We show that THP-1 human macrophage survival is compromised in the presence of E2 if ER-α but not ER-β receptor levels are down-regulated. This is because E2 signalling via ER-α mediates the anti-apoptotic event of Bcl-2 up-regulation, whereas ER-β signals for the pro-apoptotic event of Bax translocation to the mitochondria via Na\(^{+}\)-H\(^{+}\) exchanger mediated intracellular alkalinization.

**Materials and methods**

**Cell lines and culture**

THP-1, a human acute myelocytic leukaemia cell line, and MCF-7, a human breast carcinoma cell line (ATCC, Manassas, VA, USA), were maintained in RPMI-1640 (Biological Industries, Kibbutz Beit Haemek, Israel), supplemented with 10% FCS (Biological Industries, Kibbutz Beit Haemek, Israel). Differentiation of THP-1 monocytes to macrophages was induced by treatment with 10 ng/mL PMA for 36 hrs. Forty eight hours prior to experiment, the cells were transferred to phenol-red free RPMI supplemented with 10% dextran-coated charcoal stripped FCS to remove all extraneous sources of oestrogen.

**Reagents**

E2 (cyclodextrin encapsulated), E2 conjugated to BSA (E2-BSA), E2-BSA conjugated to FITC (E2-BSA-FITC), PD98.059, nigericin, amiloride and propidium iodide (PI) were obtained from Sigma-Aldrich (St. Louis, MO, USA). ICI 182.780 was obtained from Tocris Cookson (Bristol, UK). Negative control siRNA was purchased from Ambion (Austin, TX, USA), whereas Bcl-2, ER-α and ER-β siRNAs were obtained from Dharmacon (Lafayette, CO, USA). The siRNA transfection reagent Transpass R2 was purchased from New England Biolabs (Ipswich, MA, USA). All reagents for Western blotting and ECL development were from Amersham Biosciences (Piscataway, NJ, USA). SNARF (5-(and-6)-carboxy SNARF\(^{5\text{-AM}}\)-AM), Sodium Green™ tetra-acetate, secondary antiamouse IgG conjugated to Alexa fluor 488 and Hoechst 33342 nuclear dye were purchased from Molecular Probes (Eugene, OR, USA). Antibodies for oestrogen receptor α/β, oestrogen receptor-α and actin were procured from Calbiochem (Darmstadt, Germany). whereas antibodies against phospho-ERK and whole-ERK were from StressGen Biotechnologies (Victoria, BC, Canada). Anti-Bcl-2, anti-Bax and anti-cytochrome c antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GAPDH antibody was from Ambion (Austin, TX, USA), whereas anti-histone dimethyl lysine antibody was purchased from Upstate (VA, USA). Secondary antiamouse and anti-rabbit IgG conjugated to horseradish peroxidase were procured from Jackson Immunoresearch (Cambridgeshire, UK). The Vybrant apoptosis detection system was purchased from Promega (Madison, WI, USA). Anti-clusterin antibody was a kind gift from Dr. C. Yan Cheng of the Population Council, NY, USA. All other chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless mentioned otherwise.

**Intracellular pH measurement**

Intracellular pH measurement was performed with the long-wavelength fluorescent pH indicator carboxy SNARF-1 AM following manufacturer's protocol. Briefly, the cells (10\(^{6}\)/ml) were resuspended in serum-free RPMI and incubated with a final concentration of 1 μM SNARF-1 AM, diluted from a stock solution of 1 mM in DMSO for 15 min. at room temperature. Cells were washed and incubated for 20 min. at room temperature for complete deestimation of AM esters. In situ calibration of SNARF-1 AM was performed with the ionophore nigericin at 10 μM concentration in a high-K\(^{+}\) buffer to equilibrate intracellular pH with that of the controlled extracellular medium. Appropriate groups were subjected to different treatments, and fluorescence measurements were commenced in a spectrofluorometer (Perkin Elmer, Waltham, MA, USA), followed by kinetic analysis. The pH was calculated from the fluorescence measurements using the following formula:

\[
\text{pH} = pK_a - \log \left[ \frac{(R - R_0)/(R_0 - R)}{(F_0/R_0^2)/(F/R^2)} \right]
\]

where pKa of carboxy SNARF-1 AM is 7.5. R is the ratio of fluorescent intensities (F) measured at two emission wavelengths, 580 (λ1) and 640 nm (λ2), with fixed excitation at 514 nm. The subscripts A and B represent the limiting values at the acidic and basic end-points of the titration, respectively. Na\(^{+}\)-free and HCO\(_3\)\(^{-}\)-free buffer were prepared as described by Khaled et al. [24].
Intracellular Na⁺ measurement

For intracellular Na⁺ measurement, cells (10⁶/mL) were labelled for 20 min. at room temperature with the cell permeable fluorescent Na⁺ indicator Sodium Green™ tetra-acetate, diluted to 1 μM in RPMI 1640 from a 5 mM stock solution made in DMSO. After washing the cells to remove excess probe, kinetic fluorescent measurements were carried out with a spectrofluorometer at an excitation of 480 nm and emission of 520 nm (BMG Fluostar Optima, BMG technologies, Offenburg, Germany). In situ Calibration was accomplished by using the indicator in solutions of precisely known free Na⁺ concentration in the presence of the pore forming antibiotic gramicidin (10 μM). Intracellular Na⁺ was calculated using the following equation:

\[ [\text{Na}^+]_\text{intr} = K_D (F - F_{\text{free}}/F_{\text{max}} - P), \]

where \( K_D \) of the dye is 5.7 mM at 37°C. \( F \) is the fluorescence of the experimental sample. \( F_{\text{free}} \) is fluorescence in the absence of Na⁺ and \( F_{\text{max}} \) is fluorescence in the presence of saturating concentrations of Na⁺.

siRNA transfection

THP-1 macrophages were transfected with specific siRNAs using Transpass R2 transfection reagent as described previously [5]. Briefly, Bcl-2 siRNA (15 pmol), ER-α and ER-β siRNA (100 pmol) or negative control siRNA (pre-designed siRNA with no known target genes) at similar concentrations were added to transfection reagent TranspassR2, diluted in serum-free medium, and incubated for 20 min. to allow the formation of transfection complexes. The formed complexes were added to 10⁵ cells/well grown in 24-well plates and incubated for 6 hrs, 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 Nikon G2A filter cube. The percentage cell death was calculated as the number of cells with PI positive nuclei as against the total number of cells. Annexin-V-PI staining was performed as described previously [5], and cell death was assessed 24 hrs after transfection by probing extracts of transfected cells with an antibody recognizing Bcl-2 and anti ER-α/β antibody.

Subcellular fractionation

THP-1 macrophages were allowed to swell for 10 min. in hypotonic buffer (10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 7.5) followed by homogenization with a Dounce homogenizer (50 strokes). Immediately after cell lysis, the mitochondria were stabilized by addition of mitochondrial stabilization buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, pH 7.5), and the homogenate was centrifuged at 13000 × g for 15 min. to isolate the nuclear fraction. The post-nuclear supernatant was further centrifuged at 17,000 × g for 15 min. in an ultra-centrifuge (Optima XL-100K, Beckman) to isolate the mitochondria. The post-mitochondrial supernatant was centrifuged at 100,000 × g for 1 hr to obtain the membranous fraction as a pellet and the supernatant as the cytosol. The homogeneity of the obtained fractions was determined by Western blotting with probes specific for each fraction.

Cell viability assay

To assess cell viability, PI dye exclusion assay was performed by incubating the cells with 1 μg/ml PI for 5 min. at 37°C, followed by one wash with ice-cold PBS. The cells were analysed under a Nikon TE2000-E fluorescence microscope using Nikon G2A filter cube. The percentage cell death was calculated as the number of cells with PI positive nuclei as against the total number of cells. Annexin-V-PI staining was performed as described previously [5], and data acquisition was performed on a BD-LSR flow cytometer equipped with a 488 nm air-cooled argon ion laser. The acquired data was analysed using WinMDI software (Microsoft v.2.9).

Reverse transcription-polymerase chain reaction

Total RNA was isolated using TRIzol reagent (GIBCO, CA, USA) and cDNA was synthesized as described previously [5]. The specific primers used were ER-α (sense) GTGGGATAGTGAAGTGGG; ER-α (antisense) TCCAGAGCTCAAGGGCTC; ER-β (sense) TGCTCAGAGCTCAAGGG; ER-β (antisense) TGCTCAGAGCTCAAGGGATCT-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.

Immunocytochemistry

Cells were fixed with 4% formaldehyde for 20 min., followed by several washes with ice-cold PBS. Saponin (0.1%) was used for cell permeabilization, and 3% normal goat serum was used as a blocking reagent to reduce non-specific binding. The permeabilized cells were incubated with primary antibody recognizing ER-α/β (1 : 100) followed by incubation with secondary antimouse IgG conjugated to Alexa fluor 488 (1 : 200). For live-cell staining, all incubations were performed at 4°C with anti-ER-α antibody in addition to reagents as described above. For nuclear labelling of cells, Hoechst 33342 was used. All stainings were visualized using a Nikon TE2000-E fluorescence microscope using appropriate filter blocks, and the image acquisition was carried out with a high-resolution Retiga Excalibur (Q-imaging, Surrey, BC, Canada), the mask of co-localization was created using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

SDS-PAGE and Western blot

Whole cell extracts were prepared by treating the cells with lysis buffer (0.125 M Tris, 4% SDS, 30% glycerol and 10% 2-ME), and protein estimation was performed with CBX protein assay kit (G-Biosciences, St. Louis, MO, USA). Lysates were resolved on 12% SDS-PAGE gel, following which they were transferred onto nitrocellulose membrane as described before [5]. Non-specific binding sites were blocked by incubating the blots in 5% non-fat skimmed milk with 0.05% PBS-Tween 20 for 1 hr. Primary (1 : 5000) and secondary antibody (1 : 10,000) incubations were carried out for 1 hr each, and immunoreactivity was visualized by enhanced chemiluminescence using ECL reagent, as described previously [5].

Densitometry and statistical analysis

Quantitative assessment of reaction intensity in Western blots was performed using a UVP gel-documentation instrument, and the data were...
analysed with the LabWorks image analysis and acquisition software (v4.0.0.8, UVP, Upland, CA, USA). 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. Data are expressed as mean ± standard error (SE) unless mentioned. Comparisons were made between different groups using unpaired Student's t-test. The values were considered to be significantly different at P < 0.05. All analysis was performed on data acquired from three or more independent experiments.

Results

Human macrophages express both oestrogen receptor-α and β at multiple subcellular locations

E2 initiates cellular signalling pathways via interaction with its receptors expressed primarily as two subtypes – the ER-α and ER-β [25] – found in the nucleus, plasma membrane or cytosol, the distribution varying with different cell types [26]. Human macrophages are known to express both ER-α and ER-β [27, 28], however, the subcellular localization of these receptors is not known. In the current study, membrane bound E2 receptors were detected on viable differentiated THP-1 macrophages by labelling live cells at 4°C with membrane impermeable E2-BSA linked to FITC (E2-BSA-FITC), and flow cytomeric analysis showed an obvious shift in fluorescence labelling intensity in these cells compared with those labelled with only BSA-FITC used as a control (Fig. 1A). Decreased fluorescence readings obtained with cells incubated with E2-BSA-FITC in the presence of un conjugated E2 compared with cells exposed to only E2-BSA-FITC confirmed specificity of this binding (Fig. 1A).

Further analysis of presence of ERs in different subcellular locations in these cells showed surface labelling of ERs on live cells (Fig. 1B, a, i-iv) with an anti-E2 receptor antibody raised against common epitopes on ER-α and ER-β receptor proteins, thus corroborating the above data obtained with E2-BSA-FITC.

Fig. 1 Human macrophages express oestrogen receptor-α and β on the plasma membrane and cytoplasm. (A) The histogram represents flow-cytometric analysis of live THP-1 macrophages incubated with E2 conjugated to BSA-FITC (red line) or co-incubation with different concentrations of E2 (blue, 10 nM E2 and pink, 100 nM E2 lines) under similar conditions. The area shaded grey represents unlabelled cells, and the black line represents cells labelled with only BSA-FITC. Note the distinct shift in staining of the cells treated with E2-BSA-FITC demonstrating recognition of cell surface localization of ERs. (B) Indirect immunofluorescence on live as well as formaldehyde-fixed cells stained with anti-ER-α/β antibody. (a) THP-1 cells incubated with E2-BSA-FITC and BSA-FITC control. (b) THP-1 fixed; (c) secondary antibody control; (d) fixed MCF-7 cells – (i), Nomarski image; (ii), ER-α/β staining; (iii), nuclear staining with Hoechst 33342; (iv), overlap of (iii) and (ii). The MCF-7 cells (d, i-iv) used as positive controls show presence of nuclear receptors. The bar represents 10 μm. All data are representative of at least three independent experiments. (C) Western blots of subcellular fractions of THP-1 cells probed with anti-ER-α-β antibody showing presence of both forms in the cytoplasm (b, Cyto, lane 2), predominantly ER-α in membrane fraction (b, Memb, lane 1) and absence of receptors in nuclear fraction (b, Nuc, lane 3). Western blot for histone H1 and GAPDH was performed to assess the homogeneity of the obtained nuclear and cytoplasmic fractions, respectively.

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Antibody (Fig. 1 B, d, i–iv). Cytosolic ERs dimerize and migrate to the nucleus (Fig. 1 B, b, i–iv). Figure 1 B, c, i–iv shows absence of fluorescence in secondary antibody controls. MCF-7 cells, where E2 receptors are predominantly nuclear [29], were used as positive controls and showed distinct nuclear staining with the same antibody (Fig. 1 B, d, i–iv). Cytosolic ERs dimerize and migrate to the nuclei upon ligand engagement [18]; this phenomenon was used as a control for cytosolic receptors, and Figure S1 shows that upon E2 exposure the cytosolic ERs migrate to the nuclei (E2, b and d) compared with control (Control, b and d) and E2-BSA treatment (E2-BSA, b and d). Measurement of the mask of colocalization clearly shows significant colocalization of ER receptor staining with nuclear staining after E2 treatment (E2, e) compared with vehicle treated controls (Control, e) and after E2-BSA treatment (E2-BSA, e).

To determine the subtype specific distribution of receptors, Western blots of subcellular fractions of THP-1 cells were probed with the same antibody as above, and it recognized both receptor types in the cellular cytosolic fraction (Fig. 1 C, b, lane 2) whereas the membranous fraction primarily showed reactivity for ER-α subtype, the expression of ER-β being low (Fig. 1 C, b, lane 1). The nuclear fraction did not show any reactivity, suggesting the absence of nuclear oestrogen receptors in this particular cell type (Fig. 1 C, b, lane 3). Total extract of MCF-7 cells known to express both ER-α and ER-β was used as positive control (Fig. 1 C, a).

Collectively, the above data demonstrated the presence of ERs on the cell surface as well as in the cytosol of THP-1 monocyte derived macrophages, nucleus being devoid of such receptors. Both ER-α and ER-β are present intracellularly, whereas the plasma membrane appears to be primarily populated by ER-α.

E2 modulates Bcl-2 and Bax via different sets of receptors in THP-1 cells

To investigate if the receptors at different subcellular locations transmit similar or different signals for modulation of the mitochondrial apoptotic pathway, both membrane permeable and impermeable E2 were used. E2-BSA, where E2 is conjugated to BSA through a six atom hydrocarbon tether [30] restricting its diffusion through the plasma membrane, was used to distinguish signals originating from membrane bound receptors only [20, 30].

Equivalent increase in Bcl-2 levels was obtained with membrane permeable E2 as well as membrane impermeable E2-BSA at 6 hrs (Figs 2A, lanes 2 and 4). Pure E2 receptor antagonist ICI 182,780 inhibited this increase (Fig. 2A, lanes 3 and 5), confirming that in both cases, E2 receptors were involved. In contrast to its ability to modulate Bcl-2, E2-BSA was unable to exert any effect on subcellular localization of Bax (Fig. 2B, lanes 5 and 6), whereas unconjugated E2 was able to stimulate Bax translocation (Fig. 2B, lanes 3 and 4). The inability of E2-BSA to induce migration of Bax from cytosol to the mitochondria unlike free E2 suggested that Bax migration was independent of membrane receptor mediated signalling.

Based on the above data, attempt was made to identify receptor subtypes involved in mediating the above responses. ER-α and ER-β mRNA and protein were selectively down-regulated with siRNA for test the effect of this down-regulation on Bcl-2 and Bax expression. Equivalent increase in Bcl-2 levels was obtained with membrane permeable E2 as well as membrane impermeable E2-BSA at 6 hrs (Figs 2A, lanes 2 and 4). Pure E2 receptor antagonist ICI 182,780 inhibited this increase (Fig. 2A, lanes 3 and 5), confirming that in both cases, E2 receptors were involved. In contrast to its ability to modulate Bcl-2, E2-BSA was unable to exert any effect on subcellular localization of Bax (Fig. 2B, lanes 5 and 6), whereas unconjugated E2 was able to stimulate Bax translocation (Fig. 2B, lanes 3 and 4). The inability of E2-BSA to induce migration of Bax from cytosol to the mitochondria unlike free E2 suggested that Bax migration was independent of membrane receptor mediated signalling. 

Intracellular receptors were detected by staining fixed and permeabilized cells with the same antibody that demonstrated the presence of receptors within the cytosol and the membrane but not in the nucleus (Fig. 1b, i–iv). Figure 1b, c, i–iv shows absence of fluorescence in secondary antibody controls. MCF-7 cells, where E2 receptors are predominantly nuclear [29], were used as positive controls and showed distinct nuclear staining with the same antibody (Fig. 1b, d, i–iv). Cytosolic ERs dimerize and migrate to the nuclei upon ligand engagement [18]; this phenomenon was used as a control for cytosolic receptors, and Figure S1 shows that upon E2 exposure the cytosolic ERs migrate to the nuclei (E2, b and d) compared with control (Control, b and d) and E2-BSA treatment (E2-BSA, b and d). Measurement of the mask of colocalization clearly shows significant colocalization of ER receptor staining with nuclear staining after E2 treatment (E2, e) compared with vehicle treated controls (Control, e) and after E2-BSA treatment (E2-BSA, e).
A

B

C

D

E

F

G

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no decrease with ER-β siRNA (Fig. 3A, lane 3). Figure 3B shows the RT-PCR for ER-β mRNA performed with ER-β specific primers, where lane 3 shows down-regulation of ER-β mRNA levels with ER-β siRNA but not with ER-α siRNA (lane 2). Negative control siRNA did not show any interference with RNA levels of either receptors (Fig. 3A and B, lanes 1). Status of protein levels of ER-α and ER-β after ER-α siRNA (Fig. 3C, lane 3) and ER-β siRNA (Fig. 3C, lane 2) transfection shows that protein levels of both receptors were significantly down-regulated with respective siRNA treatment. A distinct reduction of surface receptor population was observed with siRNA for ER-α (Fig. 3D, b, i-iii) compared with a negative control siRNA (Fig. 3D, a, i-iii), as visualized by immunostaining of treated and untreated cells with a specific anti-ER-α antibody. Next, the effects of ER-α and ER-β down-regulation on Bcl-2 and Bax modulation were examined. E2-BSA was able to increase Bcl-2 in the presence of negative control siRNA (Fig. 3E, lane 2) but not when the cells were transfected with ER-α siRNA (Fig. 3E, lane 4), thus linking the involvement of ER-α in Bcl-2 increase. This treatment with ER-α siRNA, therefore, creates a condition within the cell, which is pro-apoptotic in nature because presence of E2 will induce Bax translocation, and in the absence of Bcl-2, Bax will induce apoptosis. In contrast, the knockdown of ER-β during E2-BSA exposure did not affect Bcl-2 increase (Fig. 3E, lane 6), showing the absence of any effect of membrane associated ER-β on Bcl-2 levels. Knockdown of ER-β (Fig. 3F, lanes 3 and 4) but not of ER-α (Fig. 3F, lanes 5 and 6) in the presence of E2 resulted in inhibition of Bax migration, thereby creating an anti-apoptotic condition because Bcl-2 will not have to counteract the effect of translocated Bax to the mitochondria. The translocation was complete in cells transfected with negative control siRNA (Fig. 3F, lanes 1 and 2). Together, the above data demonstrate a dichotomous effect of E2 on the components of the mitochondrial cell death pathway mediated through the two ER subtypes, ER-α mediating Bcl-2 increase and ER-β arbitrating Bax translocation.

Arguably, the modulation of the two receptor levels leading to changes in the pro-apoptotic Bax and anti-apoptotic Bcl-2 would affect cell survival. Annexin-V-PI staining showed that ER-β knockdown in the presence of E2 did not induce any apoptotic death (Fig. 3G, ii), whereas ER-α knockdown resulted in about 40% late apoptotic cells and 17% early apoptotic cells at 6 hrs after E2 exposure (Fig. 3G, iii). This corroborated our findings that ER-α but not ER-β was involved with the survival pathway, and interference with this pathway resulted in increased cell death in the presence of E2.

Bcl-2 modulation is mediated through ERK phosphorylation whereas Bax translocation is dependent upon intracellular alkalinization

Downstream to ER engagement by E2-BSA and E2, inhibition of ERK phosphorylation by MEK inhibitor PD 98,059 resulted in abrogation of Bcl-2 up-regulation (Fig. 4A, lane 5). Furthermore, E2-BSA was able to induce phosphorylation of ERK in as early as 10 min. (Fig. 4B, lane 4), which could be inhibited by ICI 182,780 (Fig. 4B, lane 5). Therefore, this indicated the competence of the membrane associated ERs to transmit Bcl-2 up-regulation signals. Following the observation that cytosolic ER-β was involved in Bax translocation, the mechanisms that lead to this change in subcellular localization was investigated. Clusterin
and cytosolic pH statuses were checked, as these are known to mediate Bax translocation [10, 11, 12]. E2 exposure did not suppress the expression of secretory form of clusterin in THP-1 cells (Fig. 5A), which is the pro-survival form, thereby ruling out its involvement in E2-induced Bax translocation as opposed to earlier reports in fibrosarcoma and prostate cancer cells, where suppression of clusterin was shown to induce Bax translocation [10]. Also, the level of pro-apoptotic nuclear clusterin remained unaffected (Fig. 5A).

In other cellular systems like B cells and thymocytes, it has been demonstrated that Bax translocation could occur in response to a pH change [11, 12, 31]. As shown in Figure 5B, E2 treatment resulted in an increase in intracellular pH from a basal level of 7.5 to about 7.7-7.8, but this increase was inhibited by the E2 receptor antagonist ICI 182,780 (Fig. 5B). Involvement of membrane-associated receptors was ruled out, as E2-BSA was unable to effect any change in pH. When pH change was prevented by placing the cells in high-K⁺ buffer of desired pH in the presence of nigericin, an activator of K⁺/H⁺ antiporter, E2 was not able to induce Bax translocation (Fig. 5C, lane 3 and 4). On the contrary, the translocation was complete when cells were maintained at a pH of 7.7 (Fig. 5C, lane 5 and 6), suggesting that E2-induced intracellular alkalinization acted as a signal for Bax translocation. In contrast, E2-induced Bcl-2 up-regulation was unaffected by pH alterations (Fig. 5C). To ascertain if translocation of Bax could occur whenever there was a pH change independent of other stimuli, an increase in intracellular pH was induced by nigericin treatment in high-K⁺ buffer in the absence of E2, and a change of pH to 7.7 resulted in Bax translocation but not when pH was maintained at 7.5 (Fig. 5D, lanes 5, 6 and 3, 4, respectively). These data point out that an increase in intracellular pH was sufficient to induce the translocation of Bax, independent of other signalling pathways that might be activated by E2. Also, siRNA mediated down-regulation of ER-β prevented E2 induced pH change (Fig. 5E), providing evidence that signals generated through ER-β was capable of altering the pH and also supports that data presented above that signals for Bax translocation is mediated through ER-β.

Because intracellular pH is maintained by the co-ordinated activity of a number of ion channels and their respective ions, the most important of which are the Na⁺–H⁺ exchangers (NHE) and the Na⁺- dependent transporters, involvement of each of these elements in the E2-induced increase in intracellular pH was investigated. NHE functions in the maintenance of intracellular pH by pumping out intracellular H⁺ for extracellular Na⁺, and hence its activity is indicated by both an increase in intracellular Na⁺ and alkalinization of the cytoplasm due to expulsion of H⁺ ions. When THP-1 macrophages were suspended in a Na⁺-free media, E2 was unable to induce a pH
Fig. 5 Bax translocation is dependent on E2-induced change in intracellular pH mediated via ER-β. (A) Western blot analysis of clusterin expression after E2 treatment indicates no change in the expression of the different isoform levels. Pre-sClu: precursor to secretory clusterin; nClu: nuclear clusterin; sClu: secretory clusterin. (B) Intracellular pH measurement was performed with the pH sensitive dye SNARF-1 AM. The graph represents the calculated intracellular pH of THP-1 macrophages exposed to various drug treatments as indicated over a time-course of 40 min. Note that although E2 increased the intracellular pH, this was prevented in the presence of antioestrogen ICI182,780 but was unable to alter intracellular pH. ICI: ICI182,780. (C) Appropriate groups of THP-1 macrophages were resuspended in high-K+ buffer of pH 7.5 or 7.7. The control group was left untreated whereas the other two groups were treated with 1 μM nigericin and 10 nM E2 for 6 hrs. The cytosolic (C) and mitochondrial (M) fractions of the appropriate groups were probed for Bax and Bcl-2 expression by immunoblotting. Western blotting for cytochrome c was performed to determine the homogeneity of the obtained fractions. Note the lack of Bax migration in cells maintained at basal pH (lanes 3 and 4). Cyt C: cytochrome c. (D) Appropriate groups of THP-1 macrophages were resuspended in high-K+ buffer of pH 7.5 or 7.7 and permeabilized with 1 μM nigericin to maintain the intracellular pH the same as that of the extracellular medium. Lysates of cytosolic (C) and mitochondrial (M) fractions were probed for Bax and cytochrome c by Western blotting. Note the migration of Bax at pH 7.7. Cyt C: cytochrome c. (E) Intracellular pH measurement in cells transfected with negative control siRNA and ER-β siRNA following treatment with 10 nM E2. Note the inhibition of increase in pH with knockdown of ER-β. Neg Con si: negative control siRNA; ER-β si: ER-β siRNA.
change indicating that influx of Na\(^+\) was essential for alkalinization (Fig. 6A). HCO\(_3\)\(^-\) was not required for E2-induced intracellular alkalinization because E2 was able to induce a pH change in a HCO\(_3\)\(^-\) free media (Fig. 6B). A possible role for NHE in mediating cellular alkalinization was indicated by an increase in intracellular Na\(^+\) in response to E2 as observed by an increase in Sodium Green™ fluorescence (Fig. 6C). This was further confirmed when amiloride, an NHE inhibitor lowered Na\(^+\) levels and also prevented alkalinization of the cytoplasm (Fig. 6C and D). If amiloride could prevent intracellular alkalinization, ideally then amiloride should be able to prevent translocation of Bax if pH increase and Bax translocation were linked. Western blots of cytosolic and mitochondrial fraction obtained from cells treated with E2 in the presence of amiloride showed absence of Bax translocation to mitochondria (Fig. 6E, lanes 5 and 6). Cyt C: cytochrome c. (F) Cell viability. THP-1 macrophages were transfected with negative control siRNA or Bcl-2 siRNA, and 24 hrs post-transfection cells were subjected to appropriate treatments with E2 as indicated. All cells were resuspended in high-K\(^+\) buffer and appropriate groups, where intracellular pH was to be maintained at 7.5 or 7.7, were treated with 1 \(\mu\)M nigericin. E2 treatment was given for 6 hrs and viability was analysed by propidium iodide dye exclusion method performed with fluorescence microscopy. The bar graph represents the percentage cell death in the various treatment groups. \(^*\) \(P < 0.05\), compared with cells transfected with negative control siRNA and treated with E2. \(^\#\) \(P < 0.05\) compared with Bcl-2 siRNA transfected cells treated with E2. Neg Con si: negative control siRNA; sibcl: Bcl-2 siRNA.

Fig. 6 Oestrogen increases intracellular pH by activation of Na\(^+\)-H\(^+\) exchanger. THP-1 macrophages were resuspended in Na\(^+\)-free buffer (A) or HCO\(_3\)\(^-\)-free buffer (B), and intracellular pH was measured using SNARF-1 AM dye after treatment with 10 nM E2 over a time period of 40 min. (C) Intracellular Na\(^+\) was monitored in THP-1 macrophages using the fluorescent dye Sodium Green™ that shows a distinct increase in Na\(^+\) in response to E2. Amiloride, a Na\(^+\)-H\(^+\) exchanger inhibitor, was used at a concentration of 2 \(\mu\)M 10 min prior to E2 treatment, which was able to inhibit the increase in intracellular Na\(^+\) levels. (D) Intracellular pH measurement in THP-1 macrophages exposed to E2 and pre-incubated with or without the Na\(^+\)-H\(^+\) exchanger inhibitor amiloride (2 \(\mu\)M) shows an abrogation of E2 induced increase in pH in the presence of amiloride. (E) Lysates of cytosolic (C) and mitochondrial (M) fractions prepared from the above groups after 6 hrs of incubation, and probed for Bax and cytochrome c on Western blots show the absence of Bax translocation to the mitochondria in the presence of amiloride (lanes 5 and 6). Cyt C: cytochrome c. (F) Cell viability. THP-1 macrophages were transfected with negative control siRNA or Bcl-2 siRNA, and 24 hrs post-transfection cells were subjected to appropriate treatments with E2 as indicated. All cells were resuspended in high-K\(^+\) buffer and appropriate groups, where intracellular pH was to be maintained at 7.5 or 7.7, were treated with 1 \(\mu\)M nigericin. E2 treatment was given for 6 hrs and viability was analysed by propidium iodide dye exclusion method performed with fluorescence microscopy. The bar graph represents the percentage cell death in the various treatment groups. \(^*\) \(P < 0.05\), compared with cells transfected with negative control siRNA and treated with E2. \(^\#\) \(P < 0.05\) compared with Bcl-2 siRNA transfected cells treated with E2. Neg Con si: negative control siRNA; sibcl: Bcl-2 siRNA.
To confirm that alkalinization-induced change in Bax translocation is the major pro-apoptotic event induced by E2, Bcl-2 knockdown macrophages were treated with E2, a situation that normally precipitates cell death (Fig. 6F, siBcl + E2), but when these cells are maintained at pH 7.5, the apoptosis inducing effect of E2 was abrogated (Fig. 6F, siBcl + E2, pH 7.5).

Discussion

Insights into the role of oestrogen in macrophage survival and associated mechanisms are of great relevance because the findings would have direct bearing on the development of tumour targeting therapies [32]. Our earlier study showed that E2 was able to induce apoptosis in human macrophages when Bcl-2 was down-regulated [5]. The work described in this manuscript explores the involvement of E2 receptor subtypes localized in distinct subcellular compartments in the regulation of mitochondrial death pathway in human THP-1 macrophages. We demonstrate that (i) signals for Bcl-2 increase is primarily mediated through membrane associated ER-α, (ii) the translocation of Bax to mitochondria is mediated via signalling through intracellular ER-β receptors and (iii) the E2-induced Bax translocation is dependent on intracellular alkalinization mediated through activation of Na⁺/H⁺ exchangers.

Ratio of Bcl-2/Bax is crucial in maintaining cell viability under certain conditions, and therefore the relative involvement of the ERs in regulating this ratio is important to examine. Recognition of ER-α binding sites on live cells by specific antibody and knockdown of surface ER-α by siRNA for ER-α clearly confirmed the presence of surface localized ER-α in THP-1 cells, which is in agreement with a growing body of evidence showing the presence of membrane associated ERs in various cell types [33, 34]. Interestingly, the surface located ER-α emerges as the major transducer of survival signal during E2 treatment, as demonstrated by the ability of E2-BSA, the membrane impermeable form of E2, to up-regulate Bcl-2, as well as abrogation of this effect upon siRNA mediated knockdown of surface localized ER-α resulting in cell death. Although we show that membrane associated ER-α is sufficient to transduce the survival signal, the relative contribution of cytosolic ER-α in the survival response could not be determined due to the non-availability of specific inhibitors to intracellular versus the membrane receptors. The anti-apoptotic role of ER-α as noted in our studies is in concurrence with other reports that implicate ER-α in mediating E2 induced protective role during H₂O₂ induced apoptosis in murine skeletal muscle C2C12 cells [35] or in human osteosarcoma cell line [36]. The downstream events after engagement of E2 on membrane associated ER-α involved the activation of MAPK pathway for an induction of Bcl-2 increase because E2-BSA was competent to phosphorylate ERK, and MEK inhibition could inhibit Bcl-2 up-regulation. The functional role of surface receptors in mediating survival signals was of interest because surface receptors are amenable to selective manipulation by cell impermeable agonists, providing opportunities to exploit the surface receptors for induction or inhibition of specific cellular functions.

In contrast to E2-induced Bcl-2 increase, Bax translocation was independent of membrane bound receptors because E2-BSA, which interacted with surface receptors, was unable to induce Bax migration. Because knockdown of ER-β but not of ER-α resulted in abrogation of Bax translocation, it indicated the importance of ER-β in death inducing arm of the mitochondrial apoptotic pathway. This particular function has not been demonstrated in cells of monocytic origin, but mediation of pro-apoptotic events by ER-β is known in cells of non-myeloid lineage like breast and colon cancer cells [37, 38]. As Bax translocation is the primary event that initiates changes pertaining to cell death, the mechanism of translocation consequent to ER-β mediated signalling by E2 is of importance. It is known that translocation of Bax to the mitochondria is linked to alteration in its conformation resulting in the exposure of its N-terminal or BH-3 domain [39, 40], which is under the control of various physiological factors, possibly of different natures [41]. Prior knowledge that a change in pH could trigger Bax movement [11, 12, 31] prompted us to focus on the possibility of E2 inducing a pH change in THP-1 cells. Because Bax translocation could be initiated upon intracellular alkalinization in the presence or in the absence of E2, movement of Bax was likely to be facilitated by any stimulus capable of altering cellular pH. Importantly, the consequence of Bcl-2/Bax ratio changes would affect cell survival, and a substantial decrease in cell death was observed after E2-induced pH change was blocked at the time of Bcl-2 knockdown, presumably due to lack of Bax translocation to the mitochondria, thus validating the observation that Bax migration to mitochondria due to pH change in the absence of concomitant Bcl-2 up-regulation is responsible for increased apoptosis.

Therefore, intracellular alkalinization was an important event, and this appeared to be mediated by NHE because the process was Na⁺/H⁺ dependent and could be inhibited by amiloride, a NHE inhibitor. A number of studies show that E2 can alter NHE functions [42] through a NHE regulatory factor (NHE-RF), which is a primary response gene under ER control [43]. However, the rapid increase in pH in response to E2 observed in our system makes transcriptional regulation through NHE-RF unlikely. The mechanism of NHE involvement remains unknown.

In summary, this study highlights the importance of oestrogen signalling through distinct ER subtypes in modulating the mitochondrial death pathway of human monocyte derived macrophages. The observations raise interesting possibilities of exploring the use of selective oestrogen receptor modulators specific for ER-α or ER-β or those which could signal exclusively through the membranous or cytoplasmic pool of receptors to manipulate death pathway in human macrophages. For example, estren, which is an oestrogen agonist signalling selectively on the membranous ER with no known transcriptional
effects via the classical ER mechanism [44], could be used for generating anti-apoptotic effects. The development and use of such agonists and antagonists could be utilized to target specific receptor population in target cells to achieve desired therapeutic effects like manipulation of death pathways in favour or against cell survival.

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Supporting Information

Additional Supporting information may be found in the online version of this article.

Fig. S1 Translocation of oestrogen receptors into the nucleus upon E2 treatment. Sub-cellular distribution of ERs consequent to treatment with 10 nM E2 or E2-BSA for 2 h is shown by immunofluorescence using an antibody, which recognizes both ER-alpha and ER-beta. The panel stained green represents the ER-alpha staining (b), the blue staining represents nuclear staining with Hoechst 33342 nuclear dye (c). The merge of images in panel b and c is shown in panel d. The panel "coloc mask" (e) represents the area within the cell showing colocalization of estrogen receptors with the nucleus. The value within the panel "coloc mask" represents the coefficient of colocalization of ER- alpha staining with the nuclear staining. Panel a represents phase contrast images. The bar represents 10 μm.

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