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Knockdown of receptor for advanced glycation end products attenuate 17α-ethinyl-estradiol dependent proliferation and survival of MCF-7 breast cancer cells

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ABSTRACT

Background: 17α-ethinyl-estradiol (17α-EE), a synthetic estrogen is the world's most widely and commonly used orally bioactive estrogen. Currently, 17α-EE is in use in all formulations of contraceptive pills and is implicated in the complication of breast cancer. Receptor for advanced glycation end products (RAGE) is a cell surface immunoglobulin class of molecule. RAGE is involved in the complication of various cancers.
Methods and results: This study indicates that treatment of MCF-7 breast cancer cells with 17α-EE enhances the expression of estrogen receptor related receptor gamma (ERRγ), followed by enhanced level of oxidative stress and subsequent activation of the transcription factor, nuclear factor kappa-B (NF-κB), leading to increase in RAGE expression. RAGE thus expressed by 17α-EE treatment causes further enhancement of the oxidative stress which, in turn, activates expression of cell cycle protein cyclin D1 and subsequent induction of MCF-7 breast cancer cell proliferation. RAGE also enhanced phosphorylation of prosurvival protein AKT and increased expression of Bcl_2, an antiapoptotic protein.
Conclusion: In MCF-7 breast cancer cells, 17α-EE-ERRγ interaction induces the expression of RAGE, which in turn, enhances the number of MCF-7 breast cancer cells through a multiprong action on the divergent molecules like cyclin D1, AKT and Bcl_2.
General significance: This is the first report which explains the intermediate role of ERRγ in the 17α-EE dependent RAGE expression in MCF-7 breast cancer cells. This report for the first time explains that RAGE is important not only for MCF-7 breast cancer cell proliferation but also for its survival and anti-apoptotic activities.

1. Introduction

Estrogens are a group of female steroid hormones, having a role in a wide range of physiological processes like development of female sex organs, maintaining bone density and maintenance of sexual reproduction [1]. Currently, estrogens are in use as constituent of contraceptive pills [2], and in post menopausal hormone replacement therapy (HRT) [3]. 17alpha-ethinyl-estradiol (17α-EE), a synthetic estrogen is the world's most commonly used bioactive estrogen which is in use in all formulations of oral contraceptive pills [4]. In spite of having a pivotal role in normal physiology of females, estrogens are implicated in the various types of female cancers including breast cancer [5]. Estrogen receptors (ERs) [6], and estrogen receptor related receptors (ERRs), a nuclear hormone receptor superfamily [7], are involved in estrogen dependent breast cancer complication and drug resistance [8].

Recently we have shown that in endothelial cells, interaction of 17α-EE with ERα induces the expression of receptor for advanced glycation end products (RAGE) [9]. RAGE is a cell surface immunoglobulin class of molecule [10]. RAGE interacts with divergent ligands including advanced glycation end products (AGE) [11] and S100 or calgranulin [12,13]. Thus, RAGE is a multiligand receptor molecule. RAGE is involved in proinflammatory and prooxidative reactions [13]. One recent study indicates that interaction of RAGE with S100 induces the proliferation of MCF-7 breast cancer cells [14]. However, the specific mechanism involved in the RAGE dependent MCF-7 breast cancer cells proliferation is not known. More importantly, it is not known whether 17α-EE is equally potent to induce the expression of RAGE in MCF-7 breast cancer cells as observed in endothelial cells [9]. The importance of 17α-EE generated RAGE on MCF-7 breast cancer cells proliferation and the specific mechanism involved needs to be evaluated. Moreover, the role of RAGE on AKT phosphorylation and Bcl_2 expression is not known. Of note, while AKT is a prosurvival protein [15], Bcl_2 attenuates apoptosis [16].

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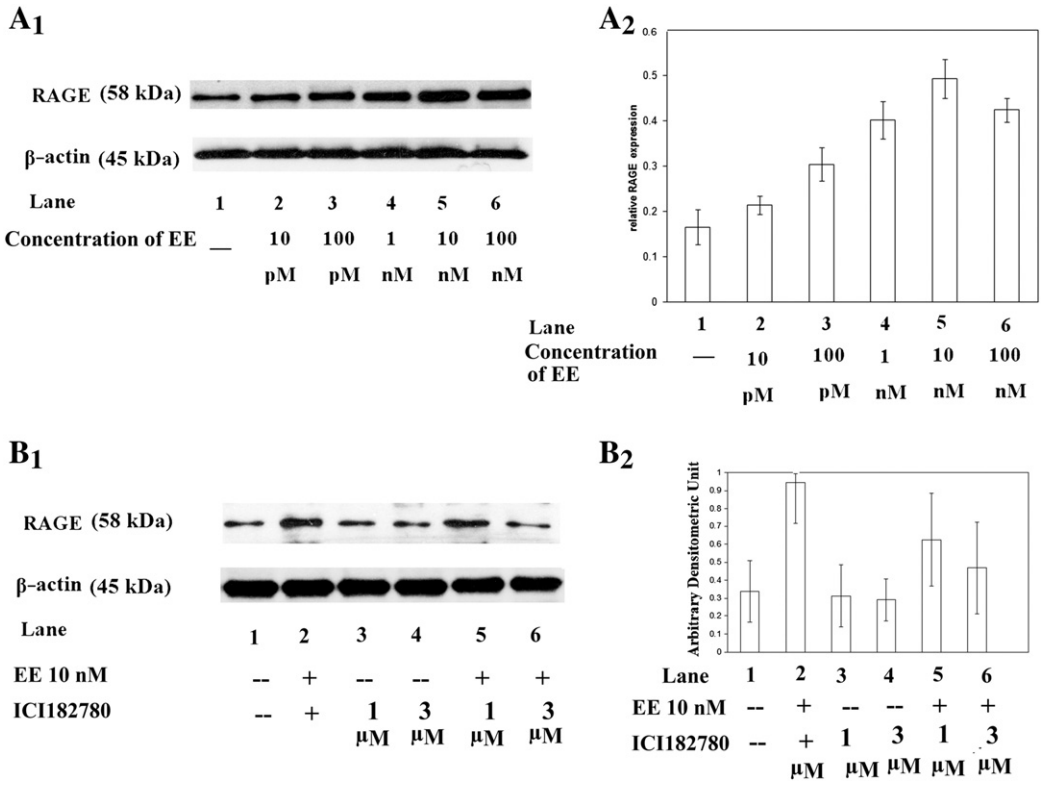

Fig. 1. A: Representative Western blot analysis showing the effect of various concentrations of 17α-EE on the expression of RAGE in MCF-7 cells: MCF-7 breast cancer cells were incubated with 17α-EE for 12 hrs. 50 μg of proteins (total cell lysate) were loaded into each lane. A 1: RAGE (top) and β-actin (bottom, internal control) were analyzed using specific monoclonal antibodies. Lane 1, untreated control; Lanes 2–4, treatment with various concentrations of 17α-EE. A 2: Densitometric plot of the above experiments. Maximum intensity of the RAGE expression was noticed when MCF-7 breast cancer cells were treated with 10 nM of 17α-EE alone for 12 hrs (lane 4). B: Representative Western blot analysis showing the effect of preincubation of ICI182780 on the 17α-EE dependent expression of RAGE in the MCF-7 cells: MCF-7 cells were incubated with ICI182780 for 6 hrs prior to treatment with 10 nM concentration of 17α-EE for 12 hrs. B 1: RAGE (top) and β-actin (bottom, internal control) were analyzed using specific monoclonal antibodies. Lane 1, untreated control; Lane 2, treatment with 10 nM concentration of 17α-EE alone for 12 hrs; Lane 3-4 incubation of MCF-7 cells with 1 μM and 3 μM of ICI182780 alone for 2 hrs; Lane 5–6 incubation of MCF-7 cells with 1 μM and 3 μM of ICI182780 for 6 hrs prior to 10 nM of 17α-EE for 12 hrs. B 2. Densitometric plot of the above experiment.

Since in a number of recent observations ERRs are implicated in antiestrogen resistance in ER positive breast cancer cells [8], the role of ERRs in 17α-EE dependent RAGE expression needs to be evaluated.

The present study, therefore, was undertaken to understand the role of 17α-EE in the expression of RAGE in the ER positive MCF-7 breast cancer cells and to evaluate the role of ERRs in this mechanism. The specific mechanism through which 17α-EE generated RAGE may enhance the number of MCF-7 breast cancer cells, was another important area of observations. In this study, we have shown that 17α-EE induces RAGE expression in the MCF-7 breast cancer cells through the activation of ERRγ. RAGE is responsible for 17α-EE mediated MCF-7 breast cancer cells survival and proliferation by activating cyclin D1, AKT and Bcl_2. We have also shown that reactive oxygen species (ROS) play a very important intermediate role in the RAGE dependent MCF-7 breast cancer cells proliferation and survival.

2. Materials and methods

Minimum essential medium (MEM), estrogens (17β-estradiol, 17α-ethinyl estradiol and 17α-epiestriol), N-acetyl cysteine (NAC), sRAGE (Santa Cruz Biotechnology), siRNA for RAGE, ERα, ERβ, ERRγ, MTT cell proliferation assay kit, antibodies for cyclin D1, Bcl_2, anti-IκBα, and HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Sigma. ICI182780 was obtained from Tocris Cookson Ltd. Antibodies for RAGE, ERα, ERβ, NF-κB, P-IκBα were purchased from Millipore Corporation Ltd. Catalase and AKT (phospho S473) were procured from Calbiochem and Abcam respectively. Glutathione peroxidase 4 (GPx-4) and ERRγ antibodies were purchased from Abcam.

2.1. Culture of MCF-7 breast cancer cells in minimum essential medium

MCF-7 breast cancer cells (human) were procured from ATCC and maintained in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS), 1 mM L-glutamine, 0.01 mg/ml insulin, 1 mM sodium pyruvate, 2.2 g/ml sodium bicarbonate, 1% antibiotic and antimycotic at 37 °C and 5% CO_2. For experiments cells were grown up to 70–75% confluency.

2.2. Treatment of MCF-7 breast cancer cells with various drugs of interest

Prior to treatment, MCF-7 breast cancer cells were washed twice with PBS and pre-incubated for 12 hrs with experimental medium containing MEM without phenol red, 2% charcoal-dextran treated FBS, 2.2 g/ml sodium bicarbonate and 1% antibiotic/antimycotic. After 12 hrs of preincubation with the experimental medium, MCF-7 breast cancer cells were treated with various experimental drugs. For treatment, MCF-7 breast cancer cells were treated with various concentrations of 17α-EE for 6 hrs. Since estrogens have a half life of only around 3 hrs [17], fresh 17α-EE was added to the cells without changing the medium and treated for another 6 hrs. This 12 hrs (6 hrs + 6 hrs) treatment time for 17α-EE was maintained for all the experiments in this study. The specific time point and treatment conditions for other drugs are discussed in their results section.

2.3. Determination of MCF-7 breast cancer cell proliferation by MTT cell proliferation assay

MCF-7 breast cancer cells were grown in 96 well plates and treated with various drugs of interest as described in Results and discussion section. Following treatment, 10 μl of MTT solution from a freshly prepared stock (5 mg/ml) was added to 90 μl of culture medium per well and incubated at 37 °C for 4–5 hrs. Following incubation, the MTT containing medium was replaced with 100 μl solvent in each well, incubated at 37 °C for 15 min with constant shaking. The absorbance was measured on an ELISA plate reader with a wavelength of 570 nm.

2.4. Transient siRNA transfection of the MCF-7 breast cancer cells

MCF-7 breast cancer cells were grown up to 60–70% confluency in 6 well plates. N-Ter nano particle based siRNA transfection reagent (Sigma) was used for transfection. Before transfection the plates were washed with phenol red free and antibiotic/antimycotic free MEM containing 2% charcoal-dextran treated FBS and incubated for 30 min at 37 °C. After incubation, MCF-7 breast cancer cells were subjected to transfection with various siRNA according to the standard protocol of the manufacturer.

2.5. Preparation of cytoplasmic and nuclear extracts of the MCF-7 breast cancer cells

Cytosolic extracts (CE) and nuclear extracts (NE) were isolated as described previously with modification. [18]. Very briefly, MCF-7 breast cancer cells were isolated by cell scraper, washed with ice cold PBS and resuspended with 100 μl ice cold CE buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.3 mM sucrose, 0.1 mM EGTA, protease inhibitor cocktail, incubated on ice for 10–15 min. 3 μl of 10% NP-40 was added, vigorously vortexed for 10–15 sec and centrifuged at 13000 rpm, 4 °C for 30 sec. The supernatant obtained was CE and pellet was nuclear material. The nuclear material was washed with ice cold CE buffer without detergent for two times at 13,000 rpm, 4 °C for 1 min. The pellet was resuspended in ice cold 100 μl of NE buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitor cocktail, rocked gently for 30 min, centrifuged at 13000 rpm, 4 °C for 30 min. The supernatant obtained was NE. Protein measurement was done by Bradford assay. 50 μg of protein was used for Western blot analysis.

2.6. Preparation of total cell lysate of the MCF-7 breast cancer cells and Western blot analysis

For Western blot analysis, MCF-7 breast cancer cells were grown in 100 mm petriplates and 17α-EE treatment was done on 70–75% confluent cells as mentioned above. The Western blot was performed as described previously [18]. Very briefly, after the treatment, MCF-7 breast cancer cells were trypsinized, pelleted, washed with ice cold PBS and resuspended in 100 μl of lysis buffer containing 5 mM HEPES (pH 7.5), 1 mM DTT, 150 mM NaCl, 1 mM EDTA, 0.5% tween-20, 10% glycerol, and protease inhibitor cocktail (Sigma). After 1 hr, the cell lysate was centrifuged, supernatant was collected and protein concentration was measured by Bradford reagent. 50 μg of protein was subjected to SDS-PAGE and separated proteins were transferred to PVDF membrane (BIORAD) by semi-dry transfer apparatus (BIORAD), membranes were treated with primary and secondary antibodies, and developed by ECL developing reagents (Amersham).

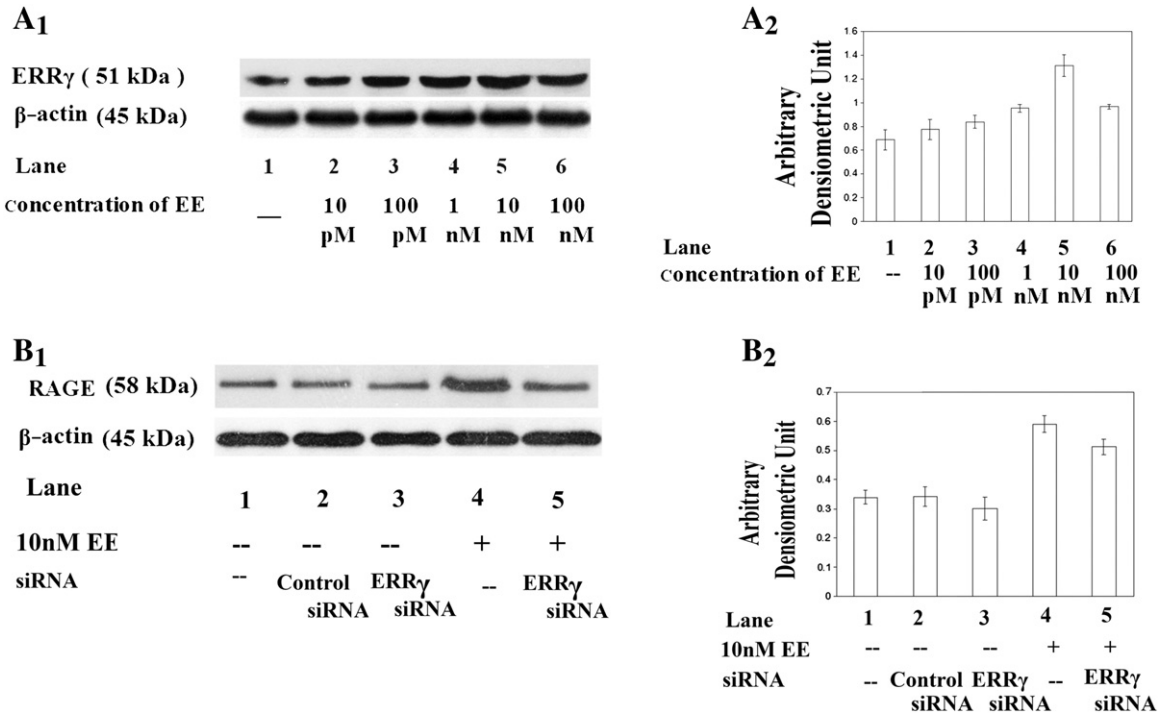

Fig. 2. A: Representative Western blot analysis and its corresponding densitometric plot showing the effect of 17α-EE on the expression of ERRγ in the MCF-7 cells. A 1: ERRγ (top) and β-actin (bottom, internal control) were analyzed using specific monoclonal antibodies. Lane 1, untreated control; Lanes 2–4, treatment with various concentrations of 17α-EE for 12 hrs. A 2. Densitometric plot of the above experiment. B: Representative Western blot analysis showing the effect of preincubation of ERRγ siRNA on the 17α-EE dependent RAGE expression in the MCF-7 cells. B 1: RAGE (top) and β-actin (bottom, internal control) were analyzed using specific monoclonal antibodies. Lane 1, untreated control; Lane 2, control siRNA; Lane 3, treatment with ERRγ siRNA alone for 6 hrs; Lane 4, treatment with 10 nM 17α-EE for 12 hrs; Lane 5, treatment with ERRγ siRNA before 10 nM 17α-EE treatment. B 2. Densitometric plot of the above experiment.

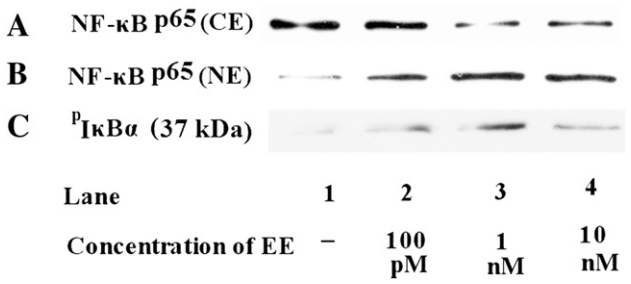

Fig. 3. Representative Western blot analysis showing the effect of 17α-EE on the translocation of NF-κB-p65 and phosphorylation of IκBα in the MCF-7 cells. Figures are in the following order: A. NF-κB-p65 in cytosolic extracts (CE). B. NF-κB-p65 in nuclear extracts (NE). C. Phosphorylated IκBα in total cell lysate. Lane 1, untreated control; Lanes 2–4, treatment with various concentrations of 17α-EE.

2.7. Determination of the generation of reactive oxygen species by 2′,7′ dichloro-fluorescence di-acetate

The level of ROS generated by MCF-7 breast cancer cells was measured as described previously [19]. Very briefly, to measure the level of ROS generation, MCF-7 breast cancer cells were treated overnight with the experimental medium as described previously. Next day, treatment with 10 nM of 17α-EE was done at various time points from 15 min to 12 hrs. After completion of the treatment with 17α-EE, the medium was aspirated, MCF-7 breast cancer cells were washed and placed in Hanks balanced salt solution (HBSS) at 37 °C incubator for 15 min. 2′,7′-dichlorofluorescin diacetate (DCFDA) solution was prepared fresh by dissolving in 100% DMSO. The MCF-7 cells were incubated in the dark with 50 μM DCFDA (Invitrogen) for 15 minutes prior to the measurement of the level of ROS. Following incubation, MCF-7 breast cancer cells were scraped and re-suspended in 2 ml of HBSS and ROS were measured using a fluorescence spectroflourimeter (Perkin Elmer) at 534 nm emission with an excitation wavelength of 488 nm.

3. Results and discussion

Receptor for advanced glycation end products (RAGE) is a cell surface immunoglobulin class of molecule [10]. RAGE binds with various ligands and therefore RAGE is recognized as a multiligand receptor molecule [11,12]. Interaction of RAGE with its ligands namely advanced glycation end products (AGE) [11] and S100 or calgranulin [12] might enhance the proinflammatory and prooxidative reactions of the cells [11,13]. Engagement of RAGE with its various ligands namely S100 complicates breast cancer [14,20]. Based on these observations, it is assumed that RAGE might be involved in the complication of breast cancer, although the exact mechanism is not known. Indeed, the use of RAGE deficient mice ($RAGE^{-/-}$) in well established mouse models of inflammation-associated carcinogenesis such as chemically induced carcinogenesis [21], and colitis associated cancer [22] provided direct genetic evidence for a novel role of RAGE in cancer.

It is now generally accepted that estrogens can complicate breast cancer [5]. Estrogens have major roles in cancer cell proliferation in ER positive breast cancer cells [23], and ERRs are held responsible for tamoxifen (an antagonist of ERs) resistance [8]. Previously, we have shown that in endothelial cells, 17α-EE and other estrogens stimulate the expression of RAGE through the activation of ERα [9]. However, it is not known whether 17α-EE is equally potent to stimulate RAGE expression in various cancer cells as observed in endothelial cells. Moreover, the role of ERRs (if any) in this mechanism is not known. Since 17α-EE is the most widely used orally bioactive estrogen, it is imperative to check the role of 17α-EE-ERs and 17α-EE-ERRs interactions in the expression of RAGE in ER positive breast cancer cells.

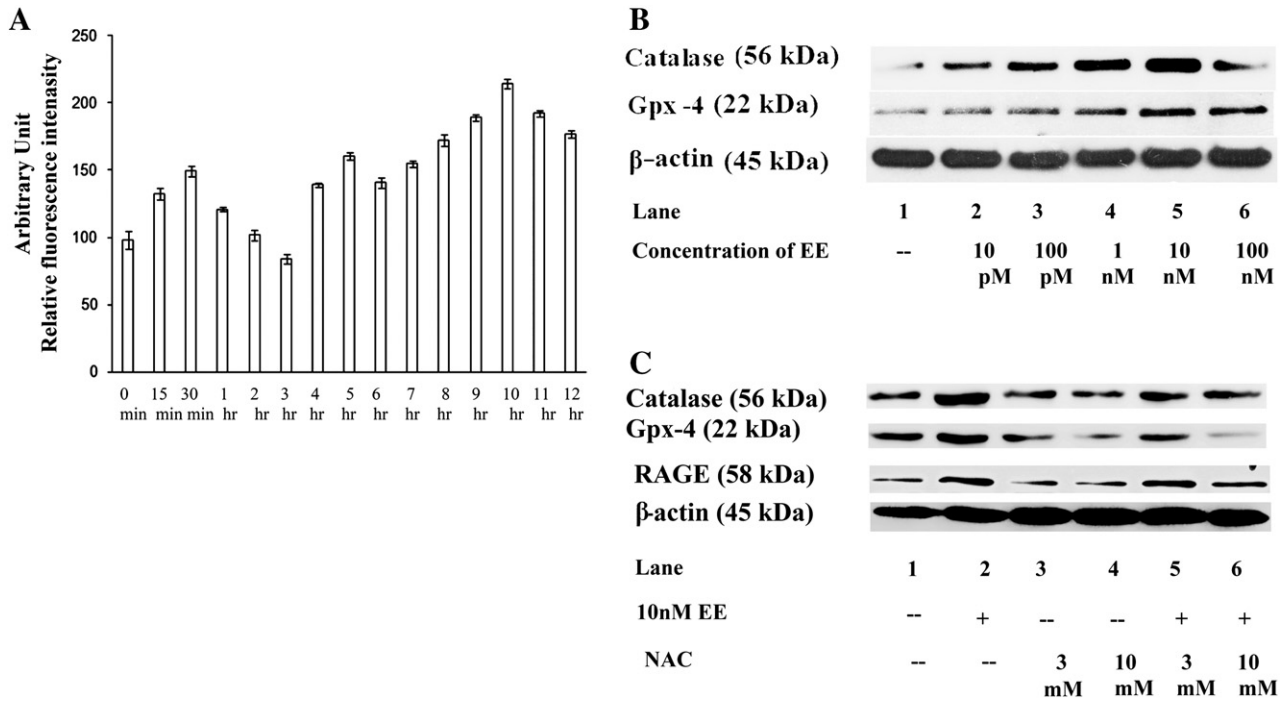

Fig. 4. A: Representative plot showing the effect of 10 nM concentration of 17α-EE at different time points on the generation of ROS in the MCF-7 cells. B. Representative Western blot analysis showing the effect of 17α-EE on the expression of catalase and GPx-4. Lane 1, untreated control; Lane 2-4, treatment with various concentrations of 17α-EE. C. Representative Western blot showing the effect of NAC on the expression of catalase, GPx-4 and RAGE. MCF-7 cells were incubated with NAC for 6 hrs prior to treatment with 10 nM of 17α-EE for 12 hrs. Lane 1, untreated control. Lane 2, treatment with 10 nM of 17α-EE alone for 12 hrs; Lanes 3–4 incubation of cells with 3 mM and 10 mM of NAC alone for 6 hrs; Lane 5-6, incubation of MCF-7 cells with 3 mM and 10 mM of NAC alone for 6 hrs prior to 10 nM of 17α-EE for 12 hrs.

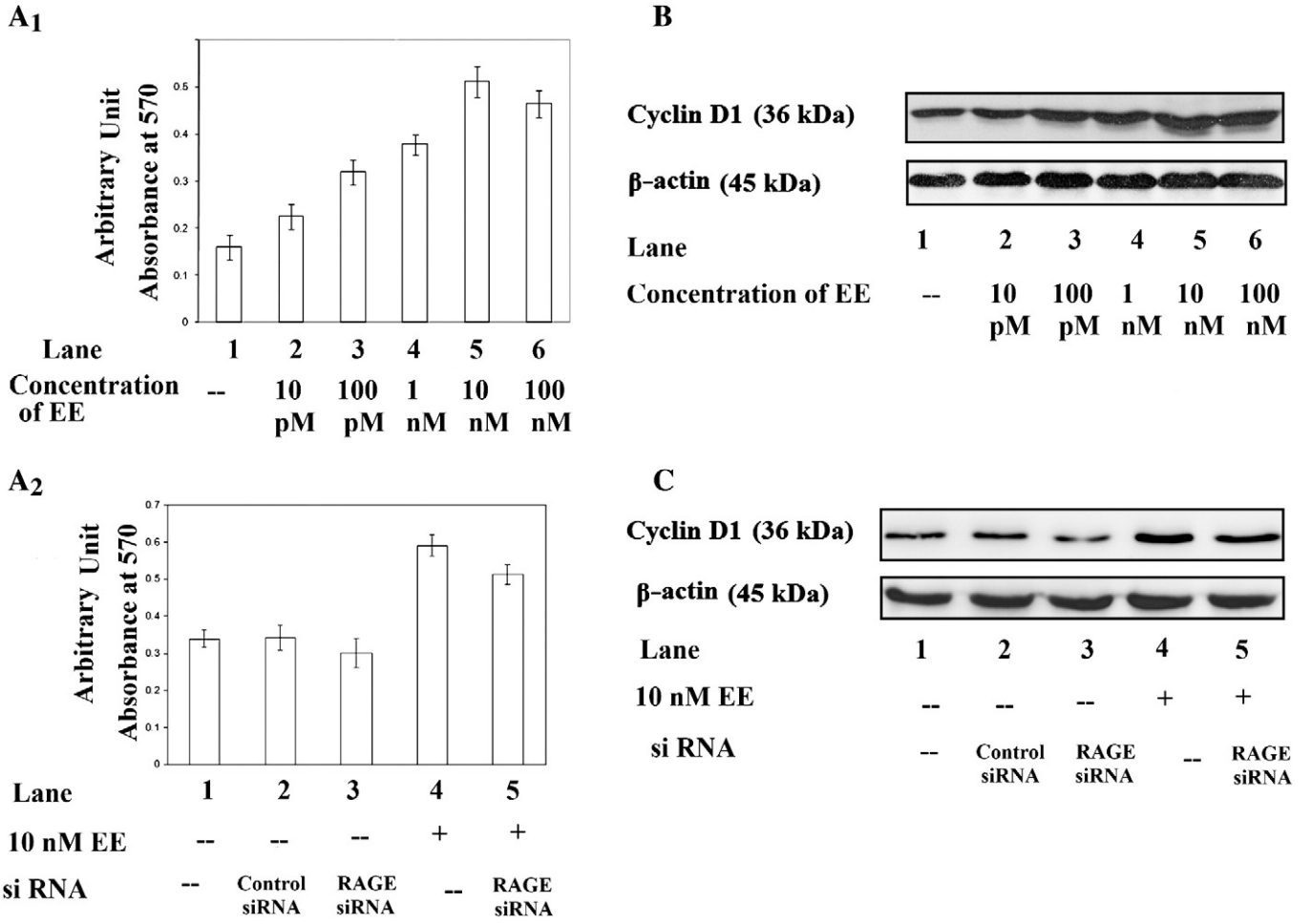

Fig. 5. A1: Representative figure showing the effect of 17α-EE on the proliferation of MCF-7 cells by MTT cell proliferation assay. A2. Representative figure showing the effect of RAGE siRNA on 17α-EE induced proliferation of MCF-7 cells by MTT cell proliferation assay. B. Representative Western blot analysis showing the effect of 17α-EE on the expression of cyclin D1 in MCF-7 cells. Lane 1, untreated control; Lanes 2–6, treatment with various concentration of 17α-EE. C. Representative Western blot analysis showing the effect of pretreatment with RAGE siRNA on the 17α-EE dependent expression of cyclin D1 on MCF-7 cells. Lane 1, untreated control; Lane 2, control siRNA; Lane 3, treatment with RAGE siRNA alone for 6 hrs; Lane 4, treatment with 10 nM 17α-EE for 12 hrs; Lane 5, treatment with RAGE siRNA before 10 nM 17α-EE treatment.

It is also essential to examine the exact mechanism through which 17α-EE possibly enhances RAGE expression in cancer cells. Our previous study indicates that in endothelial cells, TNFα generated ROS induces RAGE expression through the activation of transcription factor NF-κB [24]. Estrogens can also enhance the generation of ROS in cancer cells [25]. Therefore ROS may be the possible downstream molecules through which 17α-EE enhances RAGE expression in cancer cells.

It is also essential to understand the exact mechanism through which RAGE could affect breast cancer cells proliferation and survival. A number of recent studies indicate that interaction of RAGE with its ligands might enhance the ROS level in the cells [11]. Therefore, while RAGE is the product of enhanced oxidative stress, once expressed, RAGE itself could further enhance oxidative stress and thus might cyclically cause its own expression by a positive feedback mechanism [24]. ROS is one of the molecules that might affect cell cycle progression and increase the cell number [26]. In a separate independent study, it was also shown that estrogens affect the cell cycle progression by enhanced expression of cyclin D1, a G1 specific cell cycle protein [27]. ROS might also affect the cell survival and the expression of pro- or antiapoptotic proteins [28]. Thus, it is highly essential to check the effect of RAGE generated ROS on MCF-7 breast cancer cells proliferation, survival and apoptosis.

ER positive MCF-7 breast cancer cells are widely in use to examine the effect of estrogens on breast cancer complication. Therefore, the present study was undertaken to check the followings: (a) Role of 17α-EE, ERs and ERRs in the expression of RAGE in MCF-7 breast cancer cells, (b) Role of 17α-EE generated ROS and consequent oxidative stress on controlling the expression of RAGE in MCF-7 breast cancer cells, (c) Intermediate role of RAGE and RAGE generated ROS and subsequent oxidative stress in the 17α-EE dependent MCF-7 breast cancer cells proliferation, survival and apoptosis. The following paragraphs represent our experimental observation:

3.1. Role of 17α-ethinyl-estradiol and estrogen receptor related receptors on the expression of receptor for advanced glycation end products in MCF-7 breast cancer cells

Initially, we have checked whether estrogens can stimulate RAGE expression in MCF-7 breast cancer cells. ER positive MCF-7 breast cancer cells are widely in use to study the effects of estrogens on various estrogen dependent cell signaling pathways. In our previous studies on endothelial cells we have shown the comparative effect of 17α-EE (a synthetic estrogen and a major component of contraceptive pills), 17β-estradiol (major estrogen present in the female blood) and 17α-epiestriol (a metabolite of estrogen present in female blood) on the expression of RAGE. Of note, while 17α-EE predominantly works through ERα, 17β-estradiol is equally potent to both ERα and ERβ and 17α-epiestriol predominantly works through ERβ and shows similarity in action with genestin, a phytoestrogen [18]. Moreover, in endothelial cells 17α-EE is comparatively more potent in stimulating the expression of

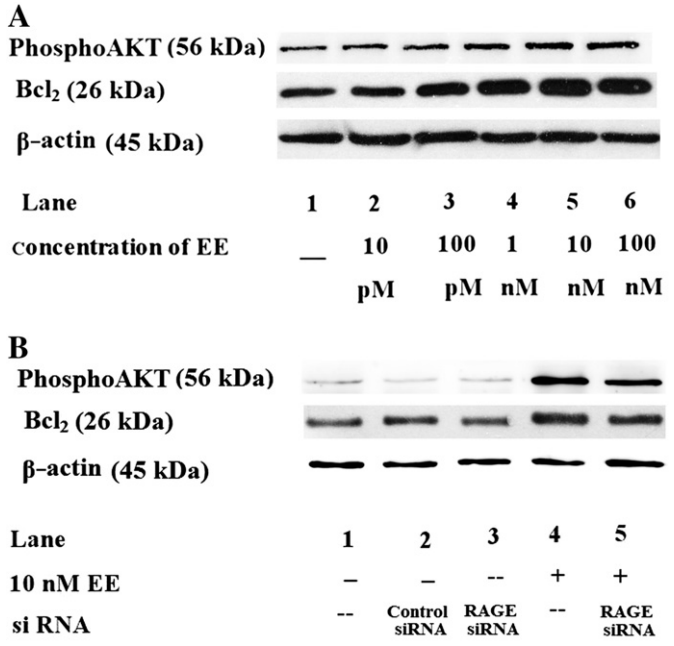

Fig. 6. A: Representative Western blot analysis showing the effect of 17α-EE on the phosphorylation of AKT and expression of Bcl_2. Lane 1, untreated control; Lanes 2–4, treatment with various concentration of 17α-EE. B. Representative Western blot analysis showing the effect of pretreatment with RAGE siRNA on the 17α-EE dependent expression of phospho Akt and the expression of Bcl_2, in MCF-7 cells. Lane 1, untreated control; Lane 2, control siRNA; Lane 3, treatment with RAGE siRNA alone for 6 hrs; Lane 4, treatment with 10 nM 17α-EE for 12 hrs; Lane 5, treatment with RAGE siRNA before 10 nM 17α-EE treatment.

RAGE in comparison to 17β-estradiol and 17α-epiestriol [9]. In the present study using MCF-7 breast cancer cells 17α-EE is the most potent estrogen to stimulate RAGE expression when compared its effect with 17β-estradiol and 17α-epiestriol (data not shown). Thus, we have chosen 17α-EE as our experimental estrogen of interest.

The results show a dose dependent induction of RAGE expression in MCF-7 breast cancer cells by 17α-EE (Fig. 1A) which was attenuated by ICI182780 (fulvestran) (Fig. 1B), a nonspecific ER antagonist. Further results confirmed that ERα is responsible for 17α-EE dependent RAGE expression in MCF-7 breast cancer cells (data not shown). These results confirm our speculation that both in endothelial cells and in MCF-7 breast cancer cells 17α-EE is equally potent in inducing the expression of RAGE.

In this context, in a number of recent observations it is claimed that ERRs, a family of nuclear orphan receptors are equally potent to bind various estrogens and show similar kind of effects as observed with estrogens [7]. It is further noticed that ICI182780 is able to attenuate ERRs binding with estrogens [29]. Additionally, tamoxifen (a nonspecific ER antagonist and breast cancer drug) dependent drug resistance is mediated at least partially through the binding of estrogens with ERRs [8]. Therefore, we examined the involvement of various ERRs in 17α-EE dependent RAGE expression in MCF-7 breast cancer cells. The results show dose dependent induction of ERRγ (Fig. 2A), but not of ERRα and ERRβ (data not shown). Further, treatment of MCF-7 breast cancer cells with siRNA against ERRγ prior to the treatment with 17α-EE attenuated the 17α-EE dependent RAGE expression (Fig. 2B), confirming the important contributory role of ERRγ in 17α-EE dependent RAGE expression.

3.2. Role of 17α-ethinyl-estradiol activated NF-κB on the expression of receptor for advanced glycation end products in MCF-7 breast cancer cells

Estrogens regulate the expression of various genes by binding to promoters of the responsive genes through either estrogen response elements (ERE), or via activation of various transcription factors namely, NF-κB and SP1 [9]. The proinflammatory and prooxidative transcription factors being activated by oxidative and inflammatory stresses regulate the expression of various proinflammatory and prooxidative genes [30]. While RAGE promoter sequence shows binding sites for transcription factors NF-κB and SP1, no ERE are observed in the RAGE promoter [31]. Compelling evidence from our two independent studies on endothelial cells indicate that proinflammatory and prooxidative cytokine TNFα and female steroids (estrogens) activate RAGE expression by two different transcription factors. While TNFα generated ROS induces

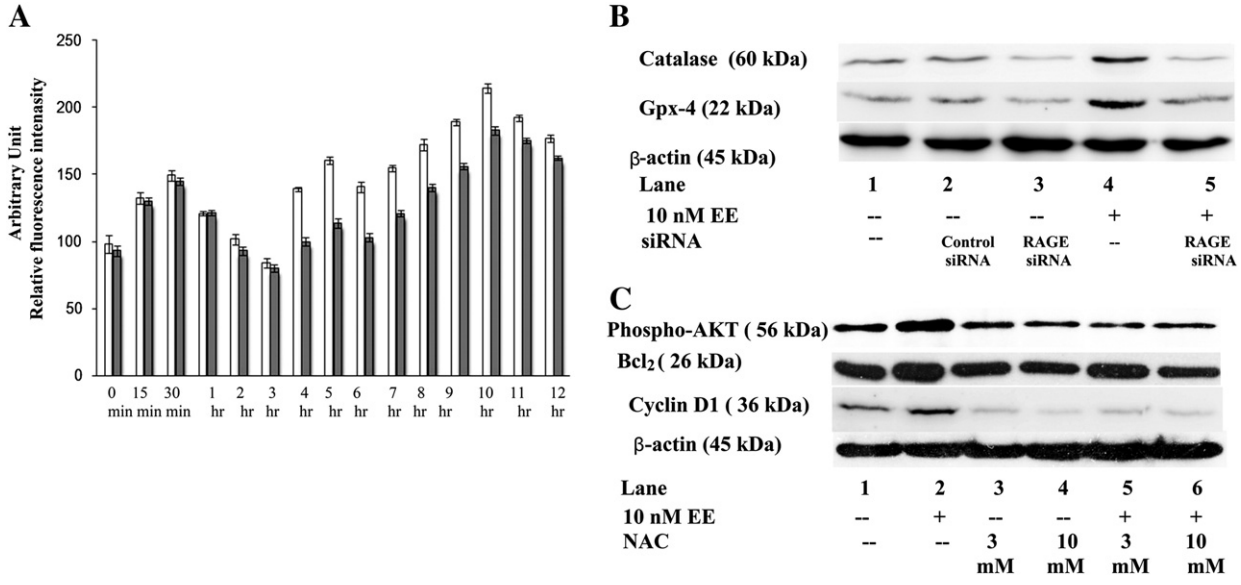

Fig. 7. A. Representative plot showing the effect of RAGE siRNA on ROS generation in the 17α-EE treated MCF-7 cells. B. Representative Western blot analysis showing the effect of pretreatment of RAGE siRNA on the 17α-EE dependent expression of catalase and Gpx-4 in the MCF-7 cells. Lane 1, untreated control; Lane 2, control siRNA; Lane 3, treatment with RAGE siRNA alone for 6 hrs; Lane 4, treatment with 10 nM 17α-EE for 12 hrs; Lane 5, treatment with RAGE siRNA before 10 nM 17α-EE treatment. C. Representative Western blot analysis showing the effect of NAC prior to 17α-EE treatment on the expression of cyclin D1, Bcl_2 and the phosphorylation of AKT in MCF-7 cells. Lane 1, untreated control; Lane 2, treatment with 10 nM concentration of 17α-EE alone for 12 hrs; Lane 3-4, incubation of cells with 3 mM and 10 mM of NAC alone for 6 hrs; Lane 5-6, incubation of cells with 3 mM and 10 mM of NAC for 6 hrs prior to 10 nM of 17α-EE for 12 hrs.

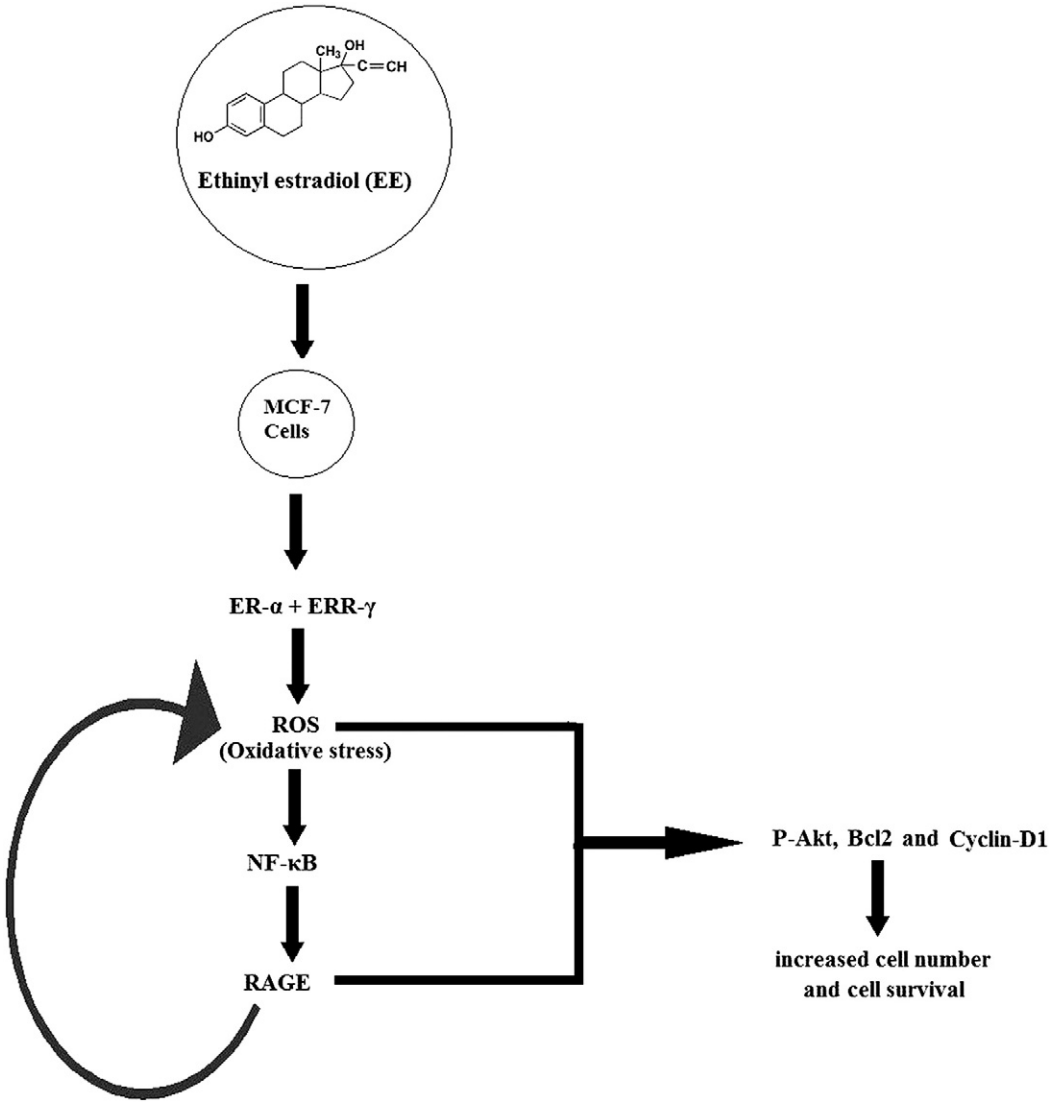

Fig. 8. A schematic diagram depicting the mechanism through which 17α-EE generated receptor for advanced glycation end products affect the proliferation and survival of MCF-7 breast cancer cells.

RAGE expression via the activation of NF-κB [24], estrogens induce RAGE expression through the activation of transcription factor SP1 and the involvement of NF-κB in this mechanism is negligible [9]. Other studies also confirmed a role of ROS in the activation of NF-κB [32].

Like TNFα, estrogens are known inducers of ROS generation and subsequent oxidative stress, particularly in various cancer cells [33]. In fact, estrogen dependent oxidative stress has a key role in breast cancer cell proliferation and survival [34]. In the present study, we have examined whether SP1 or NF-κB is responsible for 17α-EE dependent RAGE expression in MCF-7 breast cancer cells. The results indicate that following treatment of the MCF-7 breast cancer cells with 17α-EE, SP1 activity becomes negligible (data not shown) and NF-κB becomes activated and gets translocated to the nucleus (Fig. 3A and B). IκBα is the inhibitor protein which binds with NF-κB and prevents translocation of NF-κB from the cytoplasm to the nucleus. Translocation of NF-κB needs phosphorylation followed by degradation of IκBα [24]. Increased level phosphorylation of IκBα further confirmed the involvement of NF-κB in 17α-EE dependent RAGE expression in the MCF-7 breast cancer cells (Fig. 3C).

These results indicate that endothelial cells behave differently in comparison to MCF-7 breast cancer cells in terms of 17α-EE dependent expression of RAGE. In endothelial cells, SP1 is predominantly responsible for the estrogen dependent RAGE expression [9], and in MCF-7 breast cancer cells, NF-κB is responsible for 17α-EE dependent RAGE expression. While the exact reason is yet to be determine, most possibly a high level of ROS generation and subsequent oxidative stress in MCF-7 breast cancer cells favored stimulation of NF-κB by 17α-EE treatment. Of note, endothelial cells show a comparatively low level of ROS generation [35], in comparison to cancer cells [36].

To check whether the treatment of MCF-7 breast cancer cells with 17α-EE, in fact generated ROS and induced oxidative stress, we have measured the level of ROS and the expression of some of the markers of oxidative stress namely GPx-4 and catalase. 17α-EE treatment not only generated ROS (Fig. 4A), but also enhanced the expression of GPx-4 and catalase (Fig. 4B). Catalases are utilized to neutralize the excess amount of H_2O_2 [37]. Similarly, a high level of GPx-4 is necessary to neutralize the excess level of H_2O_2 [38]. Thus, in our experiments high level expression of GPx-4 and catalase indicates enhanced level of oxidative stress following treatment of MCF-7 breast cancer cells with 17α-EE.

To check whether 17α-EE dependent oxidative stress is really responsible for RAGE expression in MCF-7 breast cancer cells, we have treated them with antioxidant NAC prior to treatment with 17α-EE

and checked the level of expression of GPx-4, catalase and RAGE. Of note, in a number of investigations the antioxidant NAC is used to neutralize the excess amount of ROS [39]. Very recently it has been shown that NAC can attenuate RAGE expression, indicating the role of oxidative stress on RAGE expression [40]. In our experiments, the dose dependent attenuation of the expression of GPx-4, catalase and RAGE by NAC indicate the important contributory role of oxidative stress on 17α-EE dependent RAGE expression in the MCF-7 breast cancer cells (Fig. 4C). These results confirm that treatment of MCF-7 breast cancer cells with 17α-EE induces the generation of ROS which in turn activates RAGE expression through the activation of the transcription factor NF-κB.

3.3. Role of receptor for advanced glycation end products on 17α-ethinylestradiol dependent MCF-7 breast cancer cells proliferation and survival

Estrogens regulate cell signaling molecules involved in breast cancer cells proliferation [27]. However, role of RAGE in estrogen dependent breast cancer cells proliferation is yet to be evaluated. The present study reveals the intermediary role of 17α-EE generated RAGE on the MCF-7 breast cancer cells proliferation and the mechanism involved. Initially, we checked MCF-7 breast cancer cells proliferation by MTT cell proliferation assay following treatment with 17α-EE in presence and absence of RAGE siRNA or sRAGE. The results show that 17α-EE fails to induce MCF-7 breast cancer cells proliferation in presence of RAGE siRNA (Fig. 5A1 and A2) or sRAGE (data not shown), indicating the involvement of RAGE in the 17α-EE dependent MCF-7 breast cancer cell proliferation.

To understand the mechanism through which RAGE is involved in MCF-7 breast cancer cells proliferation, we have checked the level of cyclin D1, following treatment of MCF-7 breast cancer cells with 17α-EE in presence or absence of RAGE siRNA. In this study, cyclin D1 level was checked taking into consideration previous report which mentioned that estrogens regulate cell cycle progression by enhancing the expression of cyclin D1 [27]. The results of the present experiments indicate the involvement of cyclin D1 in RAGE dependent cell cycle progression (Fig. 5B and C). Besides activating cell cycle, estrogens also regulate cell survival by regulating the expression as well as phosphorylation / dephosphorylation of prosurvival protein AKT [41]. The pro-survival role of AKT is widely accepted [15]. Estrogens also induce Bcl_2, an antiapoptotic protein [16]. The role of RAGE (if any) in 17α-EE dependent phosphorylation of AKT and expression of Bcl_2 is not known. In the present study, RAGE siRNA attenuated 17α-EE dependent AKT phosphorylation and Bcl_2 expression (Fig. 6A and B), indicating important contributory role of 17α-EE generated RAGE in MCF-7 cells survival. Treatment of the MCF-7 breast cancer cells with sRAGE show similar kind of results (data not shown). Thus, 17α-EE induces the MCF-7 breast cancer cells proliferation through a multiprong mechanism by activating G1 specific cell cycle protein cyclin D1, prosurvival protein AKT and antiapoptotic protein Bcl_2.

3.4. Role of reactive oxygen species in receptor for advanced glycation end products dependent activation of cyclin D1 and Bcl_2 expression and the phosphorylation of AKT in MCF-7 breast cancer cells

The specific mechanism through which RAGE might affect cyclin D1, AKT and Bcl_2 levels in MCF-7 breast cancer cells needs to be evaluated. One of the possible explanations is RAGE dependent activation of cell signaling molecule MAPKs. Of note, MAPKs are one of the cell signaling molecules affecting cell cycle progression through activation of cyclin D1 [42]. Since in a number of investigations MAPKs are reported to be activated by RAGE [43], including direct binding of RAGE with MAPKs [44], this study focused on additional molecules which may affect RAGE dependent activation of cyclin D1.

Recent studies indicate that ROS could be one of the molecules which affect cell cycle progression [26]. Similarly, both AKT [45] and Bcl_2 [46] are sensitive to ROS. It is also reported that estrogen generated ROS may affect AKT and Bcl_2 [47,48]. Therefore, ROS might be suitable intermediate molecules of RAGE dependent AKT phosphorylation and Bcl_2 activation. Interestingly, in a number of nontransformed cells interaction of RAGE with its various ligands induces generation of ROS [11]. However, it is highly essential to check whether the ROS generated by RAGE is sufficient enough to affect cell cycle progression. Additionally, it is essential to distinguish RAGE generated ROS from the RAGE independent ROS generation which could be generated due to direct action of estrogens on the ROS generating system (e.g. mitochondria) of the cells [49].

In our experiments, treatment with RAGE siRNA shows no effect on the level of 17α-EE generated ROS in the time point between 1 and 4 hrs. However, there is a substantial decrease of 17α-EE dependent ROS generation within 4-5 hrs following treatment with 17α-EE (Fig. 7A). We speculate that the 1st peak of ROS generation originates from MCF-7 breast cancer cells activation by 17α-EE. This ROS generation might have started due to direct action of 17α-EE on the ROS generating system of cells [49]. However, estrogens have a half life of only around 3 hrs (17). So, there should be a substantial decrease of 17α-EE dependent ROS generation following 3 hrs of 17α-EE treatment. Consequently, RAGE is expressed which causes further generation of ROS (24), leading to the gradual formation of the 2nd peak of ROS. RAGE siRNA also attenuates the expression of catalase and GPx-4, indicating most possible involvement of RAGE in oxidative stress (Fig. 7B). Finally, treatment of the MCF-7 breast cancer cells with NAC attenuates RAGE dependent AKT phosphorylation and cyclin D1 and Bcl_2 expression (Fig. 7C). This result confirms the role of RAGE generated oxidative stress in AKT phosphorylation and Bcl_2 expression.

While in the present investigation 17α-EE generated ROS is responsible for NF-κB activation and subsequent activation of RAGE expression, we predict that 17α-EE dependent activation of high mobility group box protein 1 (HMGB1) is also involved in the MCF-7 breast cancer cells proliferation and survival. This prediction is based on our observation that in the *in vitro* cultured MCF-7 breast cancer cells estrogens stimulate HMGB1 expression which was attenuated by RAGE siRNA (data not shown). Of note, HMGB1 is one of the major ligands of RAGE [50]. HMGB1 is involved in the progression of various cancers [51]. RAGE-HMGB1 axis is implicated in the complication of various types of cancers [52,53]. A detailed role of RAGE-HMGB1 axis in the estrogen dependent breast cancer cells proliferation and survival needs to be further elaborated.

Similarly, in a recent study it was shown that under hypoxic condition MCF-7 breast cancer cells acquire a proinflammatory phenotype with an upregulation of inflammatory response genes. Proinflammatory molecule RAGE and TLRs are induced in hypoxic condition and activate NF-kB and hypoxia inducing factor (HIF-1) translocation [54]. Importance of this signaling pathway in terms of estrogen dependent breast cancer cell proliferation need to be further evaluated.

In conclusion, it appears that in MCF-7 breast cancer cells, interaction of 17α-EE with ERRγ generates ROS which activates NF-kB and ultimately leads to the activation of RAGE expression. RAGE thus expressed, causes further generation of ROS and enhances oxidative stress which increases MCF-7 breast cancer cells number in a multiprong mechanism by affecting cell proliferation (cyclin D1), cell survival (AKT) and antiapoptotic proteins (Bcl_2). A schematic diagram depicting the mechanism through which 17α-EE generated RAGE might enhance the number of MCF-7 breast cancer cells (Fig. 8). While RAGE dependent activation of ROS generation assumes a very crucial role in MCF-7 breast cancer cells proliferation and survival, other signaling pathways including RAGE-HMGB1 axis or hypoxia might also assume important contributory roles in the maintenance of the overall inflammatory status of the cancer cells. Further studies in this area will shed new light on the mechanism of RAGE dependent breast cancer cells proliferation and survival.

Conflict of interest statement

No conflict of interest.

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