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ERβ-dependent neuroglobin up-regulation impairs 17β-estradiol-induced apoptosis in DLD-1 colon cancer cells upon oxidative stress injury

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ABSTRACT

Besides other mechanism(s) 17β-estradiol (E2) facilitates neuronal survival by increasing, via estrogen receptor β (ERβ), the levels of neuroglobin (NGB) an anti-apoptotic protein. In contrast, E2 could exert protective effects in cancer cells by activating apoptosis when the ERβ level prevails on that of ERα as in colon cancer cell lines. These apparently contrasting results raise the possibility that E2-induced NGB up-regulation could regulate the ERβ activities shunning this receptor subtype to trigger an apoptotic cascade in neurons but not in non-neuronal cells. Here, human colorectal adenocarcinoma cell line (DLD-1) that only expresses ERβ and HeLa cells transiently transfected with ERβ encoding vector has been used to verify this hypothesis. In addition, neuroblastoma SK-N-BE cells were used as positive control. Surprisingly, E2 also induced NGB up-regulation, in a dose- and time-dependent manner, in DLD-1 cells. The ERβ-mediated activation of p38/MAPK was necessary for this E2 effect. E2 induced NGB re-allocation in mitochondria where, subsequently to an oxidative stress injury (i.e., 100 μM H_2O_2), NGB interacted with cytochrome *c* preventing its release into the cytosol and the activation of an apoptotic cascade. As a whole, these results demonstrate that E2-induced NGB up-regulation could act as an oxidative stress sensor, which does not oppose to the pro-apoptotic E2 effect in ERβ-containing colon cancer cells unless a rise of oxidative stress occurs. These results support the concept that oxidative stress plays a critical role in E2-induced carcinogenesis and further open an important scenario to develop novel therapeutic strategies that target NGB against E2-related cancers.

1. Introduction

Estrogens are sex steroid hormones that play an essential role for a plethora of biological responses in male and female physiology including the balance between proliferation and differentiation and/or survival and cell death. These somewhat contrasting and tissue specific estrogen actions are guaranteed by the differential expression of the two estrogen receptor subtypes (i.e., ERα and ERβ) which define the different biological cellular response to hormones [2,43]. In particular, the proliferative and anti-apoptotic effect of 17β-estradiol (E2) in cancer cells is primarily mediated by the ERα activation, whereas ERβ seems to oppose ERα actions activating pro-apoptotic cascade [1,26,41,54]. These findings indicate a tumor-suppressive function of ERβ which is functionally linked to the promotion of apoptosis, the suppression of the malignant transformation, and the inhibition of tumor cells growth [5,33,39,54]. This concept is in agreement with the ERβ levels detected in tumor tissues. In fact, the presence of ERβ in breast, endometrial, and ovarian tumors is associated with a better prognosis or a longer survival [6,40,51], whereas the level of ERβ is significantly decreased in high grade endometrial cancer [14,47]. In addition, knock-out experiments targeting ER genes in mice have revealed that ERβ is the predominant ER expressed in colonic tissues [13,37,57] and that its expression is selectively lost in human malignant colon tissue [25,34,54]. Thus, the E2 effect against cancer progression seems to be strictly linked to the expression of ERβ subtype.

Recently, it has been demonstrated that E2 binding to ERβ increases up to 300% the levels of neuroglobin (NGB), a monomeric heme-globin [9], in human neuroblastoma cell line (SK-N-BE), in mouse primary hippocampal neurons, and mouse astrocytes [15,16]. As a consequence, physiological E2 concentrations

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decrease SK-N-BE cell death and reduce the activation of the pro-apoptotic cascade under H_2O_2 induced toxicity [16]. Of note, E2 exerts its protective role in brain-derived cells by up-regulating and re-allocating NGB in mitochondria where NGB associates with cytochrome *c* (cyt*c*) to prevent stress signals induced by cyt*c* release into the cytosol and the consequent apoptosome formation [16,17,22,23].

Therefore, the interaction of E2 with ERβ is able to block apoptosis in neurons and to promote apoptosis in cancer cells. However, it is not clear if E2-induced NGB up-regulation is involved in either protective effects. Moreover, current data on the role of NGB in cancer cells are still contradictory [20,28,45,61]. In this perspective, the aim of this paper is to determine if E2-induced NGB up-regulation, which exerts an anti-apoptotic role in neurons, occurs in ERβ expressing non nervous cancer cells and if the increased level of NGB could modify the ability of ERβ to act as a tumor suppressor. Colorectal adenocarcinoma cell lines (DLD-1) and HeLa cells transiently transfected with ERβ have been used as experimental models; DLD-1 cells express only one isoform of ERβ subtype (i.e., ERβ1) without any expression of ERβ splicing form and it is well known that E2 exerts a pro-apoptotic and protective role against tumor growth in this cell context [2,27]. In addition, some experiments were performed in neuroblastoma SK-N-BE cells, as positive control for the role of E2 in NGB up-regulation.

2. Materials and methods

2.1. Reagents

E2, gentamicin, penicillin, puromycin, L-glutamine, streptomycin, actinomycin D, inhibitor protease cocktail, Dulbecco's modified Eagle medium (DMEM), RPMI-1640 medium without phenol red, charcoal-stripped fetal calf serum, and the inhibitor of palmitoyl acyl transferase 2-bromohexadecanoic acid (2-bromopalmitate; 2-Br) ($IC_{50} \sim 4.0\ \mu M$; [63]) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The ER pure inhibitor fulvestrant (ICI 182,780 or ICI), the estrogen receptor ERα-selective agonist 4,4',4"-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)trisphenol (PPT), the ERβ-selective agonist 2,3-bis(4-hydroxyphenyl)propionitrile (DPN), and the ERβ-selective antagonist (R,R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol (THC) were obtained from Tocris (Ballwin, MO, USA). The p38 inhibitor SB 203 580 (SB), the lysosomal enzymes inhibitor chloroquine (clo), and the inhibitor of 26S proteasome-mediated degradation Mg-132 were purchased from Calbiochem (San Diego, CA, USA). Bradford protein assay and chemiluminescence reagent for Western blot ECL were obtained from Bio-Rad Laboratories (Hercules, CA, USA). The polyclonal anti-NGB and monoclonal anti-poly(ADP-ribose)polymerase (PARP), anti-cytochrome *c* oxidase (COX-4), anti-protein phosphatase 2A (PP2A), anti-p38, anti-phospho-ERK, anti-phospho-AKT, anti-caspase 3 antibodies and Annexin V apoptosis detection kit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-tubulin antibody was purchased from MP Biomedical (Solon, OH, USA). The reagent fractionation kit, containing dithiothreitol (DTT) and the polyclonal anti-cytochrome *c* antibody was obtained from Clontech (San Jose, CA, USA). Anti-flag antibody was purchased from Sigma–Aldrich (St. Louis, MO, USA). Lipofectamine reagent was purchased from Invitrogen (Carlsbad, CA, USA). All the other products were from Sigma–Aldrich. Analytical or reagent grade products were used without further purification.

2.2. Cell culture

The human cervix epithelioid carcinoma cell line (HeLa) (from ATCC, LGC Standards S.r.l., Milan, Italy), the human colon adenocarcinoma cells (DLD-1) (from Sigma–Aldrich, St. Louis, MO, USA), and the human neuroblastoma cells (SK-N-BE) (from ATCC, LGC Standards S.r.l., Milan, Italy) were used. Cells were routinely grown in air containing 5% CO_2 in modified, phenol red-free, DMEM (HeLa cells) or RPMI-1640 (DLD-1 and SK-N-BE cells) media, containing 10% (v/v) charcoal-stripped fetal calf serum, L-glutamine (2×10^{-3} M), gentamicin (0.1 mg/ml) and penicillin (100 U/ml). Cells were passaged every 2 days and media changed every 2 days. Cell lines were used at passage 4–8 and were grown as previously described [2,27]. Cell line authentication was periodically performed by amplification of multiple STR loci by BMR genomics S.r.l. (Padova, Italy). Cells were treated for 24 h with either vehicle (i.e., DMSO/PBS 1:10 v/v) or E2 (10^{-8} M) or H_2O_2 (10^{-4} M) or PPT (10^{-8} M) or the specific ERβ agonist DPN (10^{-8} M). The anti-estrogen ICI (10^{-6} M), the ERβ inhibitor THC (10^{-6} M), the transcription inhibitor actinomycin D (Act 1 ng/ml), the palmitoyl acyl transferase inhibitor which impairs ER membrane localization, 2-Br (10^{-6} M), and the p38 inhibitor SB 203 580 (5×10^{-6} M) were added 30 min before E2 administration. In some experiments 10^{-5} M chloroquine (clo) or 10^{-6} M Mg-132 were added 15 min before E2 administration. Twenty-four hours after treatment cells were harvested with trypsin and centrifuged.

2.3. Annexin V with propidium iodide (PI) methodology for apoptosis with flow cytometry

Phosphatidylserine externalization was quantified by flow cytometry by using the Annexin V apoptosis detection kit including propidium iodide (PI) according to the manufacturer's guideline (Santa Cruz, CA, USA). In brief, after treatments, both attached and floating cells were collected and resuspended in 100 μl annexin-binding buffer with 2.5 μl FITC-annexin V and 10 μl PI working solution. After incubation (15 min, room temperature), 400 μl of binding buffer was added, and cells were immediately analyzed by flow cytometry with DAKO Galaxy flow-cytometer equipped with HBO mercury lamp. Analysis by flow cytometry used the FL1 (FITC) and FL3 (PI) laser lines and each sample was assessed using a collection of 10 000 events. Each experiment was carried out in triplicate the fluorescence was calculated using a FloMax© Software.

2.4. Transfection

The pcDNA 3.1 flag C ERβ was produced by PCR amplifying the ERβ ORF from the pCXN2 ERβ [44] into the Bam HI/Xho I sites in the pcDNA 3.1 flag C multiple cloning site. The resulting expression vector was sequence verified. Details are available upon request. HeLa cells were grown to ~70% confluence and then transfected with pcDNA 3.1 flag C ERβ using Lipofectamine reagent according to the manufacturer's instructions. Three hours after transfection, the medium was changed and 24 h after the cells were stimulated with various E2 concentrations. To evaluate the effective transfection of ERβ, total proteins were extracted from transfected cells and the expression of ERβ was assessed by Western blot using the specific anti-flag antibody.

2.5. Lentivirus-delivered stable gene silencing

NGB stable silencing DLD-1 cells were obtained with short hairpin RNA (shRNA) lentivirus particles (Santa Cruz, CA, USA) following the manufacturer's instructions. Two different cell types were obtained: control lentivirus cells, containing the lentivirus control particles and NGB lentivirus cells, containing lentivirus NGB-specific particles. The efficiency of lentiviral infection was checked by Western blot using anti-NGB antibody.