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Original Contribution

Estrogen receptor potentiates mTORC2 signaling in breast cancer cells by upregulating superoxide anions

Ranjana Kumari Kanchan [a], Chakrapani Tripathi [a], Khemraj Singh Baghel [a], Shailendra Kumar Dwivedi [b], Balawant Kumar [a], Sabyasachi Sanyal [c], Sharad Sharma [a], Kalyan Mitra [d], Vivek Garg [e], Kavita Singh [d], Sarwat Sultana [f], Raj Kamal Tripathi [a], Srikanta Kumar Rath [a], Smrati Bhadauria [a,*]

[a] Division of Toxicology, Central Drug Research Institute (CSIR), India
[b] Division of Endocrinology, Central Drug Research Institute (CSIR), India
[c] Drug Target and Drug Discovery Division, Central Drug Research Institute (CSIR), India
[d] Electron Microscopy Unit, Central Drug Research Institute (CSIR), India
[e] Department of Oncosurgery, Lucknow Cancer Institute, India
[f] Department of Medical Elementology and Toxicology, Jamia Hamdard, India

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ABSTRACT

The estrogen receptor (ER) plays a cardinal role in estrogen-responsive breast carcinogenesis. It is, however, unclear as to how estrogen–ER interaction potentiates breast cancer progression. Compelling evidence supports estrogen-induced redox alterations, such as augmented reactive oxygen species (ROS) levels, as having a crucial role in breast carcinogenesis. Despite ER being a biological mediator of the majority of estrogen-induced cellular responses; its role in estrogen-induced tissue-specific ROS generation remains largely debatable. We examined a panel of human breast cancer specimens and found that ER-positive breast cancer specimens exhibited a higher incidence of augmented $O_2^{\bullet-}$ levels compared to matched normal tissue. ROS are known to function as signal transducers and ROS-mediated signaling remains a key complementary mechanism that drives carcinogenesis by activating redox-sensitive oncogenic pathways. Additional studies revealed that augmented $O_2^{\bullet-}$ levels in breast cancer specimens coincided with mammalian target of rapamycin complex 2 (mTORC2) hyperactivation. Detailed investigations using in vitro experiments established that 17β-estradiol (E2)-stimulated breast cancer cells exhibited transiently upregulated $O_2^{\bullet-}$ levels, with the presence of ER being a crucial determinant for the phenomenon to take place. Gene expression, ER transactivation, and confocal studies revealed that the E2-induced transient $O_2^{\bullet-}$ upregulation was effected by ER through a nongenomic pathway possibly involving mitochondria. Furthermore, E2 treatment activated mTORC2 in breast cancer cells in a characteristically ER-dependent manner. Interestingly, altering $O_2^{\bullet-}$ anion levels through chemical/genetic methods caused significant modulation of the mTORC2 signaling cascade. Taken together, our findings unravel a novel nongenomic pathway unique to estrogen-responsive breast cancer cells wherein, upon stimulation by E2, ER may regulate mTORC2 activity in a redox-dependent manner by transiently modulating $O_2^{\bullet-}$ levels particularly within mitochondria. The findings suggest that therapies aimed at counteracting these redox alterations and/or resultant signaling cascades may complement conventional treatments for estrogen-responsive breast cancer.

Introduction

Constituting 22.9% of all female cancers diagnosed worldwide, breast cancer represent the most common form of malignancy and remains one of the most frequent cause of cancer death among women [1]. Epidemiological and experimental evidence implicates 17β-estradiol (E2)[1] in the etiology of breast carcinogenesis; however, the underlying mechanism remains largely elusive. The biological effects of estrogen are mediated through estrogen receptors (ER) α and β, which are members of a large superfamily of nuclear receptors [2]. With 60–70% of breast cancers being ER positive and thus dependent on estrogen for proliferation, the ER emerges as an important clinical target in treatment and management of breast cancer [3,4]. Consequently antiestrogens have emerged as highly effective, nontoxic endocrine therapeutic agents for women with stage IV and II ER-positive breast cancer [5]. Although antagonizing ER with antiestrogens counteracts breast cancer progression, the impairment of

Abbreviations: ER, estrogen receptor; ROS, reactive oxygen species; $O_2^{\bullet-}$, superoxide anion; mTORC2, mammalian target of rapamycin complex 2; NAC, *N*-acetyl-l-cysteine; MnTBAP, Mn(III) tetrakis-(4-benzoic acid) porphyrin; E2, 17-β-estradiol; DHE, dihydroethidium; FBS, fetal bovine serum; CSFBS, charcoal-stripped fetal bovine serum; EGF, epidermal growth factor; ERE, estrogen-responsive elements; CYP 3A4, cytochrome P450 3A4; CYP 1A2, cytochrome P450 1A2

* Corresponding author.
E-mail address: smraticdri@gmail.com (S. Bhadauria).

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ER-mediated physiological pathways accounts for undesirable side effects such as menopausal symptoms and gynecological complications [6]. Identifying and targeting ER-regulated pathways that are unique to breast carcinogenesis may prove to be a better therapeutic strategy for the treatment of ER-positive breast cancers.

Previous studies established estradiol's link to carcinogenesis via its ability to alter cellular redox status by elevating levels of cellular ROS such as hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$) [7–9]. However, the molecular framework within which physiological levels of E2 potentiate ROS generation, and how these alterations relate to breast carcinogenesis, remains obscure. Whereas some reports suggest the involvement of an ER-dependent mechanism in E2-induced ROS generation, others contradict the importance of ER. In view of these conflicting reports an in-depth experimental investigation was carried out.

E2-induced ROS generation and resultant DNA damage/genomic instability may possibly be the mechanism underlying breast carcinogenesis [10]; however, other reports implicate E2-induced ROS-mediated signaling pathways in breast carcinogenesis [11,12]. Whereas cellular signaling effects of H_2O_2 in cancer progression have long been postulated [11,13], insights about $O_2^{\bullet-}$-mediated cellular signaling have began to emerge only recently. Accumulating evidence indicates that $O_2^{\bullet-}$ serves as a growth stimulus by regulating signaling cascades leading to cell survival and proliferation [14,15]. Elevated $O_2^{\bullet-}$ levels modulate the response/resistance to anti-cancer therapy by inhibiting death execution and providing a survival advantage [16,17]. Prompted by this, we decided to examine if $O_2^{\bullet-}$-mediated signaling cascades are operational in breast carcinogenesis as well.

The mammalian target of rapamycin (mTOR), a conserved serine/threonine kinase, has emerged as one of the crucial effectors in oncogenic signaling cascades [18–20]. It exists in two functionally distinct complexes: mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 2 (mTORC2) [21,22]. Whereas mTORC1 potentiates cancer cell proliferation by promoting protein/lipid biosynthesis and counteracting autophagy [23], the precise role of mTORC2 is hitherto obscure. Apparently it promotes malignant transformation and epithelial–mesenchymal transition owing to its ability to regulate cytoskeletal organization [21,24–26]. Signaling cascades regulating mTORC1 activation are well characterized, whereas mTORC2, being discovered only recently, remains relatively less well understood [20]. Structural and biochemical characterization studies conducted on TOR (the yeast ortholog of mTOR) reveal that, owing to the presence of a redox-sensitive motif, the cellular stability and functionality of TOR is redox regulated [27]. Furthermore, using yeast TORC1, Neklesa and Davis [28] demonstrated the ability of $O_2^{\bullet-}$ to regulate the functional activity of TORC1. The evolutionarily conserved nature of TOR across species and the common presence of mTOR in both mTORC1 and mTORC2 complexes raises an obvious question as to whether mTORC2 activation is also redox regulated.

Given the propensity of E2 to elevate $O_2^{\bullet-}$, the ability of $O_2^{\bullet-}$ to regulate TOR activity, and the emerging importance of mTORC2 in cancer progression, a connection between E2-induced breast carcinogenesis and mTORC2 activation seems quite plausible. Here we address this issue by investigating whether $O_2^{\bullet-}$ acts as a mediator of breast carcinogenesis by activating mTORC2 and whether this phenomenon is regulated by ER, one of the most potent effectors of breast carcinogenesis.

Materials and methods

Antibodies and reagents

Polyclonal antibodies against mTOR, phospho-mTOR(Ser^{2481}), Akt, phospho-Akt(Ser^{473}), PKCα, phospho-PKCα(Thr^{638}), and Rictor were purchased from Cell Signaling Technology (USA). Monoclonal antibodies against ERα and superoxide dismutase-2 (SOD2) were purchased from Santa Cruz Biotechnology (USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was procured from Jackson ImmunoResearch Europe Ltd. (UK). 17β-Estradiol, fulvestrant, and rapamycin were purchased from Sigma (USA). Dihydroethidium (DHE), Mn(III) tetrakis-(4-benzoic acid) porphyrin (MnTBAP), and *N*-acetyl-L-cysteine (NAC) were procured from Calbiochem (USA). The mitochondrial superoxide anion probe MitoSOX red was purchased from Invitrogen (USA).

Cell culture and treatments

In vitro experiments were carried out employing human hepatocarcinoma-derived HuH-7 cells, human mammary cancer-derived MCF-7 and MDA-MB-231 cells, and untransformed breast epithelial MCF-10 cells. HuH-7, MCF-7, and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 100 μg/ml penicillin and 100 μg/ml streptomycin. Untransformed breast epithelial MCF-10 cells were grown in DMEM:F12 supplemented with 5% horse serum, 20 ng/ml EGF, 5 μg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, in addition to penicillin/streptomycin (100 μg/ml). Cells were grown in a humidified atmosphere (95% humidity) at 37 °C and 5% CO_2. To maintain the cells in a quiescent state and to attain defined E2 concentrations, 48 h before the treatments were initiated, the cells were switched to cell culture medium containing charcoal-stripped FBS.

Plasmids and transfection

Estrogen-responsive element (ERE)-Luc, pcDNA3-ERα, and pcDNA3-ERβ were kind gifts from Dr. Eckardt Treuter (Department of Biosciences at Novum, Karolinska Institutet, Stockholm, Sweden). HuH-7/MDA-MB-231 cells were seeded onto 24-well plates in phenol red-free DMEM supplemented with 10% charcoal/dextran-treated FBS (CSFBS). After 24 h the cells were transfected with 200 ng of reporter plasmid (ERE-Luc), 50 ng of pcDNA3-ERα/β, using Lipofectamine LTX reagent (Invitrogen) according to the manufacturer's instructions. In all transfections, pEGFPC1 (50 ng/well) was used as an internal control. Total DNA in all transfections was kept constant at 600 ng/well using the pcDNA3 empty vector. Five hours posttransfection the cells were treated with 10 nM E2 for the stipulated time periods. Treated cells were lysed and harvested. Luciferase activity was measured using a SteadyGlo kit (Promega, USA) in a GloMax-96 microplate luminometer (Promega) as described previously [29]. Green fluorescent protein (GFP) expression in each well was quantitated using a Fluostar Optima spectrofluorimeter (BMG Technologies, Germany). Luciferase values were then normalized to GFP values and were plotted as fold activity over untreated controls.

Short interfering RNA (siRNA) and transfections

All siRNA experiments were carried out using a set of four prevalidated siRNAs that were directed against Rictor/SOD2 and procured from Eurofins Analytik Germany. The sense and antisense sequences of control (scrambled) as well as Rictor/SOD2-directed siRNAs are presented in Table 1. Briefly, 4×10^5 MCF-7 cells grown in phenol red-free DMEM supplemented with 10% CSFBS and 50 μg/ml penicillin/streptomycin were transfected with 400 pM pooled prevalidated siRNAs using siPort NeoFX reagent (Ambion, USA). Twenty-four hours after transfection, the culture medium was replaced with fresh medium (CSFBS medium). The cells were further maintained for 24 h and subsequently employed for studying E2-mediated effects.