Archives of Biochemistry and Biophysics 613 (2017) 23–30

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Impact of hypoxia inducible factors on estrogen receptor expression in breast cancer cells

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ARTICLE INFO

Article history:
Received 19 July 2016
Received in revised form
31 October 2016
Accepted 3 November 2016
Available online 5 November 2016

Keywords:
Estrogen receptor (ER)
Hypoxia
Hypoxia mimetics
Breast cancer
Hypoxia inducible factor (HIF)

ABSTRACT

In women breast cancer is still the most commonly diagnosed cancer. This type of cancer is classified as a hormone-dependent tumor. Estrogen receptor (ER) expression and functional status contribute to breast cancer development and progression. Another important factor associated with cancer is hypoxia which is defined as the state of reduced oxygen availability in tissues. Intratumoral hypoxia results in the activation of the hypoxia inducible factors (HIFs). HIFs are heterodimeric transcription factors involved in the regulation of many cellular processes, such as angiogenesis, anaerobic metabolism, cell proliferation/survival, and promotion of metastasis. In this study we evaluated the interplay between hypoxia, HIF stabilization and the ER-α/β-ratio in several ER-positive breast cancer cell lines. Hypoxia was shown to inhibit ER expression in ER-positive breast cancer cells. Further experiments using the hypoxia mimetic $CoCl_2$ and HIF-1α knockdown cells indicated that the influence of hypoxia on breast cancer cells involves other pathways than the molecular oxygen sensing pathway. Moreover, we demonstrated that MCF-7 cells in long-term culture lost part of their ability to respond to hypoxic incubation. Understanding the relationships between HIF, ER-α and ER-β expression holds the promise of the development of new therapeutic agents and may provide future advances in prognosis.

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1. Introduction

Estrogens exert crucial tissue-selective effects on cellular homeostasis and play a pivotal role in the pathogenesis of breast cancer [1]. The cellular response is mediated by two different estrogen receptor (ER) subtypes, ER-α and ER-β [2]. ER belong to the family of nuclear receptors [3] and are activated by binding of specific ligands followed by dimerization of the receptor subunits [4]. The activated ER homomers or heteromers bind to estrogen response elements (EREs) which are specific recognition sites on genomic and mitochondrial DNA sequences [5,6]. As a consequence gene transcription can be either stimulated and inhibited [5,7]. The affinity of estrogens to EREs can be modulated by tissue-specific shifts in the expression of splice variants of ER-α and/or ER-β [8,9]. ER-α and ER-β differ in their ligands affinities, are expressed in a tissue-specific fashion and may act antagonistically. ER-α, formerly described as the main ER, is known to inhibit apoptosis and to stimulate cell proliferation [10–12]. ER-β has a more ubiquitous expression pattern than ER-α and is found in many different tissues in varying concentrations [13,14]. ER-β exerts specific actions in cellular homeostasis, but overall functions as an antagonist to ER-α [15,16]. This antagonism is based on different affinities for specific EREs and the capability of ER-β monomers to dimerize with ER-α monomers. Thereby heterodimers (αβ) are formed that are barely active [17]. Therefore it has been hypothesized that the response of breast tissue to estradiol (E_2) or other estrogens depends on the ER-α/β-ratio [18]. As a consequence, the ER status is thought to be a central component in the clinical evaluation of breast cancer. Indeed, ER-positive breast cancer has been associated with response to endocrine therapy.

Hypoxia is a common characteristic of locally advanced solid tumors that has been associated with diminished therapeutic response and a more malignant progression [20]. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females [21]. 25–40% of invasive breast cancers exhibit hypoxic regions [20]. In solid tumors, adaptation to

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http://dx.doi.org/10.1016/j.abb.2016.11.002
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decreased O_2 availability is achieved primarily through the stabilization of hypoxia-inducible factors (HIFs). HIFs are heterodimeric transcription factors consisting of a HIF-1β subunit and a HIF-α subunit (HIF-1α, HIF-2α) [22,23]. Under normoxic conditions the HIF-α subunits are hydroxylated by members of a family of prolyl-4-hydroxylase domain (PHD) proteins, PHD1, PHD2 and PHD3, resulting in recognition by von Hippel-Lindau protein, ubiquitination and proteasomal degradation [24]. Reduced O_2 availability causes low PHD activity and increased nuclear accumulation of HIF-α. The HIF-1β subunit is rather constitutively available. Note, however, that HIF-1β transcription is hypoxia-dependent in a cell line-dependent manner [25]. In the nucleus, HIF bind to hypoxia responsive elements (HRE) of target genes involved in angiogenesis, erythropoiesis, vasomotor control, energy metabolism and cell survival decisions [26–28]. Hypoxia mimetics such as the transition metal salt $CoCl_2$ directly inhibit PHD activity with concomitant HIF stabilization [29].

So far, the interplay between hypoxia, HIF activity and ER expression has not been clarified in cancer cells. Herein, we demonstrate that hypoxia inhibits ER expression in ER-positive breast cancer cells. Further experiments using the hypoxia mimetic $CoCl_2$ and HIF-1α knockdown cells show that this response involves other mechanisms than the molecular O_2 sensing pathway. It seems worth considering a direct link between HIF, ER-α and ER-β expression as predictive marker for the prognosis of breast cancer.

2. Materials and methods

2.1. Cell culture, hypoxia, hypoxia mimetic and tamoxifen

The cell lines MCF-7 (human breast ductal carcinoma), T47D (human breast ductal carcinoma), MX-1 (human breast adenocarcinoma), MD-A-MB 231 (human breast adenocarcinoma), SK-BR-3 (human breast adenocarcinoma) and HeLa (human cervix adenocarcinoma) were incubated in DMEM culture medium (Gibco, Darmstadt, Germany). The culture medium was supplemented with 10% fetal calf serum (Gibco) and 100 IU/ml penicillin and 100 mg/ml streptomycin (PAA Laboratories, Cölbe, Germany), respectively. To achieve hypoxic conditions, cells were placed in a humidified atmosphere at 37 °C containing 5% CO_2, 1% O_2 and balanced N_2. Control normoxic cells were placed in an incubator (5% CO_2, 21% O_2 and 74% N_2) for the same period of time. Cobalt(II)-chloride ($CoCl_2$, 50 μM) was used as hypoxia mimetic. For tamoxifen experiments cells were incubated with 100 nM 4-hydroxytamoxifen for 24 h or 48 h.

2.2. Transient transfection

MCF-7 and T47D cells were grown to 50% confluence in 6-well culture dishes before transfection. Cells were transiently transfected with siRNA using Lipofectamine™ RNAiMAX transfection reagent (Life Technologies, Darmstadt, Germany) for 48 h as described by the manufacturer. Prior to hypoxic incubation the culture medium was renewed and cells were incubated for another 6 h under normoxic conditions.

2.3. Protein extraction immunoblot analysis and densitometry

Whole cell extracts were prepared in order to analyze protein expression levels. Cells were washed with ice-cold PBS, then extracted with urea lysis buffer containing 10 mM Tris HCl (pH 6.8), 6.7 M urea, 10 M glycerin, 1% SDS and 5 mM DTT and sonicated. All protein extracts were supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentrations were determined using Bio-Rad DC Protein Assay (Bio-Rad Laboratories GmbH, München, Germany) following the manufacturer's protocol. Protein extracts were subjected to SDS-PAGE and transferred onto nitrocellulose membrane (Amersham Hybond-ECL, GE Healthcare, Freiburg, Germany) by semi-dry electroblotting. Membranes were blocked by 5% non-fat milk in PBS at 4 °C for at least 1 h. After blocking, membranes were incubated with primary antibodies (HIF-1α: BD Biosciences, Freiburg, Germany, 1:1000; HIF-2α: Santa Cruz, Heidelberg, Germany; ER-α: Santa Cruz Biotechnology, Heidelberg, Germany, 1:1000 and ER-β: Santa Cruz, 1:1000) overnight at 4 °C with cautious shaking. Then, the corresponding polyclonal horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako, Hamburg, Germany, 1:5000) were added for 2 h. A polyclonal anti-Lamin-A/C antibody (Santa Cruz) served as control for equal protein loading and transfer. ECL detection reagent (Amersham ECL Western Blotting Detection Reagents, GE Healthcare) was used for chemiluminescence detection of immunoreactive proteins. The immunoblots shown are representatives of at least three independent experiments. Densitometric quantification was done by Aida Image Analyzer v.4.27 software (Raytest, Straubenhardt, Germany). Western blot background was subtracted from the individual signal intensity. Corresponding Lamin A/C signal intensities were regarded as 100% and protein levels were calculated.

2.4. RNA isolation and quantitative real-time PCR

For the quantification of mRNA levels, total RNA was isolated from the cells using ABI Prism 6100 Nucleic Acid PrepStation (Life Technologies) following the manufacturer's instructions. 0.2 μg of RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Life Technologies). Generated cDNA was used as template in quantitative real-time PCR analysis (qRT-PCR). qRT-PCR was performed by ABI 7000 Sequence Detection System (Applied Biosystems, Darmstadt, Germany), TaqMan Universal PCR Master Mix (Applied Biosystems) and KAPA SYBR FAST Universal (Peqlab, Darmstadt, Germany). Expression values of target genes were normalized to L28 expression values. The following assays and primers were used: Taqman Gene Expression Assay for ER-α (Hs00174860_m1), Taqman Gene Expression Assay for ER-β (Hs01100353_m1) (both Applied Biosystems), human L28 forward: 5′-ATGGTCGTGCGGAACTGCT-3′ and human L28 reverse: 3′-TTGTAGCGGAAGGAATTGCG-5′. Samples were analyzed in quadruplicate and relative amounts of mRNA were calculated using the ΔΔCT method.

2.5. Statistics

If not otherwise indicated, experiments were performed in triplicate and results are presented as mean ± standard deviation of at least $n = 3$ independent experiments. Statistical comparison was performed by unpaired *t*-test with Welch's correction. Significance was accepted at $p < 0.05$. All statistics were calculated using GraphPad InStat 3.06 software (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Hypoxia reduces ER protein levels

MCF-7 and T47D breast ductal carcinoma cells express both subtypes of estrogen receptors (ER-α and ER-β). In order to verify a presumed influence of hypoxia on the ER expression in these cell lines, we examined the quantity of ER-α and ER-β protein levels in whole cell extracts. The cells were grown in normoxia (21% O_2) for 24 h followed by either 48 h of normoxic (21% O_2) or hypoxic (1%

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