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Estrogen receptor beta interacts and colocalizes with HADHB in mitochondria

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ABSTRACT

Estrogen receptors are localized in mitochondria, but their functions in this organelle remain unclear. We previously found that ERα interacted with mitochondrial protein HADHB and affected the thiolytic cleavage activity of HADHB in β-oxidation. It is known that ERβ binds to ERα. In addition, ERβ is predominately located in mitochondria. These facts led us to speculate that ERβ may also be associated with HADHB in mitochondria. In order to test this hypothesis, we performed co-immunoprecipitation and confocal microscopy analyses with human breast cancer MCF7 cells. The results demonstrated that ERβ was indeed associated and colocalized with HADHB within mitochondria. Interestingly, in contrast to the stimulatory effect of ERα on HADHB enzyme activity observed in the previous study, silencing of ERβ enhanced the enzyme activity of HADHB in the present study, suggesting that ERβ plays an inhibitory role in HADHB enzyme activity in the breast cancer cells. Our results imply that ERα and ERβ may differentially affect cellular oxidative stress through influencing the rate of β-oxidation of fatty acids in breast cancer cells.

1. Introduction

In addition to its well-recognized function in stimulating cell proliferation, steroid hormone estrogens also play other important functions, such as influencing lipid metabolism [1,2] and cellular oxidative stress [3–6]. The biological activities of estrogens are mediated by two estrogen receptors (ERs), ERα and ERβ, which are traditionally considered nuclear receptors and classical transcription factors [7]. Upon binding to the estrogen response elements, ERs affect cell proliferation through activating transcription of estrogen target genes [8]. Despite a high degree of conservation between ERα and ERβ in protein sequences (>95% and >50% homology for the DNA-binding domain and ligand-binding domain, respectively [9]), the two receptors demonstrate opposite biological functions in many cases. For example, while ERα stimulates cell proliferation, ERβ does the opposite [10]. In addition to the nucleus, ERα and ERβ were also found to be localized in the plasma membrane [11–13] and mitochondria [14–17]. The functions of ERs in the nucleus and plasma membrane have been well-studied [8,11–13]; the function of mitochondrial ERs remains unclear.

In a previous study, we found that ERα interacted and colocalized with HADHB, a mitochondrial protein that is required for β-oxidation of fatty acids in mitochondria. Furthermore, ERα and its cognate ligands 17β-estradiol and tamoxifen significantly affected the thiolytic cleavage activity of HADHB [17]. Based on the fact that ERβ can form heterodimers with ERα *in vivo* and *in vitro* [18], and that ERβ is predominately located in mitochondria [16], we speculated that ERβ might also be associated with HADHB within mitochondria. In this report, we present our findings on testing this hypothesis.

2. Materials and methods

2.1. Materials

Alpha minimal essential medium (α-MEM), Opti-MEM I reduced serum medium, and Lipofectamine™ RNAiMAX Kit were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was from Atlanta biologicals (Norcross, GA, USA). Protein A beads, acetyl-CoA, and acetoacetyl-CoA were obtained from Sigma–Aldrich (Sigma, St. Louis, MO, USA). Protein G plus agarose beads were from Calbiochem (San Diego, CA, USA). siRNA as well as the control oligos were from Shanghai GenePharma Co. Ltd. (Shanghai, China). Protease inhibitor cocktails were from Roche (Indianapolis, IN). MitoTracker RedCMXRos was from Lonza (Walkersville, MD, USA). Rabbit polyclonal anti-ERβ (H-150), goat polyclonal anti-HADHB (S-16), non-immune IgG, and secondary antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA).

Abbreviations: α-MEM, alpha minimal essential medium; ER, estrogen receptor; HADHB, hydroxyacyl-CoA dehydrogenase/trifunctional protein, beta subunit; ROS, reactive oxygen species.

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2.2. Co-immunoprecipitation

Human breast cancer MCF7 cells were maintained in α-MEM with 5% fetal bovine serum and 1% penicillin and streptomycin as described previously [17]. The cultured cells (approximately 1×10^8 cells/each) were lysed in 5 packed cell pellet volumes of lysis buffer (10 mM Hepes-KOH, pH 7.9, 0.5% NP-40, 140 mM NaCl, 10 mM KCl, 1.5 mM $MgCl_2$ and protease inhibitors) by douncing on ice. The lysate was centrifuged at 16,000*g* for 15 min at 4 °C, and the supernatant was used for immunoprecipitation. After pre-incubation of each supernatant with 40 μl protein A (or protein G) beads at 4 °C for 1 h, the pre-cleared supernatant was incubated with 5 μg anti-ERβ (or anti-HADHB) antibody and 30 μl protein A (or protein G) beads at 4 °C for 5 h with end-to-end rotation. For a negative control, non-immune IgG was used in the place of anti-ERβ (or anti-HADHB) antibody. After washing 4× with lysis buffer, the bound proteins were eluted from the beads by boiling in SDS sample buffer. The eluted proteins were analyzed by Western blotting.

2.3. Immunofluorescence staining and confocal microscopy

The immunofluorescence staining and image acquisition were performed according to our previous protocol [17] with the following specifications: (1) Anti-ERβ and anti-HADHB antibodies were both used at 1:100 dilution; (2) the fluorescence images were acquired on a Leica TCS SP2 confocal microscope (Leica Microsystems).

2.4. RNA interference

siRNA oligos (5′-GCAGACCACAAGCCCAAAU-3′) targeting ERβ [19] was used to transiently silence the expression of ERβ in MCF7 cells. A randomized siRNA sequence was used as the negative control. The siRNA oligos were transfected into MCF7 cells with Lipofectamine™ RNAiMAX using a reverse transfection protocol according to the manufacturer's instructions. Briefly, 5 μl Lipofectamine™ RNAiMAX reagent and 50 nM siRNA were gently mixed in 500 μl Opti-MEM I reduced serum medium, followed by incubation at room temperature for 20 min. The mixture was then mixed with 7.2×10^5 MCF7 cells suspended in 2 ml α-MEM in a well of a 6-well plate. The cells were incubated at 37 °C in an incubator with 5% CO_2 for 48 h before they were harvested for HADHB enzyme activity analysis.

2.5. HADHB enzyme activity assay

HADHB enzyme activity was determined through monitoring thiolytic cleavage of acetoacetyl-CoA as described previously [17]. One unit of activity was defined as the amount of enzyme that converts 1 μmol acetoacetyl-CoA per min.

2.6. Western blotting

The eluted proteins from immunoprecipitation or the total cell lysates were analyzed as described in the previous papers [17,20].

2.7. Statistical analysis

P values were calculated using a One-way ANOVA (PSI-PLOT, Pearl River, NY). Data were presented as the mean ± S.E. of three separate sample preparations.

3. Results

3.1. ERβ is associated with HADHB

In a previous study, we identified mitochondrial protein HADHB as a novel binding partner of ERα. HADHB physically bound to and was colocalized with ERα within the mitochondria of human breast cancer cells [17]. Because ERβ physically interacts with ERα [18,21], and most of ERβ is localized in mitochondria and thus is considered a resident mitochondrial protein [16], we speculated that ERβ might also be associated with HADHB in mitochondria. In order to test this hypothesis, we used co-immunoprecipitation to determine if ERβ interacted with HADHB in human breast cancer MCF7 cells. As shown in Fig. 1A, ERβ was immunoprecipitated along with HADHB by the antibody directed against HADHB in the MCF7 cells, suggesting that endogenous ERβ is specifically associated with endogenous HADHB. In a reciprocal immunoprecipitation, while non-immune IgG precipitated small amount of HADHB, the antibody specifically recognizing ERβ precipitated substantially more HADHB proteins (Fig. 1B), suggesting HADHB is specifically associated with ERβ.

3.2. ERβ colocalizes with HADHB within the mitochondria of MCF7 cells

In order to determine whether the association between ERβ and HADHB took place in the mitochondria, we performed confocal microscopy analysis using MCF7 cells (Fig. 2). Staining of the cells with anti-ERβ antibody (H-150) demonstrated that ERβ was predominately in the cytosol and exhibited a punctuate distribution (Fig. 2A), similar to that of a typical mitochondria distribution (Fig. 2B). The merged image illustrated that ERβ was localized in the mitochondria (Fig. 2C). Similarly, HADHB was also localized in the mitochondria (Fig. 2D–F). When the cells were co-stained with antibodies directed against ERβ (Fig. 2G) and HADHB (Fig. 2H), the merged image clearly demonstrated that the two proteins were colocalized, and a punctuate distribution characteristic of mitochondrial staining appeared (Fig. 2I). These results strongly suggest that ERβ is colocalized with HADHB within the mitochondria of MCF7 cells.

3.3. ERβ inhibits the thiolytic cleavage activity of HADHB

To determine whether ERβ was functionally linked to HADHB in cells, we used a double-stranded RNA sequence targeting ERβ to silence the expression of ERβ in MCF7 cells, and then assessed the effect of the suppressed expression of ERβ on the thiolytic cleavage

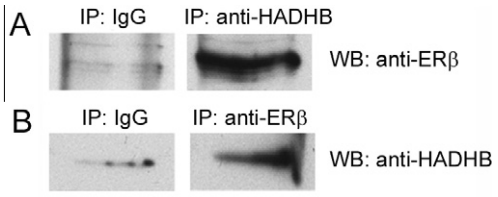

Fig. 1. ERβ is associated with HADHB. (A) ERβ was co-immunoprecipitated with HADHB. Whole cell lysates of the MCF7 cells (1×10^8/each) were immunoprecipitated with non-immune goat IgG (negative control) or anti-HADHB antibodies. The immunoprecipitated proteins were probed with anti-ERβ antibody in Western blotting. As shown, ERβ was immunoprecipitated by anti-HADHB antibody but not by non-immune IgG. (B) HADHB was co-immunoprecipitated with ERβ by anti-ERβ antibody. Whole cell lysates of the MCF7 cells (1×10^8/each) were immunoprecipitated with non-immune rabbit IgG (negative control) or anti-ERβ antibodies. The immunoprecipitated proteins were probed by anti-HADHB antibody in Western blotting. As shown, compared with control IgG, anti-ERβ antibody immunoprecipitated substantially more HADHB.

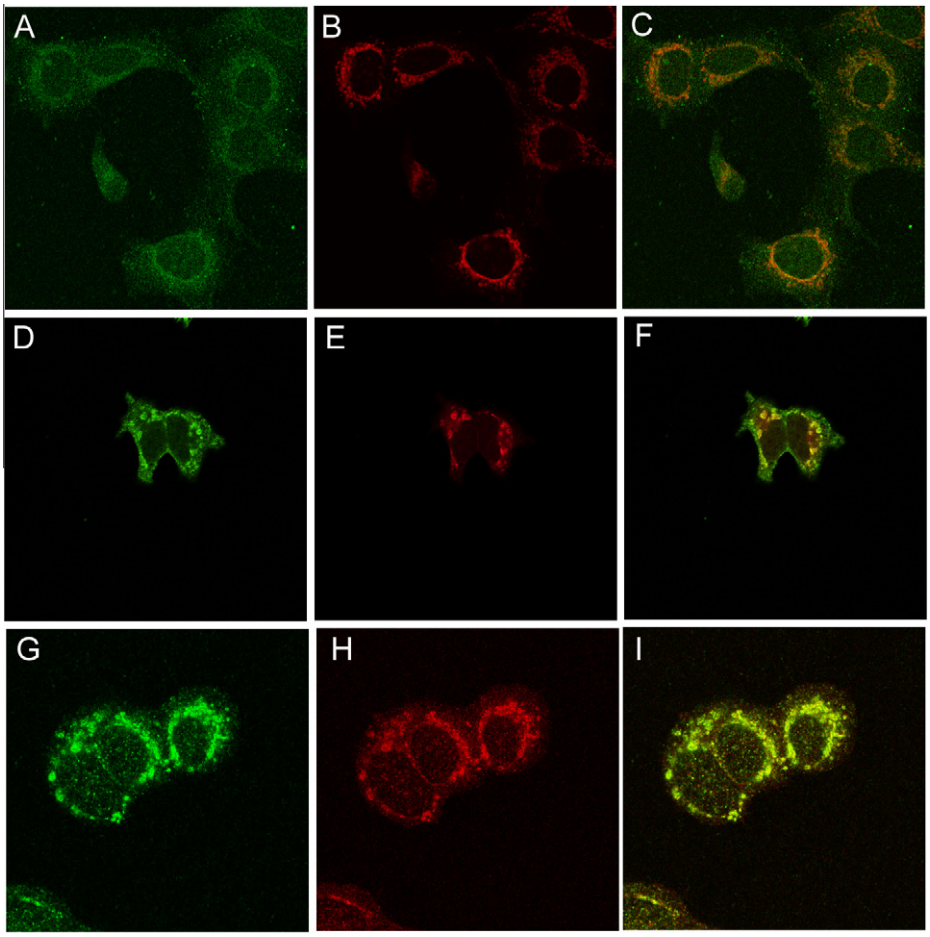

Fig. 2. ERβ colocalizes with HADHB in MCF7 cells. ERβ and HADHB were detected mostly in the cytosol with little in the nucleus (A and D, respectively). The mitochondria were visualized using MitoTracker RedCMXRos (B and E). Merged images show the overlap of ERβ and HADHB with the mitochondria (C and F, respectively). (G) The MCF7 cells were stained with ERβ antibody. (H) The MCF7 cells were stained with HADHB antibody. (I) Merge picture shows the colocalization of ERβ with HADHB.

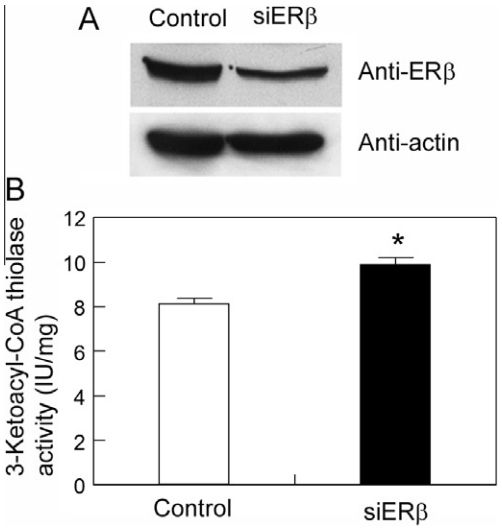

Fig. 3. Silencing of ERβ enhances the thiolytic cleavage activity of HADHB in MCF7 cells. (A) Silencing of ERβ expression in the MCF7 cells (siERβ). The MCF7 cells were transiently transfected with randomized siRNA sequence (negative control) or siRNA sequence targeting ERβ. After 48 h, whole cell lysate was probed by anti-ERβ antibody in Western blotting. Actin was used as a loading control. (B) The thiolytic cleavage activity of HADHB in the control and the siERβ cells grown in α-MEM with 5% fetal bovine serum. Values are the means ± S.E. of three separate enzyme preparations. * denotes statistical significance of $p < 0.05$.

activity of HADHB in the cells. As shown in Fig. 3, when the expression of ERβ in the MCF7 cells grown in a medium with 5% fetal bovine serum was suppressed by siRNA (Fig. 3A), the enzyme activity of HADHB was significantly enhanced ($p < 0.05$) (Fig. 3B), suggesting that ERβ plays an inhibitory role in HADHB enzyme activity.

4. Discussion

In addition to the nucleus and plasma membrane, ERα and ERβ have been found in the mitochondrion, an organelle that is responsible for generating most of the cell's energy and reactive oxygen species (ROS). Estrogens were reported to influence mitochondrial function [4] and ROS production [3,5,6,22]. Thus, it is likely that in addition to function in the nucleus and plasma membrane, estrogens and ERs may affect breast cancer development through modulating mitochondrial function and oxidative stress [3–5]. However, the exact molecular targets of estrogens and ERs in the mitochondria remain elusive. HADHB is a component of the mitochondrial trifunctional protein complex and catalyzes the last step of the thiolytic cleavage in β-oxidation of long chain fatty acids to generate acetyl-CoA in mitochondria. In the previous study, we found that ERα interacted and colocalized with HADHB within mitochondria and affected HADHB enzyme activity [17]. Results in the present study demonstrated that ERβ was also associated and colocalized with HADHB and altered the function of HADHB (Figs. 1–3). Interestingly, ERβ affected HADHB enzyme activity in the opposite way compared with ERα. In the previous study, we

found that ectopic expression of ERα in an ERα-negative breast cancer cells significantly enhanced the thiolytic cleavage activity of HADHB, suggesting that ERα plays a stimulatory role in the enzyme activity of HADHB [17]. However in the present study, rather than stimulating HADHB enzyme activity, ERβ was found to inhibit the activity (Fig. 3).

It has been shown that estrogens induce higher oxidative stress in ERα-positive breast cancer cells compared with ERα-negative cells [5,23]. Similarly, the effect of estrogens on mitochondrial function in breast cancer cells was reported to depend on the ERα/ERβ ratio: cells with a higher ERα/ERβ ratio contains lesser mitochondrial activity, whereas the cells with a lower ERα/ERβ ratio had more functional mitochondria, and the difference presumably resulted from different levels of oxidative stress between the types of cells [4]. One potential cause for the different oxidative stresses by estrogens in cells with different ERα/ERβ status is the differential expression of ROS-production/scavenging genes [3,5,6,23]. Results in our previous [17] and present studies support an alternative and/or complementary mechanism underlying the influence of estrogens on ROS production in breast cancer cells with different ERα/ERβ status. Oxidation of fatty acids is a source of cellular ROS [24,25], and increased oxidation of fatty acids was reported to increase the production of ROS in the mitochondria of cells [26–28]. Thus, it is reasonable to speculate that the increased ROS production in ERα-dominant breast cancer cells under the influence of estrogens [5] could result from the higher rate of β-oxidation due to the stimulatory effect of ERα on HADHB enzyme activity. Conversely, the lower oxidative stress observed in ERα-negative cells [5] or cells with a lower ERα/ERβ ratio [4] could be a result of lower rate of β-oxidation due to the inhibitory effect of ERβ on HADHB enzyme activity. Given the emerging importance of estrogen-induced ROS in the progression of breast cancer development [3–5,27], it will be worthy of further investigation of the interrelationship among ERα/ERβ status of cells, β-oxidation of fatty acids, ROS production in breast cancer development under the influence of estrogens.

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