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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 ER α interacted with mitochondrial protein HADHB and affected the thiolytic cleavage activity of HADHB in β -oxidation. It is known that ER β binds to ER α . In addition, ER β is predominately located in mitochondria. These facts led us to speculate that ER β 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 ER β was indeed associated and colocalized with HADHB within mitochondria. Interestingly, in contrast to the stimulatory effect of ER α on HADHB enzyme activity observed in the previous study, silencing of ER β enhanced the enzyme activity of HADHB in the present study, suggesting that ER β plays an inhibitory role in HADHB enzyme activity in the breast cancer cells. Our results imply that ER α and ER β may differentially affect cellular oxidative stress through influencing the rate of β -oxidation of fatty acids in breast cancer cells.

Keywords

Breast cancer; estrogen receptor beta; hydroxyacyl-CoA dehydrogenase/trifunctional protein; beta subunit (HADHB); mitochondria

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; 4; 5; 6]. The biological activities of estrogens are mediated by two estrogen receptors (ERs), ER α and ER β , 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 ER α and ER β 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 ER α stimulates cell proliferation, ER β does the opposite [10]. In addition to the nucleus, ER α and ER β were also found to be localized in the plasma

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membrane [11; 12; 13] and mitochondria [14; 15; 16; 17]. The functions of ERs in the nucleus and plasma membrane have been well-studied [8; 11; 12; 13]; the function of mitochondrial ERs remains unclear.

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

Materials and methods

Materials

Alpha minimal essential medium (α -MEM), Opti-MEM I reduced serum medium, and Lipofectamine^{TM} 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-ER β (H-150), goat polyclonal anti-HADHB (S-16), non-immune IgG, and secondary antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA).

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 MgCl₂ 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 preincubation of each supernatant with 40 μ l protein A (or protein G) beads at 4°C for 1 h, the pre-cleared supernatant was incubated with 5 μ g anti-ER β (or anti-HADHB) antibody and 30 μ l 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-ER β (or anti-HADHB) antibody. After washing 4 times 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.

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-ER β 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).

RNA interference

siRNA oligos (5'-GCAGACCACAAGCCCAAU-3') targeting ER β [19] was used to transiently silence the expression of ER β in MCF7 cells. A randomized siRNA sequence was used as the negative control. The siRNA oligos were transfected into MCF7 cells with Lipofectamine^{TM} RNAiMAX using a reverse transfection protocol according to the manufacturer's instructions. Briefly, 5 μ l Lipofectamine^{TM} RNAiMAX reagent and 50 nM

siRNA were gently mixed in 500 μ l 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% CO₂ for 48 h before they were harvested for HADHB enzyme activity analysis.

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 μ mol acetoacetyl-CoA per min.

Western blotting

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

Statistical analysis

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

Results

ER β is associated with HADHB

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

ER β colocalizes with HADHB within the mitochondria of MCF7 cells

In order to determine whether the association between ER β and HADHB took place in the mitochondria, we performed confocal microscopy analysis using MCF7 cells (Fig. 2). Staining of the cells with anti-ER β antibody (H-150) demonstrated that ER β 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 ER β 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 ER β (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 ER β is colocalized with HADHB within the mitochondria of MCF7 cells.

ER β inhibits the thiolytic cleavage activity of HADHB

To determine whether ER β was functionally linked to HADHB in cells, we used a double-stranded RNA sequence targeting ER β to silence the expression of ER β in MCF7 cells, and then assessed the effect of the suppressed expression of ER β on the thiolytic cleavage activity of HADHB in the cells. As shown in Fig. 3, when the expression of ER β 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 ER β plays an inhibitory role in HADHB enzyme activity.

Discussion

In addition to the nucleus and plasma membrane, ER α and ER β 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; 4; 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 ER α interacted and colocalized with HADHB within mitochondria and affected HADHB enzyme activity [17]. Results in the present study demonstrated that ER β was also associated and colocalized with HADHB and altered the function of HADHB (Figs. 1, 2 and 3). Interestingly, ER β affected HADHB enzyme activity in the opposite way compared with ER α . In the previous study, we found that ectopic expression of ER α in an ER α -negative breast cancer cells significantly enhanced the thiolytic cleavage activity of HADHB, suggesting that ER α plays a stimulatory role in the enzyme activity of HADHB [17]. However in the present study, rather than stimulating HADHB enzyme activity, ER β was found to inhibit the activity (Fig. 3).

It has been shown that estrogens induce higher oxidative stress in ER α -positive breast cancer cells compared with ER α -negative cells [5; 23]. Similarly, the effect of estrogens on mitochondrial function in breast cancer cells was reported to depend on the ER α /ER β ratio: cells with a higher ER α /ER β ratio contains lesser mitochondrial activity, whereas the cells with a lower ER α /ER β 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 ER α /ER β 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 ER α /ER β 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; 27; 28]. Thus, it is reasonable to speculate that the increased ROS production in ER α -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 ER α on HADHB enzyme activity. Conversely, the lower oxidative stress observed in ER α -negative cells [5] or cells with a lower ER α /ER β ratio [4] could be a result of lower rate of β -oxidation due to the inhibitory effect of ER β on HADHB enzyme activity. Given the emerging importance of estrogen-induced ROS in the progression of breast cancer development [3; 4; 5; 27], it will be worthy of further investigation of the interrelationship among ER α /ER β status of cells, β -oxidation of fatty acids, ROS production in breast cancer development under the influence of estrogens.

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Abbreviations

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

References

1. Chen JQ, Brown TR, Russo J. Regulation of energy metabolism pathways by estrogens and estrogenic chemicals and potential implications in obesity associated with increased exposure to endocrine disruptors. *Biochim Biophys Acta*. 2009; 1793:1128–43. [PubMed: 19348861]
2. Nemoto Y, Toda K, Ono M, Fujikawa-Adachi K, Saibara T, Onishi S, Enzan H, Okada T, Shizuta Y. Altered expression of fatty acid-metabolizing enzymes in aromatase-deficient mice. *J Clin Invest*. 2000; 105:1819–25. [PubMed: 10862797]
3. Miro AM, Sastre-Serra J, Pons DG, Valle A, Roca P, Oliver J. 17beta-Estradiol regulates oxidative stress in prostate cancer cell lines according to ERalpha/ERbeta ratio. *J Steroid Biochem Mol Biol*. 2011; 123:133–9. [PubMed: 21172438]
4. Sastre-Serra J, Nadal-Serrano M, Pons DG, Valle A, Oliver J, Roca P. The effects of 17beta-estradiol on mitochondrial biogenesis and function in breast cancer cell lines are dependent on the ERalpha/ERbeta ratio. *Cell Physiol Biochem*. 2012; 29:261–8. [PubMed: 22415095]
5. Sastre-Serra J, Valle A, Company MM, Garau I, Oliver J, Roca P. Estrogen down-regulates uncoupling proteins and increases oxidative stress in breast cancer. *Free Radic Biol Med*. 2010; 48:506–12. [PubMed: 19969066]
6. Yao J, Brinton RD. Estrogen Regulation of Mitochondrial Bioenergetics: Implications for Prevention of Alzheimer's Disease. *Adv Pharmacol*. 2012; 64:327–71. [PubMed: 22840752]
7. Jensen EV, Jacobson HI, Walf AA, Frye CA. Estrogen action: a historic perspective on the implications of considering alternative approaches. *Physiol Behav*. 2010; 99:151–62. [PubMed: 19737574]
8. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev*. 1999; 20:321–44. [PubMed: 10368774]
9. Mosselman S, Polman J, Dijkema R. ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett*. 1996; 392:49–53. [PubMed: 8769313]
10. Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA. Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc Natl Acad Sci U S A*. 2004; 101:1566–71. [PubMed: 14745018]
11. Levin ER. Cell localization, physiology, and nongenomic actions of estrogen receptors. *J Appl Physiol*. 2001; 91:1860–7. [PubMed: 11568173]
12. Levin ER. Plasma membrane estrogen receptors. *Trends Endocrinol Metab*. 2009; 20:477–82. [PubMed: 19783454]
13. Losel R, Wehling M. Nongenomic actions of steroid hormones. *Nat Rev Mol Cell Biol*. 2003; 4:46–56. [PubMed: 12511868]
14. Chen JQ, Delannoy M, Cooke C, Yager JD. Mitochondrial localization of ERalpha and ERbeta in human MCF7 cells. *Am J Physiol Endocrinol Metab*. 2004; 286:E1011–22. [PubMed: 14736707]
15. Pedram A, Razandi M, Wallace DC, Levin ER. Functional estrogen receptors in the mitochondria of breast cancer cells. *Mol Biol Cell*. 2006; 17:2125–37. [PubMed: 16495339]

16. Yang SH, Liu R, Perez EJ, Wen Y, Stevens SM Jr, Valencia T, Brun-Zinkernagel AM, Prokai L, Will Y, Dykens J, Koulen P, Simpkins JW. Mitochondrial localization of estrogen receptor beta. *Proc Natl Acad Sci U S A*. 2004; 101:4130–5. [PubMed: 15024130]
17. Zhou Z, Zhou J, Du Y. Estrogen Receptor Alpha Interacts with Mitochondrial Protein HADHB and Affects Beta-Oxidation Activity. *Mol Cell Proteomics*. 2012; 11:M111011056.
18. Ogawa S, Inoue S, Watanabe T, Hiroi H, Orimo A, Hosoi T, Ouchi Y, Muramatsu M. The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro. *Biochem Biophys Res Commun*. 1998; 243:122–6. [PubMed: 9473491]
19. Park SH, Cheung LW, Wong AS, Leung PC. Estrogen regulates Snail and Slug in the down-regulation of E-cadherin and induces metastatic potential of ovarian cancer cells through estrogen receptor alpha. *Mol Endocrinol*. 2008; 22:2085–98. [PubMed: 18550773]
20. Zhou J, Du Y. Acquisition of resistance of pancreatic cancer cells to 2-methoxyestradiol is associated with the upregulation of manganese superoxide dismutase. *Mol Cancer Res*. 2012; 10:768–77. [PubMed: 22547077]
21. Fox EM, Davis RJ, Shupnik MA. ERbeta in breast cancer--onlooker, passive player, or active protector? *Steroids*. 2008; 73:1039–51. [PubMed: 18501937]
22. Justo R, Boada J, Frontera M, Oliver J, Bermudez J, Gianotti M. Gender dimorphism in rat liver mitochondrial oxidative metabolism and biogenesis. *Am J Physiol Cell Physiol*. 2005; 289:C372–8. [PubMed: 15800054]
23. Mobley JA, Brueggemeier RW. Estrogen receptor-mediated regulation of oxidative stress and DNA damage in breast cancer. *Carcinogenesis*. 2004; 25:3–9. [PubMed: 14514655]
24. Seifert EL, Estey C, Xuan JY, Harper ME. Electron transport chain-dependent and -independent mechanisms of mitochondrial H₂O₂ emission during long-chain fatty acid oxidation. *J Biol Chem*. 2010; 285:5748–58. [PubMed: 20032466]
25. St-Pierre J, Buckingham JA, Roebuck SJ, Brand MD. Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J Biol Chem*. 2002; 277:44784–90. [PubMed: 12237311]
26. Du X, Edelstein D, Obici S, Higham N, Zou MH, Brownlee M. Insulin resistance reduces arterial prostacyclin synthase and eNOS activities by increasing endothelial fatty acid oxidation. *J Clin Invest*. 2006; 116:1071–80. [PubMed: 16528409]
27. Rosca MG, Vazquez EJ, Chen Q, Kerner J, Kern TS, Hoppel CL. Oxidation of Fatty acids is the source of increased mitochondrial reactive oxygen species production in kidney cortical tubules in early diabetes. *Diabetes*. 2012; 61:2074–83. [PubMed: 22586586]
28. Yamagishi SI, Edelstein D, Du XL, Kaneda Y, Guzman M, Brownlee M. Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A. *J Biol Chem*. 2001; 276:25096–100. [PubMed: 11342529]

Highlights

- Estrogen receptor β is associated with mitochondrial protein HADHB.
- Estrogen receptor β colocalizes with mitochondrial protein HADHB in mitochondria.
- Estrogen receptor β inhibits the thiolytic cleavage enzyme activity of HADHB.
- Estrogen receptors may affect oxidative stress through influencing β -oxidation.

Fig. 1.

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

Fig. 2.

ER β colocalizes with HADHB in MCF7 cells. ER β 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 ER β and HADHB with the mitochondria (C and F, respectively). (G) The MCF7 cells were stained with ER β antibody. (H) The MCF7 cells were stained with HADHB antibody. (I) Merge picture shows the colocalization of ER β with HADHB.

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