The Journal of Applied Pharmacology, 13, 227-2312005)

Effects of HIF-1α/VP16 Hybrid Transcription Factor on Estrogen Receptor in MCF-7 Human Breast Cancer Cells

Jungyoon CHO, Mikyung PARK, YoungJoo LEE*

College of Life Science, Institute of Biotechnology, Department of Bioscience and Biotechnology, Sejong University, Seoul, Korea

(Received July 28, 2005; Accepted December 5, 2005)

Abstract – The estrogen receptor (ER) is activated and degraded by estrogen. We have examined ER downregulation and activation under hypoxia mimetic conditions. Cobalt chloride induced ER downregulation at 24 h of treatment. This degradation involved hypoxia-inducible factor-1α (HIF-1α) as examined by using a constitutively active form of HIF-1α, HIF-1α/VP16, constructed by replacing the transactivation domain of HIF-1α with that of VP16. Western blot analysis revealed that E2-induced ER downregulation was observed within ~6 h, whereas HIF-1α/VP16-induced ER degradation was observed within 12~20 h. HIF-1α/VP16 activated the transcription of estrogen-responsive reporter gene in the absence of estrogen. These results suggest that ER downregulation and activation under hypoxia maybe mediated in part by a HIF-1α expression.

Key words □ estrogen receptor, downregulation, hypoxia-inducible factor-1α

INTRODUCTION

The estrogen receptor (ER) is a ligand-activated transcription factor, plays an essential role in promotion and progression of ER-positive breast cancer cells. On binding of estrogen, the ER undergoes conformational changes and modulates the transcription of target genes (Beato *et al.*, 2000; Reid *et al.*, 2002). Over 30 regulatory proteins affect the ER-mediated transcriptional response (McDonnell *et al.*, 2002). Furthermore, differential regulation of coactivator activity modulates ER signal transduction pathways via direct or indirect interactions (Shao *et al.*, 2004). While the magnitude of the estrogen-induced response depends on the initial concentration of ER(Alarid *et al.*, 1999), estrogen rapidly turns ER over with a half-life of about 3 h via the ubiquitin-proteasome pathway(Nawaz *et al.*, 1999). Due to this observation, it has been suggested that estrogen-induced proteolysis is associated with receptor activation leading to transactivation(Nawaz *et al.*, 2004; Alarid *et al.*, 2003). To understand the exact mechanism of estrogen-induced transcription activation, elucidation of the mechanisms governing ER regulation is critical to understanding the receptor's function.

Hypoxia plays an important role in normal physiological processes and development, as well as in tumorigenesis(Brahimi-Horn *et al.*, 2001). Hypoxia inducible factor-1 (HIF-1), a heterodimer consisting of HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT) subunits, is a key physiological transcription factor that regulates gene expression in response to changes in cellular oxygen tension(Semenza *et al.*, 1998). HIF-1 activation is dependent on HIF-1α, which usually decays under normoxia, but rapidly stabilizes under hypoxia. HIF-1 activates gene transcription via HIF-1 DNA binding sequences (Semenza *et al.*, 1998). However, some genes are regulated under hypoxia by HIF-1-independent mechanisms such as tissue factor and c-myc genes induced by hypoxia(Yan *et al.*, 1998; Mazure *et al.*, 2002).

ER is degraded by various stimuli other than estrogens, such as hypoxia, dioxin, and oxytocin (Stoner *et al.*, 2002; Wormke *et al.*, 2003; Cassoni *et al.*, 2002). AhR, a member of bHLH-PAS protein, interacted with ER in the presence of 2,3,7,8-tetrachlorodibenzo-p-dioxin and induced ER degradation (Wormke *et al.*, 2003). Ligand-occupied AhR degrades ER regardless of the presence of E2 via proteasome in the presence of the AhR-ER interaction (Wormke *et al.*, 2003). ER is degraded under hypoxia within 6-12 h via a proteasome-dependent pathway, which has implications for clinical treatment of mammary

*Corresponding author
Tel: 82-2-3408-3766, Fax: 82-2-3408-3334
E-mail: yjlee@sejong.ac.kr

tumors (Stoner *et al.*, 2002). In this study, we examined the ER downregulation under hypoxia-mimetic conditions and the role of HIF-1α on ER downregulation and ER-mediated transactivation. To examine the involvement of HIF-1α, we used an HIF-1α/VP16 hybrid, designed to be stable under normoxia. Using this system, we demonstrated that HIF-1α/VP16 was sufficient for inducing ER downregulation and ER-mediated transactivation.

MATERIALS AND METHODS

Reagents

17-β-estradiol (E2) and $CoCl_2$ were purchased from Sigma (St. Louis, MO). E2 was dissolved in 100% ethanol. All the compounds were added to the medium such that the total solvent concentration was never higher than 0.1%. An untreated group served as a control.

Plasmids

ERE2-tk81-luc constructed by inserting the fragment of the herpes simplex thymidine kinase promoter and two copies of the vitellogenin ERE into pA3luc (Gehm *et al.*, 1997). HIF-1α/VP16 DNA fragment was generated according to the methods published by Vincent et al (Vincent *et al.*, 2000). from Genzyme Corp. The hybrid DNA contains DNA binding/dimerization domain (amino acid 1 to 390) of HIF-1α and VP16 transactivation domain. Each fragment was PCR amplified using *Afl*II site at one end and used this site to join the two fragments. The insert was cloned into pShuttle-CMV adenoviral vector (AdEasy vector system, QBiogene) with *Not*I for further analysis.

Cell culture

ER-positive human breast adenocarcinoma, MCF-7 cells were from the Korean Cell Line Bank. MCF-7 cells were maintained in phenol red-free DMEM containing 1 × antibiotic/antimycotic mix (Invitrogen, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA). Cells were grown at 37°C in a humidified atmosphere of 95% air/5% CO_2 and fed every 2-3 days. Before treatment with chemicals or virus infection, the cells were washed with phosphate-buffered saline (PBS) and cultured in DMEM/5% charcoal-dextran stripped FBS (CD-FBS) for 2 days to eliminate any estrogenic source before treatment. All treatments were done with DMEM/5% CD-FBS.

Transient transfection and luciferase assay

Cells were seeded in 24-well plates at a density of 7×10^4 cells/well. After 24 h, plasmids were transiently transfected into the cell by calcium phosphate-DNA coprecipitation method. A total of 0.5 μg of DNA in 25 μl of $CaCl_2 \cdot H_2O$ (250 mM $CaCl_2$) was mixed with 25 μl of 2 × HBS (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, 12 mM dextrose, 50 mM HEPES) with constant bubbling and within 5 to 10 min this solution was added to each well. The next day, transfected cells were washed with PBS, and treated. Luciferase activity was determined 24 h after drug treatments with an AutoLumat LB9507 luminometer using the luciferase assay system (Promega, Madison, WI) and expressed as relative light units. The mean and standard deviation of triplicate samples are shown for representative experiments. All transfection experiments were repeated three or more times with similar results.

Construction of recombinant adenovirus and infection

Recombinant type 5 adenoviruses overexpressing HIF-1α/VP16 were constructed according to the manufacturer's protocol (Qbiogene, Carlsbad, USA). Preconstructed pShuttle-CMV adenoviral vector containing HIF-1α/VP16 recombinant gene together with the pAdEasy-1 vector containing the adenovirus serotype 5 genome with deletions in the E1 and E3 regions were transformed into BJ5183 (recA+) bacterial cells for homologous recombination between these two vectors. Recombinated AdEasy plasmid was transfected into human embryonic kidney 293A cells which contain the E1A and E1B Ad 5 viral gene necessary for viral replication. The resultant recombinant viruses were propagated in 293A cells, tittered, and stored at -80°C. MCF-7 cells were infected as follows. Cells were incubated in DMEM containing 2% CD-FBS for 24 h. The medium was then removed and the cells were exposed to recombinant adenoviruses in DMEM at 37°C for 2 h and cultured for additional 24 h in the media containing the serum.

Immunoblot analysis

Protein was isolated by using radioimmune precipitation buffer containing 150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 1% SDS with protease inhibitor cocktail (Sigma) on ice for 1 h and then centrifuged for 20 min at 13,000 g. Supernatant was collected and protein concentrations were measured using the Bradford method (Bio-Rad, CA, USA). Fifty microgram of protein was dissolved in sample buffer and boiled for 5 min prior to loading onto an 8% acrylamide gel. After SDS-PAGE, proteins were

transferred to a polyvinylidene difluoride membrane, blocked with 5% nonfat dry milk in Tris-buffered saline/0.05% Tween (TBST), and incubated with rabbit anti-polyclonal antibody to ERa (0.4 mg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse anti-monoclonal antibody to β-actin (Sigma) for 2 h at 1:500. After washing with TBST, blots were incubated with goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody and visualized with enhanced chemiluminescence ECL kits (Amersham Bioscience, Little Chalfont, UK).

RESULTS AND DISCUSSION

The Effects of estrogen on transactivation and downregulation of ER

Cellular levels of ER play key roles in controlling the magnitude of the estrogen induced response. Consistent with previous works (El Khissin *et al.*, 1999; Fan *et al.*, 2003), western blot analysis revealed that treatment with 10 nM E2 for 6 h significantly reduced ER levels in MCF-7 cells, a breast cancer

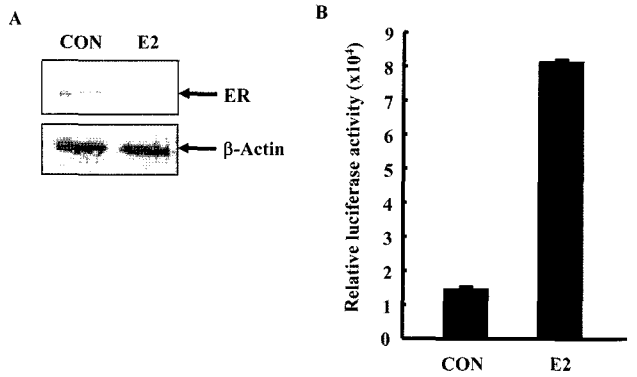

Fig. 1. Effects of E2 on ER transactivation and degradation. MCF-7 cells were incubated with 10 nM E2 for 24 h. After the incubation, the cells were lysed and total protein extracts were resolved by SDS-PAGE and immunoblotted using an anti-ERα antibody or an anti-β-actin antibody (A). MCF-7 cells were transiently transfected with ERE2-tk81-luc and treated with 10 nM E2 in phenol-red-free DMEM plus 10% CD-FBS and assayed for luciferase activity after 24 h treatments (B). Data are representative of at least three independent experiments performed in triplicate expressed as mean ± SEM.

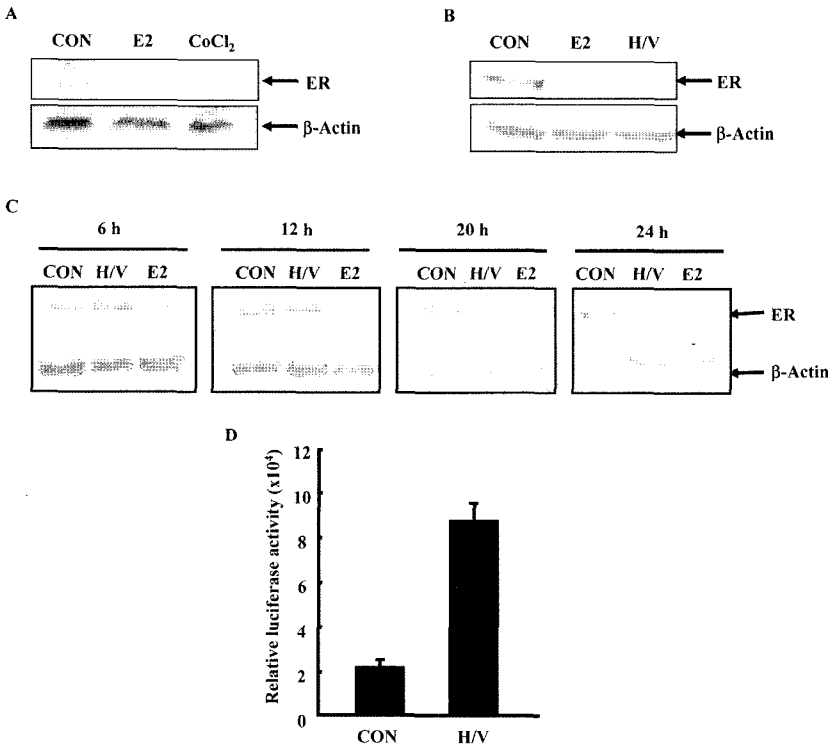

Fig. 2. Effects of HIF-1α/VP16 on ER transactivation and degradation. MCF-7 cells were incubated with 10 nM E2 or 0.5 mM $CoCl_2$ for 24 h. Total protein prepared from each cells was resolved by SDS-PAGE and analyzed for ER protein level using anti-ER or anti-β-actin antibody (A). MCF-7 cells were infected with H/V (Ad-HIF-1α/VP16) or treated with 10 nM E2 for 24 h. Total protein extracts were resolved by SDS-PAGE and immunoblotted using an anti-ER or an anti-β-actin antibody (B). MCF-7 cells were infected with H/V (Ad-HIF-1α/VP16) or treated 10 nM E2 for the indicated time. After the incubation, the cells were lysed and total protein extracts were resolved by SDS-PAGE and immunoblotted using an anti-ER antibody or an anti-β-actin antibody (C). MCF-7 cells were transiently transfected with ERE2-tk81-luc and infected with H/V (Ad-HIF-1α/VP16) and assayed for luciferase activity after 24 h infection (D). Data are representative of at least independent experiments performed in triplicate expressed as mean ± SEM.

cell line known to express both receptors endogenously (Figure-1A). However, as previously published (Nawaz *et al.*, 2004), despite the decreased level of ER protein, transactivation of ER was enhanced by E2 as examined by ERE-driven reporter assays (Figure-1B).

ER protein degradation by estrogen, cobalt chloride, HIF-1α/VP16

We examined the effects of $CoCl_2$, a hypoxic mimetic agent, on the expression of ER in the MCF-7 human breast cancer cell line. ER was downregulated by 0.5 mM $CoCl_2$ at 24 h of treatment as examined by Western blot analysis (Figure-2A). As a positive control, 10 nM 17β-estradiol (E2), a well-characterized downregulator of its own receptor, was used in parallel (Nawaz *et al.*, 1999). We then examined whether hypoxia-induced ER downregulation was dependent on the synthesis of HIF-1α. HIF-1α is rapidly degraded under normoxia via ubiquitin- and proteasome-dependent pathways and accumulates under hypoxia (Brahimi-Horn *et al.*, 2001), which hinders studies of the effects of HIF-1α expression (Hur *et al.*, 2001). To circumvent this problem, we used a constitutively stable form of HIF-1α, HIF-1α/VP16, which was initially reported by Vincent *et al* (Vincent *et al.*, 2000). This system was used to demonstrate hypoxic upregulation of the proliferator-activator-receptor γ angiopoietin-related gene mediated by HIF-1α (Belanger *et al.*, 2002). To investigate whether $CoCl_2$-induced ER downregulation occurred via HIF-1α, we examined ER proteins from MCF-7 cells that were infected with Ad-HIF-1α/VP16 for 24 h. As controls, the cells were treated in parallel with 10 nM E2 for 24 h (Figure-2B). Western blot analysis indicated that the expression of HIF-1α/VP16 hybrid protein induced ER downregulation to a level similar to that with E2 treatment (Figure-2B). These results suggest that $CoCl_2$-induced ER degradation was likely mediated by HIF-1α, and that expression of HIF-1α/VP16 under normoxia can induce ER degradation. To provide additional evidence that HIF-1α/VP16 downregulated ER, we treated cells with Ad-HIF-1α/VP16 for indicated time and analyzed Western blots for ER. As shown in Fig. 2C, ER was downregulated within 12-20 h because HIF-1α/VP16 was expressed after 12-16 h of infection. In addition, as cases with E2, HIF-1α/VP16 activated ERE-responsive luciferase plasmids (Figure-2D). These results indicate that HIF-1α/VP16 downregulates and induces ER-mediated transactivation. Activation of HIF-1 under hypoxia plays a major role in tumor biology by inducing angiogenesis (Brahimi-Horn *et al.*, 2001). It is important to elucidate the effects of hypoxia on the ER levels in breast cancer cells in treatment of breast cancer (Carherino *et al.*, 1995; Cicatiello *et al.*, 2000). Here, we report that ER is downregulated and activated by HIF-1α/VP16 in the absence of its own ligand. However, the exact mechanism of HIF-1α-induced ER degradation and the effects of that on transactivation remain to be elucidated. We are currently investigating the nature of the HIF-1-induced ER degradation and the consequence of that on the transactivation.

ACKNOWLEDGEMENTS

This work was supported by Korea research Foundation grant (KRF-2003-015-E2009, KRF-2004-041-E00383, and BK21) to YJL.

REFERENCES

Alarid, E. T., Bakopoulos, N. and Solodin, N. (1999). Proteasome-mediated proteolysis of estrogen receptor: a novel component in autologous down-regulation. *Mol. Endocrinol.*, **13**, 1522-1534.

Alarid, E. T., Preisler-Mashek, M. T. and Solodin, N. M. (2003). Thyroid hormone is an inhibitor of estrogen-induced degradation of estrogen receptor-alpha protein: estrogen-dependent proteolysis is not essential for receptor transactivation function in the pituitary. *Endocrinol.* **144**, 3469-347.

Beato, M. and Klug, J. (2000). Steroid hormone receptors: an update. *Hum. Reprod. Update*. **6**, 225-236.

Belanger, A. J., Lu, H., Date, T., Liu, L. X., Vincent, K. A., Akita, G. Y., Cheng, S. H., Gregory, R. J., and Jiang, C. Hypoxia up-regulates expression of peroxisome proliferator-activated receptor gamma angiopoietin-related gene (PGAR) in cardiomyocytes: role of hypoxia inducible factor 1alpha. *J. Mol. Cell Cardiol.*, **34**, 765-774 (2002).

Brahimi-Horn, C., Berra, E. and Pouyssegur, J. (2001). Hypoxia: the tumor's gateway to progression along the angiogenic pathway. *Trends Cell Biol.* **11**, S32-36.

Cassoni, P., Catalano, M. G., Sapino, A., Marrocco, T., Fazzari, A., Bussolati, G. and Fortunati, N. Oxytocin modulates estrogen receptor alpha expression and function in MCF7 human breast cancer cells. *Int. J. Oncol.* **21**, 375-378 (2002).

Catherino, W. H., Wolf, D. M., and Jordan, V. C. (1995). A naturally occurring estrogen receptor mutation results in increased estrogenicity of a tamoxifen analog. *Mol. Endocrinol.*, **9** 1053-1063 .

Cicatiello, L. Addeo, R. Altucci, L. Belsito, V. Petrizzi, V. Boccia, M. Cancemi, D. Germano, C. Pacilio, S. Salzano, F. Bresciani, A. Weisz, L. Cicatiello, R. Addeo, L. Altucci, Belsito, Petrizzi, V. Boccia, V. Cancemi, M., Germano, D., Pacilio, C., Salzano, S., Bresciani, F., and Weisz, A. (2000). The antiestrogen ICI 182,780 inhibits proliferation of human breast cancer cells by interfering with multiple, sequential estrogen-regulated processes required for cell cycle completion. *Mol. Cell Endocrinol.* **165**, 199-209.

Khissiin, A., El and Leclercq, G. (1999). Implication of protea-

some in estrogen receptor degradation. *FEBS Lett.* **448**, 160-166 .

Fan, M., Bigsby, R. M., and Nephew, K. P. (2003). The NEDD8 pathway is required for proteasome-mediated degradation of human estrogen receptor (ER)-alpha and essential for the anti-proliferative activity of ICI 182,780 in ERalpha-positive breast cancer cells. *Mol. Endocrinol.* **17**, 356-365.

Gehm, B. D., McAndrews, J. M., Chien, P. Y., and Jameson, J., Resveratrol, L. (1997). A polyphenolic compound found in grapes and wine, is an agonist for the estrogen receptor. *Proc. Natl. Acad. Sci. USA* **94**, 14138-14143.

Hur, E., Chang, K. Y., Lee, E., Lee, S. K. and Park, H. (2001). Mitogen-activated protein kinase kinase inhibitor PD98059 blocks the trans-activation but not the stabilization or DNA binding ability of hypoxia-inducible factor-1alpha. *Mol. Pharmacol.* **59**, 1216-1224 .

Mazure, N. M., Chauvet, C., Bois-Joyeux, B., Bernard, M. A., Nacer-Cherif, H. and Danan, J. L. (2002). Repression of alpha-fetoprotein gene expression under hypoxic conditions in human hepatoma cells: characterization of a negative hypoxia response element that mediates opposite effects of hypoxia inducible factor-1 and c-Myc. *Cancer Res.* **62**, 1158-1165.

McDonnell, D. P. and Norris, J. D. (2002). Connections and regulation of the human estrogen receptor. *Science* **296**, 1642-1644.

Nawaz, Z., Lonard, D. M., Dennis, A. P., Smith, C. L. and O'Malley, B. W. (1999). Proteasome-dependent degradation of the human estrogen receptor. *Proc. Natl. Acad. Sci. USA* **96**, 1858-1862 .

Nawaz, Z. and O'Malley, B. W. (2004). Urban renewal in the nucleus: is protein turnover by proteasomes absolutely required for nuclear receptor-regulated transcription *Mol. Endocrinol.* **18**, 493-499.

Reid, G., Denger, S., Kos, M. and Gannon, F. (2002) Human estrogen receptor-alpha: regulation by synthesis, modification and degradation. *Cell Mol. Life Sci.* **59**, 821-831.

Shao, W. and Brown, M. (2004). Advances in estrogen receptor biology: prospects for improvements in targeted breast cancer therapy. *Breast Cancer Res.* **6**, 39-52.

Semenza, G. L., Agani, F. Iyer, N. Jiang, B. H., Leung, S., Wiener, C. and Yu, A. (1998). Hypoxia-inducible factor 1: from molecular biology to cardiopulmonary physiology. *Chest* **114**, 40S-45S .

Stoner, M., Saville, B., Wormke, M., Dean, D., Burghardt, R., and Safe, S. (2002). Hypoxia induces proteasome-dependent degradation of estrogen receptor alpha in ZR-75 breast cancer cells. *Mol. Endocrinol.* **16**, 2231-2242.

Vincent, K. A., Shyu, K. G., Luo, Y., Magner, M., Tio, R. A., Jiang, C., Goldberg, M. A., Akita, G. Y., Gregory, R. J., and Isner, J. M. (2000). Angiogenesis is induced in a rabbit model of hindlimb ischemia by naked DNA encoding an HIF-1alpha/VP16 hybrid transcription factor. *Circulation* **102**, 2255-2261.

Wormke, M., Stoner, M., Saville, B., Walker, K., Abdelrahim, M., Burghardt, R. and Safe, S. (2003). The aryl hydrocarbon receptor mediates degradation of estrogen receptor alpha through activation of proteasomes. *Mol. Cell Biol.* **23**, 1843-1855.

Yan, S. F., Zou, Y. S., Gao, Y., Zhai, C., Mackman, N., Lee, S. L., Milbrandt, J., Pinsky, D., Kisiel, W. and Stern, D. (1998). Tissue factor transcription driven by Egr-1 is a critical mechanism of murine pulmonary fibrin deposition in hypoxia. *Proc. Natl. Acad. Sci. USA* **95**, 8298-8303.