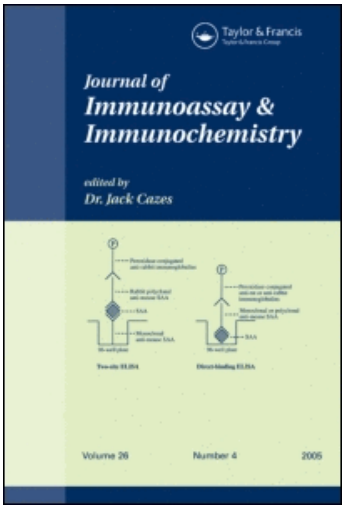

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Method to Detect Functional Estrogen Receptor Expression using Estrogen Receptor Probing Compound

Hongbiao Liu[a]; Yi Ding[a]; Rhonda R. Voskuhl[a]
[a] Department of Neurology, Reed Neurological Research Center, University of California, Los Angeles, California, USA

To cite this Article Liu, Hongbiao , Ding, Yi and Voskuhl, Rhonda R.(2005) 'Method to Detect Functional Estrogen Receptor Expression using Estrogen Receptor Probing Compound', Journal of Immunoassay and Immunochemistry, 26: 4, 295 — 301

To link to this Article: DOI: 10.1080/15321810500220894

URL: http://dx.doi.org/10.1080/15321810500220894

Journal of Immunoassay & Immunochemistry, 26: 295–301, 2005

ISSN 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810500220894

Method to Detect Functional Estrogen Receptor Expression using Estrogen Receptor Probing Compound

Hongbiao Liu, Yi Ding, and Rhonda R. Voskuhl
Department of Neurology, Reed Neurological Research Center, University of California, Los Angeles, California, USA

Abstract: A novel method to detect functional estrogen receptor expression with estrogen receptor probing compound (ERPC) has been established. ERPC is composed of estradiol-BSA-biotin. ERPC can be used to detect functional estrogen receptor expression in both Western blot and immunohistochemistry. ERPC based techniques are non-radioactive, sensitive, relatively inexpensive, and can be used with all species. The use of both ERPC and ERα- or ERβ-specific antibodies provides complementary information in characterizing estrogen receptors' expression and functional binding to ligand.

Keywords: Estradiol, BSA, Biotin, Western blot, Immunohistochemistry, Estrogen receptor

INTRODUCTION

Estrogen is a hormone which targets tissues of the reproductive system, the central nervous system, the immune system, the cardiovascular system, bone, breast, colon, and prostate. Estrogen acts on these multiple systems in a variety of ways. Estrogen exerts its effects genomically through the nuclear receptors: estrogen receptor (ER) α and ERβ. Also, ER splice variants have

Address correspondence to Rhonda R. Voskuhl, Department of Neurology, Reed Neurological Research Center, University of California, Los Angeles, CA 90095, USA. E-mail: rvoskuhl@ucla.edu

been described in both normal and pathologic tissues,[1–3] and a potential novel nuclear ER in teleosts is presently under investigation.[4] Finally, estrogen can also act non-genomically through putative membrane receptors linked to kinase cascades, calcium, and other second messenger systems.[5–8]

The importance and complexity of ERs' role in multiple systems has led to numerous investigation of ERs' tissue specific expression and function. However, existing methods of protein detection of ERs remain somewhat limited. The most widely used method detects ERs using radiolabeled estradiol (E2), specifically E2-^{125}I[9] or E2-^{3}H.[10] This method is limited by the safety disadvantages inherent in working with radioactive isotopes. A non-radioactive method, such as E2-Biotin,[11] has also been used in ELISAs. However, the sensitivity of this method (30 pg/mL) is 100-fold lower than that achieved by radioimmunoassay (0.3 pg/mL) since only one E2-Biotin binds each ER. This lower sensitivity of E2-Biotin limits its wide application in other techniques, such as Western blots or immunohistochemistry. A second non-radioactive method, widely used in flow cytometry and Western blot, employs E2 bound by FITC-labeled BSA.[12] However, this method requires the use of an expensive high performance laser scanning system, such as a Typhoon 8600 (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). A third non-radioactive and inexpensive method uses ER-specific antibodies to detect ER protein expression via Western blot and immunohistochemistry.[13,14] However, most anti-ER antibodies are not highly specific, resulting in cross reactivity in Western blot and false positives in immunohistochemistry. In addition, binding of an anti-ER antibody to an ER protein does not yield information regarding whether a given ER (or variant) in a given tissue can functionally bind E2.

Hence, there is a need for a non-radioactive, sensitive, and inexpensive method to detect ER protein expression, as well as determine ligand binding ability. Here, we propose such a method using our ER Probing Compound (ERPC).

EXPERIMENTAL

Apparatus

Bio-Rad Mini-Gel apparatus (Bio-Rad, Hercules CA) was used for SDS-PAGE. Bio-Rad Mini Trans-Blot cell (Bio-Rad, Hercules CA) was used for transferring protein to a nitrocellulose membrane. An automatic developing machine (Kodak company, Rochester, NY) was used to develop exposed film.

Materials and Reagents

Intracellular proteins were prepared using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL). ERPC was produced as

follows: biotin was conjugated to E2-BSA (Sigma, St. Louis, MO) with the EZ-link Sulfo-NHS-LC-Biotinylation kit (Pierce, Rockford, IL). Then avidin conjugated horse radish peroxidase (HRP) in VECTASTAIN ABC system (Vector Laboratories, Inc., Burlingame, CA) bound to the biotin portion of the E2-BSA-Biotin complex. SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL), a routinely used detection substrate of HRP, was then used to detect the complex. The membranes were exposed to autoradiography film (Amersham Biosciences, Piscataway, NJ). Rabbit anti-ERα primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was also used in parallel to confirm the detection of ERα by ERPC. Slides of breast fibroadenoma (Biochain, Hayward, CA) were used in immunohistochemistry.

Buffers

Washing buffer (TBS-T) consisted of Tris-buffered saline and 0.1% Tween-20. Blocking buffer was 10% non-fat milk dissolved in washing buffer.

Preparation of Conjugated E2-BSA-Biotin

Biotin was conjugated to E2-BSA with the EZ-link Sulfo-NHS-LC-Biotinylation kit (Pierce, Rockford, IL) based on the manufacturer's protocol with modification. Briefly, 1.0 mg E2-BSA was dissolved in 1.0 mL PBS and centrifuged at 10,000 rpm for 10 minutes. Supernates were collected. 1 mg Sulfo-NHS-Biotin was dissolved in 100 μL ultra-pure water. E2-BSA and Biotin solution were incubated at room temperature for 30 minutes. Biotin-labeled E2-BSA was collected based on absorbance at 280 nm.

Western Blot

To demonstrate the ability of ERPC to bind ERs, intracellular proteins were prepared using NE-PER^{TM} Nuclear and Cytoplasmic Extraction Reagents and Western blot was performed as described previously.[15] Sample proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane at 4°C, overnight, using Bio-Rad Mini Trans-Blot cell. The membranes were rinsed one time with Tris-buffered saline-0.1% Tween-20 (TBS-T), then blocked with 10% non-fat milk in TBS-T at room temperature for 2 hours. The blots were washed twice with TBS-T, then incubated with 20 ug/mL ERPC at 4°C overnight. After incubation, the blots were washed three times each, with TBS-T, for 30 minutes at room temperature. The blots were processed with the Vectastain ABC system. Then, the blots were washed four times each, with TBS-T, for 30 minutes at room temperature.

SuperSignal West Pico Chemiluminescent substrate was incubated with blots for 2 minutes at room temperature. The blots were exposed to high performance chemiluminescent film for 30 seconds. To confirm detection of ERs by ERPC, Western blot was performed in parallel with rabbit anti-ERα primary antibody.

Immunohistochemistry

Slides of breast fibroadenoma (Biochain, Hayward, CA) were deparaffinized and rehydrated. Tissue endogenous peroxidases were denatured with 3% H_2O_2 for 15 minutes at room temperature, then washed three times for 5 minutes with PBS. Sections were blocked with blocking buffer (10% BSA in PBS) for 1 hour at room temperature, then incubated with 0.5 mg/mL ERPC (or negative control: BSA-Biotin) overnight at room temperature in a humid chamber. Slides were washed 3 times for 10 minutes in PBS and developed with an ABC substrate kit. Slides were then stained with hematoxylin for 30 seconds. Slides were washed 3 times in H_2O, then mounted.

RESULTS AND DISCUSSION

Western blot was performed in parallel with ERPC and rabbit anti-ERα primary antibody. The expected size ERα (67 kDa) band in both human ovary and murine brain was identified by these two methods, and co-localized with each other, as shown in Figure 1. Results obtained with ERPC are complementary to information gained using anti-ER antibodies. ERPC demonstrates specific functional binding of an ER to estradiol, but does not differentiate between ERα and ERβ. Anti-ER antibodies do not reveal functional binding, but permit differentiation between ERα or ERβ. Hence, the use of both ERPC and ERα- or ERβ-specific antibodies provides complementary information.

In addition to Western blot, ERPC successfully detected ERs in breast fibroadenoma by immunohistochemistry, as shown in Figure 2. The ER expression was localized to the cytoplasm, not the nucleus. This observation is consistent with estrogen's mechanism of action. Unbound estrogen receptors are mainly located in the cytoplasm. Binding by ligand (E2) induces activation and a conformational change within the ERs, resulting in homodimerization or heterodimerization (α/α, β/β or α/β). The dimers are then translocated to the nucleus for gene transcription.[16] In cytoplasm, the unbound ER can bind ERPC. However, once the bound ER is translocated into the nucleus, the ligand-binding sites are occupied, thereby preventing ERPC from binding nuclear ERs.

The sensitivity of ERPC based detection methods is critically influenced by the presence of BSA within the E2:BSA:Biotin complex. Since BSA is a

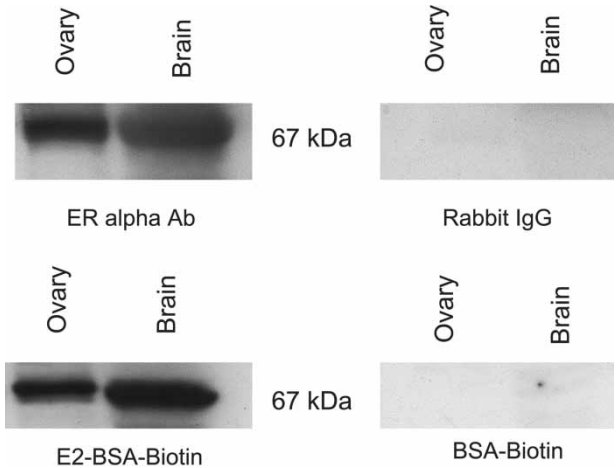

Figure 1. Detection of ERα expression in Western Blot using anti-ERα antibody and ERPC. Top panel: ERα protein expression was demonstrated in both ovary and brain by using an anti-ERα antibody to probe a Western blot. Intracellular protein was isolated, then 60 μg of human ovary and 100 μg of murine brain were loaded in each lane for Western Blot. ERα (67 KD) was demonstrated using a rabbit ERα specific primary Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) with a Biotin labeled goat anti-rabbit IgG secondary antibody. Rabbit IgG served as a primary antibody negative control. Bottom panel: ERα protein expression is confirmed, and ligand binding is shown, by probing Western blots with ERPC. Western blot of tissue proteins (as above) were probed with ERPC instead of anti-ERα antibody. A protein of the expected size for ERα (67 KD) was again detected in both human ovary and murine brain. Probing with BSA-Biotin alone served as a negative control.

bulky molecule, it could potentially partially hinder binding of E2 to the given ER.[17] However, since a single BSA molecule can bind to multiple biotin molecules, there is an amplification of signal when a single E2 binds a given ER. The fact that E2:Biotin does not have adequate sensitivity to permit its application in Western blot and immunohistochemistry, while E2:BSA:Biotin can be used in Western blot and immunohistochemistry (Figures 1 and 2), demonstrates that the effect of BSA on amplification is greater than its effect on hindrance. Thus, the net effect of the presence of BSA within ERPC is to enhance sensitivity and, thereby, permit its use in a wide range of applications.

CONCLUSION

Our novel ERPC method is not only a non-radioactive, "easy to use" detection system, but also is relevant to the investigation of function, since the binding

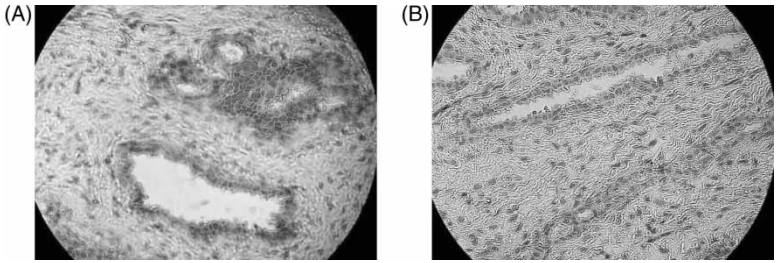

Figure 2. Detection of ERα expression by immunohistochemistry using ERPC. Light phototomicrographs of breast fibroadenoma tissue (Biochain Institute Inc. Hayward, CA) stained with ERPC is demonstrated by pink staining. Cytoplasmic staining pattern in ductal cells of breast fibroadenoma tissue (A). Negative control staining of breast fibroadenoma tissue using BSA-Biotin (B). (40X magnification).

of ERPC is dependent upon binding of the ER with its natural ligand: estradiol. ERPC can also be used to detect estrogen receptor splicing variants in numerous tissues, in various species ranging from human to mouse. ERPC also has the potential utilization in the investigation of estrogen sensitive tumors, since ER expression in some tumor tissues impacts pathogenesis and treatment. Finally, estradiol in ERPC could potentially be replaced with other estrogens (estriol, estrone) or selective estrogen receptor modulators (raloxifene, tamoxifen) to reveal the functional binding of a given detected ER to these alternative molecules.

ACKNOWLEDGMENT

The authors thank Deborah Smith for critical reading of the manuscript.

REFERENCES

1. Hirata, S.; Shoda, T.; Kato, J.; Hoshi, K. The novel isoform of the estrogen receptor-alpha cDNA (ERalpha isoform S cDNA) in the human testis. J. Steroid Biochem. Mol. Biol. **2002**, *80*, 299–305.
2. Hirata, S.; Shoda, T.; Kato, J.; Hoshi, K. Novel isoforms of the mRNA for human female sex steroid hormone receptors. J. Steroid Biochem. Mol. Biol. **2002**, *83*, 25–30.
3. Hirata, S.; Shoda, T.; Kato, J.; Hoshi, K. Isoform/variant mRNAs for sex steroid hormone receptors in humans. Trends Endocrinol. Metab. **2003**, *14*, 124–129.
4. Hawkins, M.B.; Thornton, J.W.; Crews, D.; Skipper, J.K.; Dotte, A.; Thomas, P. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc. Natl. Acad. Sci. U.S.A. **2000**, *97*, 10751–10756.
5. Levin, E.R. Cellular functions of plasma membrane estrogen receptors. Steroids **2002**, *67*, 471–475.

6. Razandi, M.; Pedram, A.; Merchenthaler, I.; Greene, G.L.; Levin, E.R. Plasma membrane estrogen receptors exist and functions as dimers. Mol. Endocrinol. **2004**, *18*, 2854–2865.
7. Toran-Allerand, C.D.; Guan, X.; MacLusky, N.J.; Horvath, T.L.; Diano, S.; Singh, M.; Connolly, E.S., Jr.; Nethrapalli, I.S.; Tinnikov, A.A. ER-X: a novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. J. Neurosci. **2002**, *22*, 8391–8401.
8. Revankar, C.M.; Cimino, D.F.; Sklar, L.A.; Arterburn, J.B.; Prossnitz, E.R. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science **2005**, *307*, 1625–1630.
9. Zheng, J.; Ramirez, V.D. Demonstration of membrane estrogen binding proteins in rat brain by ligand blotting using a 17beta-estradiol-[125I]bovine serum albumin conjugate. J. Steroid Biochem. Mol. Biol. **1997**, *62*, 327–336.
10. Tamate, K.; Charleton, M.; Gosling, J.P.; Egan, D.; Ishikawa, M.; Fottrell, P.F.; Kane, M.M. Direct colorimetric monoclonal antibody enzyme immunoassay for estradiol-17 beta in saliva. Clin. Chem. **1997**, *43*, 1159–1164.
11. Zhao, J.; Wang, Y.; Mi, J.; Li, Y.; Chang, W. Sensitive ELISA for determination of serum E2 using a new tracer E2-Biotin. J. Immunoassay Immunochem. **2003**, *24*, 369–382.
12. Monje, P.; Boland, R. Subcellular distribution of native estrogen receptor alpha and beta isoforms in rabbit uterus and ovary. J. Cell Biochem. **2001**, *82*, 467–479.
13. Kobayashi, S.; Ito, Y.; Ando, Y.; Omoto, Y.; Toyama, T.; Iwase, H. Comparison of five different antibodies in the immunohistochemical assay of estrogen receptor alpha in human breast cancer. Breast Cancer **2000**, *7*, 136–141.
14. Skliris, G.P.; Parkes, A.T.; Limer, J.L.; Burdall, S.E.; Carder, P.J.; Speirs, V. Evaluation of seven oestrogen receptor beta antibodies for immunohistochemistry, western blotting, and flow cytometry in human breast tissue. J. Pathol. **2002**, *197*, 155–162.
15. Liu, H.; MacKenzie-Graham, A.J.; Palaszynski, K.; Liva, S.; Voskuhl, R.R. "Classic" myelin basic proteins are expressed in lymphoid tissue macrophages. J. Neuroimmunol. **2001**, *116*, 83–93.
16. Hall, J.M.; Couse, J.F.; Korach, K.S. The multifaceted mechanisms of estradiol and estrogen receptor signaling. J. Biol. Chem. **2001**, *276*, 36869–36872.
17. Taguchi, Y.; Koslowski, M.; Bodenner, D.L. Binding of estrogen receptor with estrogen conjugated to bovine serum albumin (BSA). Nucl. Recept. **2004**, *2*, 5.

Received May 14, 2005
Accepted June 22, 2005
Manuscript 3170