Available online at www.sciencedirect.com

SCIENCE DIRECT®

Bioelectrochemistry 65 (2005) 149–156

Bioelectrochemistry

www.elsevier.com/locate/bioelechem

Electrochemical detection of xenoestrogenic and antiestrogenic compounds using a yeast two-hybrid-17-β-estradiol system

Adrian Schwartz-Mittelman, Anat Baruch, Tova Neufeld, Virginia Buchner, Judith Rishpon*

Department of Molecular Microbiology and Biotechnology, Tel-Aviv University, Ramat-Aviv 69978, Israel

Received 26 April 2004; received in revised form 20 July 2004; accepted 5 August 2004
Available online 28 December 2004

Abstract

The goal of this study was to determine the effects of various compounds on the 17-β-estradiol-induced dimerization of the human estrogen receptor alpha (hERα), a nuclear transcription factor. For this purpose, we used a modified yeast two-hybrid (YTH) bioassay designed to study protein–protein interactions, based on the electrochemical monitoring of hERα dimerization and detected as β-D-galactosidase reporter gene activity in a synthetic substrate *p*-aminophenyl-β-D-galactopyranoside (*p*APG). Compared with 17-β-estradiol activity, genistein, bisphenol-A (BPA), and naringenin induced dimerization to a lower extent by four, five and six magnitudes of orders of magnitude, respectively. In the presence of physiological concentrations of 17-β-estradiol, both tamoxifen and the analgesic drug acetaminophen inhibited hER dimerization in an antiestrogenic manner.

Keywords: Yeast two-hybrid; β-estradiol; Xenoestrogen; Antiestrogen; Electrochemical

1. Introduction

Estrogens are naturally occurring steroid hormones with widespread biological activities concerning growth and differentiation. Estrogens exert predominant biological effects through specific binding to the intracellular nuclear estrogen receptors (ER), ERα, and ERβ. ER activity can be modulated either by phytoestrogens and environmental pollutants known as xenoestrogens or by antiestrogenic chemicals and drugs. Endocrine-disrupting chemicals (EDCs) are found in many byproducts of industry, such as plastic, oil, and pesticides. Bisphenol-A (BPA), diethylstilbestrol (DES), and 2,4-dihydroxybenzophenone are representative of hormonally active, estrogen-mimics. Environmental exposure to xenoestrogens has been proposed as a risk factor for the disruption of reproductive development in wildlife and humans [1], as well as for the promotion of breast cancer, but the latter hypothesis remains controversial [2]. The finding of Soto et al. [3] that the EDC *p*-nonylphenol extracted from laboratory plasticware stimulates the in vitro proliferation of human estrogen-sensitive MCF-7 breast tumor cells suggested that such compounds could be potentially harmful to exposed humans and the environment at large. Similarly, Krishnan et al. [4] found that 2–5 ppb of bisphenol-A purified from autoclaved medium in polycarbonate culture flasks was sufficient to initiate the proliferation of breast cancer cells in vitro. Besides environmental EDCs, commonly used synthetic hormones and therapeutic anticancer drugs like diethylstilbestrol (DES) and tamoxifen have shown ER antagonist and agonist activity, respectively [5,6]. However, inappropriate exposure to estrogenic drugs like DES can also result in adverse effects [7].

The presence of synthetic endocrine-disrupting chemicals (EDCs), known as xenoestrogens, in the environment has led to increasing concern because such agents can modify natural endocrine function. Xenoestrogens can interfere with endocrine system function through metabolism, synthesis, secretion, and clearance [8]. Recently, limited and controversial evidence in the literature concern-

* Corresponding author. Tel.: +972 3 6409366; fax: +972 3 6409407.
E-mail address: Judithri@tauex.tau.ac.il (J. Rishpon).

1567-5394/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioelechem.2004.08.002

ing the possible antiestrogenic effects of analgesic drugs has led to an increasing awareness of their potential for estrogenlike or antiestrogenic activity. The commonly used antipyretic drug, acetaminophen, for example, exerts estrogenic or antiestrogenic effects in vitro, depending on the cell type and tissue [9]. Characterizing and comparing the mechanism of action of various drug types can contribute significantly to understanding how such agents interfere with hormone activity.

The physiological effects of estrogens are mediated by estrogen receptors. The human estrogen receptor (hERα) is a nuclear transcription factor that is naturally activated by its major endogenous ligand, 17-β-estradiol. Hormone binding to the receptor induces significant conformational changes resulting in homodimerization, translocation to the nucleus, binding to specific DNA-responsive elements (ERE), and activation or repression of gene expression [10].

One of the most powerful techniques for identifying protein–protein interactions, such as dimerization, is the yeast two-hybrid (YTH) system [6,11,12]. The assay is based on the finding that many eukaryotic transcription factors can be divided into two distinct functional domains that mediate DNA binding and transcriptional activation. Both domains contribute to dimerization. The YTH assay involves the reconstitution of beta galactosidase activity via protein–protein interactions. An ERα monomer is fused to the DNA-binding domain derived from a transcription factor (GAL*4*), and a second is fused to the activation domain of the same transcription factor. When both fused receptors are coexpressed in yeast, ER dimerization leads to the reconstitution of a functional GAL*4* transcription factor, measured as β-galactosidase activity. Commonly, the *Lac*Z gene encoding β-galactosidase is used as the reporter gene in the YTH system. The standard assays for detecting enzymatic activity are colorimetry [13], fluorometry, and luminometry [14]. Using the YTH system, Nishikawa et al. [15] identified several chemicals possessing estrogenic activity.

Highly sensitive electrochemical methods were used to detect β-galactosidase activity, using as substrate *p*-aminophenyl β-D-galactopyranoside. The product of the enzymatic reaction *p*-aminophenol (PAP) is oxidized at an electrode as shown in Scheme 1:

In the solution:

$$\text{PAPG} + H_2O + \beta\text{-galactosidase} \longrightarrow \text{PAP} + \beta\text{-D galactopyranoside}$$

and at the electrode:

$$\text{PAP} \longrightarrow \text{p-imminoquinone} + 2H + 2e^-$$

Scheme 1.

The electrochemical characteristics of PAP have been described before for immunodetection assays [16–18], as well as for bacterial systems [19,20]. The detection limit of the enzyme is 1 ng/ml.

In the present study, we used a sensitive modified YTH electrochemical bioassay developed in our laboratory [21] to characterize hERα dimerization induced by natural estrogens, phytoestrogens, xenoestrogens, EDCs, and the commonly used anticancer drug, tamoxifen. In addition, we identified ER antagonist activity of the analgestic drug, acetaminophen in the electrochemical modified YTH system. The drug inhibited the 17-β-estradiol-induced dimerization of human hERα at physiological concentrations of estradiol (10^{-11}–10^{-12} M). The inhibition was assessed by a reduction of the corresponding activity of the reporter enzyme, monitored by electrochemical measurements. The results indicate that like tamoxifen, acetaminophen inhibits hER dimerization in an antiestrogenic manner.

2. Materials and methods

2.1. Media for yeast cultures

LB, SD minimal media, YPD and YPDA (Clontech laboratories, USA): Difco peptone, agar (Difco, USA), yeast nitrogen base without amino acid, -Leu/-Trp Dropout Supplement (SD; Clontech laboratories, USA), yeast extract (Becton Dickinson, USA), adenine, and dextrose (Sigma) were prepared according to a yeast protocols handbook (Clontech). YPDA and minimal SD plates contained 2% agar.

2.2. Yeasts and plasmids

The *Saccharomyces cerevisiae* strainY-187 was purchased from Clontech Laboratories, Palo Alto, CA, USA. The plasmids pPC-86 and pPC-62 containing the cDNA of hERα were generous gifts from Prof. Sohaib A. Kahn (University of Cincinnati, OH, USA). Positive control plasmids pGBKT7-53 and pGADT7-T were purchased from Clontech Laboratories.

2.3. Chemicals

17-β-Estradiol, bisphenol A, diethylstilbestrol, genistein, naringenin, 2,4-dihydroxybenzophenone, *p*-aminophenyl-β-D-galactopyranoside (*p*APG), tamoxifen, and β-cyclodextrin were purchased from Sigma, Israel. Stock solutions of the steroids were prepared by dissolving in ethanol followed by diluting in aqueous solution. The organic solvent was diluted by at least three orders of magnitude. Sodium lauroyl sarcosinate (SLS) and acetaminophen were purchased from Fluka, Switzerland.

2.4. The electrochemical setup

Screen-printed electrodes (SPEs) were purchased from Gwent, UK. The SPEs contained three electrodes: carbon ink working electrode, carbon ink counter electrode, and

Ag/AgCl reference electrode. Multichanneled potentiostat capable of simultaneously analyzing eight samples was constructed at the Technion-IIT, Haifa, Israel. All measurements were performed at 220-mV applied potential.

2.5. Yeast two-hybrid preparation

Yeast strain Y-187 (*S. cerevisiae*) carrying the GAL1 promoter-*lac*Z reporter gene transformed with plasmids pPC-86 and pPC-62, containing the cDNA of human estrogen receptor (hER) fused to the GAL-4 DB (GAL4 DNA-binding domain–human ERα) and GAL-4 TA (GAL4 transactivation domain–human ERα) domains, were used for the yeast two-hybrid (YTH) system. As a positive control, we used plasmids pGBKT7-53 and pGADT7-T (Clontech), which encode fusion between the GAL4 DNA-binding domain/activating domain and the murine p53 tumor suppressor protein and the SV-40 large T-antigen, respectively, which interact in the YTH assay (Clontech Matchmaker 3 Manual). Transformation of the yeast was carried out using a standard, lithium acetate method with plasmid DNA, according to the instructions of the Clontech Matchmaker 3 System. Selection of transformed yeast was done on SD minimal medium lacking tryptophan and leucine, solidified with 2% agar.

2.6. Yeast cells exposure to xenoestrogens and phytoestrogens

Yeast cultivation was obtained by inoculating 50 ml sterile plastic test tubes containing 4 ml of SD medium with 100 μl of a starter culture of transformed yeast. To each test tube, one of the following chemicals was added according to the experiment: 17-β-estradiol, 2,4 dihydroxybenzophenone, diethylstilbestrol (DES), bisphenol-A (BPA), genistein, or naringenin at different concentrations ranging from 10^{-3} to 10^{-11} M, in the presence of equimolar concentrations of β-cyclodextrin. β-Cyclodextrin lowers the toxic effect of the chemical on the cells. The test tubes were vigorously shaken overnight at 30 °C. A low sublethal concentration (5 mg/l) of the membrane permeabilizer sodium lauroyl sarcosinate was also added to the liquid medium tubes during overnight growth to improve membrane permeability toward 17-β-estradiol and other chemicals.

2.7. Yeast cells exposure to antiestrogenic drugs: tamoxifen and acetaminophen

Different concentrations of tamoxifen (10^{-5} to 10^{-11} M) or acetaminophen (10^{-3} to 10^{-6} M) were added to 4 ml of SD medium inoculated with 100 μl of a transformed yeast starter culture in the presence or in the absence of different concentrations of 17-β-estradiol (10^{-10}–10^{-11} M), or as needed. A sublethal concentration (5 mg/l) of SLS was added to the yeast culture during overnight growth. The tubes were incubated overnight with vigorous shaking at 30 °C.

2.8. Preparation of yeast cells for the β-galactosidase assay

Measuring the optical density at 600 nm assessed the concentration of the overnight-cultivated yeast cells. The overnight cell concentration of the culture was approximately 1.5×10^8 cfu/ml. The concentration was equalized in all test tubes by adding growth medium to the more concentrated cultures. To remove the growth medium and any soluble organic residues, the yeast cells were separated by centrifugation for 10 min at $3000\times g$, washed, and suspended in 2 ml of PBS buffer, supplemented with 2 g/l SLS, which served as a permeabilizing agent for the yeast membranes. The test tubes were intermittently vortexed for 30 min at room temperature. Before the electrochemical assay, the cells were concentrated by a factor of 8 or 12, as necessary. The number of cells per assay was 3.2×10^8 and 4.9×10^8 cfu/270 μl, respectively.

2.9. Electrochemical determination of β-galactosidase activity

The electrochemical assay was carried out with screen-printed disposable electrodes, with 300-μl volume plastic microwells combined on top of the electrodes. Mixing of the liquid in each microwell was achieved by a suction/injection mechanism manufactured in our laboratory, operated at a frequency of approximately 12 Hz. *p*-Aminophenyl-β-D-galactopyranoside (*p*APG), at a final concentration of 0.8 mg/ml, was used as a synthetic substrate for the β-galactosidase yeast reporter gene. The product, *p*-aminophenol, was oxidized at an applied potential of 220 mV, and the electrical current generated was recorded. Results were calculated as Δcurrent/Δtime from the direct measurements during the first 20 min. The data obtained were also verified by standard optical techniques using the substrate *o*-nitrophenyl-β-D-galactopyranoside [22].

3. Results and discussion

Our goal was to determine the effects of various estrogens, xenoestrogens, phytoestrogens, as well as steroidal and nonsteroidal drugs on the estradiol-induced dimerization of human estrogen receptor alpha (hERα). Most xenoestrogens have the ability to bind the ER and interfere with the natural function of the endocrine system. We investigated this phenomenon at the molecular level, using a modified yeast two-hybrid (YTH) system with electrochemical detection, in which β-galactosidase activity is under estrogen control through ER dimerization. Using this system in an earlier study, we demonstrated that the binding of the hormone, 17-β-estradiol, to its specific ERα receptor induces its dimerization [21].

3.1. Electrochemical measurement of xenoestrogens and phytoestrogens

As the electrochemically modified YTH system selectively examines the estradiol-induced dimerization of the hormone receptor, we first evaluated the residual activity of the β-galactosidase reporter signal by applying a number of well-known xenoestrogens 2,4 dihydroxybenzophenone, diethylstilbestrol (DES), bisphenol A (BPA), and two phytoestrogens, genistein and naringenin. 2,4-Dihydroxybenzophenone is a member of the benzophenones (BPs), chemicals widely found in products for agriculture, insecticides, and pharmaceuticals. Besides being toxic, this chemical shows typical estrogenic activity [23,24]. Yeast cells were incubated overnight with different concentrations of 2,4 dihydroxybenzophenone. To increase the sensitivity of the β-galactosidase assay, we added the permeabilizing agent sodium lauroyl sarcosinate (SLS), which serves a dual purpose: (1) increases permeability during cell growth (5 mg/l) and (2) aids in permeabilization before the β-galactosidase assay (2 g/l). The test tubes were intermittently vortexed for 30 min at room temperature. This permeabilization method was more reproducible and yielded a higher signal than other established methods, such as using ethanol or toluene [21]. Figs. 1 and 2, respectively, show the amperometric measurements at a working potential of 220 mV of enzyme activity generated by yeast cultures exposed to 2,4 dihydroxybenzophenone or to 17-β-estradiol. The high sensitivity of our modified electrochemical YTH assay revealed typical estrogenic activity for 2,4 dihydroxybenzophenone. The lowest concentration of the compound detected in our system was 10^{-8} M, which is in accordance with prior results shown by Schultz et al. [25].

Bisphenol-A is an important raw material used in the plastic industry for polycarbonate resins and for varnish plating. According to the United States Environmental Protection Agency, BPA is cytotoxic, toxic to fish and amphibians, and interferes with the endocrine system by binding to the ER [4,26]. Therefore, we tested the ability of BPA to induce receptor dimerization in our electrochemical YTH system. The results (Table 1) show that compared with 17-β-estradiol, BPA induced dimerization of the hormone to a lesser extent and exhibited lower activity by five orders of magnitude. The lowest concentration measured was 10^{-6} M for BPA, whereas that of 17-β-estradiol was in the picomolar range, 10^{-11} M. Similar results were reported by Ike et al. [27] and Rehman et al. [28].

Diethylstilbestrol, a xenoestrogenic drug once prescribed during pregnancy to prevent miscarriages or premature deliveries, was subsequently discarded for human use because of teratogenicity [7]. This compound served as an example for the estrogenic activity of synthetic estrogens in our system. The DES-induced

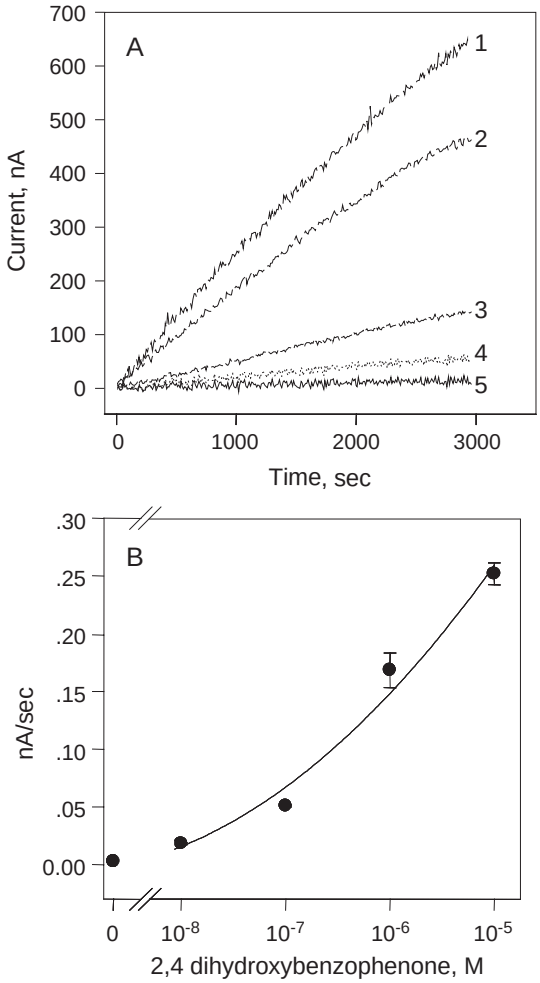

Fig. 1. (A) Electrochemical signals obtained with yeast cultures exposed overnight to different concentrations of 2,4 dihydroxybenzophenone: (1) 10^{-5}, (2) 10^{-6}, (3) 10^{-7} and (4) 10^{-8} M. (B) Enzymatic activities (●) shown as Δcurrent/Δtime.

activity of β-galactosidase expressed in yeast cell cultures incubated with different concentrations of DES is shown in Table 1. The expression of 17-β-estradiol- and DES-induced estrogenic activity at the same order of magnitude implies a similar induction of receptor dimerization by both compounds, which is in accordance with reports by Fohmar et al. [29] and Jefferson et al. [30]. Compared with 17-β-estradiol, genistein and naringenin had less estrogenic activity by four orders of magnitude (10^{-8} M) and by six magnitudes of orders of magnitude, respectively (Table 1). Similar results were reported by Schultz et al. [25].

3.2. Measurement of antiestrogenic drugs

One way that antiestrogenic drugs can interfere with dimerization is by competing with 17-β-estradiol for binding to the ER. We examined such a drug, the well-characterized anticancer agent tamoxifen in our electrochemical YTH assay. We cultured yeast cells with 10^{-11}

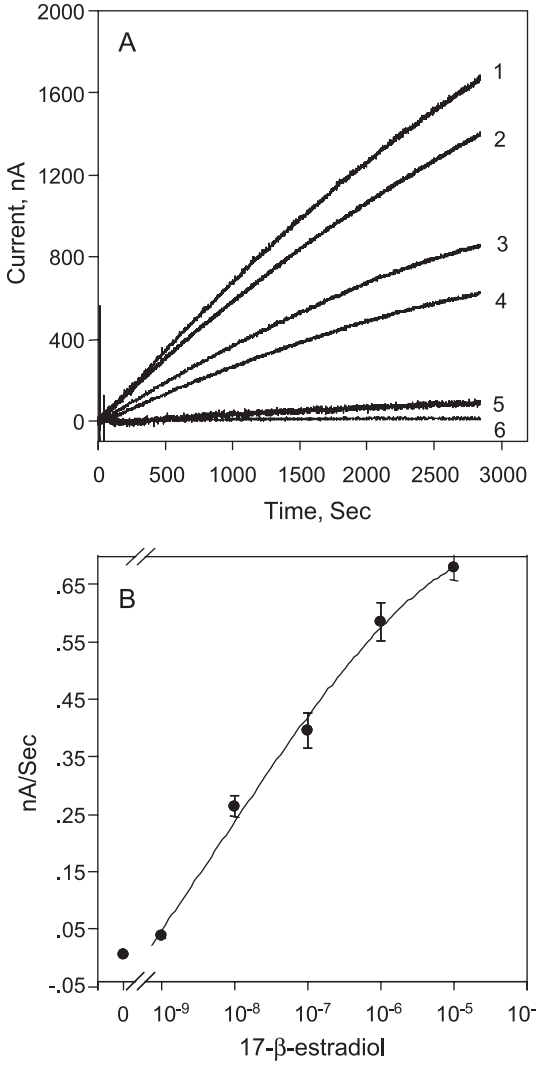

Fig. 2. Electrochemical signals obtained with yeast cultures exposed overnight to different concentrations of 17-β-estradiol: (1) 10^{-5}, (2) 10^{-6}, (3) 10^{-7}, (4) 10^{-8} and (5) 10^{-9} M. (B) Enzymatic activities (•) shown as Δcurrent/Δtime.

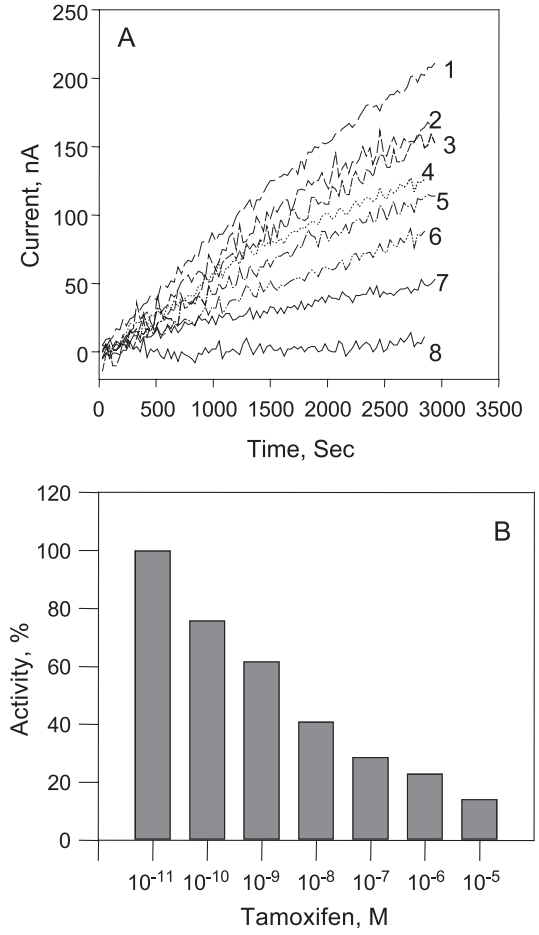

Fig. 3. (A) Electrochemical signals obtained with yeast cultures exposed overnight to constant concentration of 10^{-11} M 17-β-estradiol in the presence of different concentrations of tamoxifen: (1) 10^{-5}, (2) 10^{-6}, (3) 10^{-7}, (4) 10^{-8}, (5) 10^{-9}, (6) 10^{-10} and (7) 10^{-12} M. (B) Inhibition of hERα receptor dimerization calculated by percentage of the residual activity of the reporter enzyme, β-galactosidase.

M estradiol (physiological concentration) and measured the inhibitory effect of various concentrations of tamoxifen. The results in Fig. 3 shows that almost 25% inhibition of β-galactosidase activity occurred at a low concentration of this drug (10^{-11} M) and about 85% inhibition at the highest concentration (10^{-6} M), reflecting significant decrease of ER dimerization. The results agree with findings in other reports that tamoxifen is a partial antagonist that competes with estradiol for binding to the ER [6].

Table 1
Summary of results displayed as Δcurrent/Δtime (nA/sec), for yeast cells exposed overnight to various xenoestrogens and 17-β-estradiol measured with our electrochemical two-hybrid system

Concentration (M)	Naringenin	BPA	Genistein	2,4dihydroxy BP	DES	17-β-estradiol
10^{-3}	0.008±0.11					
10^{-4}	0.005±0.053					
10^{-5}	0.0014±0.03	0.031±0.33	0.67±0.085	0.0095±0.25		
10^{-6}		0.002±0.05	0.009±0.045	0.015±0.17	0.034±0.58	
10^{-7}			0.0035±0.035	0.0099±0.051	0.078±0.54	
10^{-8}				0.0011±0.018	0.025±0.42	
10^{-9}					0.015±0.17	
10^{-10}					0.0004±0.037	0.29±0.0027
10^{-11}						0.11±0.027

Each result represents the mean of three measurements.

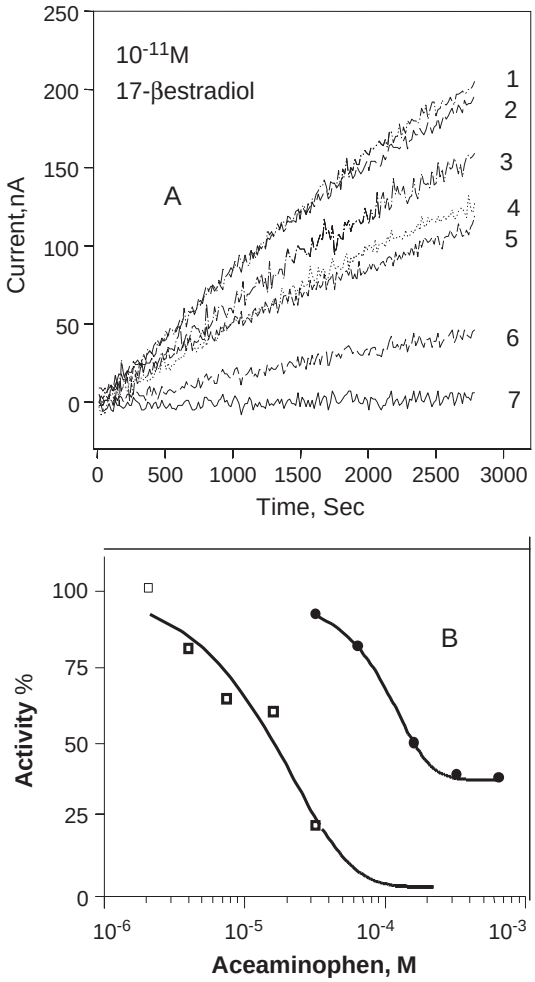

Fig. 4. Electrochemical signals obtained with yeast cultures exposed overnight to different concentrations of acetaminophen. (A) Enzyme activity obtained in the presence of 10^{-11} M 17-β-estradiol with (1) 0 M acetaminophen, (2) 2.1×10^{-6} M, (3) 4.1×10^{-6} M, (4) 8.3×10^{-6} M, (5) 1.6×10^{-5} Mm (6) 3.3×10^{-5} M and (7) control-yeast cells. (B) Inhibition of hERα receptor dimerization in the presence of 10^{-10} M (●) and 10^{-11} M (□) calculated by percentage of the residual activity of the reporter enzyme, β-galactosidase.

Although the toxic effects of high doses of a widely used analgesic and antipyretic drug, acetaminophen, on liver and kidney are well characterized [31,32], relatively few studies have been reported on the effects of acetaminophen on the endocrine system. Evidence concerning the possible estrogenic or antiestrogenic activity of acetaminophen is sparse and controversial. In vitro, acetaminophen shows both estrogenic and antiestrogenic properties, depending on the system applied. [9,33–36]. Interestingly, acetaminophen shows neither estrogenic nor antiestrogenic activity in uteri of mice or rats in vivo [20]. Therefore, we decided to examine the effect of acetaminophen in our electrochemical modified YTH system. As physiologically relevant concentrations (10^{-10} to 10^{-11} M) of 17-β-estradiol are the most interesting and noteworthy [10], we measured the effects of various concentrations of acetaminophen at physiological levels of the hormone. The assay revealed that low concentrations of acetaminophen inhibit estrogen receptor dimerization in the presence of physiological concentrations of 17-β-estradiol (Fig. 4). The inhibition was dependent on estrogen and acetaminophen concentrations and was not a result of toxic effects exerted by acetaminophen on yeast cells (Fig. 5). An acetaminophen concentration of 3.31×10^{-5} M produced almost 40% inhibition, at a hormone level of 10^{-10} M and almost 80% inhibition of the signal at a hormone level of 10^{-11} M. Significant inhibition (~20%) occurred even at extremely low concentrations of acetaminophen (as low as 4.1×10^{-6} M), which are much lower than the serum concentration of 1.32×10^{-4} M typically reported for this drug and far below toxic serum levels [37]. As dimer formation is essential for the estrogen-mediated activation of the ER [38], we assume that despite the absence of binding, acetaminophen acts as an antiestrogen in our modified YTH system. Our findings agree with the report from Miller et al. [33] demonstrating the

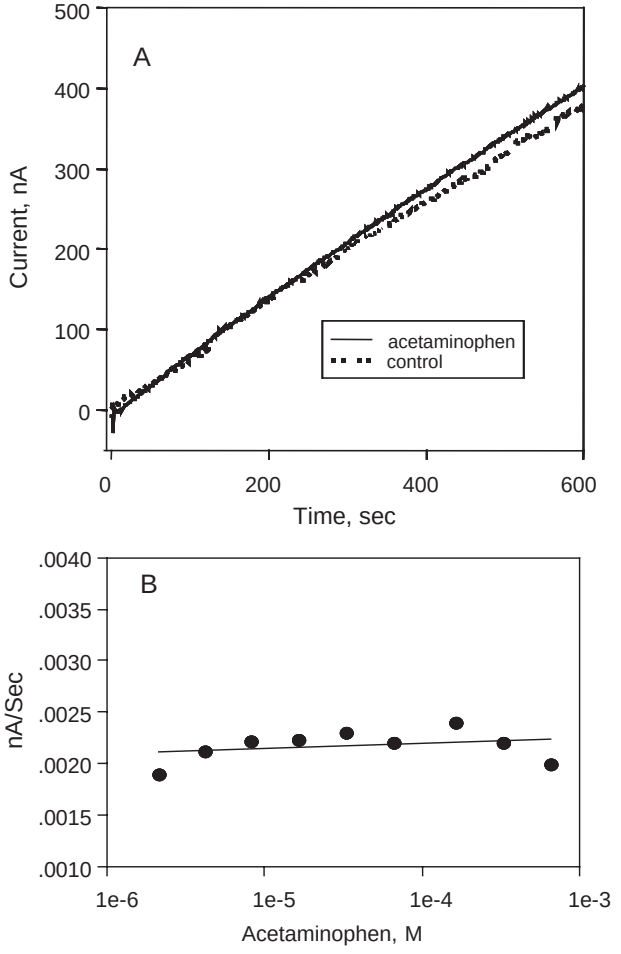

Fig. 5. (A) Electrochemical signals obtained with yeast cultures transformed with plasmids pGBT7-53 and pGADT7-7 (control yeasts) containing proteins, which react constitutively, in the absence of 17-β-estradiol and in the presence (solid line) or absence (dashed line) of 6.6×10^{-4} M acetaminophen. (B) Control yeasts grown in the presence of different concentrations of acetaminophen ranging from 6.6×10^{-4} to 2.1×10^{-6} M.

antiestrogenic properties of acetaminophen in an estrogen-dependent system.

4. Conclusions

In this paper, we offer a sensitive method based on the combination of electrochemical measurements and the YTH system especially designed for detecting the impact of estrogenic and xenoestrogenic chemicals on hERα dimerization. The results are compatible with those obtained using optical methods typical for xenoestrogenic effects in yeast systems. As electrochemical measurements contribute the advantage of working with a turbid solution, higher concentrations of yeasts can be achieved by additional preconcentration or alternatively by using electrochemical techniques to attain greater sensitivity.

Extremely important results obtained here concerned the widely used drug acetaminophen. We have shown for the first time that this drug acts at the molecular level, affecting the hER receptor. Acetaminophen markedly inhibited the dimerization of hERα cloned in the electrochemical modified YTH system, exhibiting an inhibitory effect similar to that of the common anticancer drug tamoxifen, and was not due to the toxicity of acetaminophen on yeast cells. The inhibition was mutually concentration dependent for 17-β-estradiol and acetaminophen and was measurable at a concentration as low as 4.1×10^{-6} M of the drug. Our results provide the rationale for further investigations on the clinical potential of acetaminophen in ER-dependent physiology.

References

[1] T. Colborn, F.S.V. Saal, A.M. Soto, Developmental effects of endocrine-disrupting chemicals in wildlife and humans, Environmental Health Perspectives 101 (1993) 378–384.

[2] A.K. Mitra, F.S. Faruque, A.L. Avis, Breast cancer and environmental risks: where is the link? Journal of Environmental Health 66 (2004) 24–32.

[3] A.M. Soto, H. Justicia, J.W. Wray, C. Sonnenschein, *Para*-nonylphenol—an estrogenic xenobiotic released from modified polystyrene, Environmental Health Perspectives 92 (1991) 167–173.

[4] A.V. Krishnan, P. Stathis, S.F. Permuth, L. Tokes, D. Feldman, Bisphenol-A—an estrogenic substance is released from polycarbonate flasks during autoclaving, Endocrinology 132 (1993) 2279–2286.

[5] R.M. O'Regan, V.C. Jordan, The evolution of tamoxifen therapy in breast cancer: selective oestrogen-receptor modulators and down-regulators, Lancet Oncology 3 (2002) 207–214.

[6] H. Wang, G.A. Peters, X. Zeng, M.L. Tang, I. Wallace, S.A. Khan, Yeast 2-hybrid system demonstrates that estrogen-receptor dimerization is ligand-dependent in-vivo, Journal of Biological Chemistry 270 (1995) 23322–23329.

[7] K.E. Malone, Diethylstilbestrol (Des) and breast-cancer, Epidemiologic Reviews 15 (1993) 108–109.

[8] C.J. Kirk, L. Bottomley, N. Minican, H. Carpenter, S. Shaw, N. Kohli, M. Winter, E.W. Taylor, R.H. Waring, F. Michelangeli, R.A. Harris, Environmental endocrine disrupters dysregulate estrogen metabolism and Ca^{2+} homeostasis in fish and mammals via receptor-independent mechanisms, Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology 135 (2003) 1–8.

[9] J. Dowdy, S. Brower, M.R. Miller, Acetaminophen exhibits weak antiestrogenic activity in human endometrial adenocarcinoma (Ishikawa) cells, Toxicological Sciences 72 (2003) 57–65.

[10] C.J. Gruber, W. Tschugguel, C. Schneeberger, J.C. Huber, Mechanisms of disease—production and actions of estrogens, New England Journal of Medicine 346 (2002) 340–352.

[11] S. Fields, O.K. Song, A novel genetic system to detect protein–protein interactions, Nature 340 (1989) 245–246.

[12] R.C. Newton, C.P. Decicco, Therapeutic potential and strategies for inhibiting tumor necrosis factor-alpha, Journal of Medicinal Chemistry 42 (1999) 2295–2314.

[13] J.A. Miller, Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria, Cold Spring Harbor Laboratory Press, NY, 1992, 72–74 pp.

[14] V.K. Jain, I.T. Magrath, A chemiluminescent assay for quantitation of beta-galactosidase in the femtogram range-application to quantitation of beta-galactosidase in iacz-transfected cells, Analytical Biochemistry 199 (1991) 119–124.

[15] J. Nishikawa, K. Saito, J. Goto, F. Dakeyama, M. Matsuo, T. Nishihara, New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator, Toxicology and Applied Pharmacology 154 (1999) 76–83.

[16] J. Kulys, V. Razumas, A. Malinauskas, Electrochemical oxidation of catechol and *para*-aminophenol esters in the presence of hydrolases, Bioelectrochemistry and Bioenergetics 7 (1980) 11–24.

[17] M. Masson, Z. Liu, T. Haruyama, E. Kobatake, Y. Ikariyama, M. Aizawa, Immunosensing with amperometric detection, using galactosidase as label and *P*-aminophenyl-beta-D-galactopyranoside as substrate, Analytica Chimica Acta 304 (1995) 353–359.

[18] I. Rosen, J. Rishpon, Alkaline-phosphatase as a label for a heterogeneous immunoelectrochemical sensor—an electrochemical study, Journal of Electroanalytical Chemistry 258 (1989) 27–39.

[19] D.L. Scott, S. Ramanathan, W.P. Shi, B.P. Rosen, S. Daunert, Genetically engineered bacteria: electrochemical sensing systems for antimonite and arsenite, Analytical Chemistry 69 (1997) 16–20.

[20] I. Biran, L. Klimentiy, R. Hengge-Aronis, E.Z. Ron, J. Rishpon, On-line monitoring of gene expression, Microbiology-UK 145 (1999) 2129–2133.

[21] A. Schwartz-Mittelmann, T. Neufeld, D. Biran, J. Rishpon, Electrochemical detection of protein–protein interactions using a yeast two hybrid: 17-beta-estradiol as a model, Analytical Biochemistry 317 (2003) 34–39.

[22] P.K. Birmingham, M.J. Tobin, D.M. Fisher, T.K. Henthorn, S.C. Hall, C.J. Cote, Initial and subsequent dosing of rectal acetaminophen in children—a 24-hour pharmacokinetic study of new dose recommendations, Anesthesiology 94 (2001) 385–389.

[23] H.G. Gonzalez, A. Farbrot, O. Larko, Percutaneous absorption of benzophenone-3, a common component of topical sunscreens, Clinical and Experimental Dermatology 27 (2002) 691–694.

[24] Y. Nakagawa, T. Suzuki, S. Tayama, Metabolism and toxicity of benzophenone in isolated rat hepatocytes and estrogenic activity of its metabolites in MCF-7 cells, Toxicology 156 (2000) 27–36.

[25] T.W. Schultz, G.D. Sinks, M.T.D. Cronin, Structure–activity relationships for gene activation oestrogenicity: evaluation of a diverse set of aromatic chemicals, Environmental Toxicology 17 (2002) 14–23.

[26] K. Satoh, F. Nagai, N. Aoki, Several environmental pollutants have binding affinities for both androgen receptor and estrogen receptor alpha, Journal of Health Science 47 (2001) 495–501.

[27] M. Ike, M.Y. Chen, C.S. Jin, M. Fujita, Acute toxicity, mutagenicity, and estrogenicity of biodegradation products of bisphenol-A, Environmental Toxicology 17 (2002) 457–461.

[28] K. Rehmann, K.W. Schramm, A.A. Kettrup, Applicability of a yeast oestrogen screen for the detection of oestrogen-like activities in environmental samples, Chemosphere 38 (1999) 3303–3312.

[29] L.C. Folmar, M.J. Hemmer, N.D. Denslow, K. Kroll, J. Chen, A. Cheek, H. Richman, H. Meredith, E.G. Grau, A comparison of the estrogenic potencies of estradiol, ethynylestradiol, diethylstilbestrol, nonylphenol and methoxychlor in vivo and in vitro, Aquatic Toxicology 60 (2002) 101–110.
[30] W.N. Jefferson, E. Padilla-Banks, G. Clark, R.R. Newbold, Assessing estrogenic activity of phytochemicals using transcriptional activation and immature mouse uterotrophic responses, Journal of Chromatography B—Analytical Technologies in the Biomedical and Life Sciences 777 (2002) 179–189.
[31] R.C. Blantz, Acetaminophen: acute and chronic effects on renal function, American Journal of Kidney Diseases 28 (1996) S3–S6.
[32] A. McGregor, L.J. More, K.J. Simpson, D.J. Harrison, Liver death and regeneration in paracetamol toxicity, Human and Experimental Toxicology 22 (2003) 221–227.
[33] M.R. Miller, E. Wentz, S. Ong, Acetaminophen alters estrogenic responses in vitro: inhibition of estrogen-dependent vitellogenin production in trout liver cells, Toxicological Sciences 48 (1999) 30–37.
[34] S.L. Gadd, G. Hobbs, M.R. Miller, Acetaminophen-induced proliferation of estrogen-responsive breast cancer cells is associated with increases in *c*-myc RNA expression and NF-kappa B activity, Toxicological Sciences 66 (2002) 233–243.
[35] E. Harnagea-Theophilus, S.L. Gadd, A.H. Knight-Trent, G.L. DeGeorge, M.R. Miller, Acetaminophen-induced proliferation of breast cancer cells involves estrogen receptors, Toxicology and Applied Pharmacology 155 (1999) 273–279.
[36] E. Harnagea-Theophilus, M.R. Miller, Acetaminophen alters estrogenic responses in vitro: stimulation of DNA synthesis in estrogen-responsive human breast cancer cells, Toxicological Sciences 46 (1998) 38–44.
[37] M.E. Stocker, J.E. Montgomery, Serum paracetamol concentrations in adult volunteers following rectal administration, British Journal of Anaesthesia 87 (2001) 638–640.
[38] V. Kumar, P. Chambon, The estrogen-receptor binds tightly to its responsive element as a ligand-induced homodimer, Cell 55 (1988) 145–156.