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Developing a Biosensor for Estrogens in Water Samples: Study of the Real-time Response of Live Cells of the Estrogen-sensitive Yeast Strain RMY/ER-ERE using Fluorescence Microscopy

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Abstract

Using a fluorescein di-β-D-galactopyranoside (FDG) substrate we show that in live cells of an estrogen-sensitive yeast strain RMY/ER-ERE with human estrogen receptor (ERα) gene and the *lacZ* gene which encodes β-galactosidase, the uptake of 17 β-estradiol (E2) and the subsequent production of β-galactosidase enzyme occur quite rapidly, with maximal enzyme-catalyzed product formation evident after about 30 minutes of exposure to E2. This finding which agrees with the well-known rates of enzyme-catalyzed reactions could have implications for shortening the duration of environmental sample screening and monitoring regimes using yeast-based estrogen assays, and the development of biosensors for environmental estrogens to complement quantification methods.

Keywords: Biosensor, Estrogen, Yeast, Live cell response, Environmental monitoring, Fluorescence

1. Introduction

Nuclear steroid receptors have been widely studied and their proposed mechanisms of action comprehensively reviewed (DeFranco, 2002; Losel et al., 2003; Nilsson and Gustafsson, 2000; Nilsson and Gustafsson, 2002; Shapiro, 2003). Pathways following the generally proposed model are termed classic, nuclear or genomic (Losel and Wehling, 2003; Losel et al., 2003). In the classic model, un-liganded estrogen receptors in the nucleus move rapidly. When bound with ligand the activated estrogen-estrogen receptor dimers move fast searching for hormone-binding domains in the nucleus termed estrogen response elements (EREs) located upstream of the promoters for transcription of specific proteins (DeFranco, 2002). The estrogen-induced signaling pathways are highly conserved in yeast and mammalian cells (Pham et al., 1992), making yeast-based assays promising in modeling the cellular response of mammalian cells exposed to estrogenic compounds. Yeast cells also have other advantages for use as whole cell biosensors including physical robustness, rapid growth, and ease of manipulation (Baronian, 2004).

The yeast strain RMY/ER-ERE was used in this study. The construct harbors genes for the wild-type human estrogen receptor α (ERα), estrogen response elements (EREs) and the *lacZ* gene encoding β-galactosidase (Liu and Picard, 1998). The RMY/ER-ERE strain produces β-galactosidase, an enzyme which catalyses the hydrolysis of lactose, on exposure to 17 β-estradiol (E2). The strain was used in a yeast-based assay with the chromogenic substrate ortho-nitrophenol β-D-galactopyranoside (ONPG), to detect the estrogenic activity induced by E2 exposure in aqueous samples (Figure 1).

In the assay, the yeast cells were incubated with the sample for 20 hr before the cells were lysed

and the ONPG was hydrolyzed to yellow product ortho-nitrophenol (ONP). The amount of ONP produced in the reaction catalyzed by the released β-galactosidase enzyme in 6 min was quantified to obtain an estimate of the enzyme activity. This transcription-driven incubation time is much longer than reported times of 60-180 min for gene expression of proteins observed and modeled for live cells (Hargrove et al., 1991; Rogatsky et al., 1997; Rories and Spelsberg, 1989), so we set out to establish the real-time response of the strain RMY/ER-ERE after exposure to E2. Our goal was to reduce the time requirement when using the yeast as a biosensor for estrogens in aqueous samples. This approach was based on the hypothesis that the response of live estrogen-responsive yeast cells to E2 in aqueous samples is rapidly detectable.

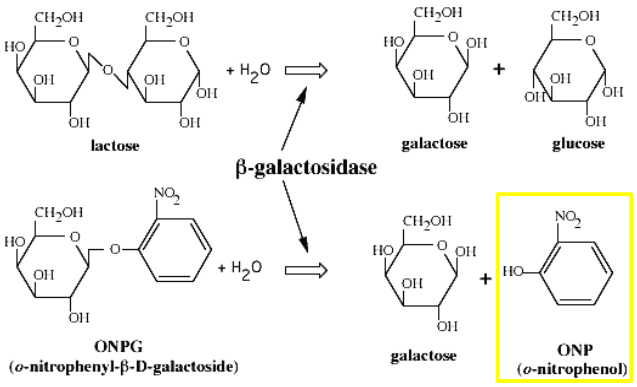

Figure 1. Hydrolysis of lactose and the lactose-analog ONPG is catalyzed by *lacZ* encoded β-galactosidase enzyme. ONP is a yellow product that can be quantified by spectrophotometry.

2. Experimental Section

2.1. Yeast construct information

The budding *Saccharomyces cerevisiae* yeast construct RMY/ER-ERE (*his3 leu2-3, 112 trp1-1 ura3-52* / hER-TRP1-2μ- [pG/ER(G)], ERE-CYC1-LacZ-URA3-2μ [pUCΔSS-ERE], HIS3-CEN/ARS [pRS423]) was a kind gift from Dr. Didier Picard (University of Geneva,

Switzerland). The construct expresses the wild-type human estrogen receptor α (ERα) and contains an estrogen response element (ERE) and a reporter gene upstream of the *lacZ* gene encoding β-galactosidase.

2.2. Yeast cell culture

The yeast construct RMY/ER-ERE was selectively cultured using a 0.2 μm pore filter-sterilized selective medium (SM) without the amino acids histidine (His), methionine (Met) and tryptophan (Trp) [20 g/L dextrose (*Fisher Scientific, USA*); 6.7 g/L yeast nitrogen base without amino acids (*Difco, USA*); 0.032 g/L adenine sulfate (*ICN Biomedical, USA*); 5 g/L casamino acids (*Fisher Scientific, USA*); 2% bacto-agar [for agar plate] (*Fisher Scientific, USA*) in nanopure water (*Barnstead nanopure system, USA*)].

Yeast from an SM agar plate streak was collected with a sterile pipette tip and mixed in 5 mL of liquid SM medium using a pipettor to obtain a homogeneous cell suspension. The suspension was poured into a polystyrene petri dish (60x15 mm; *Falcon, USA*) and the cells grown to confluence in humid air at 30°C. Growth medium was refreshed every 4 to 5 days or as required. When confluence was attained, the cells were pelleted [2000g for 15 min at 4°C], the spent medium was discarded and the cells were re-suspended in 5 mL of fresh, cold yeast-peptone-dextrose (YPD) medium [10 g/L yeast extract (*Difco, Detroit, USA*); 20 g/L peptone (*Difco, Detroit, USA*); 20 g/L dextrose (*Fluka BioChemika, Buchs, Switzerland*)] and placed on ice until use.

2.3. E2 standards preparation

A 10 mM (10^{-2} M) stock solution of E2 standard (*Calbiochem, USA*) in absolute denatured

ethanol (*Fisher Scientific, USA*) was prepared. A working stock standard of 10^{-4} M was prepared by serially diluting the 10^{-2} M stock standard in absolute ethanol. For E2–treated cells, the 10^{-4} M stock standard was diluted in Dulbecco's phosphate-buffered saline (PBS; *Gibco BRL, USA*) to 10^{-5} M for fluorescence microscopy. Unexposed control cells were sham treated with E2-free PBS.

2.4. Chromogenic assay

A volume of 250 µL of each standard in aqueous medium was added to 750 µL of SM with 50 µM $CuSO_4$ (*VWR Scientific, USA*) and an inoculum of 200 µL yeast cell suspension to give a final volume of 1.2 mL (0.2% EtOH), and the mixture incubated at 30°C for 20 hr. Then cells were collected by centrifugation (all centrifugation at 2140g for 10 min), the supernatant discarded, and the cells re-suspended in Z-buffer [(Klein et al., 1994): 60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 50 mM β-mercaptoethanol (*ICN Biomedical, USA*), 1 mM $MgSO_4.7H_2O$ (all other reagents from *EM Science, USA*)]. Cell suspension (100 µL) was added to 100 µL ONPG in Z-buffer, 40 µL chloroform, and 40 µL SDS in a 30°C water bath and incubated for 6 min. The reaction was stopped, cell debris removed by centrifugation, and the absorbance of the supernatant measured at 420 nm. All standards were assayed in triplicate.

In this study, 0.1 mM fluorescein di-β-D-galactopyranoside (FDG; *Marker Gene Technologies, Inc., USA*; Figure 2) was used as a substrate to study the time-lapsed response of live yeast cells to a dose of estrogen following a concept for a non-transcriptional fluorescent cellular sensor developed by Muddana and Peterson (Muddana and Peterson, 2003). FDG is hydrolyzed in a reaction catalyzed by β-galactosidase to give a fluorescent product (fluorescein) that can be detected by fluorescence microscopy. To start with the cells were treated with 10^{-6} M 17β-

estradiol a concentration that was found to be the optimum for maximal response to estrogen without toxicity to the yeast cells.

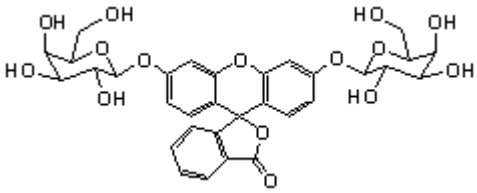

Figure 2. Fluorescein di-β-D-galactopyranoside (FDG) structure. FDG is hydrolyzed in a reaction catalyzed by β-galactosidase to give a fluorescent product that can be detected by fluorescence microscopy.

2.5. Bright-field and fluorescence microscopy

An aliquot of 2800 µL of cells, washed and re-suspended in PBS, was incubated in a 30°C waterbath for 10 min after which 100 µL of the well-mixed cell suspension was immediately added to 100 µL of pre-warmed (30°C) 0.1 mM FDG substrate (*Marker Gene Technologies, Inc., USA*) in a 2-mL amber glass vial to give the T-1 sample. A volume of 300 µL of medium with or without E2 (sham control) was then added to the remaining cells, a sample collected immediately (time = 0 hr) and incubation continued with sample aliquots removed at the desired time points [T-1, 0, 0.5, 1, 2, 4, and 24 hr]. This gave a final E2 medium concentration of 10^{-6}M to E2-exposed cells. The FDG loading was stopped with 1800µL of ice-cold stop medium [PBS containing 10mM HEPES (*VWR, USA*), 4% FBS (*HyClone Perbio, USA*), and 1 pg/mL of propidium iodide (*Sigma-Aldrich, USA*) which was added to the medium to identify dead cells, which fluoresce red] and the cells kept at 4°C in the dark until viewing. [HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, FBS = fetal bovine serum].

For imaging, 5 µL of the stained cell suspension was added to a microscope slide, a cover slip

added, and the cells viewed at 1000X magnification using a Zeiss microscope with a 10X eyepiece and 100X oil-immersion objective (*Zeiss Axioskop, Germany*). Images were captured with an in-built Leica digital camera and PictureFrame software. For fluorescent images a GFP/FITC filter set was used [Excitation = ~488 nm/blue; Emission = ~512 nm/green]. All the work was carried out in dim oblique light to avoid photobleaching. Brightness and contrast were corrected using Adobe Photoshop 7.0 and IrfanView 3.90 software. With this procedure there was no observed increase in cell death (which would have shown up as red cell fluorescence) during the 24 hr experiment period.

Preliminary trials with up to 2mM FDG substrate and up to 13 s UV light exposure time (results not shown) were carried out with and without yeast cells to establish the optimum substrate concentration and light exposure to minimize the effects of substrate fluorescence (observed at more than 3 s exposure for 1 mM FDG and above). Without E2 and FDG, some yeast cells showed autofluorescence when exposed to UV light for more than 3 s. When FDG was added to cells not exposed to E2, there was some autofluorescence for UV exposure times longer than 3 s for 0.1 mM FDG and above, and substrate fluorescence at exposure times longer than 6 s for 1 mM. Adding the stop solution diluted the FDG concentration in the medium to $5x10^{-3}$ mM at the time of viewing which eliminated substrate fluorescence, and yeast autofluorescence was minimized at the 1 s exposure time used.

3. Results and Discussion

3.1. Chromogenic assay

The yeast cell dose-response to standard dilutions of E2 when assayed with ONPG is shown in Figure 3. The β-galactosidase activity was normalized by the maximum enzyme activity

measured. The concentration of 10^{-6} M 17β-estradiol was found to be the optimum for maximum response to estrogen and was used in this study. Other estrogenic compounds behave in a similar manner to estrogen and, even though they have a different structure, may bind to the estrogen receptor eliciting a similar response (Coldham et al., 1997).

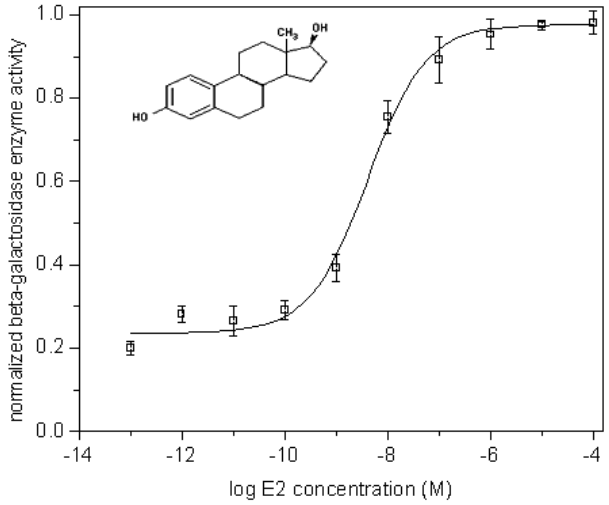

Figure 3. A typical dose-response curve of β-galactosidase production when the estrogen-responsive yeast strain RMY/ER-ERE is exposed to E2 in an aqueous medium for 20 hr and assayed with the chromogenic substrate ONPG is shown. Error bars show intra-assay variation (3 assays, 3 replicates per assay), and the sigmoid curve-fit is for visualization purposes only. Inset is the chemical structure of 17 β-estradiol (E2).

3.2 Fluorescence Assay

The experimental results show that the uptake of E2 and the generation of fluorescent product occur quite rapidly, with maximal protein production within 30 min of exposure to E2 (Figure 4). This finding is important because current yeast-based assays for estrogenic activity use incubation periods of yeast with a sample from 16 – 20 hr (Coldham et al., 1997; Klein et al.,

1994) to 5 – 8 days (Desbrow et al., 1998). The response of estrogen-responsive yeast cell to estrogens is quite rapid as expected. For sample screening, the need for long incubation of the yeast with a sample, a colorimetric assay of the protein, and for light spectroscopy could be eliminated, allowing for faster sample processing and routine assay use.

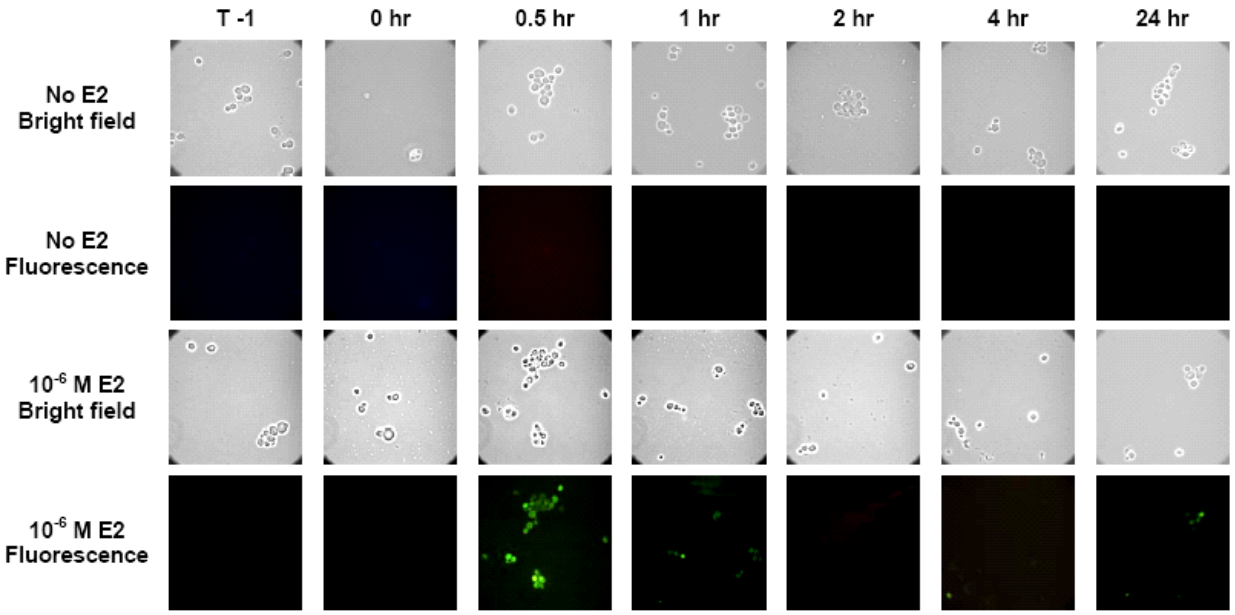

Figure 4. Real-time uptake of 17 β-estradiol (E2) by live yeast cells and fluorescent product generation in the estrogen-responsive yeast RMY/ER-ERE before exposure (T-1), immediately upon exposure (0 hr), and up to 24 hr into the experiment. Maximum fluorescence was observed after 30 min (0.5 hr) of exposure to 10^{-6} M E2 (Row 4, Column 3). After 60 min of exposure the fluorescence is markedly reduced and remains at this low level for the 24-hr exposure (Row 4, Columns 4 – 6). Unexposed cells (No E2; Row 2) did not show any fluorescence above background levels for the duration of the experiment.

4. Conclusions

When live cells of the estrogen-sensitive yeast strain RMY/ER-ERE are exposed to the estrogen

17β-estradiol (E2) in a water sample, the uptake of the estrogen and the subsequent production of β-galactosidase enzyme occur rapidly, with maximum enzyme-catalyzed product formation evident within about 30 min of exposure. The fluorescent product was imaged using fluorescence microscopy, providing a visible response of the live yeast cells to the presence of E2 in the water sample without the need to lyse or fix the cells, or require additional sample processing. This finding could lead to shorter environmental sample screening and monitoring regimes for estrogenic activity, and the development of live whole cell yeast-based biosensors for environmental estrogens to complement quantification methods.

It was important to carry out preliminary tests to determine optimal cell density, FDG substrate concentration, and UV exposure time. More work is required to calibrate the fluorescence assay response to estrogen concentration. Additional work is looking at the fluorescent response of live estrogen-responsive yeast cells incubated with different concentrations of E2 standard to obtain a standard dose-response. Lower affinity estrogenic compounds of environmental interest such as nonylphenols (Bennett and Metcalfe, 2000; Gimeno et al., 1998), and environmental samples are also being studied. These studies are part of the development of an environmental biosensor for monitoring field samples.

Acknowledgements

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