In situ DETECTION OF PROGESTERONE BINDING SITES IN THE PLASMA MEMBRANE OF THE FILAMENTOUS FUNGUS *Rhizopus nigricans*

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Abstract

Steroid hydroxylating enzymes in the fungus *Rhizopus nigricans* are induced by progesterone and by some other steroids. It is known that in higher organisms steroids exert their nongenomic action via steroid binding proteins located in the plasma membrane of the cells, thus our aim was to detect progesterone binding sites in *R. nigricans* plasma membrane. In this report membrane receptors were identified by two independent methods, analysis of progesterone binding to plasma membrane fraction by competitive binding assay using [^{3}H]- and [^{1}H] progesterone (EC_{50} = 55 nM) and *in situ* binding of fluorescein isothiocyanate-coupled cell impermeant progesterone conjugate to fungal protoplasts, detected by fluorescence microscopy.

Introduction

The filamentous fungus *Rhizopus nigricans* from the class *Zygomycetes* is a black mold that develops on state bread. Exogenous steroids exert toxic effects in this fungus.[1] In response to hostile action of these agents from the environment, the microorganism has evolved an efficient defense system of metabolizing steroids with a final goal to eliminate them from the mycelia into surrounding medium. The defense mechanism involves steroid hydroxylation enzymes containing cytochrome P450, which are inducible by their substrates.[2] Progesterone was found to be the best substrate for the induced enzymes and the most effective inducing steroid. The route of progesterone action in the fungus is not well understood. Since steroids are known to mediate their signaling and subsequent biological activities via cytoplasmic/nuclear receptors – genomic action[3] and via membrane receptors – nongenomic action,[4,5] our investigation of progesterone signaling in *R. nigricans* was oriented to both pathways. In our previous study we revealed cytosolic progesterone receptors[6] pointing to the genomic action of progesterone in this microorganism. Our preliminary results obtained on crude plasma membrane fraction of *R. nigricans* indicated the possibility of nongenomic action of

progesterone.[7] In experiments presented in this report we used a technique of *in situ* detection of labeled progesterone attached to the outer surface of fungal protoplasts. We applied progesterone 3-(O-carboxymethyl) oxime-bovine serum albumin labeled with fluorescein isothiocyanate (progesterone-BSA-CMO-FITC) for fluorescence microscopy. For comparison, we performed standard competitive binding assay in the plasma membrane fraction using [^{3}H] progesterone and radio inert progesterone.

Results and discussion

We identified the specificity of progesterone binding to plasma membrane sites using two different techniques: *in vitro* analysis of enriched plasma membrane fraction by competitive binding assay and *in situ* determination of specifically bound progesterone-BSA-CMO-FITC to the outer surface of *R. nigricans* protoplasts.

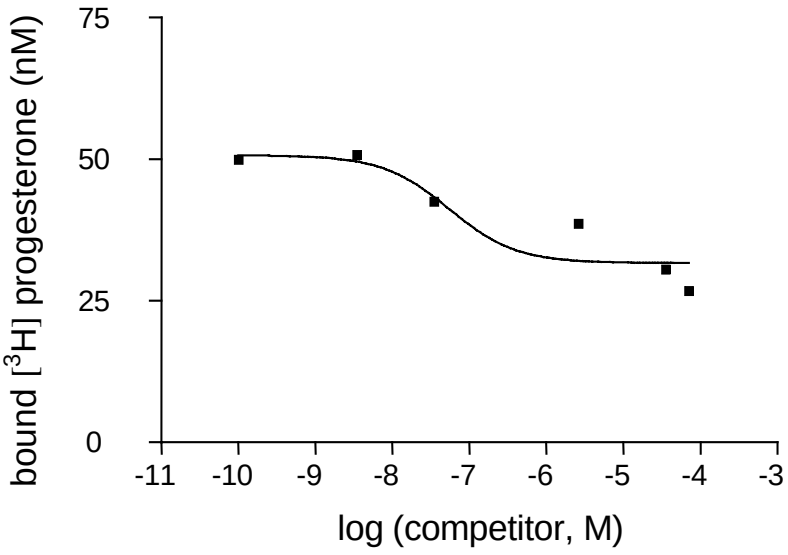

Figure 1. Displacement of [^{3}H] progesterone by radio inert progesterone from plasma membrane of *R. nigricans*. Plasma membrane fraction was incubated either with [^{3}H] progesterone (50 nM) alone or with [^{3}H] progesterone in the presence of different concentrations of unlabeled progesterone. After 50 min of incubation at 22 °C, unbound progesterone was excluded on Sephadex LH-20 mini columns and bound radioactivity counted. Results were analysed in accordance with the one site competition equation by PRISM2 Computer Package (Graphpad, San Diego, CA, USA).

Results obtained by displacement of [^{3}H] progesterone by [^{1}H] progesterone were analysed in accordance with the one site competition equation using PRISM2 computer package (Figure 1). Progesterone binding in the plasma membrane fraction was characterized by EC_{50} of 55 nM. This is comparable to results of Lutz et al. (10), who reported the presence of specific high affinity progesterone binding sites (EC_{50} = 100 nM) in membranes of *Xenopus* oocytes.

Fungal protoplasts were successfully prepared using specific lytic enzyme mixture (Figure 2). The ability of the protoplasts to bind progesterone to the plasma membrane was evaluated using progesterone-BSA-CMO-FITC. The localization of progesterone binding sites on protoplasts surface is presented in Figure 3. Protoplasts under fluorescent microscopy after incubation with progesterone-BSA-CMO-FITC are shown in Figure 3A. Progesterone conjugate binding specificity was demonstrated by strong decrease of fluorescent labeling after incubating protoplasts with a 1000-fold excess of free progesterone (Figure 3B). In addition, no fluorescence was detected when protoplasts were incubated with FITC-labeled BSA, suggesting that the fluorescence is due to binding of progesterone. These results are in accordance with studies of functional binding sites for progesterone in rat Leyding cell plasma membrane obtained with the same progesterone conjugate.[11]

Studies of further steps of nongenomic action of progesterone, possibly including heterotrimeric G-protein activation and other subsequent events, such as adenylyl cyclase activation or inositol-1,4,5-trisphosphate signaling and eventual involvement of phosphorylation cascade, are in progress.

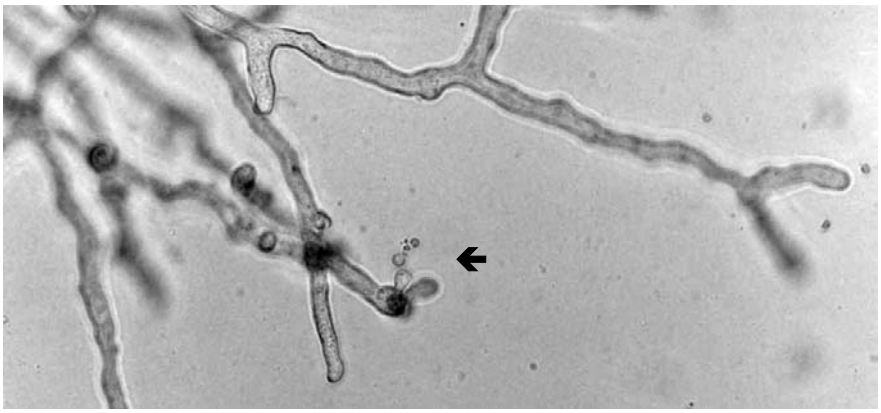

Figure 2. Protoplasts of *R. nigricans* (×40) prepared with specific lytic enzymes.

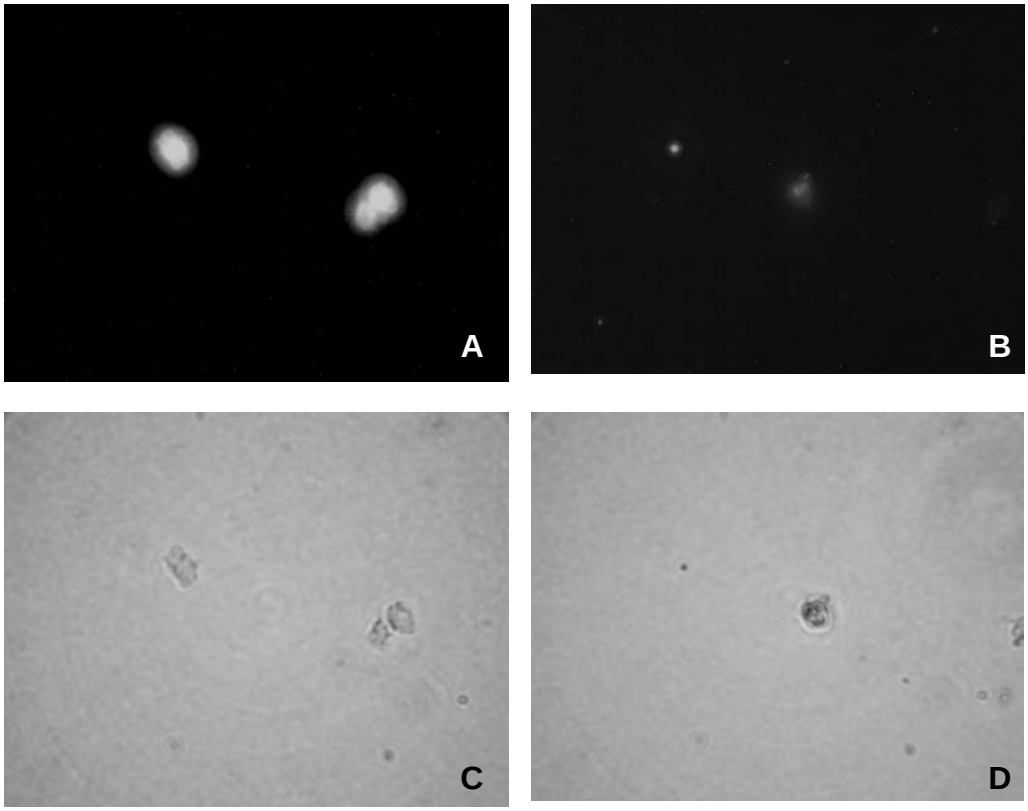

Figure 3. Fluorescence microscopy of *R. nigricans* protoplasts after incubation with progesterone-BSA-CMO-FITC (**A**; ×400). In specific binding experiments (**B**; ×400) protoplasts were incubated with a 1000-fold excess of free progesterone. **C** and **D** (×400): light microscopy of A and B, respectively.

Experimental

Materials

Progesterone-BSA-CMO-FITC (8 mol progesterone/mol BSA and 3 mol FITC/mol BSA-progesterone), FITC-labeled BSA (12 mol FITC/mol BSA) and progesterone were from Sigma. Yatalase was from Takara (Japan). Sephadex LH-20 was obtained from Pharmacia and [1,2,6,7-^{3}H] progesterone (91 Ci/mmol) was from Amersham.

Microorganism

Filamentous fungus *R. nigricans* ATCC 6227b was cultivated as described previously.[8]

Plasma membrane preparation

Plasma membrane fraction was prepared by differential centrifugation of fungal homogenate.[7]

Competitive binding assay

Progesterone binding molecules were determinated by competitive binding assay using [^{3}H]- and [^{1}H] progesterone.[9]

Preparation of protoplasts

Mycelia of *R. nigricans* were suspended in a cell wall lytic solution composed of 50 mM malate buffer (pH 5.5), 0.5 M $MgSO_4$, as an osmotic stabilizer, and 1.0% Yatalase. After incubation at 30 °C for 4 hours with gentle shaking, the residual mycelia were removed by filtration. Protoplasts were concentrated by centrifugation at 700 g for 5 min.

Analysis of progesterone receptor ligand-binding activity

Protoplasts were incubated with progesterone-BSA-CMO-FITC at concentration 50 μg/ml for 1 hour at 4 °C in the dark. A 10-fold excess of fixative (4% paraformaldehyde and 2% glutaraldehyde in PBS) was added and further incubation for 1 hour at 4 °C followed. Protoplasts were then spun down by centrifugation at 1500 g, washed from unbound conjugate with PBS three times and examined by fluorescence microscopy and transmission light microscopy. Control experiments were performed replacing progesterone-conjugate with FITC-labeled BSA alone. For specific binding experiments protoplasts were incubated with progesterone-conjugate in the presence of a 1000-fold excess of free progesterone.

Acknowledgements

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Povzetek

Progesteron in nekateri drugi steroidi inducirajo encime, ki hidroksilirajo steroide v glivi *Rhizopus nigricans*. Pri višjih organizmih je poznano t.i. negenomsko delovanje steroidov preko proteinov plazemske membrane, ki vežejo steroide, naš namen pa je bil določiti vezavna mesta za progesteron v plazemski membrani *R. nigricans*. Membranske receptorje smo v tej študiji določali z dvema neodvisnima metodama, s proučevanjem vezave progesterona na frakcijo plazemske membrane s kompetitivno vezavno analizo s [^{3}H]- in [^{1}H] progesteronom (EC_{50} = 55 nM) in z *in situ* vezavo fluorescentno označenega progesterona, ki ne prehaja v celico, na glivne protoplaste, kar smo določali s pomočjo fluorescentne mikroskopije.