Infectious Diseases in Obstetrics and Gynecology 6:176–181 (1998)

17-β-Estradiol Upregulates the Stress Response in *Candida albicans*: Implications for Microbial Virulence

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ABSTRACT

Objective: The influence of 17-β-estradiol on the stress response of *Candida albicans* was studied.

Methods: The survival of clinical isolates of *C. albicans* treated with 17-β-estradiol after heat and oxidative stress was measured by viable plate counts. Cellular proteins were analyzed via SDS-PAGE.

Results: The heat stress response induced by 17-β-estradiol in *C. albicans* grown at 25° C protected the organisms against the lethal temperature of 48.5° C, as shown by viable plate counts. 17-β-estradiol also enhanced protection of *C. albicans* against oxidative stress (menadione exposure). SDS-PAGE analysis of cytoplasmic extracts revealed proteins induced by 17-β-estradiol were similar to those induced by heat.

Conclusion: 17-β-estradiol enhances survival of *C. albicans* under heat and oxidative stresses. The proteins induced by 17-β-estradiol are probably heat shock proteins. Because heat shock proteins are considered to be virulence factors, 17-β-estradiol may function to promote in vivo survival. Infect. Dis. Obstet. Gynecol. 6:176–181, 1998. © 1998 Wiley-Liss, Inc.

KEY WORDS

Candida albicans; heat shock proteins; 17-β-estradiol; global regulation

The host environment may contain substances that serve as signals to induce virulence factors of low-virulence organisms.[1] *Candida albicans,* normally a low-virulence commensal, encounters a new set of environmental factors when entering the host. The dynamic environment inside the host may include factors such as temperature, pH, and the normal flora and its metabolic products.[2] These factors, along with the oxidative stresses associated with host defense mechanisms, require physiologic adaptation in the pathogenesis of *C. albicans.* Temperature enhances the virulence of several bacteria and influences fungal morphogenesis.[3] Increased temperature, pH, and steroids have also been shown to induce the yeast-to-hyphal transformation of *C. albicans,*[4–6] which in turn is linked to the virulence of this organism.[4,6–8]

All organisms display an inherent response, the heat shock (or stress) response, to significant changes in their environment. Heat shock proteins protect the cell(s) under stress conditions and have also been implicated as virulence factors for pathogenic bacteria and fungi.[3,9] Thus, the heat shock response exemplifies physiologic adaptation in response to changing environments.

Our laboratory has previously shown that 17-β-estradiol induces hyphal transformation in *C. albicans* and increases colony size among some yeast strains.[10,11] In a rat model, *Candida* colonization is estrogen-dependent.[12] Regardless of antibiotic treatment, pregnant women remain susceptible to

Grant Sponsor: Iowa Osteopathic Educational Foundation.

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Received 1 June 1998
Accepted 17 August 1998

Candida infection.[13] With these findings in mind, 17-β-estradiol is an attractive candidate as an environmental cue for *C. albicans* virulence regulation. To investigate this possibility, we studied the response of *C. albicans* to heat and oxidative stresses after treatment with 17-β-estradiol.

METHODS AND MATERIALS

C. albicans Strains

C. albicans strains were obtained from patients undergoing gynecologic examination. Isolates were identified as *C. albicans* by microscopic morphology, germ tube formation in human serum, and brown colonies on BIGGY agar. Isolates were maintained on Sabouraud's dextrose agar (SDA) slants at 4°C. Cultures were subcultured on fresh SDA slants every six months.

Estradiol Effect on *C. albicans'* Heat Stress Response

17-β-estradiol (1,3,5[10]-estratriene-3,17β-diol) was purchased from Sigma Chemical Company (St. Louis, MO) and a stock solution of 1×10^{-3} M in methanol was prepared. Dilutions were made into growth media as needed for all experiments. Starter cultures of three *C. albicans* strains (GT 157, 275G, and GT 158) were supplemented with 1×10^{-6} M or 1×10^{-9} M 17-β-estradiol (using methanol as the vehicle) and incubated in Sabouraud's dextrose broth (SDB) at 25° C for 24 hours. Control cultures (no estradiol) had an equivalent amount of methanol and were prepared in duplicate. After 24 hours, one control culture and 17-β-estradiol treated cultures (1×10^{-6} M or 1×10^{-9} M) were transferred directly to a 48.5° C water bath for 10 minutes for lethal heat challenge. The remaining control culture was used to quantify the heat adaptive response of *C. albicans* against the lethal heat challenge. Heat adaptation of this culture was achieved by transferring the culture to a 37° C water bath for 1 hour to elicit the heat shock response, after which it was subjected to the lethal heat challenge in the 48.5° C water bath. Before the lethal heat challenge, aliquots were plated on SDA for initial viable plate counts. Plate counts were also performed after lethal heat challenge. Postheat challenge plate counts for all three strains were averaged and presented graphically.

Effect of Estradiol on *C. albicans'* Response to Oxidative Stress

To study the response of *C. albicans* to oxidative stress after 17-β-estradiol treatment, menadione (Sigma) was used as the oxidative stress agent. Starter cultures of three *C. albicans* strains (GT 157, 275G, and GT 158) were prepared as described above. After a 24-hour incubation period, aliquots were taken from starter cultures and placed into culture tubes containing SDB containing 1×10^{-4} M menadione for 1 hour. 10 μL aliquots were taken from each sample before and after menadione treatment and plated onto SDA plates for viable counts. The colony counts for the three strains were averaged and presented graphically, with 1×10^{-6} M and 1×10^{-9} M 17-β-estradiol treated cultures compared with the control.

SDS-PAGE and Densitometry of Cellular Proteins

Isolation of cellular proteins was based on the method used by Nicolet and Craig[14] for *Saccharomyces cerevisiae. C. albicans* strain GT 157 was grown in yeast nitrogen base broth (Difco, Detroit, MI) and incubated at 25° C for 18–20 hours to midlogrithmic phase. The culture was divided into four aliquots: two of which contain 17-β-estradiol (1×10^{-6} M or 1×10^{-9} M) and the remaining two contained methanol equal to that present in the 17-β-estradiol treated cultures. After addition of 17-β-estradiol or methanol, tubes were incubated for 30 minutes at room temperature. During the final 15 minutes, one methanol-containing control was transferred to a 39° C water bath to induce a heat shock response. After this incubation, 2-mL aliquots were placed into microfuge tubes and the yeast cells were pellated at 13,000*g* for 2 minutes at room temperature. Cells were resuspended in 100 μL of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA), with 10 μL of protease inhibitor cocktail (Sigma). Roughly 100 μL of acid-washed, 425-600 μm glass beads (Sigma) were added to the tubes and shaken with a custom-made reciprocating cell disrupter operated at 3,000 strokes/minute. After cell disruption, tubes were centrifuged (13,000*g* at room temperature for 5 minutes), and the supernatant was collected. A portion of the sample was used for protein analysis by the BCA method (Pierce,

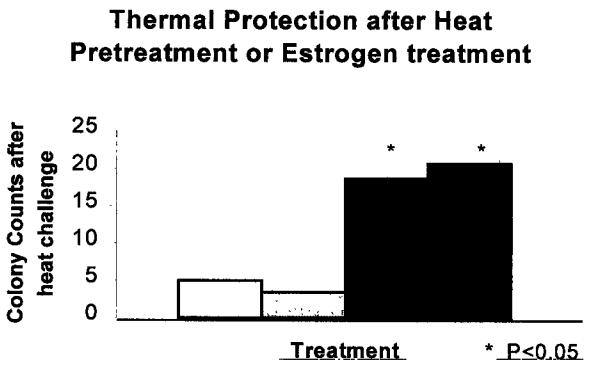

Fig. 1. Heat adaptation induced by 17-β-estradiol. Viable plate counts were taken before and after heat challenge. The graph represents the averages of postheat treatment colony counts between the three strains used in this experiment (open bar = control, shaded bar = heat adapted, hashed bar = 10^{-6} M estradiol, filled bar = 10^{-9} M estradiol). Student's *t*-test was used to compare the heat-shocked and estradiol-treated cells with the control group. $P < 0.05$ indicates significance.

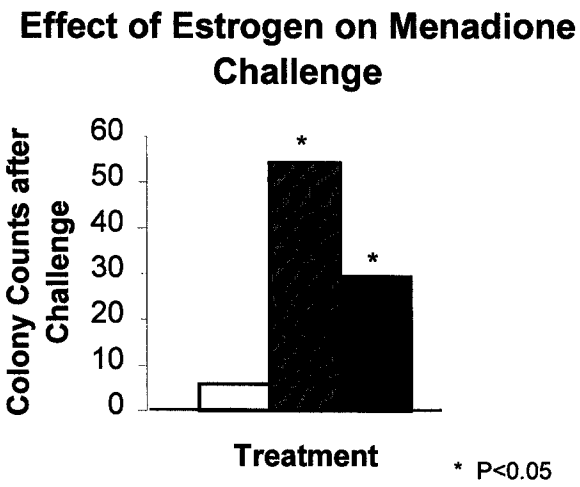

Fig. 2. Enhancement of protection against oxidative stress after 17-β-estradiol treatment. The graph shows the results of the viable plate count after oxidative stress challenge. The values shown on this graph are the averages between the three strains used in this experiment (open bar = control, hashed bar = 10^{-6} M estradiol, filled bar = 10^{-9} M estradiol) . Student's *t*-test was used to compare estradiol treated groups with the control group. $P<0.05$ indicates significance.

Chicago, IL). BCA analysis was used to normalize sample loading for SDS-PAGE. Samples were placed in an equal amount of Laemelli sample buffer. The samples were then loaded onto a 7.5% polyacrylamide gel with a 4.5% stacking layer. After the gels were electrophoresed, they were stained with GelCode® Coomassie-blue stain (Pierce). Densitometric analysis of proteins was done with the aid of a BioRad Fluor-S MultiImager and the MultiAnalyst® software (BioRad, Hercules, CA).

Statistical Analysis

For the heat and oxidative stress challenge experiments, the plate counts were graphed on Microsoft Excel. To compare the significance of the estradiol treated groups to controls, a paired Student *t*-test was used, with $P < 0.05$ considered significant. Statistical analysis was performed with the SigmaPlot software package (Jandel Scientific).

RESULTS

To evaluate the effect of 17-β-estradiol on heat stress tolerance of *C. albicans*, cultures were grown with or without 17-β-estradiol (1×10^{-6} M or 1×10^{-9} M) and subjected to a heat challenge of 48.5° C. Plate count results showed that 17-β-estradiol enhanced the survival of *C. albicans* during lethal heat challenge (Figure 1). In fact, the 17-β-estradiol treated cells showed the greatest survival. The Student *t*-test showed that there were significant differences between the estradiol treated groups and the control group (control vs. 1×10^{-6} M estradiol, $P < 0.04$; control vs. 1×10^{-9} M estradiol, $P < 0.04$). The heat adapted vs. control ($P < 0.203$) combined data failed to show a significant protective effect of heat adaptation, perhaps due to too intense a heat challenge.

The effect of 17-β-estradiol on *C. albicans'* resistance to oxidative stress was also investigated. Viable plate counts showed that 17-β-estradiol, compared with control organisms, enhanced survival of *C. albicans* against the oxidative stress (see Figure 2), and this effect was confirmed with the Student *t*-test values (control vs. 1×10^{-6} M estradiol, $P < 0.03$; control vs. 1×10^{-9} M estradiol, $P < 0.01$).

The survival of 17-β-estradiol treated *C. albicans* against the stresses studied in this report prompted the investigation of the intracellular proteins by means of SDS-PAGE. Figure 3 shows the results from gels loaded with the cellular extracts. The results indicate several proteins that are induced by heat are also induced by 17-β-estradiol. Densitometry readings of the protein bands confirm this observation (see Figure 4). For example, heat adaptation (39° C) increased the expression of the 90 kD

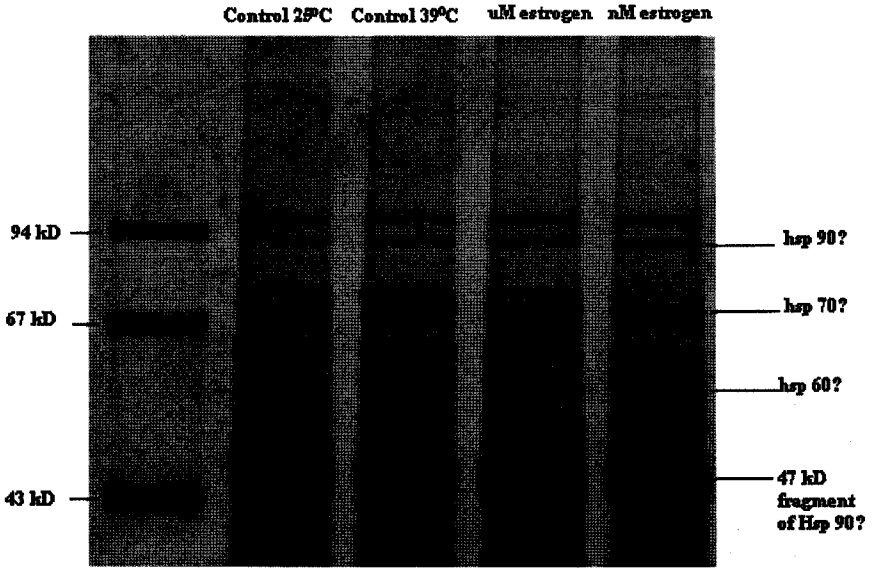

Fig. 3. SDS-PAGE of cellular extracts from 17-β-estradiol treated cells. Proteins were stained with GelCode® Coomassie blue stain, and the image was recorded with the BioRad Fluor-S Multilmager. Proteins of interest are highlighted to the right of the gel, markers (94 kD, 67 kD, and 43 kD) are labeled to the right, and the treatment for each lane is indicated above the respective lane.

protein by 50%, where as 1×10^{-9} M 17-β-estradiol increased expression of the 90 kD protein by 75%.

DISCUSSION

C. albicans is currently the most common fungal pathogen. *Candida* is a frequent cause of vaginitis in pregnant women and, in a rat model, is dependent upon the presence of estrogen.[12] Our laboratory has previously shown that 17-β-estradiol induces the yeast-to-hyphal transformation and increases colony size of *C. albicans*,[10,11] suggesting that 17-β-estradiol could serve as an environmental cue that may upregulate the virulence factors of *C. albicans*. The current study investigated the effects of 17-β-estradiol treatment on the protection of *C. albicans* subjected to heat and oxidative stresses.

The SDS-PAGE analysis, along with the densitometric readings, showed the induction of several proteins in *C. albicans* after treatment with 17-β-estradiol. Some of the proteins induced by 17-β-estradiol were also induced by heat. A group of proteins known as heat shock proteins (Hsps) are known to be induced by heat stress. These proteins protect cells from stresses, such as heat, pH extremes, and oxidative stresses; all of which may occur in vivo. In addition, Hsps have been implicated as virulence factors[3], especially the 90 kD and 70 kD Hsps (Hsp90 and Hsp70).[9,15] Hsp90 is of interest because antibodies from the serum of patients who have recovered from systemic candidiasis are specific for the 47 kD fragment of Hsp 90.[16] Hsp70 has been identified as another immunogenic antigen of *C. albicans* and has the ability to enhance systemic candidiasis.[15] Hsp90 has been reported to generate the 47 kD fragment as a breakdown product.[17] As the gel electrophoresis showed, there is strong induction of a 90 kD and a 47 kD protein by 17-β-estradiol. Densitometric analysis showed that the induction of these proteins by 17-β-estradiol is stronger than the natural induction by heat. These proteins are likely to be Hsps, although definitive proof will require more specific methods.

The results presented in this paper showed 17-β-estradiol enhanced the protection of *C. albicans* under heat and oxidative stresses. In the thermal stress experiment, it is interesting to note that the heat adapted cells appeared to be as vulnerable to the heat challenge as the controls. The cells were subjected to 37° C for 1 hour, which may have been too long of an incubation for an appropriate heat shock response, as Nicolet and Craig[14] describe that the heat shock response is time-dependent, where a 30-minute incubation in 39° C may suffice. Perhaps the smaller incubation period would afford a more favorable heat shock response. Alterna-

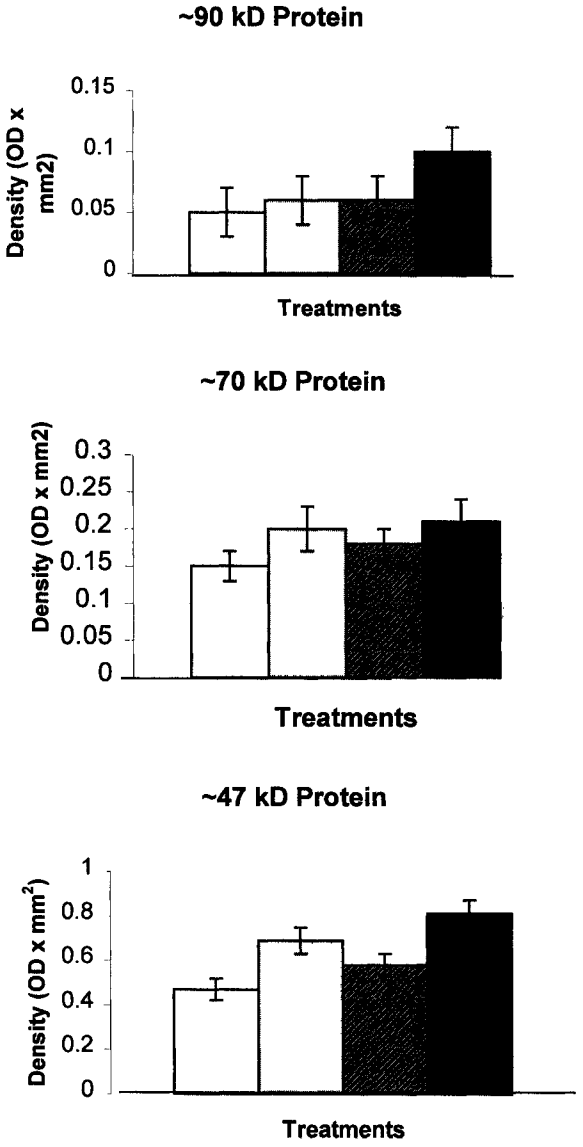

Fig. 4. Densitometric analysis of proteins induced by 17-β-estradiol. Proteins were analyzed using the program MultiAnalyst software program. Density of bands was measured as optical density (open bar = control 25° C, shaded bar = control 39° C, hashed bar = 10^{-6} M estradiol, filled bar = 10^{-9} M estradiol).

tively, a less stringent heat challenge may also be appropriate.

If the proteins induced by 17-β-estradiol are in fact Hsps, this would explain the increased protection of *C. albicans* against the lethal heat challenge but would not necessarily explain the increased resistance to oxidative stress. Hsps aid the regeneration of heat-denatured proteins. The SDS-PAGE data show that the induction of proteins is pleiotropic. Because a variety of proteins are induced by 17-β-estradiol, catalase and superoxide dismutase may be among those elicited by 17-β-estradiol, thereby explaining protection against oxidative stress. Interestingly, catalase and superoxide dismutase have been shown to be induced by heat shock.[18] Davidson et al.[18] reported that proteins such as catalase and superoxide dismutase play an immediate role in reducing the effects of reactive oxygen species during heat stress; whereas Hsps play a role in the recovery of denatured proteins due to the heat stress.[18]

Because 17-β-estradiol's enhanced protection of *C. albicans* subjected to heat and oxidative stresses may be explained by the induction of Hsps, research in our laboratory to identify the specific proteins induced by 17-β-estradiol is underway. The data in this report suggest 17-β-estradiol may act as an environmental cue that leads to the physiologic adaptation in *C. albicans* to the host environment. These physiologic adaptations could protect *C. albicans* against host defenses and adverse environmental conditions. Estrogen may also serve as a messenger by letting the organism know that it is in a human host. The prevalence of candidal vaginitis in pregnant women could be partially explained by the increased levels of estrogen and their effects on *C. albicans*. The fact that estrogen apparently enhances certain physiologic adaptations does not imply that estrogen is the only signal that enhances the virulence of *C. albicans*. There are likely to be other factors, since systemic candidiasis is prevalent in immunocompromised patients, and this setting does not show a strong estrogen dependence. Estrogen enhanced hyphal formation[10] and physiologic adaptations described in this report give support to the idea of 17-β-estradiol acting as a global regulator of *Candida* virulence factors.

ACKNOWLEDGMENTS

This work was supported in part with an equipment infrastructure grant from the Iowa Osteopathic Educational Foundation.

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