Estrogen, Heat Shock Proteins, and NFκB in Human Vascular Endothelium

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Background—We hypothesized that estrogen would increase HSP72 in human coronary artery endothelial cells (HCAEC), and that these would be more sensitive to estrogen than our previous observations in myocytes.

Methods and Results—HCAEC were treated with 17β-estradiol or tamoxifen, ranging from physiological to pharmacological (1 nM to 10 μmol/L) for either 24 hours (early) or 7 days (chronic). HSP expression was assessed by Western blots. Both early and chronic 17β-estradiol and tamoxifen increased HSP72. Electromobility shift assays (EMSA) showed activation of HSF-1 with early, but not chronic, 17β-estradiol. 17β-Estradiol activated NFκB within 10 minutes, and the ER-α selective inhibitor, ICI 182 780, abolished this effect. Transcription factor decoys containing the heat shock element blocked HSP72 induction. Estrogen pretreatment decreased lactate dehydrogenase release with hypoxia. This protective effect persisted despite blockade of HSF-1 by decoys. However, an NF-κB decoy prevented the increase in HSP72 and abolished the estrogen-associated protection during hypoxia.

Conclusions—17β-Estradiol upregulates HSP72 early and chronically via different mechanisms in HCAEC, and provides cytoprotection during hypoxia, independent of HSP72 induction. NF-κB mediates the early increase in HSP72, suggesting that estrogen activates NF-κB via a nongenomic, receptor-dependent mechanism, and this leads to activation of HSF-1. Activation of NF-κB was critical for estrogen-associated protection. Further studies are needed to elucidate the involved signaling pathways. (Arterioscler Thromb Vasc Biol. 2004;24:1628-1633.)

Key Words: estrogen ■ endothelium ■ signal transduction ■ hypoxia ■ HSP72

Gender-specific differences in the incidence of cardiovascular disease have long been recognized. Although the risk of cardiovascular disease (CVD) increases with age, premenopausal women have markedly lower risk than age-matched men. Although women’s risk for cardiac disease increases significantly after menopause, some of this risk may be attenuated by estrogen therapy, leading to a 40% to 50% decrease in CVD; however, clinical trials of estrogen therapy in postmenopausal women have not shown the expected benefit. Clearly, much more needs to be understood about the effects of estrogen at a more basic level than can be addressed in a clinical trial.

The mechanisms to explain the cardioprotective effects of estrogen remain complex and elusive. Estrogen-induced changes in blood lipid profiles can only account for <30% of the decreased risk in premenopausal women. We postulated that another means by which estrogen provides cardioprotection is via upregulation of heat shock proteins (HSPs). HSPs are endogenous protective proteins that guard cells from injury and aid in recovery after injury. We have shown that estrogen increases HSP72 in male rat cardiac myocytes. Further, we have observed that female rats have more HSP72 in their hearts than males do. Importantly, this difference is lost with surgically induced menopause. Despite the higher endogenous levels of estrogen in females and the higher baseline amounts of HSP72, supplemental estrogen still leads to a further increase in HSP72 and HSP90 in cardiomyocytes from female rats. HSP90 binds intracellular hormone receptors. It has been postulated, therefore, that the interaction between HSP90, hormone receptors, and HSF is an important element in activation of HSF-1 by hormones. Estrogen, as well as hypoxia, stimulates HSP90 binding to eNOS in cardiac myocytes and vascular endothelial cells, with subsequent regulation of NO release, PI3-Akt activation, and calcium sensitivity.

Thus, our findings in cardiomyocytes prompted us to investigate whether 17β-estradiol has a similar effect on human coronary artery endothelial cells (HCAEC). We examined the effects of both early and chronic administration of 17β-estradiol and a selective estrogen receptor modulator with mixed agonist/antagonist effects, tamoxifen, on HSP expression, and whether estrogen protected HCAEC from hypoxia. Finally, using transcription factor decoys, we examined the mechanism by which 17β-estradiol regulates HSP synthesis. Through this work, we identified a novel link between estrogen treatment, NFκB activation, and HSF-1.
activation. To our knowledge, this is the first report of the differential role of HSF-1 activation during early versus chronic estrogen and HSP induction, and the first use of transcription factor decoys to evaluate heat shock factor activation as a mechanism of action.

Methods

Endothelial Cell Culture

HCAEC were purchased from Clonetics (La Jolla, Calif). Because of availability, all experiments used male HCAEC. Cells were cultured at 37°C in a humidified incubator with 5% CO₂, 95% room air in phenol red free endothelial basal medium (Clonetics) supplemented with the following (per 500 mL): 10 mL fetal bovine serum, 0.5 mL human endothelial growth factor, 2 mL human fibroblast growth factor-B, 0.5 mL vascular endothelial growth factor, 0.5 mL ascorbic acid, 0.5 mL l-argin 3-IGF-1, and 0.5 mL gentamycin/ amphotericin-B. All experiments were performed at an early passage number.4–6

Treatment Protocols

Seventy percent to 90% confluent HCAEC were used for all studies. Cells were treated with 17β-estradiol, tamoxifen (an estrogen receptor agonist/antagonist), or vehicle (an equal volume of diluent (LabVision, Fremont, Calif) as previously described.15

Electromobility Shift Assays

Activation of HSF-1 was detected by electromobility shift assays (EMSA) as described.15 5’GCCTCGAATGTTGCAGACCTTT3’ and its complementary strand were used as a probe. Biotin-labeled 5’AGTGGAGGGACCTTTCCAGG3’ and its complementary strand were used to assess activation of the transcription factor NFκB.

Nuclear protein extracts were used for all EMSA. Nuclei were isolated by the method deMoissac et al16 Incubation with a 50-fold excess of unlabeled probe, “cold compete,” was performed to control for nonspecific binding. Supershift studies were performed with anti-HSF-1 antibody (Affinity Bioreagents) and anti-HSF-2 antibody (LabVision, Fremont, Calif) as previously described.

In later experiments, to compare NF-κB activation in multiple samples, a kit detecting activation of NF-κB and binding of p50 was used (Pierce). HCAEC were treated with 17β-estradiol, 17β-estradiol in the presence of the estrogen receptor antagonist ICI 182 780 (10 nM), tamoxifen (10 nM), or raloxifene (10 nM) for 15 minutes. Nuclear extract was prepared following the directions of the manufacturer. Samples were assayed in duplicate in a 96-well plate coated with the consensus NF-κB binding element. After incubation with anti-p50 followed by a secondary antibody linked to HRP, plates were developed with a chemiluminescent substrate and read in a luminometer. An internal positive control was used as a reference point for maximal signal. “Cold competition” for this assay was performed by adding excess consensus binding element to wells.

This reduced the signal level to near zero. A mutated binding sequence had no effect on signal.

Transcription Factor Decoy Experiments

To further investigate the role of HSF-1 activation in the 17β-estradiol-mediated increases in HSPs, transcription factor decoys were used with the consensus sequence for the heat shock element (HSE), the nuclear sequence bound by HSF on activation, and translocation into the nucleus. HCAEC were transfected with double-stranded phosphorothioate oligonucleotides (2 μmol/L) containing either the HSE consensus sequence 5’GCCTCGAATGTTGCAGACCTTT3’ or NF-κB 5’CCTGAAGGGATTTCTCCTCC3’ (Trilink, San Diego, Calif) using 5 μg/μL Lipofectin (Gibco BRL, Carlsbad, Calif), which is routinely used to facilitate efficient uptake of the oligonucleotide decoys by vascular cells.17 Twelve hours later, 17β-estradiol was added to the media to a final concentration of 100 nM. A scrambled double-stranded phosphorothioate oligonucleotide with the sequence 5’ATGGCGCTGGTTATTCACGCG3’ was used as a control. Cells were either prepared for Western blotting or subjected to the hypoxia protocol as described previously.

Statistics and Data Analysis

Values reported are means±SEM of 3 or more separate experiments with multiple data determinations in each experiment. Data were compared by 1-way ANOVA followed by a student Newman–Keuls post hoc test. When normalized values were compared with control values, data were analyzed using an ANOVA on Ranks followed by a Dunn test; if data passed tests of normality and equal variance, 1-way ANOVA was performed. A P<0.05 was considered significant.

Results

Changes in HSP Levels With Estrogen and Tamoxifen

After confirming the presence of estrogen receptors in male HCAEC (not shown), we examined the effect of early and chronic treatment with 17β-estradiol or tamoxifen on 3 HSPs. As shown in Figure 1A, early (24-hour) 17β-estradiol resulted in a significant increase in both HSP72 and HSP90. Chronic (1-week) 17β-estradiol resulted in HSP72, but not HSP90, accumulation (Figure 1B). Representative Western blots are shown in Figure I (available online at http://atvb.ahajournals.org).

The estrogen receptor antagonist/agonist, tamoxifen, had a similar effect on HSP levels. Both early and chronic (Figure IIA and IIB, available online at http://atvb.ahajournals.org) tamoxifen exposure resulted in significant increases in HSP72 and HSP90 at the higher doses; however, the highest dose given chronically was lethal to cells. Neither 17β-estradiol nor tamoxifen treatment altered HSP27 (not shown).

Transcription Factor Activation

EMSAs were used to evaluate activation of the transcription factor for the heat shock response, HSF-1, after treatment with 17β-estradiol. Early estrogen resulted in HSF activation by 3 hours of treatment, but chronic estrogen did not activate HSF (Figure 2A). Supershift experiments confirmed that HSF-1 rather than HSF-2 was activated by early estrogen treatment (Figure 2A).

Previously, we hypothesized that the delayed activation of HSF-1 might occur because of the interaction of the estrogen receptor with HSP90, which also binds HSF-1.6 Alternatively, HSF-1 activation may take several hours because

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another pathway is activated first. To test this, NF-κB activation was assessed at 5, 10, 15, 30, and 60 minutes after treatment with 17β-estradiol as well as after several hours. As shown in Figure 2B and 2C, activation of NF-κB occurred within 10 minutes of treatment with 17β-estradiol, peaking at 15 minutes, and still showing activation at 60 minutes. Later time points of several hours showed little or no activation of NF-κB. Activation of NF-κB was seen with both 1 nM and 100 nM 17β-estradiol. To further assess whether 17β-estradiol activates NF-κB via a receptor-dependent mechanism, NF-κB activation was measured after treatment with tamoxifen (10 nM), raloxifene (10 nM), or 17β-estradiol (10 nM) in the presence of ICI 182 780 (10 nM), an inhibitor of ERα. ICI 182 780 abolished the NF-κB activation observed with 17β-estradiol, whereas tamoxifen and raloxifene had no significant effect (Figure 2D).

Transcription Factor Decoy Experiments
To confirm that HSF-1 activation is an essential part of the signaling pathway by which early 17β-estradiol induces HSP72, we used a double-stranded phosphorothioate oligonucleotide with an HSE consensus sequence as a transcription factor decoy.18,19 This HSF decoy acts as a cytosolic “sponge,” binding the activated HSF-1 before it has a chance to translocate to the nucleus. The HSF decoy prevented estrogen-related HSP72 accumulation during early treatment; however, HSP72 levels remained elevated after chronic 17β-estradiol despite transfection with the HSF decoy (Figure 3A and 3D). This confirms the results of the EMSA experiments and shows that early estrogen upregulates HSP72 via activation of HSF-1; however, increased HSP72 with chronic estrogen treatment is via a mechanism other than HSF-1 activation. Transfection with the NF-κB decoy also prevented the estrogen-associated increase in HSP72 after 24 hours (Figure 3B and 3D). Transfection with the scrambled decoy had no effect on HSP72 levels (Figure 3C and 3D).

Hypoxia/Cellular Injury
Hypoxia was used to evaluate whether 17β-estradiol can prevent cell injury. Both early and chronic estrogen resulted in significant decreases in LDH release, a marker of cell injury, during hypoxia compared with controls (Figure III, available online at http://atvb.ahajournals.org). The HSF decoy did not block the protective effects of estrogen on LDH release (Figure 4A), but the NF-κB decoy abolished the protective effects of estrogen (Figure 4B).

Discussion
Investigation of the effects of estrogen on HCAEC demonstrates that: (1) 17β-estradiol increases HSP72 and HSP90 early and chronically; (2) 17β-estradiol treatment results in sequential activation of NFκB and HSF-1; (3) the mechanism underlying the early and chronic increases differ; (4) transcription factor decoys to HSF-1 and NF-κB both can block the increase in HSP72; and (5) only NF-κB inhibition blocks the protective effects of 17β-estradiol against hypoxic injury. Previously, we have observed that 17β-estradiol increases HSP72 in cardiomyocytes, but there was no change in HSP90 or HSP27. We were interested in whether estrogen would have a similar effect on the endothelial cell.20 In contrast to the myocyte, both HSP90 and HSP72 increased significantly in response to 17β-estradiol but showed a similar response time; however, the endothelial cells were much more sensitive to the effects of 17β-estradiol, and changes in HSPs were seen at much lower concentrations than in myocytes (1 nM to 10 μmol/L versus 100 nM to 10 μmol/L). HSP27, again, was unchanged despite the presence of an estrogen response element in the promoter of this gene.

Chronic treatment with 17β-estradiol had a greater effect on HSP72 protein levels, with the lowest dose (10 nM) resulting in the maximal increase. The chronic treatment with estrogen more closely reproduces the in vivo setting where endothelial cells would be continuously exposed to estrogen.

The mixed antagonist/agonist, tamoxifen, was studied for comparison. Interestingly, similar effects were observed with early treatment. With chronic treatment, much less of a response was observed in HSP levels, and the highest dose was lethal to the cells. Thus, tamoxifen-like estrogen could increase expression of HSP72 and HSP90, but the effect was less with chronic treatment, in contrast to estrogen, in which the effect was greater with chronic treatment.

To further elucidate the mechanism involved in the estrogen-associated increase in HSPs, transcription factor

Figure 1. Western blots of HCAEC exposed to 1 nM, 100 nM, or 100 μM 17β-estradiol. A, HSP72 with 24-hour estrogen. B, HSP90 with 24-hour estrogen. C, HSP72, 7-day estrogen treatment. D, HSP90, 7 days of estrogen. β-actin is shown as loading control. *P<0.05 versus control.
decoy experiments were performed. In the early setting, the activation of NF-κB and HSF-1 were necessary to increase HSP72. The decoy for HSF abolished the increase in HSP72 but did not abolish the protective effect of treatment with 17β-estradiol; however, the decoy for the NF-κB transcription factor abolished the increase in HSPs and the protective effect of treatment with 17β-estradiol. These results suggest that NF-κB is activated first, and then HSF-1 is activated. This is further bolstered by the observation that NF-κB is activated within minutes of treatment with 17β-estradiol, and HSF-1 activation takes several hours. Previously, we interpreted the lag between hormone treatment and activation of HSF-1 in cardiomyocytes to result from change in the homeostasis between HSP90–HSF-1 and the estrogen receptor, with HSF-1 being released as a result, and subsequent activation occurring spontaneously. However, the current results suggest that the signaling pathway between the addition of estrogen and the increase in HSP72 is much more complex. Our data indicate that activation of NF-κB is necessary for the increase in HSP72.

Figure 2. A, HSF activation in HCAEC treated as follows: lane 1, control, vehicle only; lane 2, 100 nM 17β-estradiol for 7 days; lane 3, 10 μmol/L 17β-estradiol for 7 days; lane 4, 1 nM 17β-estradiol for 3 hours; lane 5, 100 nM 17β-estradiol for 3 hours; lane 6, 10 μmol/L 17β-estradiol for 3 hours; lane 7, 100 nM 17β-estradiol for 3 hours plus 50-fold excess of unlabeled oligonucleotide (cold compete); lane 8, 100 nM 17β-estradiol for 3 hours plus HSF-1 antibody (supershift); lane 9, 100 nM 17β-estradiol for 3 hours plus HSF-2 antibody (no supershift). B, EMSA demonstrating activation of NF-κB after 15 minutes of treatment with 100 nM 17β-estradiol compared with ethanol only (E, lane 1). Lane 3 shows loss of signal with cold competition (CC) with excess unlabeled oligonucleotide. C, NFκB activation at 1 and 100 nM with 15 minutes of treatment using NF-κB p50 activation assay. Control is treated with ethanol only. D, Effects of antagonists (ICI 182 780) and mixed antagonists/agonists (tamoxifen [T] and raloxifene) versus 17β-estradiol (E2). *P<0.05 versus control.

Figure 3. A, HSP72 levels after transfection with HSF decoys and 100 nM 17β-estradiol for 24 hours or 7 days. B, HSP72 levels with one of the following: NFκB decoys before 24 hours with 100 nM 17β-estradiol; 24 hours with 100 nM 17β-estradiol; or vehicle. C, HSP72 with HSF decoys or scrambled (SCR) decoys before 24 hours 100 nM 17β-estradiol. D, Graph summarizes changes. *P<0.05 versus C and E. C, indicates control, ethanol only; E, 17β-estradiol; HSF, NFκB; SCR (scramble), respective decoy applied before 17β-estradiol.
and the additional intermediate steps. It should be emphasized that the activation of NF-κB was seen with physiological concentrations of estrogen.

With chronic 17β-estradiol, to model in vivo when estrogen is continuously present, only HSP72 increased. This increase in HSP72 was not abolished by treatment with a decoy for HSF-1; however, cells were treated with decoy for only the last 24 hours of estrogen. These results suggest that another mechanism, other than activation of HSF-1, was responsible for the increased HSP72. This result is consistent with our observation that after ovariectomy, it took 9 weeks for HSP72 levels in female rat hearts to decrease to the level of male rat hearts.

Estrogen pretreatment protected HCAEC from hypoxia/reoxygenation. Blocking the increase in HSP72 through the use of transcription factor decoys for HSF did not abolish the protective effects of estrogen. This is in contrast to isolated rat cardiomyocytes in which blocking the endogenous increase in HSP72 in response to simulated ischemia increases cellular injury. The maintenance of a protective effect from estrogen treatment, despite blocking the increase in HSP72, is consistent with the cytoprotective effects of estrogen being plethoric, rather than dependent on a single mechanism. The 2 cell types, endothelial cells and myocytes, are vastly different, with different susceptibilities to injury. Thus, the greater importance of HSP72 to protection in myocytes versus endothelial cells is not surprising, although HSP72 is known to be protective in the endothelial cell.

Estrogen is known to have a wide variety of biological effects. These effects are mediated by at least 2 different receptors (ERα and ERβ) and occur via genomic and nongenomic mechanisms, resulting either in direct local effects (e.g., changes in ion channel activity) or in activation of cell signaling cascades. An example of this is the activation of endothelial nitric oxide synthase (eNOS), which occurs through an IP3-kinase–dependent pathway and is independent of transcription. HSP90 is involved in this, because it complexes with eNOS in the cell, and estrogen increases the binding eNOS by HSP90. The exact mechanisms through which this occurs are still being elucidated. The rapidity of activation of NF-κB in the current work supports a nongenomic mechanism.

In contrast to HSF decoys, the NF-κB decoys abolished the estrogen-induced protection against hypoxia. This finding, in addition to our observation that the NF-κB decoy prevented the estrogen-associated increase in HSP72, suggests that this redox-sensitive transcription factor plays a key role in the estrogen-associated cellular protection against hypoxic injury.

NF-κB is implicated in the regulation of diverse cellular processes, including apoptosis, cell division, differentiation, growth, immunity, and cellular responses to stress, including hypoxia. NF-κB has been shown to be activated in atherogenesis, with angina, during transplant rejection, after ischemia/reperfusion, in congestive heart failure, dilated cardiomyopathy, after preconditioning, and with heat shock. Regulation of NF-κB is complex and involves cross-talk with other pathways, the best-described of which are cytokine-mediated pathways. The effect of estrogen and selective estrogen receptor modulators on NF-κB regulation has been primarily studied with respect to modulation of cytokine-induced NF-κB activation. In general, estrogen attenuates cytokine-induced NF-κB activation, whereas selective estrogen receptor modifiers (SERM), including raloxifene and tamoxifen, have varied effects on NF-κB activation. However, NF-κB activation was assessed several hours after estrogen exposure. We have described the effect of estrogen and 2 SERMs on rapid changes in NF-κB activation status. To our knowledge, this is the first report of rapid activation of NF-κB by estrogen via an ERα-dependent mechanism.

The mechanisms by which estrogen activates NF-κB remain speculative, with few observations published to date. Speir et al reported that the estrogen receptor and an NF-κB component (p65) compete for limited amounts of p300, a relative of the cyclic AMP response element–binding protein. In T cells, 17β-estradiol suppresses the IL-2 and IL-1 receptors, decreasing activation of NF-κB. Hence, the estrogen receptors have an indirect effect on transcriptional activation of inflammatory genes by NF-κB. However, estrogen’s effects are a mixture of protective and inflammatory responses, which reflects the complexity of the response to estrogen. Likewise, NF-κB is both protective and injurious, depending on the cell type and setting. Further work is needed to determine the role of NF-κB in estrogen-associated protection in endothelial cells as well as in the increase in HSP72.

In summary, estrogen treatment of human coronary artery endothelial cells induced HSP synthesis and attenuated cell injury during 24 hours of hypoxia. This protective effect
persisted despite blockade of HSF-1 binding. Chronic estrogen exposure had similar effects—HSF induction and protection against hypoxia-induced damage—but without HSF-1 activation. Early estrogen treatment activated NF-κB via an ERα-mediated pathway. Transfection with an NF-κB decoy before treatment with estrogen prevented the increase in HSP72 and abolished estrogen-associated protection during hypoxia. Collectively, these results suggest multiple mechanisms of estrogen-related protection, with probable cross-talk between signaling pathways. The mechanisms of HSP induction during chronic estrogen exposure and the involvement of NF-κB in estrogen-associated HSP induction and protection during hypoxia remain to be elucidated. Treatment with 17β-estradiol and tamoxifen may provide a novel means of protecting not only cardiac myocytes but also the vascular endothelium.

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References

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