Modulation of Estrogen Signaling by the Novel Interaction of Heat Shock Protein 27, a Biomarker for Atherosclerosis, and Estrogen Receptor β

Mechanistic Insight Into the Vascular Effects of Estrogens

Harvey Miller, Stephanie Poon, Benjamin Hibbert, Katey Rayner, Yong-Xiang Chen, Edward R. O’Brien

Objective—We sought to discover proteins that associate with estrogen receptor beta (ERβ) and modulate estrogen signaling.

Methods and Result—Using a yeast 2-hybrid screen, we identified heat shock protein 27 (HSP27) as an ERβ-associated protein. HSP27 is a recently identified biomarker of atherosclerosis that is secreted at reduced levels from atherosclerotic compared with normal arteries. In vitro protein-binding assays confirmed the specific interaction of HSP27 with ERβ and not ERα. HSP27 expression was absent in coronary arteries with complex atherosclerotic lesions. Interestingly, HSP27 expression was also absent in 60% of coronary arteries from young males and females (27.6±6.5 years) with normal histology or nonobstructive fatty streaks/atheromas. Moreover, the absence of HSP27 in these normal or minimally diseased arteries coincided with the loss of ERβ expression. Only 35% of arteries showed coexpression of HSP27 and ERβ. Relative to controls, estradiol-mediated transcription was reduced 20% with overexpression of HSP27 and increased 44% when HSP27 protein levels were reduced with HSP27 siRNA.


Key Words: atherosclerosis ■ hormones ■ receptors ■ signal transduction ■ women

There is a sex bias in the prevalence of cardiovascular morbidity and mortality that favors women until menopause; thereafter, this difference is lost. Although a plausible explanation for this epidemiological distinction is the presumed vasculoprotective effect of ovarian hormones, randomized primary and secondary prevention trials involving hormone replacement therapy not only are nonconfirmatory but also document ill effects. Estrogens act via at least 2 receptors that are expressed in the vessel wall (ERα and ERβ), although there is increasing evidence that receptor-associated proteins play a critical role in determining the biological responses to ligand-dependent and ligand-independent signaling.

We hypothesized that coregulators of ERs may modulate estrogen signaling in vascular tissues. In this article, we report that heat shock protein 27 (HSP27), a recently reported potential biomarker of atherosclerosis, specifically interacts with estrogen receptor beta (ERβ)—the receptor isoform that shows transient mRNA overexpression early after vascular injury. Using a differential proteomic screening approach, HSP27 secretion levels from human carotid plaques were found to be markedly diminished compared with normal arteries. Moreover, circulating blood levels of HSP27 were decreased in patients with carotid atherosclerosis relative to healthy subjects. We now demonstrate that expression of HSP27 diminishes as the stage of coronary atherosclerosis progresses and that HSP27 is capable of regulating estrogen mediated transcription in vitro.

Materials and Methods

Briefly, our studies involve 4 major components: (1) the use of a yeast 2-hybrid screen to identify proteins that associate with the unique A/B domain of ERβ; (2) the in vitro confirmation of the association of HSP27 with ERβ and not ERα; (3) the demonstration of HSP27 expression in human coronary arteries; and (4) determination of the effect of modulating HSP27 levels on estrogen signaling. For a detailed account of the methodologies used in this article, please see http://atvb.ahajournals.org.

Results

HSP27 Interacts Specifically With the A/B Domain of ERβ

Using a yeast 2-hybrid screen, we identified previously unreported protein interactions with the ERβ. We selected the A/B domain of ERβ as our "bait" because it shares only 30% sequence identity with ERα. Dual-reporter gene activity
To confirm our yeast 2-hybrid findings, we used 2 separate in vitro assays. First, a fusion protein pull-down assay was performed using MBP column precipitation. Fusion proteins of ERβ-MBP and HSP27–intein were expressed in bacteria, and then cell lysates were isolated. The ERβ-MBP protein was immobilized in a column before either HSP27–intein or nonfused intein cell lysates were run through the column. The retained product was then isolated and analyzed by Western blot with an intein-specific probe. With the empty intein construct, minimal protein was retained in the column, indicating that intein alone interacted only weakly with the ERβ–MBP product (ie, background; Figure 1D). However, the HSP27–intein fusion construct resulted in much darker band, consistent with a greater retention of protein and a strong HSP27 and ERβ interaction. Second, coimmunoprecipitation of the 2 proteins was performed. HeLa cells were transfected with either an ERβ–DsRed fusion construct (alone) or both ERβ–DsRed and HSP27–EGFP fusion protein constructs. An anti-DsRed antibody was used to immunoprecipitate proteins that bound to ERβ, and isolated proteins were analyzed by Western blot analysis using an HSP27 specific antibody. In HeLa cells transfected with the ERβ construct alone a weak band was observed—consistent with nonspecific binding (Figure 1E). In HeLa cells cotransfected with both the ERβ and HSP27 fusion protein constructs, the 50-kDa HSP27–EGFP fusion protein was immunoprecipitated, thereby indicating a specific interaction between ERβ and HSP27.

To ensure that HSP27 specifically interacted with ERβ and not ERα, coimmunoprecipitation of HeLa cell lysate from cells transfected with either an ERα or an ERβ expression vector was performed with an anti-HSP27 antibody. The precipitate was then analyzed by Western blot with both ERβ-specific and ERα-specific antibodies. The anti-HSP27 antibody clearly immunoprecipitated ERβ but failed to demonstrate an appreciable signal above background levels for ERα (Figure 1F).

HSP27 Is Coexpressed With ERβ in the Coronary Arteries of Young Individuals

The expression of HSP27 and ERβ was examined in coronary arteries with advanced atherosclerosis, as well as the proximal LAD of 20 young human subjects who died of noncardiovascular causes (14 men and 6 women; age: 27±6.5 years). HSP27 expression was absent in those arteries with advanced atherosclerotic lesions (Figure 2A and 2B). However, in coronary arteries with benign intimal hyperplasia or nonobstructive, pathological fatty streaks and/or atheromas (Stary type I and type III lesions, respectively), we noted variable expression of both HSP27 and ERβ (Figure 2C to 2F). The endothelium was present in all arteries, thereby discounting the possibility that false-negative immunolabeling results were the result of a denuded endothelium (Figure 2G). In these arteries, the following relationships were noted for the expression of HSP27 and ERβ: 7 of 20 (35%) were immunopositive for both, whereas 12 of 20 (60%) were negative for both (Figure 2H). Only one artery was immunopositive for HSP27 but lacked immunodetectable ERβ. No histological features were predictive of HSP27 or ERβ

(LEU2 and β-galactosidase) identified 77 positive screening results from a human lung fibroblast cell line cDNA library that contained 5.3×10⁸ clones. Restriction enzyme analysis identified duplicate clones, and the unique clones were sequenced. Because the A/B domain of ERβ contains the transcriptional activation function AF1, we ensured that selection gene expression was dependent on the interaction with the candidate proteins and not simply caused by auto-activation. Discounting ferritin and ribosomal proteins, which are commonly reported false-positives (http://www.fccc.edu/research/labs/golemis/), 3 unique clones remained: B8, D6, and HSP27 (BC073768; Figure 1A and 1B). All screens were repeated in triplicate to ensure the validity of the interactions. The HSP27 clone shared 98.7% sequence identity with the HSP27 protein sequence in the open reading frame (Figure 1C).
expression in these arteries; however, on serial tissue slides the expression of HSP27 and ERβ in luminal endothelial cells was clearly related (e.g., $R^2=0.94$, $R^2=0.88$; $P<0.0001$).

**HSP27 Estradiol Mediated Transcriptional Activity**

Finally, we examined the role of HSP27 in modulating the estrogen transcriptional activity in HeLa cells stably transfected with an estrogen response element (ERE)-driven EGFP reporter construct. We tested the effects of elevated and decreased levels of HSP27 on estrogen signaling. Cells were treated with 100 nM 17β-estradiol. A, $H_2O_2$ treatment to induce upregulated expression of endogenous HSP27 (inset Western blot) and resulted in a 20% decrease in ERE reporter output (relative fluorescence unit [RFU]; normalized to $\mu$g of total cell protein; *$P<0.05$; n=12/group). When used with siRNA to HSP27, dramatically reduced HSP27 levels relative to scrambled (non-sense) oligomers and oligofectamine alone. GAPDH loading of each lane is shown in the lower row. B, The resultant decrease in HSP27 protein with siRNA treatment (B) produced increases in ERE output (RFU normalized to number of cells) of 44% and 59% relative to scrambled and oligofectamine respectively (ANOVA: $P<0.001$, with $P<0.05$ for the 2 noted comparisons).

**Discussion**

Nuclear receptor-associated proteins are important determinants of the cellular response to ligand-dependent and
ligand-independent steroid hormone signaling. Because we are interested in the vascular effects of estrogens, we sought to identify possible coregulatory proteins of \( \text{ER}\beta \), the ER isoform that shows transient mRNA overexpression after vascular injury. Using a yeast 2-hybrid screen, we discovered the association of HSP27, a recently recognized biomarker of atherosclerosis, with \( \text{ER}\beta \). By way of various in vitro protein assays, we confirmed the interaction of HSP27 with \( \text{ER}\beta \) but not \( \text{ER}\alpha \). Whereas Martin-Ventura et al recently noted decreased secretion of HSP27 in the supernatant of atherosclerotic carotid plaques compared with normal human arteries, these investigators did observe HSP27 immunopositive smooth muscle cells in atherosclerotic carotid endarterectomy specimens and normal mammary arteries. In contrast, we found an absence of HSP27 expression in coronary arteries with complex atherosclerotic lesions. There are several potential explanations for this discrepancy in the HSP27 immunolabeling results: (1) different arteries were examined (carotid versus coronary); (2) perhaps the complexity of the atherosclerotic lesions differed; and (3) we used a monoclonal anti-HSP27 antibody whereas Martin-Ventura et al used a polyclonal anti-serum to HSP27. Although immunolabeling has its limitations, we were intrigued to find either an absence or presence of both HSP27 and \( \text{ER}\beta \) in all but 1 coronary artery from this population of young subjects. Whether the expression of these 2 proteins is linked requires further study. Interestingly, sex did not predict expression of \( \text{ER}\beta \) or HSP27.

Heat shock proteins are highly conserved molecular chaperones that show upregulated expression in response to a range of cellular insults (eg, heat, oxidative stress, infection, cytokines) and play an active role in the stabilization and refolding of key intracellular proteins. Although vascular smooth muscle and endothelial cells are known to express HSP27, the role of this protein in the vessel wall is only now beginning to be studied. Moreover, a number of studies report provocative associations between circulating heat shock protein levels or anti-heat shock protein antibodies and vascular disease.

Even though it has been known for more than a decade that HSP27 is induced by estrogens and in some way associated with estrogen receptors in various cells (eg, breast and endometrial tumors, platelets), these studies were completed before the discovery of \( \text{ER}\beta \). Our study is the first to report that HSP27 specifically associates with \( \text{ER}\beta \) and acts as a corepressor of estrogen signaling. Coregulatory proteins confer milieu-specific responses to steroid hormone receptors and altered levels of these factors play important roles in some diseases. For example, Gregory et al demonstrated that steroid receptor coactivators of the p160 family are expressed in the normal endometrium during the menstrual cycle but overexpressed in women with polycystic ovarian syndrome, and lead to poor reproductive function, endometrial hyperplasia, and cancer.

Similarly, as a corepressor of estrogen signaling, HSP27 may play a role in atherogenesis. However, the critical question that needs to be resolved is whether HSP27 loss contributes directly to coronary atherogenesis or if it is merely a secondary phenomenon that follows the accumulation of an atherosclerotic plaque? Given that induction of HSP27 with herbimycin A reduces neointimal hyperplasia in rat carotid arteries subjected to balloon injury, it is attractive to postulate that the relative absence of HSP27 may in fact be an important mechanistic step in atherogenesis. For example, because many important vascular growth factors and cytokines have an ERE, it is conceivable that unregulated estrogen mediated transcription of these factors might occur in the absence of HSP27 and foster atherogenesis. Hence, knowledge of an individual’s HSP27 “status,” perhaps reflected by a simple blood test, may be predictive of atherogenesis and who should receive estrogen therapy, because the development of undesirable side effects (eg, venous thrombosis, malignancy) could be caused by loss of HSP27 regulation of estrogen-mediated transcription. With studies of HSP27 ongoing in various patient populations, the usefulness of this biomarker in clinical management will soon be clarified.

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