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Journal of Molecular and Cellular Cardiology 36 (2004) 577–584
Journal of Molecular and Cellular Cardiology
www.elsevier.com/locate/yjmcc

Original Article

Estrogen and regulation of heat shock protein expression in female cardiomyocytes: cross-talk with NFκB signaling

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Received 9 December 2003; received in revised form 9 January 2004; accepted 10 February 2004

Abstract

Estrogen is associated with increased heat shock protein (HSP)72 and protection during hypoxia-reoxygenation in cardiomyocytes from adult male rats, as previously reported. We have also reported that female rats have more cardiac HSP72 than males. We hypothesized that, despite higher endogenous estrogen levels and higher baseline HSP72, 17β-estradiol treatment would still result in increased HSP72 and protection during hypoxia-reoxygenation in cardiomyocytes from females.

***Methods/Results.* –** Cardiac cells isolated from adult female rats were treated for 12 hr with 17β-estradiol (0.1, 10, or 50 μM), tamoxifen, (10 or 25 μM; estrogen receptor agonist/antagonist), geldanamycin (2, 5, or 10 μg/ml; inactivates HSP90, preventing interaction with HSF1), or vehicle. Western blot analyses revealed that treatment with 17β-estradiol (10 or 50 μM), tamoxifen (25 μM), and geldanamycin (all doses) resulted in significant increases in HSP72. Electromobility shift assays revealed activation of HSF1 by 2 to 3 hr, and NFκB activation by 15 min. HSP72 induction via HSF1 activation was confirmed using transcription factor decoys containing the heat shock element, which prevented the estrogen-related HSP72 induction. Estrogen pretreatment resulted in decreased LDH release during 24 hr hypoxia. This protective effect persisted despite decoy-mediated blockade of nuclear HSF1 binding. However, transfection with an NFκB decoy not only prevented an estrogen-associated increase in HSP72, but also abolished the estrogenrelated protection during hypoxia.

***Conclusions.* –** Despite higher endogenous estrogen, 17β-estradiol and the selective estrogen receptor modulator, tamoxifen, upregulate HSP72 in cardiomyocytes from adult females, and provide cytoprotection during hypoxia, independent of HSP induction. NFκB activation is necessary for the increase in HSP72, suggesting that estrogen treatment activates NFκB, with subsequent HSF1 activation. NFκB activation is critical for estrogen-associated HSP induction, and protection during hypoxia in female cardiocytes. Treatment with 17β-estradiol and tamoxifen may provide a novel means of protecting both male and female cardiac myocytes against hypoxia-induced damage. Further studies are needed to define the cross-talk between HSF1 and NFκB signaling pathways.

Keywords: Hormones; Cardiac myocytes; Signal transduction; Hypoxia

1. Introduction

Gender-related differences in the prevalence of cardiovascular disease (CVD) have long been recognized [1]. Premenopausal women have a markedly lower risk for CVD than age-matched men, with a significantly heightened risk after the onset of menopause. While the results of three studies published in 2002 have prompted an important re-evaluation of the appropriate use of estrogen replacement therapy [2–4], the preponderance of evidence suggests that aspects of the post-menopausal heightened risk may be attenuated by estrogen therapy, resulting in a 40–50% decrease in CVD incidence [5–8].

Given the controversies surrounding estrogen and heart disease, unraveling the complex cardioprotective mechanisms of estrogen has become a heightened priority. Estrogen-induced changes in blood lipid profiles only account for about 30% of the decreased risk [5]. We postulated that another means by which estrogen provides cardioprotection is via upregulation of heat shock proteins (HSPs). HSPs are endogenous proteins that protect cells from injury and aid in post-ischemic recovery [9–11]. We have shown that estro-

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doi:10.1016/j.yjmcc.2004.02.005

gen leads to an increase in HSP72 in male rat cardiac myocytes [12], and human coronary artery endothelial cells (Hamilton et al., in review). Importantly, the increase in cardiac HSP72 is associated with protection against cell damage during hypoxia and reoxygenation. Further, we have observed that female rats have more myocardial HSP72 than males at baseline [13]. Significantly, this difference is lost with surgically-induced menopause. These results prompted us to investigate whether 17β- estradiol has the same effect in cardiac myocytes isolated from adult female rats. We examined the effects of both 17β-estradiol and a selective estrogen receptor modulator with mixed agonist/antagonist effects, tamoxifen, on HSP expression. Using hypoxia as a physiologically relevant form of injury, we investigated whether estrogen treatment protected female cardiomyocytes as it does in males. Finally, using transcription factor decoys, we examined the mechanism by which 17β-estradiol regulates HSP synthesis. We identified a novel link between estrogen treatment, NFκB activation and HSF1 activation in cardiac myocytes. To our knowledge, this is the first report of the use of transcription factor decoys in cardiomyocytes to evaluate heat shock factor activation as a mechanism of action.

2. Methods

2.1. Isolation of adult rat cardiac myocytes

The animal protocol used for these experiments was approved by the Baylor College of Medicine Animal Research Committee in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Cardiac myocytes were isolated from 3–4 month old female Sprague-Dawley rats weighing 225–250 g as described previously [14–15]. Briefly, hearts were removed following an IP injection of ketamine, xylazine, and acepromazine and retrograde-perfused for approximately 5 min with heparin-Joklik A buffer (modified MEM; GIBCO; Grand Island, NY; plus 60 mM taurine, 20 mM creatine, 5 mM HEPES, 0.1% , 1 IU heparin/ml, pH 7.4). The perfusion solution was changed to Joklik A with 0.6 mg/ml type II collagenase (Worthington Biochemical; Lakewood, NJ). The resulting cell suspension was filtered and separated on a 6% gradient. Following reintroduction of CaCl2, myocytes were resuspended in medium 199 (M199) supplemented with 100 U penicillin, 20 μl HSA,, 100 μg insulin, and 5 μg transferrin/ml and transferred to a cell culture flask for a 2-hr 37°C incubation (5% CO2, 95% air) prior to plating on 0.2% laminin-coated dishes. This isolation procedure yields an average 70% rod-shaped cardiac myocytes.

2.2. Treatment protocols

When myocytes became adherent, the medium was exchanged for fresh supplemented M199 containing 17β-estradiol in doses ranging from 1 nM to 50 μM or tamoxifen (10 or 25 μM), a selective estrogen receptor modulator with both agonist and antagonist activity. Geldanamycin (2, 5, or 10 μg/ml) served as a positive control, known to activate HSF1 by inactivating HSP90, thereby preventing its interaction with HSF1. Control cells were treated with vehicle only (equal volume of diluent, ethanol or DMSO). Experiments were performed with 3 or 4 replicates in each of three separate experiments. For western analyses, cells were collected 12 hr following treatment and for electromobility shift assays (EMSA; activation of HSF and NFκB), cells were collected 15 to 180 min following application of treatment.

2.3. Hypoxia and cellular injury

Following 12 hrs of treatment with 17β-estradiol, cardiomyocytes were gently washed with PBS and changed to a glucose-free medium to prevent anaerobic glycolysis for energy production. Cells were subjected to 12 hrs of hypoxia in an Anaerobic Workstation (Forma Scientific, Marietta, OH) as previously described [16]. Cellular injury was estimated by measuring LDH release [10].

2.4. Western blotting

Western blotting was performed as previously described [15]. Blots were developed with a chemiluminescent system. Bands were quantified by densitometry and expressed relative to vehicle-treated controls. *Electromobility Shift Assays* Activation of HSF1 was detected by EMSA as previously described [17]. Biotin-labeled 5'GCCTCGAATGTTCGCGAACTTT3' and its complementary strand were used as a probe. Biotin-labeled 5'AGTTGAGGGGACTTTCCCAGGC3' and its complementary strand were used to assess activation of the transcription factor NFκB.

Nuclear protein extracts were used for all EMSAs. Nuclei were isolated by the method of de Moissac et al. [18]. Cells were collected in 1 ml of nuclear isolation buffer A (10 mM HEPES, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.3% Nonidet P-40, protease inhibitors, pH 7.9), and centrifuged at 400 × g for 5 min. The pellets were resuspended in 200 μl of the same buffer, allowed to swell on ice for 15 min, and centrifuged at 400 × g at 4°C for 10 min. Pellets were resuspended in nuclear isolation buffer B (20 mM HEPES, 50 mM KCl, 0.1 mM EDTA, 0.4 mM activated sodium orthovanadate; 0.4 mM NaF; 0.5 mM PMSF; 1 mM DTT, 10% glycerol, pH 7.9), rocked at 4/C for 15 min, and centrifuged at 12,000 × g for 5 min. Incubation with a 50-fold excess of unlabeled probe, "cold compete", served as a control for nonspecific binding. Supershift experiments were performed with anti-HSF1 antibody (Affinity Bioreagents) and anti-HSF2 antibody (LabVision, Fremont, CA) to determine which HSF isoform was activated.

To facilitate the rapid comparison of NFκB activation in multiple samples, a kit detecting activation of NFκB and binding of p50 was used (Pierce Biotechnology Inc., Rockford, IL). Nuclear extracts were prepared following manufac-

turer directions. Samples were assayed in duplicate in a 96 well plate containing the consensus NFκB binding element. After incubation with anti-p50 followed by a secondary antibody conjugated 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 done by adding excess consensus binding element to wells. This reduced signal level to near zero. A mutated binding sequence had no effect on the signal.

2.5. Transcription factor decoy experiments

To further investigate the role of HSF1 and NFκB activation in the estrogen-mediated increases in HSP72, transcription factor decoys were employed. Because transcription factors can recognize their relatively short binding sequences even in the absence of the surrounding genomic DNA, short doublestranded phosphorothioate oligodeoxynucleotides containing consensus sequences can prevent gene expression in culture [19,20]. Effectively, these consensus sequences act as a cytosolic sponge for activated transcription factor, binding the transcription factor before it can translocate to the nucleus and bind to a promoter. Decoys were used for both HSF and for NFκB. Myocytes were transfected (12 hr incubation) with doublestranded phosphorothioate oligonucleotides (2 μmols/L) containing either the HSE consensus sequence (5'GCCTCGAATGTTCGCGAACTTT3') or binding sites for NFκB (5'CCTTGAAGGGATTTCCCTCC3') (Trilink, San Diego, CA). This transfection protocol was based on previous experience with oligonucleotide transfection in isolated cardiomyocytes [10,21]. Following transfection, 17β-estradiol was added to the media to a final concentration of 0.10–50 μM. A scrambled, doublestranded phosphorothioate oligonucleotide with the sequence 5'ATGGCCTCGTTTATTCACGCG3' was used as a control. After a 12-hr 17β-estradiol treatment, cells were either prepared for western blotting or subjected to the hypoxia protocol as described previously. *Statistics and Data Analysis* - Data reported are means ± SEM of three or more separate experiments with multiple data determinations in each experiment. Data were compared by one-way ANOVA followed by a student Newman-Keuls *post hoc* test. Where normalized values are compared with control values, data were analyzed using an ANOVA on Ranks followed by a Dunn's test; if data passed tests of normality and equal variance, one-way ANOVA was performed. Significance was established *a priori* at $p<0.05$.

3. Results

3.1. Changes in HSP levels with estrogen and tamoxifen

As shown in Figure 1, treatment with the two higher doses of 17β-estradiol (10 and 50 μM) resulted in a significant increase in HSP72, but the lowest dose, 0.10 μM, had no effect on HSP72 levels. The selective estrogen receptor antagonist/agonist, tamoxifen, had a similar effect on HSP levels (Figure 2), with the higher dose, 25 μM, resulting in a significant increase in HSP72. As expected, geldanamycin treatment, at all doses, resulted in marked increases in HSP72 (Figure 3).

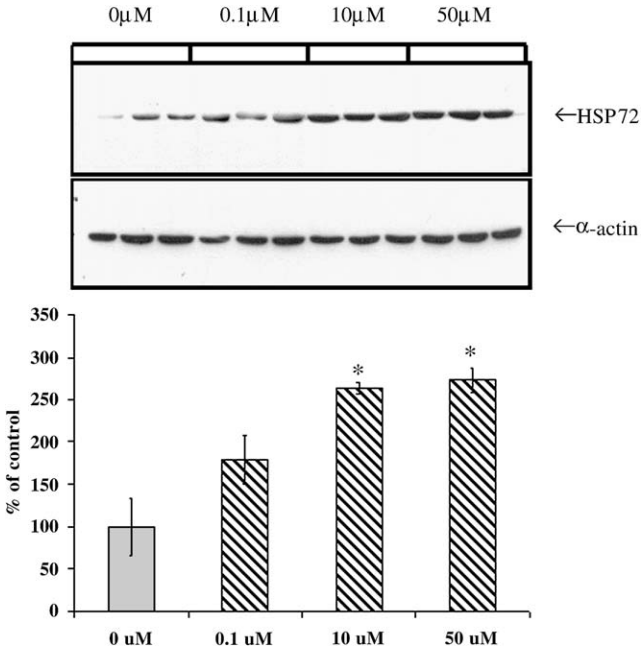

Fig. 1. HSP72 western blot analysis of cardiomyocytes exposed to 0.10, 10, or 50 μM 17β-estradiol. Actin is shown as a loading control. * $p<0.05$ vs. all other groups.

3.2. Transcription factor activation

EMSAs were used to evaluate activation of the transcription factor for the heat shock response, HSF1, following

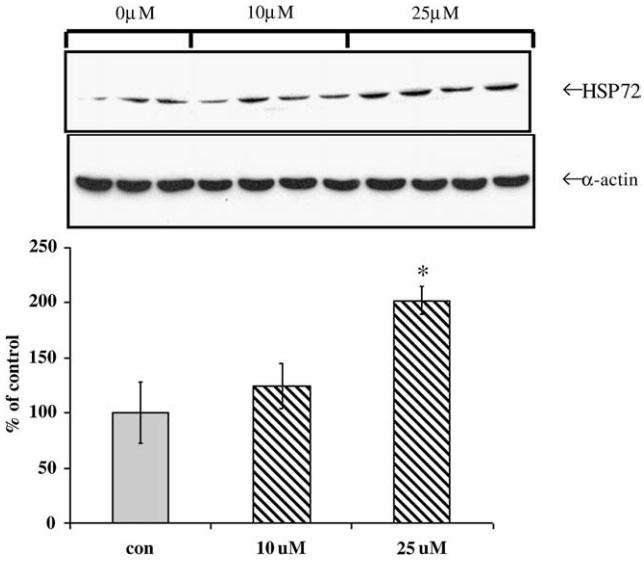

Fig. 2. HSP72 western blot analysis of cardiomyocytes exposed to 10 or 25 μM tamoxifen. Actin is shown as a loading control. * $p<0.05$ vs. all other groups.

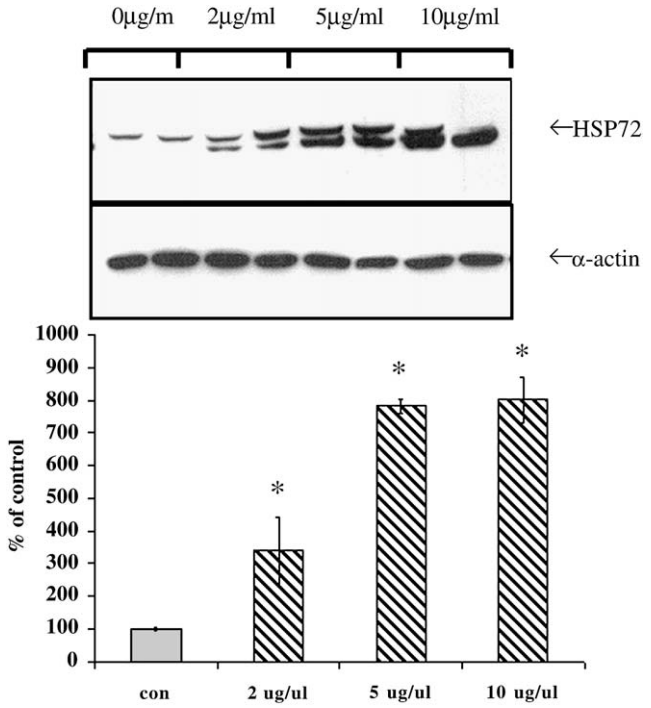

Fig. 3. HSP72 western blot analysis of cardiomyocytes exposed to 2, 5, or 10 µg/ml geldanamycin. Actin is shown as a loading control. * $p<0.05$ vs. all other groups.

treatment with 17β-estradiol (Figure 4). Supershift experiments confirmed that HSF1 rather than HSF2 was activated by estrogen. HSF1 activation was not evident until several hours after 17β- estradiol treatment. Previously, we hypothesized that this occurred because of the interaction of the estrogen receptor with HSP90, which also forms a complex with monomeric HSF1, maintaining it in an inactive state [12]. Alternatively, HSF1 activation may be delayed due to signaling via other pathways prior to HSF1 activation. To further investigate this possibility, NFκB Detection Assays for p50 were done at 15, 30 and 60 min following treatment with 17β-estradiol. As shown in Figure 5, activation of NFκB occurred within 15 min. of treatment with 17β-estradiol, and

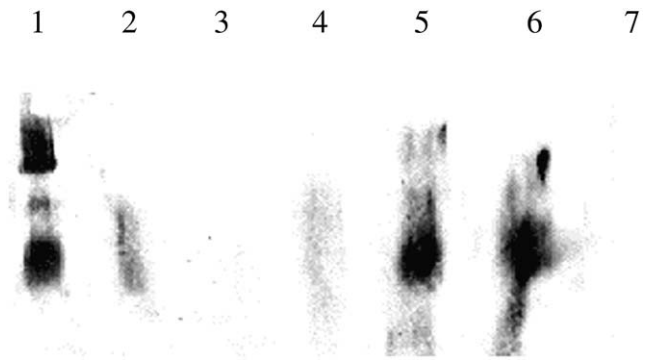

Fig. 4. HSF activation in nuclear protein isolated from cardiomyocytes with the following treatment: lane 1, 10 µM 17β-estradiol + HSF1 antibody (supershift); lane 2, 10 µM 17β-estradiol + HSF2 antibody (no supershift); lane 3, 0 µM 17β- estradiol (vehicle only); lane 4, 0.10 µM 17β-estradiol; lane 5, 10 µM 17β-estradiol; lane 6, 50 µM 17β-estradiol; lane 7, 10 µM 17β-estradiol plus 50-fold excess of unlabeled oligonucleotide

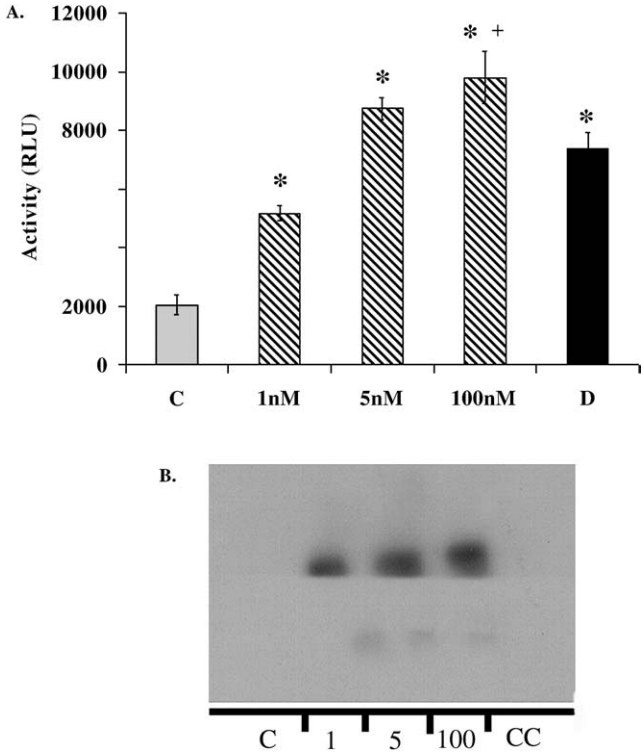

Fig. 5. Activation of NFκB by 17β-estradiol. (A) Graph summarizes the effect of different concentrations of 17β-estradiol on NFκB activity (RLU = Relative Light Units) as measured with an NFκB activation kit as described under Methods. Concentrations are as indicated. C - ethanol (vehicle) only. D - 100 nM for 60 min. All other values are at 15 min. * $p < 0.05$ vs. C. + $p < 0.04$ vs. 1 nM and 60 min. of 100 nM. No difference between 5 nM and 100 nM at 15 min. (B) Representative EMSA demonstrating activation of NFkB.

then declined, but activation was still present at 60 min. Activation of NFκB was seen with 5 nM to 100 µM 17β-estradiol. 1 nM 17β-estradiol showed less activation of NFκB at 15 min, nonetheless, activation was clearly present. Thus, NFκB activation occurred earlier and at much lower concentrations of 17β-estradiol than HSF activation.

3.3. Transcription factor decoy experiments

To confirm that HSF1 activation is an essential part of the signaling pathway by which 17β-estradiol treatment induces HSP72, we used a double-stranded phosphorothioate oligonucleotide with an HSE consensus sequence to act as a transcription factor decoy [19;20]. This dimer effectively acts as a cytosolic decoy binding all the activated HSF1 before the transcription factor translocates to the nucleus, thus preventing binding of the nuclear HSE sequence. As shown in Figure 6A, the decoy containing the HSE prevented estrogen-related HSP72 accumulation. This confirms the results of the EMSA experiments and shows that 17β-estradiol upregulates HSP72 via activation of HSF1 and binding to the HSE consensus sequence. However, transfection with the NFκB decoy also prevented the estrogen-associated increase in HSP72 after 24 hours (Figure 6B). Transfection with the scrambled decoy had no effect on HSP72 levels (Figure 6B).

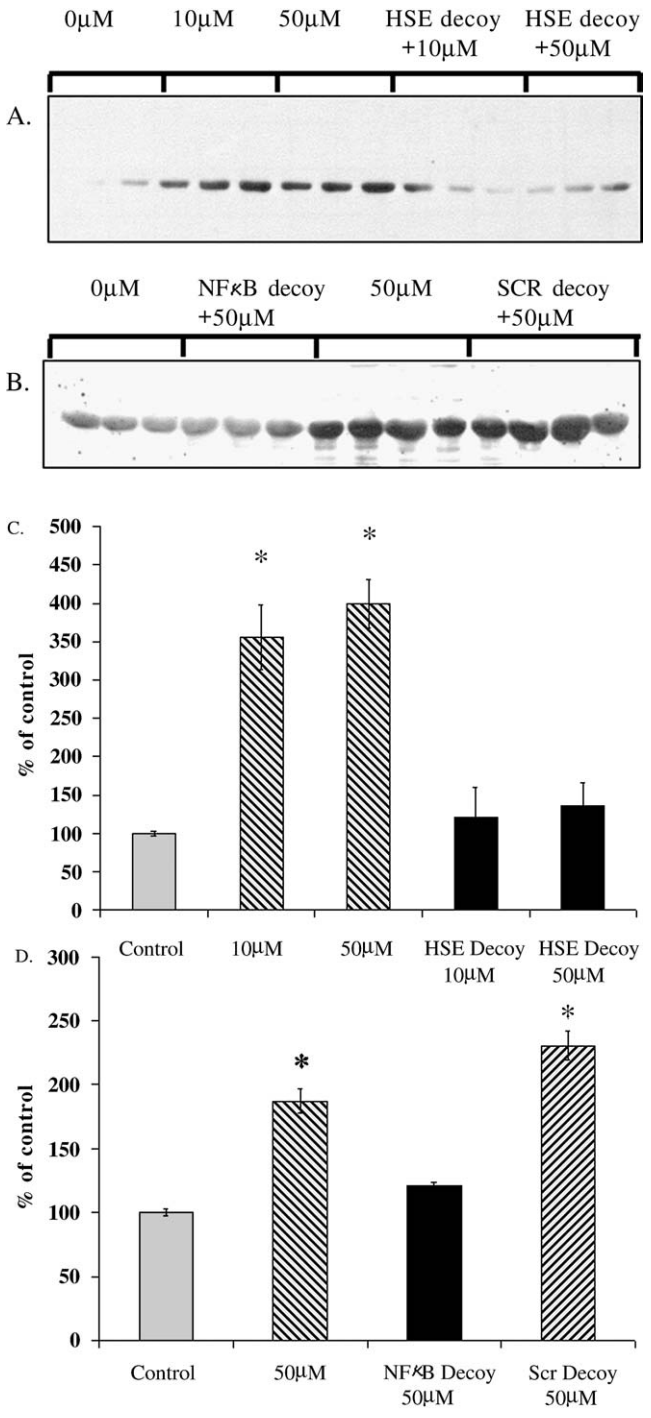

Fig. 6. Transcription factor decoys effect on HSP72 protein levels. (A) HSP72 levels with the following treatments: vehicle; 12 hour treatment with 10 μM 17β-estradiol; 12 hour treatment with 50 μM 17β-estradiol; transfected with HSF1 decoys (containing the HSE sequence) prior to 12 hour treatment with 10 μM 17β-estradiol; or transfected with HSF1 decoys prior to 12 hour treatment with 50 μM 17β-estradiol. (B) HSP72 levels with one of the following treatments: vehicle; transfected with NFκB decoys prior to 12 hour treatment with 50 μM 17β-estradiol; 12 hour treatment with 50 μM 17β-estradiol; or transfected with scrambled control decoys (SCR) prior to 12 hour treatment with 50 μM 17β-estradiol. (C) Densitometric analysis of decoy experiments reflected in A; * $p < 0.05$ vs. control (vehicle). (D) Densitometric analysis of decoy experiments reflected in B; * $p < 0.05$ vs. control (vehicle).

3.4. Hypoxia/cellular injury

Pretreatment with 17β-estradiol, even at the dose that didn't increase HSP72 (0.10 :M), prevented hypoxia-induced cell injury as evidenced by significant decreases in LDH release during hypoxia compared with vehicle-treated controls (Figure 7A). The decoy containing the HSE sequence did not block the protective effects of estrogen on LDH release (Figure 7B); however, the NFκB decoy did abolish the protective effects of estrogen (Figure 7C).

4. Discussion

Previously, we have observed that 17β-estradiol increases HSP72 in cardiac myocytes isolated from male rats.[12] We have also reported that unstressed female rats have greater myocardial HSP72 than males [22]. In the face of both higher endogenous estrogen levels and higher baseline cardiac HSP72, we were interested in whether estrogen would have similar effects on myocytes isolated from female hearts compared to males. This investigation demonstrates that: [1] Increased HSP72 expression is not necessary for the cardioprotective effects of estrogen and [2] that NFκB signaling is required for the cytoprotective effect of estrogen, but the downstream mediators are not known. These conclusions are supported by our observations that: [1] 17β-estradiol increases HSP72 in myocytes isolated from female hearts, [2] the dose of 17β-estradiol required to increase HSP72 is as much as 10-fold higher in cardiomyocytes from females compared to males, [3] 17β-estradiol treatment results in sequential activation of NFκB and HSF1, [4] transcription factor decoys for HSF1 and NFκB both block the estrogen-related increase in HSP72, and [5] only NFκB inhibition completely blocks the protective effects of 17β-estradiol during hypoxia. Interestingly, while 0.10 μM estrogen resulted in no change in HSP72 by western analysis, this dose was associated with cytoprotection during hypoxia. The selective estrogen receptor modulator, tamoxifen, also resulted in increased expression of cardiac HSP72.

To clarify the mechanism involved in the estrogen-associated increase in HSP72, transcription factor decoy experiments were performed. Activation of both NFκB and HSF1 was necessary to increase HSP72. The HSF1 decoy abolished the increase in HSP72, but did not eliminate cytoprotective effects of 17β-estradiol. Interestingly, however, the decoy for the NFκB transcription factor abolished both the increase in HSP72 and the protective effect of 17β-estradiol. These results suggest that estrogenrelated upregulation of HSP72 requires activation of NFκB prior to HSF1 activation in cardiomyocytes. This is further supported by the observation that NFκB is activated within minutes of treatment with 17β-estradiol while HSF1 activation takes hours.

Previously, we have hypothesized the delay between 17β-estradiol treatment and activation of HSF1 results from redistribution of cellular HSP90. This redistribution could, in

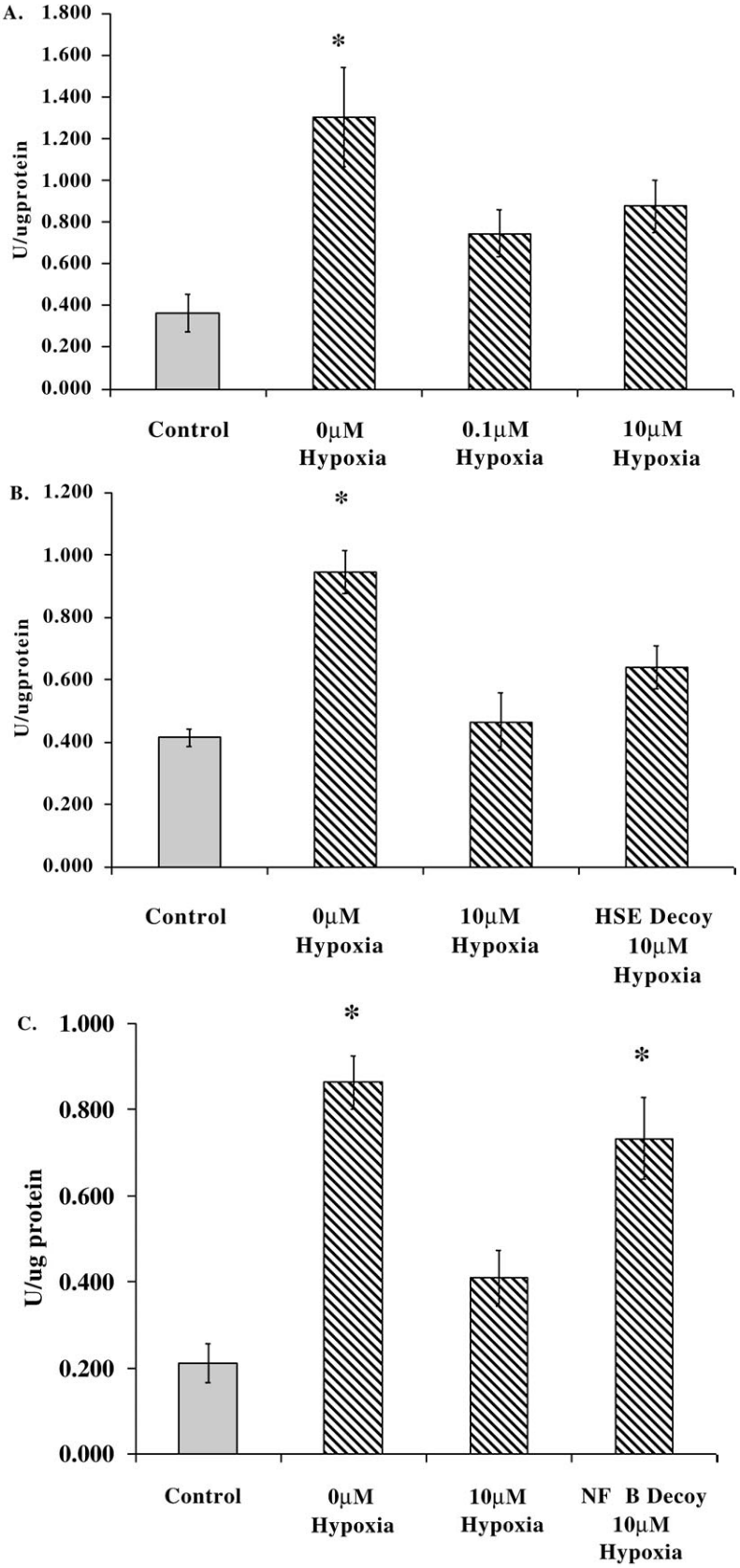

Fig. 7. Hypoxia-reoxygenation induced release of lactate dehydrogenase (LDH) (A). LDH release after 12 hours hypoxia following 17β-estradiol exposure (B). LDH release after 12 hours hypoxia following transfection with HSF1 decoys (containing the HSE sequence) and 12 hour treatment with 17β-estradiol exposure (C). LDH release after 12 hours hypoxia following transfection with NFκB decoys and 12 hour treatment with 17β-estradiol exposure. *p<0.05 vs. all other groups.

theory, result in a change in the homeostasis between HSP90-HSF1 and the estrogen receptor leading to the release and subsequent activation of HSF1 [12].

However, the current results indicate that the signaling pathway between the addition of estrogen and the increase in HSP72 is much more complex, and dependent upon NFκB activation. Of note, we have shown the same sequential activation of NFκB and HSF1 in human coronary artery endothelial cells treated with 17β-estradiol. (Hamilton et al., submitted) Further work is necessary to identify the mechanism by which estrogen activates NFκB.

Our discovery that decoy-mediated blockade of HSP72 induction did not abolish protection during hypoxia is consistent with the cytoprotective effects of estrogen being plethoric rather than dependent on a single mechanism. Estrogen is known to have a wide variety of biological effects. These effects can be exerted via both nuclear mechanisms and nongenomic mechanisms such as those resulting in activation of cell signaling cascades [23,24]. An example of this is the activation of eNOS via an IP3-kinase dependent pathway [25;26]. Estrogen also increases HSP90-eNOS binding, implicating another important heat shock in this complex signaling cascade [25].

In contrast to the observed effects with HSF1 decoys, NFκB decoy-transfection prior to estrogen treatment abolished protection during hypoxia. This finding, in addition to our observation that transfection with an NFκB decoy prevented an estrogenassociated increase in HSP72, suggests that this redox-sensitive transcription factor plays a key role in the estrogen-associated protection against hypoxic injury. The rapidity of the activation of NFκB by estradiol strongly suggests this is a nongenomic effect of estrogen. Further work is needed to determine the role of NFκB in estrogen-associated protection in cardiomyocytes as well as in signaling an increase in HSP72.

Our observation that NFκB is an intermediary between estrogen and the heat shock proteins presents a novel signaling pathway. The physical interaction between steroid receptors and NFκB, however, has been recognized for nearly ten years [27]. Several groups have described an interaction between the p65 subunit of NFκB and estrogen receptors [27–30]. The relationship between the estrogen receptor and NFκB has primarily been described as an antagonistic one in cultured cell lines [31], perhaps via competition between the estrogen receptor and the p65 subunit of NFκB for limited amounts of p300, a close relative of the CREB-binding protein [28]. However, estrogen may regulate redox sensitive transcription factors such as AP-1 and NFκB in a cell typespecific manner due to cell-specific estrogen receptor cofactors [32]. The results of the current study indicate that, at least in myocardial cells, a relationship exists between NFκB activation and estrogen-induced activation of HSF1. Whether this estrogen-related sequential activation of NFκB and HSF1 exists in other cell types, and whether it is directly mediated by the estrogen receptor, should be the focus of future investigations. Interactions between the heat shock response and the NFκB signaling pathway are thought to occur at multiple levels [33]. Induction of the heat shock response prior to a pro-inflammatory signal results in inhibition of NFκB activation. Further, the heat shock response appears to increase expression of I6B″, an inhibitory protein that masks the nuclear translocation sequence of NFκB, and decrease phosphorylation of I6B″, a presumably essential step in NFκB activation. While HSF1 is the primary transcription factor regulating expression of many HSPs, HSF1 and HSP72 may not be involved in inhibition of the NFκB pathway and pro-inflammatory responses as evidenced by studies using fibroblasts from HSF1 null mutant mice [34]. Despite markedly impaired HSP72 expression, these cells still demonstrated inhibition of NFκB signaling following heat shock. Hence, the interaction between the heat shock signaling pathway and the NFκB pathway appears to be quite complex, and may also be cell-specific in nature.

Collectively, these data indicate that the cross-talk between HSF1 activation and NFκB signaling warrants further investigation.

In summary, estrogen induced HSP72 and attenuated injury of cardiomyocytes from female rats during hypoxia. These findings were consistent with those previously reported in cardiomyocytes from males. This protective effect persisted despite blockade of nuclear HSE binding via transfection with HSF1 decoys. Estrogen activated NFκB, an event which was required for protection during hypoxia as demonstrated by transfection with NFκB decoys. These results suggest multiple mechanisms of estrogen-related protection exist with evidence of cross-talk between signaling pathways. The role of NFκB in estrogen-associated HSP induction and protection during hypoxia remains to be elucidated. Efforts should focus on identifying downstream mediators, currently unidentified, that are responsible for estrogen-induced protection in cardiomyocytes. Treatment with 17β-estradiol and tamoxifen may provide a novel means of protecting cardiac myocytes from both male and female hearts against the damaging effects of hypoxia.

Acknowledgements

This work was supported by grants from The Women's Fund, Houston, TX (AAK), the NHLBI, HL58515, AG19327, and a VA Merit Award (AAK). The authors thank Jessica Tristan for her assistance with the LDH assays.

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