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Original article

17β-estradiol modulation of angiotensin II-stimulated response in cardiac fibroblasts

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Abstract

The ovarian hormone, 17β-estradiol, has been suggested to play an important role in gender-specific differences in cardiovascular diseases. One possible cardioprotective mechanism involves the interaction between 17β-estradiol and the renin–angiotensin system. Previous studies demonstrated that fibroblast function and gene expression are regulated by biochemical factors including growth factors, hormones, and cytokines, but little is known regarding the integration of these diverse signals. Therefore, the purpose of this study was to determine the ability of 17β-estradiol to modulate angiotensin II (AngII) effects on integrin-induced collagen gel contraction, matrix metalloproteinase (MMP) activity and expression, and signal transduction pathways in isolated neonatal cardiac fibroblasts. 17β-estradiol significantly attenuated AngII-stimulated collagen gel contraction and significantly diminished the effect of AngII on the expression of β1 and not α1integrins. Active MMP-2 levels were decreased by AngII and addition of 17β-estradiol resulted in further reductions. Relative MMP-2 mRNA levels showed essentially identical patterns to protein levels. 17β-estradiol pretreatment increased AngII-mediated mitogen-activated protein (MAP) kinase p42/44 activation and slightly decreased p38 activation compared to non-pretreated fibroblasts. Simultaneous addition of 17β-estradiol and AngII had little to no effect on AngII activation of p42/44 or p38 MAP kinase. The current studies demonstrate the inhibitory role of estrogen on AngII-induced fibroblast-mediated ECM remodeling, gene expression, and signal transduction. These studies begin to elucidate the mechanisms of estrogen effects on myocardial remodeling and function.

Keywords: Heart; Fibroblasts; Angiotensin II; 17β-estradiol; Estrogen; Integrins; MMPs; MAP kinase; Collagen gel contraction

1. Introduction

Cardiovascular disease is one of the leading causes of death in both men and women in the United States; however, after menopause, the risk of developing heart failure becomes dramatically higher in women [1]. This increased incidence of cardiovascular disease in postmenopausal women has generally been attributed to decreased hormonal levels [2]. There has been an accumulating body of evidence demonstrating that estrogen exerts a direct effect on the heart resulting in gender-specific differences in both humans and animal models of cardiovascular diseases. Despite this significant amount of literature, clinical trials have failed to support the benefits of hormone replacement in humans [3,4]. The differences between these studies have been attributed to several parameters, including estrogen isoforms and the time interval between menopause and hormone replacement therapy. In spite of these discrepancies, it has been generally accepted that ovarian hormones, specifically 17β-estradiol, play an important cardioprotective role.

Many questions still remain unanswered regarding the biochemical and molecular mechanisms of the estrogen-induced effects on cardiac cells. Of the known cardioprotective mechanisms, estrogen acts through receptor-mediated pathways to inhibit the development of atherosclerosis [5,6] and increase endothelial and inducible nitric oxide synthase [7–11]. Another possible cardioprotective mechanism involves an interaction between estrogen and the renin–angiotensin system (RAS). Angiotensin II is a potent vasoconstrictor and major regulator of blood pressure and fluid and electrolyte homeostasis [12–14]. Elevated circulating and tissue levels of angiotensin II have been implicated in the development of hypertension and heart failure [12,15–17]. Estrogen has been demonstrated to reduce angiotensin converting enzyme (ACE) activity and mRNA concentrations, which leads to a decrease in the conversion of angiotensin I to angiotensin II [12–14]. In addition,

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experimental evidence showed that angiotensin type I (AT1) receptor expression and density were up-regulated in aortic smooth muscle cells in the absence of estrogen in ovariectomized rats, and estrogen replacement therapy down-regulated this receptor [12,18]. Thus, the beneficial effects of estrogen in cardiovascular disease may, in part, be a result of RAS modulation. Angiotensin II is an important mediator of cardiomyocyte hypertrophy and fibroblast gene expression in models of hypertension [19–21]. Angiotensin II treatment has been demonstrated to increase collagen levels and fibroblast density and proliferation, which are hallmarks of myocardial fibrosis [20,22,23]. Only recently has the integration of these diverse factors by cardiac fibroblasts begun to be examined. The purpose of this study was to determine whether 17β-estradiol modulates the angiotensin II effects on integrin-induced collagen gel contraction, MMP activity and expression, and signal transduction in isolated cardiac fibroblasts.

2. Materials and methods

2.1. Isolation and culture of cardiac fibroblasts

Newborn rats were obtained with foster moms from Harlan Sprague–Dawley and housed in an AAALAC-approved animal facility. All experiments with animals were approved by the University of South Carolina IACUC. For isolation of cardiac fibroblasts, rats were sacrificed by decapitation 3 days after birth. Hearts were dissected, minced, and digested with collagenase as previously described [24,25]. Fibroblasts were isolated by selective attachment to tissue culture plastic. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 5% newborn calf serum, and antibiotics. Fibroblasts were used prior to passage three and all parallel experiments were performed with cells of the same passage number. Twenty-four hours prior to treatment of fibroblasts with angiotensin II, fibroblasts were rinsed in sterile saline and culture continued in DMEM containing F12 supplement (Sigma) without serum.

In the present study, neonatal cardiac fibroblasts were chosen as a primary cell culture source due to the period of rapid growth and remodeling occurring similar to that experienced in adult pathophysiological conditions. In addition, these neonatal cells were taken prior to puberty wherein increased circulating hormones and estrous cycling are occurring. The use of neonatal cells has been a widely accepted model in numerous studies to elucidate cardiac cell response to estrogen modulation [26–29].

2.2. Collagen gel contraction

The 3-dimensional collagen gel assay has been widely used to quantify the ability of cells to interact with, migrate in, and contract the collagen matrix [30,31]. Fibroblasts were isolated from neonatal rat hearts as described above and maintained in serum-containing DMEM. Prior to the start of collagen gel experiments, fibroblasts were rinsed and culture continued in DMEM-F12 without serum for 24 h. Fibroblasts were subsequently cultured in 3-dimensional collagen gels in the presence of varying physiological doses of 17β-estradiol (10^{-8} M to 10^{-10}M) [32–34] with or without angiotensin II (10 μg/ml) [35]. Digital images were captured 24 h after addition of angiotensin II to the collagen gels. The areas of the top surface of the collagen gels were measured as a relative indicator of the degree of collagen gel contraction [30,35]. Collagen gel experiments were repeated in triplicate with each of three different fibroblast isolations.

2.3. Western blot protein analysis

Neonatal cardiac fibroblasts were cultured as described above and serum-starved 24 h prior to treatment. In some cases, as indicated in Results, 17β-estradiol was added during this period. Fibroblasts were subsequently treated with varying doses of 17β-estradiol (10^{-8} M to 10^{-10} M) in the presence or absence of angiotensin II (10 μg/ml) for 5 min to 24 h, depending on the end assay. Following treatment, fibroblasts were extracted in boiling lysis buffer containing 1 mM sodium orthovanadate, Tris Base (pH 7.4), and 1% sodium dodecyl sulfate. Culture dishes were scraped to collect cellular proteins. Pierce BCA Assays were performed to determine protein concentration in each sample. Total protein (30 μg) was separated on 4–15% sodium dodecyl sulfate polyacrylamide gels (Biorad Ready Gel). Even loading was confirmed by Coomassie Blue staining of identical replicate acrylamide gels. For Western blot analysis, protein samples were transferred onto nitrocellulose membranes. Individual membranes were checked for even transfer by Fast green staining and rinsed in Tris-Buffered Saline containing 0.1% Tween 20 (TBS-T). Membranes were blocked in 5% powdered milk in TBS-T and incubated in primary antibody solutions. Primary antibodies used for this study included Phospho-ERK 1/2 (p42/44 mitogen activated protein-MAP-kinase), total ERK 1/2 (p42/44), Phospho-p38 MAP kinase, total p38 MAP kinase, β1 integrin, α1 integrin, estrogen receptor-α (ER-α), estrogen receptor-β (ER-β), angiotensin II type-1 receptor (AT-1), angiotensin II type-2 receptor (AT-2), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). Both Phospho-ERK1/2 antibodies and ERK 1/2 antibodies (dilution 1:1000; Cell Signaling) were incubated 24 h in TBS-T containing 5% bovine serum albumin. Phospho-p38 antibody (dilution 1:1000; Cell Signaling) incubation was similar except for 48 h. GAPDH (dilution 1:1000; Chemicon), β1 integrin (dilution 1:1500) [36], α1 integrin (dilution 1:1000; Chemicon), ER-α (dilution 1:1000; Santa Cruz), ER-β (dilution 1:1000; Santa Cruz), AT-1 (dilution 1:5000; Alpha Diagnostic International), and AT-2 (dilution 1:5000; Alpha Diagnostic International) antibodies all required 24-h incubation in TBS-T containing 5%-powdered milk. Following incubation in primary antibodies, blots were rinsed several times in TBS-T and incubated in appropriate (anti-rabbit or anti-mouse; dilution 1:1500) horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) in TBS-T containing 5% milk for 1 h. Blots were rinsed again and immuno-reactive proteins were detected on X-ray film using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Western blot results were normalized to either their corresponding GAPDH (α1 and β1 integrins), total ERK 1/2 (Phospho-ERK 1/2), or total p38 (Phospho-p38). Protein bands from each Western blot were quantified using the AlphaImager 2000 Documentation and Analysis System (Alpha Innotech).