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Original article

Estrogen exerts concentration-dependent pro-and anti-hypertrophic effects on adult cultured ventricular myocytes. Role of NHE-1 in estrogen-induced hypertrophy

Ana Kilić, Sabzali Javadov, Morris Karmazyn *

Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, Medical Sciences Building, University of Western Ontario, London, Ontario, Canada N6A 5C1

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ABSTRACT

Estrogen has been shown to protect the heart and attenuate myocardial hypertrophy and left ventricular remodelling through as yet to be defined mechanisms. In the present study we examined concentration-dependent effects of estrogen on hypertrophy of adult rat cardiomyocytes, potential underlying mechanisms related to intracellular pH (pH_i) and possible sex-dependent responses. Cardiomyocytes were isolated from adult male and female Sprague–Dawley rats and used immediately for pH_i determinations or cultured and subsequently treated for 24 h with 17β-estradiol to assess hypertrophic responses. Fluorometric measurements with the pH_i-sensitive dye BCECF demonstrated that at 1 pM 17β-estradiol increased pH_i (+0.05 pH units in females and +0.12 pH units in males, $P<0.05$) by a rapid non-genomic mechanism that was blocked by the sodium-hydrogen exchange isoform 1 (NHE-1) specific inhibitor AVE-4890 (AVE, 5 μM). Treatment with 1 pM 17β-estradiol for 24 h increased cell size (females: 20%, $P<0.05$; males: 29%, $P<0.05$) and ANP expression (females: 414%, $P<0.05$; males: 497%, $P<0.05$) in a NHE-1-, and ERK1/2 MAPK-dependent manner. At 1 nM, 17β-estradiol decreased pH_i (females: −0.24 pH units, $P<0.05$; males: −0.07 pH units, $P<0.05$) which was also prevented by AVE, although at this concentration the hormone had no direct hypertrophic effect but instead prevented hypertrophy induced by phenylephrine. Our results show that low levels of estrogen produce cardiomyocyte hypertrophy through ERK/NHE-1 activation and intracellular alkalinization whereas an antihypertrophic effect is seen at high concentrations. These effects may further our understanding of the role of estrogen in heart disease particularly associated with hypertrophy.

1. Introduction

Heart failure mortality and morbidity continues to be high despite recent advances in diagnosis and treatment. Survival rate is different in men and women with heart failure, with the prognosis for women being significantly better than for men after the onset of symptomatic heart failure caused by non-ischemic heart diseases [1,2]. Gender differences have also been observed in hypertrophic and hypertensive rat models that show an accelerated progression to heart failure in males [3,4]. Furthermore, a variety of studies on transgenic mouse strains has revealed significant cardioprotection in females with cardiomyopathy or hypertrophy [5–7]. It should be pointed out that premenopausal women have a much lower incidence of cardiovascular diseases, such as myocardial infarction, hypertrophy and heart failure than men of similar age, although this advantage is lost after menopause, suggesting that a major female hormone estrogen has a cardioprotective effect [8]. Estrogen actions are known to be mediated through two estrogen receptors, α and β, which belong to the nuclear hormone receptor superfamily. Estrogen receptors α and β (ER α and ERβ), have been described in cardiomyocytes, as well as in vascular smooth muscle and endothelium [9,10]. Furthermore, the enzymes required for synthesis of estrogen are expressed in the heart [11]. The distribution of not only the receptors for steroid sex hormones, but also the synthetic enzymes for their production, suggest that local production of sex hormones and autocrine/paracrine effects may play important roles in mediating the cardiovascular effects of the sex hormones. This local synthesis could be important in post-menopausal women, when estrogen production decreases. Estrogens also have direct acute vasodilatory effects on vascular tone which is part of cardioprotective action of estrogens in premenopausal women [12]. However, estrogen has been shown to protect hearts against necrotic and apoptotic cell death and fibrosis [13,14] and to attenuate myocardial hypertrophy and left ventricular remodelling [15].

Na^+–H^+ exchanger isoform 1 (NHE-1) is one of four key sarcolemmal proteins involved in intracellular pH (pH_i) regulation in cardiomyocytes. The exchanger is ubiquitously expressed in all tissues and in addition to pH_i, it also regulates cell volume and proliferation [16,17]. Experimental and clinical studies have demonstrated the involvement of increased NHE-1 activity in cardiac remodelling

Abbreviations: ANP, atrial natriuretic peptide; AVE, AVE-4890; BCECF, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; βE2, 17β-estradiol; ICI, ICI182,780; NHE-1, Na^+-H^+ exchanger 1, pH_i, intracellular pH; PD, PD98059; PE, phenylephrine; RT-PCR, Real-time-polymerase chain reaction.

* Corresponding author. Tel.: +1 519 661 3872; fax: +1 519 661 4051.
E-mail address: morris.karmazyn@schulich.uwo.ca (M. Karmazyn).

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following ischemia as well as in hypertrophy and fibrosis of nonischemic origin [18–20]. Increased NHE-1 activity is associated with cardiac dysfunction which may be explained by increased $[Na^+]_i$ followed by intracellular Ca^{2+} overload [21,22, and reviewed in 18 and 23]. Serum concentrations of 17β-estradiol in female rats range from low pM levels in the estrus phase and can increase to 500 pM in the proestrus phase [24,25] whereas in male rats plasma levels of 38.9 pM have been reported [26]. At concentrations ranging from 1–100 pM, 17β-estradiol has been shown to increase pH_i in rat aortic smooth muscle cells by a rapid non-genomic mechanism [27]. However, there are no data concerning sex differences and NHE-1 expression or activity in cardiomyocytes. The purpose of this study was to investigate sex differences in NHE-1 expression and/or activity in cultured adult rat cardiomyocytes and to determine whether NHE-1 can be affected by 17β-estradiol. We further determined concentration- and gender-dependent hypertrophic responses to 17β-estradiol and whether these are related to NHE-1 activity. Moreover, the possible contribution of the mitogen activated protein kinase (MAPK) family was also determined. We demonstrate that 17β-estradiol exerts opposite effects on pH_i at different concentrations and that the effects are mediated through modulation of NHE-1 activity. We further show that this is associated with a pro-and anti-hypertrophic effect of the hormone which is dictated by 17β-estradiol concentration and which is more pronounced in cardiomyocytes from male animals.

2. Materials and methods

2.1. Isolation and culture of adult rat ventricular cardiomyocytes

This investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The isolation of calcium-tolerant adult rat ventricular myocytes was carried out using a modified method described previously [22]. In brief, hearts from male or female Sprague–Dawley rats (200–250 g, Charles River Canada, St. Constant, Qc, Canada) were removed and retrogradely perfused with a Ca^{2+}-free buffer. After 5 min of perfusion, the heart was switched to a buffer containing collagenase (Worthington Biochemical, Lakewood, NJ, USA) and proteases. At the end of digestion, the heart was removed and the ventricles were minced into small pieces in stop buffer. The cell suspension was filtered through a nylon mesh and the extracellular Ca^{2+} concentration was increased in a stepwise fashion (up to 1000 μM). The viability of cardiomyocytes was greater than 92% of the final cell population. Cells were resuspended in plating medium containing 5% serum, 10 mM BDM, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were plated on laminin-coated dishes, and placed in a 98% air/2% CO_2 incubator at 37 °C. Approximately 1 h after plating, damaged cells were removed by replacing plating medium with culture medium containing 10 mM BSA and 100 U/ml penicillin, without serum.

2.2. Measurement of pHi in isolated adult rat ventricular cardiomyocytes

pH_i was measured in freshly isolated cardiomyocytes using the pH-sensitive dye 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, 2 μM, Invitrogen Carlsbad, CA, USA). Cells were loaded with the dye at 37 °C for 30 min and placed on the stage of an inverted Leica microscope. Cardiomyocytes were continuously perfused at 1 ml/min with (HCO_3) free HEPES buffer solution. After equilibration with the superfusion buffer and stabilization of myocyte, cells were superfused with buffer containing estradiol (1 pM or 1 nM) in the absence or presence of the NHE-1 specific inhibitor AVE-4890 (AVE, 5 μM, gift from Sanofi-Aventis, Frankfurt, Germany), the estrogen receptor antagonist ICI182,780 (ICI, 1 μM, Tocris Bioscience, Ellisville, MO, USA) or the ERK1/2 MAPK inhibitor PD98059 (PD, 10 μM, Calbiochem, San Diego, CA, USA) for 15 min at 37 °C. The pH_i of individual cardiomyocytes was recorded by photometry at 502.5 nm and 440 nm for excitation and 528 nm for emission using a monochromator Deltascan-4000 system (PTI, Lawrenceville, NJ, USA).

After each experiment, an *in situ* calibration of the fluorescent signal was performed for each cell. Cardiomyocytes were calibrated by superfusion with calibration buffers which differed from the superfusion medium and contained (in mM) 20 NaCl, 119 KCl, 1 MgCl, 12 NaHCO3, 12 HEPES, 2 EGTA, 50 2, 3-butanedione monoxime and 0.015 of the ionophore nigericin. The pH_i for each cell was determined from a linear regression of fluorescence ratio versus the pH value of the calibration buffer.

The NH_4Cl prepulse technique was used to determine activity of NHE-1 and the effect of treatments on NHE-1 activity in cardiomyoyctes. The cells were subjected to intracellular acidification by exposure to 25 mM NH_4Cl for 5 min followed by perfusion with NH_4^+ free buffer. A similar drug addition protocol was followed as described above except that cells were allowed to equilibrate in superfusion buffer plus estradiol or in superfusion buffer plus estradiol and inhibitors for 20 min.

2.3. Treatment protocol

Cells were serum-starved 8 h prior to all experiments after which cells were treated for 24 h (for cell size, gene expression and expression of NHE-1, ERα, ERβ) or up to 60 min (for activation of MAPK) in a MEM medium without serum with the α_1-adrenoreceptor agonist, phenylephrine (PE, 10 μM), a synthetic derivative of estrogen, 17β-estradiol (1 pM or 1 nM) and/or its inactive analog 17α-estradiol (1 pM or 1 nM). When studied, ICI (1 μM), PD (10 μM) or AVE (5 μM) were added 30 min before treatment with PE or estradiol. The concentrations of drugs used in the present study reflect those that were found to exert maximal effects in initial experiments aimed at identifying optimal concentrations of each agent.

2.4. Measurement of cell surface area

Cells were treated with vehicle (control), PE, 17-β-estradiol and/or 17-α-estradiol in the presence or absence of ICI, PD or AVE for 24 h. Cell surface area was captured using a Leica inverted microscope equipped with a Polaroid digital camera at 200× magnification and measured using SigmaStat Pro 5.0 software (Systat, Inc., Richmond, CA, USA). At least 50 randomly selected cells per experiment were used to determine cell size and averaged to provide an *N* value of one.

2.5. Determination of gene expression

Reverse transcription-polymerase chain reaction (RT-PCR) was used to analyze NHE-1 and atrial natriuretic peptide (ANP) mRNA expression. The expression of NHE-1, ANP and 18 rRNA genes was analyzed in a 20-μl reaction volume using SYBR Green Jumpstart Taq ReadyMix DNA polymerase (Sigma-Aldrich, Oakville, ON, Canada), and fluorescence was measured and quantified using a DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA).

2.6. Western blotting for p38, ERK1/2 MAPKs, NHE-1 and ERα and ERβ

At the end of the treatment period, cell lysates were subjected to SDS-PAGE and transferred onto nitrocellulose membrane (0.45 μm pore size) (Amersham Biosciences Inc., Cleveland, OH, USA). The membranes were blocked in 5% milk for 1 h, primary antibody for 2 h, secondary antibody for 1 h, then detected by ECL reagent (Amersham Biosciences Inc., Cleveland, OH). Antibodies were purchased from either Chemicon, Temecula, CA, USA (NHE-1), Cell Signaling Technology Inc. Danvers, MA, USA (ERK1/2) or Santa Cruz Biotechnology, Santa Cruz, CA, USA (ERα, ERβ and GAPDH) and used at dilutions of 1:1000 for NHE-1, ERα, ERβ and 1:2000 for p38 and ERK1/2 MAPKs and GAPDH. An ECL system (Amersham Biosciences Inc., Cleveland,