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Original Article

Estrogen receptor beta mediates gender differences in ischemia/reperfusion injury

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Abstract

Under hypercontractile conditions associated with increased intracellular calcium, male hearts show enhanced ischemia/reperfusion injury compared to female hearts. Our aim in this study was to identify the specific estrogen receptor involved in this gender difference. Following brief treatment with isoproterenol, isolated mouse hearts were subjected to ischemia and reperfusion. Postischemic contractile function and infarct size were measured in wild-type (WT) male and female hearts, and female hearts lacking functional alpha estrogen receptor (αERKO), or the beta estrogen receptor (ßERKO). WT male hearts exhibited significantly less functional recovery and more necrosis than WT females. αERKO female hearts exhibited ischemia/reperfusion injury similar to that observed in WT females, whereas ßERKO females exhibited significantly less functional recovery than WT females and were similar to WT males. These data suggest that estrogen, through the ß-estrogen receptor, plays a role in the protection observed in the female heart. Furthermore, we identified genes that were differentially expressed in ßERKO female hearts compared to αERKO and WT female hearts, and found altered expression of a number of metabolism genes, which may be important in ischemic injury. We further showed that WT female hearts have increased ratio of carbohydrate to fatty acid metabolism relative to WT males.

Keywords: Heart; Metabolism; Estrogen receptor; Ischemia

1. Introduction

There are considerable data showing that pre-menopausal females have reduced risk for cardiovascular diseases [1]. However, a recent large clinical trial failed to show cardioprotection for post-menopausal females on estrogen–progestin replacement therapy [2]. In fact, the women's health initiative (WHI) study showed increased cardiovascular risk for females taking an estrogen–progestin combination [2]. The adverse cardiovascular outcomes with the hormone combination appear to be related to the thrombotic effects likely associated with progestins. It is unclear why pre-menopausal females have reduced cardiovascular disease whereas treatment with estrogen–progestin is not protective. These studies demonstrate the need for a better understanding of the mechanisms responsible for protection in females.

Cross et al. [3–5] have shown that females have a reduced susceptibility to ischemia/reperfusion injury related to increased cell calcium induced by addition of isoproterenol, elevated extracellular calcium or altered calcium transporters. Estrogen was implicated in this cardioprotection because females that were ovariectomized exhibited increased injury similar to males [4]. A goal of this study was to determine if the protection observed in females was mediated by estrogen receptors ER-α or ER-ß. We present the novel finding that the protection in females is mediated by ER-ß. We further report that hearts lacking ER-ß have altered expression of several metabolism genes, which could contribute to the differences observed in susceptibility to ischemia/reperfusion injury.

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2. Materials and methods

2.1. Mouse heart ischemia/reperfusion studies

2.1.1. Animals

Mice lacking functional ER-α (αERKO) or null for ER-ß (ßERKO) were generated as described previously and were backcrossed into C57B6 [6,7]. All animals were treated in accordance with NIH guidelines.

2.1.2. Langendorff perfusion

Hearts from αERKO, ßERKO and wild-type (WT) littermates were isolated and perfused as described previously [3]. Briefly, mice were anesthetized with pentobarbital then heparinized. The hearts were excised quickly and placed in ice-cold buffer. The aorta was cannulated for retrograde perfusion at a constant pressure of 90 cm of H_2O. Perfusate was a phosphate-free Krebs–Henseleit buffer containing in mmol/l 120 NaCl, 10 glucose, 25 $NaHCO_3$, 1.75 $CaCl_2$, 5.9 KCl, 1.2 $MgSO_4$, and 5 U/l insulin. Temperature was maintained at 37 °C and buffer was oxygenated with 95% oxygen/5% CO_2 to maintain pH at 7.4. Contractile function was monitored by inserting a latex balloon into the left ventricle, which was connected to a pressure transducer (Argon/Maxxim Medical; Athens, TX) and data were collected on a PowerLab data acquisition system (AD Instruments; Boulder, CO). The hearts were perfused for a 20 min stabilization period during which time the magnet was shimmed and control NMR spectra were acquired. Hearts were treated with isoproterenol (10 nM) for 1 min just prior to ischemia. All hearts were made globally ischemic for 20 min, followed by a 2 h reperfusion period. Following the 2 h reperfusion period, the hearts were perfused with 1% 2,3,5-triphenyltetrazolium chloride (TTC) to measure area of necrosis as described previously [8]. Recovery of postischemic function, expressed as a percentage of preischemic function, was also used as a measure of ischemic injury. We used the rate pressure product (left ventricular developed pressure, LVDP × heart rate) as a measure of function.

2.1.3. ^{31}P NMR

Intracellular pH (pH_i) and high-energy phosphates were continuously monitored during the study by acquiring 5 min ^{31}P NMR spectra. The NMR studies were carried out on a Varian Inova 500 MHz NMR spectrometer as described previously [3]. The pH_i was determined from the chemical shift difference between the phosphocreatine (PCr) and the intracellular inorganic phosphate (Pi) resonances.

2.2. RNA extraction

Hearts were snap frozen in liquid nitrogen at the time of harvest. RNA was extracted using Qiagen RNeasy kit. Hearts were pooled for each group to provide enough RNA for multiple microarray hybridizations. RNA concentration was determined by optical density at 260 nm. Formaldehyde gel electrophoresis was performed to confirm the distinct 28S and 18S ribosomal RNA. All samples had a 28S band twice the intensity of the 18S band.

2.3. Gene profiling and real time PCR

2.3.1. Microarray hybridization experiments

Oligonucleotide microarray chips containing 70mer probes for 16,463 mouse genes were prepared using standard protocols. Oligomer probes (Qiagen; Valencia, CA) were derived from unique 3′ regions of genes in the UniGene Database Build Mm 102. Probes are routinely resequenced to confirm their identity. Updated clone information can be viewed on the web site http://www.dir.niehs.nih.gov/microarray/clones. Oligomer probes were spotted onto poly-L-lysine-coated glass slides using a robotic DNA arrayer (Beecher Instruments; Bethesda, MD). Total RNA (35 μg) was labeled with Cy3- and Cy5-conjugated dUTP (Amersham; Piscataway, NJ) using a reverse transcription reaction and hybridized to the oligonucleotide microarray chip.

Chips were scanned using an Axon scanner (Axon Instruments; Foster City, CA), and images were analyzed using the Array Suite Software (Scanalytics; Fairfax, VA). Relative fluorescence intensity was measured for each labeled RNA with subtraction of local background values. A ratio of the relative intensities of each fluor bound to each probe was calculated. The distribution of the ratio of all genes was analyzed and normalized based on the median value of a panel of 100 control genes. The distribution of the ratio of all of the genes was then calculated, and intensity ratio values that differed from the median with a confidence interval >95% were scored as significant changes [9]. To increase stringency, each hybridization experiment was performed in quadruplicate.

2.3.2. Verification

Quantitative real-time PCR (RT-PCR) was used to verify altered gene expression levels. The primer sequences used in RT-PCR are shown in Table 1. First strand synthesis was conducted for 60 min at 48 °C in a 10-μl reaction containing 100 ng total RNA, 5.5 mM $MgCl_2$, 2 μM dNTPs, 4 units RNase inhibitor, and 12.5 units reverse transcriptase. The RT-PCR reaction was carried out in a total volume of 40 μl, which included the first strand synthesis reaction to which was added 4 mM $MgCl_2$, 8 mM dNTPs, 1× SYBR green PCR buffer (PE Biosystems), 0.4 μM gene specific primers, and 2.5 units AmpliTaq Gold DNA polymerase (PE Biosystems). The reaction was analyzed using an Applied Biosystems PRISM 7700 detection system. mRNA expression was normalized to GAPDH and 18S RNA (Amplicon Technologies). Each reaction was performed in triplicate.

2.4. ^{13}C NMR metabolism study

Male and female WT hearts were Langendorff perfused with Krebs–Henseleit buffer for a 20 min control period. Perfusion buffer was switched to control buffer plus 1 mM