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Original article

Gene reprogramming in exercise-induced cardiac hypertrophy in swine: A transcriptional genomics approach

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ABSTRACT

Cardiac hypertrophy of the left ventricle (LV) in response to dynamic exercise-training (EX) is a beneficial adaptation to increased workload, and is thought to result from genetic reprogramming. We aimed to determine which transcription factors (TFs) are involved in this genetic reprogramming of the LV in swine induced by exercise-training. Swine underwent 3–6 weeks of dynamic EX, resulting in a 16% increase of LV weight/body weight ratio compared to sedentary animals ($P = 0.03$). Hemodynamic analysis showed an increased stroke volume index (stroke volume/body weight +35%; $P = 0.02$). Microarray-analysis of LV tissue identified 339 upregulated and 408 downregulated genes (false discovery rate < 0.05). Of the human homologues of the differentially expressed genes, promoter regions were searched for TF consensus binding sites (TFBSs). For upregulated and downregulated genes, 17 and 24 TFBSs were overrepresented by >1.5-fold ($P < 0.01$), respectively. In DNA-binding assays, using LV nuclear protein extracts and protein/DNA array, signal intensity changes >2-fold were observed for 23 TF-specific DNA probes. Matching results in TFBS and protein/DNA array analyses were obtained for transcription factors YY1 (Yin Yang 1), PAX6 (paired box 6) and GR (glucocorticoid receptor). Notably, PAX6 and GR show lower signals in TFBS and protein/DNA array analyses upon exercise-training, whereas we previously showed higher signals for these factors in the remodeled LV of swine post-myocardial infarction (MI). In conclusion, we have identified transcription factors that may drive the genetic reprogramming underlying exercise-training induced LV hypertrophy in swine. PAX6 and GR are among the transcription factors that are oppositely regulated in LV hypertrophy after exercise-training and MI. These proteins may be at the base of the differences between pathological and physiological hypertrophy.

1. Introduction

Cardiac hypertrophy and remodeling is the response of the heart to an increased workload, stress or injury, in an attempt to maintain or increase cardiac output [1,2]. Pathological hypertrophy as it occurs in hypertension, valvular diseases or after myocardial infarction (MI), is an independent risk factor for the later development of heart failure [3,4]. In contrast, physiological hypertrophy such as that occurring during normal growth, pregnancy, or after prolonged exercise-training, is not associated with an increased risk for cardiovascular disease [5,6]. Exercise-induced hypertrophy is not associated with increased fibrosis and apoptosis, and decreased capillary density, as is seen in various forms of pathological hypertrophy [7]. Physiological hypertrophy and pathological hypertrophy therefore have different cellular and molecular phenotypes which are thought to result from different genetic reprogramming [7–9].

The majority of studies into the molecular mechanisms underlying physiological hypertrophy have employed rodents subjected to treadmill running, voluntary wheel running or swim training. Various models of physiological hypertrophy in rodents have identified an important role for growth factors such as insulin-like growth factor (IGF-1), and phosphatidylinositol-3-kinase/Akt signaling [10]. Gene expression profiling has shown that expression of cell survival and fatty acid oxidation genes is altered after physiological hypertrophy, whereas genes involved in apoptosis and inflammation were mainly affected in

Abbreviations: MI, myocardial infarction; LV, left ventricle; EX, exercise-training; SED, sedentary; TFBS, transcription factor binding site; GR, glucocorticoid receptor; FDR, false discovery rate; DE, differentially expressed.

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pathological hypertrophy [8,11–13]. However, there are significant differences in cardiac physiology between rodents and large laboratory animals [14,15], such as swine, which in many aspects mimics human cardiac physiology and hemodynamics more closely than rodents [14]. Thus, similar to humans, prolonged exercise-training in swine produces cardiac hypertrophy [16,17], increases coronary blood flow capacity [18], increases stroke volume [17], and decreases heart rate [16]. However, compared to murine models, studies in large animals into the molecular mechanisms of physiological hypertrophy, and the differences with pathological hypertrophy are scarce.

We hypothesized that LV hypertrophy results from genetic reprogramming through a limited number of transcription factors. We have previously reported on the changes in gene expression profiles, and implicated transcription factors, that underlie hypertrophy of the left ventricle (LV) during recovery from a MI [19]. Here, we studied the molecular pathways that underlie physiological hypertrophy and the TFs that drive these changes in LV hypertrophy caused by 3–6 weeks of dynamic exercise-training in swine. Using the changes in gene expression profiles, we explored the differentially expressed genes for common transcription factor binding sites (TFBSs), and combined these data with protein/DNA array analysis of protein extracts prepared from LV nuclei.

2. Methods

2.1. Experimental animals

Studies were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996) and with approval of the Erasmus University Medical Center Animal Care Committee. Cardiac tissue was collected from 2 to 3 months old prepubescent female and neutered male Yorkshire × Landrace swine.

Swine underwent a 3–6 week long (average 31 days, range 18–42 days) incremental dynamic exercise protocol (EX, n = 11). Swine ran 5 days/week on a treadmill starting at 3 km/h for 30 min with an increase of 0.1 km/h and 1 min per day to a maximum of 5 km/h for 50 min. Sedentary animals served as a control group (SED, n = 10). At the end of the study period, hemodynamic and echocardiographic analyses were performed as previously described in detail [20–22]. Hemodynamic measurements could not be obtained from 1 SED animal and 2 EX animals. After completion of the measurements, animals underwent a sternotomy, the heart was arrested and immediately excised. Subendocardial tissue from the anterior wall of the left ventricle was dissected and snap-frozen in liquid nitrogen (i.e. within 3 min of excising the heart) and stored at −80 °C until use for RNA and protein analysis.

2.2. Gene expression profiles

RNA isolation and subsequent microarray analysis was performed for eight exercise-trained animals and for eight sedentary animals (four females and four males in each group) as described elsewhere [19]. Microarray analysis was performed using GeneChip Porcine Genome Arrays (Affymetrix, Santa Clara, CA, USA). All microarray data have been submitted to the NCBI GEO database with GEO accession number GSE37922. Gene expression was considered significantly different at a false discovery rate (FDR) of <0.05. The annotation of ANEXdb [23] was used. The homologous human RefSeq IDs were obtained by alignment to the NCBI RefSeq RNA database.

To confirm the microarray data for selected genes, RNA was isolated from additional tissue LV biopsies from the 8 EX and 8 SED animals using Trizol reagent (Invitrogen). cDNA was prepared from 0.5 μg tissue RNA using the AB (Applied Biosystems) High-capacity cDNA RT kit and random primers, according to the manufacturer's instructions. Forward and reverse primers were designed for selected genes using the cDNA sequence in Ensembl version 70 (January 2013; www.ensembl.org) and the free online Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) to span at least one exon (Supplemental data, Table S1). Quantitative PCR was performed in an AB StepOnePlus system on 10 ng of cDNA using the Sybr Green PCR master mix (AB) and selected primer pairs (0.3 μM each) in a total volume of 20 μl (10 min 95 °C; 40 cycles of 15 s 95 °C; 60 s 60 °C). For each gene, serial 3-fold dilutions of mixed cDNA (10 ng from each animal) in the range of 100 till 0.4 ng per PCR reaction was amplified in parallel, to generate a standard curve. With the various primer pairs, amplification efficiency ranged from 87 till 100%. Quantification was done by the ΔC_T method, using the PPIA (cyclophilin A) gene as reference; data were expressed relative to cyclophilin A mRNA expression level. For each PCR reaction, a melting curve was made to confirm the generation of a single PCR product. For each gene, identity of the PCR product with the proper cDNA sequence was confirmed by double-strand sequencing with the primers used in qPCR, after PCR clean-up with ExoSAP-IT reagent (USB) and the BigDye terminator cycle sequencing kit (Invitrogen) in an ABI 3500 capillary sequencer.

2.3. TF binding site analysis

Of the human homologues of the differentially expressed (DE) genes, regions between 500 bp upstream and 100 bp downstream of the transcription start site were scanned for the presence of putative TF binding sites (TFBSs), as described [19]. Overrepresentation of TFBS in the up- and downregulated genes was determined using a one-tailed Fisher exact probability test and considered significant when more than 1.5-fold and at $P < 0.01$.

2.4. Protein/DNA array analysis

Nuclear protein extracts were prepared from LV tissue as previously described [24]. Extracts from four animals per group were pooled and then semi-quantitatively assayed for DNA binding activity to a protein/DNA array (Combo array, Panomics/Affymetrix, Milan, Italy) containing 345 distinct DNA probes [19]. TF binding activity was considered significant when there was at least a 2-fold signal difference between both nuclear protein pools.

2.5. Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA, USA) was performed to explore the biological functions and molecular networks of the DE genes, using the human RefSeq IDs as input. Biological groups were identified with which the DE genes were significantly associated ($P < 0.001$). Interconnectivity of DE genes was visualized by construction of molecular networks. The TFs from the protein/DNA array were used as input for Ingenuity Pathway Analysis, to link them to genes identified in the microarray analysis. In the 'build' function and with the 'grow' tool, DE genes were linked to the TFs.

2.6. Statistics

Data are presented as mean ± SEM. Differences between groups were analyzed by unpaired Student's *t*-test. $P < 0.05$ (two-tailed) was considered to be statistically significant.

3. Results

3.1. Anatomical and hemodynamical data

In swine that were exercise-trained for 3–6 weeks physiological hypertrophy was observed. A 16% increase ($P = 0.03$) in LV weight to body weight ratio was observed compared with sedentary animals (SED), indicative of LV hypertrophy (Fig. 1A). This was accompanied by a trend towards a lower basal heart rate (−12%; $P = 0.083$;