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Altered expression of FHL1, CARP, TSC-22 and P311 provide insights into complex transcriptional regulation in pacing-induced atrial fibrillation

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Abstract

Atrial fibrillation (AF) is the most common progressive disease in patients with cardiac arrhythmia. AF is accompanied by complex atrial remodeling and changes in gene expression, but only a limited number of transcriptional regulators have been identified. Using a low-density cDNA array, we identified 31 genes involved in transcriptional regulation, signal transduction or structural components, which were either significantly upregulated or downregulated in porcine atria with fibrillation (induced by rapid atrial pacing at a rate of 400–600 bpm for 4 weeks that was then maintained without pacing for 2 weeks). The genes for four and a half LIM domains protein-1 (*FHL1*), transforming growth factor-β (TGF-β)-stimulated clone 22 (*TSC-22*), and cardiac ankyrin repeat protein (*CARP*) were significantly upregulated, and chromosome 5 open reading frame gene 13 (*P311*) was downregulated in the fibrillating atria. FHL1 and CARP play important regulatory roles in cardiac remodeling by transcriptional regulation and myofilament assembly. Induced mRNA expression of both *FHL1* and *CARP* was also observed when cardiac H9c2 cells were treated with an adrenergic agonist. Increasing TSC-22 and marked P311 deficiency could enhance the activity of TGF-β signaling and the upregulated *TGF-β1* and *-β2* expressions were identified in the fibrillating atria. These results implicate that observed alterations of underlying molecular events were involved in the rapid-pacing induced AF, possibly via activation of the β-adrenergic and TGF-β signaling.

Keywords: Atrial fibrillation; Low-density cDNA array; Transcriptional regulator; Transforming growth factor-β signaling

1. Introduction

Atrial fibrillation (AF), the most common sustained arrhythmia, causes progressive alterations in atrial electrical, contractile and structural properties, which are associated with changes in cardiac gene expression [1,2]. Recent studies have focused on the molecular basis of atrial remodeling using candidate gene and genome-wide approaches in AF patients [3–5]. Gene expression profiling studies on dedifferentiation [3], apoptosis [5], fibrosis and thromboembolic events [4] have highlighted the complex regulation of gene expression during AF formation and maintenance. However, thus far, transcriptional regulators involved in regulating transcription in AF have been proposed [6], but attempts to identify the regulators have been lacking. Activation of transcriptional regulators during AF may coordinately lead to sequential transcriptional control events that regulate the phenotype of cardiomyocytes in response to AF disease conditions [6]. In fact, AF is often associated with other cardiovascular diseases such as hypertension, thyrotoxic heart disease, coronary artery disease, rheumatic valve disease, and heart failure [7]. Moreover, changes in gene expression in clinical AF may be the result of AF combined with other underlying heart diseases [4]. Therefore, functional studies of transcriptional regulators in rapid pacing-induced AF or transgenic animal AF models [8] may clarify the underlying molecular mechanisms of AF because the potential confounding effects of other underlying heart diseases have been eliminated.

In the present study, we investigated 84 candidate genes specifically associated with fibrillating atria of a porcine AF

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induced by rapid atrial pacing using a low-density cDNA array. Three genes, four and a half LIM domains protein-1 (*FHL1*), transforming growth factor beta-stimulated clone 22 (*TSC-22*) and cardiac ankyrin repeat protein (*CARP*), encoding transcriptional regulators, were significantly upregulated, and chromosome 5 open reading frame gene 13 (*P311*), an anti-fibrotic gene, was downregulated in the fibrillating atria. Along with confirmation of the selected genes for differential expression profiles, the further identification of protein expression and implicated mechanism in the fibrillating atria were subsequently evaluated. The results provide more insights into the underlying molecular events in pacing-induced AF and evidence of complex transcriptome changes that may accompany AF development.

2. Materials and methods

2.1. AF induced by rapid atrial pacing

A porcine model of AF was used as described previously [9]. Eighteen adult Yorkshire-Landrace strain pigs were used (12 in the AF group and 6 in the sham control group), with mean body weight 62±5 kg. In this study, six pigs in the AF group were added besides the specimens of 6 AF and 6 sham control that were sampled in our previous study [10]. The experimental protocol conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of the National Taiwan University College of Medicine. All pigs were provided by the Animal Technology Institute in Taiwan (ATIT) and housed at the animal facility in the ATIT. Each animal was transvenously implanted with either a high-speed atrial pacemaker (Itrel-III; Medtronic Inc., Minneapolis, MN) for the AF group or an inactive pacemaker for the sham control group (i.e., sham hearts maintained normal sinus rhythm, SR). The atrial pacing lead (Medtronic) was inserted through the jugular vein and screwed to the right atrium. The atrial high-speed pacemaker was programmed to a rate of 400–600 beats per min for 4 weeks in the AF group. After continuous pacing, the atrial pacemaker was turned off and the animals remained in AF. The animals were sacrificed 2 weeks after the pacemaker was turned off, and thus the total duration of atrial depolarization was 6 weeks. In the sham control group, the pacemaker remained off for the entire 6 weeks after implantation.

2.2. Tissue processing

The pigs were anaesthetized and sacrificed at the end of the experimental period. The right atrial appendages (RAA) and left atrial appendages (LAA) were excised and immediately frozen in liquid nitrogen and then stored at −80 °C until use for RNA or protein extraction for later experiments.

2.3. RNA isolation

Total RNA was extracted and quantified from the pig LAA and RAA according to our previous report [11]. In brief, 200 mg of atrial tissue was homogenized on ice by a rotor–stator-type tissue homogenizer in 1 ml TRIzol reagent (GIBCO BRL, Gaithersburg, MD). Cellular debris was removed by centrifugation for 10 min at 12,000×*g* at 4 °C, and RNA was precipitated by adding equal volumes of isopropanol and then washing with 75% ethanol. The resultant RNA was further purified with the RNeasy Midi kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The amount of total RNA was determined spectrophotometrically at 260 nm, and the integrity was confirmed by analysis on a denaturing agarose gel. The RNA was used in the cDNA microarray and quantitative real-time RT-PCR analysis.

2.4. Specialized AF Chip design and preparation

A total of 84 gene sequences were selected for a low-density cDNA array, named the AF Chip. Selection of these genes was based on the following three considerations: the gene had been reported to be associated with a cardiomyopathy [12], the gene was significantly and differentially expressed in fibrillating tissue of a rapid pacing-induced AF model in our previous report on a microarray containing 6032 human cDNA clones (UniversoChip, AsiaBioinnovations, Newark, CA) [10], and the gene clone was available from the IMAGE consortium (Open Biosystems, Huntsville, AL). All symbols and accession numbers of the selected genes used in the AF Chip are shown in Fig. 1. Additionally, two control cDNAs, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-tubulin, as well as pUC19, were included in the chip. The selected clones were purchased from IMAGE consortium, and the sequences were verified in our laboratory. The clones were amplified using PCR with 36 cycles of a denaturing temperature of 95 °C for 30 s, annealing temperature of 55 °C for 30 s, and extension temperature of 72 °C for 45 s. The commercial primers for amplification were as follows: T7 primer (5′-TAA TAC GAC TCA CTA T AG GG-3′), Sp6 primer (5′-CAT ACG ATT TAG GTG ACA CTA TAG-3′), T3 primer (5′-AAT TA A CCC TCA CTA AAG-3′), M13 forward primer (5′-GTA AAA CGA CGG CCA G-3′), and M13 reverse primer (5′-CAG GAA ACA GCT ATG AC-3′) (Invitrogen, Carlsbad, CA). After amplification, the quality and specificity of the PCR products were confirmed by agarose gel electrophoresis.

PCR-amplified DNA products were mixed with dimethyl sulfoxide (1:1, v/v) and then spotted onto amino-coated glass slides (TaKaRa Mirus Bio Inc., Madison, WI) using a robotics SpotArray 24 (PerkinElmer Life Sciences, Boston, MA). To evaluate the reliability of the AF Chip, PCR-amplified DNA products were spotted in duplicate onto a slide. Spotted DNA was crosslinked and denatured according to the manufacturer's instructions (Takara Mirus Bio Inc., http://www.takaramirusbio.com).

2.5. Hybridization and imaging of fluorescently labeled cDNA

Twenty μg of total RNA extracted from the SR and AF subjects was reverse-transcribed with an oligo-dT primer to prepare fluorophore-labeled SR and AF cDNA with Cyanine-3 dUTP (Cy3) and Cyanine-5 dUTP (Cy5) (PerkinElmer Life Science, Boston, MA), respectively. Fluorophore-labeled cDNA pairs were precipitated together with ethanol and purified using Microcon YM-30 purification columns (Millipore, Bedford, MA). The labeled cDNAs were resuspended in the hybridization buffer of 20% formamide, 5× SSC, 0.1% SDS and 0.1 mg/ml salmon sperm DNA (Ambion, Austin, TX), and then denatured by heating at 95 °C for 3 min. The mixture of labeled cDNA pairs was applied to the AF Chips under a 22-mm^2 cover slip and allowed to hybridize for 16 h at 55 °C in a hybridization chamber (GeneMachines, San Carlos, CA). The chips were washed at 45 °C for 10 min in 2× SSC, 0.1% SDS, followed by two washes at room temperature in 1× SSC (10 min) and 0.2× SSC (15 min). After hybridization, the slides were scanned with a dual-laser scanner GenePix 4000B at 10-μm resolution (Axon Instruments Inc., Union, CA). Results of the competitive hybridization were imaged at different photomultiplier settings to yield a balanced and applicable signal. Spots with saturated signal intensity or with signal less than background were excluded from the final data set. The data were converted from image to signal using GenePix Pro 4.1 software (Axon Instruments Inc.) for further statistical analysis.

2.6. Data analysis and gene ontology

The fluorescence intensity of the local mean background was calculated for each spot, and we accepted only those cDNA spots with a fluorescence signal intensity more than the mean local background plus 2 standard deviations (SD) and greater than the intensity of the negative control. The signals from all the experimental arrays were normalized based on the *GAPDH* probe sets in which the average signal from *GAPDH* (as an internal control) was defined as unchanged. On the same AF Chip, the two intensity values of the duplicate cDNA clone spots were averaged and used to determine the ratio between the AF and sham control subjects (i.e., the ratio of Cy5/Cy3 intensities). Genes were classified as differentially expressed when the value of the mean ratios was ≥1.5 or ≤0.67. Functional classification of the differentially expressed genes was according to the GeneCards web site (http://www.genecards.org/ index.shtml) and the Gene Ontology Consortium (http://www.geneontology.org).