International Journal of Cardiology 168 (2013) 2100–2108

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Effects of irbesartan on gene expression revealed by transcriptome analysis of left atrial tissue in a porcine model of acute rapid pacing in vivo

Ravi Kumar Chilukoti [a,1], Jörg Mostertz [a,1,2], Alicja Bukowska [b], Christoph Aderkast [c], Stephan B. Felix [d], Matthias Busch [d], Uwe Völker [a], Andreas Goette [b,e], Carmen Wolke [c], Georg Homuth [a,1], Uwe Lendeckel [c,*]

[a] University Medicine, Ernst-Moritz-Arndt-University Greifswald, Interfaculty Institute for Genetics and Functional Genomics, Greifswald, Germany
[b] Working Group: Molecular Electrophysiology, University Hospital Magdeburg, Otto-von-Guericke University, Magdeburg, Germany
[c] University Medicine, Ernst-Moritz-Arndt-University Greifswald, Institute of Medical Biochemistry and Molecular Biology, Greifswald, Germany
[d] University Medicine, Ernst-Moritz-Arndt-University Greifswald, Department of Internal Medicine B, Greifswald, Germany
[e] Cardiology and Intensive Care Medicine, St. Vincenz-Hospital, Paderborn, Germany

ARTICLE INFO

Article history:
Received 21 August 2012
Received in revised form 18 December 2012
Accepted 13 January 2013
Available online 12 February 2013

Keywords:
Atrial fibrillation
Acute atrial tachyarrhythmia
Angiotensin
CTGF
Endothelin
SGK1

ABSTRACT

Background: Atrial fibrillation (AF) is characterized by electrical and structural remodeling of the atria with atrial fibrosis being one hallmark. Angiotensin II (AngII) is a major contributing factor and blockage of its type I receptor (AT1R) prevents remodeling to some extent. Here we explored the effects of the AT1R antagonist irbesartan on global gene expression and profibrotic signaling pathways after induction of rapid atrial pacing (RAP) in vivo in pigs.

Methods and results: Microarray-based RNA profiling was used to screen left atrial (LA) tissue specimens for differences in atrial gene expression in a model of acute RAP. RAP caused an overall expression profile that reflected AngII-induced ROS production, tissue remodeling, and energy depletion. Of special note, the mRNA levels of *EDN1*, *SGK1*, and *CTGF* encoding pro-endothelin, stress- and glucocorticoid activated kinase-1, and of connective tissue growth factor were identified to be significantly increased after 7 h of rapid pacing. These specific expression changes were additionally validated by RT-qPCR or immunoblot analyses in LA, RA, and partly in LV samples. All RAP-induced differential gene expression patterns were partially attenuated in the presence of irbesartan. Similar results were obtained after RAP of HL-1 cardiomyocytes in vitro. Furthermore, exogenously added endothelin-1 (ET1) induced *CTGF* expression concomitant to the transcriptional activation of *SGK1* in HL-1 cells.

Conclusions: RAP provokes substantial changes in atrial and ventricular myocardial gene expression that could be partly reversed by irbesartan. ET1 contributes to AF-dependent atrial fibrosis by synergistic activity with AngII to stimulate *SGK1* expression and enhance phosphorylation of the SGK1 protein which, in turn, induces *CTGF*. The latter has been consistently associated with tissue fibrosis. These findings suggest ETR antagonists as being beneficial in AF treatment.

1. Introduction

Atrial fibrillation (AF) induces significant electrophysiological and structural changes in the myocardium, and substantially compromises microvascular blood flow [1]. Atrial fibrosis represents a hallmark of AF-dependent structural remodeling [2–6]. In addition to experimental data, histological studies have also shown that the amount of fibrous tissue is increased in fibrillating human atria [7–10]. In particular, patients with permanent AF show severe alterations in tissue architecture [7,11] accompanied by increased atrial expression of *ACE*, *MEK-1*, and *-2*, and *ERK-1*, and *-2*. Fibrosis isolates groups of atrial myocytes as well as individual myocytes which impairs cell-to-cell coupling leading to inhomogeneities in intra- and inter-atrial conduction and slowed conduction velocity. Both mechanisms favor the inducibility of prolonged episodes of AF [12]. AF itself is clearly associated with – and may under certain conditions promote – atrial fibrosis [2]. In the heart failure model, development of atrial fibrosis was associated with increased atrial angiotensin II levels [2]. AF has also been associated with increased plasma and tissue levels of angiotensin II (AngII) [2,6,13,14] which were shown to result from increased expression/activity of ACE [7].

At the atrial level, it is well established that AngII upon binding to its preferred receptor, AT1R, leads to the activation of NADPH oxidase [15–17]. In the early phase of AF, increased NADPH oxidase activity contributes to elevated ROS production [18–20], protein modification, and subsequent alterations in redox-related gene expression patterns [18]. Although in long-lasting AF mitochondrial enzymes become a

* Corresponding author at: Universitätsmedizin Greifswald, Ernst-Moritz-Arndt-University Greifswald, Institute of Medical Biochemistry and Molecular Biology, Fleischmannstraße 42-44, D-17475 Greifswald, Germany. Tel.: +49 3834 865425; fax: +49 3834 865402.
E-mail address: uwe.lendeckel@uni-greifswald.de (U. Lendeckel).
[1] These authors contributed equally to this work.
[2] Present address: Competence Centre Functional Genomics, Junior Research Group Pathoproteomics, Ernst-Moritz-Arndt University, Greifswald, Germany.

0167-5273/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ijcard.2013.01.007

dominating source of ROS [21], the activation of the atrial angiotensin II system, or concomitant cardiovascular disease, can provide a "morphologic, arrhythmogenic substrate" for re-entry arrhythmia and allows AF to persist.

Recent studies have identified connective tissue growth factor (CTGF) as a potent profibrotic factor in various pathophysiological conditions, including AF [20,22]. Increased amounts of CTGF in the left atrial tissue from patients with AF were associated with increased fibrosis and increased tissue levels of AngII [18]. AngII has been shown to induce *CTGF* via activation of Rac1 and NADPH oxidase, leading to increased expression of target genes [18]. Similarly, AngII induced *CTGF* expression in perfused rat hearts [22]. In a porcine AF model, both the tissue levels of AngII and CTGF were increased in the left and right atria [22].

Recent data suggest, however, that CTGF alone is not sufficient to promote the development of fibrosis in all pathophysiological settings. Despite the fact that enhanced *CTGF* expression is regularly associated with fibrosis, in vivo evidence clearly demonstrating causality between elevated *CTGF* expression and induction of fibrosis is still lacking. Of note, over-expression of *CTGF* in cardiomyocytes failed to induce fibrosis [23]. Likewise, in a rapid pacing-induced model of heart failure, there was no evidence of fibrosis despite of substantially increased levels of CTGF protein [24].

In the present study we investigated the expression of *CTGF* in a porcine in vivo model of acute rapid pacing (RAP) and characterized the underlying mechanisms by comparative transcriptome analysis. We took advantage of new developments in pig transcriptomics including improved annotation of the porcine transcriptome [25]. For validation of specific candidate genes as well as determination of time- and frequency-dependency of *CTGF* induction, HL-1 cardiomyocytes subjected to RAP in vitro were used.

Besides the demonstration of a RAP-induced overall atrial expression profile that reflects AngII-dependent induction of ROS production, tissue remodeling, and energy depletion, our data indicate that AngII-dependent up-regulation of cardiac *CTGF* is mediated by a pathway involving transcriptional induction of *ET1* as well as *SGK1* and post-transcriptional activation of SGK1 by phosphorylation.

2. Material and methods

2.1. Rapid atrial pacing (RAP) model

Tissue samples used in this study were from the same animals as described previously [16]. The animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Magdeburg. Briefly, a total of 14 pigs were subjected to closed chest, rapid atrial pacing (RAP). In five animals RAP was performed for 7 h at a rate of 600 bpm (twice diastolic threshold, 2-ms pulse duration; RAP-group). In five additional animals RAP was performed in the presence of irbesartan infusion (1 mg/kg bolus injection followed by 0.3 $mg \times kg^{-1} \times h^{-1}$ i.v.; RAP + Irb-group), and four pigs were instrumented without further intervention (sham). After the 7 hour pacing period, the chest and the pericardial sac were opened and the heart was exposed. Tissue samples were immediately frozen in liquid nitrogen and used for RNA profiling and immunoblot analyses as described below.

2.2. Cardiomyocyte cell culture

The murine cardiomyocyte cell line HL-1 was kindly provided by Dr. William Claycomb (Louisiana State University Health Science Center, New Orleans, LA). Cells were passaged and cultured in Claycomb-Medium (Sigma-Aldrich, Taufkirchen, Germany) as described [26].

For rapid pacing in vitro, HL-1 cells were seeded into gelatin/fibronectin-coated 6-well plates at a density of 1×10^6 cells/well/4 ml. Pacing was performed with carbon electrodes using the culture cell pacer system C-Pace EP (IonOptix, Milton, MA). Cells were paced at 20 Hz (if not indicated otherwise) for 7 or 24 h (5 V/cm, 4 ms bipolar pulse).

2.3. RNA isolation and quality control

Total RNA was extracted from frozen specimens of the left and right atrium (LA, RA), and left ventricle (LV), and also from cultivated HL-1 cardiomyocytes by performing a modified phenol extraction using the TRIzol reagent (Invitrogen, Karlsruhe, Germany) [27]. For tissue samples, homogenization of snap-frozen tissue was carried out using a bead mill dismembrator (Braun) at 2600 rpm for 2 min. RNA was further purified using the RNA Clean-Up and Concentration Micro Kit (Norgen, Canada) and concentrations were measured using a ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA). For transcriptome analysis, RNA integrity was validated by means of the lab-on-chip capillary electrophoresis technology (Bioanalyzer 2100, Agilent Technologies, Santa Clara, CA, USA). Only RNA samples with an RNA integrity number (RIN) > 7.5 [28], 260/280 nm ≥ 1.8, 260/230 nm ≥ 1.9 were used for microarray analyses and RT-qPCR experiments.

2.4. Microarray expression analysis

The purified total RNA samples from the left atrium (LA) were subjected to transcriptome analysis using the GeneChip Porcine Genome Arrays (Affymetrix Inc., Santa Clara, CA, USA). Expression profiling was done at the level of single RNA samples for RAP (n = 3), RAP/I (n = 5), and sham (n = 4). Target preparation and hybridization were performed according to the manufacturer's instructions using the one-cycle target labeling and control reagents kit (GeneChip® Expression Analysis Technical Manual, Affymetrix Inc.). For each sample, 5 μg of total RNA was reverse transcribed into cDNA. After subsequent in vitro transcription, the concentration and purity of the resulting biotinylated cRNA were assessed by using the ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.) and the cRNA pattern was examined by gel electrophoretic separation using the Bioanalyzer 2100 (Agilent Technologies). Hybridization was achieved by incubating target-filled array cartridges at 45 °C for 16 h. The arrays were washed and stained with streptavidin–phycoerythrin using the standard fluidics protocol for Fluidics Station 450 (Affymetrix Inc.) and subsequently scanned with a GeneChip Scanner 3000 (Affymetrix Inc.).

2.5. Microarray data analysis

Quality assessment of all hybridizations was carried out by inspecting scan images and by carefully reviewing external and endogenous controls using the Expression Console software (Affymetrix Inc.). For all processed arrays, the available control parameters passed the default threshold tests and all arrays were considered to be of good quality. Microarray data analysis was done using the Rosetta Resolver® system for gene expression data analysis (Rosetta Bio software, Seattle, WA, USA). In brief, normalized intensity signals were calculated by processing the Affymetrix CEL files using the Affymetrix Rosetta intensity data summarization. Samples were analyzed based on fold change calculations and signal statistics after direct comparison of different samples (sham vs. RAP, sham vs. RAP/I). Genes exhibiting significantly different expression on the mRNA level were identified using the following cut-off criteria: one-way analysis of variance with subsequent Benjamini and Hochberg false discovery rate multiple-testing correction on pair-wise comparisons (ANOVA, $p \leq 0.05$), signal correction statistics (Ratio Builder, $p \leq 0.05$) and fold-change ≥ 1.5-fold (for a complete list of significantly regulated genes, see Supplemental Material 1). A heat map was generated using the k-NN classifier with Euclidean distance similarity measure in order to display specific gene expression signatures including those associated with ROS production and redox-signaling, tissue remodeling, and energy depletion.

2.6. In silico-pathway- and functional analysis of microarray data

In-silico-pathway- and functional analysis of differentially expressed genes was carried out using the commercial systems biology oriented package *Ingenuity Pathways Analysis* (Ingenuity Systems, Inc. CA, USA) using the annotation details provided by Christopher K. Tuggle [25] with their corresponding gene identifiers and expression values.

2.7. Reverse transcription quantitative PCR (RT-qPCR)

To validate several specific candidate genes resulting from the microarray analyses, the fluorescent reporter probe method was used. Specific probes and primers were used as listed in Table 1 (pig). Probes and primers for qPCR were designed and functionality tested by Microsynth AG (Balgach, Switzerland) on the basis of the appropriate *Sus scrofa* DNA-sequences. All samples were analyzed in duplicate. Briefly, 400 ηg of the purified total RNA was converted into cDNA using SuperScript™ II RT kit (Invitrogen, Life Technologies GmbH, Darmstadt, Germany). Gene expression was then measured with the 7900HT Fast Real-Time PCR System (Applied Biosystems, CA, USA). Thermocycling was performed for all samples in a 25 μl PCR master-mix containing 2.5 μl cDNA. Initial denaturation at 95 °C for 10 min was followed by 50 cycles of denaturation at 95 °C for 15 s, and annealing/elongation at 60 °C for 30 s. Specific cDNA signals were normalized to those of GAPDH. The obtained threshold cycles were then used to calculate relative gene expression differences as fold-change. Significance of the gene expression changes was tested by using Mann–Whitney *U* test (Wilcoxon rank-sum test).

For the HL-1 cell experiments, qPCR was performed using the CFX96 (BioRad, Munich, Germany). All samples were analyzed in triplicate. A 25 μl reaction mixture consisted of 1× SensiMix SYBR (Quantace, Bioline, UK), 1 μl cDNA, and 0.3 μmol/l specific primers (listed in Table 1). Initial denaturation at 95 °C for 10 min was followed by 40 cycles with denaturation at 95 °C for 15 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 30 s. Quantities of *RPL0* mRNA were used to normalize cDNA contents. The fluorescence intensity of the double-strand specific SYBR-Green, reflecting the amount of PCR product actually formed, was obtained in real-time at the end of each elongation step. The amounts of specific initial template mRNA were calculated by means of the