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Inhibition of aldosterone synthase (CYP11B2) by torasemide prevents atrial fibrosis and atrial fibrillation in mice

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ABSTRACT

Loop diuretics are used for fluid control in patients with heart failure. Furosemide and torasemide may exert differential effects on myocardial fibrosis. Here, we studied the effects of torasemide and furosemide on atrial fibrosis and remodeling during atrial fibrillation. In primary neonatal cardiac fibroblasts, torasemide (50 μM, 24 h) but not furosemide (50 μM, 24 h) reduced the expression of connective tissue growth factor (CTGF; 65 ± 6%) and the pro-fibrotic miR-21 (44 ± 23%), as well as the expression of lysyl oxidase (LOX; 57 ± 8%), a regulator of collagen crosslinking. Mineralocorticoid receptor (MR) expression and activity were not altered. Torasemide but not furosemide inhibited human aldosterone synthase (CYP11B2) activity in transfected lung fibroblasts (V79MZ cells) by 75 ± 1.8%. The selective CYP11B2 inhibitor SL242 mimicked the torasemide effects. Mice with cardiac overexpression of Rac1 GTPase (RacET), which develop atrial fibrosis and spontaneous AF with aging, were treated long-term (8 months) with torasemide (10 mg/kg/day), furosemide (40 mg/kg/day) or vehicle. Treatment with torasemide but not furosemide prevented atrial fibrosis in RacET as well as the up-regulation of CTGF, LOX, and miR-2, whereas MR expression and activity remained unaffected. These effects correlated with a reduced prevalence of atrial fibrillation (33% RacET + Tora vs. 80% RacET). Torasemide but not furosemide inhibits CYP11B2 activity and reduces the expression of CTGF, LOX, and miR-21. These effects are associated with prevention of atrial fibrosis and a reduced prevalence of atrial fibrillation in mice.

1. Introduction

Loop diuretics, such as furosemide and torasemide, are used for symptomatic fluid control in patients with chronic heart failure (CHF) [1]. Both diuretics exert similar diuretic effects [2,3] but their effect on clinical outcomes remains uncertain. Interestingly, previous studies have reported that torasemide — but not furosemide — decreased collagen volume fraction in the hearts of patients with chronic heart failure (CHF) [4], and early evidence suggests that torasemide treatment was associated with lower mortality compared to furosemide in the TORasemide In Chronic Heart Failure (TORIC) study [5].

Myocardial fibrosis is a hallmark of both left ventricular dysfunction and of atrial arrhythmogenic structural remodeling [6–8] which represents an important substrate for atrial fibrillation (AF) [9]. An important mediator of left ventricular and atrial fibrosis is the activation of mineralocorticoid receptors (MRs) by aldosterone [10–15]. Interestingly, experimental and human studies have reported that torasemide, but not furosemide, may exert anti-aldosterone effects [16–18]. However, the underlying mechanisms and the consequences of these observations are only partially understood, especially in the context of atrial fibrillation.

Our previous studies found that the fibrosis in left atria of patients with AF is characterized by increased angiotensin II tissue concentrations as well as increased expression and activity of the small Rho-GTPase Rac1, increased expression of the connective tissue growth factor (CTGF) and increased expression of lysyl oxidase (LOX), a key enzyme of collagen crosslinking [19–23], as well as microRNA-21 (miR-21), a regulator of ventricular [24] and atrial fibrosis [25]. This pro-fibrotic signaling pathway can be activated by aldosterone [26]. Based on these findings, the present study was undertaken to test the hypothesis of a potential differential effect of the loop-diuretics torasemide and furosemide on myocardial fibrosis and atrial remodeling and to characterize the underlying signaling events. To this end,

Abbreviations: AF, atrial fibrillation; AngII, angiotensin II; CTGF, connective tissue growth factor; CY11B2, aldosterone synthase; LA, left atrium; LOX, lysyloxidase; miR-21, microRNA-21; MRs, mineralocorticoid receptors; RacET, transgenic mice with cardiac overexpression of Rac1 GTPase; SR, sinus rhythm; WT, wild-type mice.

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cultured primary cardiac cells and Rac1 transgenic mice were studied that spontaneously develop atrial fibrosis and atrial fibrillation with aging.

2. Materials and methods

2.1. Cell isolation and culture

Cardiomyocytes and cardiac fibroblasts were isolated from the atria and the ventricles of 5 day old neonatal Sprague–Dawley rat hearts [27]. Purity of fibroblasts was confirmed by vimentin staining. After 48 h in culture, myocytes exhibited regular spontaneous contractions. The primary cells were used for experiments after 3–6 days of culture.

HL-1 cells, a cardiac muscle cell line from the AT-1 mouse atrial cardiomyocyte tumor lineage, were a gift from William C. Claycomb, Ph.D.; Q7 Professor of Biochemistry and Molecular Biology (LSU Health Sciences Center; New Orleans) [50]. HL-1 cells maintain the ability to contract and retain differentiated cardiac morphological, biochemical, and electrophysiological properties [50].

Ventricular rat neonatal cells were used for the signaling experiments, the results were confirmed using pooled atrial neonatal cardiomyocytes and fibroblasts as well as HL-1 cells.

2.2. Animal studies

Mice with cardiac overexpression of constitutively active (V12) Rac1 under the control of the α-myosin heavy chain (MHC) promoter (RacET) and wild type controls (WT; FVB-N-strain) promoter [28] were fed with normal chow (ssniff, Germany) or normal chow supplemented with 10 mg/kg/day of commercially available torasemide (Meda Pharma, Germany) or 40 mg/kg/day furosemide (STADA, Germany) for 8 months. Heart rate, regularity and the presence of p-waves were documented by ECG (Picker, Schwarzer CU 12 system) under anesthesia with ketamine (ketavet, 100 mg/kgKG) and xylazine (rompun, 10 mg/kgKG) ip. Transthoracic echocardiography (Vevo 770® high-resolution imaging system (Visual Sonics, Canada) with a center frequency of 30 MHz and a focal depth of 12.6 mm) was performed in all mice under anesthesia with ketamine (ketavet, 100 mg/kgKG) and xylazine (rompun, 10 mg/kgKG) ip; fractional shortening (FS), enddiastolic thickness of interventricular septum (IVSd) and the left posterior wall (LPWd) as well as the left ventricular enddiastolic diameter (LVDd) were taken. The characteristics of the mice are depicted in Table 1. None of the wild type mice exhibited atrial arrhythmias [19,20,29].

The study was approved by the animal ethics committee of the Universität des Saarlandes and is in accord with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Pub. No. 85-23, revised 1996). The study was not funded by the pharmaceutical industry.

2.3. Reverse-transcriptase polymerase-chain reaction (RT-PCR)

Total RNA isolation, reverse transcription, and competitive PCR was performed according to standard techniques. The sense, (5′-GATATCTTCAAAAGAGAGG-3′) and anti-sense (5′-TACTGTTCAG CTAATCACG-3′) primers were used to amplify a CYP11B2 cDNA and (5′-TACTGTTCAGCTAATCACG-3′) and anti-sense (5′-TACTGTTCAG CTAATCACGC-3′) primers were used to amplify a MCR cDNA fragment. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an external standard. Each PCR cycle consisted of denaturing at 94 °C for 30 s, annealing at 53 °C for 30 s, and elongation at 72 °C for 60 s. The linear exponential phases for CYP11B2, MCR, and GAPDH PCR were 40 and 22 cycles, respectively. Equal amounts of corresponding CYP11B2, MR, and GAPDH RT-PCR products were loaded on 1.5% agarose gels and optical densities of ethidium-bromide stained DNA bands were quantitated.

2.4. Micro-RNA isolation and RT-PCR

MicroRNAs were isolated by a miRNA isolation kit (mirVana, Ambion). For real-time PCR, a target-specific stem loop structure and reverse transcription primers were used. After reverse transcription miR-21 expression was quantified with specific TaqMan hybridization probes (TaqMan miR-21 microRNA assay, Applied Biosystems). The small RNA molecule U6 small nuclear (*Rnu6-2*) was amplified as a control.

2.5. Western analysis

Protein lysates were prepared as described [27]. Immunoblotting was performed using Anti-Rac1 (Rac1, Upstate, clone 23A8), CTGF (sc-14939, Santa Cruz; USA), LOX (ab31238, Abcam, UK), and GAPDH (ab8245, Abcam; UK). Immunodetection was accomplished using goat anti-rabbit or goat anti-mouse secondary antibody (1:4000 dilution, Sigma) and an enhanced chemiluminescence kit (Amersham) followed by densitometry.

2.6. Rac1 GST-PAK pull-down assay

Pull-down assays were performed using agarose labeled PAK-1 fusion protein (Upstate, USA) as described (4). Q8

2.7. CYP11B2 activity

The untransfected V79MZ Chinese hamster cells (provided by Prof. Bernhardt, Saarland University) and the human CYP11B2 expressing V79MZ cell line were grown as monolayer culture in Dulbecco's modified Eagle medium (DMEM; c.c.pro, Oberdorla, Germany) supplemented with 5% of fetal calf serum (FCS; Sigma), penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), and sodium pyruvate (1 mM) at 37 °C in 5% CO_2 in air.

The cDNA of CYP11B2 was amplified from total RNA extracted from NCI-H295R cells (ATCC, Manassas, USA). Total RNA was isolated with the GenElute™ Total Mammalian Miniprep Kit (Sigma-Aldrich, Munich, Germany), following the manufacturer's instructions. cDNA synthesis was performed using Improm-II™ Reverse Transcriptase (Promega, Madison, USA) according to Promega's standard reverse transcription protocol. This procedure was followed by a polymerase chain reaction (PCR) to amplify the CYP11B2 gene with specific sense (5′-GCCACCAT GGCACTCAGGGCAAAGGCAGAGG-3′) and anti-sense (5′-CTAGTTAATC GCTCTGAAAGTGAGGAGGGGGGACG-3′) primers. The purified PCR-product was cloned into MCS of pcDNA3.1/V5-His© TOPO® TA expression vector from invitrogen (Carlsbad, USA).

One day before transfection, 2×10^5 untransfected V79MZ cells were seeded into 35 mm culture dishes (Nunc, Wiesbaden, Germany) and incubated overnight in complete growth medium. On the following day the approximately 70% confluent culture was transfected with the generated plasmid using the liposomal transfecting reagent Roti®-Fect (Carl Roth, Karlsruhe, Germany) following the manufacturer's recommendations. Stably transfected clones carrying the Neomycin resistance marker were selected with 750 μg/ml G418 sulfate.

For determination of inhibitory effects on aldosterone synthase, V79MZ cells expressing human CYP11B2 gene were grown on 24-well cell culture plates (8×10^5 cells per well) with 1.9 cm^2 culture area per well (Cellstar®, Greiner Bio-One, Frickenhausen, Germany) in 1 ml DMEM culture medium until confluence. Before testing, the DMEM culture medium was removed and 450 μl of fresh DMEM with 50 μM torasemide or 50 μM furosemide was added. Every value was determined in duplicate. After a preincubation step of 60 min at 37 °C, the reaction was started by the addition of 50 μl of DMEM containing the substrate 11-deoxycorticosterone (containing 0,15 μCi of [1,2-^{3}H] 11-deoxycorticosterone, dissolved in ethanol, final concentration 100 nM) and cells were incubated for further 45 min. Controls were treated in

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