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A Cardiac Mouse Model for Nongenomic Mineralocorticoid Receptor Effects

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Authors' contributions

This work was carried out in collaboration between all authors. Author SW managed and performed the experiments and the analyses of the study, managed literature searches and wrote the first draft of the manuscript. Author UG managed analyses of the study, managed literature searches and wrote the final draft of the manuscript. Authors BS, SR, CG and IBB managed experiments and analyses of the study. Authors MG and JN designed the study, wrote the protocol and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: As a ligand-dependent transcription factor the mineralocorticoid receptor (MR) regulates water and electrolyte homeostasis in epithelial tissues but also plays a crucial role in the pathogenesis of cardiovascular diseases. In addition to its genomic effects, via the glucocorticoid response elements, rapid interactions with cytosolic signaling cascades have been described, but the physiological or pathophysiological role of this nongenomic MR pathway is still hardly known.
Study Design: Transgenic and wild type mice (FVB/N background) of random sex were used in this study. Experimental groups (n = 8-10 for each subgroup) were: A) Cardiac function and gene

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expression at six months of age; B) Cardiac function and gene expression at twelve months of age; C) Cardiac function at six months of age after 4 weeks aldosterone/NaCl treatment.
Place and Duration of Study: Institute for Pharmacology and Toxicology, Medical Faculty of the Martin Luther University Halle-Wittenberg, between June 2008 and November 2011.
Methodology: To investigate the cardiac nongenomic MR effects *in vivo*, we generated a transgenic (TG) mouse model with cardiomyocyte-specific overexpression of a truncated variation of the human MR (hMR^{DEF}). Characterization of six and twelve months old mice focused on cardiac function, electrical activity, and gene transcription under baseline and stimulation conditions by either isoproterenol or aldosterone/NaCl treatment.
Results: Whereas overexpression of a full-length MR in the heart was lethal, the phenotype of the hMR^{DEF}-TG mouse seemed inconspicuously. Noteworthy, the nongenomic MR effect modulated the cardiac transcription of the α-subunit of the voltage-gated potassium channel ERG, which resulted in prolonged intraventricular electrical activity. Therefore, nongenomic MR signaling pathways may be responsible for MR-associated cardiac arrhythmias.
Conclusion: Our findings demonstrate that nongenomic MR effects can modulate cardiac electrophysiology *in vivo* and therefore indicate an involvement of nongenomic MR signaling pathways in the pathogenesis of cardiac dysfunction.

Keywords: Mineralocorticoid receptor; nongenomic effects; transgenic mice; real time PCR; ERG potassium channel; cardiac electrophysiology; cardiovascular diseases.

1. INTRODUCTION

The mineralocorticoid receptor (MR) is a steroid hormone receptor and therefore a member of the nuclear receptor subfamily [1]. The MR takes part in blood pressure regulation by controlling salt and water homeostasis [2,3]. As a ligand-dependent transcription factor, with its endogenous ligand aldosterone (aldo), the MR modulates protein expression via hormone response elements (HRE) [2,4]. In addition to its physiological role in epithelial tissues, the MR is involved in the pathogenesis of several diseases [3,5-13]. Of special interest is its role in the development of cardiovascular dysfunction [14-17]. In the cardiovascular system, the MR promotes fibrosis, endothelial dysfunction, hypertrophy and structural remodeling as well as inflammation [18-26]. Furthermore, the MR decreases outward rectified potassium currents and enhances the L-type calcium current in cardiomyocytes, which increases the incidence of arrhythmias [27-32]. The underlying mechanisms are not completely understood. Besides its classical genomic signaling pathway the MR shows rapid cytosolic effects which are unaffected by inhibition of transcription [33].

The nongenomic MR effects activate protein kinase C (PKC), which raises cytosolic Ca^{2+} concentration and activates the Na^+/H^+ exchanger (NHE) [34-37]. This results in changes of intracellular pH and Na^+ concentration, increased cell volume and activity of pH-dependent potassium channels [3]. Furthermore, nongenomic proinflammatoric and arteriosclerosis-promoting MR effects as well as a rapid aldosterone-dependent increase of reactive oxygen species (ROS) via extracellular signal-regulated kinase 1 and 2 (ERK1/2) phosphorylation and PKC activity have been reported [38-41].

Moreover, activation of cytosolic signaling cascades by the nongenomic MR signaling pathways could modulate transcription. This can be HRE-independent via interaction with the cytosolic phosphatase calcineurin PP2BAβ and the CREB/cAMP-pathway regulating for example proinflammatoric genes [42,43] or via MR-dependent transactivation of the membrane-associated epidermal growth factor receptor (EGFR) that induces activation of mitogen activated protein (MAP) kinase cascade followed by phosphorylation of transcription factors, thus enhancing the transcription of other growth factors, which promotes proliferation [44-46]. In addition, transcriptional effects of the cytosolic MR signaling pathways can also be mediated by interaction with genomic signaling pathways [37,47]. For example, MR-dependent ERK phosphorylation leads to increased MR translocation into the nucleus, elevating genomic MR effects [46].

To examine the pathophysiological role of the nongenomic MR signaling pathways in the heart isolated from the classical genomic effects, we generated a new transgenic mouse model with cardiomyocyte-specific expression of a truncated

human MR (hMR^{DEF}) which only mediates the nongenomic effects [2]. We show that the nongenomic MR signaling pathways do not lead to enhanced lethality like the genomic MR effects. In addition, our data indicate that the nongenomic signaling pathways decreases transcription of the α-subunit of the outward rectified potassium channel ERG (Ether-a-go-go-Related Gene), promoting cardiac dysfunction and the incidence of arrhythmias. Taken together, our findings identify nongenomic MR effects as possible factors in the pathogenesis of cardiac arrhythmia.

2. MATERIALS AND METHODS

2.1 Generation of the Transgenic Mouse Model

Using the peGFP-C1-hMR vector [48], containing the human MR coupled with the gene of the enhanced green fluorescent protein (eGFP), the truncated hMR fragment (hMR^{DEF}, 921 bp) was created as previously described [2]. Transgenic mice (FVB/N background) with cardiomyocyte-specific overexpression of the hMR^{DEF} were generated as described [49]. Heart-specific expression was achieved by the use of the α-myosin heavy chain promoter. Similarly, a transgenic mouse line was generated with a cardiac specific overexpression of the full-length MR (hMR).

Investigations were performed on six and twelve months old transgenic animals of each gender and wild type littermates as controls. All mice were housed under conditions of optimum light, temperature and humidity with food and water provided ad libitum. Transgene-positive mice were identified by PCR assay of tail genomic DNA. One founder lineage was established and used for further studies.

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Animals were handled and maintained according to approved protocols of the animal welfare committee of the University of Halle-Wittenberg, Halle, Germany (approval reference number 42502-02-691 MLU).

2.2 Cardiac mRNA Expression

Total RNA was extracted from cardiac ventricle tissue samples using the TRIzol reagent protocol (Ambion®, Life Technologies, Darmstadt, Germany). Subsequently, after the digestion with DNaseI, cDNA synthesis was performed with RevertAidTM H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Darmstadt, Germany) according to the manufacturer's instructions for the reverse transcriptase-polymerase chain reaction (RT-PCR) and with a modified protocol for the quantitative real-time PCR (qPCR).

Qualitative expression of hMR^{DEF} in the heart was analysed by RT-PCR. The following primer pair was used: sense [5'-gaaattgtatatgcaggcta-3'] and antisense [5'-tgcagtcgaacgaactgaag-3']; product size: 350 bp.

Quantitative real-time PCR (qPCR) was performed in a Stratagene Mx3005P System by using the Platin SYBR Green KIT (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions with the specific couple of primers (Supplement data, Table S1) to examine the relative mRNA expression of the endogenous MR- (*Nr3c2*), hypertrophy- (*Nppa*, *Nppb*, *Myh6*, *Myh7*), fibrosis- (*Col1a1*, *Col3a1*, *Fn1*) and inflammation- (*Serpina3*) related genes as well as the transcription of the α-subunits of the cardiac L-type calcium channel (*Cacna1c*) and the voltage-gated potassium channel mERG (*Kcnh2*). For the relative mRNA amounts of hMR^{DEF} the tagged eGFP was detected. The 18S rRNA was used for normalization and the relative mRNA expression was calculated according to the $2^{(-\Delta\Delta C(t))}$ method as described before [50].

2.3 Western Blot Analysis

Preparation of homogenates was performed as described previously [51]. Double strength sample buffer (pH 7.8) containing 20 mmol/l Tris-base, 2 mmol/l EDTA, 20% (w/v) SDS, 10% (v/v) glycerol, 1.2% (w/v) dithiothreitol, and 0.004% (w/v) bromophenol blue was added and further processed as reported [52]. Equal amounts of protein were loaded per lane. Minigels were run using 10% polyacrylamide separating gels. Proteins were electrophoretically transferred to nitrocellulose membranes (Whatman, Dassel, Germany) in 50 mmol/l sodium phosphate buffer (pH 7.4) 2.5 h at 1.5 A at 4℃. As loading control, reversible staining of total proteins by Ponceau S was used directly after the transfer (Supplemental data, Fig. S1). Next, membranes were blocked with TRIS-buffered saline containing 5 % non-fat dry milk powder and 0.01% Tween 20 for 2 h at room temperature

followed by incubation with a polyclonal antiGFP antibody (1:300, GFP (FL):sc-8334, Santa Cruz Biotechnology, Santa Cruz, USA) in blocking buffer (5 % non-fat dry milk powder) at 4℃ over night. Subsequently the membrane was treated with a alkaline phosphatase-conjugated secondary antibody (anti-rabbit IG, 1:1000, Sigma-Aldrich Chemical Co, St Louis, MO, USA). Bands were detected using nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate as alkaline phosphatase substrate.

2.4 Immunohistochemistry

Tissue probes of mouse ventricles were fixed in buffered 4% formaldehyde and routinely embedded in paraffin. Phosphate buffered saline (PBS) was used for all washings and dilutions. Four-µm-thick paraffin tissue sections were deparaffinized with xylene and graded ethanol. For pathohistological analysis, tissue sections were routinely stained with H&E and with Masson's trichrome stain for fibrosis. For immunohistochemical labeling, antigen retrieval was achieved by heating the sections in 10 mM sodium citrate buffer, pH 6.0, at 95℃ for 30 min in a domestic vegetable steamer. Immunostaining was performed according to the standard protocol routinely used for immunohistology [53]. We recently reported that endogenous Fc receptors in routinely fixed cells and tissue probes do not retain their ability to bind Fc fragments of antibodies [54]; therefore, blocking the endogenous Fc receptors prior to incubation with antibodies was omitted. The unusually strong autofluorescence of the probes did not permit to visualize GFP in the heart probes directly. Therefore, GFP was detected indirectly using rabbit polyclonal antiGFP antibodies. After immunoreaction with primary antibodies and following washing in PBS, sections were treated for 10 min with methanol containing 0.6% H_2O_2 to quench endogenous peroxidase. Bound primary antibodies were detected with AmpliStain™ Horse Radish Peroxidase (HRP) conjugate (SDT, Baesweiler, Germany), according to manufacturers' instructions. The HRP label was visualized using NovaRed substrate kit (Vector Laboratories, Burlingame, CA, USA). All steps were preceded by rinsing with PBS (pH 7.4).

2.4.1 Microscopy and image processing

Immunostained sections were examined on a Zeiss microscope "Axio Imager Z1". Microscopy images were captured using AxioCam digital microscope cameras and AxioVision image processing (Carl Zeiss Vision, Germany). Images shown are representative of three independent experiments which gave similar results. The images were acquired at 96 DPI and submitted with the final revision of the manuscript at 300 DPI.

2.5 Electrocardiography

Surface electrocardiogram (ECG) measurements (bipolar extremity derivation, Einthoven I.) were performed on mice, anaesthetized with midazolam (18 mg/kg) and ketamine (160 mg/kg) allowing spontaneous breathing, using a commercially available electrocardiographic system (BioAmp; ADInstruments, Spechbach, Germany). All recordings were analyzed for heart rate, P, PQ, QRS, QT times by the PowerLab software program (ADInstruments, Spechbach, Germany). To calculate QT_C time, Bazett formula was used to correct QT time ($QT_C = {}^{QT}/_{\sqrt{RR}}$).

2.6 Echocardiography

Two-dimensional transthoracic echocardiography was performed on anaesthetized mice (intraperitoneally with a mixture of midazolam (18 mg/kg) and ketamine (160 mg/kg)) using the Vevo® 2100 Sonograph (Visual Sonics, Toronto, Canada) as previously described [52].

2.7 Stimulation Conditions

2.7.1 β-Adrenoceptor stimulation

During electrocardiographic and echocardiographic investigations, anaesthetized mice received an intraperitoneal injection of the β-adrenoceptor agonist isoproterenol (1 mg/kg) for short-term stress-induction.

2.7.2 Aldosterone/NaCl treatment

Five months old animals received subcutaneous aldosterone (aldo) releasing pellets (releasing rate 0.75 µg/h = 600 µg/kg/d, Innovative Research of America, Sarasota, USA) combined with high-salt intake (1% sodium chloride (NaCl) in tap water ad libitum) about four weeks.

2.8 Statistics

Data are presented as mean ± SEM. Statistical significance was estimated by a Mann-Whitney test or by analysis of variance (ANOVA) followed by Bonferroni´s t-test as appropriate using the

software Prism 5 (GraphPad Software, La Jolla, CA, USA) at a *P*-value = 0.05.

3. RESULTS

3.1 The hMR^{DEF} in the Heart

Overexpression of the truncated (hMR^{DEF}) and full-length (hMR) human mineralocorticoid receptor restricted to the heart of transgenic mice was achieved by means of the α-myosin heavy chain (αMHC) promoter. While breeding of the hMR^{DEF} transgenic mouse line was without difficulty, the offspring of the hMR mice showed enhanced lethality within the first fourteen days after birth (Supplement data, Fig. S2). This time frame reflects the characteristics of the αMHC promoter which is mainly activated (thyroid hormone-dependent) after birth. Therefore, no hMR mouse was alive at the time of examination and the hMR^{DEF} mice had to be compared to wild type littermates.

hMR^{DEF} mRNA measured by RT-PCR was detectable in hMR^{DEF} but not in WT hearts (Fig. 1A), indicating transcriptional activity of the transgene. Using the hematoxylin and eosin and Masson's trichrome staining to detect fibrosis, no pathological alterations could be detected between ventricles of hMR^{DEF} and WT samples (Supplement data, Fig. S3). For comparison: a higher grade of fibrosis was detected in hMR myocardium (Supplement data, Fig. S3). To visualize hMR^{DEF} expression in the myocardium, we carried out indirect immunostaining of hMR^{DEF} using an anti-GFP antibody in ventricular sections. Fig. 1C demonstrates localization of hMR^{DEF} in cardiac myocytes of TG mice, where the staining, specific for hMR^{DEF}, was prominent in the sarcoplasm (Fig. 1C). For comparison, staining was also performed in hMR heart sections where GFP immunostaining was found mainly in the nuclei indicative for an increased genomic activity of the overexpressed MR (Supplement data, Fig. S3). Histology was studied for at least three ventricular sections per group (Fig. 1C and Supplemental Fig. S3 show typical results for histological staining). Western blot analysis confirmed the desired cardiac-specific expression of the transgene at the protein level; no corresponding protein band could be detected in lung, liver or kidney of hMR^{DEF} mice (Fig. 1B). Furthermore, electrolyte levels in blood (sodium, potassium, and calcium) were in the normal range and unchanged in TG mice compared to WT.

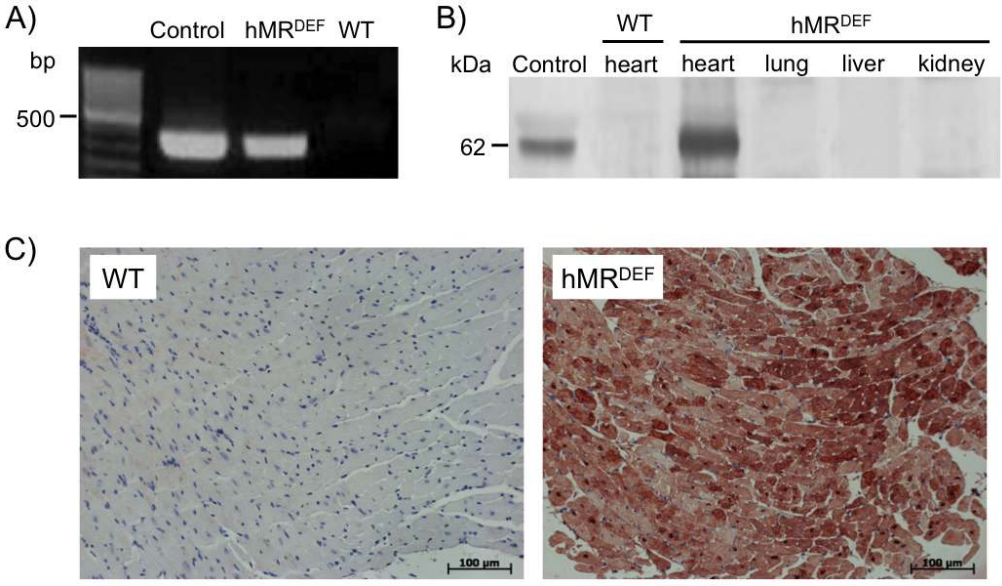

Fig. 1. Cardiac hMR^{DEF} expression in adult transgenic mice. (A) Representative RT-PCR analysis of total RNA isolated from ventricles of wild type (WT) and transgenic (hMR^{DEF}) mice revealed expression of human MRDEF mRNA only in hMR^{DEF} mice. As control a hMR^{DEF} cDNA-containing plasmid was used as template for PCR (Ctr). (B) Representative Western blot analysis of ventricular homogenates (50 µg protein per lane) demonstrates that protein expression of hMR^{DEF} is restricted to the heart. (C) By immunohistology, hMR^{DEF} was detected only in hMR^{DEF} heart sections but not in WT hearts

3.2 Cardiac Function, Electrical activIty and Transcription of Six Months old Mice

Cardiac hMR^{DEF} overexpression did not affect survival of six months old hMR^{DEF} mice (Supplement data, Fig. S2) and did not increase relative heart weight compared to wild type (WT) littermates at the time of examination (Supplement data, Table S2).

To investigate cardiac function and electrical activity in hMR^{DEF} and WT littermates, we recorded echocardiography and surface electrocardiography (ECG) in six months old mice under baseline condition and under β-adrenoceptor stimulation. No differences could be detected in left ventricular function under baseline condition or under short-term β-adrenoceptor stimulation between hMR^{DEF} and WT mice by using two-dimensional echocardiography (Supplement data, Fig. S4A and B). Also the ECG, in rest and after isoproterenol injection, was unchanged between hMR^{DEF} and WT animals (Supplement data, Fig. S5A and B).

To investigate whether the presence of hMR^{DEF} in the heart affected cardiac transcription, qPCR studies were performed. Next to the mRNA expression of the endogenous MR, encoded by *Nr3c2*, transcription of several hypertrophy, fibrosis and inflammation related genes was analyzed. Moreover, the mRNA expression levels of the α-subunits of the cardiac L-type calcium channel (*Cacna1c*) and the voltage-gated potassium channel ERG (*Kcnh2*) were measured (Fig. 2). In hMR^{DEF} mice, mRNA expression of endogenous MR was increased compared to WT littermates as well as the transcription of *Nppa*, *Nppb*, *Myh7*, *Col1a1*, *Col3a1* and *Fn1*. Furthermore, the mRNA amount of the α-subunit (*Cacna1c*) of the L-type calcium channel was also enhanced in the hMR^{DEF} heart. Interestingly, *Myh6* mRNA expression level, encoding cardiac α-myosin heavy chain (αMHC), showed no alteration in the presence of hMR^{DEF}. The transcription of the α-subunit of the voltage gated potassium channel hERG (*Kcnh2*) in hMR^{DEF} hearts was also not changed compared to WT mice (Fig. 2).

3.3 Cardiac Function, Electrical Activity and Transcription Under aldo/NaCl Stimulation

Aldo/NaCl treatment about four weeks did not increase relative heart weight of hMR^{DEF} mice compared to WT aldo/NaCl (Supplement data, Table S3).

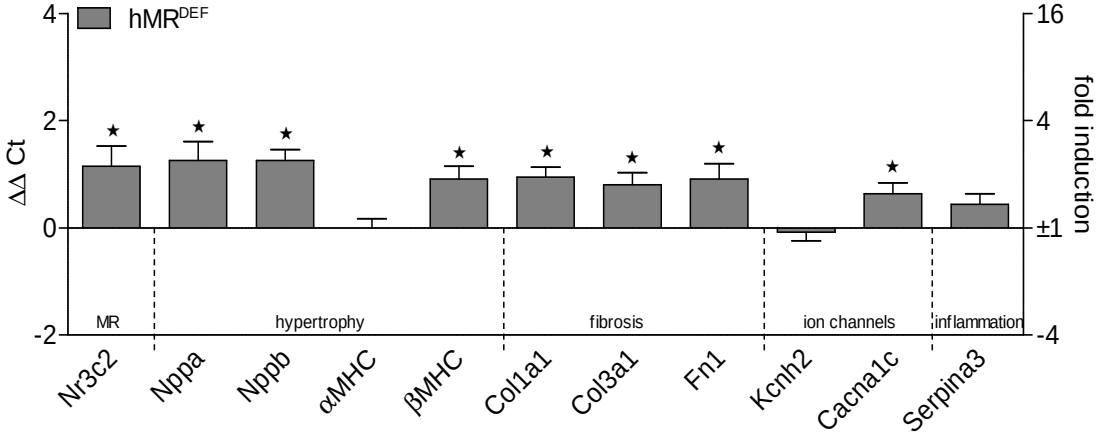

Fig. 2. Cardiac gene expression in six months old hMR^{DEF} mice. mRNA expression of endogenous MR (*Nr3c2*) and several hypertrophy, fibrosis and inflammation related genes like atrial natriuretic peptide (*Nppa*), natriuretic peptide type B (*Nppb*), myosin heavy chain isoforms α (*Myh6*) and β (*Myh7*), collagen subtypes (*Col1a1, Col3a1*), fibronectin (*Fn1*) and antichymotrypsin (*Serpina3*) as well as the α-subunits of the L-type calcium channel (*Cacna1c*) and the voltage gated potassium channel hERG (*Kcnh2*) were investigated in mice expressing the transgene hMR^{DEF} in the heart compared to WT

*Data are means ± SEM. *p < 0.05 vs. WT. n = 8 per group*

After the four-week-treatment, two-dimensional echocardiography of hMR^{DEF} mice showed a higher cardiac output appeared for hMR^{DEF} aldo/NaCl mice compared to the WT aldo/NaCl group (Fig. 3A). This might be due to the slightly enhanced heart rate in hMR^{DEF} aldo/NaCl mice, because all detected volumes were almost equal between treatment groups (Fig. 3A). Surface ECGs showed an increased heart rate in hMR^{DEF} aldo/NaCl mice compared to WT aldo/NaCl mice (Fig. 3B). Besides, a prolonged frequency-corrected QT interval (QT_C interval) occurred in hMR^{DEF} aldo/NaCl mice compared to the WT aldo/NaCl group (Fig. 3B). The enhanced intraventricular electrical activity seemed to base on elongated repolarization because the QRS complex (ventricular depolarization) was not different between the treated groups (Fig. 3B). This hypothesis was supported by the reduced transcription of the α-subunit of voltage-gated potassium channel ERG (*Kcnh2*), important for repolarization, in hMR^{DEF} aldo/NaCl hearts compared to WT aldo/NaCl mice (Fig. 3C). In the untreated control groups, no difference in cardiac function or electrical activity was noted between WT and hMR^{DEF} mice (Supplement data, Fig. S6).

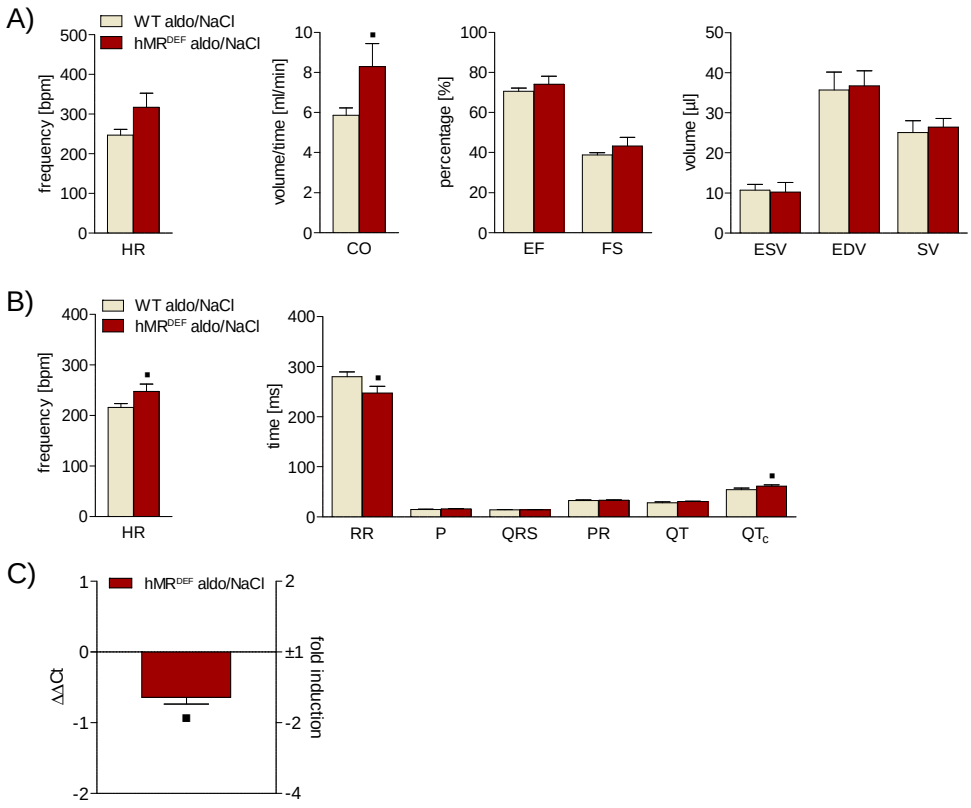

Fig. 3. Cardiac function, electrical activity and transcription under aldo/NaCl stimulation. (A) Cardiac function after four weeks of treatment was measured by two-dimensional echocardiography. hMR^{DEF} mice showed an enhanced cardiac output (CO) compared to WT. (B) Electrical activity after aldo/NaCl stimulation recorded by electrocardiography. Next to increased heart rate (HR), hMR^{DEF} mice showed prolonged intraventricular conduction time (QT_C interval) compared to WT aldo/NaCl. (C) qPCR analysis of voltage gated potassium channel hERG (*Kcnh2*) after the four weeks treating period. mRNA amount of the hERG subunit was decreased in hMR^{DEF} hearts compared to WT aldo/NaCl. CO, cardiac output; EDV, endiastolic volume; EF, ejection fraction; ESV, endsystolic volume; FS, fractional shortening; HR, heart rate; P, atrial depolarization; PQ, impulse transmission from atria to ventricles; QT, intraventricular conduction time (frequency-dependent); QT_C, corrected QT interval; QRS, ventricle depolarization; RR, interval from heart beat to heart beat; SV, stroke volume

Data are means ± SEM. ▪$p < 0.05$ vs. WT aldo/NaCl. n = 8 per group

3.4 Cardiac Function, Electrical Activity and Transcription of Twelve Months Old Mice

Cardiac hMR^{DEF} overexpression did not affect survival of twelve months old hMR^{DEF} mice (Supplement data, Fig. S2) and did not increase relative heart weight compared to WT littermates at the time of examination (Supplement data, Table S2).

To investigate cardiac function and electrical activity in hMR^{DEF} and WT littermates, echocardiography and ECG were performed under baseline condition as well as under β-adrenoceptor stimulation. Again no differences were detected in left ventricular function under baseline condition or under short-term β-adrenoceptor stimulation between hMR^{DEF} and WT mice by using echocardiography (Supplement data, Fig. S7A and B). Also resting ECGs were unchanged between hMR^{DEF} and WT animals (Supplement data, Fig. S8). In contrast, after short-term β-adrenoceptor stimulation by isoproterenol injection, twelve months old hMR^{DEF} showed a prolonged QT and frequency-corrected QT interval (QT_C interval) compared to WT (Fig. 4A). Here, the QRS complex (ventricular depolarisation) between WT and hMR^{DEF} mice was not different, therefore, the enhanced intraventricular electrical activity was assumed to base on elongated repolarization (Fig. 4A). This hypothesis was once more supported by the slightly reduced transcription of the α-subunit of voltage-gated potassium channel ERG (*Kcnh2*), in hMR^{DEF} hearts compared to WT mice (Fig. 4B).

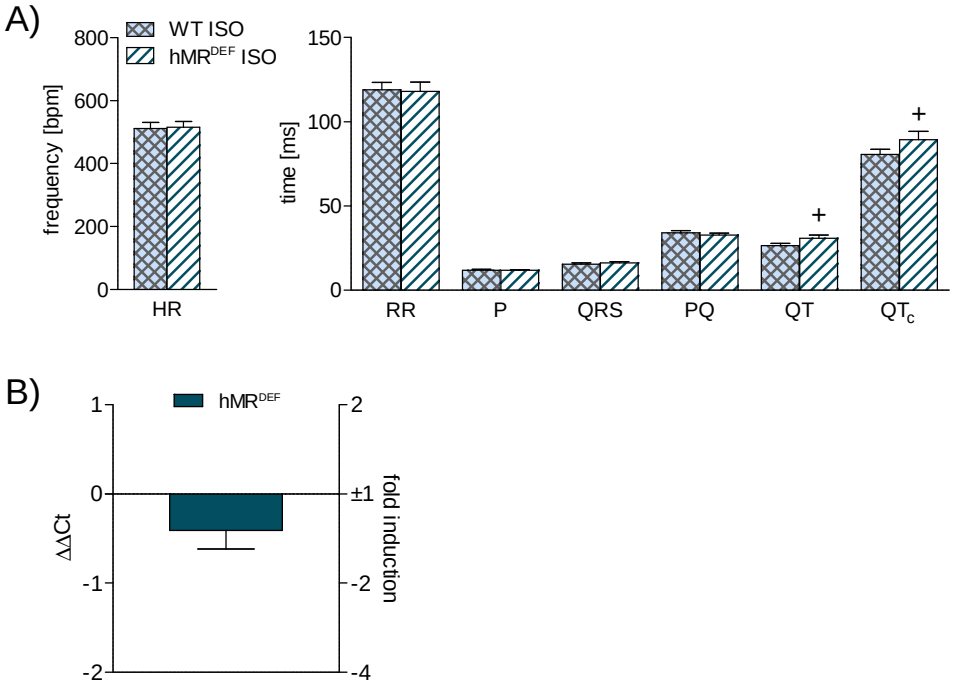

Fig. 4. Cardiac electrical activity and gene transcription of twelve months old mice. (A) Electrical activity of twelve months old mice recorded by electrocardiography after short-term isoproterenol (ISO) injection. Under β-adrenoreceptor stimulation, hMR^{DEF} mice showed prolonged intraventricular conduction time (QT and QT_C interval) compared to WT ISO. Basal data are presented in supplemental Fig. S8. (B) qPCR analysis of voltage gated potassium channel hERG (*Kcnh2*) of twelve months old mice. mRNA amount of the hERG subunit was slightly decreased (p = 0.066) in hMR^{DEF} hearts compared to WT littermates. HR, heart rate; P, atrial depolarization; PQ, impulse transmission from atria to ventricles; QRS, ventricle depolarization; QT, intraventricular conduction time (frequency-dependent); QT_C, corrected QT interval; RR, interval from heart beat to heart beat

Data are means ± SEM. $^{+}p < 0.05$ vs. WT ISO. n = 9 per group

4. DISCUSSION

As mentioned in the introduction, the MR mediates not only genomic but also nongenomic effects, which are dependent on the C-terminal domains (D,) E and F [2]. So far, the physiological and/or pathophysiological relevance of the nongenomic signaling cascade for the heart *in vivo* is not well understood. Therefore, we generated a new mouse model to investigate the rapid MR effects *in vivo* in a transgenic mouse heart. As transgene, we used a fusion protein of eGFP with the DEF domain of the MR. Previous experiments in cell culture revealed no influence of the eGFP tag on MR function [2]. Even if we cannot exclude an impact of eGFP, we renounced to establish a transgenic model with solely eGFP expression. Moreover, some time ago it was demonstrated that expression of eGFP alone in mice had no implications for cellular function [55].

Cardiac hMR^{DEF} overexpression did not increase mortality of up to twelve months old TG mice. In contrast, cardiac overexpression of the full-length hMR caused premature death. Therefore, the nongenomic MR effects do not have the dramatic life-threatening outcome like the genomic MR signaling pathway.

Our data show that relative heart weight of the hMR^{DEF} mice was not increased at the age of six month compared to wild type (WT) littermates. At this time point, no ECG or functional abnormalities could be observed, either under baseline conditions or after short-term isoproterenol injection. But, remarkably, an increased mRNA expression level of endogenous MR (*Nr3c2*) was noticeable, which might be due to the presence of the hMR^{DEF}. Therefore, hMR^{DEF} may have affected transcription of the endogenous MR via crosstalk with other membrane receptors, e.g. receptor tyrosine kinases like EGFR or the angiotensin II receptor 1 (AT1R) [44-46]. Additionally, cardiac gene expression patterns in hMR^{DEF} mice showed a shift to prohypertrophic, profibrotic and proinflammatoric genes compared to WT hearts, indicating that nongenomic MR effects promote hypertrophy, fibrosis and inflammation. Here, the underlying pathway remains still unclear but, apparently, there is a switch from nongenomic to genomic effects, probably, by increasing the endogenous MR expression and activity. Furthermore, the observed elevated mRNA expression of the α-subunit of the L-type calcium channel could be due to this interaction as well and might be responsible for enhanced cardiac $[Ca^{2+}]i$ current found in mice overexpressing the full-length human MR in the heart [30].

Aldosterone treatment combined with high-salt intake led to detrimental effects on cardiovascular functions [20,30,56,57]. Besides, aldosterone caused ionic channel remodeling in isolated rat adult cardiomyocytes with upregulation of calcium currents, a decrease in the Ito potassium currents and lengthening of the late phase of AP repolarization [27,58]. But the underlying mechanisms are still unclear [30]. In the present study, we demonstrate that pathological activation of nongenomic MR effects by aldosterone/NaCl led to prolonged intraventricular electrical activity, which may cause ventricular arrhythmias and fibrillation [30,32]. Interestingly, the cardiac transcription of the potassium channel ERG (*Kcnh2*), responsible for repolarization, was decreased in hMR^{DEF} hearts under aldosterone/NaCl stimulation compared to treated WT mice. If the decrease of *Kcnh2* expression is directly mediated by nongenomic MR signaling or indirectly by modulation of endogenous MR activity is currently unclear, but this result merits attention, because the reduced transcription of *Kcnh2* could be an underlying mechanism of the observed prolonged QT_C interval in the treated TG mice, which might be one reason for ventricular arrhythmias and fibrillation.

Furthermore, relative heart weights of twelve months old hMR^{DEF} mice were not different compared to WT littermates. At this time point, neither under baseline conditions nor after short-term isoproterenol injection functional abnormalities could be observed. Resting ECG revealed no different electrical activity as well. But under short-time β-adrenoceptor stimulation by isoproterenol injection, intraventricular conduction time (QT and QT_C interval) was elongated in hMR^{DEF} hearts, correlating with slightly reduced cardiac transcription of *Kcnh2*, indicating that in older animals, short-term stress-stimulation is able to influence cardiac conduction system when nongenomic MR activity is enhanced.

Taken together, our results indicate an involvement of both, the genomic and the nongenomic MR signaling pathway in the pathogenesis of heart failure. The classical genomic signaling pathway promotes hypertrophy, fibrosis and inflammation [20,24-26] and is associated with an increase in calcium

current [30,58] possibly by raising the cardiac transcription of α-subunit of L-type calcium channel (*Cacna1c*). In addition, nongenomic MR signaling seems to be an alternative pathway to modulate genomic MR activity and to influence hypertrophy, fibrosis and inflammation via a nongenomic-genomic interaction.

Pathological nongenomic activation by long-term aldosterone/NaCl stimulation reduces cardiac transcription of the α-subunit of the voltage-gated potassium channel (*Kcnh2*), which might lead to alterations in the cardiac conduction system, especially of repolarization, increasing the incidence of cardiac arrhythmias. Moreover, along with reduced *Kcnh2* expression the heart may be more vulnerable to stress-induced arrhythmias.

5. LIMITATIONS OF THE STUDY

Additionally, the crosstalk with other receptors should be studied in future work. Putative candidates are receptor tyrosine kinases like EGFR, insulin receptor (IR)/insulin-like growth factor receptor (IGF-1 receptor), platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR). But also the AT1R may be involved. Therefore, it needs to be elucidated in which way intracellular signaling pathways including ERK1/2, JNK and NF-κB activation are involved in the nongenomic MR signaling in our transgenic mouse model [2,44,46,59]. Moreover, in the last years, the G protein-coupled receptor GPR30 was proposed as a new membrane-bound aldosterone receptor that is also expressed in the heart. Signaling of the GPR30 involves transactivation of the EGFR leading to stimulation of the ERK1/2 and/or PI3K/AKT pathways [44,60]. The involvement of the GPR30 was not examined in this study. Finally, protein expression of important genes analyzed here by qPCR and channel functions in isolated mouse cardiomyocytes were not examined in this study and need to be addressed in future work.

6. CONCLUSION

Thus, from our experiments we hypothesize that also nongenomic MR effects might be involved in the pathogenesis of MR-dependent hypertrophy, fibrosis and inflammation. Furthermore, nongenomic MR signaling pathways may to be responsible for MR-associated arrhythmias and may present new targets for drug therapy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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