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Anti-inflammatory effects of endothelin receptor blockade in left atrial tissue of spontaneously hypertensive rats

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ABSTRACT

Objective: In spontaneously hypertensive rats (SHR) atrial remodeling has been shown to involve increase in endothelin (ET) signaling. Furthermore, inflammatory processes may further contribute to tissue remodeling. The aimed of this study was to investigate whether an endothelin receptor antagonist, macitentan, could reduce left atrial (LA) remodeling in arterial hypertension.

Methods: Molecular characterization of atria was performed in SHR at the age of 8 months and their age-matched normotensive control rats (WKY). SHR were treated with macitentan and, for comparison with a blood pressure reducing drug, with doxazosin. After two months of treatment, molecules involved in endocardial inflammation and atrial calcium handling were assessed. The molecular changes provoked by rapid-pacing (RAP) were analyzed in atrial tissue slices.

Results: Doxazosin reduced the systolic blood pressure compared with the untreated SHR (159 ± 26 vs. 176 ± 17; $P < 0.05$) or macitentan (vs. 189 ± 21; $P < 0.05$). Macitentan lowered the increased levels of atrial ET-1 and abrogated the pacing-induced upregulation of preproET-1-mRNA in atrial slices from SHR. Macitentan reduced the elevated levels of atrial 8-isoprostanes, the increased expression of pro-inflammatory ICAM-1 and IL-8, the phosphorylation of MAP kinases, ERK and p38, the phosphorylation of NF-κB and the expression of VCAM-mRNA. Major Ca^{2+}-regulating proteins and markers of hypertrophy and fibrosis, however, were not affected. Doxazosin elicited similar changes, except for the alterations in ET-1 levels, NF-κB phosphorylation and VCAM-mRNA.

Conclusion: Macitentan reversed pro-inflammatory remodeling in hypertensive atria in a blood pressure-independent manner, which might prevent endocardial dysfunction and thereby, thrombogenesis in arterial hypertension.

1. Introduction

The pathophysiology of pressure-induced atrial cardiomyopathy, possibly due to activation of the endothelin system, remains to be fully elucidated [2]. Endothelin (ET-1) is a 21-amino acid peptide hormone implicated in a multitude of biological processes and linked to cardiac pathology. Besides its prominent role as a vasoconstrictor, ET-1 exerts potent hypertrophic and profibrotic effects and mediates pro-inflammation as well as elevated ROS (reactive oxygen species) production [3]. ET-1 is synthesized as an inactive precursor, preproET-1, which is then converted into an active form by proteases like ECE, NEP and chymase. ET-1 mediates its biological effects via two distinct G-protein-coupled receptors, ET_AR and ET_BR. The expression pattern of members of the ET system indicates that the source and target cells are localized in the same tissues. ET-1 has been implicated in the pathogenesis of atrial cardiomyopathy and, in particular, of atrial fibrillation (AF) [4]. Multivariate analysis of the human left atrium (LA) confirms associations of ET-1 with AF, hypertension, and LA size [5]. PreproET-1 mRNA levels correlate with genes involved in cardiac dilatation, hypertrophy, and fibrosis. ET-1 contributes to AF-dependent atrial fibrosis by synergistic activity with angiotensin II (Ang II) to enhance the expression of CTGF [6].

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ET-1 overexpression in vessel walls has been consistently observed in different animal models of hypertension, including DOCA rats, stroke-prone SHR, Dahl salt-sensitive rats and AngII-infused rats [7]. In the DOCA-model of hypertension, ET-1 is also overexpressed in the heart [8]. In in vivo experiments, ET-1 favors ventricular arrhythmias by prolonging action potential duration and exciting afterdepolarizations [9–10]. Recent reports highlight the potentially pro-arrhythmic effects of ET-1 on intracellular Ca^{2+} handling in atrial myocytes and trabeculae via activation of inositol-1,4,5 trisphosphate (IP_3) receptors [11–14]. In addition, ET-1 can act pro-arrhythmic on human atrial tissue [15].

At the tissue level, ET-1 and AngII might act synergistically to promote atrial remodeling and AF. Both peptides induce myocyte hypertrophy and interstitial fibrosis, thereby creating a substrate for AF. In addition to structural changes of the atrial wall, a process of endocardial remodeling has been introduced, which might provide the basis for atrial thrombogenesis and stroke in AF [16]. In particular, patients with arterial hypertension are at high risk to suffer a stroke during AF and, therefore, characterization of the underlying pathophysiology is of clinical importance. Thus, we aimed to investigate whether the blockade of the ET system by means of a dual endothelin receptor antagonist, macitentan, might avert or reverse LA remodeling in hypertensive rats. Based on the aforementioned actions of ET-1 and the role of the endothelin system in atrial remodeling and atrial cardiomyopathy, we studied whether macitentan might modulate the atrial endothelin and angiotensin systems, inflammatory state, oxidative stress, hypertrophy and fibrosis, and myocyte calcium handling in LA tissue from hypertensive rats. As an animal model, we chose spontaneously hypertensive rats (SHR), which are an established model of hypertensive heart disease and atrial cardiomyopathy [17–19]. In order to compare the effects of endothelin receptor blockade with those of "pure" blood pressure reduction, we also studied atrial remodeling processes in SHR treated with doxazosin, a selective α1-adrenergic receptor blocker.

2. Materials and methods

2.1. Experimental animals and tissue sampling

For an initial characterization of molecular changes in atria, male hypertensive SHR at the age of 8 months (weighing 369 ± 19 g, n = 18) and age-matched normotensive WKY rats (weighing 477 ± 24 g, n = 24) were used (JANVIER Laboratories, France). For treatment experiments, 8 months old male SHR were distributed randomly into three groups. Two groups were subjected to an oral macitentan (n = 18) (kindly provided by Actelion) or doxazosin treatment (n = 18) (Hexal AG) each 30 mg/kg/day in food. A group of untreated animals was run in parallel (SHR-CTL, n = 23). The rats were housed in temperature- and light-controlled animal quarters in the central animal lab at the University Hospital Magdeburg according to guidelines of experimental animal care of the European Community. All experiments were approved by the Council on Animal Care Committee in Halle, Saxony Anhalt, Germany (permit: 42502-2-1259 UniMD). Systolic, diastolic, mean blood pressure and heart rate were estimated within the 7-th or 8-th week of experiment by use of a tail-cuff CODA System, as described previously [17]. After 2 months the animals were sacrificed, body weight was determined, serum/plasma were collected and hearts were explanted and either snap-frozen for further analysis or used for in vitro pacing.

2.2. Culturing tissue slices and in vitro pacing

The atrial tissue slices were prepared as previously described [20]. Tissue pacing was performed at rates of 0.6 and 5.0 Hz. The viability of atrial tissue was processed as previously reported [20].

The atrial tissue slices were prepared in cold, oxygenated (95% O_2/5% CO_2) DMEM medium (Life Technologies GmbH, Darmstadt, Germany) using a vibratome (VT1200S; Leica Microsystems, Wetzlar, Germany). The 350 μm slices were placed on top of the membrane of tissue culture inserts (0.4 μm Merck Millipore, Darmstadt, Germany) in a petri dish, which was filled with DMEM medium for up to 24 h in an incubator (21% O_2, 5% CO_2, 37 °C). For stimulation in an electrical field, a pair of custom-built carbon electrodes was submersed at the opposite ends of a petri dish and connected to a stimulation unit (GRASS Stimulator) as previously described [20]. Pacing of the tissue slices was performed up to 24 h at 37 °C at rates of 0.6 and 5.0 Hz (10 V/cm, 5 ms bipolar pulse). The viability of cultured rat atrial tissue slices was confirmed using an established viability staining technique (CellTracker Green CMFDA, Invitrogen, Karlsruhe, Germany). The living slices were incubated for 2 h with the final concentration of 5 μM dye in serum-free medium and imaged as stitched mosaic with a fluorescent microscope (Axiovert 200 m; Carl Zeiss Microscopy GmbH, Jena, Germany) using an excitation wavelength filter of 480/40 nm, a diachroic filter 505 nm long pass and emission wavelength between filter of 535/50 nm.

2.3. Determination of plasma BNP, ANP and atrial levels of 8-isoprostanes, AngII and ET-1

Plasma concentrations of rat BNP-45 (Assay Pro) and rat ANP (Bio-Cat) were measured according to the manufactures's protocol. Absorbance was recorded at 450 nm, and the concentrations were calculated from a standard curve obtained with BNP-45 or ANP respectively. Atrial extracts were prepared according to the manufacturer's recommendations for AngII ELISA kit (BioVendor), ET assay kit (Caymann Chemicals), and 8-isoprostanes ELISA (EnzoLifeScience). Optical density was determined using a TECAN Reader (Swiss).

2.4. Quantitative RT-qPCR

Isolation of total RNA from atrial tissue slices, cDNA synthesis, and RT-qPCR were performed exactly as previously described (20). The specific primers that were used are listed in Table 1.

2.5. Western blot analysis

Preparation of atrial tissue slices for immunoblot analyses and Western Blotting itself were performed as previously described [20]. Briefly, atrial slices were homogenized in modified RIPA buffer, the homogenates cleared by centrifugation, and the supernatants stored at −20 °C for further use. Samples of 20 μg protein each were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were

Table 1
Primer used for quantitative RT-PCR.

Gene	E* (%)	Sequences	
		Upstream: 5′-3′	Downstream: 5′-3′
ACE	105	GTT CGT GGA GGA GTA TGA CCG	CCG TTG AGC TTG GCG ATC TTG
ß-Actin	90	AAG ATG ACC CAG ATC ATG TTT GAG	AGG AGG AGC AAT GAT CTT GAT CTT
ETAR	90	CCC TTT TTG CAG AAG TCG TC	GTA CCA TGA CGA AGC CGA TT
ICAM-1	96	AGG TAT CCA TCC ATC CCA CA	GCC ACA GTT CTC AAA GCA CA
IL8	108	GAA GAT AGA TTG CAC CGA	CAT AGC CTC TCA CAC ATT TC
LOX-1	106	TCC ATC ACA CTC AAG GTC CC	TCT GCC TCA CAG AGG TTT CC
NOX2	90	CAT CTG GCC TGA TCC TCA TC	AGC AGG TAG ATC ACA CTG GC
NOX4	101	GAA CAG TTG TGA AGA GAA GC	GTG TTT GAG CAG AGC TTC TG
Prepro-ET	100	ACT TCT GCC ACC TGG ACA TC	GGC TCG GAG TTC TTT GTC TG
VCAM-1	91	CAG AGA TCC AAT TCA GTG GC	GAG GTG TAG ACT TGT AGT TC

E = Efficiency in % = {[10^(−1/slope)]−1} × 100.

incubated with primary antibody against phospho-44/42 ERK, 44/42ERK, phospho-p38 and p38 (1:1000; Cell Signaling, USA), NOX-2 (1:1000, BD, USA), ICAM-1 (1:500, Santa Cruz, USA), GAPDH (1:5000, Santa Cruz, USA). An appropriate horseradish peroxidase (HRP)-conjugated secondary antibody was used facilitating the subsequent detection by means of enhanced chemiluminescence (Pierce, USA). The protein expression was quantified using Quantity One Software (BioRad, USA). Signal intensities were normalized to GAPDH.

For calcium-regulating proteins, expression and phosphorylation was determined by immunoblot as described previously [17]. Briefly, left atria were homogenized and protein concentration was quantified by means of the BCA method. Equal amounts of protein were loaded on gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes. Quality of transfer was probed using Ponceau S. Primary antibodies used for detection of protein expression and phosphorylation were 1) on 4–20% gradient gels (BioRad, Germany): anti-CaMKII (50 kD, Badrilla, A010-55AP, 1:5,000), anti-CSQ (55 kD, Thermo Scientific, PA1-913, 1:2,500), anti-GAPDH (34 kD, Calbiochem, CB1001, 1:50,000), anti-IP_3R2 (313 kD, Abcam, ab77838, 1:1,000), anti-NCX1 (120 kD, Thermo Scientific, MA1-4672, 1:1,000), anti-SERCA2a (100 kD, Badrilla, A010-20, 1:5,000), anti-RyR (565 kD, Thermo Scientific, MA3-916, 1:5,000), anti-pSer2808-RyR2 (Badrilla, A010-30, 1:5,000); 2) on 8% glycine gels: anti-Cav1.2a, i.e. cardiac type α1C subunit of the L-type Ca channel (250 kD, Alomone Labs, ACC-013, 1:200); and 3) on 14% tricine gels: anti-actin (44 kD, clone C4, MP Biomedicals #69100, 1:50,000), anti-PLB (25 kD, Badrilla, A010-14, 1:5,000), anti-Ser10-PLB (Badrilla, A010-10AP, 1:1,000), anti-pSer16-PLB (Badrilla, A010-12, 1:5,000), anti-pThr17-PLB (Badrilla, A010-13, 1:5,000). Afterwards, suitable HRP-conjugated secondary antibodies were used. Chemiluminescence was detected by the Chemidoc-XRS Imaging System (BioRad, Germany). Bands were quantified using ImageJ (NIH, Bethesda, USA). GAPDH or actin were used for normalization. In order to detect various proteins from a single membrane (and thus save sample material), membranes were cut appropriately between bands, so that one housekeeping protein (e.g. GAPDH) could be used for normalization of various proteins. In some cases, stripping was performed.

2.6. Statistical analysis

Normality of data distribution was assessed with Shapiro-Wilk test. For data analyses, ANOVA tests followed by post-hoc Tukey test or Dunnett's multiple comparison test were applied. Statistical analyses were performed using Origin 8.5 (OriginLab Corporation, USA). If not indicated otherwise, all values are given as mean ± SEM. A P value of <0.05 was considered to be statistically significant.

3. Results

3.1. Macitentan treatment does not affect systolic blood pressure in SHR rats

To differentiate atrial tissue effects related to the endothelin receptor blockage from effects linked to the lowering of systemic blood pressure we introduced an additional SHR group treated with doxazosin. In contrast to angiotensin-converting enzyme inhibitors, beta-blockers or calcium-channel blockers, doxazosin is a selective α1-adrenergic receptor blocker that effectively lowers blood pressure in SHR rats [21] and does not show any influence on the remodeling of heart tissue [22]. In our study, only chronic treatment with doxazosin decreased systolic blood pressure as compared with the untreated SHR group (159 ± 26 vs. 176 ± 17 mmHg; $P < 0.05$) or macitentan group (vs. 189 ± 21 mmHg; $P < 0.05$). Both treatments had no effects on heart rate (MAC: 378 ± 36, DOX: 404 ± 30 vs. SHR 386 ± 32 1/min).

3.2. Activation of atrial endothelin and Renin-Angiotensin system in SHR.

The activation of the ET and the AngII system has been implicated in the pathophysiology of hypertension. Indeed, in the SHR we detected increased amounts of preproET-1-mRNA (4.7 ± 0.7 vs. WKY: 1.0 ± 0.1) and ACE (angiotensin-converting enzyme)-mRNA (1.6 ± 0.1 vs. WKY: 1.0 ± 0.1) (Fig. 1 (a), (b)). Of note, the increase in both genes occurred preferentially in the LA and was independent of age (data not shown). In agreement with these results, we found elevated levels of both peptides, ET-1 (SHR: 0.15 ± 0.02 vs. WKY: 0.09 ± 0.01 pg/mg) and AngII (SHR: 8.7 ± 0.9 vs. WKY: 6.1 ± 0.8 pg/mg) (Fig. 1(c) (d)). The treatment with macitentan lowered the atrial level of ET-1 (MAC: 0.07 ± 0.02 pg/mg) without changing the expression of preproET-mRNA (Fig. 1(a), (c)). Furthermore, the amounts of ET_AR-mRNA were elevated in the SHR compared to normotensive rats. The Western blot analysis supported the increased expression of ET_AR (Fig. 1(e), (f)). Neither macitentan nor doxazosin affected the ET_AR-mRNA and protein levels.

In order to study molecular changes in response to high frequency electrical stimulation (mimicking AF) we prepared atrial slices from WKY and SHR with and without treatment with macitentan or doxazosin. Fig. 1(g) demonstrated viability of left atrial slices after stimulation in the electrical field. As shown in Fig. 1 ((h), (i)), stimulation in the electrical field caused a significant increase in the preproET-1-mRNA and ACE-mRNA in atrial slices from SHR and WKY rats. Of note, macitentan abrogated the stimulation dependent upregulation in preproET-1-mRNA (Fig. 1(h)). Interestingly, both treatments showed a tendency to lower the increased ACE-mRNA in respond to stimulation in the electrical field (Fig. 1(h), (i)).

3.3. Inflammatory state in left atrial tissue from SHR.

Inflammatory state is involved in the pathophysiology of human hypertension and AF. The hypertension in SHR was characterized by the upregulation of intracellular adhesion molecule, ICAM-1-mRNA (1.78 ± 0.04 vs. WKY: 1.00 ± 0.07) and IL-8-mRNA (2.98 ± 0.24 vs. WKY: 1.0 ± 0.2, $P < 0.01$) in LA (Fig. 2(a) to (c)). Western blot analysis supported the elevated expression of ICAM (1.8 ± 0.2 vs. WKY: 1.0 ± 0.2, $P = 0.057$) (Figure (d), (e)). The treatment with macitentan and doxazosin lowered the expression of ICAM-mRNA (MAC: 1.41 ± 0.03, DOX: 1.44 ± 0.07, $P < 0.05$), IL-8 (MAC: 1.55 ± 0.06, DOX: 1.90 ± 0.05, $P < 0.05$). However, only macitentan reduced the VCAM-1 mRNA significantly. Doxazosin had no effect on VCAM-mRNA (Fig. 2(c)).

As demonstrated in Fig. 2(d), the inflammatory state in SHR was accompanied by an activation of MAPK kinases: p38, ERK, and an increase in the phosphorylation of NF-κB (SHR: 3.9 ± 0.9 vs. WKY 1.0 ± 0.1, $P < 0.01$). Whereas the increase in the phosphorylation of MAPK-p38 and MAPK-ERK was attenuated by both treatments, the upregulation in the NF-κB was lowered only with macitentan treatment (MAC: 2.3 ± 0.1, $P < 0.05$) (Fig. 2(e-h)).

Next, we evaluated the possible differences in the expression of inflammatory molecules in atrial slices in the response to the stimulation in the electrical field. Interestingly, there were no changes in the response between hypertensive and normotensive atrial slices in view of expression of ICAM-1-mRNA, IL-8 mRNA and VCAM-mRNA. However, both treatments abolished the upregulation of ICAM-1-mRNA in response to pacing (Fig. 2(i-k)).

3.3.1. Oxidative stress in left atrial tissue from SHR

8-isoprostanes are products of peroxidation of arachidonic acid. The atrial 8-isoprostanes increased significantly in SHR (7.5 ± 0.8 vs. WKY 3.5 ± 0.5 ng/mg) (Fig. 3(a)). Both treatments effectively reduced levels of 8-isoprostanes (MAC: 5.0 ± 0.7, DOX: 4.8 ± 0.5) in hypertensive atrial tissue (Fig. 3(a)). Moreover, atrial tissue from SHR showed upregulated amounts of Lox-1-mRNA, a receptor for oxidized low-density lipoproteins. Surprisingly, neither macitentan nor doxazosin regulated Lox-1-mRNA (Fig. 3(c)). Then, we attempted to find the source of

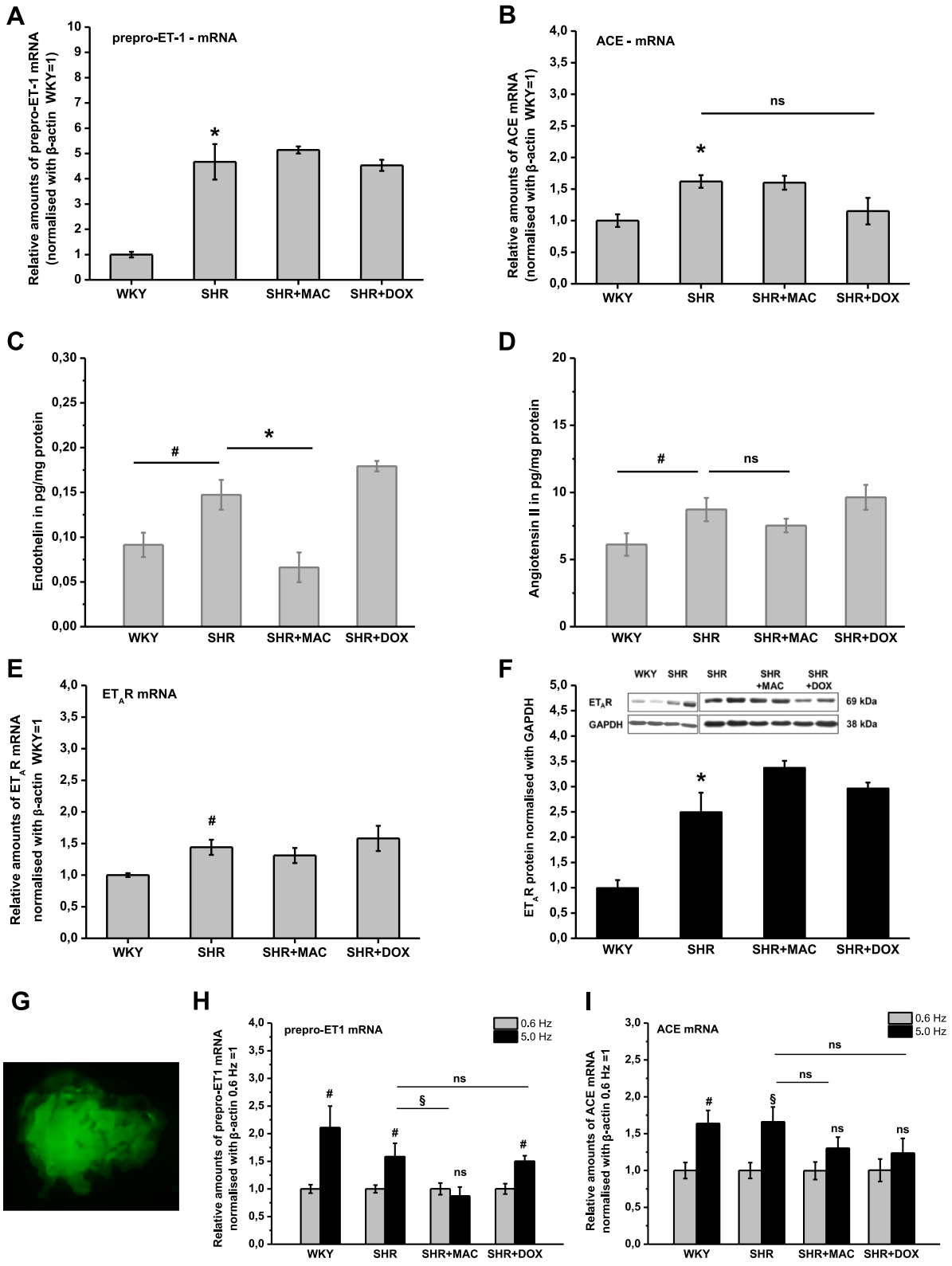

Fig. 1. Activated ET-1 and Ang II system in the left atria from SHR. (a-b)(e) RT-qPCRs detecting prepro-ET-1, ACE, and ET_AR in LA of WKY, SHR without or with treatment with MAC or DOX (N = 6 per group) (c) Increased atrial level of ET-1 (N = 8–12) (d) Augmented atrial level of AngII (N = 8–12) (f) Western blot for ET_AR and quantification in the LA (g) Representative viability of atrial slices after 24 h of pacing. (h, i) Atrial slices from WKY and SHR with and without treatments were paced at 5.0 Hz vs. 0.6 Hz. RT-qPCRs of prepro-ET-1 and ACE in the response to the stimulation (N = 4 per group). $^*P < 0.01$, $^{\#}P < 0.05$; §<0.07.

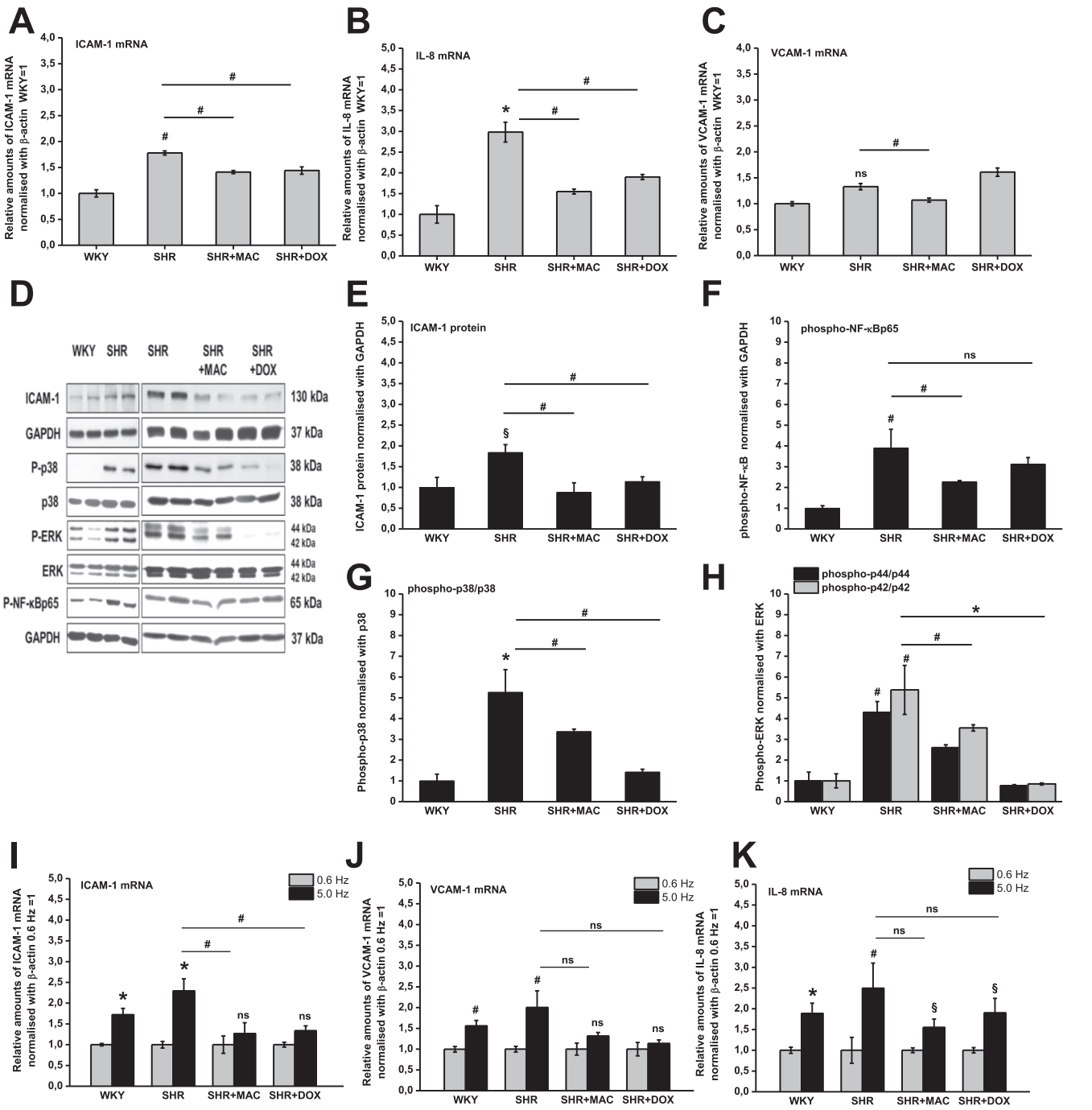

Fig. 2. Anti-inflammatory effects of macitentan in atria. (a-c) RT-qPCRs were performed to determine changes of ICAM-1, IL-8 and VCAM-1 in LA of WKY, SHR without or with treatment with MAC or DOX (N = 6 per group) (d-h) Immunoblots and their Quantification (N = 6) (i-k) Atrial slices from WKY and SHR with and without treatments were paced at 5.0 Hz vs 0.6 Hz up to 20 h. RT-qPCRs of ICAM, VCAM-1 and IL8 in the response to the stimulation (N = 4 per group). $^{*}P < 0.01$, $^{\#}P < 0.05$; $^{\S}P < 0.07$.

oxidative stress in hypertensive atrial tissue. Our RT-qPCR analysis revealed an increase in NOX4-mRNA (Fig. 3(d)). The expression of NOX2 was moderately elevated and did not reach statistical significance (Fig. 3 (b) (e)). Neither macitentan nor doxazosin affected the expression of NOX2 or NOX4.

3.4. *Increased expression of fibrotic and hypertrophic molecules in left atrial tissue from SHR.*

As previously demonstrated hypertension is associated with fibrotic changes in LA [17]. Whether changes in the expression of the fibrosis-associated genes, CTGF (connective tissue growth factor), TGF-β1 (transforming growth factor-beta 1), HB-EGF (heparin-binding epidermal growth factor), and bFGF (basic fibroblast growth factor) are abolished by treatment with macitentan, has been determined by RT-qPCR. Our analysis revealed that neither macitentan nor doxazosin prevented the hypertension-mediated upregulation (Fig. 4 (a) to (d)). In addition, both treatments did not affect the molecular markers of hypertrophy, MYH7-mRNA (β-myosin heavy chain) and BNP-mRNA (brain natriuretic peptide) (Fig. 4 (e) (f)).

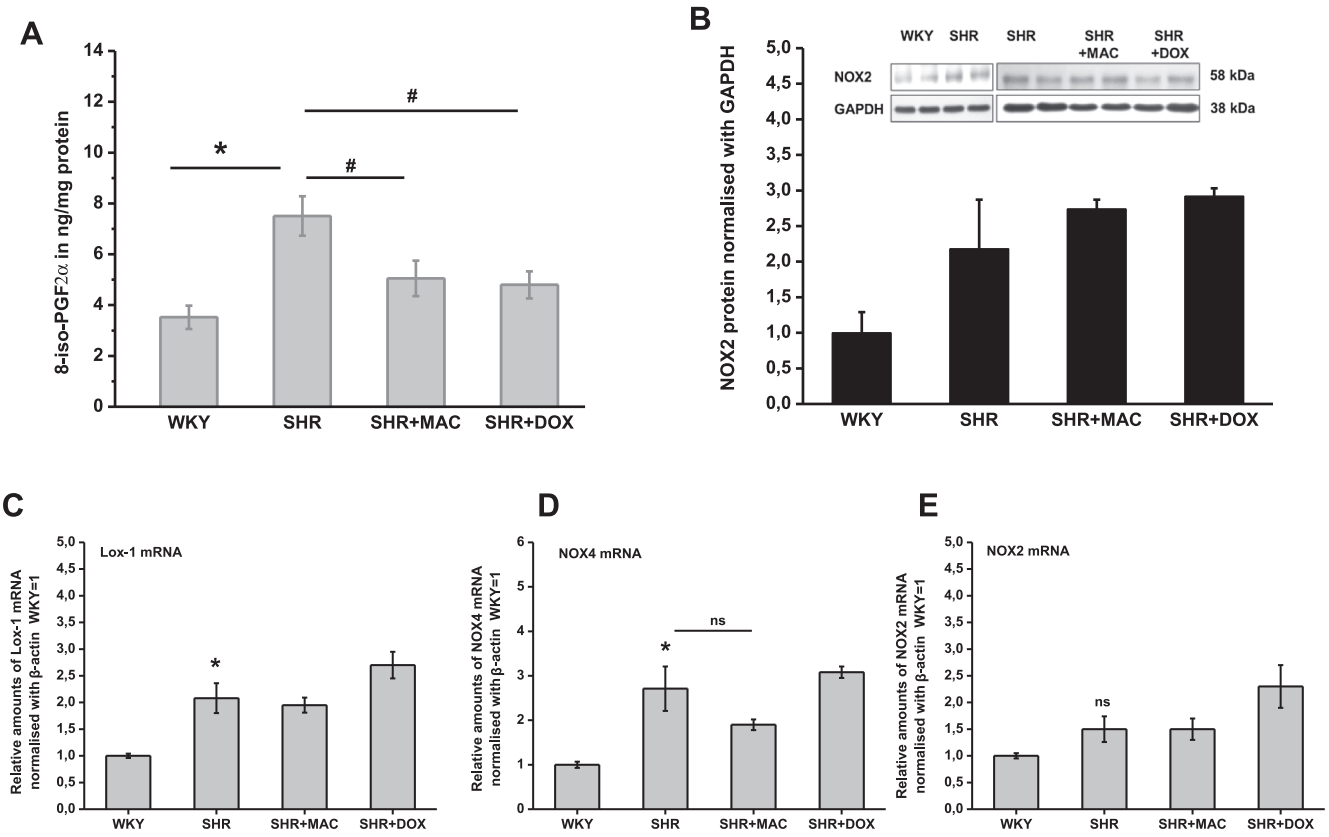

Fig. 3. Anti-oxidative effects of macitentan and doxazosin in atrial tissue from SHR. (a) Atrial 8-isoprostanes (N = 8–10 per group) (b) Quantification of immunoblots for NOX2 and (c-e) RT-qPCRs for Lox-1, NOX4, and NOX2 in LA (N = 6); *P < 0.01, #P < 0.05.

3.5. Calcium handling in atria

At the age of 6–8 months SHR exhibited altered Ca^{2+} handling. This was accompanied by functional changes and altered expression and phosphorylation of Ca^{2+}-regulating proteins [17]. Specifically, expression of the α1C subunit of L-type Ca^{2+} channels and of RyR2 were reduced, whereas phosphorylation of RyR2 at the PKA site, S2808, was increased in SHR. In order to examine whether macitentan or doxazosin treatment normalizes Ca^{2+} handling, we probed for the expression and phosphorylation of major Ca^{2+}-regulating proteins. Western blot analysis for α1C, calsequestrin (CSQ), RyR2, SERCA2a and phospholamban (PLB) did not reveal any significant alterations in either expression or phosphorylation (RyR2 S2808, PLB) in the treatment groups compared to the SHR control group (Fig. 5). However, protein expression of CaMKIIδ (Ca^{2+}/calmodulin-dependent protein kinase II), a kinase involved in arrhythmogenesis and remodeling, was significantly lower in both treatment groups (MAC: 0.69 ± 0.08, DOX: 0.60 ± 0.08 vs. SHR: 1.00 ± 0.08) (Fig. 5 (h).

4. Discussion

The present study demonstrates protective effects achieved by chronic blockade with the dual endothelin receptor antagonist, macitentan, in a rat model of hypertensive heart disease. The treatment with macitentan reduced the increased atrial level of ET-1 in the absence of blood pressure (BP) lowering effects. The blockade of endothelin receptors elicited anti-inflammatory effects via reduction in the phosphorylation of MAP kinases, p38 and ERK, and transcription factor NF-κB. As a consequence, downstream effectors like ICAM-1, VCAM-1 and IL-8 were downregulated. Moreover, the blockade of ET receptors attenuated the enhanced level of 8-isoprostanes, a marker of oxidative stress, and led to downregulation of CaMKIIδ. Other important calcium handling proteins and fibrosis markers were not affected by the blockade.

Dual ET_A/ET_B endothelin receptor antagonists (ERAs) display different efficiency in lowering BP in animal models of hypertension [23,24]. Trensz et al. [24] demonstrated that salt-dependent/low-renin animal models of hypertension (e.g. DOCA-salt rats) are more sensitive to BP lowering effects of aprocitentan (an active metabolite of macitentan) than normal-renin models of hypertension (SHR). In addition, the BP lowering effects of ERAs are dose-dependent. Thus, in our study, the chronic blockade of endothelin receptor with 30 mg/kg/day of macitentan did not affect BP in SHR. Independently of BP lowering effects, treatments with ERAs achieve tissue protective effects in SHR. Thus, Karam et al. [25] described that the chronic treatment with bosentan normalized renal function and slightly decreased left ventricular hypertrophy and fibrosis. McDonald et al. [26] reported that the treatment with bosentan abolished various microvascular lesion formation during hypertensive retinopathy in SHR. In our study, effects observed by the chronic ET receptor blockade might be related exclusively to the tissue effects of macitentan.

At the cardiac level, ET-1 might closely interact with local paracrine systems in order to mediate its long-term effects on heart remodeling [27–29]. In various cell types, ET-1 acts as a pro-inflammatory mediator that activates NF-κB [30–33]. Importantly, a long-term blockade of ET receptors inhibited NF-kappaB and AP-1 activation and the downstream effectors ICAM-1, VCAM-1, and TF in the heart and kidney of an hypertensive rat model [30]. In the present work, we demonstrated that LA in the hypertensive rat model is characterized by the presence of an inflammatory state and displayed an activated AngII and ET system. The chronic treatment with macitentan did not affect the expression of ACE and preproET-1, but reduced the level of ET-1. The effect seems to be sufficient to interfere with MAP kinase signaling and NF-κB. Consequently, macitentan abolished the inflammatory state in the LA from

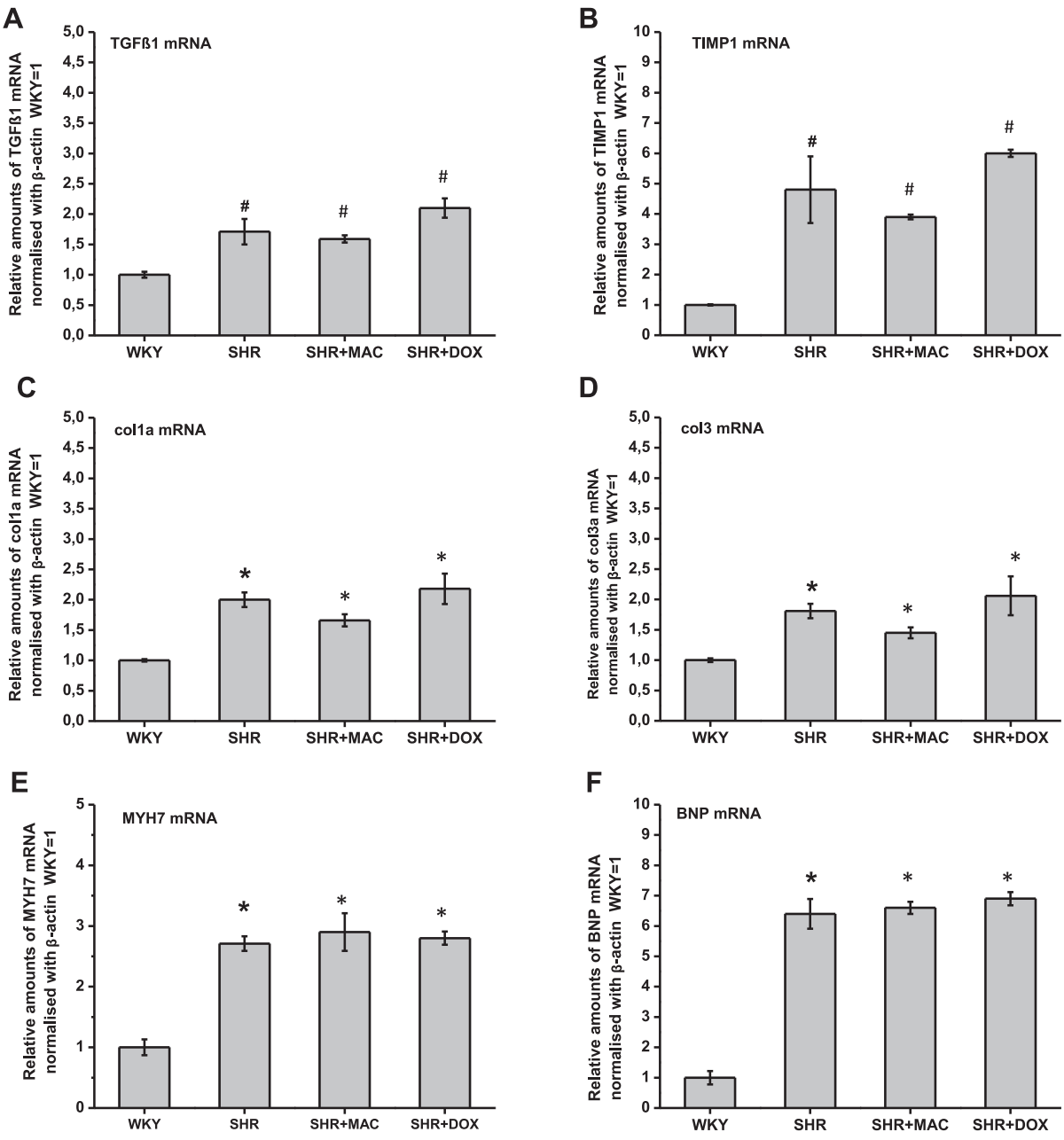

Fig. 4. Macitentan does not affect markers of fibrosis and molecular hypertrophy in LA of SHR. RT-qPCRs were performed to determine whether treatment with macitentan or doxazosin avert increased expression of fibrosis markers: (a) TGFß, (b) TIMP-1, (c) collagen 1a and (d) collagen 3, and upregulated markers of molecular hypertrophy: (e) MYH7 and (f) BNP. (N = 6 per group). $^{*}P < 0.01$, $^{\#}P < 0.05$; versus WKY. Difference between SHR, SHR + MAC and SHR + DOX were not significant.

SHR. Of note, the hypotensive effect caused by doxazosin also averted the activation of MAP kinase signaling.

The interaction between the AngII and ET system might play an important role in oxidative signaling. Cingolani et al. reported that the Ang II–induced production of ROS could be blocked not only by the AngII type 1 receptor blocker, losartan, but also by the nonselective ET-1 receptor blocker TAK044 or, the selective ET_A receptor blocker BQ-123 [27]. Of note, exogenous ET-1 induced a similar increase in ROS production as Ang II. On that note, Wedgwood et al. [34] reported that ET-1 enhanced production of peroxynitrite, thereby contributing to development and progression of endocardial dysfunction during pulmonary hypertension. In support of this notion, we detected increased atrial 8-isoprostanes in SHR. Isoprostanes, prostaglandin-like compounds that are formed by free radical-catalyzed lipid peroxidation of, preferentially, PUFA (polyunsaturated fatty acids). Their presence, especially at elevated concentrations, has been associated with chronic states of oxidative stress. Interestingly, the treatment with macitentan and doxazosin attenuated the levels of atrial 8-isoprostanes. In an attempt to elucidate the source of ROS we investigated expression of major isoforms of NADPH oxidase and Lox-1. At the age of 8 months, NOX4-mRNA and Lox-1-mRNA were significantly increased in the LA of SHR. Remarkably, macitentan did not affect the expression of the pro-oxidative molecules. In agreement with our results, in hypertensive DOCA rats the elevated expression of NADPH oxidase was not influenced by ET antagonist. However, this antagonist normalized the increased mitochondrial generation of ROS [35]. According to several reports, NADPH oxidase is the major source of ROS generation when stimulated by AngII [2,19,27], whereas ET-1 preferentially increases

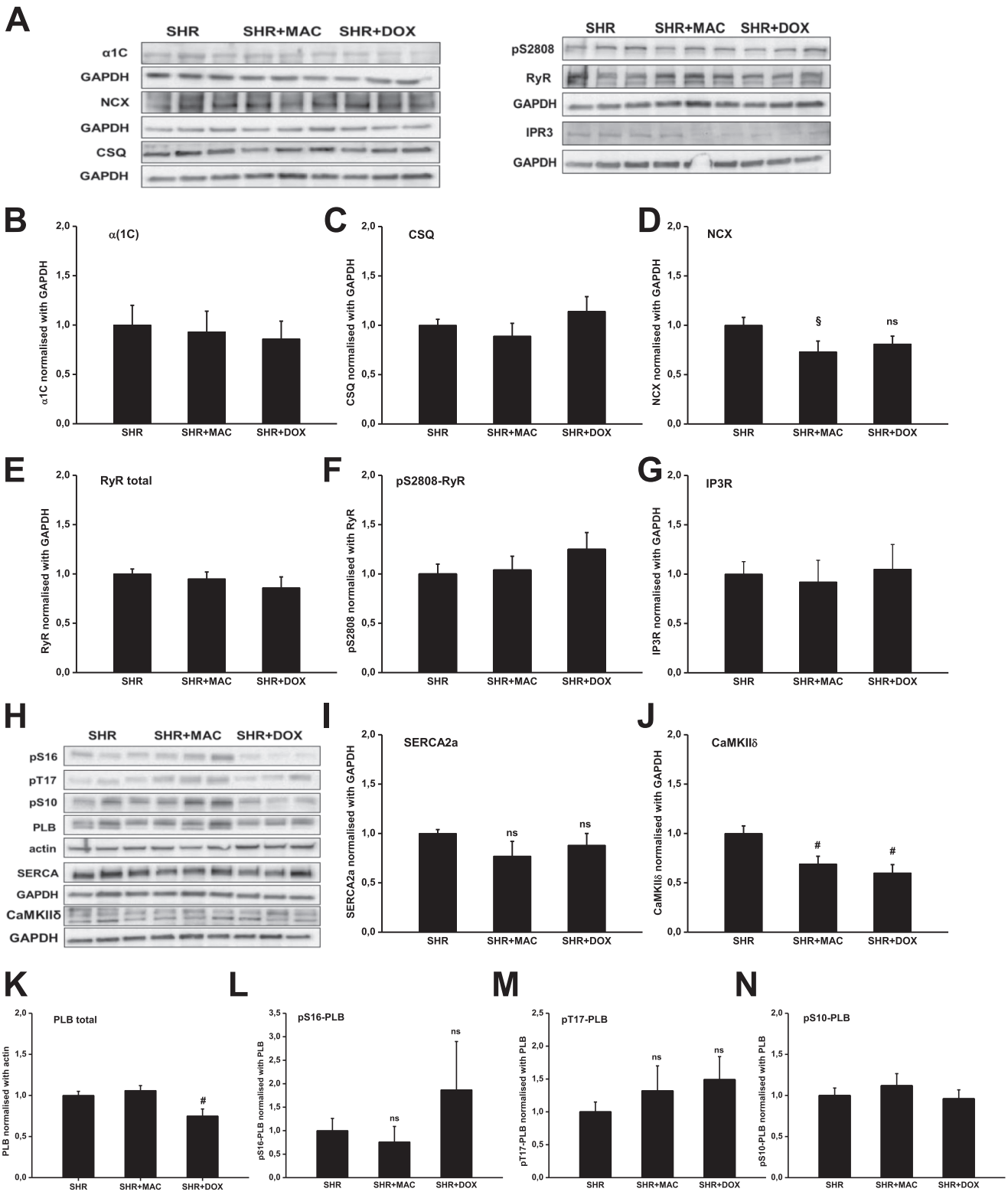

Fig. 5. Effects of macitentan on the calcium handling in LA in SHR. (a) Representative immunoblots showing expression of α1C, NCX1, CSQ and RyR2 from SHR treated with MAC or DOX versus SHR-CTL (b-g) Quantification of immunoblots (N = 6–9 per group) (h) Representative immunoblots showing expression of SERCA2a, CaMKIIδ and PLB. Actin controls for pS16, pT17 and pS10 are not shown (i-m) Quantification of immunoblots (N = 6–9); #$P < 0.05$. Note that some blots/bands of housekeeping proteins used for normalization are identical (e.g. in (a) GAPDH blot/bands shown below CSQ and GAPDH blot/bands shown below RyR) as they are derived from the same membrane.

ROS production through mitochondrial-derived pathways [27,35–37]. Of note, the ET-1-induced increase in contractility is mediated by an intracellular pathway triggered by mitochondrial ROS [36]. Inflammation state as well as occurrence of redox–regulated signaling has been linked to "endocardial remodeling" [2,38–39]. Recent studies emphasized the importance of endocardial inflammation and dysfunction for the development of atrial thrombogenesis [1,40].

At the tissue level, ET-1 and AngII could act synergistically to promote AF. Both peptides induce myocyte hypertrophy and interstitial fibrosis, thereby creating a *substrate* for AF. In addition, both peptides exert direct, presumably Ca^{2+}-mediated, pro-arrhythmic effects [11,32] that may serve as a *trigger* for AF. As reported recently LA of SHR rats display an increased expression of fibrosis markers as well as an enhanced level of markers for molecular hypertrophy [17]. However, the treatment with macitentan did not affect these markers. These results are in contrast with the majority of reports, in which blockade of the ET system counteracted hypertrophic effect [29–30,41]. The possible explanation for different impact of ET blockade might in part depend on tissue type. Muller at al. showed that bosentan was more effective in kidney than in heart of hypertensive rats [30].

ET-1 mobilizes Ca^{2+} by increasing Ca^{2+} release from different intracellular stores [12–14,42]. Recently, we showed evidence for remodeling of Ca^{2+} handling in LA myocytes from SHR, which consisted of reduced L-type Ca^{2+} current and altered sarcolemmal and SR Ca^{2+} handling [17]. The blockade of ET receptors by macitentan, however, did not affect the expression of the major Ca^{2+}-handling proteins (except for reduced expression of CaMKII) suggesting no substantial effects on intracellular Ca^{2+} handling remodeling in SHR atria. CaMKII has been demonstrated to be strongly implicated in calcium-dependent regulation of pathological cardiac structure and function [43,44].

Comparison of the effects of macitentan treatment with doxazosin treatment reveals some important results. For most (yet not all) of the parameters studied, doxazosin treatment elicited similar alterations as observed with macitentan treatment, including anti-inflammatory and anti-oxidative effects and a lack of effect on major Ca^{2+}-handling proteins and markers of hypertrophy and fibrosis. Importantly, however, only doxazosin treatment reduced systolic blood pressure. This implies that "pure" inhibition of the endothelin system by dual ET receptor blockade with macitentan (in the absence of blood pressure reduction) is as effective in altering some aspects of adverse atrial remodeling in hypertensive heart disease as "pure" blood pressure reduction by α1 receptor blockade with doxazosin. Thus, levels of atrial 8-isoprostanes, expression of pro-inflammatory ICAM-1 and IL-8, phosphorylation of MAP kinases, phosphorylation of NF-κB, and expression of VCAM may reflect a final common pathway, which can be affected either by blockade of atrial ET receptors or by (unspecific) blood pressure lowering drugs. The lack of treatment effects on markers of atrial fibrosis or hypertrophy were unexpected. This "paradox" might be explained by different treatment thresholds to change expression of inflammatory markers compared to makers of atrial fibrosis and hypertrophy. Thus, the induced lowering of blood pressure (doxazosin group) or degree of ET blockade (macitentan group) was sufficient to reduce isoprostanes, IL-8, ICAM, VCAM, and NF-κB, but insufficient to reduce atrial fibrotic and hypertrophy markers.

Prior experimental and clinical data confirmed a high potential of endothelin receptor blockade in treatment of essential hypertension, resistant hypertension, heart failure [45]. In addition, there is convincing evidence for similarities of actions of RAS effectors and ET-1 in processes of tissue remodeling. This provides rationale for testing possible additive effects of the combination of dual ETA/ETB antagonist with RAS blockers in treatment of hypertension [24]. However, the number of clinical studies investigating the benefits of combined treatment ERAs with RAS blockers is limited yet. In contrast to previous results, in our study we could not see a significant effect of macitentan on systemic blood pressure. Thus, the presented molecular changes in the macitentan treated animals appear to be caused by direct atrial ET receptor blockade.In might be speculated, that higher macitentan doses (macitentan doses to reduce systemic blood pressure) might induce additional effects on fibrosis and hypertrophy.

4.1. The present study has some limitations

We determined endpoint measurement of hemodynamic parameters by using the tail-cuff method. Unfortunately, we did not have access to telemetry device that would provide continuous and more precise recordings. Secondly, we were not able to assess atrial remodeling in hypertensive rats by means of Echo or MRI. Thirdly, we analyzed male tissue and tissue from left atria, only. Thus, we may have missed gender/atrial-differences in tissue remodeling, and furthermore, we did not include analyses of complete signal transduction pathways in our animal experiments. However, the effect of endothelin and endothelin receptor blockade has been extensively studied. Thus, we chose several molecular markers as read-out in the present study to validate the effect of macitentan in atrial tissue [2,7–15]. However, we did not confirm all findings at the mRNA level at the protein levels by western blotting. Oxygen levels might have varied in the in vitro pacing model within the tissue slices and might differ from in vivo conditions. Although viability staining was performed, we cannot rule out that experimental conditions applied do not fully represent physiological pO2 levels.

5. Conclusions

The present study demonstrates that the blockade of ET-1 receptors by macitentan caused anti-inflammatory and anti-oxidative effects in hypertensive atria in a blood pressure-independent manner. Macitentan reversed the pro-inflammatory tissue remodeling in hypertensive atria. Nevertheless, macitentan did not affect the hypertension-dependent alterations of major Ca^{2+}-regulating proteins and markers of atrial hypertrophy and fibrosis, which both are relevant components of structural atrial remodeling and atrial cardiomyopathy.

6. Contribution

AB, YN, CW, UL, JK and AG have worked on the experiments and analyses of the results. All members have participated to write the manuscript. Figures were done by AB, YN and JK

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: AG: Speaker fees from Abbott, Astra Zeneca, Bayer Health Care, Berlin Chemie, Biotronik, Boehringer Ingelheim, BMS/Pfizer, Boston Scientific, Daiichi-Sankyo, Medtronic, Menarini, Omeicos, Sanofi-Aventis, and Viofor.

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