Online Supplement

Cathepsin A mediates ventricular remote remodeling and atrial cardiomyopathy in rats with ventricular ischemia/reperfusion.

Short title: Cathepsin A mediates atrial cardiomyopathy

Mathias Hohl (a), Katharina Erb (a), Lisa Lang (a), Sven Ruf (b), Thomas Hübschle (b), Stefan Dhein (c), Wolfgang Linz (b), Adrian D. Elliott (d), Prashanthan Sanders (d), Olesja Zamyatkin (a), Michael Böhm (a), Ulrich Schotten (e), Thorsten Sadowski (b), Dominik Linz (a,d).

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(1) Klinik für Innere Medizin III, Universität des Saarlandes, 66421 Homburg/Saar, Germany
(2) Sanofi-Aventis Deutschland GmbH, 65926 Frankfurt, Germany
(3) Herzzentrum Leipzig Abt. Herzchirurgie, Leipzig, Germany
(4) Centre for Heart Rhythm Disorders, South Australian Health and Medical Research Institute, Royal Adelaide Hospital, University of Adelaide, SA 5005 Adelaide, Australia
(5) Department of Physiology, university Maastricht, Maastricht, The Netherlands

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Correspondence:
Dominik Linz, MD, PhD

Centre for Heart Rhythm Disorders,

Department of Cardiology, Royal Adelaide Hospital, University of Adelaide

Adelaide, 5000, AUSTRALIA

Telephone: +61882222723; Facsimile: +61882222722

Email: Dominik.Linz@adelaide.edu.au

Methods

Human tissue

Human failing myocardium was obtained from patients with end stage ischemic cardiomyopathy scheduled for heart transplantation (ICM, n=8). Eight donor hearts of patients with no signs of heart disease that could not be used for transplantation due to ABO-mismatch were used as non-failing controls (Ctr, n=8). Detailed information about clinical parameters and medication were not available. Clinical data of comparable patients was published previously[1]. This investigation conforms with the principles outlined in the Declaration of Helsinki and was reviewed by an ethics committee.

Animal models

All animal studies were performed in accordance to the German law for the protection of animals. The investigation conforms with the guide for the Care and Use of laboratory Animals published by the United States National Institutes of Health (Eighth edition; revised 2011). The procedures followed were in accordance with current Sanofi-Aventis Laboratory Animal Science and Welfare guidelines. The study was approved by the regional animal ethics commission in Darmstadt, Germany.

90 rats (male Wistar, 260 – 300g body weight, 12 weeks old) were purchased from Harlan GmbH (Borchen, Germany) and housed three per cage under standardized conditions (room temperature 24ºC, relative humidity 55%, 12 hours dark/light cycle). They had free access to a standardized diet (#1320, sodium content 0.2 %, Altromin, Lage, Germany) and tap drinking water.

Permanent ligation of left anterior descending coronary artery in rats

Permanent ligation (PL) of left anterior descending (LAD) coronary artery in rats was performed according to the method described previously[2]. Briefly, 30 male Wistar rats were anesthetized with 5% isoflurane in 95% oxygen followed by a deep anesthesia via intraperitoneal injection of ketamine hydrochloride (80mg/kg bodyweight) and xylazine hydrochloride (6 mg/kg bodyweight). For post-operative pain management carprofen (5 mg/kg) was administered subcutaneous. After intubation and ventilation left thoracotomy was performed under aseptic conditions via the third intercostal space. The pericardium was opened. In 20 rats (PL-rats), the LAD was ligated permanently 2 mm distal to the aortic origin. Occlusion was signaled by

blanching of the epicardial surface distal to the suture. Afterwards, lungs were inflated by increasing positive end-expiratory pressure, and the chest was closed. Sham operation in 10 additional rats (Sham-rats) was identical, without coronary occlusion. To prevent acute lung edema, the rats received furosemide (Lasix®, 2 mg/kg, twice daily for 3 days) via drinking water. Four PL-rats died during the 24-h postoperative period. Heart tissue of PL-rats and Sham-rats were preserved for biochemical analysis at 2 weeks (8 PL-rats, 5 Sham-rats) and 8 weeks (8 PL-rats, 5 Sham-rats) after permanent LAD ligation. For euthanasia, rats were anesthetized as described above, the diaphragm was cut under deep anesthesia and hearts were quickly removed and preserved for biochemical analysis.

Ischemia/Reperfusion in rats

Ventricular ischemia and reperfusion (I/R) was performed as described previously[3]. Briefly, 60 male Wistar rats were anesthetized with 5% isoflurane in 95% oxygen followed by a deep anesthesia via intraperitoneal injection of ketamine hydrochloride (80mg/kg bodyweight) and xylazine hydrochloride (6 mg/kg bodyweight). For post-operative pain management carprofen (5 mg/kg) was administered subcutaneous. Afterwards rats were intubated and mechanically ventilated with room air. A left-sided thoracotomy was performed. In 52 rats, myocardial ischemia was induced by temporary occlusion of the left coronary artery for 30 min followed by reperfusion upon release of the ligation. Nine rats underwent sham operation. Eight I/R-rats died during the 24-h postoperative period. The day after surgery, animals were randomized in 4 groups: Sham operated rats (I/R-Sham, n=9), placebo treated I/R-rats (I/R-Placebo, n=15), ramipril treated I/R-rats (I/R-Ramipril, n=14), cathepsin A (CatA) -inhibitor ((S)-3-{[1-(2-Fluoro-phenyl)-5-methoxy-1H-pyrazole-3-carbonyl]-amino}-3-o-tolyl-propionic-acid), SAR) treated I/R-rats (I/R-SAR, n=14).

Ramipril and SAR were synthesized in chemical departments of Sanofi. SAR is a new orally active CatA inhibitor. For more details on pharmacokinetic and pharmacodynamic see reference[4]. SAR (30 mg/kg/day) was administered via oral gavage once every day. An oral dose of 30 mg/kg once daily has been shown to display optimal CatA inhibition for 24h in pharmacokinetic studies in rats[4]. Ramipril (1mg/kg/day) was administered in chow. Ramipril at 1mg/kg/day gave optimal results in previous studies and was shown to produce up to 24 h inhibition of angiotensin converting enzyme activity in plasma, aorta, heart and brain cortex and a 48 h inhibition of the enzyme in kidney and lung in rats. [5,6,7].

Magnetic resonance imaging

This magnetic resonance imaging (MRI) protocol has been used previously.[8,9] After 9 weeks treatment, rats were anaesthetized for MRI of the heart with 1.5-2.5% isoflurane in an oxygen - nitrous oxide mixture (30/70 %). Rats were then positioned prone and horizontally in a

custom-manufactured animal holder in the magnet and allowed to breathe freely. The holder setup included a nose-cone, lines for delivering and scavenging anesthetic gases, a built-in warm water circuit, a rectal temperature probe, and a respiratory monitoring system. Rats were secured within the holder by using surgical tape, without compressing their abdomen and chest regions. All experiments were performed with a Bruker Biospec 70/30 (Bruker Biospin, Ettlingen, Germany) MR system, operating at 300 MHz with a bore of 30 cm. The used assembly consisted of the gradient system BGA-12S and a Rapid-QUAD-Birdcage (112/90) resonator.

Tuning, matching and shimming were performed manually for each animal. Scout images of the individual rat heart were measured in coronal (4-chamber-view) and sagittal (2-chamber-view) orientation. Sixteen contingent slices were acquired in short-axis orientation covering the entire heart. The retrospective CINE MRI sequence is a modified FLASH sequence with an in-slice navigator echo with the following parameters: Gaussian-shaped RF pulse, 1000 µs; flip angle, 20°; repetition time, 7.1 ms; echo time, 1.93 ms; sample rate, 75.75 kHz; echo position, 30%; navigator echo points, 256; field of view, 60 x 60 mm^2; matrix, 128 x 128; in-plane resolution, 234 x 234 μm^2; slice thickness, 1.2 mm; number of repetitions, 120; total acquisition time, approximately 30 min (16 slices, 1´54 min / slice). To cover the cardiac cycle ten-time frames for each slice of the heart were retrospectively reconstructed with the customized program IntraAngio within the Bruker ParaVision 5.0 software. The basal function of the heart was investigated first, followed by a cardiac stress which was induced by infusion of dobutamine (10 µg/kg/min) into the jugular vein over 5 min.

MRI images were converted to DICOM format. A stack of short-axis image slices was acquired (thickness, 1.0mm), fully covering the left ventricle (LV) and left atrium (LA) (planned at end-diastole) from the very LA roof to the apical LV. After the end-diastolic and end-systolic phases were identified on a slice-by slice basis, the endocardial and epicardial borders were traced.

LV function and mass: LV end-systolic volume (ESV), LV end-diastolic volume (EDV), LV stroke volume (SV), cardiac output (CO), and LV ejection fraction (EF) were computed based on the traced borders. LV mass was calculated by slice summation by using the cardiac NMR (local software package, taking a specific gravity of 1.05 g/cc. LV mass was measured, including the most basal image slice and papillary muscles. LV epi- and endocardium were delineated in all data sets. Image analyses were performed off-line by using Mass4Mice software (Medis medical imaging systems, 2300 AJ Leiden, The Netherlands). Two slices were selected from the midventricular level of each heart for analysis of regional contractile function. The midventricular level was chosen because the infarct pattern in this region typically includes all three tissue types of interest (i.e., enhanced, adjacent, and remote). Slices closer to the base of the heart contain typically no or less infarct, whereas slices closer to the apex are

nearly circumferentially infracted. The circumference of each short-axis slice was divided into 100 equiangular radial segments, each of 20 degrees, taking right ventricular corner as the starting point, with clockwise rotation, and divided in septal, anterior, lateral and posterior sections. Papillary muscles were excluded in the endocardial borders during planimetry.

LA function: LA-area was manually encircled. The point of insertion of the mitral valve leaflets was taken as the atrioventricular border. Pulmonary veins were excluded at their ostia and the left atrial appendage was excluded at its base. Minimal LA- (LAmin) and maximal LA-volume (LAmax) and their difference (cyclical change volume) were determined from the LA-volume/time curves. The minimal volume at the end of rapid passive emptying (LAre) and the volume before active emptying (LApc) were determined from the volume-time curves. LA-emptying function parameters (total percent emptying ((LAmax-LAmin)/LAmax), active percent emptying ((LApc-LAmin)/LApc), passive percent emptying ((LAmax-LAre)/LAmax) were computed. To measure the maximal LA diameter a typical horizontal long axis four chamber image was used for analysis. All dimensions were measured throughout the cardiac cycle[8]. The cardiac cycle was divided into 10 equal phases with an interphase time difference of the spontaneous cycle length / 10. Heart rate during MRI scan was not different between the groups (see **Table 1**, main manuscript). The MRI-images of all LAs were analyzed by the same blinded investigator. This protocol has been used previously to determine LV and LA function[8,9].

Atrial electrophysiological studies

After 10 weeks treatment, electrophysiological measurements were performed during general anesthesia by intraperitoneal injection of pentobarbital (100 mg/kg i.p.). Surface ECG (lead II) was recorded via subcutaneous needle electrodes. To analyze atrial conduction, a custom-made mapping electrode with 4*5 unipolar electrodes (with 1.0 mm interelectrode distance) was placed on the LA free wall. Unipolar signals were recorded using a custom-made channel mapping amplifier (filtering bandwidth 0.1-408 Hz, sampling rate 1.0 kHz, A/D resolution 16 bits). Unipolar pacing was performed from the surface of the LA (pulse width of 1 ms at twice the diastolic threshold, cycle length: 150 ms). Maps from 5 consecutive beats were analyzed. Local activation times were identified by maximum negative dV/dt in each unipolar electrogram. Local activation time differences were calculated between neighboring electrodes (conduction times). Conduction times of ≥ 3 ms (equivalent to conduction velocities ≤ 33 cm/s) were considered as being prolonged. Total-atrial activation time was defined as the time difference between the right atrial activation time, visualized by a custom made multiple action potential (MAP)-catheter (Franz-like electrode) next to the pacing electrode, and the latest left atrial activation time recorded by the mapping electrode during right atrial-pacing (cycle length:

150 ms). Atrial effective refractory period (AERP) was measured at a basic cycle length of 150 ms at the free wall of the LA. The mean of 3 AERP measurements was used for analysis. Susceptibility to AF was tested using repetitive 1 s bursts of stimuli (cycle length: 10 ms). When atrial electrograms showed a rapid atrial rate, cycle length < 70 ms and duration >5 beats, AF was diagnosed. The duration of the longest of three induced subsequent episodes of AF was taken as AF-duration. One I/R-Sham, four I/R-Placebo and one I/R-SAR rat died during open-chest experiments. Hearts were removed and quickly preserved for biochemical and histological analysis.

Left ventricular and atrial histology and immunohistochemical staining

To visualize LV and LA tissue fibrosis amount and LA cardiomyocyte diameter, tissue-preparation was performed as described previously[8], and 5 µm sections were either stained with picro-sirius red (#13422.00500, Morphisto, Frankfurt am Main, Germany) or hematoxylin and eosin (H.E.; #2C-163, Waldeck, Münster, Germany), respectively. For LA stained with picro-sirius red, polarization microscopy was performed to visualize collagen type I (yellow-red fibers) and type III (green fibers) based on the birefringence properties of collagen[10]. For the analysis Nicon Instruments Software (NIS)-Elements (BR 3.2, Nikon instruments) was used. Immunostaining of Cx43 in LA tissue was performed as described elsewhere[8]. Briefly, rat LA tissue was fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin. Tissue sections of 5 µm were mounted on glass slides, deparaffinized with xylene and hydrated in descending concentrations of ethanol. Following hydration, sections were incubated for 1 hour in 0.05 % Citraconic anhydrid (Sigma-Aldrich, Germany) at 98°C in a water bath and washed afterwards in 1xPBS-Tween (Phosphate-Buffered Saline: 137 mmol/L NaCl; 2.7 mmol/L KCl; 4.3 mmol/L Na_2HPO_4; 1.47 mmol/L KH_2PO_4, pH 7.4 containing 0.1 % Tween). Slices were incubated overnight with primary antibody (mouse anti-Cx43, Merck Millipore #MAB3068, Darmstadt, Germany), diluted (1/30) in PBS-T (Phosphate-Buffered Saline: 137 mmol/L NaCl; 2.7 mmol/L KCl; 4.3 mmol/L Na_2HPO_4; 1.47 mmol/L KH_2PO_4, pH 7.4 containing 0.1 % Tween) in a moisture-chamber at 4°C. Anti-mouse-IgG-FITC (Dianova) was used as secondary antibody, dituted 1/30 in PBS-T for 2 hours at 37°C. Slides were mounted with DAPI mounting medium (#H-1200, Vector Laboratories Inc, Burlingame, CA). Expression of Cx43 at the lateral and polar membrane of cardiomyocytes from rat atrium was investigated. Per animal 2-3 sections were analyzed, and per section 5-10 cells, so that each data point of the columns is representing 100-250 cells.

Blood samples

BNP 32 (biomarker for heart failure) determination was performed according the respective ELISA-kit description (BNP 32-Phoenix Pharmaceuticals, Inc. Karlsruhe, Germany).

Gene expression analysis

Gene expression analysis from human left ventricle, rat left ventricle and rat left atrial tissue was performed by TaqMan PCR. Total RNA was extracted using peqGold TriFast (#30-2010; PeqLab, Erlangen, Germany) extraction reagent per manufacturer´s protocol. Genomic DNA impurities were removed by DNase treatment (Peqlab), and cDNA was synthesized by reverse transcription using the HighCap cDNA RT Kit (#4368814; Applied Biosystems) according to the manufacturer´s protocol. TaqMan PCR was conducted in a StepOne plus thermocycler (Applied Biosystems) using TaqMan GenEx Mastermix (#4369016, Applied Biosystems). Signals were normalized to corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) controls. No template controls were used to monitor for contaminating amplifications. The ΔCt was used for statistical analysis and $2^{-\Delta\Delta Ct}$ for data presentation. Probes used to amplify the transcripts were as follows (purchased by Applied Biosystems Life Technologies): rat Collagen1a1 (Col1a1): Rn01463848_m1; rat Col1a2: Rn00670286_m1; rat Col3a1: Rn01437681_m1; rat Col4a1: Rn01482927_m1 rat Ctsa (Cathepsin A) Rn01424035_g1; rat Nppb (BNP) Rn00580641_m1; rat Nppa (ANP) Rn00664637_g1, rat Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Rn01775763_g1. Human Nppa Hs00383230_g1, human Ctsa Hs00264902_m1, human GAPDH Hs02758991_g1.

Cathepsin A ELISA

Black microtiter plates were coated overnight at 4°C with anti-mouse cathepsin A antibody (R&D Systems). The next day wells were washed with 1xPBS and blocked with 5 % BSA in 1xPBS for 1 h at room temperature. In 1xPBS diluted heart lysates were given into the wells and incubated for 2 h at room temperature. Recombinant mouse cathepsin A (R&D Systems) served as control. After washing in DELFIA wash buffer (Perkin Elmer) biotinylated anti-cathepsin A antibody (R&D Systems) was pipetted to the wells and incubated for another 2 h at room temperature. After washing in DELFIA wash buffer europium-labeled streptavidin (Perkin Elmer) was pipetted into the wells and incubated for 1 h at room temperature. After extensive washing in DELFIA wash buffer 200 µl/well DELFIA enhancement solution (Perkin Elmer) was given to the wells and fluorescence was measured after 20 and 60 min at 340/620 nm in a Tecan Ultra plate reader.

Statistical analysis

All data are expressed as mean±SEM. An unpaired Student´s ttest (two-tailed) was used for statistical analysis comparing 2 groups. For assessment of statistical significance between four groups, one-way ANOVA followed by Tukey´s multiple comparison test was applied. A two-way ANOVA on raw data for factor treatment and repeated measures factor cord was used for the regional parameters wall motion (WM) and systolic wall thickness (SWTS) under basal conditions and during dobutamine stress testing followed by the Newman-Keuls test for multiple pairwise comparisons. P-values <0.05 were regarded as statistically significant. P-values that reached a value less than 0.0001 were reported as $p<0.0001$. Statistical analysis was carried out using Prism software (GraphPad Version 6.01).

References

1. Hohl M, Wagner M, Reil JC, Müller SA, Tauchnitz M, Zimmer AM, Lehmann LH, Thiel G, Böhm M, Backs J, Maack C. HDAC4 controls histone methylation in response to elevated cardiac load. *J Clin Invest* 2013;**123**:1359-1370.
2. Wiemer G, Itter G, Malinski T, Linz W. Decreased nitric oxide availability in normotensive and hypertensive rats with failing hearts after myocardial infarction. *Hypertension* 2001;**38**:1367-1371.
3. Wohlfart P, Linz W, Hübschle T, Linz D, Huber J, Hess S, Crowther D, Werner U, Ruetten H. Cardioprotective effects of lixisenatide in rat myocardial ischemia-reperfusion injury studies. *J Transl Med* 2013;**11**:84.
4. Ruf S, Buning C, Schreuder H, Horstick G, Linz W, Olpp T, Pernerstorfer J, Hiss K, Kroll K, Kannt A, Kohlmann M, Linz D, Hübschle T, Rütten H, Wirth K, Schmidt T, Sadowski T. Novel β-amino acid derivatives as inhibitors of cathepsin A. *J Med Chem* 2012;**55**:7636-7649.
5. Wohlfart P, Linz W, Hübschle T, Linz D, Huber J, Hess S, Crowther D, Werner U, Ruetten H. Cardioprotective effects of lixisenatide in rat myocardial ischemia-reperfusion injury studies. *J Transl Med* 2013;**11**:84.
6. Unger T, Fleck T, Ganten D, Lang RE, Rettig F. 2-[N-[(S)-1-ethoxycarbonyl-3-phenylpropyl]-L-alanyl]-(1S,3S,5S)-2- azabicyclo[3.3.0]octane-3-carboxylic acid (Hoe 498): antihypertensive action and persistent inhibition of tissue converting enzyme activity in spontaneously hypertensive rats. *Arzneimittelforschung* 1984;**34**:1426-1430.
7. Linz W, Jessen T, Becker RH, Schölkens BA, Wiemer G. Long-term ACE inhibition doubles lifespan of hypertensive rats. *Circulation* 1997;**96**:3164-72.
8. Linz D, Hohl M, Dhein S, Ruf S, Reil JC, Kabiri M, Wohlfart P, Verheule S, Böhm M, Sadowski T, Schotten U. Cathepsin A mediates susceptibility to atrial tachyarrhythmia and impairment of atrial emptying function in Zucker diabetic fatty rats. *Cardiovasc Res* 2016;**110**:371-380.
9. Hohl M, Lau DH, Müller A, Elliott AD, Linz B, Mahajan R, Hendriks JML, Böhm M, Schotten U, Sanders P, Linz D. Concomitant Obesity and Metabolic Syndrome Add to the Atrial Arrhythmogenic Phenotype in Male Hypertensive Rats. *J Am Heart Assoc* 2017;**6**.
10. Nagaraju CK, Dries E, Popovic N, Singh AA, Haemers P, Roderick HL, Claus P, Sipido KR, Driesen RB. Global fibroblast activation throughout the left ventricle but localized fibrosis after myocardial infarction. *Sci Rep* 2017;**7**:10801.

Supplemental Figure S1

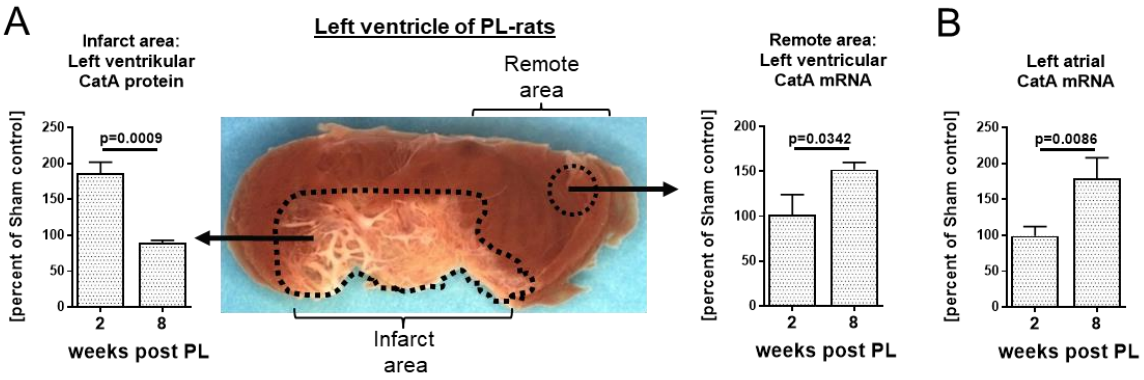


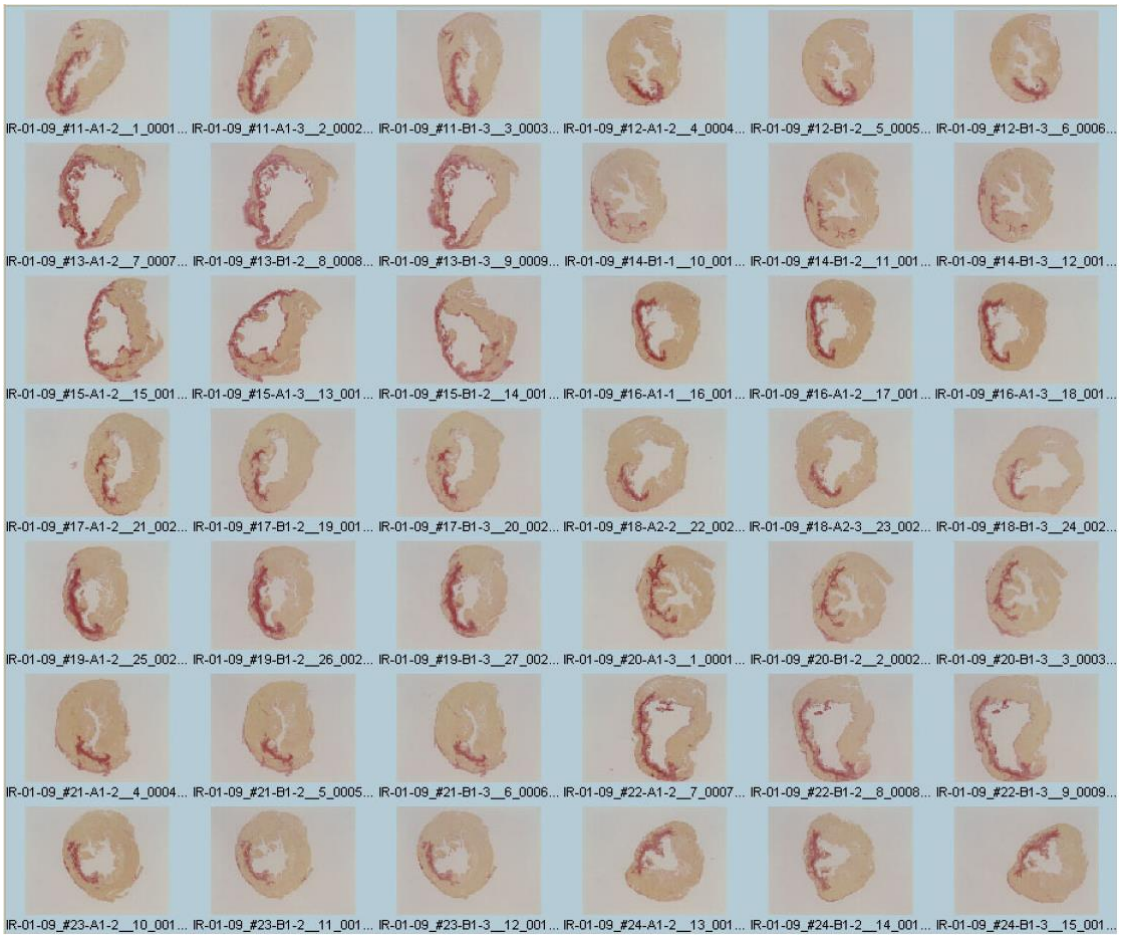
Figure S1: Differential expression pattern of cathepsin A in rats with permanent ligation

Rats with permanent ligation (PL) of the left anterior descending coronary artery. **A left**: CatA protein expression (determined by ELISA) in LV tissue isolated from the infarct area 2 weeks (n=5 sham, n=8 PL) and 8 weeks (n=5 sham, n=8 PL) after PL. **A right**: CatA mRNA levels (determined by TaqMan PCR analysis) in LV tissue remote from the infarct area and (**B**) in left atrial (LA) tissue 2 and 8 weeks after PL. Values are shown as mean±SEM. For statistical analysis an unpaired students ttest was used.

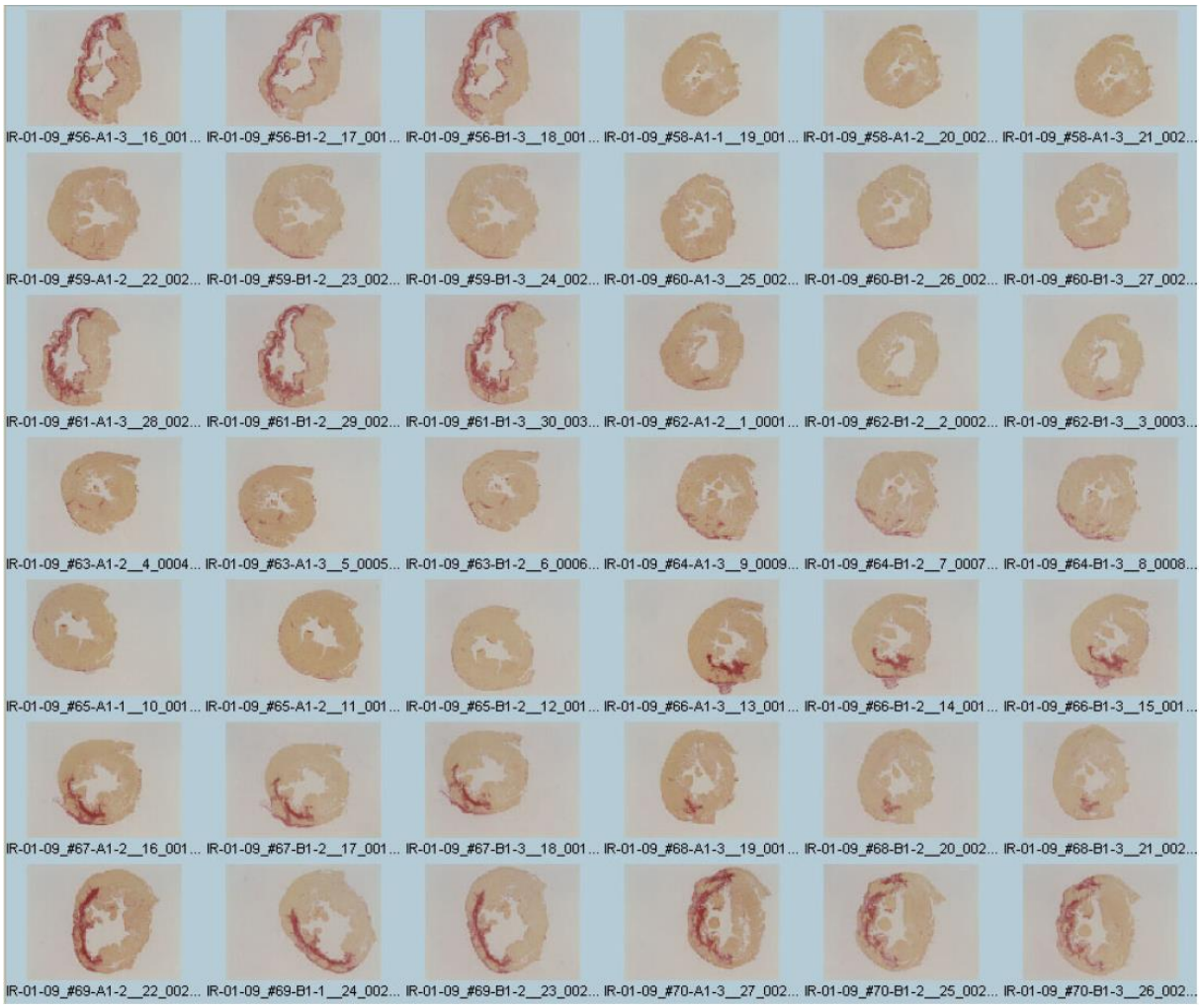
Supplemental Figure S2:

Picro-Sirius Red stained heart sections of I/R-Sham control animals

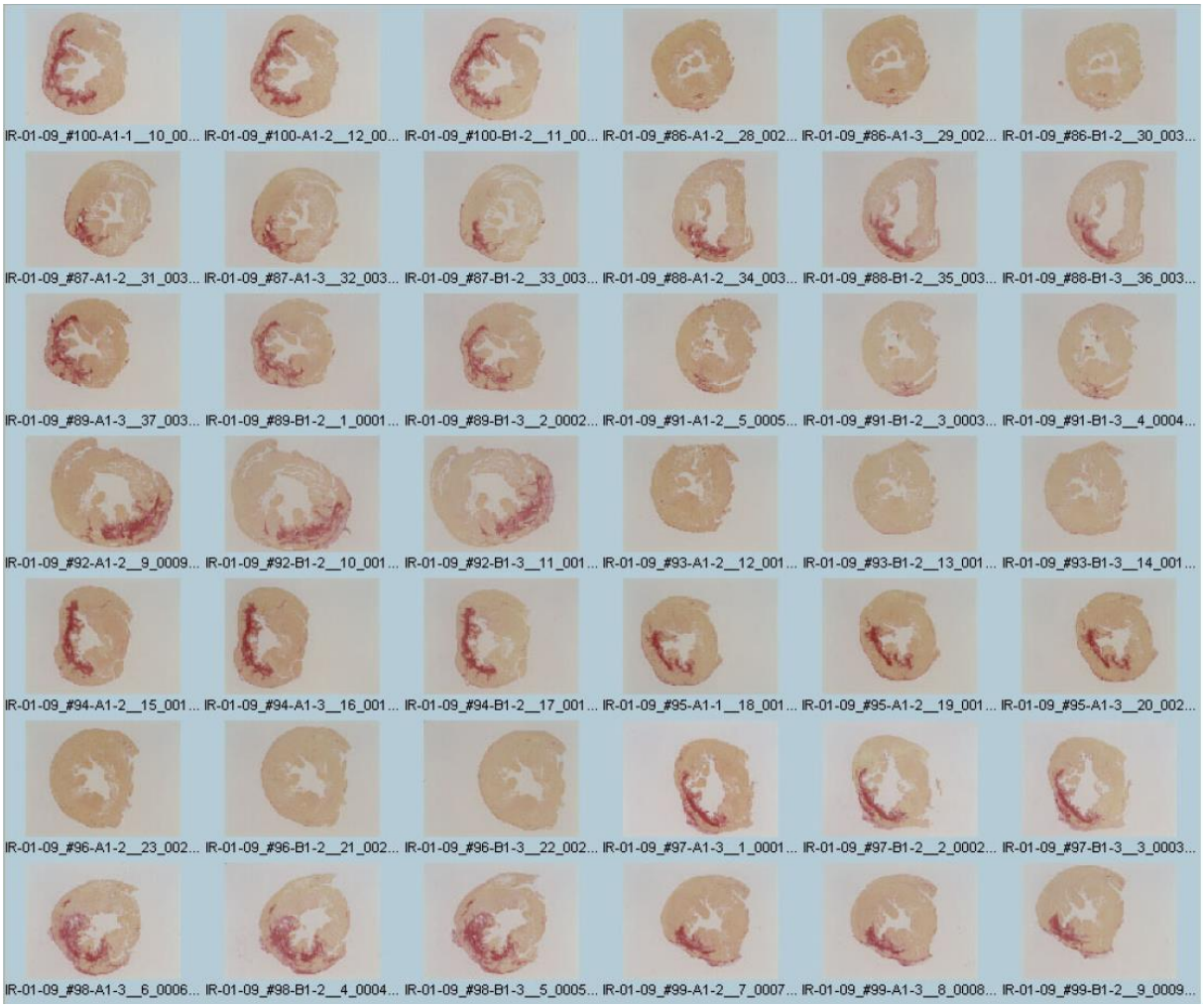
Picro-Sirius Red stained heart sections of I/R-Placebo treated animals



Picro-Sirius Red stained heart sections of I/R-Ramipril treated animals



Picro-Sirius Red stained heart sections of I/R-SAR treated animals



Supplemental Figure S3

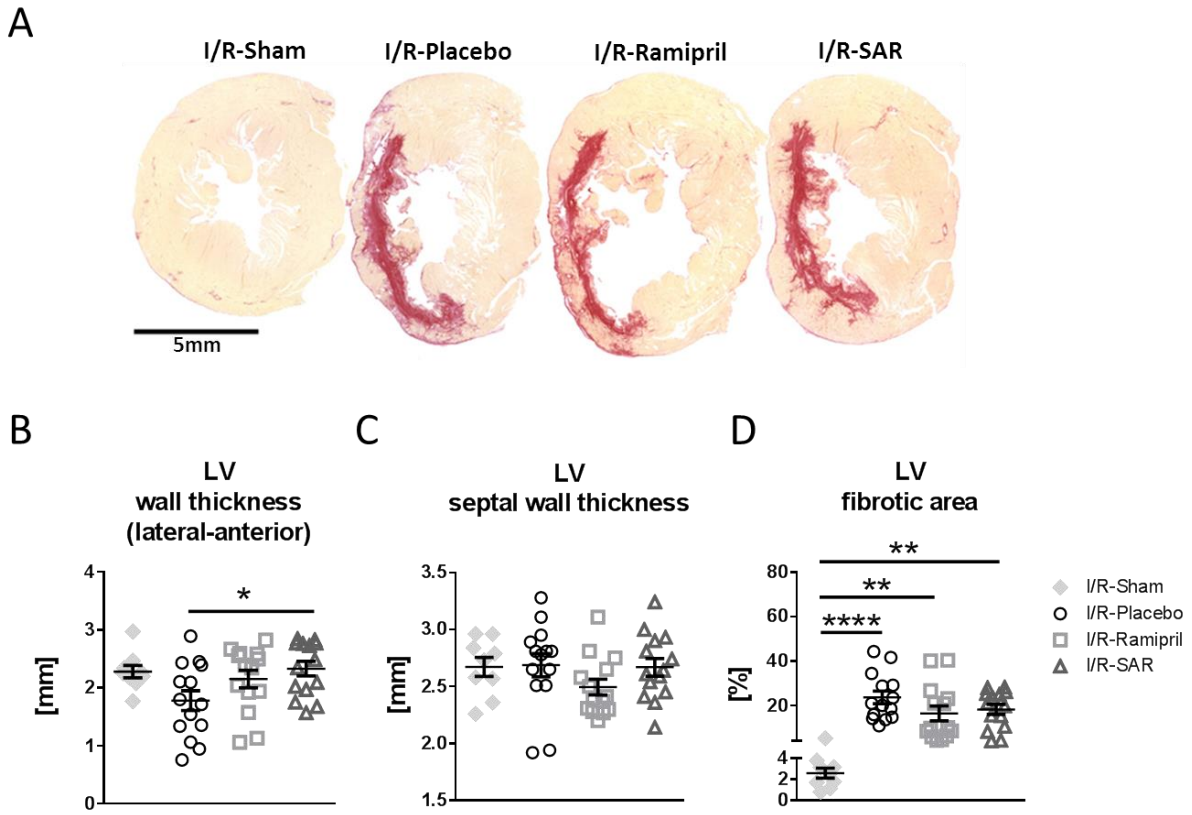


Figure S3: (A) Representative left ventricles of I/R-Sham, I/R-Placebo- and I/R-rats treated with ramipril or SAR, stained with picro-sirius red to quantify (**B)** LV wall thickness, **(C)** LV septal wall thickness and **(D)** LV fibrosis formation.
I/R-Sham (n=9), placebo-treated I/R-rats (n=14), I/R-rats treated with Ramipril (n=14) or SAR (n=14). Values are shown as mean±SEM. *****=p<0.05, **=p<0.01, ****=p<0.0001. For statistical analysis one-way ANOVA followed by Tukey´s multiple comparison test was used.