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Research Article

Excess ischemic tachyarrhythmias trigger protection against myocardial infarction in hypertensive rats

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Increased level of C-reactive protein (CRP) is a risk factor for cardiovascular diseases, including myocardial infarction and hypertension. Here, we analyzed the effects of CRP overexpression on cardiac susceptibility to ischemia/reperfusion (I/R) injury in adult spontaneously hypertensive rats (SHR) expressing human CRP transgene (SHR-CRP). Using an *in vivo* model of coronary artery occlusion, we found that transgenic expression of CRP predisposed SHR-CRP to repeated and prolonged ventricular tachyarrhythmias. Excessive ischemic arrhythmias in SHR-CRP led to a significant reduction in infarct size (IS) compared with SHR. The proarrhythmic phenotype in SHR-CRP was associated with altered heart and plasma eicosanoids, myocardial composition of fatty acids (FAs) in phospholipids, and autonomic nervous system imbalance before ischemia. To explain unexpected IS-limiting effect in SHR-CRP, we performed metabolomic analysis of plasma before and after ischemia. We also determined cardiac ischemic tolerance in hearts subjected to remote ischemic perconditioning (RIPer) and in hearts *ex vivo*. Acute ischemia in SHR-CRP markedly increased plasma levels of multiple potent cardioprotective molecules that could reduce IS at reperfusion. RIPer provided IS-limiting effect in SHR that was comparable with myocardial infarction observed in naïve SHR-CRP. In hearts *ex vivo*, IS did not differ between the strains, suggesting that extra-cardiac factors play a crucial role in protection. Our study shows that transgenic expression of human CRP predisposes SHR-CRP to excess ischemic ventricular tachyarrhythmias associated with a drop of pump function that triggers myocardial salvage against lethal I/R injury likely mediated by protective substances released to blood from hypoxic organs and tissue at reperfusion.

Introduction

The inflammatory response plays a crucial role in the pathophysiology of many cardiovascular disorders, including acute myocardial infarction and post-ischemic heart failure. Previous research has shown that C-reactive protein (CRP), a protein of the acute phase of inflammation, enhances the extent of myocardial damage associated with ischemic heart disease [1]. In humans, increased CRP production is a predictive marker for future coronary events, recurrent myocardial infarction, chronic heart failure, and cardiovascular death [2,3]. It has been shown that CRP, together with a CRP-activated complement, is deposited in atherosclerotic plaques and infarcted myocardium [4].

To investigate the effect of increased levels of CRP on pathogenetic mechanisms of cardiovascular diseases, various animal and experimental models have been used. In rodents, CRP is not an acute-phase

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reactant and their CRP does not activate complement [5]. Therefore, humanized CRP transgenic animals that activate rodent complement were created [6]. Transgenic expression of human CRP exacerbated the progression of post-ischemic heart failure in mice [7]. Similarly, antisense oligonucleotides designed to specifically hybridize to either rat or human CRP mRNA improved left ventricular systolic function in rats after myocardial infarction [8]. Finally, the administration of human CRP immediately after myocardial infarction activated complement, increased infarct size (IS), and impaired the left ventricle (LV) systolic function in normotensive Wistar rats [6,9].

Recently, Pravenec et al. [10] created the humanized CRP transgenic rat strain SHR-CRP from spontaneously hypertensive rats (SHR). SHR is a widely studied rat model of human essential hypertension that is genetically predisposed to the development of the metabolic syndrome and characterized by an increased myocardial sensitivity to acute ischemia/reperfusion (I/R) injury [11–13]. In SHR-CRP, the overall concentration of CRP (endogenous rat and transgenic human) was markedly elevated compared with SHR controls. The increased CRP level was accompanied by multiple features of the metabolic syndrome including insulin resistance, hypertriglyceridemia, and increased blood pressure [10,14,15]. Concerning the heart, the increased level of human CRP resulted in a perivascular accumulation of monocytes and oxidative stress in the LV of SHR-CRP [14,15]. However, the effect of transgenic expression of human CRP on myocardial I/R injury in hypertensive rats with the metabolic syndrome has not been determined.

In the present study, we assessed the incidence and severity of ischemic and reperfusion ventricular arrhythmias and myocardial IS in hearts *in vivo* of transgenic SHR-CRP and SHR controls. Then, subsequent physiological, biochemical, and molecular biological analyses were performed to explain the observed changes in cardiac ischemic tolerance caused by the CRP transgene. Although counterintuitive, the results indicated that repeated and prolonged ventricular tachyarrhythmias occurring during coronary artery occlusion in SHR-CRP can trigger protective mechanisms that lead to IS reduction. Therefore, we revealed a novel link between ventricular arrhythmias and acute myocardial infartion caused by an I/R insult in hypertensive rats.

Materials and methods

Animal model

Adult male rats of SHR and SHR-CRP strains [16–20 weeks old, n=99 per strain, body weight (BW) 300–370 g] were used. Transgenic SHR-CRP were derived by microinjections of SHR fertilized ova with a construct containing cDNA for human CRP under control of the apoE promoter with the objective of driving expression of the CRP transgene in the liver where CRP is normally produced [10,15]. Rats were bred in the Institute of Physiology of the Czech Academy of Sciences, and housed in an air-conditioned animal facility and allowed free access to their standard chow and water. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (published by the National Academy of Science, National Academy Press, Washington, D.C.). Experimental protocols were approved by the Animal Care and Use Committee of the Institute of Physiology, the Czech Academy of Sciences (Permit Number: 75/2016).

Myocardial I/R *in vivo*

Rats were subjected to acute I/R as described previously [16]. Briefly, anesthetized (sodium pentobarbital, 60 mg/kg i.p.; Sigma–Aldrich, Czech Republic) animals were intubated with a cannula connected to a rodent ventilator (Ugo Basile, Italy) and ventilated with room air at 68–70 strokes/min (tidal volume of 1.2 ml/100 g body weight). The rectal temperature was maintained between 36.5 and 37.5°C by a heated table throughout the experiment. Left thoracotomy was performed and a silk braided suture 5/0 (Chirmax, Czech Republic) was placed around the left anterior descending (LAD) coronary artery at approximately 1–2 mm distal to its origin. Regional myocardial ischemia was induced after 15-min stabilization by the tightening of the suture threaded through a polyethylene tube. After a 20-min occlusion period, the ligature was released and reperfusion of previously ischemic tissue continued. Chest was closed after 3 min of reperfusion, air was exhausted from thorax and spontaneously breathing animals were maintained in deep anesthesia following 180 min by i.p. administration of pentobarbital (one third of the initial dose per hour). In the separate group of SHR, remote ischemic perconditioning (RIPer) was induced by three cycles of 4 min occlusion of both hindlimbs (by the tourniquet) followed by 4-min reperfusion starting 2 min after onset of ischemia. Dark-blue skin color was taken to indicate leg ischemia. A single-lead electrocardiogram (ECG) was registered during ischemia and the first 3 min of reperfusion.

Myocardial I/R *ex vivo*

Animals were anesthetized as above. Hearts were rapidly excised and perfused according to Langendorff under constant flow (adjusted to approximately 10 ml/min per g) as described [17] with non-recirculating Krebs–Henseleit

solution containing (in mmol/l): NaCl 118.0; KCl 3.2; $CaCl_2$ 2.5; $MgSO_4$ 1.2; $NaHCO_3$ 25.0; KH_2PO_4 1.2; glucose 7.0. The medium was saturated with 95% O_2 and 5% CO_2 (pH 7.4) and maintained at 37°C. Expected heart weights were calculated from the regression equations established on the basis of our previous data for heart-to-body weight ratio. Epicardial ECG were recorded with electrodes attached to the right atrium and the apex of the heart. After 20-min stabilization, regional 40 min no-flow ischemia was induced by occlusion of the LAD coronary artery followed by 60 min reperfusion. ECG was registered during ischemia and the first 10 min of reperfusion. In both *in vivo* and *ex vivo* hearts, rats exhibiting ventricular arrhythmias before I/R (during stabilization) were excluded from experiment.

IS determination and analysis of arrhythmias

In open-chest rats, hearts were excised and washed with saline via aorta at the end of reperfusion. The area at risk (AR) was delineated by perfusion with 5% potassium permanganate in both experimental settings, as described earlier [16]. Frozen hearts were cut into slices 1 mm thick, stained with 1% 2,3,5-triphenyltetrazolium chloride (pH 7.4; 37°C; Sigma–Aldrich, Czech Republic) for 30 min and fixed in formaldehyde solution. Four days later, both sides of the slices were photographed. The IS, the size of AR and LV were determined by computerized planimetric method using the software Ellipse (ViDiTo, Slovakia). The incidence and severity of ventricular arrhythmias during the ischemic insult and at the beginning of reperfusion were analyzed offline from ECG records by custom-designed software according to the Lambeth Conventions as previously described [18]. Breifly, premature ventricular complexes (PVCs) occurring as singles, salvos, or tachycardia (VT, a run of four or more consecutive PVCs) were counted separately. The incidence and the number of episodes of ventricular tachycardia (VT) and fibrillation (VF) were also evaluated. A single VF episode lasting more than 2 min was considered as sustained VF (sVF). Rats/hearts exhibiting sVF during ischemia were excluded from further evaluations. The duration of tachyarrhythmias was assessed as the duration of VT plus reversible VF.

Echocardiography

In the separate sets of SHR and SHR-CRP, evaluation of geometrical and functional parameters of the hearts was performed using echocardiographic system GE Vingmed System Seven with 14 MHz linear matrix probe [19]. Anesthesia was induced with 3% of isoflurane (Aerrane, Baxter SA) and then maintained at 2% during the ultrasound procedure. Rectal temperature was maintained within 36.5 and 37.5°C by a heated table throughout measurements. Diastolic and systolic dimensions of LV were measured during echocardiographic evaluation including anterior and posterior wall thickness (AWTd, PWTd, AWTs, PWTs) and LV cavity diameter (LVDd, LVDs). From these dimensions, the following functional echocardiographic parameters were derived: fractional shortening (FS) = (LVDd − LVDs)/LVDd * 100, ejection fraction (EF) = 100 * ($LVDd^3 - LVDs^3$)/$LVDd^3$, diastolic and systolic LV volumes (EDV, ESV) were calculated based on prolate spheroid geometry using the formula EDV = 0.001 * (4 * π/3) * k * $LVDd^3$/8 and ESV = 0.001 * (4 * π/3) * k * $LVDs^3$/8, where (k) is a ratio of long to short axis, stroke volume (SV) = EDV − ESV and cardiac output (CO) = SV * HR, where (HR) is heart rate.

After the measurement of baseline parameters, the functional reserve with serially infused dobutamine (1, 5, and 25 ng/g/min i.v. for 4 min each; Sigma–Aldrich, Czech Republic) was assessed [20].

Blood pressure measurements

In the separate sets of SHR and SHR-CRP, heart rate (HR) and mean arterial pressure (MAP) were determined after 3-day treatment by parasympathomimetic pyridostigmine (acetylcholinesterase inhibitor; 40 mg/kg/day in tap water; Sigma–Aldrich, Czech Republic) and in untreated conscious rats. For blood pressure measurement and drug application, polyethylene catheters (PE50 resp. PE10, Portex, Smith Medical International Ltd., U.K.) were inserted into the left carotid artery and jugular vein under isoflurane anesthesia as mentioned above. Both catheters were filled with heparinized saline (500 I.U./ml, Heparin Léčiva, Zentiva, Czech Republic), tunnelled subcutaneously and exteriorized in the interscapular region. One day after the surgical procedures, the experiments were carried out in conscious rats kept in small transparent cages as described previously [21]. The signal from pressure transducer connected to bridge amplifier was digitalized with a computer-based monitoring PowerLab system and recorded by LabChart software (ADInstruments Ltd, Australia). HR was derived from arterial pressure signal as the reciprocal of pulse interval, which was computed as the interval between two consecutive systolic peaks. All animals were allowed to stabilize for a period of 30-min before measurements. To estimate the cardiac parasympathetic tone, methylatropine [a muscarinic acetylcholine receptor (AChR) antagonist, which does not cross the blood–brain barrier; 2 mg/kg i.v.; Sigma–Aldrich, Czech Republic] were acutely administered to non-treated and pyridostigmine-treated SHR and SHR-CRP.

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Electrophysiological measurements and analysis

ECG recordings were acquired in urethane (10 mg per 1 g of body weight i.p.; Sigma–Aldrich, Prague, Czech Republic) anesthetized non-infarcted rats. Needle 5-min electrocardiograms (lead II) were recorded by Biopac System (Biopac Systems Inc., Santa Barbara, CA, U.S.A.) with a sampling rate of 1000 Hz. ECG intervals (RR, PR, QRS, QT) were measured by a blinded observer manually in five consecutive beats, averaged, and the mean values were used for further analyses and comparisons. Corrected QT intervals (QTc) were computed using Bazett's formula normalized to average RR interval ($QTc = QT/(RR/f)^{1/2}$, f = 240 ms) [22].

Membrane potentials in the LV trabeculae were recorded as described previously [23]. Briefly, immediately after the animals were killed by cervical dislocation, the hearts were excised and the papillary muscles with adjacent trabeculae were cut from the LV. The preparations were placed into a tissue bath perfused with oxygenated warm (37°C) Tyrode solution (in mmol/l: NaCl 137, KCl 4.5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, and HEPES 5; the pH was adjusted to 7.4 with NaOH; all chemicals were purchased from Sigma–Aldrich, Czech Republic) and stimulated at various frequencies (1, 2, 3, and 5 Hz; Pulsemaster Multi-Channel Stimulator A300, World Precision Instruments, Inc., FL, U.S.A.). Action potentials were measured using glass microelectrodes filled with 3 M KCl (resistance > 20 MΩ; Microelectrode Puller P-1000, Sutter Instruments, CA, U.S.A.). Action potential duration at 90% repolarization was measured offline in ten consecutive cycles by in-house software made in MATLAB 2014b (MathWorks Inc., MA, U.S.A.). The results were averaged and used for statistical analyses.

Optical mapping

Intact animals of both strains were anesthetized by sodium pentobarbital as mentioned above. Hearts were rapidly excised and Langendorff-perfused in non-recirculating constant flow mode (10 ml/min per g) with Tyrode's solution (in mmol/l: NaCl 145, KCl 5.9, $CaCl_2$ 1.1, $MgCl_2$ 1.2, glucose 11, HEPES 5; pH 7.4; 37°C) saturated with 100% O_2. After 10-min stabilization, the hearts were bolus stained with 200 μl of 0.125% di-4-ANNEPS (Invitrogen, Thermo Fisher Scientific, Czech Republic) in DMSO (Sigma–Aldrich, Czech Republic) injected into a compliance chamber of the perfusion system as described earlier [24]. Additionally, reversible myosin II inhibitor blebbistatin (Sigma–Aldrich, Czech Republic) was added (50 μl in 0.4% DMSO) into the perfusion circuit to reduce undesirable heart motion. Recording was performed during sinus rhythm and during stimulation from the middle of the LV (300 ms cycle length, 2 ms duration, twice threshold) using the ULTIMA L camera fitted to BX51 WI epifluorescence microscope (Olympus, Japan) equipped with a 150 W Xe arc lamp (Cairn, U.K.) as described [25]. Activation maps were constructed and conduction velocity (CV) parallel (longitudinal CV) and perpendicular (transversal CV) to the fiber direction were determined from the paced recordings. Heart rate was determined using BV_Analyzer software tools.

Phospholipids fatty acids analysis

In the separate sets of SHR and SHR-CRP, rats were killed by cervical dislocation; the heart was dissected on ice into the right ventricle (RV), LV and septum (S), each part was separately weighted, and LV samples were immediately frozen in liquid nitrogen and stored at −80°C until use for biochemical and metabolomic analyses.

Phospholipids from the LV were extracted according to Folch et al. [26] and lipid classes were separated by thin layer chromatography, fatty acids (FAs) converted into methyl esters and analyzed by gas chromatography as desribed earlier [19] using gas chromatograph platform consisting of GC-FOCUS with automatic sampler AI 1310 (Thermo Fisher Scientific, Italy). The FA profile of the phospholipids was used to calculate index reflecting the degree of unsaturation.

Receptor density and enzyme activity

Frozen LVs were combined and placed in the five-fold volume of ice-cold TMES buffer (20 mM TRIS, 3 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, pH 7.4) containing protease and phosphatase inhibitors (cOMPLETE and PhosSTOP, Sigma–Aldrich, Czech Republic) and homogenized using Ultra Turrax (15 s, 24000 rpm) on ice and then in a glass homogenizer with a motor-driven Teflon pestle at 1200 rpm for 2 min on ice. The homogenate was centrifuged (2100 rpm, 10 min, 4°C), supernatant was collected and pellet re-homogenized in TMES using glass-teflon homogenizer. After then, it was centrifuged again under the same conditions. Both supernatants were pooled and subsequently centrifuged (23500 rpm, 30 min, 4°C, Beckman Coulter Optima L.90K ultracentrifuge). Pellets (crude membrane fractions) were suspended in TME buffer (20 mM TRIS, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.4) and washed. The resulting crude membranes were aliquoted and stored at −80°C. Total number of β-adrenergic (β-AR) and muscarinic (AChR) receptors in crude membranes was measured using radioligand binding method in saturation arrangement with the non-selective β-AR antagonist [^{3}H]CGP12177 and the AChR antagonist [^{3}H]QNB, respectively, as described

previously [27,28]. Adenylyl cyclase (AC) activity was determined by measuring the conversion of [α-^{32}P]ATP into [^{32}P]cAMP [29].

Metabolomic and lipidomic analyses

Metabolomic and lipidomic profiling was conducted using a combined targeted and untargeted workflow for the lipidome, metabolome, and exposome analyses (LIMeX). For detailed description, see the recent study [30]. Briefly, LV samples (≈100 mg) were homogenized with 650 µl MeOH containing internal standards for 2 min using a grinder (MM400, Retsch, Germany), proteins precipitated at −80°C for 30 min and separated from the supernatant by centrifugation (12000×***g***, 10 min, 4°C). Then, 600 µl of the supernatant was mixed with 3.38 ml of cold water with 0.01 mM HCl, the tubes were shaken for 1 min and samples purified using solid phase extraction columns (Strata-X 200 mg, Phenomenex, Torrance, CA, U.S.A.). Lipid metabolites were extracted from tissue samples and plasma, respectively, using a biphasic solvent system of cold methanol, methyl *tert*-butyl ether and water with some modifications. [31]. The bottom (polar) phase was collected and used for two LC-MS platforms. For hydrophilic interaction chromatography (HILIC) one aliquot was evaporated, resuspended in an acetonitrile/water (4:1, v/v) mixture followed by separation on an Acquity UPLC BEH Amide column (50 × 2.1 mm; 1.7 µm, Waters, The Netherlands) using gradient elution with acetonitrile/water (95:5, v/v) and water both solvents supplemented with 10 mM ammonium formate and 0.125% formic acid. The second aliquot was cleaned up using an acetonitrile/isopropanol mixture (1:1, v/v) and after evaporation, the dry extract was resuspended in 5% methanol with 0.2% formic acid followed by separation using reversed-phase liquid chromatography on an Acquity UPLC HSS T3 column (50 × 2.1 mm; 1.7 µm, Waters, The Netherlands) using gradient elution with water and methanol both solvents supplemented with formic acid (0.2 and 0.1%, respectively). Lipid metabolites were extracted using Strata-X 33u reverse phase extraction columns (200 mg/3 ml, Phenomenex, Torrance, CA, U.S.A.) according to manufacturer's instructions.

In the separate sets of rats, animals were subjected to I/R *in vivo* as described above. One milliliter of blood was taken out from the carotid artery 15 min before coronary occlusion and 1 min before start of reperfusion. Blood was centrifuged and plasma aliquots were frozen in liquid nitrogen, and stored at −80°C until use. The metabolites were extracted as described above. For LC-MS analysis, the systems consisted of a Vanquish UHPLC System (Thermo Fisher Scientific, Bremen, Germany) coupled to a QExactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used.

LC-MS was used to detect oxylipins [32,33]. For data processing, MS-DIAL software was used [34].

Statistics

The results are expressed as means ± SEM from the indicated number of experiments. Unpaired *t* test or two-way ANOVA with subsequent Bonferroni's test were used for comparison of differences in normally distributed variables between groups. Not normally distributed data (arrhythmias) are expressed as median ± interquartile range. Differences in the number of PVCs between the groups were compared by the Mann–Whitney test. The incidence of tachycardia and fibrillation was examined by Fisher's exact test. Differences were assumed statistically significant when $P<0.05$.

Results

Ischemic and reperfusion ventricular arrhythmias and myocardial infarction in hearts *in vivo*

Detail analysis of I/R arrhythmias revealed a distinct proarrhythmic phenotype in SHR-CRP. The median of the total number of ischemic PVCs was significantly increased to 3281 in SHR-CRP compared with 666 in SHR. Similarly, the median of tachyarrhythmias duration [VT + reversible VF] was significantly higher in SHR-CRP (336 s) than in SHR (32 s) (Figure 1A, Supplementary Table S1). One SHR-CRP died due to sustained VF (excluded from the study). Transgenic expression of CRP also tended to increase the incidence of VT and reversible VF. The median of the number of reperfusion PVCs was significantly higher in SHR-CRP than in SHR controls (Figure 1B).

The AR normalized to LV did not differ between SHR-CRP (41.6 ± 4.0%) and SHR (41.9 ± 1.9%). Surprisingly, the IS normalized to AR was significantly lower in SHR-CRP (48.5 ± 5.1%) compared with SHR (66.5 ± 4.9%; Figure 1C). Hence, the excess I/R arrhythmias were associated with the reduction of myocardial infarction in SHR-CRP hearts *in vivo*.

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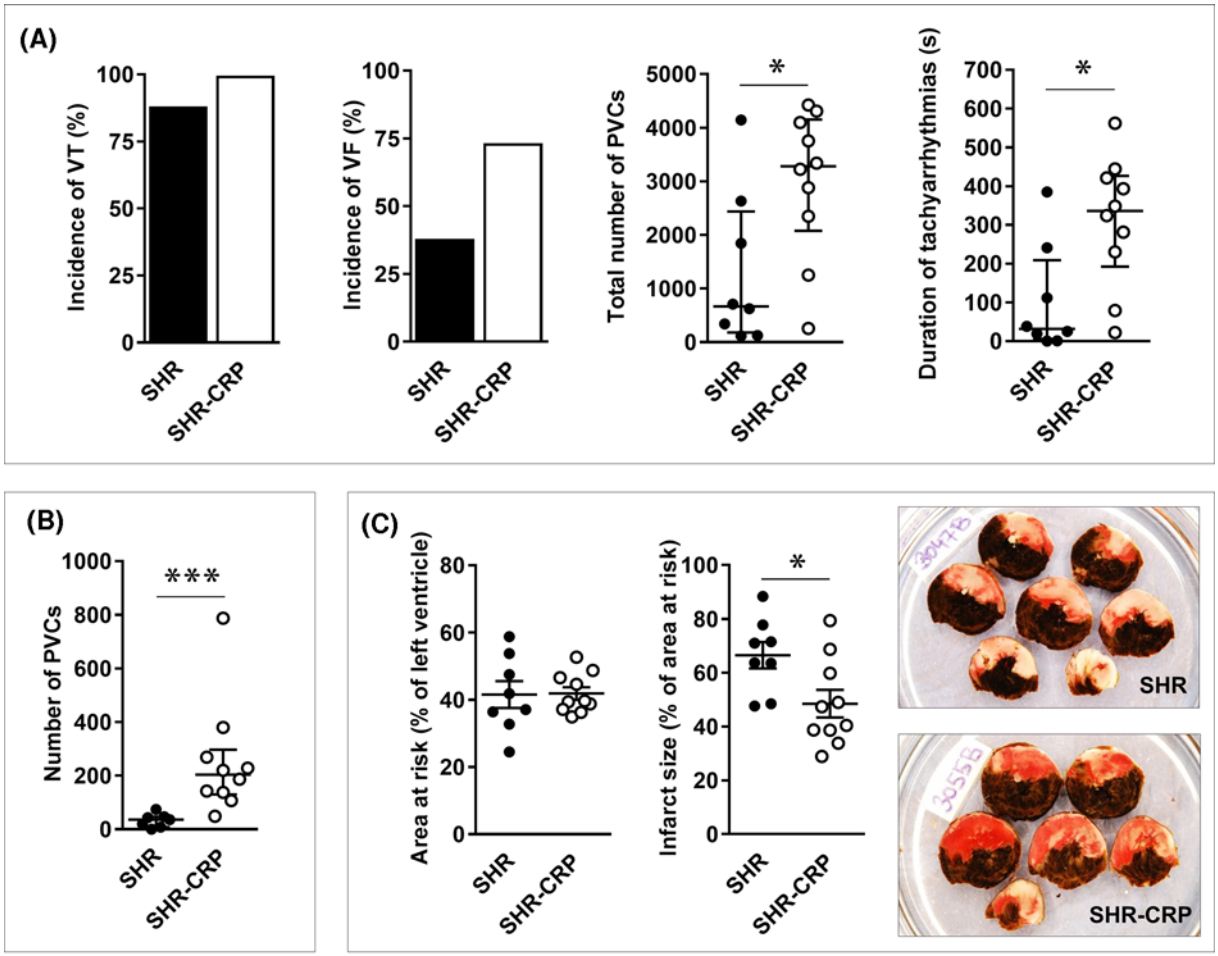

Figure 1. Ischemic and reperfusion ventricular arrhythmias and myocardial infarction in hearts *in vivo*

The incidence of VT and VF, the total number of PVCs, and the duration of tachyarrhythmias (VT + reversible VF) (**A**); the number of reperfusion PVCs (**B**); the relative size of the AR and the IS in hearts *in vivo* with representative images of staining (**C**) in control SHR and transgenic SHR-CRP. Values are shown as median with interquartile range (number of PVCs and duration of tachyarrhythmias) and as mean ± SEM of 8–10 hearts in each group. $^{*}P<0.05$ and $^{***}P<0.001$ vs. SHR by Mann–Whitney test (number of PVCs and duration of tachyarrhythmias), by Fisher's exact test (incidence of VT and VF), and by unpaired *t* test (relative size of the AR and IS).

ECG, epicardial CVs, and action potential duration analyses

We tried to explain a predisposition of SHR-CRP hearts to increased I/R arrhythmias using various electrophysiological approaches. ECG analysis did not show any significant differences in ECG intervals between the strains in non-infarcted hearts *in vivo*. However, the RR interval tended to decrease ($P=0.059$) in SHR-CRP (Figure 2A). Optical mapping analysis of *ex vivo* perfused hearts revealed that the strains did not differ in HR, longitudinal and transversal epicardial CVs, and the anisotropy ratio (longitudinal-to-transversal CV; Figure 2B). Further analysis showed that transgenic expression of CRP did not affect the action potential duration at 90% repolarization values measured in the LV trabeculae paced at various rates (Figure 2C). In short, these experiments did not reveal any substantial electrophysiological abnormalities that would predispose SHR-CRP to a higher incidence of I/R arrhythmias.

Myocardial FA composition and levels of eicosanoids

Given negative findings in the electrophysiological experiments, we focused on biochemical analysis of potential pro- and antiarrhythmic lipid mediators in the heart and plasma of non-infarcted rats. For these analyses, we prepared myocardial tissue and plasma samples from separate sets of animals; Supplementary Table S2 summarizes the body and heart weights of SHR-CRP and SHR.

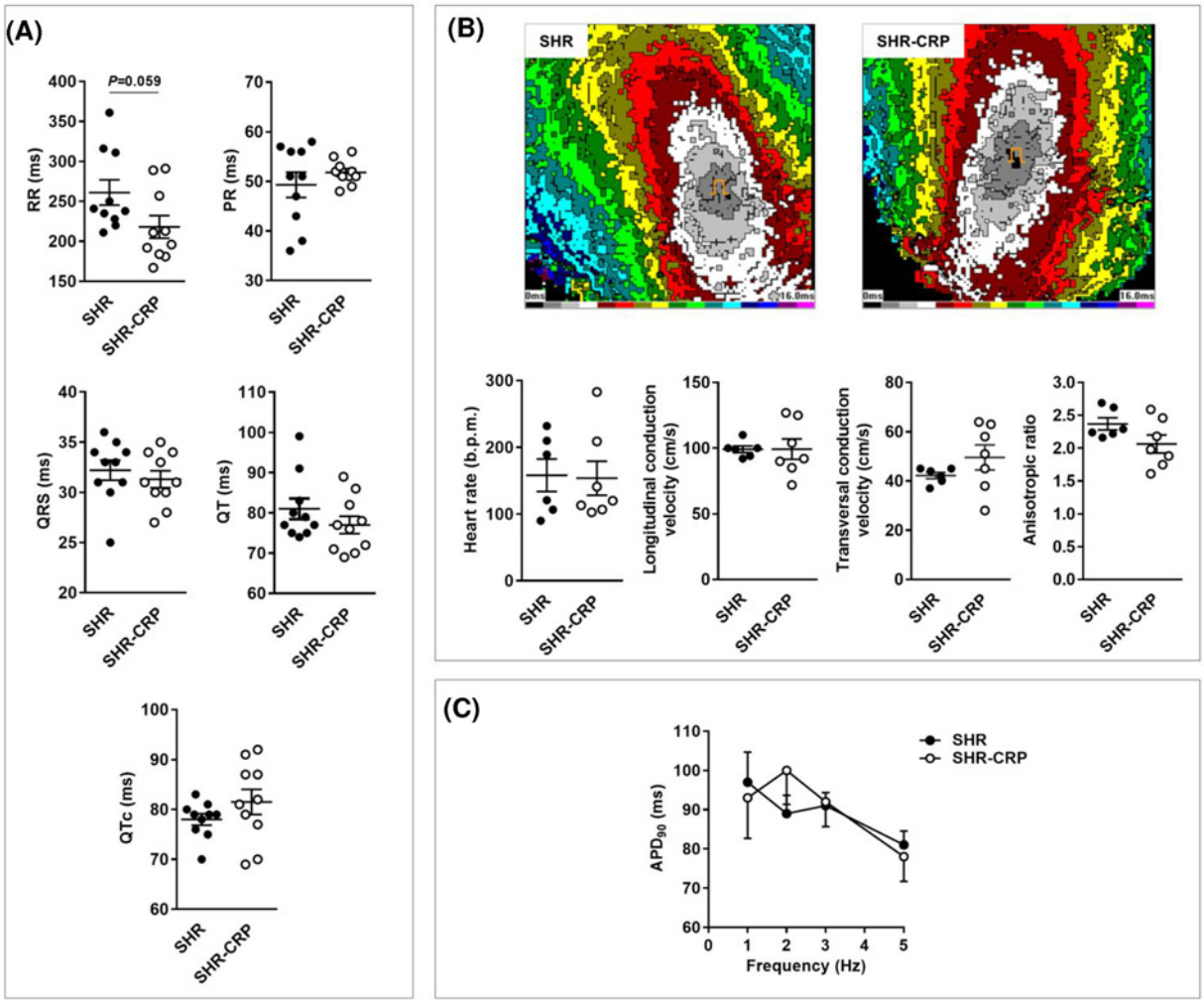

Figure 2. ECG, epicardial CV, and action potential duration analyses

RR and PR intervals, QRS complex, QT and QTc intervals derived from ECG recordings in hearts *in vivo* (**A**). Typical examples of LV epicardial activation maps, heart rate, longitudinal and transversal CV, and anisotropic ratio (longitudinal-to-transversal CV) in hearts *ex vivo* (**B**), and action potential duration at 90% repolarization (APD_{90}) at stimulation frequencies of 1–5 Hz in left ventricular papillary muscles of the control SHR and transgenic SHR-CRP (**C**). b.p.m., beats per minute. Values are mean ± SEM of 6–10 hearts in each group. Data were analyzed by unpaired *t* test.

The myocardial proportion of n-3 polyunsaturated fatty acids (PUFAs) in phospholipids was significantly lower in SHR-CRP than in SHR, while n-6 PUFAs and both monounsaturated and saturated FAs did not differ between the strains. The lower myocardial proportion of n-3 PUFA was reflected in the significant increase in n-6 to n-3 PUFAs ratio by 13% in SHR-CRP (Figure 3A). A detailed FAs analysis revealed that this effect was due to a significantly lower concentration of the docosahexaenoic acid, a predominant n-3 PUFA (Figure 3A; Supplementary Table S3). Further, myocardial abundances of antiarrhythmic epoxyeicosatrienoic acids (EETs) were decreased (for 14,15-EET significantly) in SHR-CRP compared with SHR. Moreover, the levels of EETs metabolites 11,12- and 14,15-dihydroxyeicosatrienoic acid increased by 93 and 117%, respectively, but the differences did not reach statistical significance due to high variability (Figure 3B,C). In plasma, we did not observe any significant changes at the levels of analyzed epoxides (Figure 3B,C). Finally, the level of 6-keto prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), a non-enzymatic hydrolysis product of antiarrhythmic prostaglandin I_2 (PGI_2), significantly decreased by 44% in plasma of SHR-CRP (Figure 3D). On the other hand, the level of thromboxane B_2 (TXB_2), an inactive metabolite of proarrhythmic thromboxane A_2 (TXA_2) produced by platelets, increased by 78% in plasma of SHR-CRP compared with SHR, but the difference did not reach statistical significance. In the LV myocardium, we did not observe any significant changes at

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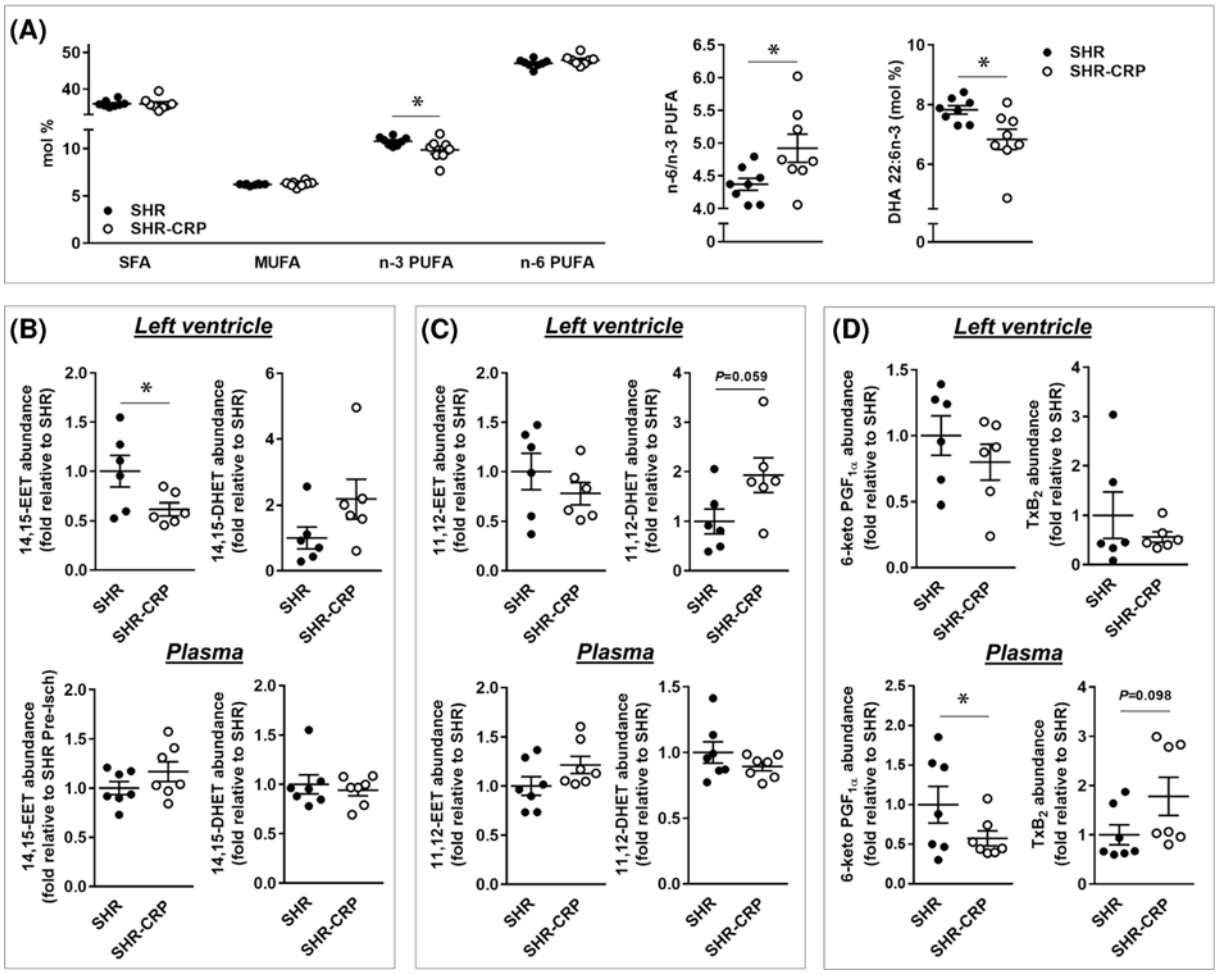

Figure 3. Myocardial FA composition and levels of eicosanoids

The total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), n-3 and n-6 PUFA, n-6/n-3 PUFA ratio, and docosahexaenoic fatty acid (DHA) proportion in the total phospholipids in the left ventricle (**A**). Myocardial and plasma levels of 14,15-epoxyeicostrienoic (EET), 14,15-dihydroxyeicosatetraenoic acids (DHET) (**B**), 11,12-EET and 11,12-DHET (**C**), and 6-keto $PGF_{1\alpha}$ and TXB_2 (**D**) in the control SHR and transgenic SHR-CRP. Values are mean ± SEM of six to eight hearts in each group. *$P<0.05$ vs. SHR by unpaired *t* test.

the level of 6-keto-$PGF_{1\alpha}$ and TXB_2 (Figure 3D). These findings suggest that an altered composition of myocardial and plasma lipids can be responsible, at least in part, for the proarrhythmic phenotype of SHR-CRP.

Autonomic nervous system activity

Autonomic nervous system activity exerts potent effects on the sensitivity of the heart to arrhythmias. Therefore, we determined the total number of muscarinic cholinergic and β-adrenergic binding sites in myocardial membrane preparations using saturation binding with the AChR agonist [^{3}H]QNB and β-AR agonist [^{3}H]CGP12177 (Figure 4A,B). Analysis of saturation binding curves indicated that myocardial membranes prepared from SHR-CRP had a significantly lower number of AChRs than those from SHR. There was no difference in the binding affinity of these receptors (Figure 4A). The levels of β-ARs were similar in both strains, but the dissociation constant (K_D) was significantly lower in SHR-CRP (Figure 4B). Competition binding experiments with the β_2-AR selective antagonist (ICI 118.551) did not show any differences in the proportion of β_1- and β_2-ARs between the strains (Figure 4B).

To evaluate the functional state of the myocardial AC signaling system, we investigated the activity of AC under various experimental conditions. The basal activity of AC and the AC activity stimulated by NaF (a G protein activator) or by forskolin (a direct activator of the catalytic subunit of AC) were comparable in myocardial preparations from both SHR-CRP and SHR. On the other hand, the AC activity stimulated by the β_1-AR agonist dobutamine and

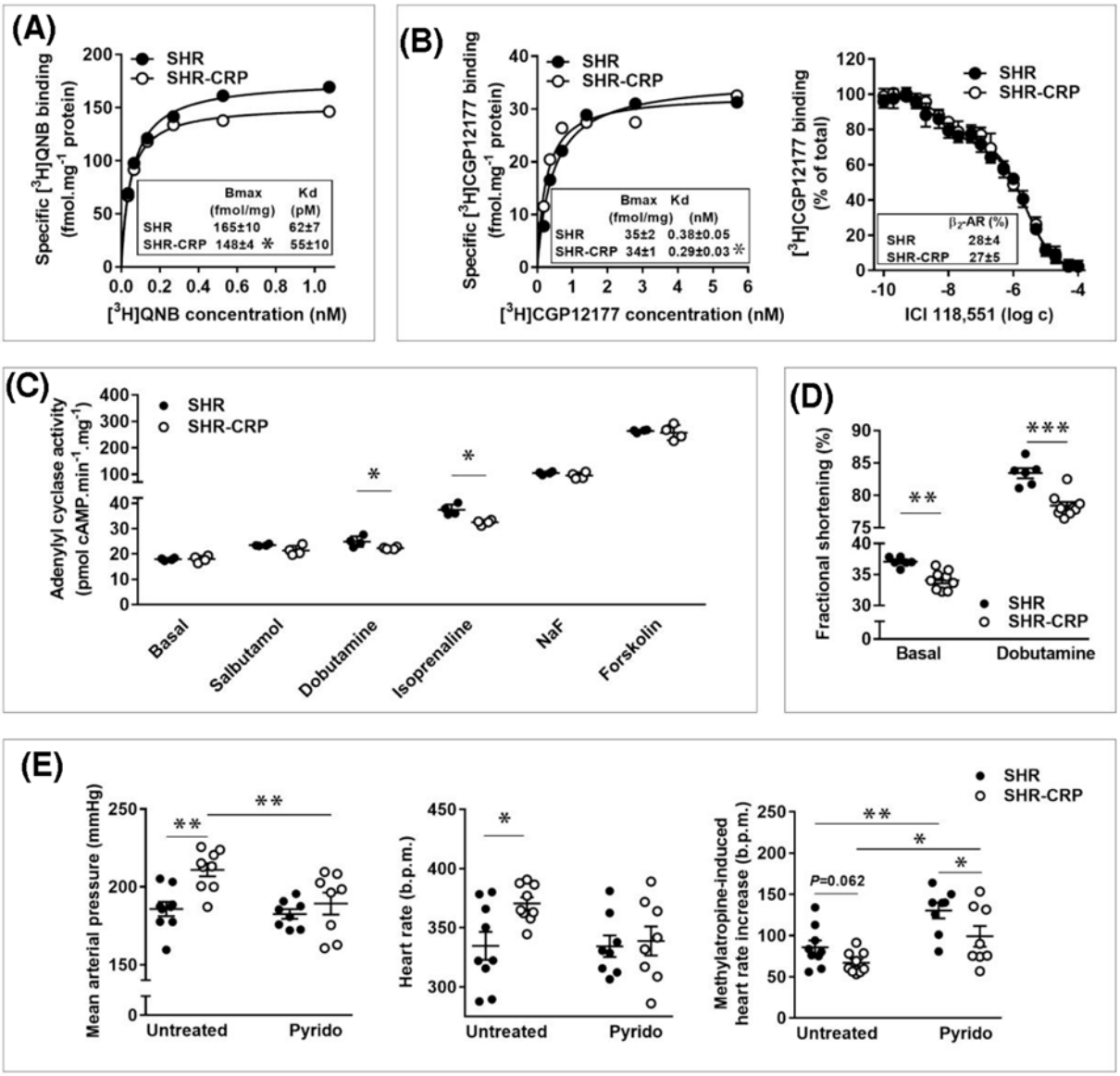

Figure 4. Autonomic nervous system activity

Saturation binding curves of AChRs (**A**) and β-ARs (**B**) constructed by measurement of [^{3}H]QNB and [^{3}H]CGP 12177, respectively, binding to crude myocardial membranes using increasing concentrations of the radioligand. The distribution of β-ARs subtypes in myocardial preparations was assessed by competitive displacement of [^{3}H]CGP 12177 binding by the β_2-AR selective antagonist ICI 118.551 (**B**). AC activity under basal conditions and after stimulation by salbutamol, dobutamine, isoproterenol, NaF, and forskolin in LV of control SHR and transgenic SHR-CRP (**C**). Baseline values of the LV FS and maximal values determined by dobutamine stress echocardiography (**D**). Mean arterial pressure and heart rate in conscious SHR and SHR-CRP treated by pyridostigmine (Pyrido; 40 mg/kg/day in drinking water, 3 days) and the effect of acute administration of methylatropine (2 mg/kg, i.v.) (**E**). b.p.m., beats per minute. Values are shown as mean ± SD of four measurements (competitive study, AC activity) and as mean ± SEM of six to nine rats in each group (FS, mean arterial pressure, heart rate). *P<0.05, **P<0.01, and ***P<0.001 vs. SHR or untreated group by unpaired *t* test (saturation binding curves, AC activity, FS) and by two-way ANOVA with Bonferroni's test (mean arterial pressure, HR).

β_1/β_2-AR agonist isoprenaline was slightly but significantly lower in SHR-CRP than in SHR. The enzyme activity stimulated by the β_2-AR agonist salbutamol did not significantly differ between the groups (Figure 4C).

The response of LV function to β-AR stimulation was examined by dobutamine stress echocardiography in a separate set of rats. Supplementary Table S4 and Figure 4D summarize echocardiographic parameters of LV. In untreated

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rats, the cardiac index did not differ between the strains (32.2 ± 1.8 vs. 32.2 ± 1.0 ml/min/100 g BW). Nevertheless, due to a slight LV dilation, transgenic expression of CRP significantly decreased the LV systolic function expressed as fractional shortening (FS) to 34.0 ± 0.5% compared with 37.1 ± 0.3% in SHR (Figure 4D, Supplementary Table S4). Dobutamine increased maximal FS in both strains, but mild systolic dysfunction persisted in SHR-CRP (Figure 4D). Altogether, these findings suggest that myocardial β-AR signaling did not substantially differ between the strains.

In additional experiments, MAP and HR were analyzed in conscious rats affected by pharmacological modulators of parasympathetic activity. In untreated SHR-CRP, MAP and HR were significantly higher (by 13.6 and 10.7%, respectively) compared with untreated SHR. Acute administration of methylatropine increased HR by 25.6% in SHR and by 18.1% in SHR-CRP (Figure 4E). Three-day treatment with pyridostigmine did not affect SHR but significantly reduced MAP and tended to decrease HR in SHR-CRP to the values comparable with SHR. In both pyridostigmine-treated SHR and SHR-CRP, acute administration of methylatropine significantly increased HR compared with untreated groups; this effect was more pronounced in SHR (38.9%) than in SHR-CRP (29.3%) (Figure 4E). Altogether, these data suggest that transgenic expression of human CRP in SHR resulted in autonomic nervous system imbalance due to suppressed parasympathetic activity.

Relationship among the duration of tachyarrhythmias, blood pressure drop, and IS

As the proarrhythmic phenotype of SHR-CRP hearts *in vivo* was associated with the reduction in myocardial infarction (Figure 1), we supposed that these manifestations of acute I/R injury can be in a close relationship. To test this hypothesis, we performed a retrospective analysis of our previous data [17,35] obtained in SHR under completely identical experimental conditions to expand the results of the present study.

Figure 5A shows the relationship between the duration of ischemic ventricular tachyarrhythmias and IS normalized to AR in both strains. The majority of SHR (25 out of 29 rats) exhibited the duration of tachyarrhythmias lower than 200 s without any apparent influence on IS. However, four SHR (14%) with the duration of tachyarrhythmias exceeding 200 s had lower IS than the rest of SHR. In SHR-CRP, eight out of ten rats had the duration of tachyarrhythmias longer than 200 s that was associated with an IS-limiting effect (Figure 5A).

Severe ischemic arrhythmias can lead to episodes of blood pressure drop resulting in insufficient organ and tissue perfusion. Figure 5B shows the individual values of time when MAP dropped below 40 mmHg due to ischemic ventricular tachyarrhythmias in SHR and SHR-CRP. A total of 40 mmHg was chosen based on the previous animal studies that determined this value of MAP as close to the critical closing pressure for the whole circulation [36,37]. During ischemia, MAP dropped below 40 mmHg in three out of eight SHR and in nine out of ten SHR-CRP (Figure 5B). In these animals (three SHR and nine SHR-CRP), the myocardial infarction was significantly reduced to 47.1 ± 3.5% compared with 75.3 ± 3.5% in those rats that did not exhibit sufficient MAP drop (Figure 5B). The AR normalized to LV did not differ between the groups with or without MAP drop episodes (38.7 ± 3.7 vs. 43.3 ± 2.6%). In short, severe ischemic arrhythmias caused transient blood pressure drops associated with insufficient organ and tissue perfusion, which was accompanied by IS-limiting effect.

Plasma metabolomic analysis

We hypothesized that severe ischemic arrhythmias result in a release of hypoxic metabolites from underperfused organs and tissue to the blood that can reduce myocardial injury at reperfusion. To test this hypothesis, we performed metabolomic analysis of plasma before and at the end of myocardial ischemia in SHR and SHR-CRP (Supplementary Table S5). Of all analyzed plasma metabolites collected before ischemia, 97.1% did not differ between the strains. Acute myocardial ischemia significantly affected (predominantly elevated) 76.0% of plasma metabolites in SHR-CRP and 36.5% in SHR. In ischemic SHR-CRP, levels of most plasma metabolites (66 out of 79) were significantly different from those of ischemic SHR (Supplementary Table S5). Specifically, myocardial ischemia increased plasma levels of compounds associated with tissue hypoxia and metabolites, reflecting the inhibition of oxidative metabolism in the organism. Indeed, lactate and pyruvate levels significantly increased in SHR-CRP (but not in SHR), suggesting organ and tissue hypoxia (Figure 6A). Plasma levels of succinate, which is the Krebs cycle metabolite, significantly increased in both strains, but the effect was more pronounced in SHR-CRP than in SHR (Figure 6A). The level of urea cycle substrate glutamate and its metabolites citrulline significantly increased at the end of ischemia in SHR-CRP only (Figure 6B). The plasma level of orotate, the product of an alternative pathway of glutamate metabolism and substrate for pyrimidines synthesis, increased significantly, more in SHR-CRP than in SHR (Figure 6B). Likewise, urate abundance, the product of purine degradation, significantly increased at the end of ischemia in SHR-CRP but not in SHR (Figure 6C). Finally, the plasma level of acetoacetate [ketone bodies reacting with NADH and hydrogen

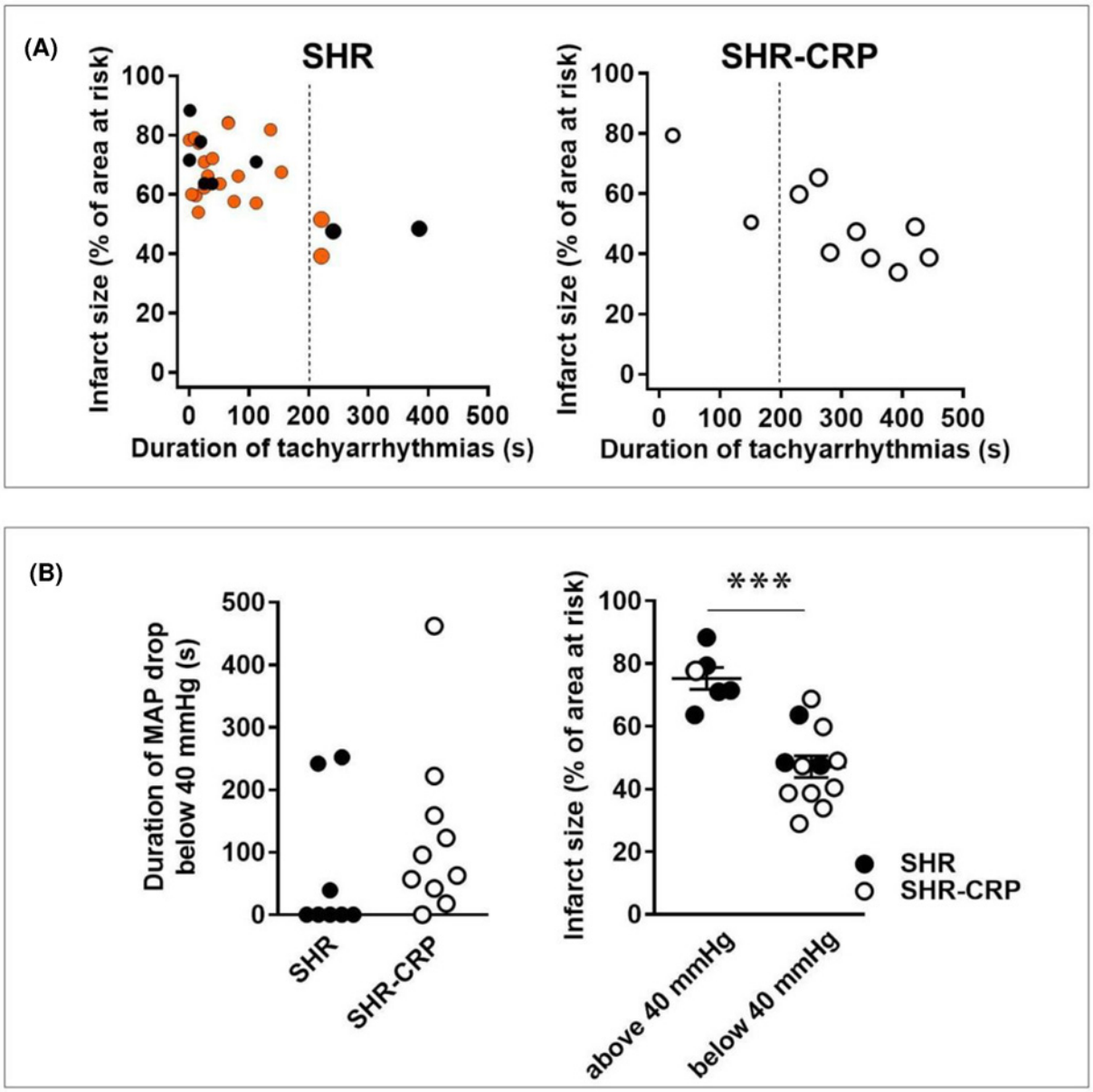

Figure 5. Relationship between duration of tachyarrhythmias and IS

Relationship between IS (normalized to the AR) and the total duration of ischemic ventricular tachyarrhythmias in SHR (n=29) and transgenic SHR-CRP (n=10). Data are from Neckar et al. [17,35; with permissions] (red circles) and the present study (SHR, black circles; SHR-CRP, open circles) obtained under completely identical experimental conditions (**A**). The individual values of time when mean arterial pressure (MAP) dropped below 40 mmHg, and the IS in hearts of SHR and transgenic SHR-CRP with or without MAP drops below 40 mmHg (**B**). Values are shown as mean ± SEM of eight to ten hearts in each group. ***P<0.001 vs. SHR by unpaired t test.

ion and forming β-hydroxybutyrate (BHB)], significantly decreased in plasma at the end of ischemia in SHR-CRP only (Figure 6D). Accordingly, the BHB level significantly increased, and the effect tended to be more pronounced in SHR-CRP than in SHR (Figure 6D).

RIPer

IS-limiting effect triggered by excess ischemic arrhythmias and mediated by a release of various hypoxic metabolites to the blood can imitate the cardioprotective action of RIPer [38,39]. To test this hypothesis, we analyzed myocardial

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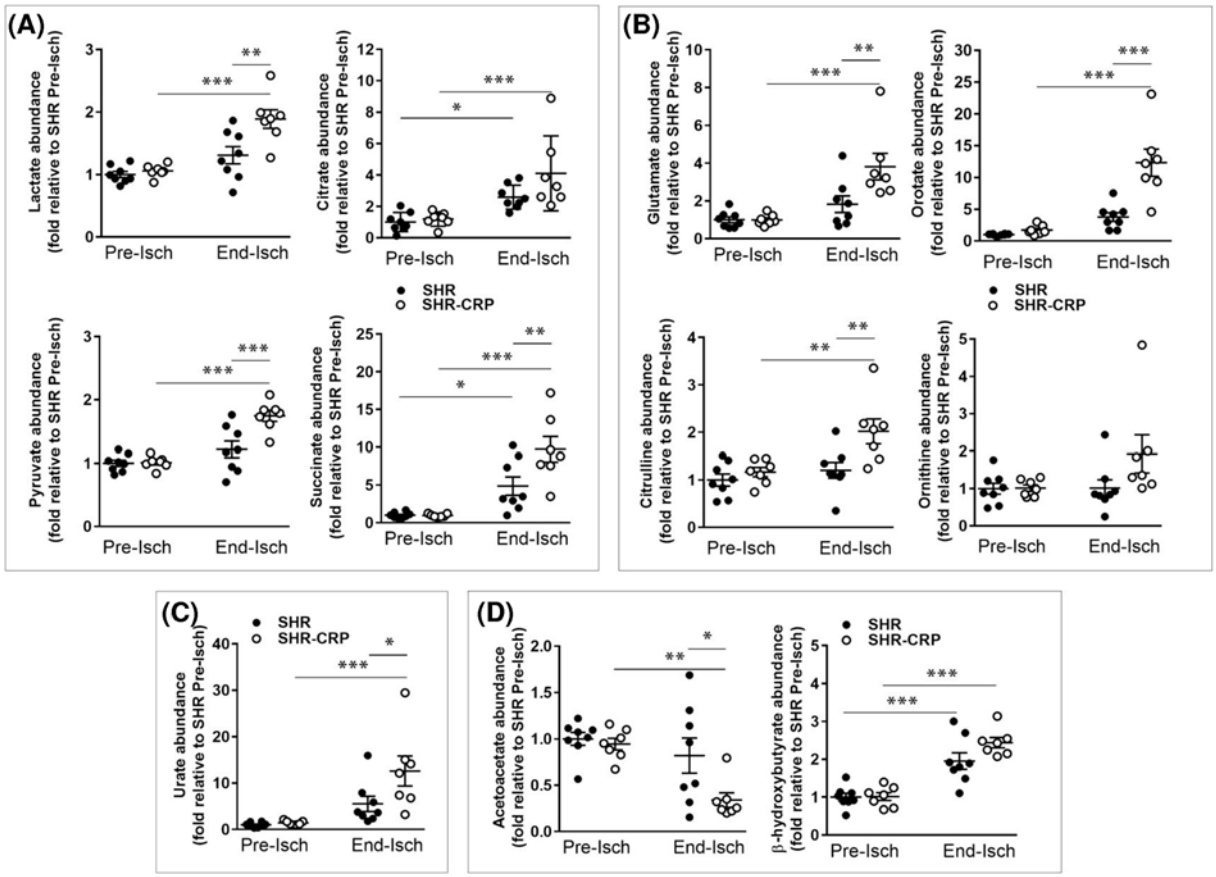

Figure 6. Metabolomic analysis of plasma

Plasma levels of selected metabolites associated with tissue hypoxia and oxidative metabolism (**A**), urea cycle (**B**), catabolism of purine nucleotides (**C**) and formation of ketone bodies (**D**) before (Pre-Isch) and at the end (End-Isch) of 20-min ischemia in the control SHR and transgenic SHR-CRP. Values are mean ± SEM of seven to eight hearts in each group; *$P<0.05$, **$P<0.01$, and ***$P<0.001$ vs. SHR or Pre-Isch group by two-way ANOVA with Bonferroni's test.

ischemic tolerance in SHR subjected to RIPer (i.e., repeated occlusions of both hindlimbs during acute myocardial ischemia).

The incidence and severity of ischemic and reperfusion ventricular arrhythmias did not differ between perconditioned and naïve control SHR. RIPer only tended to reduce the incidence and number of severe ischemic arrhythmias (VT and VF) compared with controls (Figure 7A,B and Supplementary Table S1). The size of AR normalized to LV was similar in both groups (41.9 ± 1.9 and 40.3 ± 5.8%). In perconditioned SHR, the IS normalized to AR was significantly smaller than in controls (46.1 ± 3.9 vs. 66.5 ± 4.9%; Figure 7C). Thus, RIPer resulted in an IS-limiting effect in SHR (without significant changes in I/R arrhythmias), which was comparable with myocardial infarction observed in SHR-CRP.

Ischemic and reperfusion ventricular arrhythmias and myocardial infarction in hearts *ex vivo*

If our hypothesis of the IS-limiting effect caused by metabolites released to plasma due to hypoxia (as a consequence of severe ischemic tachyarrhythmias) is correct, the isolated perfused hearts of SHR-CRP subjected to ischemia will not show smaller IS than the hearts of SHR.

Figure 8A,B and Supplementary Table S1 demonstrate ischemic and reperfusion arrhythmias in SHR and SHR-CRP hearts *ex vivo*. In SHR, the incidence of VT reached 100%, and the incidence of VF reached 12.5%. One of SHR-CRP hearts had sustained VF (excluded from the study) and the incidences of VT and reversible VF were similar to those of SHR (100 and 18.2%, respectively; Figure 8A). The incidence and severity of both ischemic and reperfusion arrhythmias did not differ between the strains in any parameter (Figure 8A,B and Supplementary Table S1).

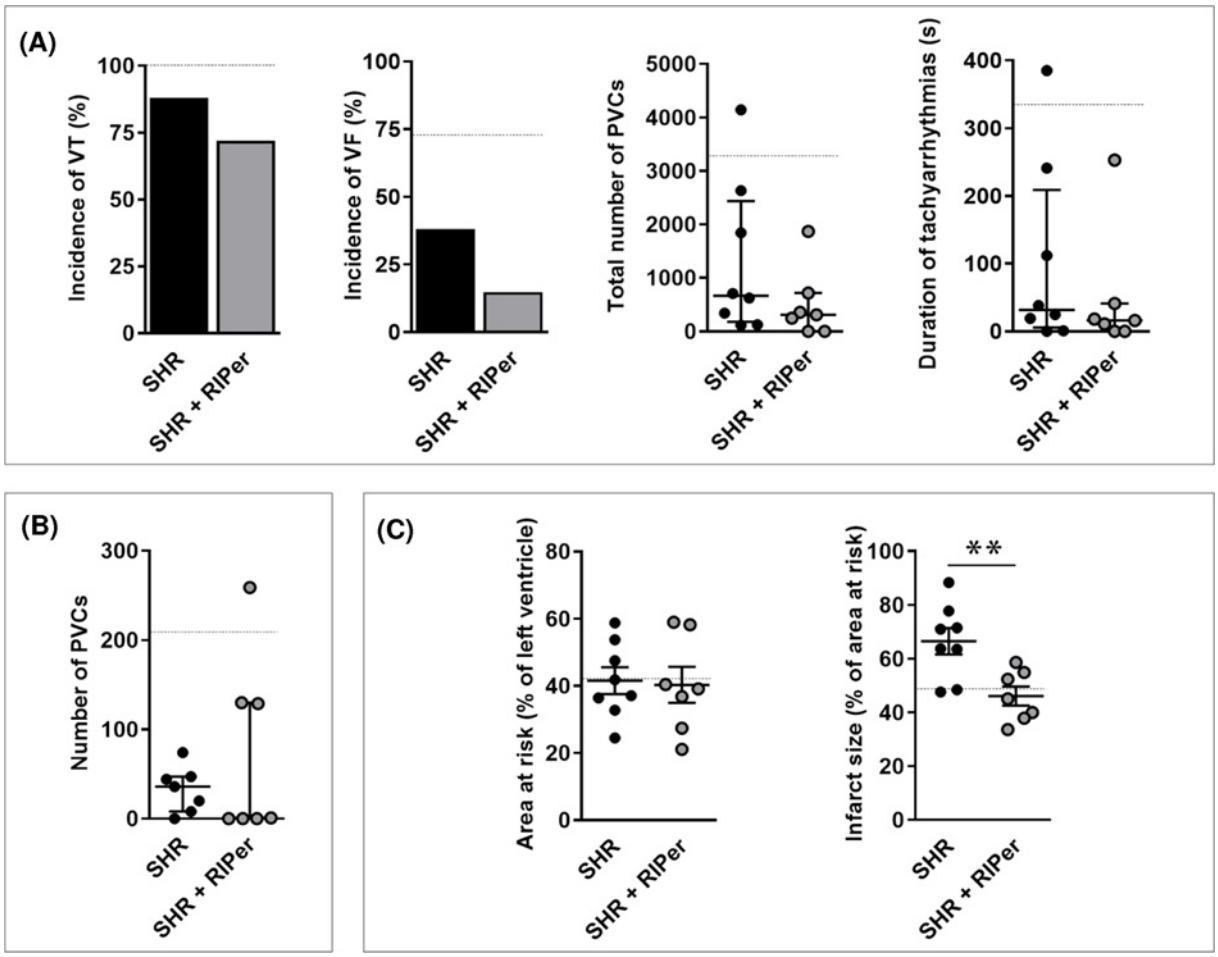

Figure 7. Ischemic and reperfusion ventricular arrhythmias and myocardial infarction in SHR hearts *in vivo* subjected to RIPer

The incidence of VT and VF, the total number of PVCs and duration of tachyarrhythmias (VT + reversible VF) (**A**); the number of reperfusion PVCs (**B**); the relative size of the AR and the IS (**C**) in hearts of control SHR *in vivo* and SHR subjected to RIPer. Data for SHR group were copied from Figure 1. Values are shown as median with interquartile range (number of PVCs and duration of tachyarrhythmias) and as mean ± SEM of seven to eight hearts in each group. Data were analyzed by Mann–Whitney test (number of PVCs and duration of tachyarrhythmias), by Fisher's exact test (incidence of VT and VF) and by unpaired *t* test (relative size of the AR and IS). $^{**}P<0.01$ vs. SHR by unpaired *t* test. Dashed lines represent the respective values of naïve SHR-CRP hearts shown in Figure 1.

In SHR-CRP and SHR, the size of AR normalized to LV was comparable (38.5 ± 2.8 and 43.8 ± 2.8%, respectively; Figure 8C). The IS normalized to AR reached 50.0 ± 4.3% in SHR-CRP and did not significantly differ from that in SHR (43.8 ± 2.5%; Figure 8C). Finally, we did not observe any relationship between the duration of ventricular tachyarrhythmias and IS normalized to AR in either strain subjected to I/R *ex vivo*. The duration of tachyarrhythmias exceeding 200 s (3 out of 18 hearts) did not reduce myocardial infarction (Figure 8D). Therefore, ventricular tachyarrhythmias *per se* were unable to trigger an IS-limiting effect without the response of other organs and tissue.

Discussion

In the present study, transgenic expression of human CRP predisposed SHR-CRP to repeated and prolonged ischemic ventricular tachyarrhythmias. The proarrhythmic phenotype was associated with an altered composition of myocardial and plasma lipids and suppressed parasympathetic activity. Unexpectedly, the excessive ischemic arrhythmias in SHR-CRP hearts *in vivo* were connected with a significant reduction in myocardial infarction. It seems unlikely that the IS-limiting effect in SHR-CRP is a direct consequence of transgenic expression of human CRP. Our data indicate

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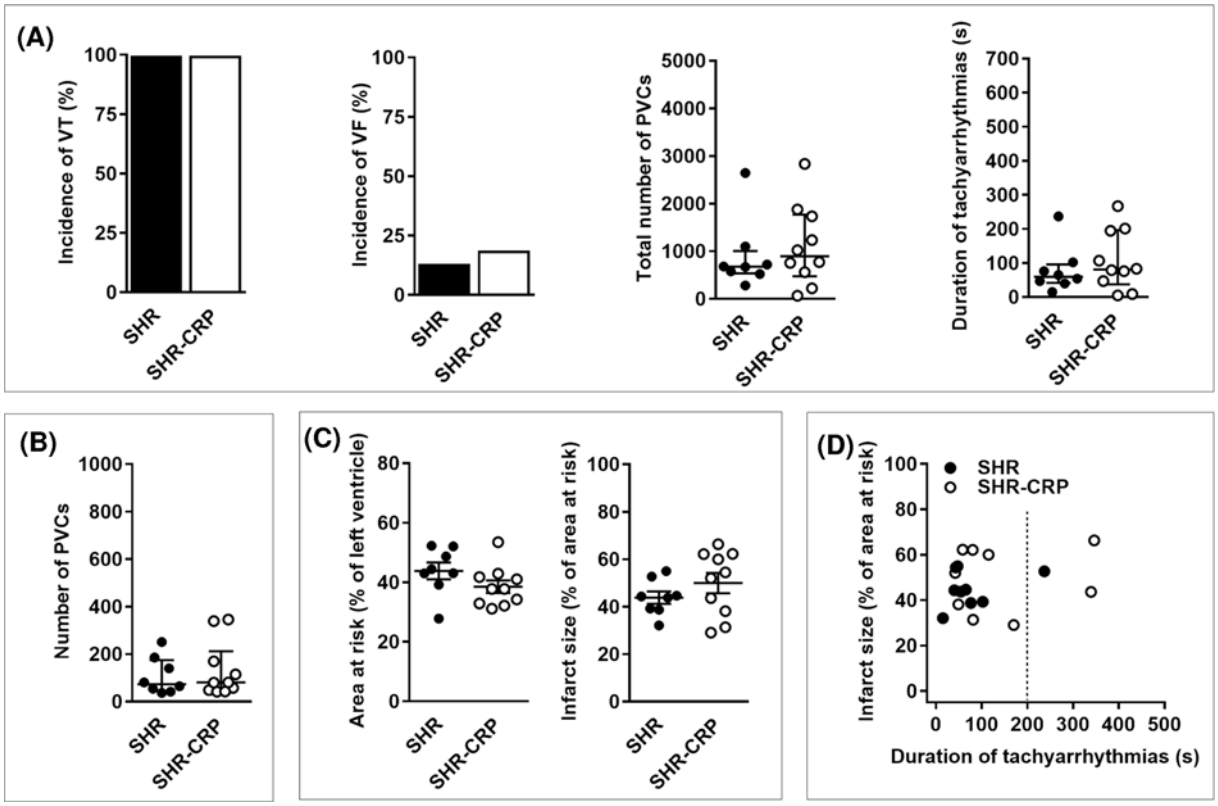

Figure 8. Ischemic and reperfusion ventricular arrhythmias and myocardial infarction in hearts *ex vivo*

The incidence of VT and VF, the total number of PVCs and duration of tachyarrhythmias (VT + reversible VF) (**A**); the number of reperfusion PVCs (**B**); the relative size of the AR and the IS (**C**); the relationship between IS (normalized to the AR) and the total duration of tachyarrhythmias (**D**) in hearts *ex vivo* of the control SHR and transgenic SHR-CRP. Values are shown as median with interquartile range (number of PVCs and duration of tachyarrhythmias) and as mean ± SEM of eight to ten hearts in each group. Data were analyzed by Mann–Whitney test (number of PVCs and duration of tachyarrhythmias), by Fisher's exact test (incidence of VT and VF), and by unpaired *t* test (relative size of the AR and IS).

that insufficient organ and tissue perfusion caused by severe ischemic tachyarrhythmias triggers the release of various hypoxic metabolites in blood, which could reduce myocardial injury at reperfusion.

Proarrhythmic phenotype in SHR-CRP

As electrophysiological experiments did not reveal any abnormalities related to high ischemic and reperfusion arrhythmogenesis in SHR-CRP, we focused on biochemical analyses of selected myocardial and plasma lipids that could be responsible for the proarrhythmic phenotype. In experimental animal models, a long-lasting intake of food enriched with n-3 PUFAs decreases the myocardial phospholipid n-6/n-3 PUFA ratio and diminishes the incidence and severity of ventricular arrhythmias during I/R [40–43]. In patients, higher consumption of n-3 PUFAs reduces the incidence of coronary heart disease and sudden cardiac death [44,45]; the inverse relationship between plasma CRP and n-3 PUFAs levels was reported [46–48]. Our study expands previous research by demonstrating that the excessive I/R arrhythmias in SHR with transgenic overexpression of human CRP is associated with lower content of n-3 PUFAs in myocardial phospholipids.

Myocardial or plasma eicosanoids, biologically active metabolites of arachidonic acid (AA), can modify heart sensitivity to ventricular arrhythmias. It has been shown that increased levels of EETs, epoxides produced from AA, protect hearts against electrical instability in mice subjected to pressure overload or I/R [49–52]. Similarly, pharmacological interventions that increase availability of EETs reduce the incidence of life-threatening VF in hypertensive Ren-2 transgenic rats subjected to I/R [53]. In our SHR-CRP transgenic rats, myocardial, but not plasma, levels of antiarrhythmic EETs decreased, while their less biologically active metabolites dihydroxyeicosatrienoic acids increased

compared with SHR. Thus, we speculate that lower myocardial levels of EETs could contribute to the increased ischemic arrhythmogenesis in SHR-CRP. Concerning EETs in plasma, their role in cardiac sensitivity to acute I/R arrhythmias seems unlikely. Accordingly, no association of plasma EETs with CRP levels in patients with established coronary artery disease was observed [54].

TxA_2 and PGI_2 represent important pro- and antiarrhythmic lipids derived from prostaglandin H_2 [55,56]. Platelet-synthetized TxA_2 has proarrhythmic action and pharmacological inhibition of its synthesis protects the heart against I/R-induced arrhythmias [57,58]. In contrast with TxA_2, acute exogenous administration of PGI_2 reduces the incidence of I/R arrhythmias [59–61]. In this study, lower plasma levels of 6-keto-$PGF_{1\alpha}$ (a non-enzymatic hydrolytic product of antiarrhythmic PGI_2) and higher TXB_2 levels (a stable and inactive metabolite of proarrhythmic TxA_2) were detected in SHR-CRP with the excessive ischemic arrhythmias. It is in-line with the above-mentioned reports and it also corresponds with studies determining TxA_2 and PGI_2 as important metabolites predisposing to ventricular arrhythmias in other experimental settings [62–66]. Concerning the relationship between CRP, TxA_2, and PGI_2, Grad et al. [67] showed that transgenic overexpression of human CRP suppressed PGI_2 synthase and augmented TxA_2 activity through an increase in the expression of TxA_2 receptor in mice. An increased plasma level of CRP was also associated with an elevated plasma level of TxB_2 in smokers with chronic coronary artery disease [68] and with increased urinary TxB_2 excretion in SHR [69]. Therefore, these findings suggest that CRP can modulate TxA_2 and PGI_2 activity and their biosynthesis or degradation and that could modify risk of excees ischemic arrhythmias.

High incidence of ischemic and reperfusion ventricular arrhythmias in SHR-CRP was also associated with an autonomic nervous system imbalance, as suggested by a lower number of AChRs in LV and by decreased HR and MAP after parasympathomimetic treatment. Indeed, enhanced sympathetic or insufficient parasympathetic nervous activity can substantially contribute to the occurrence of severe ventricular arrhythmias under I/R [70] and during heart failure progression [71]. It has been shown that systemic inflammation (measured as a plasma level of CRP) is associated with decreased vagal heart rate control in patients with coronary artery disease [72]. In large animal models, vagal nerve stimulation reduced an elevated plasma CRP level and attenuated heart failure progression [73], suppressed acute ischemic and reperfusion ventricular arrhythmias [74], and diminished an increased post-MI ventricular arrhythmias inducibility [75]. Thus, autonomic nervous system imbalance and inflammation are mutually related [76]. In line with our present findings, it has been shown that increased TxA_2/PGI_2 plasma ratio can limit vagal tone [77] while n-3 PUFAs diet can increase vagal tone [78]. Taken together, our data suggest that altered composition of myocardial and plasma lipids as well as autonomic nervous system imbalance can potentially contribute to the excessive ischemic arrhythmogenesis in SHR-CRP hearts *in vivo*. However, the fact that I/R arrhythmias did not differ between the strains when assessed in hearts *ex vivo* argues for a dominant role of extracardiac factors. Further targetted experiments are needed to unravel the mechanism of increased arrhythmogenesis in SHR-CRP.

IS limitation in SHR-CRP

In contrast with the excessive ischemic arrhythmias, myocardial infarction under *in vivo* conditions was significantly smaller in SHR-CRP compared with SHR. This result is in contradiction to generally considered harmful role of CRP in ischemic heart diseases [1–4]. To the best of our knowledge, no experimental or clinical data suggesting CRP-associated cardioprotective action are available. Therefore, we tried to explain this unexpected finding by including a retrospective analysis of our previous data on ischemic arrhythmias and IS in SHR subjected to I/R *in vivo*. Like SHR-CRP, those few SHR that showed an extreme total duration of ischemic tachyarrhythmias were also protected against myocardial infarction. Therefore, we hypothesized that insufficient organ and tissue perfusion caused by repeated and prolonged ischemic tachyarrhythmias led to hypoxia, resulting in a release of cardioprotective metabolites in blood, which could reduce myocardial injury at reperfusion. The following observations support this view. First, we show that the IS-limiting effect manifested only in those animals that exhibited episodes of repeated and prolonged ventricular tachyarrhythmias resulting in substantial blood pressure drops, which temporally compromised blood supply to tissue. Second, RIPer induced by repeated hindlimbs occlusions during myocardial ischemia reduced the IS in SHR (without significant changes in I/R arrhythmias) to a value comparable with IS observed in naïve SHR-CRP. Third, the I/R insult in *ex vivo* hearts did not reveal any differences in IS between the strains, indicating that the body hypoxic response was required for the manifestation of improved myocardial ischemic tolerance.

In line with this view, our metabolomic analyses showed that acute myocardial infarction significantly affected plasma levels of many metabolites at the end of ischemia in SHR-CRP. The increased levels of some of these metabolites reflect not only organ and tissue hypoxia, but they were also identified as potent cardioprotective molecules in previous studies. For example, pyruvate was revealed as a powerful protectant against acute myocardial I/R injury

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[79]. It has been suggested that the accumulation of succinate may improve ischemic energetics. Moreover, extracellular succinate may serve as a signaling molecule at reperfusion [80], as it promotes hypoxia-inducible factor-1α (HIF-1α) activation by inhibition of HIF-1α-degrading enzymes prolyl hydroxylases [81], thereby mediating cardioprotection [82,83]. Further, high plasma levels of glutamate or orotate at the start of reperfusion can also provide cardioprotection [84–86]. It has also been shown that acute elevation of urate mediates antioxidant defense against peroxynitrite- and tyrosine nitration-induced damage and protects hearts against I/R injury [87,88]. Last but not least, exogenously administered ketone body BHB before reperfusion reduced I/R injury in mice and rats [89,90]. Similarly, 3-day fasting associated with BHB level elevation limited the extent of acute myocardial infarction in rats [91]. Further studies are needed to reveal individual contributions of these metabolites to cardioprotective signaling elicited by excess ischemic arrhythmias.

The fact that our experiments were performed on rats can be considered a certain limitation for the potential translation of the results. Indeed, there are major species differences, in particular between small rodents, large animals and humans, in ventricular ion channel function, susceptibility to arrhythmias and their lethality [92,93]. For example, while VF episodes induced by coronary occlusion usually self-terminate in rats, myocardial ischemia in pigs and humans often leads to sustained VF, which without external defibrillation results in death [93,94]. Malignant ischemic ventricular tachyarrhythmias are a common cause of sudden cardiac death in humans [95]. However, it cannot be excluded that our finding also applies to other species and may have potential clinical relevance in patients with acute MI accompanied by excess ventricular arrhythmias. Elucidation of the underlying mechanism may be beneficial in the search for novel protective strategies against acute myocardial I/R injury.

Conclusion

Our experiments demonstrate a new form of myocardial protection against infarction that is likely mediated by protective substances released from body organs and tissue made hypoxic due to exaggerated ischemic tachyarrhythmias. This form of cardioprotection is initiated by the heart itself without any external intervention (i.e., 'self-conditioning') such as a targeted short occlusion of another organ as was demonstrated for various forms of remote conditioning. It seems unlikely that a decline in coronary perfusion and myocardial hypoxia play a major role in the protective mechanism because we did not observe any reduction of IS in isolated SHR-CRP hearts perfused under *ex vivo* conditions.

This mechanism of IS reduction is most likely not limited to SHR-CRP strain, as the same effect was observed in some progenitor SHR. Moreover, in our ongoing study, excess ischemic tachyarrhythmias were associated with the reduced IS also in transgenic rats with ANG II-dependent malignant hypertension (unpublished). It seems, therefore, that the IS limitation triggered by repeated and prolonged ventricular tachyarrhythmias during ischemia may represent a more general phenomenon. Based on our present data, we strongly encourage performing an analysis of arrhythmias occurring during I/R experiment *in vivo* in addition to IS determination. It can help to avoid misleading interpretations of cardiac ischemic tolerance, in particular in transgenic animal models with various comorbidities.

Clinical perspectives

- The role of human CRP in acute myocardial I/R injury was studied in SHR with transgenic expression of CRP (SHR-CRP) and SHR controls.
- SHR-CRP exhibited exaggerated ischemic tachyarrhythmias that reduced myocardial IS due to a drop of perfusion pressure and subsequent release of protective substances from hypoxic organs and tissues.
- This new form of myocardial protection, i.e. the IS limitation triggered by the heart itself without any external intervention, may represent a more general phenomenon. Elucidation of its mechanism may be beneficial in the search for novel protective strategies against acute myocardial I/R injury.

Data Availability

The authors confirm that the data supporting the findings of the present study are available within the article (and/or) its supplementary materials.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Jan Neckář: Conceptualization, Supervision, Funding acquisition, Validation, Investigation, Methodology, Writing—original draft, Project administration, Writing—review and editing. **Petra Alánová:** Investigation. **Veronika Olejníčková:** Investigation, Methodology. **František Papoušek:** Software, Investigation, Methodology. **Lucie Hejnová:** Investigation. **Jan Šilhavý:** Investigation, Methodology. **Michal Behuliak:** Investigation. **Michal Bencze:** Investigation. **Jaroslav Hrdlička:** Investigation. **Marek Vecka:** Investigation. **Dagmar Jarkovská:** Investigation. **Jitka Švíglerová:** Investigation. **Eliška Mistrová:** Investigation. **Milan Štengl:** Supervision, Investigation. **Jiří Novotný:** Supervision, Investigation, Methodology. **Bohuslav Oštádal:** Conceptualization, Writing—review and editing. **Michal Pravenec:** Conceptualization, Funding acquisition. **František Kolář:** Conceptualization, Supervision, Investigation, Writing—original draft, Writing—review and editing.

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Abbreviations

β-AR, β-adrenergic receptor; 6-keto-$PGF_{1\alpha}$, 6-keto prostaglandin $F_{1\alpha}$; AA, arachidonic acid; AC, adenylyl cyclase; AChR, acetylcholine receptor; AR, area at risk; BHB, β-hydroxybutyrate; CRP, C-reactive protein; CV, conduction velocity; EET, epoxyeicosatrienoic acid; FA, fatty acid; I/R, ischemia/reperfusion; IS, infarct size; PGI_2, prostaglandin I_2; PUFA, polyunsaturated fatty acid; PVC, premature ventricular complex; RIPer, remote ischemic perconditioning; SHR, spontaneously hypertensive rat; SHR-CRP, spontaneously hypertensive rats expressing human CRP transgene; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; VF, ventricular fibrillation; VT, ventricular tachycardia.

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