Cellular electrophysiological mechanism of arrhythmias in athlete's heart

Doctoral (PhD) dissertation thesis

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1. Introduction

Physical activity and competitive sport are two of the most effective strategies for improving fitness, and they are linked to positive improvements in the majority of cardiovascular disease risk factors (including blood lipids, blood pressure, insulin sensitivity, and weight). Moderate aerobic activity has reliably been linked to a lower risk of coronary heart disease (CHD) and death in a significant number of epidemiological studies. Even a small amount of exercise will greatly reduce the risk as compared to a sedentary lifestyle. Compared with a sedentary lifestyle, even a small amount of exercise can significantly reduce the risk. Although the debate about potential in general, the link between vigorous endurance exercise and the increased risk of certain heart diseases (such as atrial fibrillation, ventricular fibrosis) is clearly health-promoting for most people. Exercise, on the other hand, may increase the risk of sudden death in a small number of people with cardiac conditions. Fortunately, sudden death among top athletes is very rare, approximately 1:50,000-1:100,000 per year, occurring 2-4 times more often than in the age-matched control population. It commonly affects young, seemingly healthy athletes and has a significant emotional and social effect on the group. The 3-6 % of the underlying cause of sudden cardiac death events remains unclear. Hypertrophic cardiomyopathy, which causes high electrical instability exists in the athlete's heart as a consequence of the intensive physical training, additionally, several drugs that are in

daily use among athletes may further facilitates arrhythmias. Therefore, great efforts have been made to better understand the causes of sudden cardiac death (SCD) in athletes.

2. Aims of the Thesis

(i) We assumed that the sudden cardiac death of top athletes may be the result of the concurrent existence of trigger mechanisms, such as delayed afterdepolarization and repolarization inhomogeneity, which represent an increased substrate for arrhythmia.
(ii) Furthermore we hypothesized the different ordinary drugs could influence the potassium channels and aggravates the arrhythmia propensity.

3. Material and Methods

3.1. Exercise training protocol

The rats were randomly divided into control (n = 18) and trained groups (n = 18) after the acclimatization. Trained group underwent a 12-week-long swimming training protocol to provoke physiological myocardial hypertrophy as described previously by Radovits et al. It was a 150 liter water container which was divided into 6 lanes with a surface area of 20x25 cm and a depth of 45 cm per lane and filled with tap water maintained at 30-32°C. The dimensions of lanes were selected to avoid reclining to the walls and floating of rats.

Swimming training was performed 5 days/week. For the rats adaptation, the duration of swimming was raised by 15 minutes every second training day from a baseline of 15 minutes on the first day, until obtaining the maximal 200 min/day. Control rats were placed into the water for 5 minutes/day via 12 weeks to reduce the possible differences caused by the stress of water contact.

3.2. Echocardiography

At the completion of the swimming training program, LV morphological alterations in control (n =18) and trained (n = 18) rats were observed by echocardiography as described before, except that rats were anesthetized with isoflurane (5% induction dose, 1–2% maintenance dose). Animals were placed on controlled heating pads, and therefore the core temperature was maintained at 37°C. After shaving the anterior chest, transthoracic echocardiography was performed within the supine position employing a 13 MHz linear transducer (12LRS,GE Healthcare, Horten, Norway), connected to an echocardiography system (Vivid i, GE Healthcare). Standard two-dimensional and M-mode long-and short axis (at mid-papillary level) images were acquired. Recordings were analyzed off-line employing a dedicated software (EchoPac, GE Healthcare). We counted heart rate (HR) on images recorded by M-mode. On two-dimensional recordings of the short-axis at the mid-papillary level, LV end-diastolic (LVEDD) and end-systolic diameter (LVESD) also as LV anterior (AWT) and posterior (PWT) wall thickness in diastole

(index: d) and systole (index: s) were measured. End systole was defined because the time point of minimal LV dimensions, while end-diastole because the time point of maximal dimensions. All values were averaged over three consecutive cycles.

Fractional shortening (FS) was examined from the measurements of LV chamber diameters: FS =[(LVEDDLVESD)/LVEDD] Å~ 100. LV mass was consistent with the subsequent formula: LV mass = [(LVEDD + AWTd + PWTd)3 − LVEDD3] Å~ 1.04 Å~ 0.8 + 0.14. To calculate left ventricular mass index, we normalized the left ventricular mass values to the tibial length (TL) of the animal.

3.3. Morphometric assessment

Standard morphometric measurements were obtained including bodyweight and post-mortem heart weight, also as tibial length. All animals body weight were weighed before termination. After Langendorff isolated heart measurements, the dry heart weights were measured (n = 12/group). Tibias were prepared and length were measured after termination. For morphometric analysis, we were utilizing a conventional analytical balance and a ruler.

3.4. Isolated heart experiments

After the training protocol, 20-week-old male Wistar rats were used (12 control and 12 trained). ECG and left ventricular pressure (LVP) of isolated hearts were measured in Langendorff-perfused hearts as described before. Animals were anaesthetized with Na-pentobarbital

(300 mg/kg,i.p.), and were injected with heparin sodium (300 IU) into the hepatic portal vein. Hearts were rapidly excised, placed via the aorta on a Langendorff apparatus and retrogradely perfused with warm (37°C) modified Krebs–Henseleit bicarbonate buffer (KHB) at a continuing pressure (80 mmHg). The KHB solution contained (in mmol/L):$NaHCO_3$25; KCl 4.3;NaCl 118.5; $MgSO_4$1.2; $KH_2PO_4$1.2; glucose 10; $CaCl_2$1.8, having a pH of 7.4 ± 0.05 when gassed with 95% O_2+ 5% CO_2. The LVP was measured by a water-filled latex balloon which was inserted into the left ventricular cavity and inflated to get an impact state end-diastolic pressure (LVEDP) of 4–8 mmHg. A pump (Masterflex) provided continous exchange of the KHB and the constant column pressure. The electrical activity as electrocardiogram (ECG) detected by the three lead self-made electrodes and signal amplifier (Experimetria, Hungary). The LVP and the ECG were simultaneously recorded using the WinWCP software(V4.9.1. Whole Cell Electrophysiology Analysis Program, John Dempster, University of Strathclyde,UK). Ventricular extrasystoles were generated by hypokalemic (2.7 mM K^+) KHB solution.

3.5. Measurement of ionic currents

Rat ventricular cardiomyocytes were isolated enzymatically from the left ventricular as described in our previous study. The L-type Ca^{2+}current, K^+ currents, Ca^{2+} transient measurements were also described earlier. The estimation of sarcoplasmic reticulum Ca^{2+}content by caffeine method was applied as previously described.

3.6. Determination of phospho-PKA C, phospho-phospholamban and SERCA2 by western blot

The pan and phosphorylated forms of PKAC, phospholamban (PLN) and SERCA2 were measured in myocardial tissue samples taken from the left ventricle (n = 6/group). The fresh LV tissue samples were immediately frozen in liquid nitrogen and stored at -80°C. 30 μg (PKA C, pPKA C), 50 μg (PLN, pPLN) and 20 μg (SERCA2) total protein extracts were fixed using 10% (PKA C, pPKA C), 15% (PLN, pPLN) and 8% (SERCA2) sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. After blocking in 5% milk-TBS-T, the membranes were immunolabeled with the respective primary antibodies supported by the Calcium Ion Regulation Antibody Sampler Kit (Cell Signalling Technology; Danvers, MA,USA; overnight, at 4°C; dilutions: anti-PKA C, antipPKA C (-α, -β, and -γ when phosphorylated at Thr197):1:7000, anti-PLN, anti-pPLN (when phosphorylated at Ser16/Thr17): 1:2500, anti-SERCA2: 1:7000). We used a secondary antibody Horseradish peroxidase which conjugated goat anti-rabbit IgG (Southern Biotech, Birmingham, AL, USA; 1 h, RT; 1:8000). The membranes were developed with an ECL kit (Advansta; SanJose, CA, USA)and exposed to X-ray film. The equivalent protein loading was verified by coomassie blue staining and normalized to total protein. The equivalent protein loading was verified by coomassie blue staining. Use Image J(FIJI; NIH, Bethesda, MD, USA) to evaluate the

integrated optical density values(sum of every band corrected to the background).

3.7. Gene expression analysis by qRT-PCR

All mRNA analyses were applied as described previously. Fresh left ventricular tissue samples (n =6/group) were excised and snap-frozen in liquid nitrogen and stored at -80°C. We homogenized the myocardial samples in a lysis buffer (RLT buffer; Qiagen, Hilden, Germany), total RNA was separated from the tissue using the RNeasy Fibrous Tissue Mini Kit (Qiagen) consistent with the manufacturer's instructions and quantified by measuring optical density at 260 nm.1 μg total RNA was used for reverse transcription [QuantiTect Reverse Transcription Kit (Qiagen)]. Quantitative real-time PCR was performed with the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in triplicates of every sample, in the total volume of 10 μl in each well containing cDNA, TaqMan Universal PCR MasterMix and a TaqMan Gene Expression Assay for the subsequent genes: alpha-1 subunit of a voltage-dependent calcium channel (Cacna1c, assay ID: Rn00709287_m1), alpha-2 and delta subunits of the voltage-dependent Ca^{2+} channel complex (Cacna2d1, Rn01442580_m1), beta-2 subunit of the voltage-dependent Ca^{2+} channel complex (CACNB2, Rn00587789_m1), ryanodine receptor 2 (Ryr2, Rn01470303_m1), calsequestrin 2 (CASQ2, Rn00567508_m1), Na^{+}/Ca^{2+}exchanger (NCX) SLC8A1, Rn04338914_m1), sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2) (Atp2a2,

Rn00568762_m1) and phospholamban (PLN, Rn01434045_m1) purchased from Applied Biosystems. Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; assayID: Rn01775763_g1). We calculated the expression levels using by the CT comparative method (2-ΔCT). All results were expressed as values normalized to the average values of the control group.

4. Results

4.1. Echocardiographic results

Compared with the control group, the resting heart rate (HR) of the trained group was significantly lower. Echocardiography also showed significant myocardial hypertrophy, and the thickness of the anterior and posterior walls of the left ventricle (LV) during systole and diastole and the LV mass index also increased. Left ventricular end-diastole was unchanged and end-systolic size reduction resulted in a considerably higher fractional shortening in trained rats, suggesting increased systolic performance.

4.2. Morphometric results and Langendorff-perfused experiments

(i)The unchanged length of tibia confirmed that the control animals and the trained animals were at the same age. The body weight of sedentary rats was significantly larger, and the physical dimensions of the heart including total weight, body mass index, and ventricles weight and index of the trained rats were significantly increased.

(ii) Langendorff experiment: Compared with the control group, the ECG recordings showed that the long-term R-R variability of the training group increased significantly, while the R-R interval between the two groups remained unchanged. Similarly, there are no differences in QT intervals, while long-term QT variability was decreased in trained rats. Consistent with ECG data, it was found that the left ventricular end-systolic pressure became greater in the trained group. Arrhythmia analysis showed that the extra ventricular beats increased significantly in the trained group. There were no significant differences between the two groups in bigeminy.

(iii) Characteristics of premature beats: In all experiments, a 5-minutes-long section was evaluated. The data analysis involved only single extra beats that were clearly separated, and bigeminy and salvo were excluded. The coupling interval was determined as the time between the initiation of the extra beat and the initiation of the upstroke of the previous steady-state beat. Compared with the control group, the coupling interval were significantly shorter in the trained group. The amplitude ratios of premature beats/steady-state beats were compared at 3 discrete coupling intervals, where we could collect a sufficient amount of data (130, 141, and 149 ms). Since the control group produced only a small number of additional beats during these time intervals, we extended the analysis of the control group to 10 minutes. The ratio of amplitudes was slightly larger in trained animals at 141 ms and 149 ms compared with control.

4.3. Spontaneous Ca^{2+} release measurement

Spontaneous Ca^{2+} release was measured in single cardiomyocytes field-stimulated at 4 Hz for 15 s. Although we observed spontaneous Ca^{2+} release events in both groups, the amount of spontaneous events within the trained group increased significantly.

4.4. Measurements of Ca^{2+} transient, SR Ca^{2+} content and $I_{Ca,L}$

The voltage-current relationship of L-type Ca^{2+} current ($I_{Ca,L}$) within the presence of buffered intracellular solution: 50 ms long depolarization pulse with a holding potential from -80 mV to -40 mV were applied to inactivate the sodium current, and then voltage steps to 30 mV were applied to induce a Ca^{2+} current. $I_{Ca,L}$ density was not different between groups. Rapid application of 10 mM caffeine with a holding potential of -80 mV was used to estimate the SR Ca^{2+} content. Before caffeine is applied, 10 consecutive pulses from -80 to 0 mV were used to reach a steady-state SR Ca^{2+} level. We analyzed the integral of caffeine-induced NCX current as an indicator of SR Ca^{2+} content and we found that SR Ca^{2+} content was significantly increased in the trained group compared to the control group. Ca^{2+} transients were measured at a pacing frequency of 4 Hz to approximate the physiological heart rate of rats. We found that the Ca^{2+} transient amplitudes obtained from the trained group were increased. The half-decay time of the Ca^{2+} transients, measured at 50% of the transient decay, was faster in the trained group.

4.5. Ion channel gene expression level

The expression levels of genes involved in Ca^{2+} handling were analyzed by qRT-PCR. We found that the relative mRNA expression of ryanodine receptor 2 and calsequestrin were significantly higher in the trained group compared to the control. There were no differences in the mRNA levels of NCX, SERCA2, LTCC genes and PLN.

4.6. Phosphorylation of PKA C, PLN and SERCA2 protein expression

The pan and phosphorylated forms of key proteins involved in regulation of Ca^{2+} homeostasis, including PKA C, PLN and SERCA2 protein expression, were compared by using left ventricular biopsies from training and control rats. In the trained animals the phosphorylation of phospholamban oligomers was significantly increased. There were no significant differences in PKA C phosphorylation and SERCA expression between the two groups

4.7. Repolarizing potassium currents: I_{to} and I_{K1}

In the presence of 10 mM EGTA and I_{CaL} inhibition, the possible remodeling-induced I_{to} and I_{K1} density changes were examined. From a holding potential of -80 mV, voltage steps were used up to 60 mV by 300 ms-long to measure Ito. The currents were identical between groups. I_{K1} was measured by using a 300 ms-long depolarizing pulses between -140 and -30 mV from a holding

potential of -80 mV. Similarly to the I_{to}, there was no difference in I_{K1} between the control group and the trained group. Compared with control cells, the average cell size of the cardiomyocytes of the trained animals (estimated from the whole cell capacitance obtained from our patch clamp experiment) was increased significantly.

4.8. The effect of ibuprofen on the rapid delayed rectifier (I_{Kr}) potassium current in rats and dogs

It was mentioned in the Introduction section that different drugs could have marked effect on the repolarization kinetics. It was previously demonstrated that a non-steroidal anti-inflammatory (NSAID) compound, diclofenac, inhibited the I_{Kr}, and lengthened the action potential duration when the repolarization reserve was attenuated. Since NSAID (such as ibuprofen), and other compounds with extracardiac indication are often used by top athletes, it is feasible that they increase the arrhythmogenic propensity during a training-induced electrical remodeling. Since our rat model has markedly different repolarization process than the human, furthermore it did not show difference between control and trained groups, it is unsuitable for the analysis of drug-induced repolarization changes. Therefore, our further experiments were performed on normal dogs that have comparable repolarization process to the human.

At first, the NSAID compound ibuprofen was investigated in 250 μM (51.5μg/ml) concentration in canine ventricular myocytes by using the whole-cell configuration of the patch clamp technique. The

applied concentration of solvent DMSO did not affect the amplitude or kinetics of the measured transmembrane ion current. However, in canine ventricular myocytes, 250 μM ibuprofen significantly reduced the rapid delayed rectifier (I_{Kr}) potassium current.

4.9. Effects of cisapride and terfenadine on IKr current in rabbits

The antihistamine terfenadine, and the gastroprokinetic agent cisapride was also analyzed on the I_{Kr} current, by using rabbit ventricular myocytes and whole-cell patch clamp technique at 37°C. We found that both cisapride and terfenadine significantly reduces the I_{Kr}.

5. Conclusion

(i) Our results obtained from swimming trained rats lead us to the conclusion that sudden cardiac death related to training-induced remodeling could be based on the unfavorable results of Ca^{2+} homeostasis adaptation. The increased Ca^{2+} content of SR provides larger available Ca^{2+} when it is released, which is an adaptive response of Ca-cycling to meet the improved cardiac output demand during exercise. However, the increased Ca^{2+} load of the SR in the trained hearts may also serve as a potential arrhythmia trigger source, leading to spontaneous Ca^{2+} release events. During training, the spontaneous Ca^{2+} releases might be facilitated by increased sympathetic tone or electrolyte disturbances, causing extra Ca^{2+} load.

(ii) Our results obtained from dogs and rabbits indicate that different ordinary drugs could influence the potassium channels, aggravating repolarization inhomogeneity and providing/worsening arrhythmogenic substrate.

Taken together our results, the improved arrhythmogenic susceptibility of the athlete's heart could be attributed to the electrical remodeling of the heart but other factors that are closely coupled to the lifestyle of the competitive athletes – such as excessive drug use - could further aggravate the arrhythmia propensity.

STUDIES RELATED TO THE THESIS

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