Chronic Skeletal Muscle Ischemia in Rats Decreases the Inducibility of Ventricular Tachyarrhythmias after Myocardial Infarction

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Abstract. *Background: Chronic hind-limb ischemia confers cytoprotection after coronary occlusion, but it is unclear whether it ameliorates substrate formation for ventricular tachyarrhythmias (VTs). Materials and Methods: Chronic hind-limb ischemia was generated by femoral artery excision in 50 rats, while 25 animals were sham-operated. Left coronary artery ligation was performed after 3 weeks and infarct size was measured 24 hours thereafter. The inducibility of VTs was assessed by programmed electrical stimulation (PES) 4 weeks post-ligation. A score was assigned, based on protocol stage and tachyarrhythmia duration. Monophasic action potentials (MAP) were recorded prior to and 4 weeks after ligation. Results: The infarct size was smaller (p=0.000079) in the ischemic rats (25.7±2.1%) than in the controls (41.7±2.2%), accompanied by a lower (p=0.029) arrhythmia score (1.05±0.38 versus 2.70±0.68, respectively). The action potential duration (APD) was shorter (p<0.05) in the ischemic rats prior to ligation and remained stable after 4 weeks. Conclusion: Chronic hind limb ischemia limits infarct size and decreases inducible ventricular tachyarrhythmias.*

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Myocardial infarction (MI) remains a significant health-related problem worldwide (1). In the long term, the loss of contractile tissue compromises left ventricular (LV) function, resulting in chronic cardiac failure. In addition, LV dilatation facilitates the formation of re-entrant circuits, mostly at the rim of the infarct scar (2). These regions are the site of origin of sustained ventricular tachyarrhythmias (VTs) that often herald sudden cardiac death (2).

The most important parameter that determines long-term prognosis after acute coronary occlusion is the extent of myocardial necrosis (3). Large infarcts are associated with LV dysfunction, arrhythmogenesis and increased mortality (2, 3). Thus, there have been substantial research efforts towards the advent of treatments (that will act over and above acute phase reperfusion) aiming towards the salvage of ischemic myocardium.

Short ischemic episodes of peripheral tissues, such as the mesenterium, the kidney and the skeletal muscle, have been previously shown to act remotely in the heart, rendering LV myocardium more resistant to prolonged ischemia (4-7). Out of these tissues, skeletal muscles can be accessed and manipulated without major risk in the clinical setting, should this method prove of therapeutic value. Indeed, experimental studies have demonstrated decreased infarct size after coronary artery ligation, when preceded by brief skeletal muscle ischemia (8-12). This concept has attracted considerable scientific interest, for its potential to unravel novel cytoprotective pathways.

The cytoprotective effects of skeletal muscle ischemia may be augmented if such episodes are sustained for long periods of time. In our previous study, four-week skeletal muscle

ischemia in rabbits (13), decreased myocardial necrosis after ischemia/reperfusion; this beneficial effect was secondary to collateral vessel growth, evidenced by an approximately 10% increase in subendocardial and intramyocardial capillaries (13). However, the effects of this intervention on the substrate formation for VTs have not been examined.

In the present study, the hypothesis that chronic skeletal muscle ischemia may decrease infarct size, which, in turn, may lower the susceptibility for VTs in the chronic phase of MI was examined this hypothesis in rats. To shed light on the underlying mechanisms, monophasic action potentials (MAPs) which represent the summation of the action potentials of a small area and provide information on tissue viability and electrophysiology (14), were recorded from the ventricular epicardium.

Materials and Methods

Animal study population. The study cohort consisted of 75 Wistar rats (8-10 weeks of age, weighing 250-350g). The animals were housed 2-3 per cage in our animal facilities, under optimal laboratory conditions (controlled temperature, humidity and 12:12 hour-light: dark cycles) and were given free access to standard rodent chow and water. All the animals received humane care, according to the 'Guiding Principles in the Care and Use of Animals', approved by the Council of the American Physiological Society, and the study protocol complied with national and international legislation (European Union directive for the protection of animals used for scientific purposes, 2010/63/EU).

The experimental animals were randomly assigned into two groups (in a 2:1 allocation ratio), namely limb-ischemic and controls. This unbalanced randomization design minimizes the number of experiments in the control group, without compromising the statistical power (15). The study protocol is graphically depicted in Figure 1.

Generation of hind-limb ischemia. Hind-limb ischemia was generated in 50 rats (280±12g), while 25 rats (285±14g) were sham-operated. Under ether anesthesia, the animals were intubated and mechanically ventilated using a rodent ventilator (model 7025, Ugo Basile, Comerio, Italy). Anesthesia was maintained with a mixture of oxygen and 2% isoflurane. A longitudinal incision was made in the left thigh and the femoral artery (including major branches) was dissected free along its entire length. After ligation of the external iliac, the popliteal and saphenous arteries, the femoral artery was completely excised from its proximal origin until its bifurcation (Figure 2). Antibiotic prophylaxis (cephazolin, 15 mg/kg daily intramuscularly for 4 days), and analgesia (buprenorphine, 0.04 mg/kg daily intramuscularly for 10 days) were administered post-operatively.

Myocardial infarction induction. Three weeks after limb ischemia generation, MI was induced by permanent left coronary artery ligation. The procedure followed in our laboratory has been described previously in detail (16-18). Briefly, using the same anesthesia protocol as described above, a left thoracotomy was performed, the pectoralis muscle groups were dissected and the thoracic cage was opened with a blunt curved forceps. The pericardium was dissected, the heart was exteriorized and the left

coronary artery was encircled 2mm from its origin and ligated with a 6-0 suture. Ligation extended from the pulmonary cone to the left atrial appendage; following these anatomical landmarks reproducibly produces a large anterior MI (16-18). A 6-lead electrocardiogram (ECG) was recorded (QRS-Card digital PC-ECG, PBI Pulse Biomedical Inc., Norristown, PA, USA) after amplification by software (QRS Card Cardiology Suite version 4.05, PBI Pulse Biomedical Inc.); The ST-segment elevation in two or more leads was considered proof of induced MI. The incision was closed in 3 layers and pneumothorax was evacuated, allowing the resumption of spontaneous respiration. The animals regained consciousness within 2-3 minutes after cessation of anesthesia.

Risk area and infarct size measurement. Infarct size was measured 24 hours post-ligation, using a previously described method (19). After anesthesia with ketamine and xylazine, the heart was harvested and mounted on a reperfusion apparatus. The coronary ligature was released and the heart was perfused *via* the aorta with normal saline at room temperature for 2 min. When all residual blood had been removed from the coronary arteries, the normally perfused tissue was delineated from the risk (ischemic) zone as follows: the coronary ligature was retightened at the same site and 5 ml of green fluorescent microspheres (Duke Scientific Corp., Palo Alto, CA, USA), 2-9 μm in diameter, suspended in saline, were infused over 5 min. Subsequently, the hearts were frozen at –20°C for 24 hours and were sliced into 2 mm sections from apex to base. The slices were incubated in 1% triphenyltetrazolium chloride (TTC), which reacts with dehydrogenase enzymes and nicotinamide adenine dinucleotide (NADH) in viable tissue in isotonic phosphate buffer solution (at 37°C, pH 7.4) for 20 min and were immersed in 10% formaldehyde solution for 24 hours. The slices were placed between glass-plates and the risk zone (light brown), the infarcted area (dark brown) and the normal myocardium (brisk red) were identified under ultraviolet light (λ=366 nm). These areas were traced on to an acetate sheet placed over the top glass plate, scanned into Adobe Photoshop 6.0 and measured with a Scion Image software program (Scion Corporation, Frederick, MD, USA). Volumes were calculated by multiplying the corresponding areas by the slice thickness. The volume of infarct area and area-at-risk were expressed in cm^3 and the percent ratio of infarct area/area-at risk (%I/R) was calculated.

Monophasic action potential recordings. MAPs were recorded at baseline, *i.e.*, prior to MI induction and after 4 weeks, *i.e.*, prior to programmed electrical stimulation (PES). Recordings were made from the lateral LV epicardium, while recordings from the right ventricle (RV) served as reference. The method used in our laboratory has been described previously (16,17). In brief, a probe (model 200, EP Technologies, Sunnyvale, CA, USA) was placed on the epicardium, exerting mild, constant pressure to eliminate electrical artefacts. The signal was amplified (preamplifier model 300, EP Technologies) and filtered at 50 Hz using a digital notch-filter (for elimination of power line interference). The signal was further filtered using a band-pass filter, allowing a signal range between 0.05-500 Hz. A continuous data stream was fed into a computer equipped with an analog-to-digital converter (BNC 2110, National Instruments Corporation, Dallas, TX, USA) and two-minute recordings were obtained, as soon as a clear, steady state signal was achieved. This recording duration is deemed optimal, since it provides a sufficient number of analyzable beats, whilst causing minimal interference with the experimental procedure (16, 17).
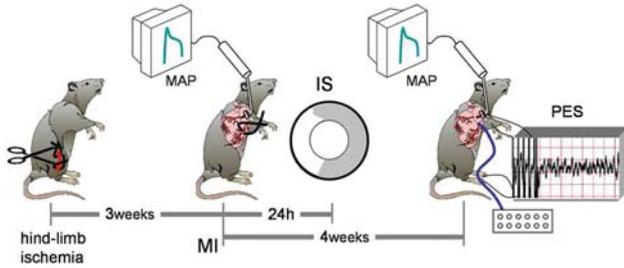
Figure 1. *Study protocol. MAP: Monophasic action potential; MI: myocardial infarction; IS: infarct size; PES: programmed electrical stimulation.*

During chronic MI, special care was taken to obtain MAP recordings from the peri-infarct region, according to prior guides (14, 20) and our previous experience (17,18). In particular, the LV was inspected and the probe was positioned in an area adjacent to pale and akinetic tissue. The probe was gently moved towards the infarcted area, until signals of very low amplitude were encountered. Subsequently, it was moved back and recording was performed at the site where an abrupt change in signal morphology and amplitude re-emerged.

The software utilized in this study, developed and validated at the University of Ioannina (21), permits recording and off-line analysis. For purposes of this study, the action potential duration (APD) was measured at 90% and 75% of repolarization. During analysis, non-sinus beats were excluded and 50 consecutive sinus beats per recording were analyzed.

The heart rate (HR) in beats per minute (bpm) was recorded at baseline and at the chronic stage of MI from MAP recordings.

Programmed electrical stimulation. Four weeks after ligation, the anesthesia protocol and the surgical procedure for heart exposure were repeated. A bipolar epicardial electrode (model 6495 Medtronic Inc., Minneapolis, MN, USA) was sutured to the RV outflow tract and was connected to a programmable electrical stimulator (D330 MultiStim, Digitimer Ltd, Letchworth Garden City, UK). A 3-lead ECG was continuously recorded on-line and saved for subsequent analysis. The PES protocol has been described previously (22). In brief, pacing was performed at twice diastolic threshold at a pulse width of 1 msec; the effective refractory period was determined by a single extra stimulus after 20 paced beats, at a basic cycle length of 100 msec. Induction of VTs was attempted by PES at the same cycle length with double and triple extra-stimuli. The end-point was the induction of sustained VT, defined as >15 consecutive ventricular ectopic beats. As in previous reports (16-18), distinction between various VTs was not attempted, because they can occur interchangeably in the rat heart. A preparation was considered non-inducible, when PES produced either no VTs or self-terminating salvos of <5 beats. A VT was considered non-sustained when it consisted of ≥5 but ≤15 beats, before spontaneous self-termination.

A previously validated (23) arrhythmia score was assigned for each experiment, based on the duration of the induced VT and PES stage; this score ranges from 0 for non-inducible preparations to 6 for sustained VTs induced with one extra-stimulus, a detailed description is shown in Table I.
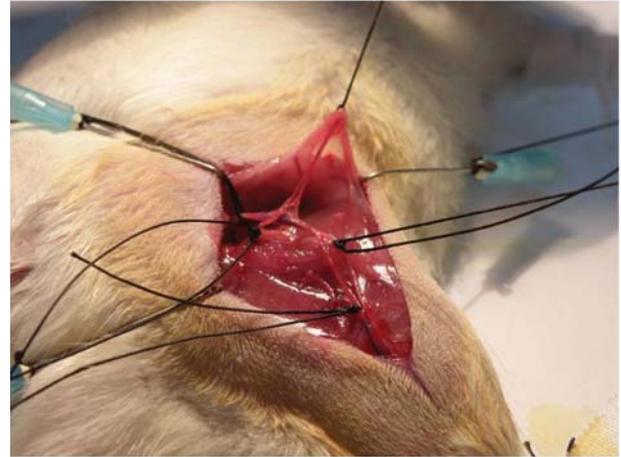

Figure 2. *Femoral artery excision.*

Table I. *Inducible tachyarrhythmia score.*

Score	Description
0	Non-inducible (<5 ventricular ectopic beats)
1	Non-sustained (5≤VT ≤15 beats) with 3 extrastimuli
2	Sustained (VT>15 beats) with 3 extrastimuli
3	Non-sustained (5≤VT ≤15 beats) with 2 extrastimuli
4	Sustained (VT>15 beats) with 2 extrastimuli
5	Non-sustained (5≤VT ≤15 beats) with 1 extrastimulus
6	Sustained (VT>15 beats) with 1 extrastimulus

Statistical analysis. All values are presented as mean±standard error of the mean. Categorical variables were compared with two-tailed Fisher's exact test. Numerical variables were normally distributed (as per Kolmogorov-Smirnov test for normality) and were compared with Student's *t*-test for independent variables, while changes in variables over time were compared with *t*-test for dependent variables. Statistical significance was defined at an alpha level of 0.05.

Results

Two animals died after hind-limb ischemia generation and were excluded from further analysis. Three weeks after the procedure, signs of severe limb ischemia, namely skin necrosis and limb atrophy, were evident in all the remaining animals in the ischemic group. No complications were observed in the sham-operated group.

Mortality. Coronary artery ligation was performed in 48 rats with limb-ischemia and in 25 controls. Three rats (2 limb-ischemic and 1 control) died during surgical manipulations and were excluded. Twenty rats died post-MI, namely 12 limb-ischemic (12/46; 26.0%) and 8 controls (8/24; 33.3%),
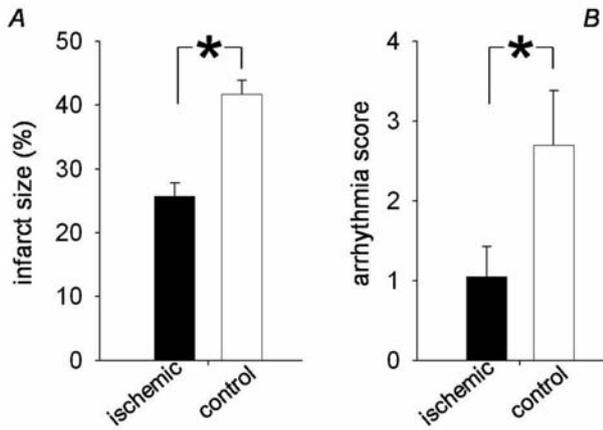
Figure 3. *Infarct size and programmed electrical stimulation. Infarct size (% infract area/ area at risk) (A) and arrhythmia score (as Table I) (B) in limb-ischemic rats (black bars) and controls (white bars). *: significant (p<0.05) differences.*
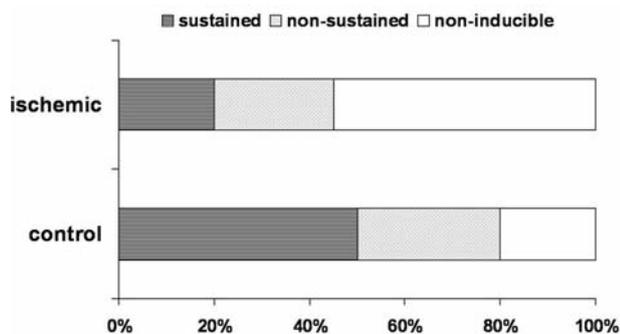

Figure 4. *Induced arrhythmias.*

Figure 5. *Example of programmed electrical stimulation- induced ventricular fibrillation (VF), which organizes into polymorphic and subsequently into monomorphic ventricular tachycardia (VT), (S= Stimulus)*

Figure 6. *Heart rate in limb-ischemic rats (black circles) and controls (white circles).*

but this difference in mortality failed to reach statistical significance (*p*=0.58).

Infarct size. The infarct size was measured in 20 survivors, namely in 14 limb-ischemic rats and 6 controls. Infarct size was smaller (*p*=0.000079) in the limb-ischemic (25.7±2.1%) than in the control animals (41.7±2.2%), as depicted in Figure 3A.

Programmed electrical stimulation. PES was performed in 30 rats, namely in 20 limb-ischemic and 10 controls. The arrhythmia score was lower (*p*=0.029) in the limb-ischemic group (1.05±0.38) than in the controls (2.70±0.68), (Figure 3B). In the control group, sustained VT was induced in 5 (50%) animals, non-sustained VT in 3 (30%), while 2 (20%) were non-inducible. In the limb-ischemic group, sustained VT was induced in 4 (20%) rats, non-sustained VT in 5
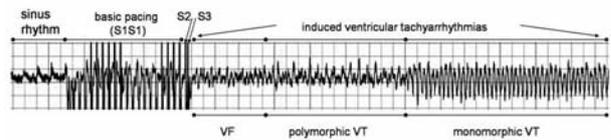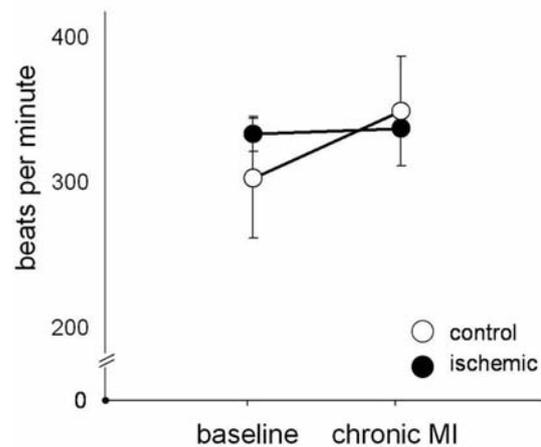
(25%), while no VTs were induced in 11 (55%) (Figure 4). An example of induced sustained VT is given in Figure 5.

Heart rate. HR at baseline and at the chronic stage of MI was comparable, but the trend differed (*p*=0.026) in the two groups. Specifically, HR decreased by a mean of 19±11 bpm in the ischemic group, while it increased by a mean of 54±22 bpm in the controls (Figure 6).

Monophasic action potentials. No difference between the two groups was noted in the RV action potentials, either at baseline, or during chronic MI (data not shown). In contrast, significant differences were found in the LV MAPs. At baseline, the APD at 90% (*p*=0.027) and 75% (*p*=0.030) of repolarization was shorter in the ischemic group (55.0±11.7 msec and 48.0±9.8 msec, respectively) than in the controls (71.5±16.8 msec and 61.5±13.6 msec, respectively). However, these differences were absent at the chronic stage of MI; in the MAPs from the peri-infarct region, the APD at 90% (*p*=0.44) and 75% (*p*=0.33) of repolarization was comparable in the ischemic (62.5±11.5 msec and 53.6±9.6 msec, respectively) and control rats (56.3±12.0 msec and 47.3±8.5 msec, respectively), (Figure 7).
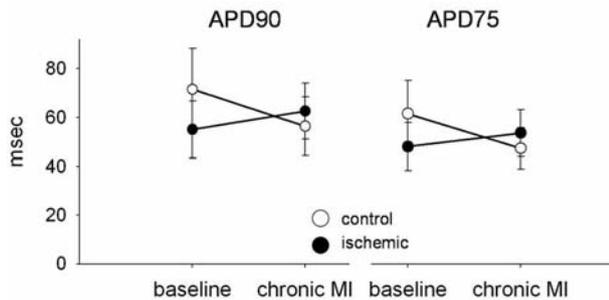
Figure 7. *Action potential duration at 90% and 75% of repolarization in limb-ischemic rats (black circles) and controls (white circles).*

Discussion

This study demonstrated that chronic hind-limb ischemia in the rat decreased the inducibility of VTs in the chronic phase of MI. This beneficial effect appeared to be secondary to limitation of myocardial necrosis, evidenced by a nearly 40% decrease in infarct size.

The present findings were in line with our previous work (13), in which four-week skeletal muscle ischemia in rabbits resulted in smaller infarcts after 30 minutes of regional ischemia, followed by 3 hours of reperfusion. Although neovascularization in the ventricular myocardium is a likely mechanism (13), the downstream pathways responsible are incompletely understood. Previous studies (24, 25) demonstrated that chronic limb-ischemia resulted in local collateral circulation, aiming at preserving blood supply. This collateral vessel growth was characterized by the enlargement of incomplete pre-existing anastomoses towards the large peripheral arteries (26), driven by increased local expression of pro-angiogenic growth factors (27, 28). Therefore, it can be postulated that these stimuli, produced locally in the ischemic skeletal muscle, may circulate and may exert their effects on other arterial beds, such as the coronary circulation.

An interesting finding of the present study, favouring this hypothesis, was the decreased LV action potential duration prior to MI induction, in the rats subjected to chronic hind-limb ischemia. In fact, previous observations have suggested significant electrophysiological actions of growth factors (29); more specifically, vascular endothelial growth factor has been shown to enhance intercellular coupling in the ventricular myocardium (30) and to modify calcium and potassium currents in vascular endothelial cells (29, 31). However, further data are necessary, before any conclusions can be drawn.

Irrespective of the mechanism of myocardial salvage, the decreased susceptibility to VTs observed in the limb-ischemic rats should be attributed to the limitation of myocardial necrosis. In agreement with previous studies in rats (32) and humans (2, 3), the present findings indicated a

relationship between infarct size and VTs. Large myocardial infarcts lead to LV enlargement and fibrosis, thereby enhancing VT substrate formation (33).

While this may contribute to current understanding of post-MI cytoprotection, the main limitation was the lack of measurements of pro-angiogenic growth factors in the LV myocardium.

Conclusion

Decreased myocardial necrosis after coronary ligation is translated into decreased substrate formation for VTs in the chronic stage of MI, confirming the cytoprotective effects of chronic skeletal muscle ischemia on the LV myocardium.

Acknowledgements

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