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Research Paper

Low-flow ischaemia and reperfusion in rat hearts: energetic of stunning and cardioprotection of genistein

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Abstract

Objectives Low-flow ischemia (LFI) is consequent to coronary disease and produces cardiac stunning during reperfusion (R). Energetic performance and mechanisms of Ca^{2+} handling during LFI/R are not known. Moreover, cardioprotection of the phytoestrogen genistein (Gen) remains to be demonstrated in LFI/R. The aim was to study the mechanisms of the stunning consequent to LFI/R and the effects of Gen on both sexes.

Methods Rat ventricles were perfused inside a calorimeter to measure maximal pressure development (P) and total heat rate (Ht) before and during exposition to LFI/R. The mechanisms of stunning were evaluated with selective drugs.

Key findings Female hearts (FH) developed higher postischemic contractile recovery (PICR) and muscle economy (P/Ht) than males (MH). Cardioprotection was sensitive to blockade of mKATP channels, UCam and NOS. Perfusion of 20 μmol/l Gen reduced PICR and P/Ht during LFI/R in FH, and dysfunction was increased by mNCX blockade with mPTP opening. However, intraperitoneal 5 mg/kg Gen (Gen-ip) was cardioprotective in both sexes, and the beneficial effect of Gen-ip was blocked by 100 μmol/l 5-HD.

Conclusions FH are more protected than MH against the LFI/R dysfunction, which involves mitochondrial Ca^{2+} loss; Gen-ip was more cardioprotective in MH than in FH, mainly by activation of the mKATP channels.

Introduction

The clinical situation of cardiac ischaemia is generally triggered by a partial obstruction of coronary arteries which reduces the perfusion flow in certain myocardial region.[1–3] Nevertheless, some of the mechanisms underlying ischaemia-reperfusion were evaluated under experimental models of no-flow ischaemia. So, it is important to understand the mechanisms involved in the stunning consequent to a low-flow ischaemia (LFI) and reperfusion. Stunning was defined as a depressed myocardial function following an ischaemic event, while cardiac hibernation is the consequence of a chronical limitation of the coronary flow which maintains viability but not contractility and is reversible by revascularization.[4] Very few works explain the genesis of cardiac dysfunction induced by LFI. In isolated rat hearts, the reduction of perfusion flow to 1.2 ml/min produced downregulation of the cardiac function, with lower fall in high-energy phosphates and contractile recovery than in a similarly hypoxemic condition at normal flow.[5] Moreover, although LFI did not induce calcium-regulating protein loss in adult and in senescent hearts, it increased RNAs coding for calcium-handling proteins (SERCA2, NCX, RyR2).[6] Energetically, we demonstrated that the heat released during isolated beats in rat hearts was progressively reduced under LFI mainly by the fall in contractility and metabolism.[7] However, this energetical reduction under low-flow was less than under no-flow ischaemia because this one also reduced the energy associated to Ca^{2+} removal.[7] The fall in aerobic metabolism seem to be compensated by the anaerobic, as reported in isolated mouse hearts in which reducing the perfusate flow to 10% increased the anaerobic metabolism and leakage of lactate dehydrogenase as well as depressed contractility with 20–25% during reperfusion.[8] In the same species, reducing perfusion flow to 5% of initial induced a mild sublethal injury without cell death, which was independent on the genetical reduction of hexokinase levels contrarily to no-flow ischaemia.[9] The influence of metabolic substrates was also studied in LFI models induced by coronary

stenosis in instrumented pig. Pigs with metabolic syndrome were more sensitive to the stunning than normal pigs, partially due to abnormal activation of Akt and excessive utilization of fatty acid substrates.[10] Connecting all, the energetic metabolism plays an important role in low-flow stunning, still not completely known.

Premenopausal women exhibit endogenous cardioprotection in comparison with men, which is thought to result from the beneficial effects of estrogen. The risk of coronary dysfunction is generally increased in women after menopausia.[11] The vasomotor symptoms associated to this condition are frequently treated with soy flavonoids.[12] Although these phytoesterols are traditionally used, their health benefits are still under study, as well as their mechanisms as phytoestrogens, epigenetics, and on cellular targets as PKA and TK in several tissues.[13] These compounds could help to reduce the risk of coronary diseases after the endogenous estrogen decline, being genistein (Gen) one of them. It is known that Gen strongly inhibits tyrosin-kinases (TK)[14] and modifies several cellular mechanisms in the heart. Under physiological conditions, Gen inhibits L-type Ca^{2+} current,[15–17] increases the Ca^{2+} content of the sarcoplasmic reticulum (SR), reduces Ca^{2+} efflux by the Na^{+}/Ca^{2+}-exchanger (NCX) and increases myofilaments Ca^{2+} sensitivity.[18,19] We have demonstrated that perfusing Gen before no-flow ischaemia induced different effect depending on sex, being more important the negative inotropism in male rats at low temperature (30 °C), which was sensitive to TK inhibition.[20] The *ex vivo* perfusion of Gen did not show an important cardioprotection against moderate stunning induced by the no-flow ischaemia-reperfusion model in female rat hearts.[20]

Calorimetry is a methodology that allows to evaluate cardiac energetics in a physiological situation of a whole perfused heart, with the advantage of simultaneously having all functions as contractility, Ca^{2+} and other ionic transporters and metabolism, in the presence or absence of perfusion flow of different magnitudes.[7,21] The use of selective pharmacological drugs allows to evaluate the "*in situ*" role of each mechanism, and the ratio between contractility and total heat rate gives an estimation of total muscle economy.[22–24]

Then, the aim of this work was to study the sex differences and the energetical consequences and mitochondrial mechanisms underlying the stunning consequent to a brief episode of LFI and reperfusion. Moreover, this model of preliminar coronary dysfunction was used to evaluate the influence of the phytoestrogen genistein as a putative strategy to prevent the stunning in hearts of both sexes. The *in vivo* administration of genistein to rats before LFI/R was more cardioprotective than cardiac perfusion of it, and the responsible mechanisms were evaluated.

Methods

Ethical approval

This work was done following the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, Eight edition; 2011), the latest directives of the European Union for laboratory animal care (2010), and the Resolution 1047, Annex II of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). Procedures were approved by the Laboratory Animal Care and Research Committee (CICUAL, protocol number 015-5-2015, approved on August 2015) of the School of Exact Sciences, National University of La Plata (UNLP), Argentina.

Animals and procedures

Male and female adult Sprague-Dawley rats (230–280 g weight) were obtained from the Biotery of the School of Exact Sciences. Animals were housed in up to four rats by cage, with water and standard pellet food *ad libitum*, up to the day of the experiment. Some *ex vivo* protocols were done in non-treated rats, while others were done on rats treated with genistein 5 mg/kg intraperitoneally administered 24 h before the *ex vivo* experiment (Gen-ip). Rats were anaesthetised with pentobarbital overdose (60 mg/kg I.P.) and heparinized (2000 IU). After loss of consciousness and reflexes, with muscle relaxation, the thorax was opened and the heart was quickly excised and perfused.

Low-flow ischaemia/reperfusion model in isolated perfused rat ventricles

Hearts were retrogradely perfused by Langerdorff technique with Krebs solution, at a constant flow of 8 ml/min per gram with a peristaltic pump (Gilson Minipuls, Villiers-le-Bel, Paris, France).[20,23,24] As described in previous works, perfusion flow was calculated by the equation $CF = 7.43*HW^{0.56}$ (where CF is coronary flow and HW is the heart weight) to prevent heart edema.[25,26] Atria and any focus of spontaneous beating in the right ventricle were removed. A latex balloon filled with water was introduced in the left ventricle and connected by a cannula to a Bentley DEL900 pressure transducer. While continuously perfused, heart was introduced into the calorimetrical chamber, which was submerged in a water bath at a controlled temperature of 37 ± 0.01 °C. It was electrically stimulated at 2 Hz, 5 V-5 ms, by means of two electrodes connected to an electrical stimulator (Letica LE 12406, Spain). Two signals were continuously recorded by a PowerLab 2/26 amplification system (AD Instruments, Bella Vista, New

South Wales, Australia): the calorimetrical one and the left intraventricular pressure (LVP) at optimal diastolic volume. From the LVP it was calculated the maximal pressure developed during the isovolumetric contraction (P) and the changes in diastolic pressure with respect to the initial condition in Krebs-C (ΔLVEDP), both of which were expressed in mmHg after calibration. Also the maximal rates of contraction (+P) and relaxation (−P) were calculated from the first derivative (dP/dt), as well as the four times of contraction delimited by the derivative recording (tC1, tC2, tR1 and tR2). To compare the postischaemic contractile recovery (PICR), P was also expressed as a percentage of the initial P in Krebs-C.

After stabilization of mechanical and calorimetrical signals, a treatment was perfused (see Protocols) and after a new stabilization the LFI was induced by quickly changing perfusion flow to 1.5 ml/min per gram (mean weight of hearts was 0.746 ± 0.020, $n = 91$), as previously used in some works.[5,7] This low-flow ischaemic condition (LFI) was maintained by 45 min and followed by reperfusion (R) at 6 ml/min with Krebs-C. The period of LFI was chosen to obtain stunning with about 50% of contractile recovery during reperfusion without significant infarct areas.[19]

Calorimetrical measurements

As described in previous works,[19–23] the calorimeter made of a copper mass has an internal chamber containing two ceramic modules with 127 thermosensitive units each one (Melchor Thermoelectrics, Trenton, NJ, USA). They detect differences in temperature between the inside (heart) and the external bath (at constant temperature). The calorimetrical signal (in microvolts) was amplified and digitized with the A/D system, simultaneously with the LVP signal. Total heat rate (Ht expressed as mW/g of wet ventricular weight) was continuously recorded either, in the normal initial flow and in the low-flow perfusion during the LFI/R protocol. Calorimetrical baselines were obtained before and after introducing the heart in the chamber, in both flow values. Calibration of the calorimetrical signal (in mW/mV) was done by applying a constant electrical power (2 mW) on the ventricle at the end of the experiment, for each perfusion flow, as it was previously described.[7,20,21] Results were also expressed as a percentage of the initial Ht after stabilization in Krebs-C. Total muscle economy was calculated as the P/Ht ratio (in mmHg g/mW).

Protocols

After a stabilization period of about 30 min with Krebs-C (C), each one of the following protocols with drugs (at concentrations in which they are relatively selective) was applied in the isolated ventricles during 20 min before LFI. When two drugs were used, the first one was perfused during 5 min before Gen and with it during the following 15 min. Protocols shown in Figure 1 were:

In non-treated rats: (1) Perfusion with C in female and male rats; (2) perfusion with C + 10 μmol/l clonazepam (Clzp, to block the mNCX)[23,27] in female rats; (3) perfusion with C + 100 μmol/l 5-hydroxydecanoate (5HD, to selectively inhibit the mitochondrial mKATP channels)[28,29] in female and male rats; (4) perfusion with C + 1 μmol/l Ru-360 (to selectively block the mitochondrial Ca^{2+}-uniporter, UCam)[30]; (5) perfusion with C + 30 μmol/l L-NAME (to block the NO-synthase); (6) perfusion with C + 20 μmol/l genistein (Gen) in male and in female rats; (7) perfusion with C + 10 μmol/l clonazepam (Clzp) followed by C + Clzp + 20 μmol/l Gen in female rats (to evaluate whether the effects of Gen involve mNCX activation); (8) perfusion with C + 0.2 μmol/l ciclosporine-A (Cys-A, to block the mPTP)[31] followed by C + Cys-A + Clzp + 20 μmol/l Gen in female rats (to evaluate whether the effects of Clzp + Gen involve mPTP activation); (9) perfusion with C + 0.2 μmol/l ciclosporine-A in female rats.

In rats treated with genistein 5 mg/kg vía I.P. in female and male rats 24 h before the *ex vivo* experiment (Gen-ip): (10) perfusion with C; (11) perfusion with C + 100 μmol/l 5HD (to evaluate whether the effects of Gen involve the mKATP activation).

After 45 min of ischaemia the hearts exposed to all the treatments were reperfused during 45 min with Krebs-C, except those pretreated with Cys-A which were reperfused with C + Cys-A, as previously reported.[24,26,31] Figure 2 shows a typical mechano-energetical recording of a control heart exposed to LFI/R (up) and a heart from a Gen-ip treatment (down).

To evaluate whether this model of LFI/R induces myocardial infarction, in some experiments, the reperfused hearts were cut in transverse slices from apex to base. Sections were incubated for 20 min in 1% triphenyltetrazolium chloride (pH 7.4, 37 °C) and immediately scanned. Applying this technique, viable sections were stained red and the infarct area remained unstained. Infarct area was measured and expressed as percentage of the left ventricular area (Image Pro Plus, Rockville, Maryland, USA).

Solutions and drugs

The composition of control Krebs (Krebs-C) to perfuse entire ventricles was as follows (in mmol/l): 1 $MgCl_2$, 125 NaCl, 0.5 NaH_2PO_4, 7 KCl, 25 $NaHCO_3$, 2 $CaCl_2$ and 6 dextrose, bubbled with 95% O_2–5% CO_2. It was similar to that used in previous works.[20,23,24]

Genistein (Gen; Sigma-Aldrich de Argentina, Buenos Aires, Argentina) was prepared at 20 mmol/l in

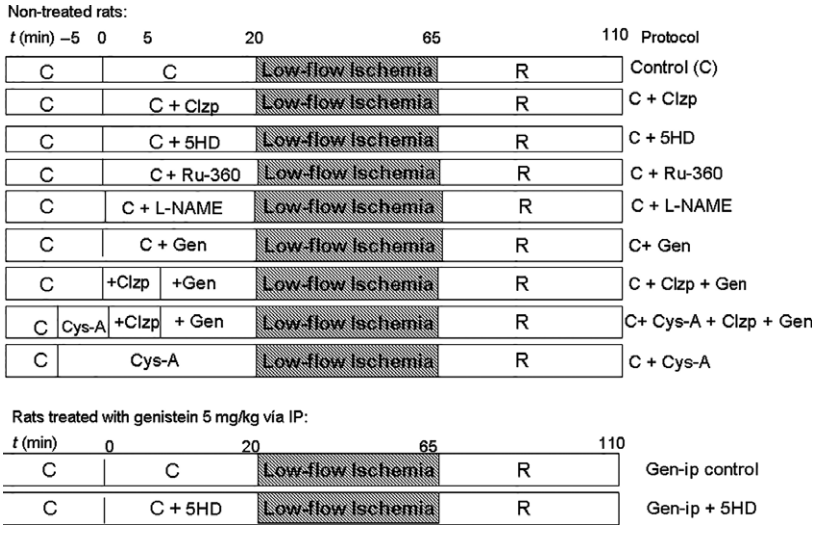

Figure 1 Protocols applied on isolated hearts coming from non-treated or treated with intraperitoneal 5 mg/kg genistein (Gen-ip) rats. Periods assigned to each treatment are indicated in the upper scale in min, considering time 0 when changing to low-flow perfusion (1.5 ml/min per gram; LFI). Stabilization (C) and reperfusion (R) were done with control Krebs. Treatments perfused before and during LFI are in the second box (C: Krebs-C; Gen: 20 μmol/l genistein; Clzp: 10 μmol/l clonazepam; 5HD: 100 μmol/l 5-hydroxydecanoate, Ru360: 1 μmol/l Ru360, Cys-A: 0.2 μmol/l ciclosporine A, L-NAME: 30 μmol/l L-NAME).

dymethylsulfoxide (DMSO) conserved at −20 °C and either, diluted to 20 μmol/l in Krebs-C for perfusing isolated hearts or administered in saline at 5 mg/kg via I.P. in rats. Before diluting (1 : 1000) in Krebs-C for perfusion, clonazepam (Clzp, Saporiti, Buenos Aires, Argentina) was prepared as an aqueous solution at 10 mmol/l, ciclosporine-A (Cys-A, Fluka; Sigma-Aldrich) was prepared at 0.2 mmol/l in DMSO, sodium 5-hydroxydecanoate (5HD; ICN Biomedicals Inc., Santa Ana, CA, USA) was prepared as at 100 mmol/l in DMSO, Ru-360 (μ-oxo- bis trans-formatotetramine ruthenium; Calbiochem, San Diego, California, USA) was prepared in water at 1 mmol/l.[24] All the drugs were diluted in Krebs-C at the moment of the experiment.

Statistical analysis

Results were expressed as media ± SEM for any time in the n samples of each treatment, which were considered normally distributed. Multiple comparisons of treatments vs time were done by two-way ANOVA, evaluating the hypothesis that there is at least one difference among the treatments in each variable (treatment and time). *Post hoc* paired tests were done among treatments (Sidak tests at each time for comparing two conditions, and Tukey tests among the means of effect over time when there were more than two treatments). Always a significance level of $P < 0.05$ was considered. All the statistical analysis was performed by using the Graph Pad Prism v.6.0 software.

Results

Role of sex and mitochondrial Ca^{2+} transporters in the stunning due to LFI and reperfusion

At the initial condition of 8 ml/min per gram of perfusion flow with Krebs-C, isolated hearts from non-treated rats developed a steady contractility (P) of 86.94 ± 6.0 mmHg (n = 60) in female rat hearts (FH) and 77.1 ± 4.6 mmHg (n = 31) in males (MH), and a heat rate (Ht) of 18.0 ± 1.1 mW/g in FH and 17.1 ± 0.7 mW/g in MH. When perfusion flow was reduced to 1.5 ml/min per gram during 45 min, P fell more quickly in MH than in FH (Figure 3a) while Ht fell to similar values of 1.1 ± 0.4 (n = 5) and 2.1 ± 0.5 mW/g (n = 6), respectively. During reperfusion P was partially recovered more in FH than in MH (up to about 72% vs 33%; Figure 3a), Ht recovered to about 80% of initial in both sexes (Figure 3b) and the economy (P/Ht) recovered much more in FH than in MH (Figure 3c). The stunning was accompanied by diastolic dysfunction (increase in LVEDP) during the hypoperfusion in MH but not in FH, while both of them similarly contracted during reperfusion (Figure 3d). There were no significative differences in the relative rates of contraction (+P/P) and relataxion (−P/P) between FH and MH.

To evaluate the role of mitochondrial transporters in the higher cardioprotection of FH, some of them were specifically blocked. The role of mNCX was evaluated by perfusing FH with the selective inhibitor clonazepam (Clzp) at

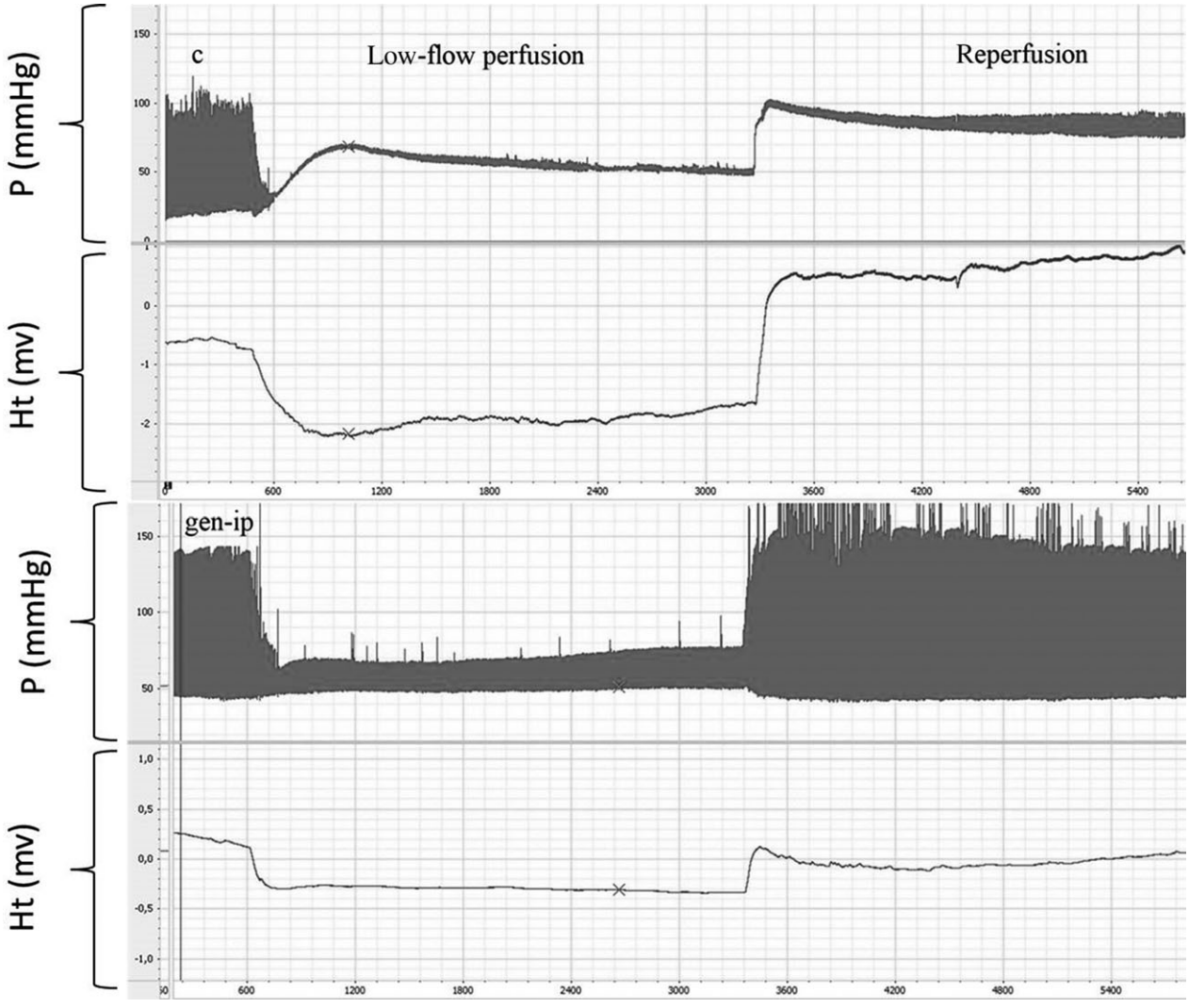

Figure 2 Typical digitized recordings of the left ventricular pressure (upper, in mmHg) and heat rate (lower, in volts, still not converted to Ht) obtained from a control male rat heart exposed to low-flow ischaemia and reperfusion (upper) and from a male rat heart treated with Gen-ip (lower).

10 μmol/l before and during the exposition to LFI, and then reperfusing with Krebs-C. Clzp contributed to significantly reduce P (Figure 4a) and total muscle economy (P/Ht; Figure 4b) during the hypoperfusion, but during reperfusion it did not affect their recovery in comparison with the control FH group. Clzp neither significantly changed the diastolic contracture of FH during the whole LFI/R cycle (Figure 4c). The role of the Ca^{2+} uniporter (UCam) during LFI/R in FH was assessed by blocking it with 1 μmol/l Ru360 before and during LFI. Figure 4 shows that Ru360 reduced P and P/Ht during the LFI and R of FH, and increased the diastolic contracture during the hypoperfusion.

To evaluate the role of nitric oxide (NO) production in FH, 1 μmol/l L-NAME was perfused in FH before LFI. Figure 4 also shows that L-NAME reduced P and P/Ht during both, LFI and R, and increased the diastolic contracture during R.

Ex vivo effects of genistein in hearts exposed to LFI

Considering that Gen is a phytoestrogen, its effects were separately assessed in FH and MH. Perfusion of 20 μmol/l Gen induced a small negative inotropism before LFI in both FH and MH. Then, during the LFI/R cycle Gen significantly reduced the PICR in FH (Figure 5a) but not in MH (Figure 6a). Similar behaviour had the total muscle economy (P/Ht) which was strongly reduced only in FH

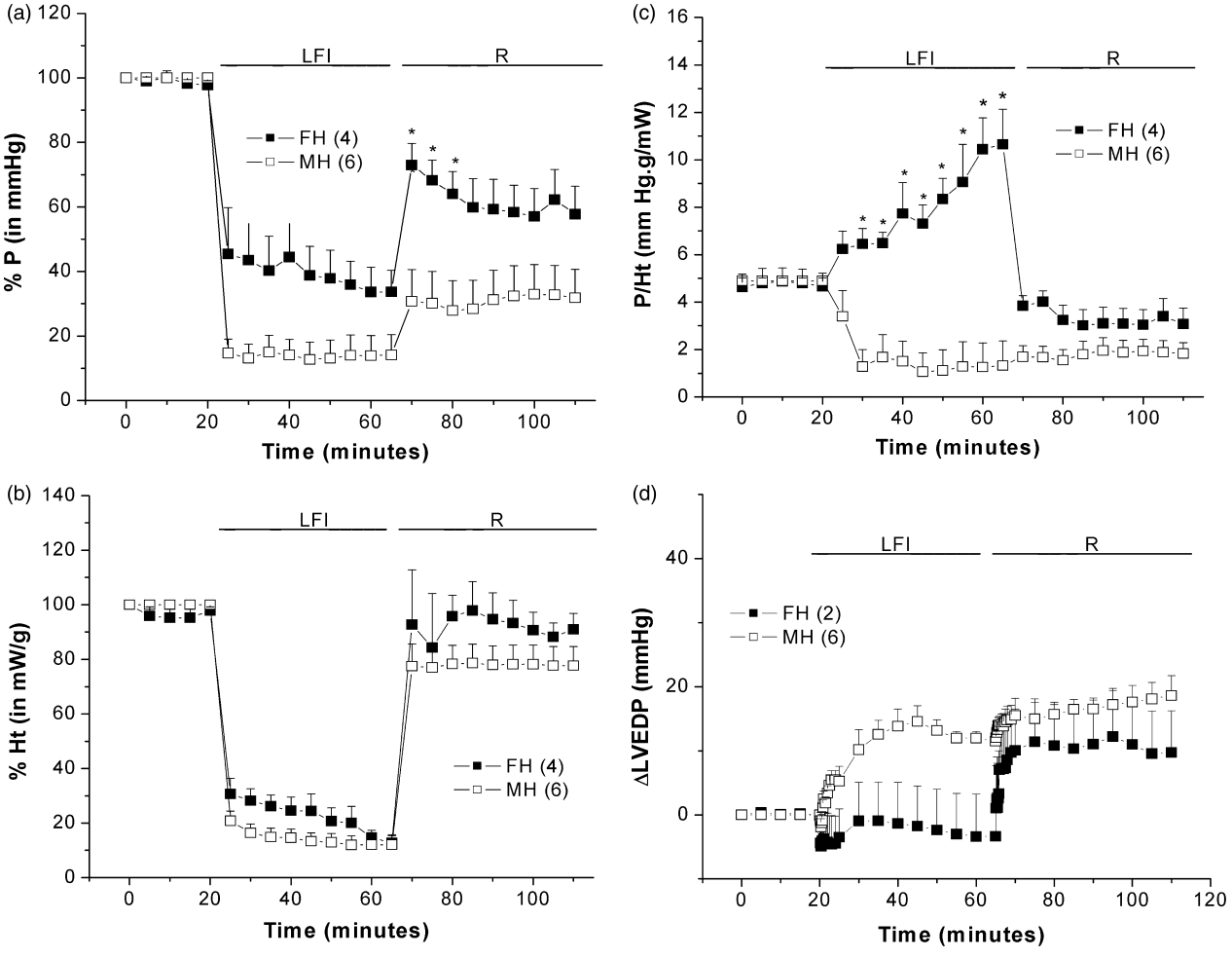

Figure 3 Differences in the responses of female (FH) and male (MH) rat hearts to a period of 45 min low-flow ischaemia (LFI) followed by reperfusion (R). (a): maximal pressure development (P, as % of initial value). (b): total heat rate (Ht, as % of initial value). (c): total muscle economy (P/Ht in mmHg g/mW). (d): changes of left ventricular end diastolic pressure (ΔLVEDP in mmHg) over initial value. Results are shown as mean ± SEM (number of experiments). Two-way ANOVA by treatment $F = 97.16$, 197.9 and 88.29 ($P < 0.0001$), respectively, for (a), (c) and (d), $F = 3.24$ for (b) (NS); by time: $F = 27.70$, 84.59, 5.25 and 4.73 (all $P < 0.0001$), respectively, for (a–d). *Post hoc* tests $^*P < 0.05$.

(Figure 5b). Nevertheless, Gen increased the diastolic contracture during the LFI/R, in both FH (Figure 5c) and MH (Figure 6c).

To understand whether the effects of Gen on LVEDP and PICR of FH could involve the mitochondrial Ca^{2+} extrusion through the mNCX, FH were perfused with 10 μmol/l Clzp before and during the perfusion of 20 μmol/l Gen and exposed to LFI. Figure 5a shows that perfusion with Clzp + Gen strongly reduced P during the whole LFI/R cycle in comparison with the FH perfused only with Gen or Clzp. Nevertheless, since the Clzp + Gen group did not change the recovery of Ht, it reduced the total muscle economy (P/Ht) during all the LFI/R (Figure 5b). This loss of economy could be associated to the increase in diastolic contracture induced by Clzp over that of Gen especially during the end of R (Figure 5c). Such contractile dysfunction suggested a mitochondrial damage. Then, to evaluate whether the mitochondrial permeability transition pore (mPTP) was responsible of it, 0.2 μmol/l ciclosporine-A (Cys-A) was perfused during the treatment with Clzp and Gen including the LFI, and remaining alone during R since it is known that mPTP is opened during it. Figure 5a and 5c show that the mechanical dysfunction in FH exposed to Clzp + Gen was attenuated by Cys-A, since PICR was increased and ΔLVEDP was reduced especially at the end of R, while muscle economy was improved (Figure 5b). The group of FH treated only with Cys-A was similar to control FH in P, P/Ht and ΔLVEDP, which shows that mPTP was not opened during LFI/R. These results allow to conclude that simultaneous perfusion of Clzp and Gen evoked a dysfunction during LFI associated to the mPTP opening.

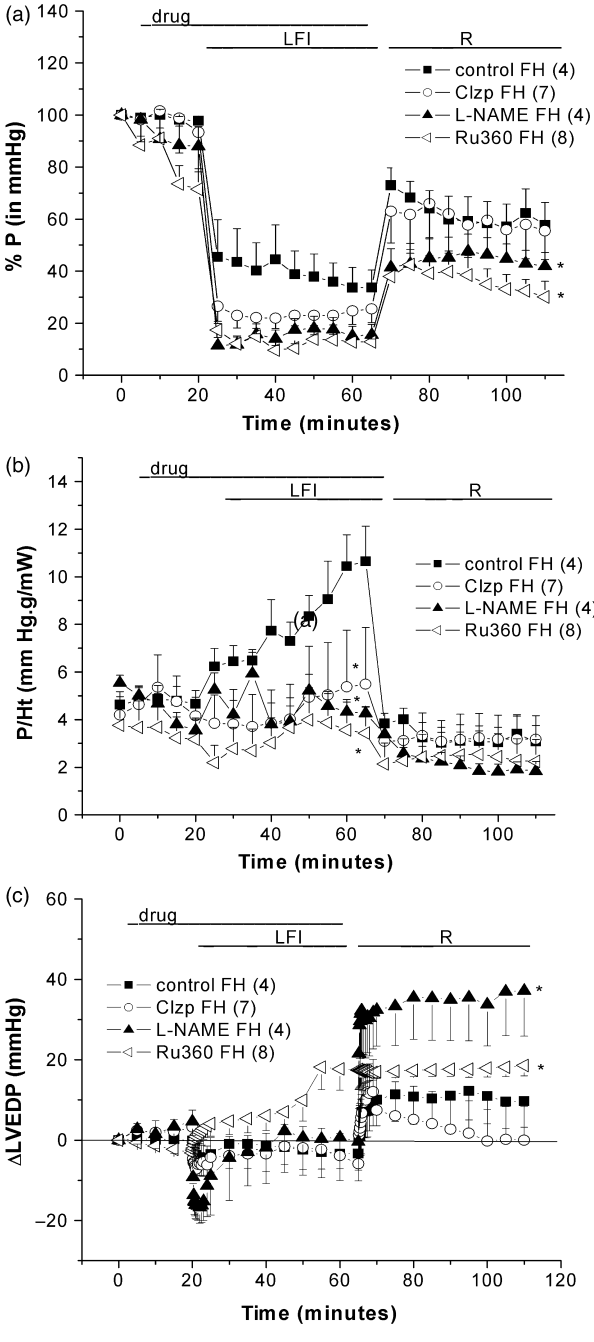

Figure 4 Participation of mitochondrial Ca^{2+} transporters in the stunning associated to low-flow ischaemia (LFI) and reperfusion (R) in female rat hearts (FH). Effects of 10 μmol/l clonazepam (Clzp), 100 μmol/l 5-hydroxydecanoate (5HD), 1 μmol/l Ru360 (Ru360) and 30 μmol/l L-NAME (L-NAME). (a): maximal pressure development (P, as % of initial value). (b): total muscle economy (P/Ht in mmHg g/mW). (c): changes of left ventricular end diastolic pressure (ΔLVEDP in mmHg) over initial value. Results are shown as mean ± SEM (number of experiments). Two-way ANOVA by treatment $F = 31.96$, 16.89 and 44.54 (all $P < 0.0001$), respectively, for (a–c); by time: $F = 42.76$, 3.52 and 11.96 (all $P < 0.0001$), respectively, for (a–c). *Post hoc* tests for the whole condition: $^*P < 0.05$ vs control.

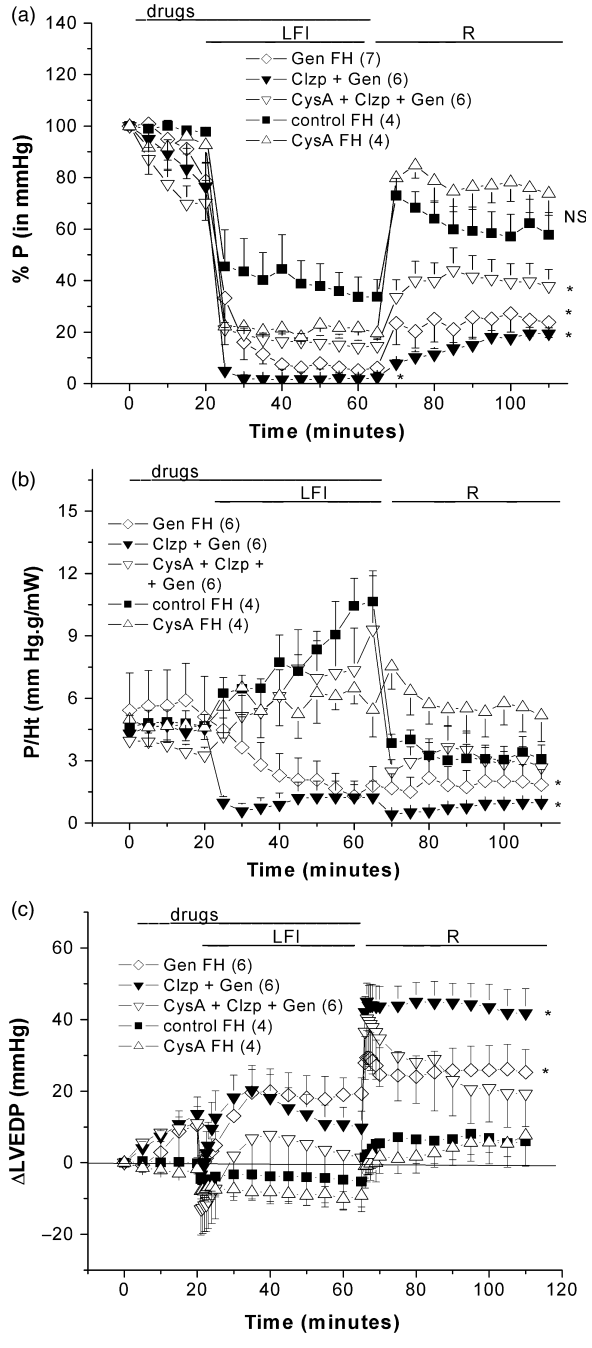

Figure 5 Effects of perfusing 20 μmol/l genistein (Gen) before and during the exposition to low-flow ischaemia (LFI) in female rat hearts (FH), and consequences of the mNCX inhibition with 10 μmol/l clonazepam (Clzp) and of mPTP inhibition with 0.2 mg/ml ciclosporine-A (Cys-A). (a): maximal pressure development (P; as % of initial value). (b): total muscle economy (P/Ht in mmHg g/mW) and (c): changes of left ventricular end diastolic pressure (ΔLVEDP in mmHg) over initial value. Results are shown as mean ± SEM (number of experiments). Two-way ANOVA by treatment $F = 114.7$, 64.32 and 76.70, respectively, for (a–c) (all $P < 0.001$); by time: $F = 86.68$, 4.182 and 10.42, respectively, for (a–c) (all $P < 0.0001$). *Post hoc* tests $^*P < 0.05$ vs others, as described in the text.

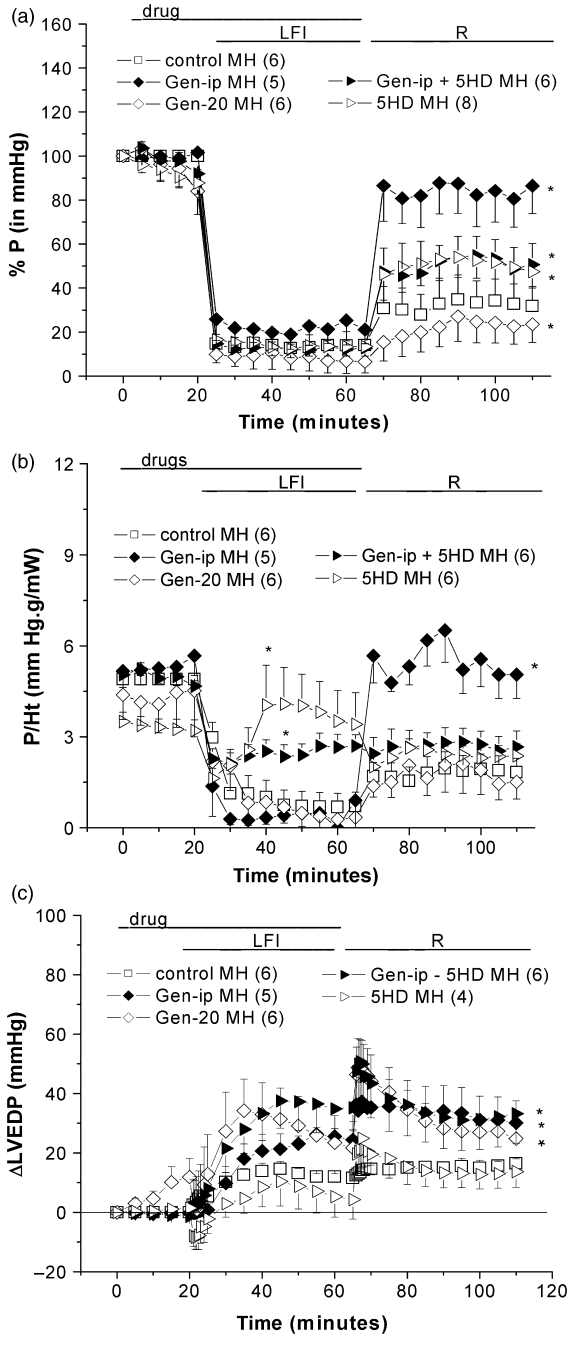

Figure 6 Effects of perfusing 20 μmol/l genistein (Gen-20) or administering Gen 5 mg/kg (Gen-ip) before the exposition to low-flow ischaemia (LFI) in male rat hearts (MH), and consequences of the mKATP inhibition with 100 μmol/l 5-hydroxydecanoate (5HD) on: (a) maximal pressure development (P, as % of initial value), (b) total muscle economy (P/Ht in mmHg g/mW) and (c) changes of left ventricular end diastolic pressure (ΔLVEDP in mmHg) over initial value. The group treated only with Gen was added for comparison. Results are shown as mean ± SEM (number of experiments). Two-way ANOVA by treatment: $F = 44.6$, 23.06 and 55.05 (all $P < 0.0001$), respectively, for (a–c); by time: $F = 86.99$, 16.98 and 17.56, respectively, for (a–c) (all $P < 0.0001$). *Post hoc* tests: *$P < 0.05$ vs control, as described in the text.

In vivo effects of genistein on LFI on both sexes

When Gen was intraperitoneally injected at 5 mg/kg 24 h before the experiment of LFI/R (Gen-ip) the PICR was improved more in males (Figure 6a) than in females (Figure 7a) comparing to the respective control group. The energetic recovery was not significantly changed by Gen-ip, and muscle economy (P/Ht) increased during R in both MH (Figure 6b) and FH (Figure 7b), as well as ΔLVEDP (Figures 6c and 7c) in comparison to the respective control groups. Moreover, in MH but not in FH, Gen-ip reduced the relative rates of contraction (+P/P) and relaxation (−P/P) and accordingly prolonged the times of contraction (tc1 and tc2) and relaxation (tR1 and tR2; Table 1).

In order to evaluate whether the cardioprotection induced by Gen-ip in the LFI/R was due to the activation of mKATP channels, 100 μmol/l 5HD was perfused in hearts isolated from rats of both sexes treated with Gen-ip. Results showed that 5HD decreased the PICR induced by 5 mg/kg Gen in both, MH (Figure 6a) and FH (Figure 7b). Hearts maintained the high energetical output, by which 5HD reduced P/Ht during R in MH (Figure 6b) and in FH (Figure 7b). The reduction in economy may be associated to the further increase in diastolic contracture induced by 5HD in hearts treated with Gen-ip during the whole LFI/R cycle in both sexes (Figures 6c and 7c). As a control, perfusion of 5HD in non-treated hearts before LFI/R did not significantly change the postischaemic P, P/Ht or ΔLVEDP comparing to the respective control of MH (Figure 6) but in FH it strongly reduced PICR and muscle economy (Figure 7). Results suggest that the beneficial effects of Gen-ip during LFI/R and the female cardioprotection are related to activation of mKATP channels.

The model of LFI/R did not suffer significant infarct, since it represented a 7.2 ± 1.1% ($n = 6$) of total area in non treated hearts, and it was significantly reduced to 1.8 ± 0.4% ($n = 8$) in ventricles perfused with 20 μmol/l Gen, and to 3.5 ± 0.7% ($n = 7$) in ventricles isolated from rats treated with 5 mg/kg Gen (ANOVA: $F = 17.93$, $P < 0.0001$, $P < 0.05$ by Tukey *post hoc* tests).

Discussion

This work gives new insights about the underlying mechanisms of the stunning consequent to hypoperfusion, as a model of an incipient coronary dysfunction, as well as about the cardioprotective mechanisms of the phytoestrogen genistein.

Mechanisms underlying stunning consequent to LFI and reperfusion

Results show that hypoperfusion (20% of initial flow) reduced not only inotropism but also energetic output, but

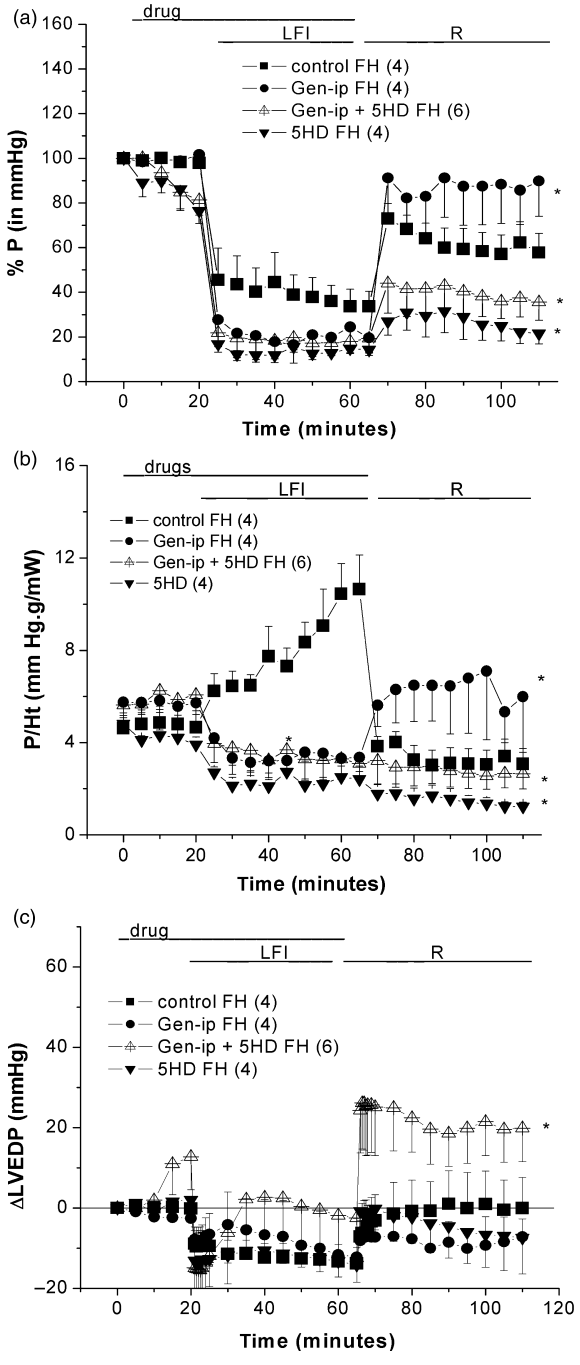

Figure 7 Effects of intraperitoneal administration of 5 mg/kg genistein (Gen-ip) in female rat hearts (FH), and consequences of the mKATP channels inhibition with 100 μmol/l 5-hydroxydecanoate (5HD) perfused before and during low-flow ischaemia (LFI) on: (a) maximal pressure development (P, as % of initial value), (b) total muscle economy (P/Ht in mmHg g/mW) and (c) changes of left ventricular end diastolic pressure (ΔLVEDP in mmHg) over initial value. Results are shown as mean ± SEM (number of experiments). Two-way ANOVA by treatment: $F = 53.81$, 49.12 and 33.01 (all $P < 0.0001$), respectively, for (a–c); by time: $F = 37.43$, 2.38 and 2.27, respectively, for (a–c) (all $P < 0.0001$). *Post hoc* tests: $^*P < 0.05$ vs control.

females were more protected than males during LFI and R. Total muscle economy (P/Ht) was reduced in males, suggesting that the energetical consumption (evidenced by Ht) was less affected than contractility. This high energetical consumption was observed in rat hearts exposed to the same low-flow perfusion under a very low cardiac demand (single beats at 25 °C).[7] In that situation, Ht fell less than P because low-flow reduced the tension-dependent and metabolic heat fractions without affecting the tension-independent heat related to Ca^{2+}-cycling (activation heat). Moreover, this relative maintaining of energetic consumption agrees with Assayag *et al.*[6] who described that in beating rat hearts a 15% reduction in perfusion flow induced a great fall in tension while the energy consumer systems such as SERCA2, NCX and RyR2 protein levels were unaltered. Other work reported that beating hearts were adapted to LFI by down-regulation of myocardial performance while established a new equilibrium between the energy supply and demand, preserving hearts from diastolic contracture better than a similar degree of hypoxia, but increasing lactate release and ATP turnover.[32] So, LFI induces little changes in Ca^{2+} transport and metabolic adaptation, while the low contractility is associated with the loss of diastolic stretching.

The lesser contractile recovery of male with respect to female hearts under LFI/R agree with previous reports suggesting that in more severe conditions of no-flow ischaemia and reperfusion (global *ex vivo* ischaemia, and *in vivo* occlusion of coronary artery) female rat hearts were less infarcted than males.[33] Those authors attributed the difference to estrogenic effects since males reduced the postischaemic injury after administration of estradiol. Also, they showed that females had higher phosphorylation of aldehide deshydrogenase (ALDH2) and α-ketoglutarate dehydrogenase (αKGDH), which reduce ROS accumulation in mitochondria, and those effects depend on activation of the phosphatidylinositol 3-kinase.[33] Since changes in metabolism could affect the mitochondrial Ca^{2+} transporters, we explored their role in the cardioprotection of FH during LFI/R. That is, FH had a rise in P and muscle economy (P/Ht) during LFI and higher PICR with respect to MH. Results suggested that blockade of mitochondrial Ca^{2+} uptake through the UCam with Ru360 reduced P during both LFI and R in FH, with a fall in muscle economy during the hypoperfusion (Figure 4). On this model, P was less affected than in a model of rat hearts exposed to no-flow ischaemia and R with the same degree of stunning, in which Ru360 reduced PICR from about 75–20%.[26] It was also reported that Ru360 reduced the rise in $[Ca^{2+}]$m suffered by rabbit cardyomyocytes under simulated I/R.[34] The increase in LVEDP induced by Ru360 during LFI agree with the consequent accumulation of cytosolic Ca^{2+}, which maintain high the energetical consumption and Ht. Considering toghether these evidences suggest that mitochondrial Ca^{2+} uptake contributes to maintain metabolism and

Table 1 Effects of Gen-ip 5 g/kg on the relative rates of contraction (+P/P) and relaxation (−P/P) and the periods of contraction (tC1 and tC2) and relaxation (tR1 and tR2) in male heart rats (MH)

	Control MH	Gen-ip	Control MH	Gen-ip	Control MH	Gen-ip
Condition/Time	+P/P		tC1		tC2	
C	19.98 ± 1.53	14.33 ± 0.034	0.048 ± 0.006	0.126 ± 0.034	0.027 ± 0.003	0.044 ± 0.007
LFI 45	17.44 ± 1.20	17.74 ± 0.014	0.043 ± 0.003	0.082 ± 0.014	0.033 ± 0.002	0.042 ± 0.007
R 5	17.40 ± 1.54	13.63 ± 0.037	0.045 ± 0.003	0.180 ± 0.037	0.038 ± 0.005	0.048 ± 0.005
R 25	16.30 ± 1.33	13.23 ± 0.036	0.050 ± 0.006	0.128 ± 0.036	0.045 ± 0.008	0.052 ± 0.004
R 45	16.30 ± 1.67	12.91 ± 0.036	0.053 ± 0.004	0.122 ± 0.036	0.045 ± 0.008	0.050 ± 0.004
2-way ANOVA	By condition: $F = 11.42$, $P < 0.01$ By time: $F = 1.77$, NS		By condition: $F = 35.39$, $P < 0.0001$ By time: $F = 1.38$, NS		By condition: $F = 6.793$, $P < 0.05$ By time: $F = 2.035$, NS	
	−P/P		tR1		tR2	
C	17.19 ± 1.11	11.92 ± 0.64	0.053 ± 0.003	0.058 ± 0.002	0.057 ± 0.006	0.112 ± 0.006
LFI 45 min	16.67 ± 1.45	15.17 ± 2.35	0.045 ± 0.003	0.066 ± 0.005	0.050 ± 0.003	0.086 ± 0.014
R 5 min	18.71 ± 2.02	11.68 ± 0.56	0.045 ± 0.004	0.054 ± 0.006	0.055 ± 0.006	0.100 ± 0.008
R 25 min	19.50 ± 2.60	12.87 ± 1.26	0.048 ± 0.003	0.058 ± 0.004	0.058 ± 0.012	0.100 ± 0.011
R 45 min	17.88 ± 1.98	12.78 ± 0.79	0.048 ± 0.006	0.060 ± 0.003	0.058 ± 0.012	0.098 ± 0.012
2-way ANOVA	By condition: $F = 22.47$, $P < 0.0001$ By time: $F = 0.28$, NS		By condition: $F = 17.49$, $P < 0.0001$ By time: $F = 0.70$, NS		By condition: $F = 49.85$, $P < 0.0001$ By time: $F = 0.737$, NS	

indirectly the cellular Ca^{2+} level, myofilaments activation and muscle economy during LFI/R. However, this role of uniporter resulted more critical under no-flow or hypoxic conditions than under LFI. During the LFI in FH, the UCam maintained the mitochondrial metabolism down-regulated at a threshold rate that preserved contractility (high P/Ht ratio). At least in part, the preservation is related to the fact that low-flow perfusion maintains a low oxygen provision and avoids the accumulation of metabolites and protons in the extracellular medium, at a difference of no-flow.

Moreover, the inhibition of the mKATP channels with the selective blocker 5HD reduced the contractile performance and muscle economy of FH under LFI/R (Figure 7). It is known that these channels open when the ATP levels fall, and contribute to reduce the mitochondrial electrochemical gradient as driving force for Ca^{2+} uptake and its overload.[28,29] It was reported that their selective inhibition with 5HD reduced the cardioprotection in preconditioning.[35,36] Nevertheless, the mKATP channels opening not always participate in cardioprotection.[24,37] Our results in MH showed that 5HD did not reduce the PICR and P/Ht nor increased LVEDP during LFI/R (Figure 6). The comparison of these results suggests that cardioprotection in FH is at least partially due to activation of mKATP channels, in agreement with a previous report about mechanisms of estrogens.[38] The mKATP channels contribute to maintain a low mitochondrial $[Ca^{2+}]$, which reduces Ca^{2+} overload and the diastolic dysfunction, both of which are characteristics of more severe conditions of ischaemia.

On the other hand, selective inhibition of mitochondrial Ca^{2+} extrusion through the mNCX with clonazepam[23,27] did not affect the PICR of FH but reduced muscle economy (P/Ht) during hypoperfusion. The only beneficial effect of clonazepam was the reduction in diastolic contracture (ΔLVEDP) during all the LFI/R. Results suggest that the mNCX inhibition increases the mitochondrial Ca^{2+}-dependent metabolism and the associated Ht (P/Ht falls) while reduces the cytosolic Ca^{2+} and consequently the ΔLVEDP, in agreement to that suggested for these selective drugs.[27] We have found that Clzp increased PICR in guinea-pig hearts[39] and in rat hearts[26] exposed to no-flow ischaemia. Results suggest that mitochondria contribute to the stunning because under LFI the mitochondria may be losing Ca^{2+} through the mNCX. This extrusion could contribute to down-regulate the Ca^{2+}-activated metabolism and improve P/Ht ratio in FH. However, despite the cytosolic Ca^{2+} increase, P and LVEDP are reduced by the fall in muscle length and turgency. When perfusion flow is restored (R) the elevated Ca^{2+} level activates myofilaments generating diastolic contracture while the low ATP levels and SR Ca^{2+} loading reduce P with high energetical consumption (stunning). As it was discussed females rat cardiomyocites have lower diastolic Ca^{2+} and Ca^{2+} transients than males[40] and this difference could contribute to the cardioprotection of FH during LFI/R respect to MH.

Other mechanism which has been proposed as responsible for the cardioprotection in female hearts is the protein S-nitrosylation induced by estrogens.[41] The authors showed that female mice hearts exposed to global no-flow ischaemia and reperfusion recovered more than males,

because they received the post-translational S-nitrosylation of a higher number of proteins than males. Among them the mitochondrial F_1F_0-ATPase and cyclophylin D are exclusively S-nitrosylated in females, and more sarcorreticular Ca^{2+}-ATPase (SERCA-2a) was S-nitrosylated in females than in males. Those labile protein modifications protect from mitochondrial ROS accumulation. Also, the L-type Ca^{2+} channels were S-nytrosylated in female hearts with the consequent decrease in Ca^{2+} influx.[42] Moreover, female hearts increased the expression and phosphorylation of the endothelial nitric oxide synthase (eNOS) and the total production of NO, which is related to S-nitrosylation, cardioprotection and up-regulation by estrogens.[41,42] In the FH exposed to LFI/R we found that perfusion of L-NAME (non-selective blocker of eNOS and iNOS) reduced contractile recovery and muscle economy (P/Ht ratio) with diastolic contracture (Figure 4). These results suggest that NO production is part of the cardioprotection of females under LFI/R. This role of NOS agrees with that showed in previous reports from mice hearts exposed to no flow-ischaemia and reperfusion, in which genetical removal of phospholamban (PLB) was more detrimental in males than in females, and the difference was abolished by L-NAME independently on the greater sarcorreticular Ca^{2+} content.[43] As well as in that report, after L-NAME the FH recovered a low mechano-energetical performance which was similar to that of untreated MH, suggesting that female cardioprotection depends on NOS. The fact that cardioprotection in FH exposed to LFI/R was inhibited with inhibition of both, mKATP channels and NO production, suggest a possible relation between these mechanisms. Indeed, Sasaki *et al.*[44] has shown that NO can selectively activate the mKATP channels, in part by oxidation of channels as peroxynitrite. So, results suggest that estrogens in FH could be activating a pathway involving both cardioprotective mechanisms.

Effects of genistein during LFI/R

Ex vivo effects

In a previous work, we demonstrated that perfusion of genistein induced sex- and temperature-dependent effects (negative inotropism in male rat hearts evident at 30 but not at 37 °C).[20] Temperature and sex affect the transporters and Ca^{2+} homeostasis in rat hearts,[40] and could unbalance the several effects of genistein, such as Ca^{2+} channels inhibition,[14–16] myofilaments sensitivity and SR Ca^{2+} uptake.[17,18] Similarly to that obtained in no-flow I/R,[20] perfusion of 20 μmol/l Gen before LFI slightly reduced the contractile performance without affecting muscle economy, according to the balance between those mechanisms of Gen which increase or decrease the Ca^{2+} transient. However, in FH perfusion of Gen reduced P and P/Ht during the whole LFI/R cycle (Figure 5), but it did not affect them in MH (Figure 6). However, in both of them, Gen increased the diastolic contracture during the whole LFI/R period, suggesting that it increases the diastolic $[Ca^{2+}]$i. When blocking mNCX with Clzp to evaluate whether the diastolic contracture of Gen was due to mitochondrial Ca^{2+} extrusion, the dysfunction was increased since PICR fell from about 60% to 20% of initial in FH. As Clzp increases the $[Ca^{2+}]$m,[27,39] this one could reach a detrimental higher level with Clzp and Gen during LFI in FH. The addition of Cys-A to the Clzp + Gen perfusion partially reverted the fall in P and P/Ht and reduced the diastolic contracture during R (Figure 5). This result suggests that the cause of such dysfunction was a cytosolic and mitochondrial Ca^{2+} overload which triggered the mPTP opening. However, the LFI/R did not open the mPTP by itself, since Cys-A alone did not alter the mechano-energetic recovery. The fact that Gen increased LVEDP at the start of R similarly when it was alone or with the presence of Clzp and Cys-A, suggests that Gen rises the cytosolic Ca^{2+} by an independent mechanism. According to this, we have previously shown that 20 μmol/l Gen reduced the mitochondrial Ca^{2+} uptake in hearts and cardiomyocytes.[20] Thus, combined effects of Gen and Clzp, respectively, increased the cytosolic and mitochondrial $[Ca^{2+}]$ during LFI/R so that to trigger the mPTP opening.

In vivo effects

Finally, Gen was cardioprotective only when it was administered *in vivo* at 5 mg/kg 24 h before the ex-vivo experiment of LFI/R, more in males (PICR from about 30% to 90% of initial, Figure 6) than in females (PICR from about 60% to 90%, Figure 7), in agreement with the estrogenic cardioprotective effect previously existent in females.[38] The fact that in males Gen-ip reduced the rates of contraction and relaxation, and prolonged the respective times agrees with the increase of PICR and P/Ht, and can be respectively, related to the partial blockade of Ca^{2+} channels[17] and reduction in cytosolic Ca^{2+} efflux,[18,19] as found in cardiomyocytes treated with Gen.

The possibility that cardioprotection of Gen-ip during LFI/R would be associated to the activation of mKATP channels was demonstrated by the negative effects produced by their selective blocker 5HD. As previously discussed, the opening of mKATP channels reduces the driving force for Ca^{2+} influx through the UCam, and thereby attenuates the mitochondrial Ca^{2+} overload.[29,35,45] It was shown that activation of mKATP channels prevents both necrosis and apoptosis presumibly by inhibition of the mPTP opening.[46] Here, 5HD reduced the cardioprotective effects of Gen in both sexes, increasing the LVEDP and reducing PICR and muscle economy during LFI/R. Our results agree with a previous report in which the mKATP channels were found as one of the

mechanisms responsible for the reduction of the infarct size when Gen was intravenously injected in rabbits exposed to coronary occlusion as a more severe model of no-flow I/R.[47] Thus, Gen would activate mKATP channels as estrogens do.[38] The mechanism of Gen would be independent on the ischaemic insult degree, from the stunning triggered by low-flow to the infarct consequent to prolonged no-flow ischaemia. Nevertheless, the fact that cardioprotection was only developed by *in vivo* administration suggests either that a metabolite is the responsible, or that the effect requires a slow protein synthesis. The work of Couvreur *et al.*[47] described that in rabbits the cardioprotection of Gen involves the phosphorylation of Akt and inhibition of GSK3β. Nevertheless, these pathways are characteristic of a drastic ischaemic damage such as infarct[48] but we did not find their role in the stunning consequent to no-flow ischaemia (still unpublished results). Comparing our results obtained with 5-HD it can be concluded that the mKATP channels were activated by Gen-ip as cardioprotective, as well as they participate by reducing the stunning by LFI/R in FH with respect to MH. The slow mechanism of Gen before opening the mKATP channels could involve an activation sequence similar to that reported in the preconditioning, with adenosine and PKC.[28,49]

Even when it is not easy the extrapolation among species, these results obtained in the model of LFI/R in rat hearts explain the mechanisms of Ca^{2+} homeostasis underlying cardioprotection of females over males in hibernation. This female preponderance has been reported for women in clinical ischaemic episodes.[11] Few works studied human hearts, but in byopsies of hibernating and stunned myocardium authors found that the expressions of Ca^{2+} regulation-proteins such as phospholamban, SERCA-2a, calsequestrin, troponin-I, or p38 MAPK were not changed.[50] Stunning reduced the cardioprotective Hsp72 protein, while hibernation increased cAMP levels as a sign of adaptation.[50] However, in our model LFI was induced in the isolated hearts, by which the adrenergic receptors were not activated, and we analysed the functional consequences for Ca^{2+} homeostasis and contractility. Moreover, this work demonstrates that a natural product with phytoestrogenic properties as genistein, used to treat menopausical symptoms, has prevented the consequences of a coronary insufficiency in males, by an effect similar to that of estrogens in females. The perpective is that females with less endogenous estrogens had the same increase in cardioprotection by receiving genistein or other isoflavone. As in other situations in which mitochondrial Ca^{2+} handling is the target,[51] the mechano-energetical studies allow to evaluate the underlying mechanisms under different models of ischaemia/reperfusion and to evaluate the cardioprotection of drugs.

Conclusions

In summary, this work describes for first time the role of mitochondrial Ca^{2+} transporters in the mechano-energetic performance of the beating rat heart suffering stunning by the low-flow perfusion and reperfusion. Females recovered more than males, and the cardioprotection involves the mitochondrial [Ca^{2+}] regulation by UCam, mNCX and mKATP-channels, as well as the NO production. These mechanisms activated during LFI/R promote a mitochondrial Ca^{2+} loss which down-regulates metabolism and ATP synthesis but prevents postischaemic Ca^{2+} overload in FH, reducing the stunning. Moreover, this work shows that the phytoestrogen genistein is cardioprotective in this LFI/R model when it was *in vivo* administered by i.p. injection, but not when it was directly perfused before the LFI. The cardioprotection of Gen-ip involves an increment in the PICR and the muscle economy, and is greater in MH than in FH. The sensitivity of cardioprotection to the selective blocker 5HD in both MH and FH allowed to demonstrate that the treatment of Gen *in vivo* involves mainly the activation of mKATP channels, which prevents the mitochondrial Ca^{2+} overload responsible of diastolic dysfunction, reduced muscle economy and stunning during LFI/R.

Declarations

Conflict of interest

There is no conflict of interest.

Acknowledgements

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