Clinical and Experimental Pharmacology and Physiology (2016) **43**, 102–115 doi: 10.1111/1440-1681.12500

ORIGINAL ARTICLE

Sex differences in the mechano-energetic effects of genistein on stunned rat and guinea pig hearts

Germán A Colareda,* María I Ragone*† and Alicia E Consolini*

**Experimental Pharmacology Group (GFEYEC), Department of Biological Sciences, School of Exactas Sciences, National University of La Plata, and †National Council of Scientific and Technical Research (CONICET), La Plata, Argentina*

SUMMARY

Although the phytoestrogen genistein (Gen) is considered protective in cardiovascular diseases, its direct effects on stunned hearts after transient ischemia-reperfusion (I/R) are unknown. This report studied the effects of 20 μmol/L Gen on the mechano-calorimetric behaviour during I/R of rat and guinea pig hearts to evaluate the energetics of Ca^{2+} homeostasis. Isolated beating hearts were perfused with control Krebs solution inside a calorimeter with or without perfusion of Gen before a transient period of I/R. Left ventricular pressure development (P) and total heat rate (Ht) were continuously measured. At 37°C, Gen did not change post-ischemic contractile recovery (PICR), but it increased the relaxation rate. However, PICR was reduced in hearts of male rats and guinea pigs at 30°C. Total muscle economy (P/Ht) showed the same behaviour as P at each temperature. Inhibition of phosphatases with orthovanadate during Gen perfusion prevented a decrease in PICR in male rat hearts, suggesting that this effect is due to tyrosine kinase inhibition. Reperfusing ischemic hearts with 10 mmol/L caffeine-36 mmol/L Na^+-Krebs induced contracture dependent on the sarcoreticular Ca^{2+} content. Contracture relaxation depends on mitochondrial Ca^{2+} uptake and Gen reduced the relaxation rate. Moreover, Gen prevented the increase in Rhod-2 fluorescence (free $[Ca^{2+}]$m) of rat cardiomyocytes. In guinea pig hearts, Gen maintained ischemic preconditioning, but was reduced by 5-hydroxydecanoate, suggesting the participation of mitochondrial adenosine triphosphate (ATP)-dependent K channels. Results suggest that Gen acts on several mechanisms that regulate myocardial calcium homeostasis and energetics during I/R, which differ in a temperature- and sex-dependent manner.

Key words: calorimetry, genistein, heart, ischemia/reperfusion, phytoestrogen.

INTRODUCTION

Genistein (Gen) is a phytoestrogen extracted from soy and it is considered to prevent cardiovascular diseases, mostly due to its vasodilator properties.[1,2] However, there is no conclusive evidence of Gen providing direct cardioprotection to the ischemic myocardial tissue. It is well known that Gen inhibits tyrosine kinases (TK).[3] Moreover, several effects of Gen were reported in guinea pig myocardium under physiological conditions, such as the inhibition of L-type Ca^{2+} current,[4–6] an increase in Ca^{2+} content of the sarcoplasmic reticulum (SR), a decrease in Ca^{2+} efflux by the Na^+/Ca^{2+} exchanger and an improvement in myofilament Ca^{2+} sensitivity.[7,8] These effects allow us to hypothesize that Gen could reduce the Ca^{2+} overload induced during ischemia/reperfusion (I/R) that results in diastolic contracture, and this could lead to an improved contractility of the reperfused heart.

There are no studies on the effects of Gen on 'stunned' hearts, a condition defined as depressed myocardial function following a transient ischemic event and reperfusion without infarct.[9] This model resembles the clinical situation of a first transient ischemia, when it is important to prevent further complications. Gen has been studied in models of severe ischemia, such as in ovariectomized rats[10,11] and rabbit hearts with coronary occlusion used as a postconditioner.[12] In these models, Gen reduced the resulting myocardial infarct. These reported effects were influenced by several factors, such as ischemic degree, animal species and sex, taking into account that Gen is a phytoestrogen. Under physiological conditions, Gen proved to modulate mechanisms at the sarcolemmal and sarcoreticular levels. It is hypothesized that Gen could differently affect the stunning of two species that differ in the relative participation of sarcoreticular and sarcolemmal sources of Ca^{2+}. Sarcoreticular Ca^{2+} content is larger in rat than in guinea pig cardiomyocytes[13] and the SR contributes more to the cytosolic Ca^{2+} transient in rat than in guinea pig hearts.[14] Since Gen is included in the normal diet (as soy) or as a natural supplement, it was of interest to evaluate whether Gen could prevent the stunning caused by a transient ischemic episode. In order to define the impact of Gen on several cellular targets, its effects were assessed in both species, rats and guinea pigs, and in both sexes.

Global I/R induces contractile and energetic dysfunction, in degrees from stunning to infarction, depending on the ischemic period and tolerance of oxygen deprivation.[15–17] Calcium homeostasis and metabolism are affected by I/R, resulting in energetic

Correspondence: Prof. Dra. Alicia E Consolini, Cátedra de Farmacología, Depto de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 y 115 (1900) La Plata, Argentina. Email: dinamia@biol.unlp.edu.ar

Received 14 April 2014; revision 12 September 2015; accepted 2 October 2015.

failure and metabolic recovery. Since the heart directly converts a fraction of biochemical enthalpy into external work and heat, under isometric conditions (zero external work) the rate of heat production helps estimate the rate of metabolic enthalpy expenditure. The continuous, simultaneous measurement of contractile and calorimetric output of the isolated heart provides information about the energetics of Ca^{2+} handling and muscle economy during I/R. Whole-heart on-line calorimetry is sensitive enough to detect changes that depend on exothermic events, such as Ca^{2+} transport, contractility and aerobic metabolism.[18–20] Myocardial ischemia reduces the metabolic restoration of adenosine triphosphate (ATP) and the associated heat fraction (known as recovery heat).[21] Consequently, there is a reduction in the activity and heat release of active transports such as Na,K-ATPases and Ca-ATPases (known as tension-independent heat), as well as in the actomyosin cross-bridge (known as tension-dependent heat).[21] Whole-heart on-line calorimetry has been used extensively to study the energetics of Ca^{2+} homeostasis in the stunned heart after a transient ischemia, and the cardioprotection of cardioplegic solutions of high $[K^+]o$[22–24] or pyruvate (Pyr),[25] and the effects of hyperthyroidism.[26] Microcalorimetry has also been performed by other authors using a more sensitive device to estimate the energetics of the tension-dependent heat of superfused trabeculae in diabetes.[27,28] Nevertheless, the physiologically perfused whole heart is the only preparation capable of simulating ischemia by flow cessation and reperfusion.

The aims of this study were to evaluate whether the phytoestrogen Gen prevents the typical mechano-energetic performance of stunning due to ischemia and reperfusion in isolated rat and guinea pig hearts, as well as to assess the influence of sex on this effect. Moreover, the study evaluated whether Gen could modify the preconditioning (PC) in guinea pig hearts, which is another prevention strategy that can be combined with estradiol. Pharmacological tools were used to explore the underlying mechanisms in isolated hearts and cardiomyocytes. Left ventricular pressure (LVP) and its maximum during contraction (P), energetic output (Ht) and muscle economy (P/Ht) were measured in isolated hearts over the period of I/R. Besides, fluorometric signals of free mitochondrial and cytosolic $[Ca^{2+}]$ were measured in isolated cardiomyocytes exposed to Gen.

RESULTS

Sex differences in the effects of Gen in rat hearts under I/R

This study analyzed the effects of pre-treating the heart with 20 μmol/L Gen before inducing ischemia and different models of cardiac stunning. Figure 1 summarizes the protocols used. In each control protocol (C, without any treatment), a duration of ischemia (I) was chosen that produced a recovery of about 50% contractility during reperfusion (R). In the more physiological condition (37°C and 3 Hz stimulation frequency), the isolated rat heart developed stunning after exposure to 20 min of I followed by 45 min of R. Since the underlying mechanisms of stunning at subphysiological conditions (30°C and 1 Hz) had been studied previously, this study assessed the mechanisms of Gen in hearts exposed to this model of stunning, consisting in 45-min I/45-min R for isolated rat hearts and 30-min I/45-min R for guinea pig hearts. Figure 1 also shows typical images of slices of reperfused

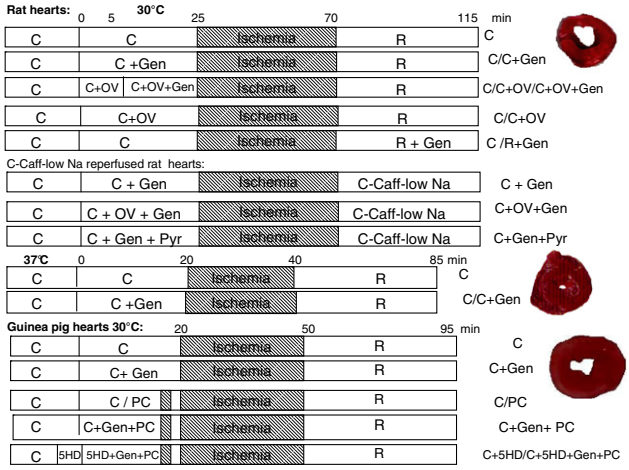

Fig. 1 Protocols applied on isolated rat (at 30 and 37°C) and guinea pig (at 30°C) hearts. Periods assigned to each treatment are indicated in the upper scale in minutes, considering start of treatment before no-flow ischemia (I) time 0. Stabilization (C) and reperfusion (R) were done with control Krebs, except when adding 20 μmol/L Gen (R+Gen) or when reperfusing with 10 mmol/L caffeine-36 mmol/L Na^+-Krebs (C-caff-low Na). Pre-treatments are in the second box (C, Krebs-C; Gen, 20 μmol/L genistein; OV, 10 μmol/L orthovanadate; 5HD, 30 μmol/L 5-hydroxydecanoate; Pyr, 10 mmol/L pyruvate; PC, preconditioning). Transverse sections of rat and guinea pig hearts obtained at the end of control protocol (C-I/R) are shown to the right, in order to demonstrate the absence of infarcted areas in these models of stunning.

rat and guinea pig Krebs-C treated hearts. These images show that the models of stunning did not produce significant myocardial infarction. In protocols at 30°C, the infarct size of rat hearts was as low as $4.5 \pm 0.5\%$ ($n = 8$), while guinea pig hearts developed $16.6 \pm 5.2\%$ ($n = 8$) of the left ventricle area. At 37°C, the infarct size of rat hearts was also $5.1 \pm 0.7\%$ ($n = 10$). These results are similar to the values reported under cardioprotection in other models of I/R that developed an infarct.[29]

When perfusing 20 μmol/L Gen before ischemia, P was reduced in rat hearts at both 37°C and 30°C. Table 1 shows absolute values of P, Ht and muscle economy (P/Ht) in basal conditions after stabilization in Krebs-C and in the presence of 20 μmol/L Gen. The total muscle economy (P/Ht) of non-treated (C) hearts was reduced at higher temperature, in agreement with the rise in the rate of active transporters such as sarcoreticular Ca-ATPase (SERCA). Besides, P/Ht was reduced by Gen in male rat hearts (MRH), but not significantly in female rat hearts (FRH) at both 30°C and 37°C. At 30°C, Gen induced in FRH an increased relative relaxation rate $\Delta(-P/P)$ ($+0.42 \pm 0.21$/s, $t = 1.94$, $n = 10$, $P < 0.05$, basal $-P/P$: 5.1 ± 0.9/s), but not in MRH (basal $-P/P$: -6.0 ± 0.9/s).

After I, sex differences were found in post-ischemic contractile recovery (PICR) in rat hearts pretreated with Gen at 30°C, but not at 37°C (Fig. 2a). At 30°C, Gen induced a significantly lower PICR in MRH when compared with the group of non-treated hearts (control), which recovered similarly to FRH (Fig. 3a). The energetic output during R (as percentage of initial Ht) was similar in MRH and FRH, and higher than in control hearts at both temperatures. In consequence, Gen reduced total muscle economy (P/Ht) during R (Figs. 2b and 3c). The higher PICR in MRH at 37°C could be due to an increase in sarcoreticular Ca^{2+} uptake,

Table 1 Absolute values of maximal pressure development (P) and total heat rate (Ht) obtained after stabilization in Krebs-C (C) and after perfusing hearts with 20 μmol/L genistein (Gen) in the three models before ischemia: female and male rat hearts at 37°C (FRH_{37} and MRH_{37}, respectively), female and male rats hearts at 30°C (FRH_{30} and MRH_{30}, respectively), and female and male guinea pig hearts at 30°C (FGH_{30} and MGH_{30}, respectively) (paired *t*-test: $^{*}P < 0.05$ *vs* C, $^{\#}P < 0.01$ *vs* C)

Treatment	P (mm Hg)		Ht (mW/g)		P/Ht (mmHg/mW per g)	
	C	Gen	C	Gen	C	Gen
FRH_{37} (6)	84.4 ± 12.6	67.3 ± 11.6	19.0 ± 2.0	15.9 ± 2.0	4.1 ± 0.4	3.9 ± 0.6
MRH_{37} (7)	62.5 ± 11.7	37.4 ± 5.0*	15.7 ± 2.2	12.1 ± 1.3	3.8 ± 0.3	3.1 ± 0.3
FRH_{30} (16)	105 ± 10	83.5 ± 9.6$^{\#}$	13.5 ± 0.7	12.7 ± 1.0	8.1 ± 0.8	6.8 ± 0.8
MRH_{30} (17)	86.4 ± 12.0	69.6 ± 10.4*	13.2 ± 0.8	13.9 ± 1.4	8.0 ± 1.0	5.9 ± 0.8*
FGH_{30} (9)	30.6 ± 5.0	36.1 ± 5.9	15.3 ± 1.2	12.8 ± 1.2	1.9 ± 0.3	2.8 ± 0.4
MGH_{30} (15)	32.5 ± 5.2	32.1 ± 4.7	16.7 ± 1.1	15.5 ± 1.2	2.0 ± 0.3	2.0 ± 0.3

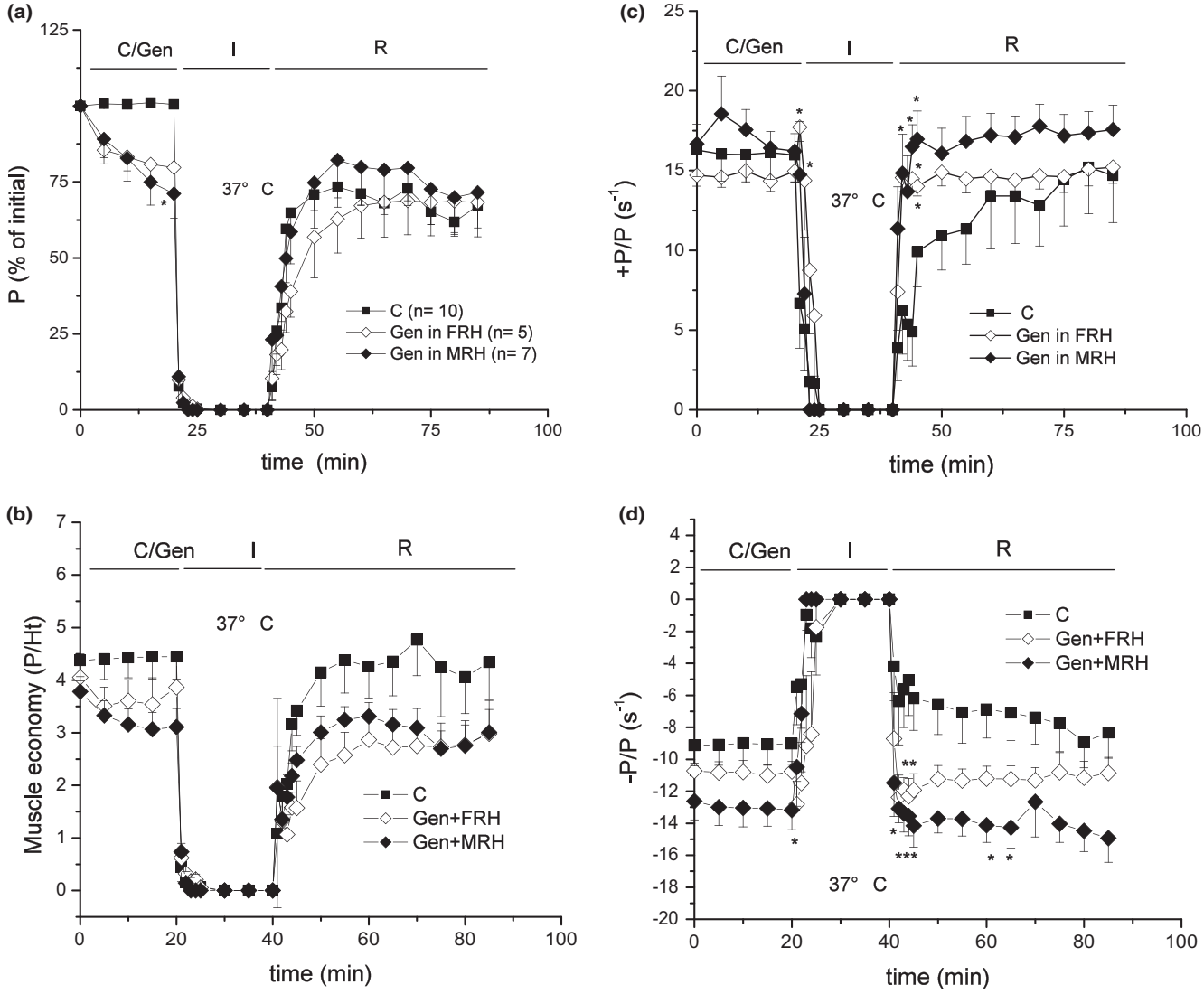

Fig. 2 Effects of 20 μmol/L genistein (Gen) on isolated rat hearts exposed to 20 min of ischemia (I) and 45 min of reperfusion (R) at 37°C with a stimulation rate of 3 Hz. (a) Maximal pressure development (P) (as % of initial value). (b) Total muscle economy (P/Ht in mmHg.g per mW). (c) relative contraction rate (+P/P in 1/s). (d) Relative relaxation rate (−P/P in 1/s). The effects of the phytoestrogen were studied separately in male (MRH) and female (FRH) rat hearts. Results are shown as mean ± SEM (number of experiments). Two-way ANOVA by treatment $F = 6.41$ ($P = 0.0018$), 15.76, 21.97 and 23.58 (all $P < 0.0001$), respectively for a, b, c and d; by time: $F = 90.6$, 25.23, 22.1 and 16.33, respectively for a, b, c and d, all $P < 0.0001$. *Post-hoc* tests $^{*}P < 0.05$ versus C.

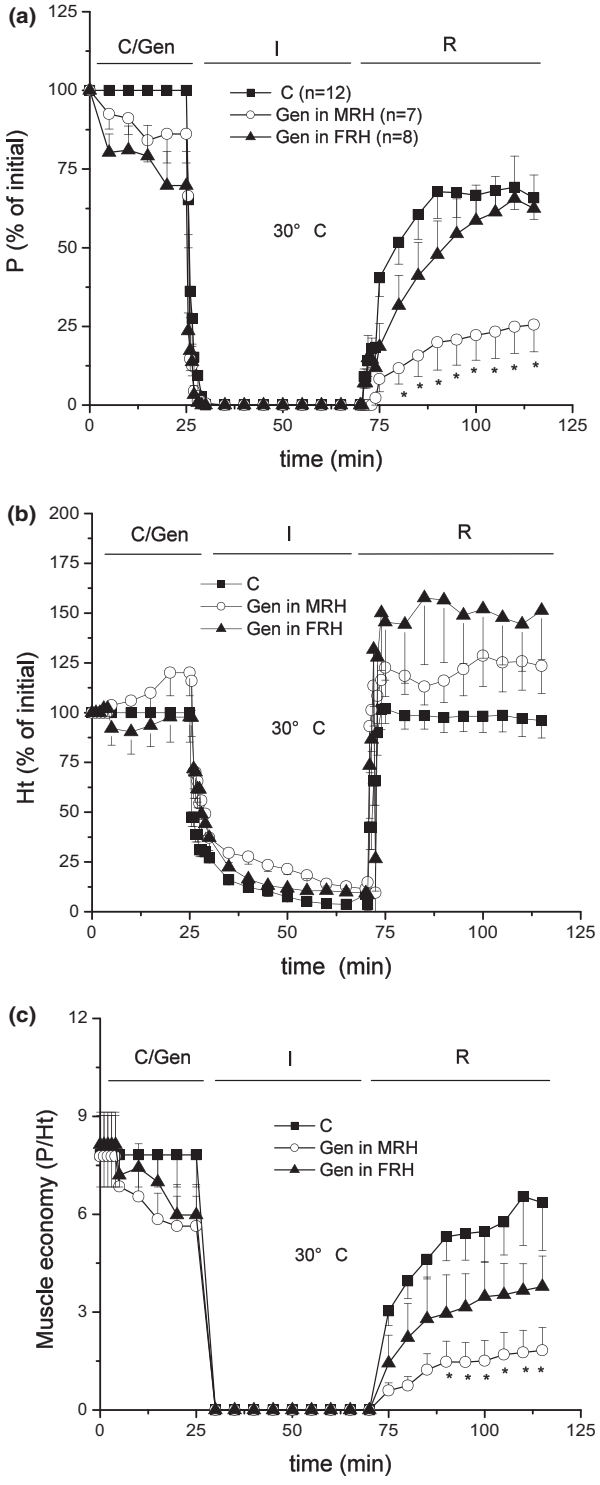

Fig. 3 Effects of 20 μmol/L genistein (Gen) on isolated rat hearts exposed to 30 min of ischemia (I) and 45 min of reperfusion (R) at 30°C with a stimulation rate of 1 Hz. (a) maximal pressure development (P) (as % of initial value). (b) total heat rate (Ht as % of initial value). (c) total muscle economy (P/Ht in mmHg.g per mW). Note the different effects induced in male (MRH) and female (FRH) rat hearts by the phytoestrogen when compared with untreated hearts of both sexes (control or C). Results are shown as mean ± SEM (number of experiments). Two-way ANOVA by treatment $F = 68.87$, 47.52 and 25.19, respectively for a, b and c, all $P < 0.0001$; by time: $F = 90.96$, 41.76 and 42.58, respectively for a, b and c, all $P < 0.0001$. *Post-hoc* tests $^*P < 0.05$ versus C.

since the relative relaxation rate (−P/P) and the relative contraction rate (+P/P) increased during R (Fig. 2c–d). Besides, Gen significantly reduced the diastolic contracture that appears at the end of I and start of R at 30°C, with a more pronounced effect in MRH than in FRH. However, Gen increased left ventricular end-diastolic pressure (LVEDP) at 37°C, suggesting that Ca^{2+} was lost to cytosol (Table 2).

Mechanisms underlying the Gen effect in rat hearts

In order to evaluate whether Gen was preventive of PICR failure in female hearts at 30°C, or if it reverted the failure during R, a group of FRH was treated with Gen only during R. This protocol did not change PICR (Fig. 4a) or muscle economy (P/Ht, Fig. 4b) when compared with hearts treated with Gen before inducing I. The results suggest that Gen could be activating a protective mechanism in FRH at 30°C, which compensates the negative effect seen in MRH. Since at 37°C the reduced PICR was not found either in FRH or MRH, it was inferred that the compensatory mechanism could be mediated by an active transport, which is known to be activated by temperature.

In order to study whether the negative effects of Gen in MRH were due to the inhibition of TK, we used the TK inhibitor sodium orthovanadate (OV), which enhances protein tyrosine phosphorylation.[30] During perfusion with 10 μmol/L OV at 30°C, P was not significantly changed in FRH or MRH (Fig. 4a), but OV prevented the negative inotropism developed by Gen in MRH before ischemia, as well as the reduction in PICR (67.1 ± 11.6% *vs* 25.5 ± 8.6% of initial P at 45 min of R; Fig. 4a). Ht was reduced by the presence of OV with Gen before I (from 16.6 ± 2.9 to 10.9 ± 2.3 mW/g), but no differences were found during the I/R period (123.4 ± 13.8%, 114.8 ± 11.1% and 110.0 ± 19.5% of initial values, for groups treated with Gen, OV+Gen and OV, respectively). Muscle economy (P/Ht) was similarly reduced during R, independently of the pretreatment with Gen and/or OV (Fig. 4b). Besides, OV partially inhibited the prevention of diastolic contracture induced by Gen at the end of R (Table 2). All these results suggest that the reduction in both contractile recovery and diastolic contracture induced by Gen in MRH at 30°C is mainly mediated by TK inhibition.

In FRH at 30°C, OV did not alter the effects of Gen on PICR (Fig. 4c) or diastolic pressure (+ΔLVEDP; Table 2), with only a slight increase in muscle economy (P/Ht) appearing at the end of R (Fig. 4d). These results suggest that in females Gen compensates the negative effects seen in males and both mechanisms are mediated by TK inhibition.

Finally, Gen could release nitric oxide, which is part of a cardioprotective mechanism mediated by the estrogenic receptor under estradiol stimulation.[31] In order to evaluate this, the hearts were treated with the selective inhibitor of NO-synthase L-NAME before and during perfusion with Gen. Figure 3c,d shows that this treatment transiently increased PICR without changing P/Ht, suggesting that the cardioprotection of Gen in FRH was not due to NO release.

Gen effects on signals of sarcoreticular Ca^{2+} release

To evaluate whether the negative inotropic effects of Gen reverted by OV in MRH were due to a reduction in sarcoreticular Ca^{2+}

Table 2 Changes in the left ventricular end diastolic pressure (ΔLVEDP in mmHg) induced by ischemia and reperfusion at 30°C and at 37°C in rat hearts of both sexes (MRH, male rat hearts; FRH, female rat hearts). The hearts were perfused with the different treatments summarized in Fig. 1 (*post-hoc* tests: $^{*}P < 0.05$ versus C, $^{\#}P < 0.05$ versus C+Gen-I-R-MRH, number of experiments in parenthesis). The pre-ischemic LVEDP under the respective treatment just before ischemia is in the first column

Treatment	Preischemic LVEDP	5 min I	End of I	5 min R	45 min R
2-way ANOVA at 30°C of changes in LVEDP: By treatment: $F = 3.368$, $P = 0.0021$; By time: $F = 30.61$, $P < 0.0001$					
C (12)	28.3 ± 4.2	−8.5 ± 2.9	11.8 ± 6.6	43.6 ± 10.1	22.4 ± 10.1
C+Gen-I-R MRH (7)	26.4 ± 5.6	−19.5 ± 8.7	−6.9 ± 10.2	13.9 ± 10.0*	−1.4 ± 3.9*
C+OV+Gen-I-R MRH (5)	21.5 ± 5.7	−5.4 ± 3.1	17.1 ± 7.3	17.4 ± 7.8*	10.7 ± 8.5
C+OV-I-R MRH (6)	20.6 ± 5.8	−15.1 ± 4.3	−6.6 ± 5.9	−12.3 ± 9.7*	0.3 ± 7.6
C+Gen-I-R FRH (8)	18.3 ± 5.4	−0.28 ± 3.3	26.0 ± 8.3 #	24.5 ± 6.0	7.2 ± 3.0
C+OV+Gen-I-R FRH (6)	22.0 ± 2.5	−25.7 ± 15.5	7.7 ± 15.4	50.6 ± 8.0	24.5 ± 17.2
C-I-R+Gen FRH (7)	20.7 ± 3.7	−24.3 ± 8.9	4.3 ± 10.9	43.2 ± 5.9	25.3 ± 7.3
C+L-NAME+Gen-I-R FRH (5)	26.3 ± 4.8	−8.6 ± 0.8	17.6 ± 4.9	32.6 ± 6.6	11.9 ± 7.1
2-way ANOVA at 37°C of changes in LVEDP: By treatment: $F = 20.44$, $P < 0.0001$; By time: $F = 17.40$, $P < 0.0001$					
C (11) at 37°C	15.5 ± 1.9	1.8 ± 3.5	5.8 ± 4.1	10.0 ± 2.7	2.0 ± 4.5
C+Gen-I-R MRH (8) at 37°C	19.7 ± 4.8	15.4 ± 5.2	33.0 ± 4.8*	32.3 ± 4.7*	18.7 ± 3.7*
C+Gen-I-R FRH (6) at 37°C	18.0 ± 5.3	−2.1 ± 2.3	23.4 ± 9.9*	27.6 ± 6.2*	9.5 ± 5.6

content, the hearts were pretreated with Gen or Gen+OV and ischemia, and then reperfused with Krebs containing 10 mmol/L caffeine (to release SR Ca^{2+}), and 36 mmol/L Na^{+} (to avoid efflux through sarco-lemmal (SL-NCX)). With such a protocol, R induces a contracture and a rise in Ht, the latter showing the active removal of the cytosolic Ca^{2+} lost from SR.[32] Figure 5a,b shows that the contracture induced by 10 mmol/L caffeine-low (Na^{+}) (ΔLVP *vs* time) in MRH was not significantly changed by Gen and/or OV addition, but both drugs together increased the associated Ht (see area under curves in Table 3). Moreover, Gen reduced the relaxation rate in both FRH and MRH (the fitted exponential k was calculated as: −0.0260 ± 0.0120, −0.0055 ± 0.0018 and −0.0122 ± 0.003/min for control, Gen+FRH and Gen+MRH, respectively, non-parametric comparison: $P = 0.05$). Since under 10 mmol/L caffeine-36 mmol/L Na^{+}-Krebs only the mitochondrial uniporter (UCam) is still able to remove cytosolic Ca^{2+}, these results suggest that Gen reduced UCam flux. In order to assess this hypothesis, 10 mmol/L Pyr was added to Gen before I, and reperfused with 10 mmol/L caffeine-36 mmol/L Na^{+}-Krebs in FRH. It is known that Pyr stimulates the aerobic metabolism and the phosphorylation potential, favoring mitochondrial Ca^{2+}-uptake.[25,33] Figure 5c,d shows that Pyr reduced the ischemic contracture in FRH, suggesting that diastolic contraction was due to inhibition of the UCam. Pyr increased Ht and its area under the curve (AUC-ΔHt) during R, but it did not change the AUC-LVP (Table 3), which is consistent with mitochondrial metabolism stimulation.

In isolated rat cardiomyocytes without stimulation (not separated by sex), the change of perfusion from control HEPES-Krebs (C) to HEPES-Krebs with 10 mmol/L caffeine-36 mmol/L Na^{+} (C-caff-lowNa) induced a transient increase in the basal signal of Fluo-4 (ΔF/Fo), followed by an abrupt fall to basal level (Fig. 6a). The same protocol induced a slow ΔF/Fo increase in Rhod-2 at the time in which the Fluo-4 signal fell. The rise in Rhod-2 signal continued even when the perfusion was back to C (Fig. 6b). The addition of Gen delayed 1.5 min the Fluo-4 ΔF/Fo fall, and significantly reduced the rise in Rhod-2 ΔF/Fo when turning back to C (Fig. 6b). These results also suggest that Gen reduces mitochondrial Ca^{2+} uptake.

Sex influence on Gen effects in guinea pig hearts under I/R and PC

In guinea pig hearts, the treatment with 20 μmol/L Gen did not induce significant changes in inotropism before ischemia in FGH or MGH (Table 1). When compared with control hearts (C), Gen induced a PICR reduction in MGH, but no changes in FGH (Fig. 7a). Nevertheless, muscle economy was not significantly changed during I/R in both groups (Fig. 7b).

In order to evaluate whether the phytoestrogen Gen induces a similar effect to estradiol[34] in ischemic PC, a model was used consisting of one episode of PC (3-min no-flow ischemia followed by 5-min reperfusion before the long ischemia). PC improved PICR during the first 5 min of R in comparison with control hearts (compare group C in Fig. 7 with group PC in Fig. 8; two-way ANOVA for P%: by treatment: $F = 16.08$, $P < 0.0001$; by time: $F = 31.60$, $P < 0.0001$). Moreover, PC improved muscle economy (P/Ht) at 5 min R (two-way ANOVA: by treatment: $F = 21.88$, $P < 0.0001$; by time: $F = 4.416$, $P < 0.0001$). Perfusion of Gen before PC maintained the increase in PICR induced by PC in males, but it was reduced by blockade of mitochondrial ATP-dependent K^{+} channels (mK_{ATP}) with 5-hydroxydecanoate (5HD) (Fig. 8a) without changes in muscle economy (P/Ht) (Fig. 8b). In females, there was a positive inotropism due to Gen and PC before ischemia but there was no improvement in PICR, while 5HD reduced the preischemic effect (Fig. 8c). Muscle economy (P/Ht) was not changed during R (Fig. 8d).

DISCUSSION

This work describes the direct effects of Gen on the mechano-energetic behaviour of isolated hearts stunned as a consequence of a transient ischemia and reperfusion. Gen did not prevent stunning, but under relatively low temperature conditions there was evidence of different effects of this phytoestrogen in a sex-dependent manner in two species, rats and guinea pigs. Although these results were obtained in an *ex vivo* model, they provide elements to understand the mechanism of Gen on myocardial stunning,

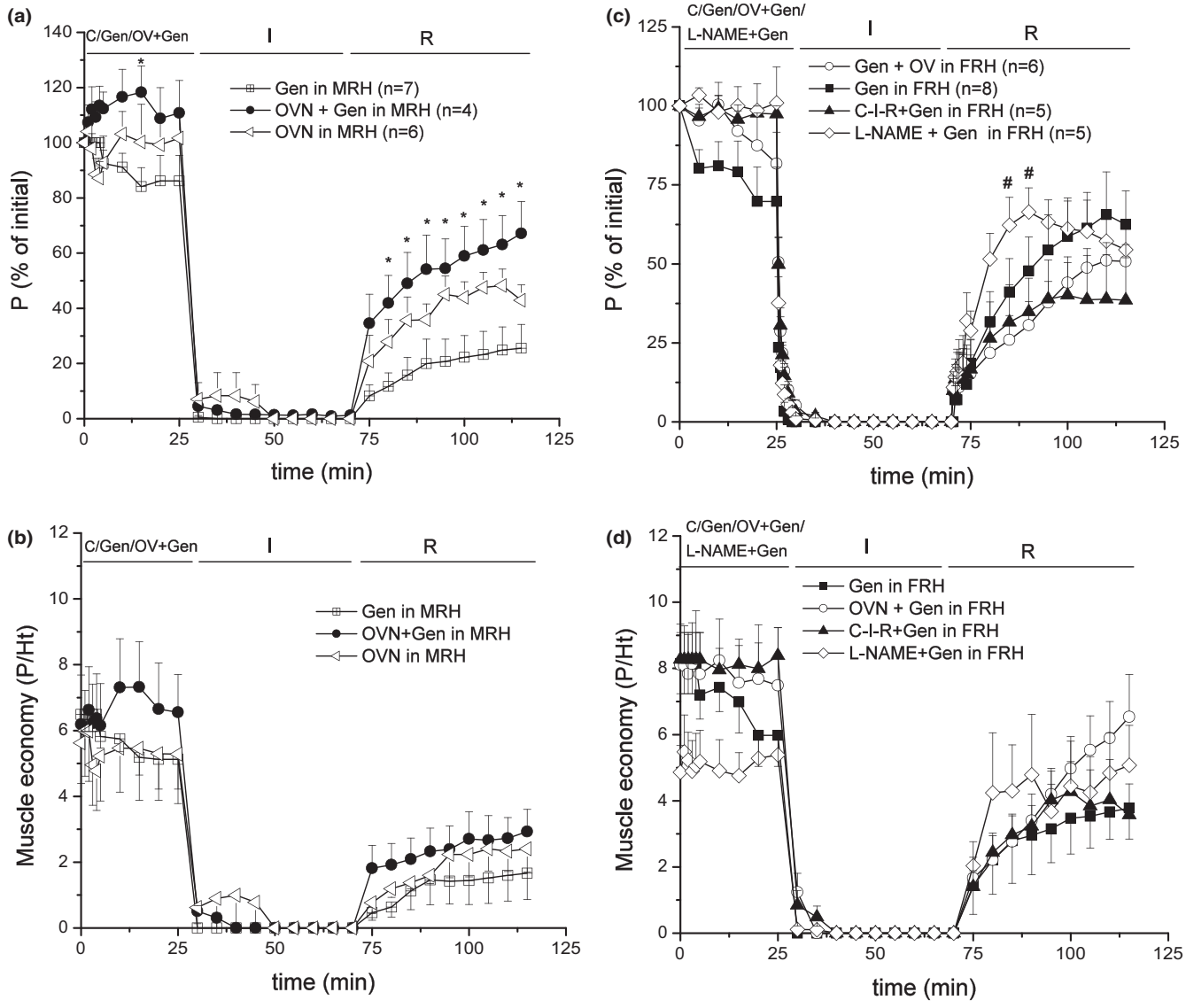

Fig. 4 Effects of 10 μmol/L orthovanadate (OV) with and without 20 μmol/L genistein (Gen) on maximal pressure development (P) (as % of initial value) and muscle economy (P/Ht) in male (a and b) and female (c and d) rat hearts exposed to ischemia (I) and reperfusion (R) at 30°C with stimulation rate of 1 Hz. In FRH (c and d), the effects of adding 30 μmol/L L-NAME to Gen and those of reperfusing with 20 μmol/L Gen are also shown. Results are shown as mean ± SEM (number of experiments). *Post-hoc* tests: $^{*}P < 0.05$ versus C in a–b (Two-way ANOVA: by treatment $F = 50.26$, $P < 0.0001$ for a and $F = 4.854$, $P = 0.0083$ for b; and by time: $F = 123.7$ and $F = 32.31$, both $P < 0.0001$, respectively for a and b), and $^{\#}P < 0.05$ versus C+Gen+OV in c–d (Two-way ANOVA: by treatment $F = 7.88$ and $F = 7.222$, both $P < 0.0001$, and by time: $F = 92.66$ and $F = 47.44$, both $P < 0.0001$, respectively for c and d).

and its properties when used as a supplement to prevent cardiovascular diseases.

Gen was perfused at 20 μmol/L, a concentration that has been demonstrated to be beneficial in some myocardial preparations.[5,11] This concentration is higher than the one found in the plasma of humans consuming the phytoestrogen (around 2 μmol/L). However, it is possible for Gen to accumulate in the heart, as it has been reported to occur in the brain.[35] At physiological temperature, Gen did not modify PICR and had a negative inotropic effect before ischemia in males. Since temperature changes the rate of active transporters, it can be expected that the experiments at 30°C will show a reduced contribution of Ca^{2+} removal mechanisms, without significantly altering channel activity (e.g., Ca^{2+} influx). In fact, the reduction in muscle economy (P/Ht) at 37°C suggests that the heart consumed more chemical energy to maintain an accelerated Ca^{2+} removal from the cytosol. This was made evident by the increase in relative relaxation and contraction rates (−P/P and +P/P) seen in control, an effect that was more prominent after perfusion with Gen (Fig. 2c,d). These results suggest that at 37°C in MRH the SR Ca^{2+} uptake and content must be increased and so it would be able to provide more Ca^{2+} for a higher PICR than at 30°C. Accordingly, the increase in LVEDP induced by Gen at 37°C suggests that more Ca^{2+} was released by SR during I/R. This mechanism seems to compensate the negative effects of Gen seen at low temperature in MRH before and after ischemia. At 30°C the reduction in

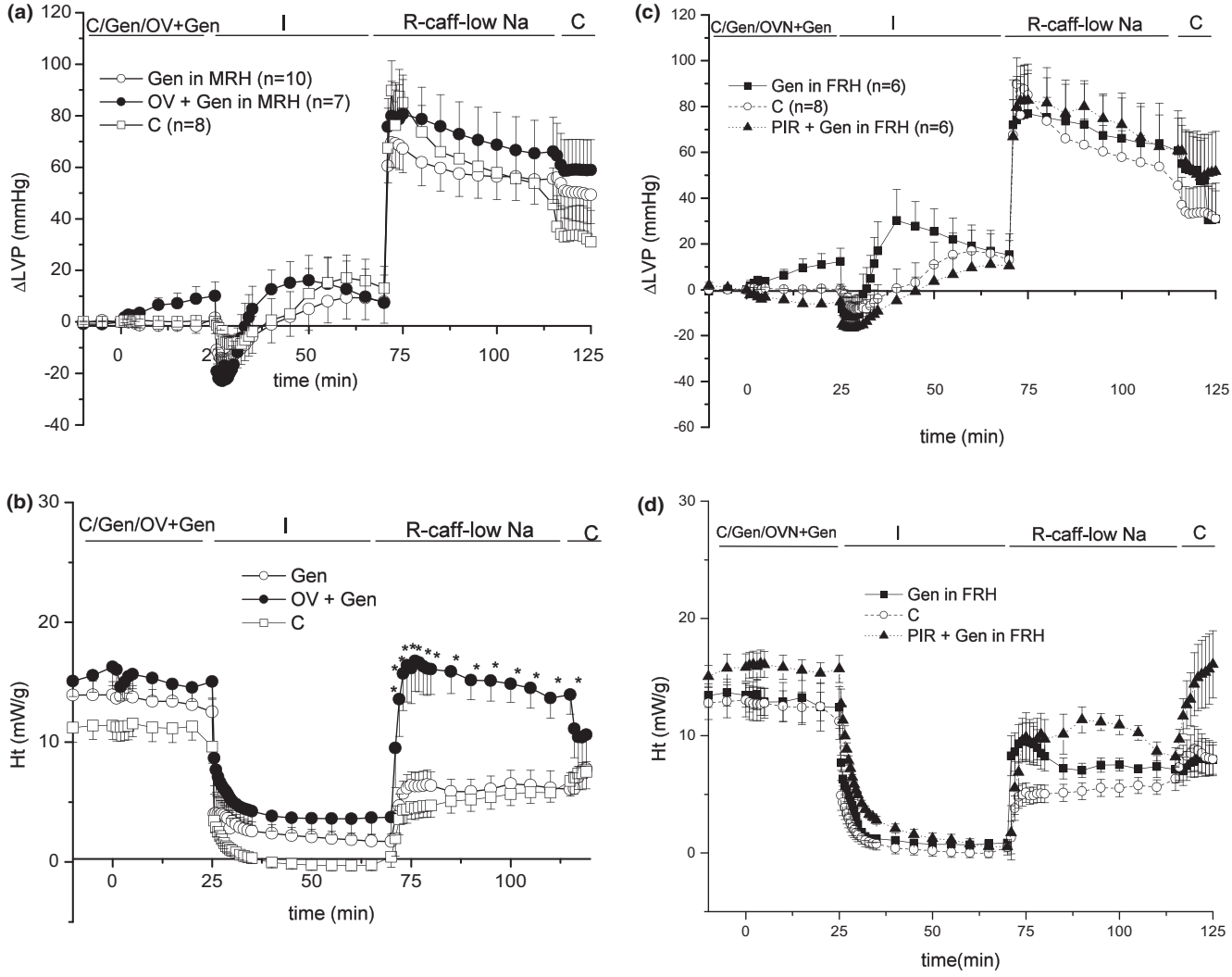

Fig. 5 Effects of 20 μmol/L genistein (Gen) on contracture and total heat rate evoked by post-ischemic reperfusion with 10 mmol/L caffeine-36 mmol/L Na-Krebs, in the absence and presence of 10 μmol/L orthovanadate (OV) on male rat hearts (MRH, a–b) and female rat hearts (FRH, c–d). (a,c) Changes in LVP measured over control preischemic condition (in mmHg). (b,d) Absolute values of heat rate (Ht in mW/g). Results are shown as mean ± SEM (number of experiments) (Two-way ANOVA by treatment $F = 6.411$, $P = 0.0017$ and 273.4, $P < 0.0001$, and by time: $F = 41.92$ and 28.37, respectively for a and b, both $P < 0.0001$. *Post-hoc* tests: $^*P < 0.05$ versus C). In FRH, the effect of 10 mmol/L pyruvate (Pyr) added to Gen perfusion is also shown (Two-way ANOVA by treatment $F = 12.9$ and 224.8, both $P < 0.0001$, and by time: $F = 25.25$ and 37.89, respectively for c and d, both $P < 0.0001$. *Post-hoc* tests: $^*P < 0.05$ *vs* c).

Condition (*n*)	AUC-ΔLVPr (mmHg.min)		AUC-ΔHt (mW. min/g)	
	MRH	FRH	MRH	FRH
$C^{(+)}$	2243.7 ± 291.3 (8)		210.3 ± 20.7 (8)	
Gen	2336.1 ± 255.9 (10)	2419.6 ± 544.0 (6)	169.2 ± 21.2 (10)	237.7 ± 36.8 (6)
Gen + OV	2580.0 ± 376.5 (7)		479.1 ± 75.9* (7)	
Gen + Pyr		2552.9 ± 481.6 (6)		392.5 ± 46.9* (6)
ANOVA	$F = 0.3892$	$F = 0.15$	$F = 13.43$	$F = 9.355$
	$P = 0.68$	$P = 0.86$	$P < 0.0001$	$P = 0.0012$

+, non-treated (C) condition was done independent on sex, and was used for comparing with both, MRH and FRH in the respective column.

Table 3 Area under the curves of intraventricular pressure (ΔLVPr) and heat rate (*ΔHt*) over the line at the end of ischemia, obtained during reperfusion with Krebs-C-10 mmol/L caffeine-36 mmol/L Na^+ of male (MRH) and female (FRH) rat hearts pretreated with the different conditions described in the text. ANOVA results for each column (including the comparison with C in both sexes) are shown, as well as the *post-hoc* tests ($^*P < 0.05$ *vs* the others).

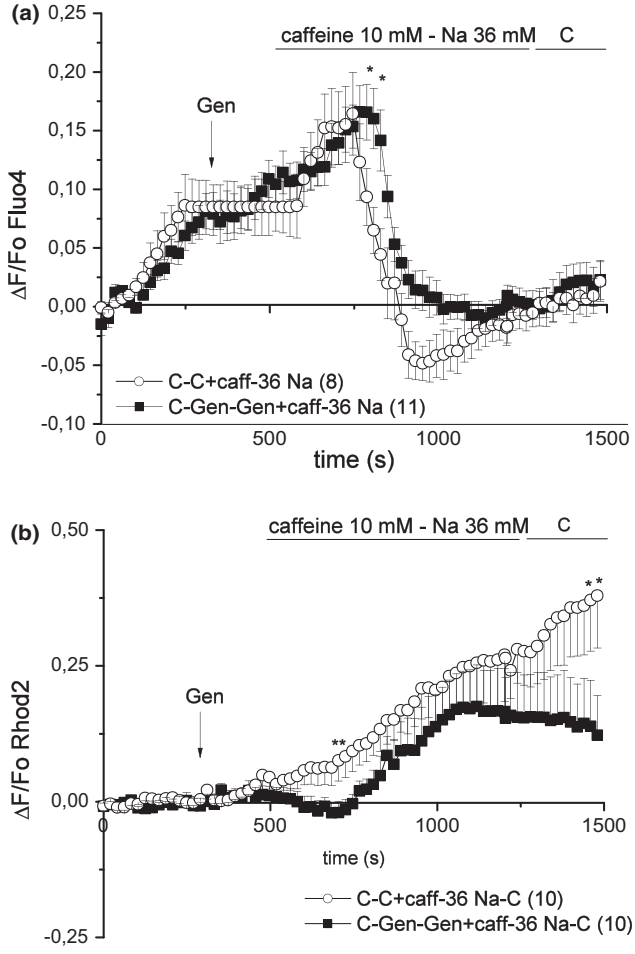

Fig. 6 Changes in free [Ca^{2+}] induced by 20 μmol/L genistein (Gen) in rat cardiomyocytes measured by confocal microscopy and using Fluo-4 to estimate cytosolic [Ca^{2+}] (a) or Rhod-2 to estimate mitochondrial [Ca^{2+}] (b) in cells perfused with Krebs-HEPES containing 10 mmol/L caffeine-36 mmol/L Na^+ and reversion to Krebs-C. Comparison with condition without Gen (c). Results are shown as mean ± SEM (number of experiments). Two-way ANOVA by treatment $F = 16.93$ and 79.07 (both $P < 0.0001$), and by time: $F = 19.78$ and 7.51 (both $P < 0.0001$), respectively for a and b. *Post-hoc* tests: $^*P < 0.05$ versus C without Gen.

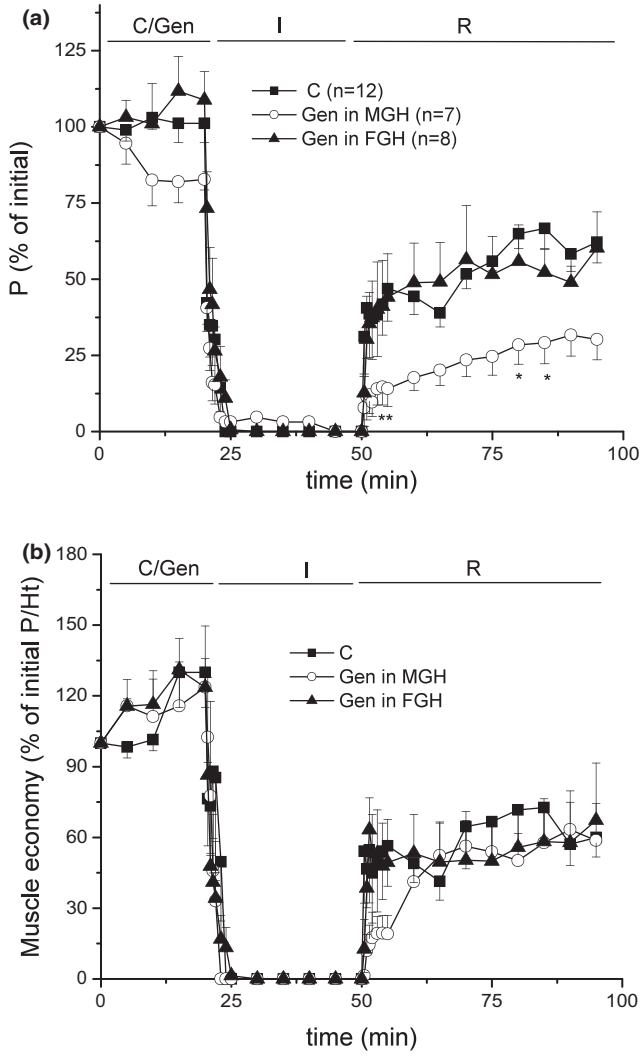

Fig. 7 Effects of 20 μmol/L genistein (Gen) on maximal pressure development (P) (as % of initial value, (a) of male (MGH) and female (FGH) guinea pig hearts exposed to ischemia (I) and reperfusion (R), and respective heat rate (as % of initial Ht, b). Gen was perfused before and during I and the result was compared to control hearts (without pretreatment). Results are shown as mean ± SEM (number of experiments). For a, two-way ANOVA: by treatment: $F = 54.91$, $P < 0.0001$, by time: $F = 46.96$, $P < 0.0001$. For b two-way ANOVA: by treatment: $F = 6.426$, $P = 0.018$, by time: $F = 17.45$, $P < 0.0001$). *Post-hoc* tests are shown at a given time as $^*P < 0.05$ versus C.

PICR and diastolic contracture in MRH suggests a predominant inhibition of Ca^{2+} influx over the increased SR uptake, both induced by Gen. The fact that these effects have been reversed by OV indicates that they depend on the inhibition of TK. OV blocks tyrosine phosphatases, a group of enzymes that permanently counteract the cellular effects of TK.[36] It is known that TK phosphorylates various proteins, producing inactivation of K_{ATP} channels in cardiomyocytes[36] and activation of L-type Ca^{2+} channels.[6,37,38] As an inhibitor of TK, Gen could activate K_{ATP} channels and inhibit Ca^{2+} influx and thus contribute to shortening the action potential and protect the heart from Ca^{2+} overload during I/R. Gen-induced negative inotropism has been described in cardiomyocytes and papillary muscles.[37] Some studies report that the Ca^{2+} current blockade is independent of TK inhibition,[4,6] while others report reversion by inhibition of phosphatases with OV.[5] Belevych *et al.*[4] describe a 50% inhibition of Ca^{2+} current in guinea pig cardiomyocytes with 20 μmol/L Gen. In our experiments, the negative inotropism induced by Gen in MRH before ischemia and the reduction in diastolic contracture and PICR seen at 30°C were all sensitive to OV. On the other hand, in FRH Gen did not modify the PICR and this effect was not changed by OV, suggesting that the Ca^{2+} channel blockade was balanced by a mechanism that improved contractility, and that both effects were mediated by TK inhibition. As for the possible effects mediated by TK inhibition, the increase in SR Ca^{2+} content and/or uptake and the reduction in Ca^{2+} efflux through SL-NCX were described.[8] As previously discussed, Gen increased the relaxation rate (−P/P) in FRH at 30°C before I and in both sexes at 37°C during R, suggesting that Gen improved the SR Ca^{2+} uptake. Sex influence on cardiac Ca^{2+} handling is a recent subject of study

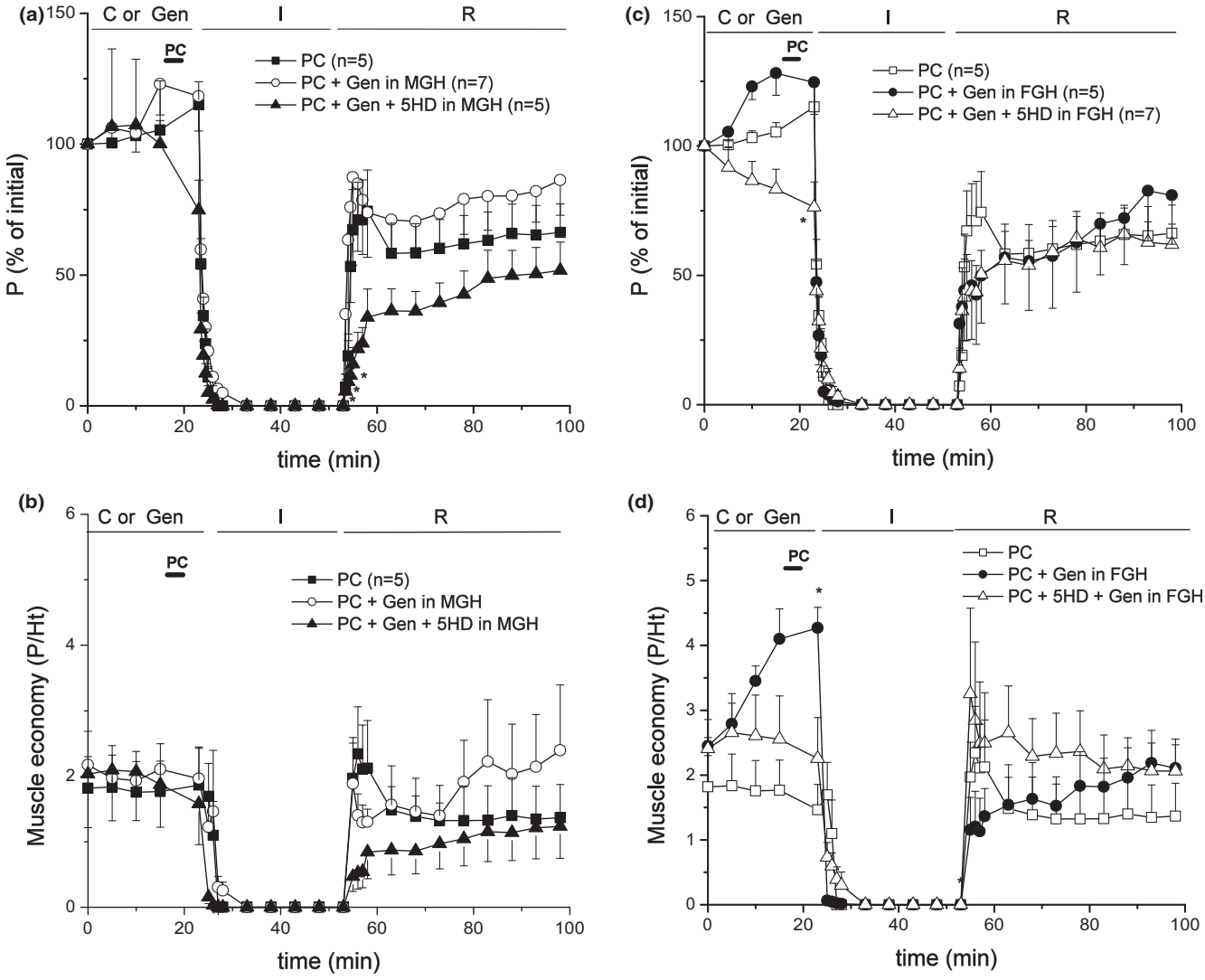

Fig. 8 Effects of 30 μmol/L 5-hydroxydecanoate (5HD) on female guinea pig hearts treated with 20 μmol/L genistein and preconditioning (PC) on maximal pressure development (as % of initial P) and muscle economy (P/Ht, in mmHg.g per mW) of male (a,b) and female (c,d) guinea pig hearts exposed to ischemia (I) and reperfusion (R). Results are shown as mean ± SEM (number of experiments). Two-way ANOVA: in male guinea pig hearts (MGH): by treatment: $F = 30.75$, $P < 0.0001$ and $F = 8.214$, $P = 0.0003$ and by time: $F = 21.26$ and $F = 27.86$, both $P < 0.0001$, respectively for a and b; in female guinea pig hearts (FGH): by treatment: $F = 3.857$, $P = 0.0218$ and $F = 7.345$, $P = 0.0007$, by time: $F = 33.57$ and $F = 10.48$, both $P < 0.0001$, respectively for c and d. *Post-hoc* tests $^*P < 0.05$ versus C.

approached by several methodologies.[39] This is important to understand the basis of the cardiovascular protection present in women before menopause, as well as the consequences of post-menopausal oestrogen replacement therapy. It was reported that the Ca^{2+} transient and the gain of excitation-contraction coupling in cardiomyocytes is lower in young females than in males, while their Ca^{2+} current densities are similar.[39] According to this, the increase in SR Ca^{2+} uptake induced by Gen could relatively improve contractility more in female than in male hearts, thus explaining the sex differences found in PICR at 30°C.

Reperfusion of ischemic hearts with Krebs-10 mmol/L caffeine-36 mmol/L Na^+ causes SR Ca^{2+} content release. Under these conditions, relaxation mainly depends on mitochondrial Ca^{2+} uptake, since neither SL-NCX nor SERCA are functional under caffeine.[24,32] The fact that Gen did not modify AUC-LVP or AUC-Ht in rat hearts suggests that SR content and Ca^{2+} cycling were not affected by the drug after ischemia. This absence of effect of the phytoestrogen resembles an earlier report in non-ischemic FRH and MRH that developed the same sarcoreticular Ca^{2+} content estimated by caffeine.[40] Ht is related to the ATP hydrolysis used in the futile SERCA cycle that is induced by caffeine, as well as to the increased mitochondrial metabolism in response to the $\Delta\Psi m$ dissipation produced by the UCam flux. The futile Ca^{2+} cycling through SERCA would release at least 1.25 mW/g, since caffeine moves about 31 nmol Ca^{2+}/g per second in guinea pigs[14] and even more in rat hearts (SERCA cycling dissipates 40 kJ/mol Ca^{2+}, moving 2 Ca^{2+} for each ATP hydrolyzed). Mitochondria consume 477 kJ/mol O_2[41] and the electron chain moves 12 H^+ by O_2 and 2 H^+ by Ca^{2+}. Thus, UCam fluxes of 32 to 350 nmol Ca^{2+}/g/s, estimated under physiological and overload conditions in isolated mitochondria, respectively,[42] could dissipate between 3 and 31 mW/g. In fact,

during the reperfusion with caff-low Na^+-Krebs, Ht reached 4.5 and 6.5 mW/g for non-treated and Gen groups, respectively, and 16 mW/g for the OV+Gen group in MRH (Fig. 5b). In FRH treated with Gen, the same reperfusion increased Ht to 9.5 mW/g, while the addition of Pyr increased Ht to 11.3 mW/g (Fig. 5d). Pyr also caused the rise of the caffeine-induced contracture relaxation rate. Both effects suggest an increased stimulation of UCam flux.[25,34] On the other hand, in non-ischemic rat cardiomyocytes, the perfusion with 10 mmol/L caffeine-36 mmol/L Na^+-Krebs induced a biphasic response in the ΔF/Fo of Fluo-4. Thus, free cytosolic (Ca^{2+}) was transiently increased during Ca^{2+} release from SR, but then it fell, indicating Ca^{2+} being removed. The simultaneous slow increase in Rhod-2 signal confirmed that Ca^{2+} removal was driven by UCam, resulting in mitochondrial Ca^{2+} accumulation. Gen delayed the fall in cytosolic Ca^{2+} during caff-low Na^+ perfusion, and reduced the rise in $(Ca^{2+})m$, suggesting again that Gen inhibited the UCam flux during cytosolic Ca^{2+} overload. Reduced mitochondrial Ca^{2+} uptake could contribute to the protection of mitochondria from Ca^{2+} overload, but will reduce metabolism and ATP resynthesis in post-ischemic recovery.

The positive inotropism induced by Gen in FGH agrees with the results of contractility in non-ischemic guinea pig papillary muscles, which increased sarcoreticular Ca^{2+} content.[7,8] The beneficial effect of Gen has been reported in models of severe ischemia, where Gen reduced infarct size in rabbits and guinea pigs.[12] Nevertheless, it is important to evaluate whether Gen could prevent stunning in transitory ischemia, which may be the first event of cardiac disease in a patient. To our knowledge, this is the first report in which Ca^{2+} handling was energetically evaluated during stunning to study Gen effects. Another cardioprotective situation explored in cardiology is PC, and we have shown that Gen maintained the beneficial effect of one episode of PC in guinea pig hearts. It is known that at least one ischemic episode of 3–5 min, followed by a minimal 5-min reperfusion is cardioprotective. The mechanism involves the activation of PKC and the sarcolemmal and mitochondrial K_{ATP} channels, as well as intracellular MAPK and TK pathways.[43,44] This mechanism was involved in the increase of PICR obtained by the combination of Gen and PC in guinea pig hearts, since it was prevented by blocking mK_{ATP} channels selectively with 30 μmol/L 5HD. The activation of K_{ATP} channels was also described for estradiol, which promoted a cardioprotection in rabbit hearts similar to PC and was caused by a non-genomic effect.[44] More recently, it has been shown that estradiol acutely perfused in isolated rat hearts induced cardioprotection by reducing the accumulation of TNF-α as PC did, but without additive effects.[34] As it was suggested for rat hearts,[45] neither PC nor Gen+PC changed muscle economy during R, but 5HD slightly reduced it at the start of R, in agreement with an increased mitochondrial metabolism that is the consequence of an increased $(Ca^{2+})m$, as previously shown.[23]

In models of severe ischemia with infarct, mitochondria were essential for cardioprotection and the critical mediator was PKCε, which phosphorylates mitochondrial permeability transition pore (MPTP) proteins, preventing their opening during the reperfusion that follows severe ischemia. Estrogens activate PKCε and endothelial nitric oxide synthase (eNOS) through pathways triggered by different estrogenic receptors (ERα, ERβ, and GPER), which include the activation of Akt/PI3K and ERK1/2, inhibition of GSK-3β, and activation of mK_{ATP} channels.[46,47] The positive inotropism of Gen in non-ischemic guinea pig papillary muscles was not attributed to effects on ERα or ERβ, or to the PI3K pathway.[7] Nevertheless, Gen intravenously injected after ischemia in rabbits was preventive of infarct and involved PI3K activation and two genomic receptors (ERα, ERβ).[12] The pathways involved differ with the severity of ischemia. In this stunning model, MPTP was not opened[23,24] and so it cannot be involved in the cardioprotective effect of Gen. Possible pathways could be PI3K/Akt and its downstream effector eNOS, as described in PC[48] and in the cardioprotection of estradiol via GPER.[31] Nevertheless, the effects of Gen were unaffected by NOS inhibition with L-NAME in our stunned rat hearts. Then, the effects of Gen in stunned hearts seem more related to mechanisms of Ca^{2+} handling between SR and mitochondria, as we have previously discussed.

In conclusion, this work demonstrates that Gen prevents diastolic contracture during stunning, but does not improve PICR. Gen induces changes in Ca^{2+} handling that are sex and temperature dependent. The mechano-energetic behaviour in the presence of Gen suggests a reduction in Ca^{2+} influx, an increase in sarcoreticular Ca^{2+} uptake and the inhibition of mitochondrial Ca^{2+} uptake in stunned rat and guinea pig hearts. The first two mechanisms were associated with TK inhibition, and the third mechanism reduced the risk of mitochondrial Ca^{2+} overload during ischemia/reperfusion. Also, Gen maintained the cardioprotective effect of PC related to the activation of mK_{ATP} channels. It can be concluded that the beneficial effects of Gen as a preventive of stunning are connected with the preservation of a low mitochondrial Ca^{2+} level, and a high relaxation rate. The consequences of these effects are the prevention of diastolic contracture, but a reduction in muscle economy due to the high energy consumption.

METHODS

Animals

All procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and Resolution 1047, Annex II of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) of the Argentine Republic. They also agree with the latest directives of the European Union for laboratory animal care (2010) and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition; 2013).

Isolated heart preparation and contractile measurements

Adult Sprague-Dawley rats (200–250 g in weight) were heparinized (unfractionated heparin; 2000 IU) and anaesthetized with an overdose of pentobarbital (60 mg/kg, via intraperitoneal (i.p.) injection). Adult guinea pigs were also heparinized (2000 IU) and anaesthetized with 25% urethane (0.6 mL/100 g, via i.p. injection), because it provides better skeletal muscle relaxation than pentobarbital.[24] Hearts were rapidly excised and perfused through coronary arteries by the Langendorff method, as previously described.[23,25,32] In rats, atria were removed and the spontaneous beating was stopped by applying pressure on the focus; in guinea pigs, the hearts continued beating spontaneously.

A latex balloon was introduced in the left ventricle, connected by a flexible cannula to a Bentley Nevada pressure transducer (Model 900; Bentley DEL900, Minden, NV, USA). While continuously perfused, the heart was introduced into a calorimetric chamber, which was then closed and submerged in water kept at a controlled temperature (± 0.01°C). Two electrodes electrically stimulated the rat hearts with a 5 V–5 ms stimulus, using an electrical stimulator (Letica LE12406, Barcelona, Spain). The spontaneous heart rate in guinea pig hearts at 30°C was 2.29 ± 0.08 beats per second (n = 37).

Rat hearts were perfused with control Krebs (C) under two conditions: 30°C and 1 Hz of stimulation, or 37°C and a 3-Hz rate. The perfusion flow rate was kept constant at 7 mL/min/g by a peristaltic pump (Gilson Minipuls, Villiers Le Bel, France). To prevent the heart oedema caused by a high flow rate, the perfusion flow was calculated using the equation $CF = 7.43*HW^{0.56}$ (where CF is coronary flow and HW is heart weight).[49] This flow was sufficient to develop an optimal maximal pressure (P) without significant oedema. Its absence was confirmed in rat hearts by the water content after I/R, either at 30°C (81.0 ± 0.3%, n = 78, 0.750 ± 0.026 g wet weight) or at 37°C (81.1 ± 0.8%, n = 22, 0.634 ± 0.02 g wet weight). These values are not different from the water content reported in rat hearts without I/R at 25°C.[19] In guinea pigs, the water content after I/R at 30°C was similar to the one in rats (82.9 ± 1.2%, n = 39, 1.108 ± 0.055 g wet weight). The experimental conditions of 30°C and 1 Hz were chosen from previous studies[23,24] in which the energetics and cellular mechanisms of stunning were characterized in rat and guinea pig hearts. Nevertheless, since those conditions were subphysiological, the effects of Gen in rat hearts were also evaluated at 37°C and 3 Hz. In both conditions, the I/R periods were chosen to obtain about a 50% PICR without significant infarct (model of stunning).

Isovolumic LVP was continuously recorded at optimal volume, simultaneously with total heat rate (Ht). LVP was recorded either in a Beckman R511A polygraph (Braintree, MA, USA) A/D converted through a National Instruments P-516 acquisition system, or directly in a PowerLab 2/26 two-channel digital acquisition system (AD Instruments, Bella Vista, NSW, Australia). The maximal pressure development of contraction over the diastolic level (P), and the changes in diastolic pressure over the steady initial condition in Krebs-C (ΔLVEDP) were calculated from LVP and expressed in mmHg. The maximal contraction and relaxation rates were calculated from the first derivative and normalized by P (+P/P = +dP/dt/P and −P/P = −dP/dt/P). Also, during I/R, contractility was expressed as a percentage of the steady initial value of P in each heart.

Calorimetric measurements

The calorimetric system has been described previously.[18,19,24–26] Briefly, the internal chamber has two ceramic modules, each with 127 thermosensitive units (Melchor Thermoelectrics, Trenton, NJ, USA), which detect changes in temperature between the inside (heart) and the outside (bath). The calibration procedure can be found in previous publications.[18,19,23,24,32] The calorimeter was submerged in a water bath at a controlled temperature (± 0.01°C). After stabilization, the calorimetric signal was continuously measured in the presence and absence of perfusion and corrected with the baseline obtained without the heart present in the chamber. After the experiment, the heat rate (Ht) was calculated and expressed in mW/g wet weight. Also, the ischemic and post-ischemic measurements were expressed as a percentage of the steady initial Ht obtained in Krebs-C. In turn, muscle economy was calculated as P/Ht ratio over time of reperfusion.

Experimental protocols

After a 40-min stabilization with Krebs-C, an initial value of P and Ht was recorded. The hearts were then exposed to different treatments, followed by the respective model of stunning for a given period of no-flow ischemia (I) and 45 min of reperfusion (R). Gen concentration (20 μmol/L) was chosen from previous studies.[5,11] Gen I/R protocols were performed in four groups, unless otherwise indicated: female rat hearts (FRH), male rat hearts (MRH), female guinea pig hearts (FGH) and male guinea pig hearts (MGH). Control I/R (no drugs) protocols were performed independently of sex.

Protocols are shown in Fig. 1. In rat hearts at 37°C, the treatments before ischemia were: (i) C (Krebs-C); (ii) C/C+Gen (Krebs-C with 20 μmol/L Gen) for 25 min. In rat hearts at 30°C, the treatments before ischemia were: (i) C (Krebs-C); (ii) C/C+Gen (Krebs-C with 20 μmol/L Gen) for 25 min; (iii) C/C+OV (Krebs-C with 10 μmol/L sodium OV) for 5 min/C+OV+Gen (Krebs-C with 10 μmol/L sodium OV and 20 μmol/L Gen) for 20 min; (iv) C/C+OV (Krebs-C with 10 μmol/L sodium OV) for 25 min; (v) C/C+L-NAME (Krebs-C with 30 μmol/L L-NAME) for 5 min/C+L-NAME+Gen (Krebs-C with 30 μmol/L sodium OV and 20 μmol/L Gen) for 20 min. All these protocols were reperfused with Krebs-C for 45 min. Furthermore, in order to assess if the effects of Gen revert during R, one group with Krebs-C before ischemia was reperfused with 20 μmol/L Gen (C/I/R+Gen).

To evaluate whether Gen and/or OV had an effect on the sarcoreticular Ca^{2+} content, the treated hearts were reperfused with Krebs containing 10 mmol/L caffeine and 36 mmol/L Na^+ (R-caff-low Na^+) as in previous studies.[24,32] Preischemic treatments were: C+Gen, C+OV+Gen and C+Gen+Pyr (with 10 mmol/L Pyr).

In guinea pig hearts, at 30°C, the preischemic treatments were: (i) C (Krebs-C); (ii) C/C+Gen (Krebs-C with 20 μmol/L Gen) for 25 min; (iii) C/PC (preconditioning of 3-min no-flow ischemia and 5-min reperfusion before prolonged ischemia); (iv) C/C+Gen+PC (Krebs-C with 20 μmol/L Gen for 15 min, followed by preconditioning of 3-min no-flow ischemia and 5-min reperfusion with Gen); (v) C/C + 5HD/C + 5HD+Gen+PC (Krebs-C with 30 μmol/L 5HD for 5 min, followed by Krebs-C with 30 μmol/L 5HD and 20 μmol/L Gen for 15 min and preconditioning).

In order to evaluate whether this model of I/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. By 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, Media Cybernetics, Rockville, MD, USA).

Isolation of cardiac myocytes

Ventricular myocytes were isolated from adult rat hearts (200–280 g in weight) as previously described.[24] Once the animal was anaesthetized, the heart was quickly removed and placed in a Langendorff system. The heart was perfused with a modified Krebs-24-HEPES solution virtually free of Ca^{2+} for 5 min. Subsequently, the solution was changed to Krebs-24-HEPES with 50 μmol/L $CaCl_2$, 0.1 mg/mL collagenase P (Roche, Mannheim, Germany) and 0.02 mg/mL protease XIV (Sigma-Aldrich, St Louis, MO, USA). After 14 min, the solution was changed again to an enzyme free-50 μmol/L Ca^{2+} Krebs-24 HEPES solution. All solutions were bubbled with O_2 and maintained at 37°C. Following this, the ventricles were removed and minced, and the pieces were shaken in the low-Ca^{2+} solution for 10 min and then filtered. The Ca^{2+} concentration was raised in steps to 1 mmol/L Ca^{2+} and myocytes were stored for up to 6 h in this HEPES-buffered saline solution.

Confocal microscopy of cardiomyocytes

To measure intracellular free Ca^{2+} signals, isolated rat cardiomyocytes were loaded with 12 μmol/L Fluo-4 AM (Molecular Probes/Invitrogen, Carlsbad, CA, USA) for 15 minu at 35–37°C. Another group of cells was loaded with 3 μmol/L Rhod-2 AM (Molecular Probes/Invitrogen) for 1 h at 4°C, followed by a wash-up at 37°C for at least 1 h in order to load the fluorophore only in the mitochondrial compartment.[24,50] Myocytes were then placed in a laminin-precoated perfusion chamber and superfused with Krebs-24 HEPES solution containing 2 mmol/L Ca^{2+} until stabilization, at 30°C. A Leica SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) was used to measure changes in fluorescence while cells were at rest. The images were analyzed using the Leica LAS AF Lite 2.2.1 software. When loaded with Fluo-4, cells were excited at 488 nm, and we measured the changes in fluorescence emission at wavelengths higher than 505 nm in a defined area in the cell (ROI). When loaded with Rhod-2, cells were excited at 540 nm, and the changes in fluorescence emission from one ROI per cell were monitored through time at wavelengths higher than 560 nm. Results were expressed as self-ratio emission fluorescence intensity (F/F_o), where F_o is the initial value. Changes in F/F_o over time were calculated by nonlinear adjustment of the baseline obtained during C perfusion at the beginning and end of each protocol, using Origin 7.0 (OriginLab, Northampton, MA, USA). Signals were recorded each 20 s for 20 or 25 min, while myocytes were perfused with the following protocol: control Krebs-24 HEPES solution (C) for 5 min, followed by C + 20 μmol/L Gen (C+Gen) for 5 min, and then by C with 10 mmol/L caffeine-36 mmol/L Na (C-caff-low Na) in the presence of Gen for 10 min, and finally return to C for 5 mins. Another analogue protocol in the absence of Gen was done. Each protocol was applied to cells loaded with Fluo-4 or Rhod-2.

Solutions and drugs

The Krebs-C solution contained (in mmol/L): 1 $MgCl_2$, 125 NaCl, 0.5 NaH_2PO_4, 7 KCl, 2 $CaCl_2$, 25 $NaHCO_3$, and 6 dextrose, bubbled with 95% O_2-5% CO_2. Gen concentration was chosen as 20 μmol/L from previous studies.[5] Gen (Sigma) was prepared as a 20 mmol/L aqueous solution, OV (Sigma-Aldrich) was prepared in water at 10 mmol/L, L-NAME (Sigma) was prepared in water at 30 mmol/L, and 5HD (ICN Biomedicals, Aurora, OH, USA) was prepared in dimethylsulfoxide at 30 mmol/L. All the drugs were added to Krebs-C the day of the experiment. Pyruvic acid sodium salt and caffeine (ICN Biomedicals) were directly dissolved in Krebs the day of the experiment. The osmolality of Krebs C-caff-low Na^+ medium was maintained by the addition of 217 mmol/L sucrose.

In order to isolate cardiomyocytes, the solution was (in mmol/L): 126 NaCl, 4.4 KCl, 1.0 NaH_2PO_4, 5 $MgCl_2$, 24 HEPES, 22 dextrose, 20 taurine, 5 creatine, 0.5 Na-Pyr, adjusted with NaOH to pH 7.4 and bubbled with O_2 100%. During the experiment, cardiomyocytes were superfused in Krebs-24-HEPES solution with the following composition (in mmol/L): 126 NaCl, 4.4 KCl, 1 $MgCl_2$, 24 HEPES, 2 $CaCl_2$ and 11 dextrose, adjusted with NaOH to pH 7.4 and bubbled with O_2 100%. For Fluo-4 experiments, 0.5 mmol/L probenecid was added.

Statistical analysis

Results were expressed as mean ± SEM. Multiple comparisons by two-way ANOVA for repeated measures (factors were treatment and time) were done for the respective groups of experiments. Also, paired *t*-tests and one-way ANOVA were used when appropriate. Post-hoc Bonferroni paired tests were done between the multiple treatments when a significant difference was found by ANOVA, and their results are shown in each figure. A $P < 0.05$ was considered significant. All statistical analyses were performed using GRAPHPAD PRISM 4 software.

ACKNOWLEDGEMENTS

This work was supported by grants from the Universidad Nacional de La Plata (11X-513, 2009–2012, and 11X-642, 2013–2016) and from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET, PIP 00213/2011–2013). The authors thank their colleague Natalia Torres, Ph.D. (CVRTI, University of Utah, USA) for her generous help in the edition of our manuscript and Magdalena Ponce for her English editing and proofreading services.

REFERENCES

1. Figtree GA, Griffiths H, Lu YQ, Webb CM, MacLeod K, Collins P. Plant-derived estrogens relax coronary arteries *in vitro* by a calcium antagonistic mechanism. *J. Am. Coll. Cardiol.* 2000; **35**: 1977–85.
2. Li HF, Wang LD, Qu SY. Phytoestrogen genistein decreses contractile response of aortic artery *in vitro* and arterial blood pressure *in vivo*. *Acta Pharmacol. Sin.* 2004; **25**: 313–8.
3. Akiyama T, Ishida J, Nakagawa S *et al.* Genistein, a special inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* 1987; **262**: 5592–5.
4. Belevych AE, Warrier AS, Harvey RD. Genistein inhibits cardiac L-type Ca^{2+} channel activity by a tyrosine kinase-independent mechanism. *Mol. Pharmacol.* 2002; **62**: 554–65.
5. Ji E, Wang C, He R. Effects of genistein on intracellular free-calcium concentration in guinea pig ventricular myocytes. *Acta Physiol. Sin.* 2004; **56**: 204–9.

6. Yokoshiki H, Sumii K, Sperelakis N. Inhibition of L-type calcium current in rat ventricular cells by the tyrosine kinase inhibitor, genistein and its inactive analog, daidzein. *J. Mol. Cell. Cardiol.* 1996; **28**: 807–14.
7. Li H, Zhang Y, Tian Z, Qiu X, Gu J, Wu J. Genistein stimulates myocardial contractility in guinea pigs by different subcellular mechanisms. *Eur. J. Pharmacol.* 2008; **597**: 70–4.
8. Liew R, Stagg MA, Chan J, Collins P, MacLeod KT. Gender determines the acute actions of genistein on intracellular calcium regulation in the guinea pig heart. *Cardiovasc. Res.* 2004; **61**: 66–76.
9. Kloner RA, Jennings RB. Consequences of brief ischemia: Stunning, preconditioning, and their clinical implications: Part 1. *Circulation* 2001; **104**: 2981–9.
10. Deodato B, Altavilla D, Squadrito G, Campo GM, Arlotta M, Minutoli L. Cardioprotection by the phytoestrogen genistein in experimental myocardial ischemia-reperfusion injury. *Br. J. Pharmacol.* 1999; **128**: 1683–90.
11. Al-Nakkash L, Markus B, Bowden K, Batia LM, Prozialeck WC, Broderick TL. Effects of acute and 2-day genistein treatment on cardiac function and ischemic tolerance in ovariectomized rats. *Gend. Med.* 2009; **6**: 488–97.
12. Tissier R, Waintraub X, Couvreur N *et al.* Pharmacological postconditioning with the phytoestrogen genistein. *J. Mol. Cell. Cardiol.* 2007; **42**: 79–87.
13. Terracciano CMN, MacLeod KT. Measurements of Ca^{2+} entry and sarcoplasmic reticulum Ca^{2+} content during the cardiac cycle in guinea pig and rat ventricular myocytes. *Biophys. J* . 1997; **72**: 1319–26.
14. Bers DM. *Excitation-Contraction Coupling and Cardiac Contractile Force*, 2nd edn. Kluwer Academic Publishers, Dordrecht, the Netherlands, 2001.
15. Bolli R, Marban E. Molecular and cellular mechanisms of myocardial stunning. *Physiol. Rev.* 1999; **79**: 609–34.
16. Bernardi P, Di Lisa F. The mitochondrial permeability transition pore: Molecular nature and role as a target in cardioprotection. *J. Mol. Cell. Cardiol.* 2015; **78**: 100–6.
17. Garcia-Dorado D, Ruiz-Meana M, Inserte J, Rodriguez-Sinovas A, Piper HM. Calcium-mediated cell death during myocardial reperfusion. *Cardiovasc. Res.* 2014; **94**: 168–80.
18. Ponce-Hornos JE, Ricchiuti NV, Langer GA. On-line calorimetry in the arterially perfused rabbit interventricular septum. *Am. J. Physiol.* 1982; **243**: H289–95.
19. Ponce-Hornos JE, Bonazzola P, Marengo F, Consolini AE, Márquez MT. Tension-dependent and tension-independent energy components of heart contraction. *Pflugers Archiv-Eur. J. Physiol.* 1995; **429**: 841–51.
20. Consolini AE, Ragone MI, Bonazzola P. Mitochondrial and cytosolic calcium in rat hearts under high-K^+ cardioplegia and pyruvate: Mechano–energetic performance. *Can. J. Physiol. Pharmacol.* 2011; **89**: 485–96.
21. Consolini AE, Márquez MT, Ponce-Hornos JE. A comparison of no-flow and low-flow ischemia in the rat heart: An energetic study. *Can. J. Physiol. Pharmacol.* 2001; **79**: 551–8.
22. Consolini AE, Bonazzola P. Energetics of the Ca^{+2} homeostasis during ischemia-reperfusion on neonatal rat hearts under high-K^+ cardioplegia. *Can. J. Physiol. Pharmacol.* 2008; **86**: 866–79.
23. Ragone MI, Consolini AE. Role of the mitochondrial Ca^{2+} transporters in the high-$[K^+]_o$ cardioprotection of rat hearts under ischemia and reperfusion: A mechano-energetic study. *J. Cardiovasc. Pharmacol.* 2009; **54**: 213–22.
24. Ragone MI, Torres NS, Consolini AE. Energetic study of cardioplegic hearts under ischaemia/reperfusion and $[Ca^{2+}]$ changes in cardiomyocytes of guinea pig: Mitochondrial role. *Acta Physiol (Oxf).* 2013; **207**: 369–84.
25. Bonazzola P, Ragone MI, Consolini AE. Effects of pyruvate on the energetics of rat ventricles stunned by ischemia-reperfusion. *Can. J. Physiol. Pharmacol.* 2014; **92**: 386–98.
26. Ragone MI, Bonazzola P, Colareda GA, Consolini AE. Cardioprotection of hyperthyroidism on the stunned rat heart under ischaemia/reperfusion: Energetics and role of mitochondria. *Exp. Physiol.* 2015; **100**: 680–97.
27. Han JC, Taberner AJ, Kirton RS, Nielsen PM, Smith NP, Loiselle DS. A unique micromechanocalorimeter for simultaneous measurement of heat rate and force production of cardiac trabeculae carneae. *J. Appl. Physiol.* 1985; **2009**: 946–51.
28. Han JC, Tran K, Nielsen PM, Taberner AJ, Loiselle DS. Streptozotocin-induced diabetes prolongs twitch duration without affecting the energetics of isolated ventricular trabeculae. *Cardiovasc. Diabetol.* 2014; **13**: 79.
29. Donato M, Buchholz B, Rodríguez M *et al.* Role of the parasympathetic nervous system in cardioprotection by remote hindlimb ischaemic preconditioning. *Exp. Physiol.* 2013; **98**: 425–34.
30. Di Salvo J, Semenchuk LA, Lauer J. Vanadate-induced contraction of smooth muscle and enhanced protein tyrosine phosphorylation. *Arch. Biochem. Biophys.* 1993; **304**: 386–91.
31. Fukumoto T, Tawa M, Yamashita N, Ohkita M, Matsumura Y. Protective effects of 17beta-estradiol on post-ischemic cardiac dysfunction and norepinephrine overflow through the non-genomic estrogen receptor/nitric oxide-mediated pathway in the rat heart. *Eur. J. Pharmacol.* 2013; **699**: 74–80.
32. Consolini A, Ragone MI, Conforti P, Volonté MG. Mitochondrial role in ischaemia-reperfusion of rat hearts exposed to high-K^+ cardioplegia and clonazepam: Energetic and contractile consequences. *Can. J. Physiol. Pharmacol.* 2007; **85**: 483–96.
33. Mallet RT, Sun J, Knott EM, Sharma AB, Olivencia-Yurvati AH. Metabolic cardio-protection by pyruvate: Recent progress. *Exp. Biol. Med. (Maywood)* 2005; **230**: 435–43.
34. Babiker FA, Hoteit LJ, Joseph S, Mustafa AS, Juggi JS. The role of 17-beta estradiol in ischemic preconditioning protection of the heart. *Exp. Clin. Cardiol.* 2012; **17**: 95–100.
35. Setchell KD. Phytoestrogens: The biochemistry, physiology, and implications for human health of soy isoflavones. *Am. J. Clin. Nutr.* 1998; **68**: 1333S–46S.
36. Kwak YG, Park SK, Cho KP, Chae SW. Reciprocal modulation of ATP-sensitive K^+ channel activity in rat ventricular myocytes by phosphorylation of tyrosine and serine/threonine residues. *Life Sci.* 1996; **58**: 897–904.
37. Ogura T, Shuba LM, McDonald TF. L-type Ca^{2+} current in guinea pig ventricular myocytes treated with modulators of tyrosine phosphorylation. *Am. J. Physiol.* 1999; **276**: H1724–33.
38. Katsube Y, Yokoshiki H, Nguyen L, Yamamoto M, Sperelakis N. Inhibition of Ca^{2+} current in neonatal and adult rat ventricular myocytes by the tyrosine kinase inhibitor, genistein. *Eur. J. Pharmacol.* 1998; **345**: 309–14.
39. Parks RJ, Howlett SE. Sex differences in mechanisms of cardiac excitation-contraction coupling. *Pflugers Arch-Eur. J. Physiol.* 2013; **465**: 747–63.
40. Farrell SR, Ross JL, Howlett SE. Sex differences in mechanisms of cardiac excitation-contraction coupling in rat ventricular myocytes. *Am. J. Physol, Heart Circ. Physiol.* 2010; **299**: H36–45.
41. Curtin NA, Woledge RA. Energy changes and muscular contraction. *Physiol. Rev.* 1978; **58**: 690–761.
42. Arieli Y, Gursahani H, Eaton MM, Hernandez LA, Schaefer S. Gender modulation of $Ca(^{2+})$ uptake in cardiac mitochondria. *J. Mol. Cell. Cardiol.* 2004; **37**: 507–13.
43. Das M, Das DK. Molecular mechanism of preconditioning. *IUBMB Life* 2008; **60**: 199–203.
44. Sarkar BCh. Similarities between ischemic preconditioning and 17 beta-estradiol mediated cardiomyocyte KATP channel activation leading to cardioprotective and antiarrhythmic effects during ischemia/reperfusion in the intact rabbit heart. *J. Cardiovasc. Pharmacol.* 2006; **47**: 277–86.

45. Eisen A, Fisman EZ, Rubenfire M *et al.* Ischemic preconditioning: Nearly two decades of research A comprehensive review. *Atherosclerosis* 2004; **172**: 201–10.
46. Deschamps AM, Murphy EM, Sun JS. Estrogen receptor activation and cardioprotection in ischemia reperfusion injury. *Trends Cardiovasc. Med.* 2010; **20**: 73–8.
47. Korzick DH, Lancaster TS. Age-related differences in cardiac ischemia-reperfusion injury: Effects of estrogen deficiency. *Pflugers Arch.* 2013; **465**: 669–85.
48. Bell JR, Bernasochi GB, Varma U, Raaijmakers AJA. Sex and sex hormones in cardiac stress-Mechanistic insights. *J. Steroid Biochem. Mol. Biol.* 2013; **137**: 124–35.
49. Dhein S. The Langendorff Heart. In: Practical Methods in Cardiovascular Research. Dhein S, Mohr FW, Delmar M. (eds). Springer, Heidelberg, Germany. 2005; pp. 155–72.
50. Trollinger DR, Cascio WE, Lemasters JJ. Selective loading of Rhod 2 into mitochondria shows mitochondrial Ca^{2+} transients during the contractile cycle in adult rabbit cardiac myocytes. *Biochem. Biophys. Res. Commun.* 1997; **236**: 738–42.