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Research Paper

Cardioprotective effect of hyperthyroidism on the stunned rat heart during ischaemia–reperfusion: energetics and role of mitochondria

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New Findings

- **What is the central question of this study?**
  Hyperthyroidism is a cardiac risk factor, but thyroid therapy is used on myocardial stunning. What is the consequence of hyperthyroidism for mitochondrial metabolism and Ca^{2+} handling of the postischaemic stunned heart?
- **What is the main finding and its importance?**
  Hyperthyroidism reduced stunning and improved muscle economy of the postischaemic rat heart. The activities of the mitochondrial sodium–calcium exchanger and mitochondrial K^+ channel in hyperthyroid rat hearts were different from those in the euthyroid rat hearts. These findings contribute to the understanding of mitochondrial bioenergetics in pathology and support thyroid therapy in the stunning induced by ischaemia.

Transient ischaemia and hyperthyroidism are cardiovascular risk factors. Nevertheless, 3,5,3′-triiodothyronine/thyroxine therapy has been used to revert myocardial stunning. We studied the influence of hyperthyroidism on the role played by mitochondria in myocardial stunning consequent to ischaemia–reperfusion. Rats were injected s.c. daily with 20 μg kg^{-1} triiodothyronine for 15 days (HpT group). Isolated ventricles from either HpT or euthyroid (EuT) rats were perfused in a calorimeter, and left intraventricular pressure (in millimetres of mercury) and heat release (*Ht*; in milliwatts per gram) were measured. Stunning was evoked by 20 min of no-flow ischaemia and 45 min reperfusion. The HpT hearts developed higher postischaemic contractile recovery (PICR) and improved total muscle economy (*P*/*Ht*) with lower diastolic contracture (ΔLVEDP) than EuT hearts. Release of Ca^{2+} from the sarcoplasmic reticulum during reperfusion with 10 mM caffeine in low-[Na^+] Krebs solution evoked a higher contracture in EuT than in HpT hearts. Blockade of the mitochondrial sodium–calcium exchanger with clonazepam increased ΔLVEDP and reduced *P*/*Ht* and PICR in HpT but not in EuT hearts. The clonazepam-induced dysfunction in HpT hearts was reduced by ciclosporin, suggesting a dependance on activation of the mitochondrial permeability transition pore. Blockade of the mitochondrial Ca^{2+} uniporter with Ru360 reduced *P*/*Ht* and PICR to ~10% in both HpT and EuT hearts. Blockade of mitochondrial K^+ channels with 5-hydroxydecanoate increased LVEDP and reduced PICR and *P*/*Ht* in HpT hearts, while it only increased LVEDP in EuT hearts. The results suggest that hyperthyroidism prevents the stunning with high dependence on the mitochondrial sodium–calcium exchanger and mitochondrial K^+ channels.

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Both HpT and EuT hearts showed a similar and critical role of the uniporter. The HpT hearts have a slow sarcoplasmic reticulum Ca^{2+} loss and low mitochondrial Ca^{2+} uptake.

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Introduction

Thyroid dysfunction, either clinical or subclinical, has been related to the increase in morbidity and mortality associated with cardiovascular episodes (Klein & Danzi, 2007). In particular, hyperthyroidism predisposes to long-term cardiac conditions such as hypertrophy and cardiac remodelling. Severe and untreated hyperthyroidism induces tachycardia, high cardiac workload and a risk of atrial fibrillation related to the upregulation of β-adrenergic receptors (Nyirenda *et al.* 2005). Some patients suffer symptoms of cardiac ischaemia because of the high oxygen demand related to the increased cardiac output (Klein & Ojamaa, 2001), which depends on the existence of previous cardiac illness and the severity of the thyroid hyperfunction (Kahaly & Dillman, 2004; Choi *et al.* 2005). Some patients, however, suffer cardiac 'stunning' as a consequence of the euthyroid sick syndrome characterized by low 3,5,3′-triiodothyronine (T_3) and normal TSH, following an acute condition risky for life (Novitzky & Cooper, 2014). 'Stunning' was defined as depressed myocardial function following an ischaemic event (Kloner & Jennings, 2001). Several authors have shown cardiac benefits of levothyroxine (T_4) or T_3 therapies in critical transient conditions of regional ischaemia–reperfusion (I/R; Heusch, 2013), global I/R in patients with cardiopulmonary bypass (Siribaddana, 2012) and I/R in brain-dead potential organ donors (Novitzky & Cooper, 2014). All these conditions induce variable degrees of myocardial stunning. In spite of some controversies (Pappa *et al.* 2011), T_4 replacement therapy was recommended in acute euthyroid sick syndrome (Novitzky & Cooper, 2014), as well as in recipients of transplantation (Novitzky *et al.* 2014), in order to obtain a rapid restoration of energy stores and myocardial function.

Although the systemic metabolic effects of thyroid hormones partly explain such clinical evidence, only some mechanisms are known in postischaemic hearts, with different consequences. Both T_3 and T_4 induce genomic and non-genomic effects, which regulate cellular targets, either positively [sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), Na^+,K^+-ATPase, α-myosin, β-receptors and voltage-gated K^+ channels] or negatively (phospholamban and sarcolemmal sodium-calcium exchanger; Cernohorský *et al.* 1998; Takeuchi *et al.* 2003; Klein & Danzi, 2007). Thus, it is expected that either T_3/T_4 therapy or hyperthyroidism will change Ca^{2+} homeostasis during I/R. It was shown that T_4 administration to rats immediately after an infarct protected hearts from injury and increased postischaemic functional recovery via overexpression of heat shock protein 70 (Pantos *et al.* 2006). Moreover, acute perfusion with T_3 during reperfusion after prolonged ischaemia was shown to be anti-apoptotic (Pantos *et al.* 2009). At low concentrations, T_3, but not T_4, improved postischaemic function, which may partly implicate alpha-1 thyroid receptor (Pantos *et al.* 2011). Nevertheless, it was shown that T_4 evoked cardioprotection similar to the preconditioning in rats, with reduction of p38 mitogen-activated protein kinase (Pantos *et al.* 2003) and inhibition of the mitochondrial permeability transition pore (mPTP; Kumar *et al.* 2012). Conversely, it was reported that subcutaneous treatment of rats with T_3 for 10 days induced a reduction of mitochondrial oxygen consumption and catalase activity, with accumulation of reactive oxygen species and swelling during severe I/R (Venditti *et al.* 2002). While most experimental reports considered severe ischaemia with infarct, apoptosis and mitochondrial swelling, the T_3/T_4 effects must depend on the degree of ischaemic damage. Thus, owing to the possible clinical and subclinical applications, it was interesting to evaluate the consequences of hyperthyroidism for the functional and energetic behaviour and Ca^{2+} homeostasis in a model of myocardial stunning. We previously showed that mitochondrial transporters contribute to the regulation of Ca^{2+} homeostasis and muscle economy during stunning induced by I/R in isolated rat hearts (Ragone & Consolini, 2009; Consolini *et al.* 2011; Bonazzola *et al.* 2014).

The experiments present in the present manuscript show that the hearts from hyperthyroid (HpT) rats had improved contractile recovery and reduced the fall in muscle economy during reperfusion compared with those from euthyroid (EuT) rats. Differences were detected between hyperthyroid and euthyroid hearts in the roles that mitochondria and the sarcoplasmic reticulum (SR) played during postischaemic Ca^{2+} homeostasis.

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, eighth edition, 2010). The procedures used in this study were approved by the Animal Care and Research Committee of the School of Medicine, University of Buenos Aires.

Animals and model of hyperthyroidism

Both male and female adult Wistar rats (280–380 g body weight, >2 months older) were obtained from the Biotery of the Institute of Cardiological Research (ININCA). Animals were housed with up to four rats per cage, with water and pelleted food *ad libitum*, until the day of the experiment. A group was made HpT by a daily S.C. injection of 20 μg kg^{-1} triiodothyronine for 15 days, as previously described (Takeuchi *et al.* 2003). Some protocols were also carried out in control non-treated EuT Wistar rats. As a test to evaluate the hyperthyroid state, blood was sampled at the moment of excising the heart in some animals, and plasma levels of T_3, T_4 and thyroid-stimulating hormone (TSH) were determined by an enzyme-linked fluorescent assay technique in an automated quantitative test used for humans (Vidas Instruments, BioMerieux, Mary l Étoile, France). Hyperthyroid rats developed a total T_3 level of 5.15 ± 1.28 nmol l^{-1}, which was about 5.6 times higher than that of EuT control animals (0.91 ± 0.08 nmol l^{-1}, $P = 0.0259$, $n = 12$ and 9, respectively). Free T_4 in HpT rats was 2.63 ± 0.6 pmol l^{-1}, which was 37% of that of EuT animals (7.0 ± 0.5 pmol l^{-1}, $P = 0.0003$), thus demonstrating the hormonal negative feedback. Plasma TSH was undetectable by this method in all HpT rats assessed (<0.05 mIU l^{-1}, $n = 12$), while it was 0.22 ± 0.04 mIU l^{-1} in four EuT rats but undetectable in another five (these low values of TSH could be caused by contamination with lung proteases during blood sampling). Also, HpT rats developed a cardiac-to-body mass ratio higher than the EuT rats (2.99 ± 0.07 *versus* 2.5 ± 0.1 mg g^{-1}, respectively, $P = 0.0002$) as well as typical behaviour of anxiety. On the day of the experiment, either HpT or EuT rats were anaesthetized with a pentobarbital (Fada-Pharma, Buenos Aires, Argentina) overdose (60 mg kg^{-1} I.P.) and heparinized. After loss of reflexes and muscle relaxation, the heart was quickly excised.

Ischaemia–reperfusion in isolated perfused rat ventricles

The heart was perfused retrogradely by the Langerdorff technique with Krebs solution, at a constant flow of 7 ml min^{-1} g^{-1} with a peristaltic pump (Gilson Minipuls 3, Villiers Le Bel, France; Ragone & Consolini, 2009). This perfusion flow was calculated using the equation $CF = 7.43 \times HW^{0.56}$ (where CF is coronary flow and HW the heart weight), which is valid for different species, and it has been recommended to prevent heart oedema caused by a high flow rate of saline perfusion (Dhein *et al.* 2005). This flow was sufficient to develop an optimal maximal pressure (P), because the pressure remained constant when perfusion flow rate was increased from 7 to 9 and then to 10 ml min^{-1} (being, respectively, 98.3 ± 2.1 and 100.9 ± 4.9% of that obtained at 7 ml min^{-1}, $n = 6$, n.s.). With a flow of <7 ml min^{-1} g^{-1}, the hearts developed 82.9 ± 1.0% of water content after I/R ($n = 47$), which is similar to the water content reported in hearts without I/R at 25°C (Ponce-Hornos *et al.* 1995), thus suggesting that I/R did not induce significant oedema. Atria were removed, and any focus of spontaneous beating in the right ventricle was turned off by applying either positive pressure or a little cut on it. A latex balloon filled with water was stitched in the left ventricle and connected by a cannula to a Bentley DEL900 Bently DEL900, Nevada, USA or a Gould Statham P23, Oxnard, California, USA pressure transducer. While continuously perfused, the heart was introduced into the calorimetric chamber, which was submerged in a water bath at a controlled temperature of 37 ± 0.01°C. It was electrically stimulated at 3 Hz, 5 V for 5 ms, by means of two electrodes connected to an electrical stimulator (either Letica 12406, Barcelona, Spain, or Grass model SD9, Braintree, MA, USA). Two signals were continuously recorded by either a PowerLab 2/26 amplification system (AD Instruments, Bella Vista, New South Wales, Australia) or an A/D converter (Axon Instruments Inc., Foster City, CA, USA). One signal was the calorimetric one and the other was the left intraventricular pressure (LVP) at optimal volume. From the LVP, we calculated the maximal pressure developed during isovolumetric contraction (P) and the changes in diastolic pressure (ΔLVEDP) with respect to the initial conditions in control Krebs solution (Krebs-C), which were expressed in millimetres of mercury after calibration. In order to compare the postischaemic recovery among the protocols, P was also expressed as a percentage of the initial P obtained after the stabilization in Krebs-C.

Calorimetric measurements. As described in previous studies (Ponce-Hornos *et al.* 1982, 1995; Consolini *et al.* 2007; Ragone *et al.* 2013), the calorimeter made of a mass of copper has an internal chamber containing two ceramic modules, each with 127 thermosensitive units (Melchor Thermoelectrics, Trenton, NJ, USA). They are sensitive to differences in temperature between the inside (heart) and the external bath (at constant temperature). The calorimetric signal (in microvolts) was amplified and digitized with the A/D system, simultaneously with the LVP signal. Total heat rate (*Ht*; expressed as milliwatts per gram of wet ventricular weight) was obtained continuously either in the presence or in the absence of perfusion during the I/R protocol. Calorimetric baselines

were obtained before and after introducing the heart into the chamber, either in the presence or in the absence of perfusion. Calibration of the calorimetric signal (in milliwatts per millivolt) was done by applying a constant electrical power (2 mW) to the ventricle at the end of the experiment, in the presence or absence of perfusion, as previously described (Ragone & Consolini, 2009; Bonazzola *et al.* 2014). The results were also expressed as a percentage of the initial *Ht* after stabilization in Krebs-C for comparison among different protocols. Total muscle economy was calculated as the ratio of *P*/*Ht* (in millimetres of mercury grams per milliwatt).

Protocols. After a stabilization period of ∼30 min with Krebs-C (C) at a beating rate of 3 Hz, each of the following protocols was applied in the isolated ventricles from either hyperthyroid (HpT) or euthyroid (EuT) rats during 30 min before ischaemia. When two drugs were used, the first one was applied for 15 min and both drugs were perfused for the following 15 min. Protocols (shown in Fig. 1) were as follows: (i) perfusion with C; (ii) perfusion with C + 10 μmol l^{-1} clonazepam [Clzp; to block the mitochondrial sodium–calcium exchanger (mNCX); Cox & Matlib, 1993]; (iii) perfusion with C + 30 μmol l^{-1} ouabain (Ouab; to increase $[Na^+]_i$ without inducing arrhythmogenesis; Gupta *et al.* 1986; Gonano *et al.* 2011); (iv) perfusion with C + 30 μmol l^{-1} Ouab followed by C + Ouab + Clzp (to block mNCX in a condition of high $[Na^+]_i$); (iv) perfusion with C + 1 μmol l^{-1} Ru360 [to block the mitochondrial Ca^{2+} uniporter (UCam) selectively; García Rivas *et al.* 2005]; (v) perfusion with C + 0.2 μmol l^{-1} ciclosporin (Cys A; to block the mPTP; Griffiths & Halestrap, 1993) followed by C + Cys A + Clzp (to evaluate whether the effects of Clzp involve mPTP activation); (vi) perfusion with C + 100 μmol l^{-1} 5-hydroxydecanoate [5HD; to block the mitochondrial K^+ (mK_{ATP}) channels; Murata *et al.* 2001]; (vii) perfusion with C + 0.2 μmol l^{-1} Cys A, followed by C + Cys A + 100 μmol l^{-1} 5HD (to evaluate whether the effects of 5HD involve mPTP activation); and (viii) perfusion with C + 0.2 μmol l^{-1} Cys A (as a control). After 20 min of ischaemia, all the hearts were reperfused for 45 min with Krebs-C, except those pretreated with Cys A, which were reperfused with C + Cys A, as previously reported (Griffiths & Halestrap, 1993; Ragone & Consolini, 2009).

Whenever indicated, the ischaemic hearts were reperfused with Krebs solution that contained 10 mmol l^{-1} caffeine and 36 mmol l^{-1} Na^+ (Caff-low Na). The resultant contracture (ΔLVP from the control pre-ischaemic level) *versus* time was determined, as well as the associated *Ht*. Their areas under the curve (AUC-ΔLVP and AUC-*Ht*) over the ischaemic line were estimated and considered proportional to the SR Ca^{2+} store, as in previous studies (Consolini *et al.* 2007; Ragone & Consolini, 2009; Ragone *et al.* 2013; Bonazzola *et al.* 2014).

There were 62 successful experiments in EuT hearts and 61 in HpT rats. About 5% of the total number of experiments were discarded owing to inappropriate basal parameters.

In order to evaluate whether this model of I/R induces myocardial infarction, in some experiments the hearts were frozen after 45 min of reperfusion and cut into 4 mm 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 while the infarcted area remained unstained. The infarcted area was measured and expressed as a percentage of free downloaded the left ventricular area (Image Pro Plus, USA). Figure 1*B* shows a typical recording, where it can be seen that after 20 min ischaemia and 45 min reperfusion, hearts were stunned without significant myocardial infarction.

Confocal microscopy in cardiomyocytes

Isolation of cardiac ventricular myocytes. Ventricular myocytes were isolated from HpT and EuT rat hearts by the method previously described for guinea-pigs (Bridge *et al.* 1990; Ragone *et al.* 2013). Briefly, the heart was quickly placed in a Langendorff system and perfused with a modified Krebs–24-Hepes solution virtually free of Ca^{2+} for 5 min. The solution was then changed to Krebs–24-Hepes with 50 μmol l^{-1} $CaCl_2$, 0.1 mg ml^{-1} collagenase P (Roche) and 0.02 mg ml^{-1} protease XIV (Sigma) and perfused for 13 min. Finally, the perfusion was changed again to an enzyme-free 50 μmol l^{-1} Ca^{2+} solution for 5 min. All solutions were bubbled with O_2 and maintained at 37°C. After this treatment, the ventricles were removed and minced into pieces, which were shaken in a low-Ca^{2+} solution for 10 min and then strained. The isolated cells were stored for up to 6 h in a 1 mmol l^{-1} Ca^{2+} Hepes-buffered saline solution.

Confocal microscopy. Isolated rat cardiomyocytes were loaded with 12 μmol l^{-1} fluo-4 AM (Molecular Probes/Invitrogen, Carlsbad, CA, USA) for 15 min at room temperature for measuring cytosolic free Ca^{2+} signals. In other group, in order to detect only the mitochondrial compartment, the myocytes were loaded for 1 h at 4°C with 3 μmol l^{-1} rhod-2 AM (Molecular Probes/Invitrogen) followed by wash-up for at least 1 h at 37°C (Trollinger *et al.* 1997). Cells were placed in a laminin-precoated perfusion chamber to be superfused with Krebs–24-Hepes solution containing 2 mmol l^{-1} Ca^{2+} (C) until stabilization. Most cells did not contract during the protocol; those did were not considered for the analysis. Changes in fluorescence

were recorded during the protocols using a confocal microscope (Leica SP5; Leica Microsystems, Mannheim, Germany). Data were analysed using the Leica LAS AF Lite version 2.2.1 software. Results were expressed as the self-ratio emission fluorescence intensity (F/F_0) every 20 s during the 20 or 25 min protocol. The changes in F/F_0 over time were calculated after non-linear adjustment of the baseline (initial and final C perfusion) by using Origin 7.0 (OriginLab Corporation, Northampton, MA, USA).

To evaluate cytosolic free Ca^{2+}, fluo-4-loaded cells were excited at 488 nm, every 20 s, and the fluorescence emission was detected at wavelengths >505 nm. To investigate mitochondrial free Ca^{2+}, rhod-2-loaded cells were excited at 540 nm, every 20 s, and the fluorescence emission was monitored at wavelengths >560 nm. Always, the signals were detected in a selected, defined area (one region of interest per cell) of uniform fluorescence without movements in the resting cells.

Protocols. Cardiomyocytes from HpT rats loaded with rhod-2 were perfused with the following sequence of solutions in each protocol with five to 12 cells originating from at least three hearts for each protocol: (i) C (5 min), C + 10 mmol l^{-1} Clzp (5 min), C + 10 mmol l^{-1} caffeine + 36 mmol l^{-1} Na^+ + Clzp (10 min), C (5 min); (ii) analogous to (i) without Clzp; (iii) C (5 min), C + 10 mmol l^{-1} Clzp (5 min), C + 10 mmol l^{-1} Clzp + 30 μmol l^{-1} Ouab (5 min), C (5 min); and (iv) C (5 min), C + 30 μmol l^{-1} Ouab (5 min), C + 30 μmol l^{-1} Ouab + 10 mmol l^{-1} Clzp (5 min), C (5 min). Myocytes loaded with fluo-4 were exposed to protocols similar to (i) and (ii). Cardiomyocytes from EuT rats loaded with rhod-2 or fluo-4 were perfused with the protocol similar to (ii), as follows: C (10 min), C + 10 mmol l^{-1} caffeine + 36 mmol l^{-1} Na^+ (10 min), C (5 min).

Solutions and drugs

The composition of control Krebs (Krebs-C) to perfuse entire ventricles was as follows (in mmol l^{-1}): 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 studies (Consolini *et al.* 2007; Ragone & Consolini, 2009). Krebs solution with 10 mmol l^{-1} caffeine and 36 mmol l^{-1} Na^+ was kept isosmotic with Krebs-C by the addition of 217 mmol l^{-1} sucrose.

For the isolation of cardiomyocytes, the composition of the Krebs–24-Hepes was as follows (in mmol l^{-1}): 126 NaCl, 4.4 KCl, 1.0 NaH_2PO_4, 5 $MgCl_2$, 24 Hepes,

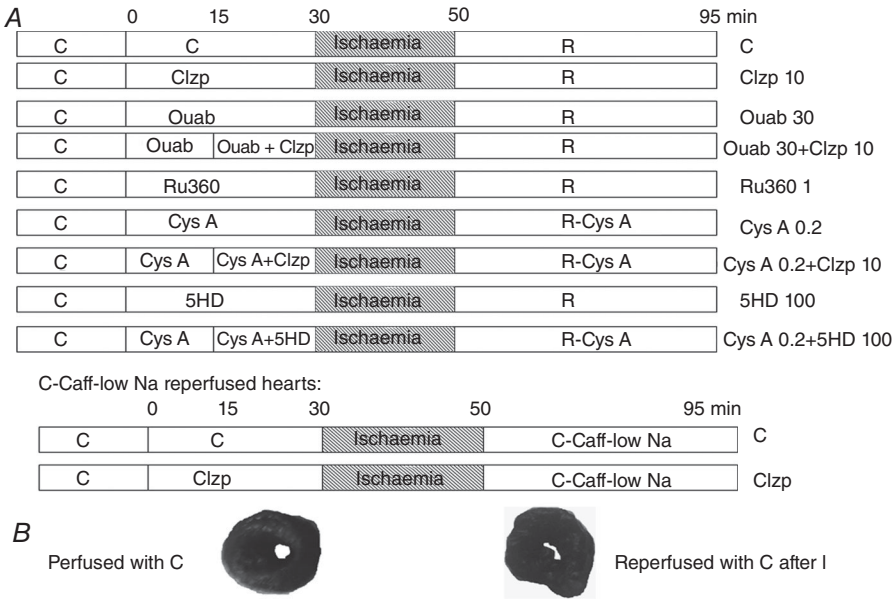

Figure 1. Protocols done in isolated ventricles
A, schematic representation of protocols carried out on isolated ventricles of euthyroid (EuT) and hyperthyroid (HpT) rats exposed to no-flow ischaemia (I; shaded bar) and reperfusion (R) with control Krebs solution (C) or with Krebs + 10 mmol l^{-1} caffeine + 36 mmol l^{-1} Na^+ (C-Caff-low Na) with several treatments. Treatments are C (control Krebs) and the following drugs: 10 μmol l^{-1} clonazepam (Clzp), 30 μmol l^{-1} ouabain (Ouab), 100 μmol l^{-1} 5-hydoxydecanoate sodium salt (5HD), 0.2 μmol l^{-1} ciclosporin (Cys A) and 1 μmol l^{-1} Ru360. Above the bars, the time of any perfusion change (in minutes) is indicated. See Methods for details. *B*, typical image of two slices from hearts exposed to triphenyltetrazolium chloride to evaluate the myocardial infarct, either in hearts perfused with C or reperfused with C after ischaemia. The absence of white areas indicates the absence of infarct in this model.

Table 1. Changes in the resting intraventricular pressure (ΔLVEDP; in millimetres of mercury) with respect to basal values in hyperthyroid (basal, 21.8 ± 2.2 mmHg) and euthyroid hearts (basal, 15.5 ± 1.9 mmHg) at 5 and 20 min ischaemia and at 5 and 45 min of reperfusion in the different protocols assessed

ΔLVEDP (mmHg)	Hyperthyroid hearts				Euthyroid hearts			
Treatment	Ischaemia 5 min	Ischaemia 20 min	Reperfusion 5 min	Reperfusion 45 min	Ischaemia 5 min	Ischaemia 20 min	Reperfusion 5 min	Reperfusion 45 min
C	−4.0 ± 0.9 (6)†	0.8 ± 2.9	0.7 ± 1.9	−1.6 ± 2.4	−3.5 ± 0.9 (7)†	−0.5 ± 0.8	7.7 ± 1.9†	1.7 ± 0.6†
C + Clzp	9.2 ± 5.0 (7)	14.5 ± 4.7†	45.7 ± 9.9*†	55.4 ± 11.9*†	−10.0 ± 4.9 (6)†	−7.1 ± 4.9	6.0 ± 2.9†	4.3 ± 2.7
C + Ouab + Clzp	−8.2 ± 1.0 (6)†	−1.0 ± 1.8	18.8 ± 2.2†	36.9 ± 10.5*†	−3.5 ± 0.6 (6)†	−2.9 ± 1.7	13.9 ± 2.5†	8.2 ± 1.2†
C + Ouab	−5.3 ± 1.7 (6)†	−4.7 ± 1.2†	6.1 ± 2.0†	7.0 ± 1.7†	0.3 ± 0.4 (6)	−1.7 ± 0.4†	15.1 ± 2.0†	14.7 ± 2.2†
C + Cys A+ Clzp	−0.8 ± 4.9 (5)	−0.2 ± 2.7	4.1 ± 2.8	−1.3 ± 1.4	22.6 ± 8.4 (6)*†	36.4 ± 2.7*†	64.1 ± 5.6*†	40.7 ± 8.1*†
C + Ru360	0.0 ± 0.2 (6)	0.7 ± 0.9	13.3 ± 2.1†	10.4 ± 2.0†	0.50 ± 0.3 (6)	0.5 ± 0.4	25.0 ± 2.0*†	24.4 ± 3.3*†
C + 5HD	19.0 ± 8.1 (7)*†	21.0 ± 4.5*†	26.2 ± 6.2*†	22.3 ± 9.0*†	30.9 ± 5.1 (7)*†	45.5 ± 3.3*†	69.4 ± 4.1*†	69.6 ± 5.3*†
C + Cys A + 5HD	4.0 ± 5.0 (6)	19.5 ± 6.0†	44.5 ± 9.5*†	32.2 ± 9.0*†	—	—	—	—
C + Cys A	—	—	—	—	22.5 ± 6.0 (7)*†	35.2 ± 6.1*†	58.5 ± 5.3*†	49.2 ± 5.0*†
Two-way ANOVA	By treatment: F = 19.75, DFn = 7, DFd = 152, $P < 0.0001$				By treatment: F = 124.02, DFn = 7, DFd = 172, $P < 0.0001$			
	By time: F = 22.73, DFn = 3, DFd = 152, $P < 0.0001$				By time: F = 67.28, DFn = 3, DFd = 172, $P < 0.0001$			

Abbreviations: control Krebs solution, standard Krebs solution; Clzp, clonazepam; Cys A, ciclosporin; 5HD, 5-hydroxydecanoate; and Ouab, ouabain. The treatment before ischaemia is indicated, and the number of experiments is in parentheses in column 'Ischaemia 5 min'. Two-way ANOVA results are shown, as well as the tests *a posteriori* (* $P < 0.05$ *versus* C at each time point); † $P < 0.05$ *versus* zero (by Student's *t* test).

22 dextrose, 20 taurine, 5 creatine, 0.5 sodium pyruvate, adjusted with NaOH to pH 7.4. For the superfusion of cardiomyocytes during the protocol, the Krebs–24-Hepes (C) solution composition was as follows (in mmol l^{-1}): 126 NaCl, 4.4 KCl, 1 $MgCl_2$, 24 Hepes, 2 $CaCl_2$ and 11 dextrose, adjusted with NaOH to pH 7.4. For the experiments with fluo-4, 0.5 mmol l^{-1} probenecid was added.

Clonazepam (Clzp; Saporiti, Buenos Aires, Argentina) was prepared as an aqueous solution at 10 mmol l^{-1} and diluted to 10 μmol l^{-1} in Krebs-C for perfusion. Ouabain (Ouab; Sigma, USA) was prepared as an aqueous solution at 0.25 mg ml^{-1} and diluted to 30 μmol l^{-1} in Krebs-C. Ciclosporin (Cys A; Fluka, Sigma-Aldrich, Saint Louis, MO, USA) was prepared in DMSO at 0.2 mmol l^{-1} and diluted in Krebs-C to 0.2 μmol l^{-1}. Sodium 5-hydroxydecanoate (5HD; ICN Biochemicals and Reagents, Santa Ana, CA, USA) was prepared as a 100 mmol l^{-1} solution in DMSO and diluted to 100 μmol l^{-1} in Krebs-C. All the drugs were diluted from their stock solutions in Krebs-C at the moment of the experiment. Caffeine (ICN, Costa Mesa, CA, USA) was directly dissolved in Krebs solution on the day of the experiment.

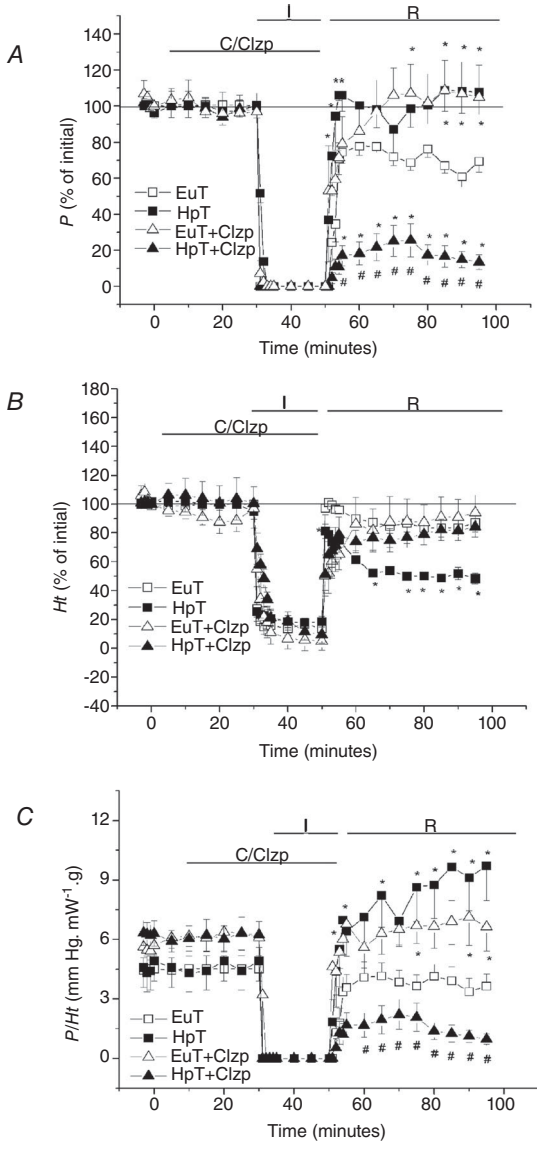

Figure 2. Mechano-energetic performance before, during and after ischaemia in hearts from euthyroid and hyperthyroid rats after perfusion with standard Krebs solution (C) or 10 μmol l^{-1} clonazepam (Clzp)
A, maximal pressure developed (*P*; expressed as a percentage of initial maximal pressure). *B*, total heat rate (*Ht*; expressed as a percentage of initial steady heat rate). *C*, total muscle economy (*P*/*Ht*; in millimetres of mercury grams per milliwatt). Results are shown as means ± SEM ($n = 7$ in EuT and 6 in HpT). $^{*}P < 0.05$ *versus* EuT and $^{\#}P < 0.05$ *versus* HpT by Bonferroni tests *a posteriori* of the two-way ANOVA ($P < 0.0001$ by treatment and time, respectively for *A*, *B* and *C*). See supporting information for further information.

Statistical analysis

Results were expressed as medians ± SEM. Multiple comparisons of treatments *versus* time were made by two-way ANOVA. *A posteriori* Bonferroni paired tests were carried out among treatments, and their results are shown in each figure. A one-way ANOVA was used for comparing one variable among more than two treatments. Also, Student's paired or unpaired *t* test were used for comparing two conditions. A value of $P < 0.05$ was always considered significant. All the statistical analysis was performed by using the GraphPad Prism v.4 software (San Diego, La Jolla, CA, USA).

Results

Ischaemia–reperfusion in hyperthyroid and euthyroid ventricles

Before any treatment or ischaemia, the HpT hearts developed a basal *P* (87.6 ± 3.1 mmHg, $n = 48$) similar to that developed by the EuT hearts (92.6 ± 2.6 mmHg, $n = 51$, $P = 0.2244$ by Student's unpaired *t* test). Simultaneously, the total heat rate (*Ht*) in HpT hearts (15.1 ± 0.1 mW g^{-1}) was lower than that of EuT hearts (18.2 ± 0.6 mW g^{-1}, $P = 0.0009$). However, total muscle economy (*P*/*Ht*) was not significantly different in HpT and EuT hearts (6.05 ± 0.04 *versus* 5.5 ± 0.3 mmHg mW^{-1} g, respectively, $P = 0.1868$). During global interruption of perfusion (I), both non-treated (C) groups of HpT and EuT hearts initially reduced resting pressures (ΔLVEDP compared with the pre-ischaemic values) and recovered them during 20 min of I (see Table 1), while *P* disappeared and *Ht* fell (Fig. 2). During reperfusion (R), HpT hearts reached a higher postischaemic contractile recovery (PICR) than EuT hearts (up to 108.8 ± 11.6 *versus* 77.5 ± 3.2% of initial *P*, $n = 6$ and 7, respectively, $P < 0.05$;

Fig. 2*A*). Simultaneously, *Ht* recovered more in EuT than in HpT hearts (up to 89.1 ± 6.0 *versus* 53.8 ± 2.3% of the initial *Ht*, respectively, $P < 0.05$; Fig. 2*B*). As a result of that, the HpT hearts developed a more economical contractile state during R than EuT hearts (*P*/*Ht* of 9.7 ± 1.7 *versus* 3.6 ± 0.6 mmHg mW^{-1} g at the end of R, respectively, $P < 0.05$; Fig. 2*C*). Moreover, the diastolic contracture seen in EuT was prevented in HpT hearts (Table 1).

Role of the mNCX

In order to evaluate the role of the mNCX in this model of stunning in HpT and EuT hearts, this exchanger was selectively blocked with 10 μmol l^{-1} Clzp before I/R. The presence of Clzp did not affect *P* or *Ht* before I (Fig. 2*A* and *B*). Nevertheless, Clzp induced dysfunction in HpT but not in EuT hearts, with PICR of up to 13.0 ± 3.8 *versus* 98 ± 14% of initial *P*, respectively (Fig. 2*A*). Also, Clzp induced less increment in the diastolic tone (ΔLVEDP) during I/R in EuT than in HpT hearts (Table 1). While both HpT and EuT hearts recovered the energetic consumption in R to a similar extent (*Ht* as a percentage of the initial value), Clzp reduced it (Fig. 2*B*). Then, the postischaemic muscle economy (*P*/*Ht*) was strongly reduced by Clzp in HpT hearts, but it improved slightly in the EuT hearts (up to 7.1 ± 1.4 mmHg mW^{-1} g; Fig. 2*C*).

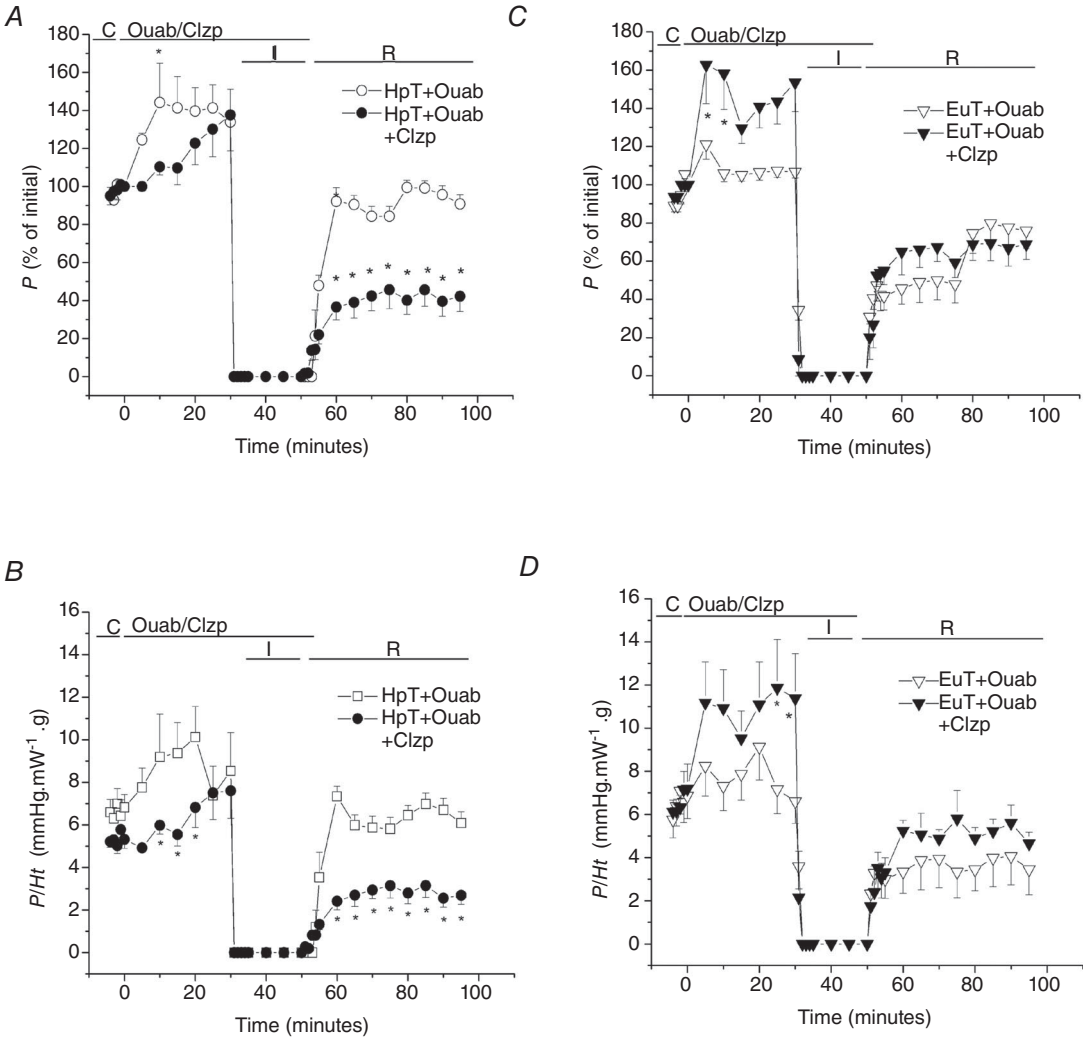

Figure 3. Effects of 30 μmol l^{-1} Ouab and 10 μmol l^{-1} Clzp on maximal pressure developed (as a percentage of initial pressure; *A* and *C*) and total muscle economy (*P*/*Ht*; in millimetres of mercury grams per milliwatt; *B* and *D*) before, during ischaemia (I) and during reperfusion (R) in hearts from HpT (*A* and *B*) and EuT rats (*C* and *D*)
Results are shown as means ± SEM ($n = 6$ in each group); two-way ANOVAs: $P < 0.0001$ by treatment and time, respectively, $^*P < 0.05$ *versus* the other treatment by *a posteriori* Bonferroni tests. See supporting information for further information.

In order to evaluate whether the effect of Clzp was due to the inhibition of a mitochondrial Ca^{2+} contribution, the gradient for mitochondrial Ca^{2+} extrusion through the mNCX was increased by raising $[Na^+]_i$ before adding Clzp. Ouabain was used at a high concentration (30 μmol l^{-1}),

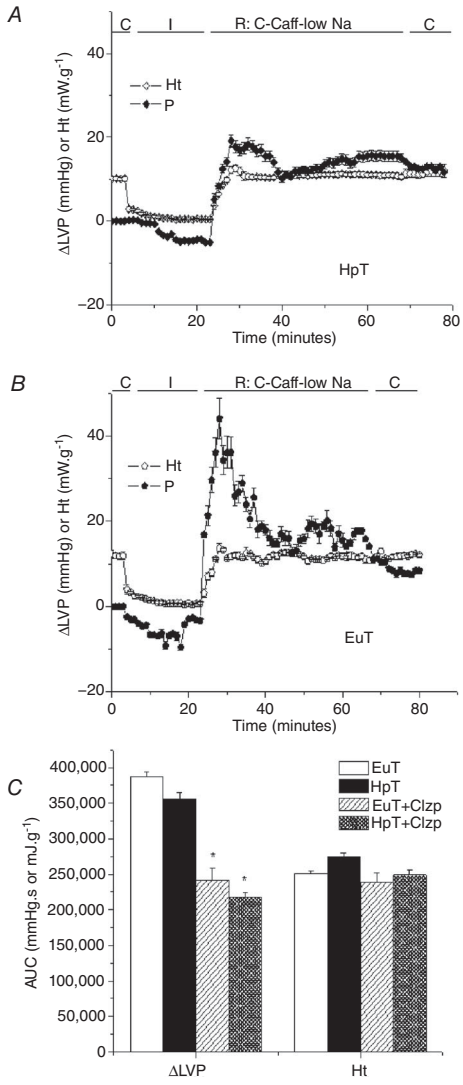

Figure 4. Effects of hyperthyroidism and clonazepam on the sarcorreticular calcium content after ischaemia in rat hearts
Effects of reperfusing ischaemic hearts with Krebs solution–10 mM caffeine–36 mM Na^+ (C-Caff-low Na) on the changes in left ventricular pressure over the initial baseline (ΔLVP) and on the heat rate (*Ht*) in HpT (*A*) and EuT (*B*) ventricles (*B*). In *C* is shown the areas under the curve (AUC) of ΔLVP and *Ht* compared with the respective end-ischaemic baselines throughout the reperfusion period with C-Caff-low Na for HpT and EuT ventricles treated with 10 μmol l^{-1} Clzp or not treated. All results are means ± SEM ($n = 5$ or 6, respectively); two-way ANOVA: $P < 0.0001$ by treatment and thyroid condition, $^*P < 0.05$ *versus* the respective condition without Clzp. See supporting information for further information.

which is sufficient to induce positive inotropism in the rat heart (Gupta *et al.* 1986; Gonano *et al.* 2011). Cardiotonic effects of Ouab were higher in HpT than in EuT hearts before I (+44.4 ± 12.1 *versus* +22.8 ± 7.0% of initial *P*, respectively). Simultaneously, *Ht* increased from 14.0 ± 0.9 to 19.9 ± 2.6 mW g^{-1} in HpT hearts ($n = 5$, $P = 0.017$) and from 15.3 ± 1.2 to 17.2 ± 1.6 mW g^{-1} in EuT hearts ($n = 6$, $P = 0.014$). Addition of Clzp did not significantly change the pre-ischaemic inotropism (Fig. 3). After I, Ouab maintained the PICR at a high level in HpT hearts (up to 99.5 ± 3.9% of initial *P*) but reduced it in EuT ventricles (up to 45.6 ± 8.7% of initial *P*). Likewise, Ouab maintained the muscle economy of HpT ventricles (*P*/*Ht* up to 7.0 ± 0.5 mmHg mW^{-1} g) but reduced it in EuT hearts (up to 4.0 ± 1.3 mmHg mW^{-1} g). In contrast, the addition of Clzp to Ouab in HpT hearts reduced PICR (up to 45.7 ± 8.7% of the initial *P*; Fig. 3*A*), increased the diastolic contracture during R (see ΔLVEDP in Table 1) and reduced the postischaemic *P*/*Ht* (up to 3.1 ± 0.5 mmHg mW^{-1} g). On the contrary, in EuT ventricles the addition of Clzp to Ouab prevented the reduction in PICR (up to 69.3 ± 9.0% of initial; Fig. 3*C*), kept the LVEDP constant (Table 1) and maintained *P*/*Ht* (5.6 ± 0.8 mmHg mW^{-1} g; Fig. 3*D*). Thus, the effects of Ouab and Clzp were opposite in HpT and EuT hearts.

Effects on the sarcoplasmic Ca^{2+} store

To evaluate whether blocking the mNCX with Clzp affects the SR Ca^{2+} store in order to explain the reduced PICR in HpT rat hearts, massive SR Ca^{2+} release was induced by reperfusing the ventricles with Krebs C + 10 mmol l^{-1} caffeine + 36 mmol l^{-1} Na^+ (C-Caff-low Na), which also minimizes the Ca^{2+} efflux by SL-NCX. Figure 4*A* and *B* shows that EuT ventricles developed a higher initial contracture than HpT hearts in the same time of ~6 min (initial rate as $\Delta P/\Delta t$ of 8.7 ± 1.0 *versus* 4.3 ± 0.4 mmHg min^{-1}, respectively, $P < 0.001$ by the test *a posteriori* of the ANOVA for the four protocols with Caff-low Na reperfusion). Clonazepam also reduced the initial rate of contracture in both EuT (3.2 ± 0.3 mmHg s^{-1}, $P < 0.001$ *versus* EuT without Clzp) and HpT hearts (2.6 ± 0.1 mmHg min^{-1}, $P < 0.001$ *versus* HpT without Clzp). Figure 4*C* shows that both EuT and HpT ventricles developed similar AUC-ΔLVP and AUC-*Ht*, and Clzp reduced the AUC-ΔLVP in both types of ventricles to a similar extent without changing AUC-Ht. The results suggest that HpT and EuT ventricles have similar SR Ca^{2+} content sensitive to caffeine, but they release it at different rates. The energetic consumption was not changed by Clzp, so that muscle economy during the contracture was reduced.

Effects on mitochondrial and cytosolic Ca^{2+}

To understand the effect of Clzp and Ouab on mitochondrial $[Ca^{2+}]$ before the induction of ischaemia, isolated cardiomyocytes of HpT ventricles loaded with rhod-2 were perfused with 30 μmol l^{-1} Ouab and/or 10 μmol l^{-1} Clzp in the chamber of the confocal microscope. Figure 5 shows that either Clzp or Ouab increased the $\Delta F/F_0$ of rhod-2 in HpT cardiomyocytes, but both together had a less than additive effect (Fig. 5*A*, *B* and *D*). The same behaviour was induced in the fluo-4 signal, which estimates changes in free cytosolic $[Ca^{2+}]$, showing that Clzp reduced the effect of Ouab (Fig. 5*C* and *D*). On the other hand, perfusion of myocytes with Krebs containing 10 mmol l^{-1} caffeine and 36 mmol l^{-1} Na^+ (C-Caff-low Na) increased the rhod-2 $\Delta F/F_0$ in EuT myocytes, but it was not significant in HpT myocytes (Fig. 6*A* and *C*). The cytosolic free $[Ca^{2+}]$ estimated by fluo-4 signal in HpT myocytes was increased by perfusing C-Caff-low Na but fell, more slowly than the respective signal in EuT cardiomyocytes, in both types of cells before the C perfusion was resumed (Fig. 6*B* and *D*). Clonazepam

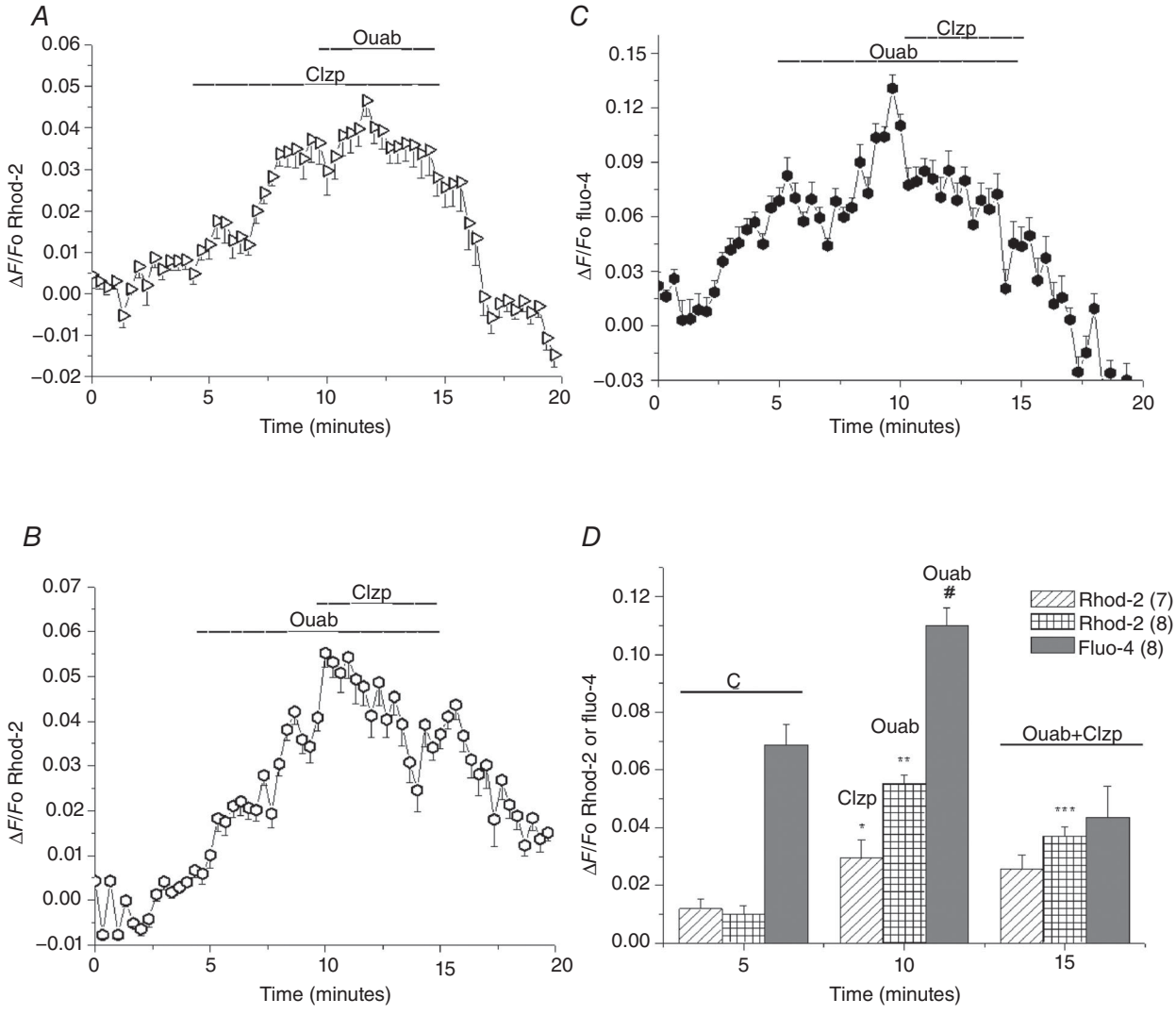

Figure 5. Changes in cytosolic and mitochondrial free calcium induced by clonazepam and ouabain in cardiomyocytes from hyperthyroid rats
Changes in the mitochondrial $[Ca^{2+}]$ (*A*) and (*B*) and cytosolic $[Ca^{2+}]$ (*C*) estimated by the relative fluorescence signal ($\Delta F/F_0$) of rhod-2 and fluo-4, respectively, in isolated cardiomyocytes perfused with control Krebs solution (C) or clonazepam (Clzp) and ouabain (Ouab). See the order of interventions in each protocol and the comparison of them at three time points (5, 10 and 15 min) in *D*. Results are means ± SEM ($n = 7$, 8 and 8 for *A*, *B* and *C*, respectively). ANOVA for each protocol in *D*: $P = 0.047$ for *A*, $P < 0.0001$ for *B* and $P < 0.0001$ for *C*; *a posteriori* tests: $^{*}P < 0.05$ *versus C*, ** and $^{***}P < 0.001$ *versus C* but $P < 0.01$ between them, and $^{\#}P < 0.01$ *versus C*). See supporting information for further information.

perfusion also slightly increased the fluo-4 signal before the increase induced by C-Caff-low Na, but it did not significantly change the rhod-2 signal.

Role of the mPTP

In order to evaluate whether the dysfunction induced by blocking the mNCX with Clzp was due to activation of the mPTP, the ventricles were treated before and during ischaemia and reperfusion with Cys A at 0.2 μmol l^{-1}. Table 1 shows that the presence of Cys A before, during and after perfusion with Clzp prevented the diastolic contracture (ΔLVEDP) induced by Clzp in HpT hearts, but increased it in EuT hearts. Figure 7 shows that Cys A also improved the PICR and the muscle economy of HpT hearts treated with Clzp (Fig. 7*A* and *B*). In contrast, Cys A did not change the PICR or the muscle economy in reperfused EuT ventricles independently of the presence of Clzp (Fig. 7*C* and *D*).

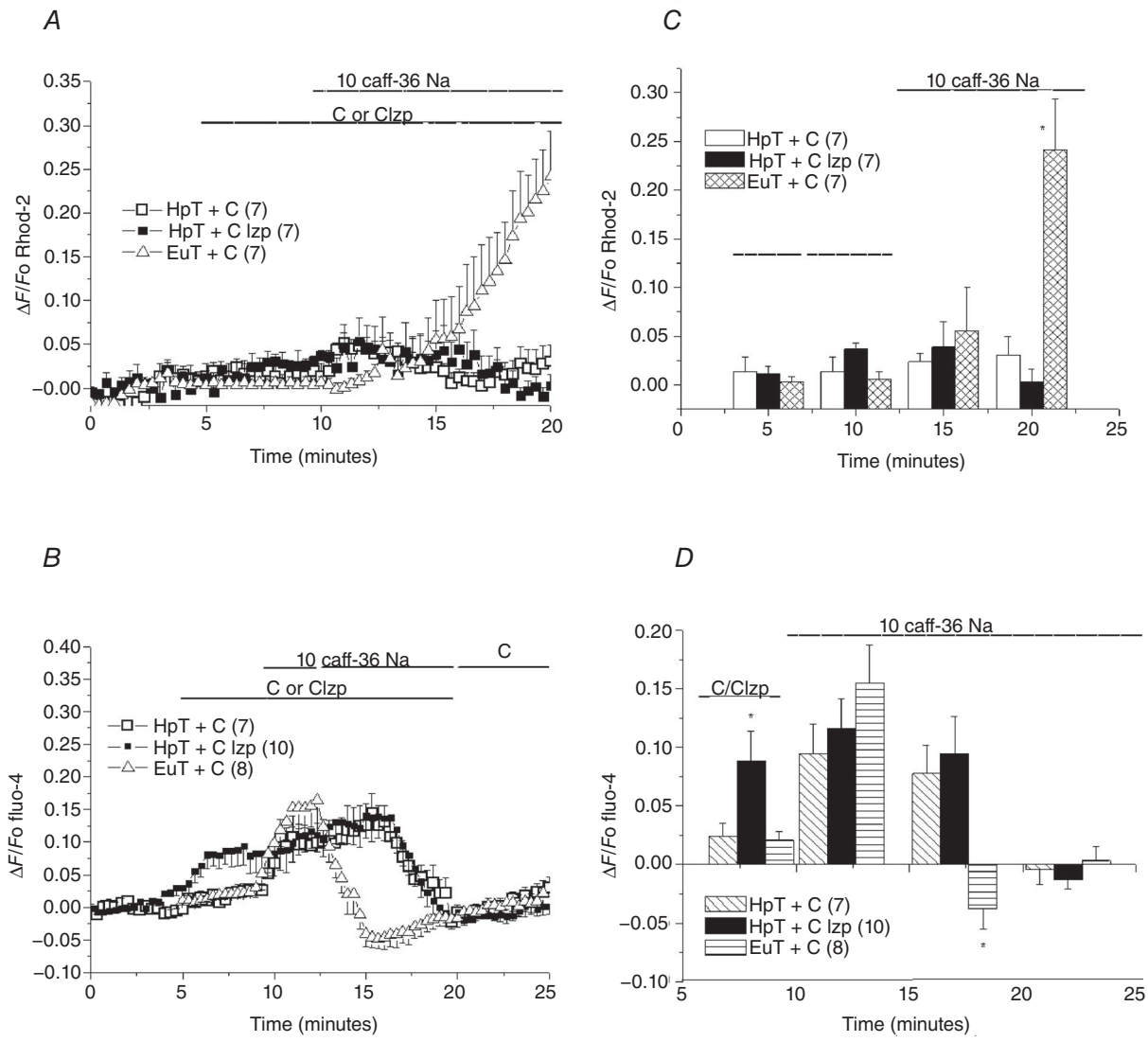

Figure 6. Changes in mitochondrial and cytosolic free calcium developed by sarcoreticular calcium release in cardiomyocytes of hyperthyroid and euthyroid rats
Changes in the mitochondrial [Ca^{2+}] (*A*) and cytosolic [Ca^{2+}] (*B*) estimated by the relative fluorescence signal ($\Delta F/F_0$) of rhod-2 and fluo-4, respectively, in isolated cardiomyocytes from HpT or EuT rats. Myocytes were perfused with control Krebs solution (C) or clonazepam (Clzp) and Krebs solution with 10 mmol l^{-1} caffeine and 36 mmol l^{-1} Na^+ (10 caff-36 Na) without or with Clzp, respectively. See the order of interventions in each graph and the respective comparisons of them at certain times in *C* and *D*. Results are means $\pm$ SEM (number of experiments is given in parentheses; $^*P < 0.05$ *versus* the others). See supporting information for further information.

Role of the UCam

In order to evaluate whether the mitochondrial Ca^{2+} uniporter has different participation in stunned HpT and EuT hearts, the ventricles were pretreated with the selective blocker Ru360 at 1 μmol l^{-1}. Ru360 did not significantly change contractility before ischaemia. Ru360 induced a reduction of the PICR in both EuT and HpT hearts (up to 16.8 ± 2.4 and 17.8 ± 2.1% of initial *P*, respectively), with a similar reduction in muscle economy (*P*/*Ht*; compare Figs 8*B* and 2*C*). Also, Ru360 developed diastolic contracture during reperfusion in both types of hearts, which was significantly higher than that of non-treated ventricles (Table 1). Thus, the inhibition of UCam induced postischaemic dysfunction in both EuT and HpT hearts.

Role of the mK_{ATP} channels

The role of mK_{ATP} channels in stunned HpT and EuT hearts was studied by perfusing the ventricles with the selective blocker 5HD at 100 μmol l^{-1}. Figure 9*A* shows that 5HD reduced the PICR in HpT ventricles, reaching

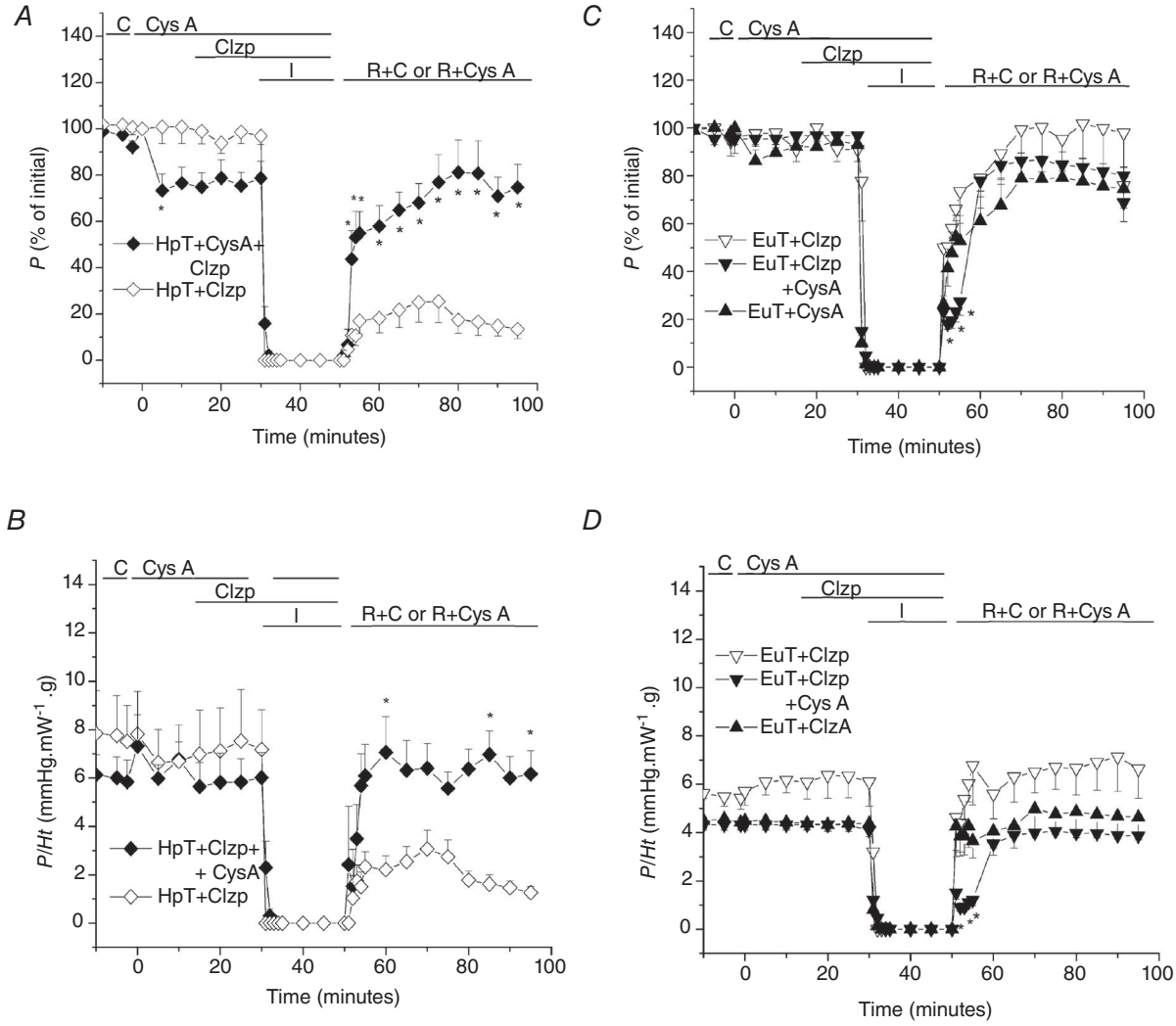

Figure 7. Role of the mitochondrial permeability transition pore (mPTP) evidenced by its inhibition with 0.2 μmol l^{-1} ciclosporin (Cys A), in hearts treated with 10 μmol l^{-1} clonazepam (Cys A + Clzp) in comparison to those treated only with Clzp or Cys A in HpT (n = 5 and 5 for treatments in *A* and *B*) and EuT rats (n = 6, 6 and 7 for the three treatments in *C* and *D*)
A and *C* show maximal pressure developed (*P*; as a percentage of initial maximal pressure); *B* and *D* show total muscle economy (*P*/*Ht*; in millimetres of mercury grams per milliwatt). Results are means ± SEM. Two-way ANOVAs: $P < 0.0001$ by treatment and time, respectively; $^{*}P < 0.05$ *versus* other treatments by *a posteriori* Bonferroni tests. See supporting information for further information.

up to 70.6 ± 6.7% of initial *P* (compare with non-treated HpT hearts in Fig. 2*A*). Table 1 shows that 5HD increased the diastolic contracture (ΔLVEDP) during I/R compared with non-treated (C) hearts. These changes became in less economical reperfused HpT hearts when mK_{ATP} channels were blocked, with *P*/*Ht* up to 4.3 ± 1 mmHg mW^{-1} g, which is lower than that reached by C hearts (Fig. 9*B*). Nevertheless, in EuT hearts 5HD did not change either PICR (up to 68.1 ± 5.2% of initial *P*) or postischemic *P*/*Ht* (to 3.4 ± 0.3 mmHg mW^{-1} g; compare with non-treated EuT hearts in Fig. 2*A* and *C*, respectively), but it increased ΔLVEDP (Table 1). To evaluate whether the dysfunction produced by 5HD in HpT hearts was due to activation of the mPTP, hearts were treated with Cys A + 5HD and reperfused with Cys A. This intervention aggravated the dysfunction, because PICR reached up to 26.8 ± 7% of initial *P*, and *P*/*Ht* was lower than 1.4 ± 0.2 mmHg mW^{-1} g (Fig. 9), while ΔLVEDP was increased (Table 1). The results suggest that mK_{ATP} channels have a more important role in Ca^{2+} homeostasis during contractile recovery of HpT than of EuT postischaemic hearts.

Discussion

The findings of this study indicate that the hyperthyroid rat heart is more resistant to a short period of ischaemia than the euthyroid rat heart, thus preventing stunning. Compared with euthyroid hearts, the hyperthyroid

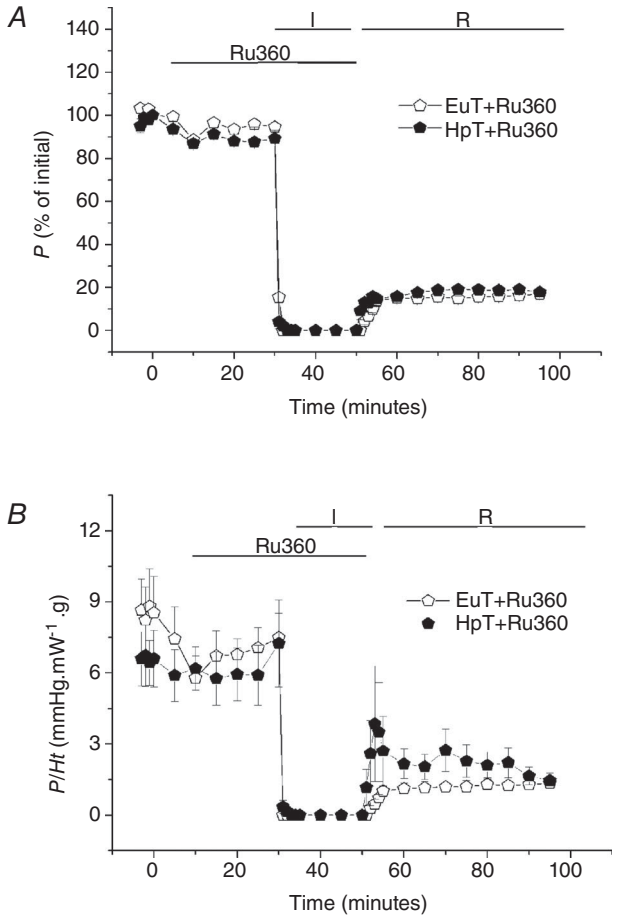

Figure 8. Role of the mitochondrial Ca^{2+} uniporter (Ucam) evidenced by blockade with 1 μmol l^{-1} Ru360 in isolated hearts from HpT (*n* = 6) and EuT rats (*n* = 6)
A, maximal pressure developed (*P*; as a percentage of initial pressure). *B*, total muscle economy (*P*/*Ht*; in millimetres of mercury grams per milliwatt). Results are means ± SEM. Two-way ANOVAs: $P > 0.05$ by treatment and $P < 0.0001$ by time, respectively. See supporting information for further information.

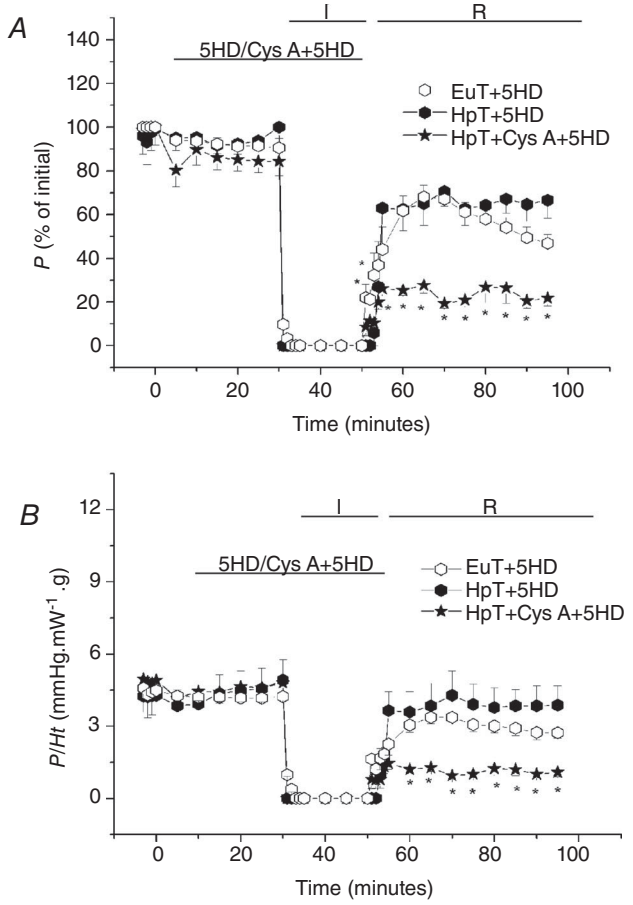

Figure 9. Role of mitochondrial K^+ (mK_{ATP}) channels evidenced by blockade with 100 μmol l^{-1} 5-hydroxydecanoate (5HD) in isolated hearts from HpT (*n* = 7) and EuT rats (*n* = 7), and effect of adding 0.2 μmol l^{-1} ciclosporin (Cys A + 5HD, *n* = 6) in HpT hearts
A, maximal pressure developed (*P*; as a percentage of initial pressure). *B*, total muscle economy (*P*/*Ht*; in millimetres of mercury grams per milliwatt). Results are means ± SEM. Two-way ANOVAs: $P < 0.0001$ by treatment and time, respectively; $^*P < 0.05$ *versus* HpT + 5HD, by *a posteriori* Bonferroni tests. See supporting information for further information.

ventricles developed a higher contractile recovery with lower energetic consumption. Consequently, hyperthyroidism or T_3 therapy rendered a more economical post-ischaemic heart. Moreover, the main difference between the two conditions was associated with mitochondrial Ca^{2+} extrusion by the mNCX, which plays an important role in Ca^{2+} homeostasis during postischaemic recovery in hyperthyroid but not in euthyroid hearts.

Before ischaemia, the hyperthyroid ventricle developed contractility and muscle economy (*P*/*Ht*) similar to that of euthyroid ventricles, in spite of the well-known upregulation of the β-adrenergic receptors (Tielens *et al.* 1996; Nyirenda *et al.* 2005). Given that our protocols did not include any addition of adrenergic agonists, the β-adrenergic pathway would not be involved, which was also suggested by the increased muscle economy of the HpT ventricle. In fact, it was reported that the addition of a β-adrenergic agonist in guinea-pig papillary muscle reduced the economy of isometric contraction (Holubarsch *et al.* 1994), and similar findings were observed with isoprenaline in left ventricular rat papillary muscles (Hasenfuss *et al.* 1987). The reduction in muscle economy was explained by the fact that the energy used for calcium cycling and cross-over of myofilaments increased more than the mechanical output elicited by β-adrenergic stimulation in the human myocardium (Hasenfuss *et al.* 1994).

In contrast, thyroid hormone has been associated with enhanced cardiac mass, mitochondrial population and protein content per mitochondrion (de Martino Rosaroll *et al.* 1988), as well as mitochondrial enzymatic activity (Martín-García, 2010). In agreement with this, our results showed both an increase in heart-to-body mass ratio and increased participation of the mNCX in the postischaemic hyperthyroid rat heart. The selective blockade of mNCX with 10 μmol l^{-1} Clzp (Cox & Matlib, 1993) strongly affected both the PICR and muscle economy (*P*/*Ht*) in HpT ventricles, which were only changed slightly in the EuT heart. These differences allowed to suggest two possible scenarios. The first possibility is that Clzp reduces the mitochondrial Ca^{2+} contribution to the sarcorreticular store, as we had previously found in cardioplegic rat hearts (2009Ragone & Consolini, 2009). The second possibility is that Clzp could induce a mitochondrial Ca^{2+} overload large enough to trigger mPTP opening with the consequent rise in $[Ca^{2+}]_i$ and diastolic contracture during reperfusion.

The first scenario was evaluated by comparing the muscle contracture developed when reperfusing with 10 mmol l^{-1} caffeine and low-$[Na^+]$ Krebs solution, which was considered proportional to the SR Ca^{2+} content (Bers, 2001). The associated *Ht* would mainly include the energy dissipated by cytosolic Ca^{2+} removal, either by mitochondria or by a futile cycle of SERCA (Consolini *et al.* 2007; Ragone & Consolini, 2009). Both HpT and EuT hearts developed similar AUC-ΔLVP and AUC-*Ht*, such that both tissues would be cycling a similar caffeine-sensitive SR Ca^{2+} content. Nevertheless, at the start of reperfusion the EuT ventricle developed a higher peak of contracture than that of the HpT heart, suggesting that Ca^{2+} is quickly lost from the SR in the postischaemic EuT heart, but slowly in the HpT heart. This difference is consistent with the fact that the EuT heart developed a higher diastolic contracture than the HpT heart during reperfusion. In isolated cardiomyocytes, the increase in free mitochondrial $[Ca^{2+}]$ ($\Delta F/F_0$ of rhod-2) evoked by 10 mmol l^{-1} caffeine–low $[Na^+]_o$ was smaller in HpT myocytes than in EuT myocytes. In contrast, the changes in free cytosolic $[Ca^{2+}]$ (fluo-4 $\Delta F/F_0$) were similar in amplitude for both types of myocytes, although the fluo-4 signal still fell during the caffeine–low Na^+ perfusion more slowly in HpT than in EuT ventricles. Given that SERCA and NCX were both ineffective during caffeine–low Na^+ perfusion, the reduction in cytosolic $[Ca^{2+}]$ must be due to the functioning of the mitochondrial Ca^{2+} uniporter (UCam). All these results suggest that during a situation of SR release, the mitochondria of EuT hearts retain more Ca^{2+} more rapidly than those of HpT hearts. Then, during reperfusion of EuT hearts, both mitochondria and the SR contribute to develop Ca^{2+} overload, resulting in higher diastolic contracture than in HpT hearts. Given that mitochondrial $[Ca^{2+}]$ determines the metabolic rate and a heat fraction independent of contractility (Consolini *et al.* 1997), the low mitochondrial $[Ca^{2+}]$ in HpT heart agrees with a higher muscle economy (*P*/*Ht*) during reperfusion compared with the EuT ventricle. In contrast, Clzp reduced the AUC-ΔLVP induced by caffeine–low Na^+ perfusion in both HpT and EuT hearts, suggesting that the mNCX contributes to regulate the SR Ca^{2+} content, as we had described in cardioplegic hearts (Ragone & Consolini, 2009). The AUC-*Ht* represents the energy consumed during the caffeine-induced contracture associated with the ATP hydrolysis used either by myofilaments or by Ca^{2+}-removal transporters. Given that at such low $[Na^+]_o$ the Ca^{2+} extrusion by the SL-NCX is not favoured, heat is released mainly by the Ca^{2+} cycling through SERCA (in the caffeine-induced leaky SR) and Ca^{2+} uptake by the UCam (dissipating the mitochondrial electrochemical potential; Ragone *et al.* 2013). When the mNCX is blocked by Clzp, an increase in mitochondrial $[Ca^{2+}]$ is expected, as observed in cardiomyocytes in the present and previous studies (Consolini *et al.* 2011), with the consequent further increase in metabolism and *Ht*. In fact, estimating muscle economy during the caffeine-induced contracture as the ratio AUC-ΔLVP/AUC-*Ht*, we can see that in the presence of Clzp the muscle was less economical than in its absence. The effect of Clzp on the mNCX was antagonized with 30 μmol l^{-1} ouabain, a concentration higher than that used in guinea-pig hearts (Ragone *et al.* 2013) owing to the low sensitivity of rat hearts to digitalis

(Gupta *et al.* 1986). This drug blocks the Na^+,K^+-ATPase and increases $[Na^+]_i$, which should enable mitochondria to increase Ca^{2+} extrusion by the mNCX (Liu *et al.* 2010; Boyman *et al.* 2013). Ouabain demonstrated the well-described positive inotropism before ischaemia, due to the reduced driving force for the sarcolemmal Ca^{2+} efflux by the SL-NCX, in both HpT and EuT ventricles. After ischaemia, ouabain maintained both the PICR and muscle economy of HpT hearts but reduced them in EuT ventricles according to the increase in Ca^{2+} overload of SR and mitochondria. Moreover, in HpT hearts Ouab improved the PICR that had been reduced by Clzp (up to 45.7 ± 8.7 *versus* 13.0 ± 3.8% of the initial *P* in the presence of Ouab and in its absence, respectively) and it reduced the diastolic contracture during I/R. These results demonstrate converse effects of blocking the mNCX and favouring its activation through the increase in Na^+ gradient. The results also show the more important role of the mNCX in HpT than in EuT hearts as a regulator of the SR Ca^{2+} content, and consequently, of Ca^{2+} transients and contractility. Moreover, despite the fact that mitochondrial $[Ca^{2+}]$ was increased by both Clzp and Ouab, the effects were not additive (Fig. 5). Coherently, HpT hearts treated with Ouab and Clzp suffered a reduction in PICR and *P*/*Ht* and increment in ΔLVEDP compared with the treatment with Ouab alone. The results also suggest that mitochondria could trigger a Ca^{2+} overload aggravated by the increase in $[Na^+]_i$ during I/R, which develops contractile dysfunction. All these results emphasize the critical role of mNCX in the HpT hearts.

The second scenario suggests that the negative effect of Clzp in the HpT heart could be due to an indirect activation of mPTP. This was confirmed by addition of Cys A, which prevented the dysfunction induced by Clzp in HpT ventricles. In EuT hearts, however, Cys A did not change PICR but instead increased ΔLVEDP, an effect also developed in the absence of Clzp. This effect of Cys A had been described and attributed to a non-specific binding (Griffiths & Halestrap, 1993; Ragone & Consolini, 2009; Xie *et al.* 2014). The findings suggest that the inhibition of mNCX produced a mitochondrial Ca^{2+} overload sufficient to open the mPTP in HpT hearts, but not in this model of stunning in EuT ventricles. Our results agree with other reports that did not find mPTP opening with an ischaemia shorter than 30 min in rat hearts at 37°C, with low damage (Griffiths & Halestrap, 1993). The activation of mPTP causes loss of mitochondrial content, swelling and dysfunction (Bernardi & Di Lisa, 2015).

Given that mitochondria play an important role in the Ca^{2+} homeostasis of postischaemic HpT hearts, we also evaluated the function of the UCam by its selective blocking with 1 μmol l^{-1} Ru360 (García Rivas *et al.* 2005; Foskett & Philipson, 2015). As in our previous studies in ischaemic rat hearts (Ragone & Consolini, 2009), blockade of the UCam strongly affected PICR and *P*/*Ht* during reperfusion in both HpT and EuT hearts, with diastolic contracture. The effect of Ru360 may be explained by both the critical role of Ca^{2+} as a mediator between cytosolic demand and activation of enzymes from mitochondrial metabolism (O'Rourke & Blatter, 2009) and the consequent increase in $[Ca^{2+}]_i$ and LVEDP (Harrington & Murphy, 2015). The results are in agreement with another report, where Ru360 was less protective than Cys A in the Ca^{2+}-induced dysfunction (Yarana *et al.* 2012).

We also evaluated the role of mK_{ATP} channels on mitochondrial energetics, because this transport mechanism helps to maintain the mitochondrial ΔΨ and prevent Ca^{2+} overload by UCam influx (Garlid *et al.* 2009; Sato & Marban, 2000). The mK_{ATP} channels were selectively blocked with 100 μmol l^{-1} 5HD, a concentration that does not affect sarcolemmal K_{ATP} channels (Murata *et al.* 2001). These conditions predispose to mitochondrial energy dissipation to maintain the mitochondrial ΔΨ, which has been described previously in beating hearts (Garlid *et al.* 2009) and in cardioplegic rat hearts exposed to I/R (Ragone & Consolini, 2009). The treatment with 5HD reduced PICR and muscle economy of HpT but not EuT ventricles, again suggesting that mitochondria influence the postischaemic Ca^{2+} transients more in HpT than in EuT hearts. However, 5HD induced diastolic contracture in both HpT and EuT hearts, suggesting that mitochondrial $[Ca^{2+}]$ contributes to the regulation of cytosolic resting Ca^{2+}. To evaluate whether the dysfunction induced by 5HD in HpT hearts was triggered by activation of mPTP by high mitochondrial $[Ca^{2+}]$, hearts were treated with Cys A + 5HD and reperfused with Cys A, considering that mPTP is opened during the first minutes of reperfusion (Griffiths & Halestrap, 1993). Unexpectedly, this treatment aggravated stunning, reducing muscle economy and increasing diastolic contracture. This is in agreement with a recent report on the functional cross-talk between mPTP and mK_{ATP} channels because both are targets of protein kinase C; consequently, the blockade of mK_{ATP} channels abolished the protective effect of Cys A (Xie *et al.* 2014). The findings suggest that 5HD is likely to have induced mPTP activation in HpT hearts.

In conclusion, this study demonstrates that hyperthyroidism prevents the stunning of rat hearts challenged by a short episode of I/R, because the contractile recovery it improves the postischaemic muscle economy and the contractile recovery. This phenomenon may be related to differences in the mitochondrial mechanisms associated with the regulation of Ca^{2+} homeostasis in hyperthyroidism compared with the euthyroid heart. The main differences are the reduced mitochondrial Ca^{2+} accumulation consequent to high Ca^{2+} extrusion by the mNCX and mK_{ATP} channel functioning to maintain the

electrochemical gradient. These 'low-Ca^{2+}' mitochondria are in functional interaction with a SR with slow Ca^{2+} release in hyperthyroid hearts. These results contribute to a better understanding of the role of mitochondria in the regulation of bioenergetics in pathological situations and suggest the possible benefits of thyroid therapy in the treatment of such pathologies as myocardial stunning by coronary ischaemia.

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Additional information

Competing interests

None declared.

Author contributions

All authors approved the final version of the manuscript, all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Supporting information

Table S2A. Statistics of Figure 2A, about percentage of the initial maximal pressure development (P) of hyperthyroid and euthyroid hearts without and with clonazepam.
Table S2B. Statistics of Figure 2B, percentage of the initial total heat rate (Ht) of hyperthyroid and euthyroid hearts without and with clonazepam.
Table S2C. Statistics of Figure 2C, muscle economy (P/Ht) of hyperthyroid and euthyroid hearts without and with clonazepam.
Table S3A. Statistics of Figure 3A, percentage of the initial maximal pressure development (P) of hyperthyroid hearts treated with ouabain without and with clonazepam.
Table S3B. Statistics of Figure 3B, muscle economy (P/Ht) of hyperthyroid hearts treated with ouabain without and with clonazepam.
Table S3C. Statistics of Figure 3C, percentage of the initial maximal pressure development (P) of euthyroid hearts treated with ouabain without and with clonazepam.
Table S3D. Statistics of Figure 3D, muscle economy (P/Ht) of euthyroid hearts treated with ouabain without and with clonazepam.
Table S4AB-Ht. Statistics of Figure 4A-B in total heat rate (Ht) of hyperthyroid and euthyroid hearts under ischemia and reperfusion with 10 mM caffeine-36 mM Na^+-Krebs.
Table S4AB-LVP. Statistics of Figure 4A-B in maximal pressure development (P) of hyperthyroid and euthyroid hearts under ischemia and reperfusion with 10 mM caffeine-36 mM Na^+-Krebs.
Table S4C. Statistics of Figure 4C, area under the curve of LVP and Ht of hyperthyroid and euthyroid hearts with and without clonazepam during the reperfusion with 10 mM caffeine-36 mM Na^+-Krebs.
Table S5D. Statistics of Figure 5D, ΔF/Fo of Fluo-4 and Rhod-2 in hyperthyroid cardiomyocytes during perfusion with clonazepam and/or ouabain.
Table S6CD. Statistics of Figure 6C-D, ΔF/Fo of Fluo-4 and Rhod-2 in hyperthyroid and euthyroid cardiomyocytes with or without clonazepam and perfusion with 10 mM caffeine-36 mM Na^+-Krebs.
Table S7A. Statistics of Figure 7A, percentage of the initial maximal pressure development (P) of hyperthyroid hearts treated with clonazepam and cyclosporin A.
Table S7B. Statistics of Figure 7B, muscle economy (P/Ht) of hyperthyroid hearts treated with clonazepam and cyclosporin A.
Table S7C. Statistics of Figure 7C, percentage of the initial maximal pressure development (P) of euthyroid hearts treated with clonazepam and cyclosporin A.
Table S7D. Statistics of Figure 7D, muscle economy (P/Ht) of euthyroid hearts treated with clonazepam and cyclosporin A.
Table S8A. Statistics of Figure 8A, percentage of the initial maximal pressure development (P) of euthyroid and hyperthyroid hearts treated with Ru360.
Table S8B. Statistics of Figure 8B, muscle economy (P/Ht) of euthyroid and hyperthyroid hearts treated with Ru360.
Table S9A. Statistics of Figure 9A, percentage of the initial maximal pressure development (P) of euthyroid and hyperthyroid hearts treated with 5HD.
Table S9B. Statistics of Figure 9B, muscle economy (P/Ht) of euthyroid and hyperthyroid hearts treated with 5HD.