RESEARCH

Role of AMPK in the protective effects exerted by triiodothyronine in ischemic-reperfused myocardium

Romina Hermann[1,2], Victoria Evangelina Mestre Cordero[1,2], María de las Mercedes Fernández Pazos[1], Mailen Florencia Córdoba[1], Federico Joaquín Reznik[1], Débora Elisabet Vélez[1,2], Andrea Lorena Fellet[1,2] and **María Gabriela Marina Prendes[1,2]**

[1]Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Departamento de Ciencias Biológicas, Cátedra de Fisiología, Buenos Aires, Argentina
[2]CONICET – Universidad de Buenos Aires, Instituto de Química y Metabolismo del Fármaco (IQUIMEFA), Buenos Aires, Argentina

Correspondence should be addressed to R Hermann: rhermann@ffyb.uba.ar

Abstract

Recent studies have provided evidence that triiodothyronine (T3) might play an effective role in the recovery of ischemic myocardium, through the preservation of mitochondrial function and the improvement of energy substrate metabolism. To this respect, it has been suggested that T3 could activate AMP-activated protein kinase (AMPK), the cellular 'fuel-gauge' enzyme, although its role has yet to be elucidated. The aim of the present study was to investigate the effects produced by acute treatment with T3 (60 nM) and the pharmacological inhibition of AMPK by compound C on isolated rat left atria subjected to 75 min simulated ischemia-75 min reperfusion. Results showed that T3 increased AMPK activation during simulated ischemia-reperfusion, while compound C prevented it. At the end of simulated reperfusion, acute T3 treatment increased contractile function recovery and cellular viability conservation. Mitochondrial ultrastructure was better preserved in the presence of T3 as well as mitochondrial ATP production rate and tissue ATP content. Calcium retention capacity, a parameter widely used as an indicator of the resistance of mitochondrial permeability transition pore (MPTP) to opening, and GSK-3β phosphorylation, a master switch enzyme that limits MPTP opening, were increased by T3 administration. All these beneficial effects exerted by T3 acute treatment were prevented when compound C was co-administrated. The present study provided original evidence that T3 enhances intrinsic activation of AMPK during myocardial ischemia-reperfusion, being this enzyme involved, at least in part, in the protective effects exerted by T3, contributing to mitochondrial structure and function preservation, post-ischemic contractile recovery and conservation of cellular viability.

Key Words

- triiodothyronine
- myocardium
- AMP-activated protein kinase
- simulated ischemia-reperfusion
- compound C
- cardiprotection

Journal of Molecular Endocrinology (2021) **66**, 207–221

Introduction

It has been described that certain severe extrathyroid diseases produce changes in the metabolism and peripheral concentrations of thyroid hormones. To this respect, a decrease in peripheral conversion of thyroxine (T4) in triiodothyronine (T3) and, concomitantly, an increase in the synthesis of reverse T3, due to a lower activity of the enzyme 5'-monodeiodasa, has been reported in patients suffering from acute myocardial infarction,

https://jme.bioscientifica.com
https://doi.org/10.1530/JME-20-0314

occurring rapidly within the onset of symptoms, reaching the lowest values after approximately 72 h (Pingitore *et al.* 2016). This entity, commonly known as 'euthyroid patient syndrome' or 'low T3 syndrome' (Ojamaa *et al.* 2000, Friberg *et al.* 2001), has been reported as the more frequent mild alteration of thyroid hormones metabolism in acute myocardial infarction (Li *et al.* 2011). If this condition constitutes an adaptive metabolic response or if it aggravates the patient's condition, it is yet to be elucidated.

It is well known that thyroid hormones have a significant impact on energy metabolism and cardiac function, both at ventricular and atrial level, which are mediated by both genomic and non-genomic effects. Based on this, studies in cells, animals and even in humans postulate that thyroid hormone supplementation could be implemented as an additional therapeutic option for the treatment of patients who have suffered acute myocardial infarction (Ranasinghe *et al.* 2006, Pantos *et al.* 2011, Pingitore *et al.* 2012), offering hope for the development of novel cardioprotective strategies that contribute to attenuate ischemia-reperfusion injury.

Recent studies have provided evidence that T3 could enhance the recovery of ischemic myocardium through the preservation of mitochondrial function and the improvement of energy substrate metabolism. In this regard, AMP-activated protein kinase (AMPK) is a widely known enzyme for its critical role in the regulation of energy metabolism against different types of stress, both physiological and pathological. Like many other protein kinases, AMPK has been shown to be significantly activated when phosphorylated by kinases, in a threonine referred to as Thr^{172} due to its position in the sequence of the catalytic α subunit (Hawley *et al.* 2003, Shaw *et al.* 2004). Once AMPK is activated, it promotes acute control of cellular energy homeostasis through the phosphorylation of enzymes directly involved in the regulation of the corresponding metabolic pathways, contributing to ATP production. Although numerous studies have shown that AMPK is activated during ischemia, and remains activated during early reperfusion, the role played by this enzyme in the myocardium undergoing ischemia-reperfusion is recently beginning to be understood.

Moreover, it has been suggested that AMPK, among other protein kinases, could be responsible for promoting the phosphorylation of glycogen synthase kinase 3β (GSK-3β) (Tian *et al.* 2019). This serine/threonine kinase has been proposed as a particularly important target of drug therapy for ischemia-reperfusion injury because the phosphorylation and concomitant inhibition of GSK-3β during ischemia-reperfusion is believed to be a final common mechanism mediating myocardial protection by many interventions (Juhaszova *et al.* 2004). Regarding this, phosphorylation of GSK-3β has been related to the suppression of the mitochondrial permeability transition pore opening (MPTP) (Nishihara *et al.* 2007), a non-specific and high conductance pore that opens in the mitochondrial inner membrane precipitated by several different factors, including calcium matrix and phosphate overload, ATP depletion, oxidative stress, and rapid correction of intracellular pH from the acidification induced by myocardial ischemia (Di Lisa *et al.* 2010). Massive and irreversible MPTP opening at the onset of myocardial reperfusion is widely considered as a critical mediator of ischemia-reperfusion injury (Halestrap & Richardson 2015). On this basis, any intervention capable to prevent or at least reduce the extent of MPTP opening results critical to preserve mitochondria and myocardial function after ischemia-reperfusion.

From this background, the possible protective role that thyroid hormones could exert on the myocardium undergoing ischemia-reperfusion emerges. Such effects could determine an important role, especially for T3, in the early recovery of myocardial tissue. To this respect, we hypothesized that AMPK might be involved, at least in part, in the acute effects that T3 develops in the ischemic-reperfused myocardium. In this concern, the aim of the present study was to gain a deeper insight into the role of AMPK in the effects exerted by acute treatment with T3, in the myocardium subjected to ischemia-reperfusion.

Methods

Experimental protocol

This study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; http://oacu.od.nih.gov/regs/guide/guide_2011.pdf) and Argentine Law No. 14346 concerning animal protection. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (EXP-FYB No 54965/17).

Female Sprague–Dawley rats weighing 220–270 g fed *ad libitum* and maintained on a 12 h light:12 h darkness cycle were used in this study. Rats were sacrificed by carbon dioxide inhalation with the purpose of inducing unconsciousness and death. Afterwards, hearts were excised quickly and cooled in ice-cold saline until contractions stopped. Left atria were excised and mounted

https://jme.bioscientifica.com
https://doi.org/10.1530/JME-20-0314

isometrically in 20 mL organ baths at a resting force of 750 mg, and paced at 1 Hz with 5–10 V, 0.6 ms square pulses applied through bipolar punctuate electrodes during the whole experiment. The bathing medium was a Krebs–Ringer bicarbonate solution of the following composition (mmol/L): NaCl 120; $NaHCO_3$ 25; KCl 4.8; $MgSO_4$ 1.33; KH_2PO_4 1.2; $CaCl_2$ 1.6; Na_2EDTA 0.02, continuously bubbled with 95% O_2–5% CO_2, pH 7.4 and kept at 31°C. As metabolic substrate, the bathing medium contained 10 mmol/L D-glucose.

After 60-min stabilization period, atria were subjected to 75-min simulated ischemia followed by 75-min simulated reperfusion. For the induction of simulated ischemia, the medium was bubbled with 95% N_2:5% CO_2 (pH 6.8–7) and 10 mmol/L D-glucose was replaced with 10 mmol/L 2-deoxy-D-glucose (Sigma–Aldrich) (Hermann *et al.* 2015, 2018). Simulated reperfusion was initiated by a buffer exchange to normoxic Krebs–Ringer bicarbonate solution.

Pharmacological interventions

60 nM 3,3′,5-Triiodo-L-thyronine (Sigma–Aldrich) was added to the bathing medium at the onset of simulated ischemia and maintained throughout the experiment (Pantos *et al.* 2009, 2011).

10 µM compound C, (6-(4-(2-piperidin-1-yl-ethoxy) phenyl)-3-pyridin-4-yl-pyrazolo(1,5-a)pyrimidine) (Sigma–Aldrich), a potent, reversible and widely used pharmacological inhibitor of AMPK (Zhou *et al.* 2001) was added to the bathing medium at the onset of simulated ischemia and maintained throughout the experiment.

Measurement of contractile function

Mechanical variables were recorded with stress transducers coupled to an amplifier and Grass polygraph. Systolic function was assessed by peak developed force (F), peak rate of contraction (+dF/dt) and force-time index (FTI), which reflects the mechanical energy of contraction per beat. Peak developed force was calculated as the difference between the peak of developed force curve and the resting force. FTI was obtained from the integration of the area under the systolic portion of the developed force curve. Diastolic function was assessed by developing contracture, measured as the rise in resting force, and the peak rate of relaxation (−dF/dt).

F, FTI and ±dF/dt were expressed as a percentage of the respective basal values at the end of the 60-min aerobic stabilization period. The rise in resting force was expressed as a percentage of the peak developed force at the end of the 60-min aerobic stabilization period.

Tissue ATP content

Atria samples were homogenized in 3% (mass/volume) cold perchloric acid for determination of tissue ATP. After removal of the denatured protein by centrifugation at 12,000 ***g*** for 5 min, aliquots of supernatant were neutralized (Hermann *et al.* 2015, 2018) and tissue ATP concentration was determined by luciferin–luciferase luminometry assay (ABCAM).

Protein concentrations were determined in the solubilized pellet by Lowry's method (Lowry *et al.* 1951). The concentration of ATP was expressed as picomoles of ATP per milligram of tissue protein.

Measurement of cellular viability

The reduction of 3-(4,5 dimethylthiazol-2yl)-2.5 diphenyltetrazolium bromide (MTT) (Sigma–Aldrich) to blue formazan by cellular dehydrogenases was assessed at the end of each experimental protocol to measure cellular viability.

For this aim, atria were incubated in 2 mL PBS containing 1 mg/mL MTT for 90 min at 37°C. Atria were then homogenized in 2 mL DMSO at 9500 rpm for 1 min and the homogenate was centrifuged at 1000 ***g*** for 10 min. The absorbance of the colored supernatant was measured spectrophotometrically at 520 nm. Optical density per milligram of wet weight was calculated (Hermann *et al.* 2015). The results were expressed as a percentage of the pre-ischemic value.

Western blotting

Atria were homogenized in lysis buffer (18.2 mmol/L HEPES, 1 mmol/L EDTA, 0.28 mol/L sucrose, 2 mmol/L dithiothreitol, 2 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L sodium fluoride, 1× protease inhibitor cocktail, pH 7.4), an aliquot was saved to measure proteins by the method of Bradford and the remaining sample was subjected to SDS-PAGE.

Equal amounts of protein were mixed with LAEMMLI sample buffer (BioRad) and resolved on 12% SDS-PAGE gels at 120 V. Next, proteins were transferred to polyvinylidene difluoride membranes at 15 V for 50 min and then blocked for 1 h with 5% BSA in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). Membranes were then incubated at 4°C overnight with polyclonal

https://jme.bioscientifica.com
https://doi.org/10.1530/JME-20-0314

rabbit anti-total AMPKα (Thermo Scientific) at 1:1000, polyclonal rabbit anti-phospho AMPKα Thr^{172} (Thermo Scientific) at 1:1000, polyclonal rabbit anti-GSK-3β (Santa Cruz Technologies) at 1:1000, polyclonal rabbit anti-phospho GSK-3β Ser 9 (Santa Cruz Technologies) at 1:1000 and polyclonal rabbit anti-β-actin antibody (Thermo Scientific) at 1:1000 dilution. HRP conjugated donkey anti-rabbit antibody (Thermo Scientific) at 1:250 dilution was then incubated for 2 h at room temperature. Bands were detected with Bio-Lumina detection reagent (Kalium Technologies) and exposed to an autoradiography film (Kodak). The films were scanned for band quantification using a Hewlett–Packard scanner and the intensity of the immunoblot bands was analyzed by densitometry using Image J software and normalized to β-actin. Results were expressed in arbitrary units.

Electron micrographs

Atria (~1mm^2 thick) were fixed for 4 h with 2.5% glutaraldehyde in phosphate buffer (pH 7.4) and then postfixed for 1 h in 1% osmium tetroxide. After this, staining en bloc with 2% uranyl acetate, dehydration in a graded series of ethanol, and imbibition in Durcupan resin were performed. Thin sections were prepared with a diamond knife and stained with lead citrate. Grids were examined under a Zeiss 109 electron microscope (Laboratorio Nacional de Investigación y Servicios en Microscopía Electrónica, Universidad de Buenos Aires).

Measurement of mitochondrial ATP synthesis rate

Mitochondria were isolated by differential centrifugation after tissue homogenization in ice-cold sucrose buffer solution (300 mmol/L sucrose, 10 mmol/L Tris–HCl, 2 mmol/L EGTA, 5 mg/mL BSA, pH 7.4) (Solem & Wallace 1993). The mitochondrial pellet was then washed three times in sucrose isolation buffer solution lacking BSA (Hermann *et al.* 2015).

Since it is well documented that complex I of the respiratory chain is most sensitive to reperfusion injury (Solaini & Harris 2005), mitochondrial ATP synthesis rate was measured in the presence of the complex I substrates pyruvate and malate. Mitochondria (750 µg protein/mL) were incubated for 5 min in a medium containing (mmol/L): KCl 125, Mops 20, Tris 10, EGTA 0.5, KH_2PO_4 2.4, $MgCl_2$ 2.5, malate 2.5, pyruvate 2.5, pH 7.4, in a metabolic shaker at 30°C. Aliquots were taken from the incubation mixture and ATP synthesis was then initiated by the addition of 2.5 mmol/L ADP. Produced ATP was measured at 5-s intervals for 120 s by luminescent method based on luciferin–luciferase reaction in which luciferase converts ATP and luciferin to oxyluciferin and light in the presence of oxygen, being the emission of light proportional to the concentration of ATP present in the sample (ABCAM). Mitochondrial protein concentration was determined by the method of Lowry using BSA as a standard. The rate of mitochondrial ATP synthesis was calculated and expressed as nanomoles per minute per milligram of mitochondrial protein.

Mitochondrial calcium retention capacity

The mitochondrial calcium retention capacity (CRC) was determined as the amount of calcium required to trigger a massive calcium release by isolated mitochondria. This parameter is used as an indicator of the resistance of the MPTP to opening after matrix calcium accumulation (Pardo *et al.* 2015). For this aim, the fluorometric probe Calcium Green-5N (Sigma–Aldrich) was used (Obame *et al.* 2008). This probe does not cross the mitochondrial membrane and functions as a low affinity indicator exhibiting high rate of dissociation, being these characteristics advantageous for evaluating rapid changes in the movement of Ca^{2+} in the medium.

To accomplish this goal, successive pulses of 6 µmol/L Ca^{2+} were added to samples of 0.5 mg/mL of isolated mitochondria suspended in buffer composed of 120 mmol/L KCl, 5 mmol/L MOPS, 5 mmol/L KH_2PO_4, 5 mmol/L malate, 5 mmol/L pyruvate, pH 7.4. After sufficient calcium loading, the MPTP opens and the concentration of this ion abruptly increases in the incubation medium, which was recorded by fluorescence changes of Calcium green-5N (100 nM) with excitation and emission wavelengths set at 506 and 532 nm, respectively. The results were expressed as nmol $CaCl_2$ per mg of mitochondrial proteins that induces massive MPTP opening.

Statistical analysis

All data are presented as mean ± S.E.M. Changes in the contractile function were statistically compared using a two-factor ANOVA for repeated measures in one factor followed by Tukey's test. All biochemical parameters were evaluated using one or two-way ANOVA followed by Tukey's test. Statistical analysis was carried out using GraphPad 8 (GraphPad Software). The probability level of 0.05 or lower was used as a criterion for biological significance.

https://jme.bioscientifica.com
https://doi.org/10.1530/JME-20-0314

Results

The diagrams presented in Fig. 1 detail the experimental protocols employed in the present study. Atria were randomly assigned to one of the experimental groups.

Contractile function

We first examined the effects produced by 75-min simulated ischemia and 75-min simulated reperfusion in isolated rat left atria. Figure 2 shows an abrupt decrease in the systolic parameters within the first 10 min of simulated ischemia. Following this early period, the decrease in these parameters occurred gradually. During the 75-min of simulated reperfusion, contractile parameters gradually recovered. Atria subjected to Is-Rs achieved a recovery of pre-ischemic systolic function of 35% at the end of the simulated reperfusion period. Acute treatment with T3 increased contractile function parameters recovery, reaching at 75-min of simulated reperfusion, the 50% of pre-ischemic systolic function. Although the administration of AMPK inhibitor did not affect *per se* systolic function recovery, it prevented the beneficial effects exerted by treatment with T3.

Figure 3 shows that acute treatment with T3 increases the development of ischemic contracture. This effect was more pronounced in the presence of T3 and compound C, without affecting the time when this peak appeared. On the other hand, during simulated ischemia, compound C generated a less pronounced decrease in the relaxation rate. After 75-min of simulated reperfusion, atria subjected to Is–Rs and treated acutely with T3 showed a significant increase in the recovery of peak rate of relaxation, compared with atria non-treated with the thyroid hormone. Compound C reversed the beneficial effect exerted by T3 over this functional parameter.

Tissue ATP content

Tissue ATP content was determined at 20-min and after 75-min of simulated ischemia. Table 1 shows that at 20-min of simulated ischemia, ATP levels fell to lower values when atria were treated with T3 or when the intrinsic AMPK activation was inhibited. However, after 75-min simulated ischemia tissue, ATP content was significantly reduced in all experimental groups.

At the end of 75-min of simulated reperfusion, tissue ATP content partially recovered. On the one hand, atria acutely treated with T3 exhibit a greater recovery of this high energy phosphate content, compared with non-treated atria. On the other hand, compound C significantly reduced tissue ATP level recovery at the end of simulated reperfusion period, both in T3 treated and not treated simulated ischemic-reperfused atria.

Cellular viability

Atria subjected to 60-min aerobic stabilization period were considered 100% of viable cells. After complete simulated ischemia-reperfusion protocol, atria showed a reduction

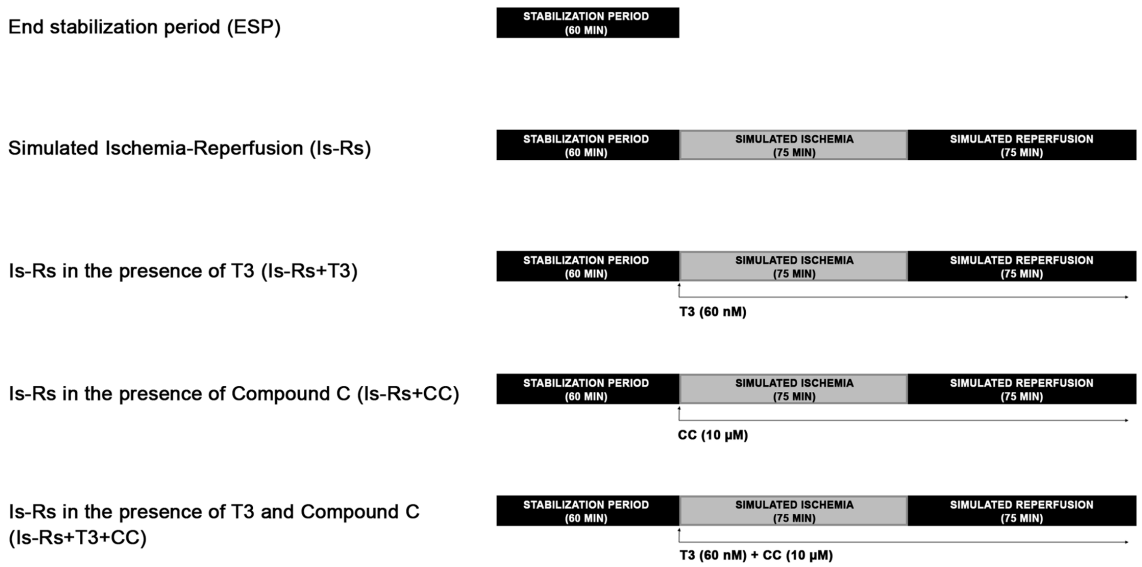

Figure 1
A schematic representation of the experimental protocols. Atria were randomly assigned to one of the groups showed in the figure.

https://jme.bioscientifica.com
https://doi.org/10.1530/JME-20-0314

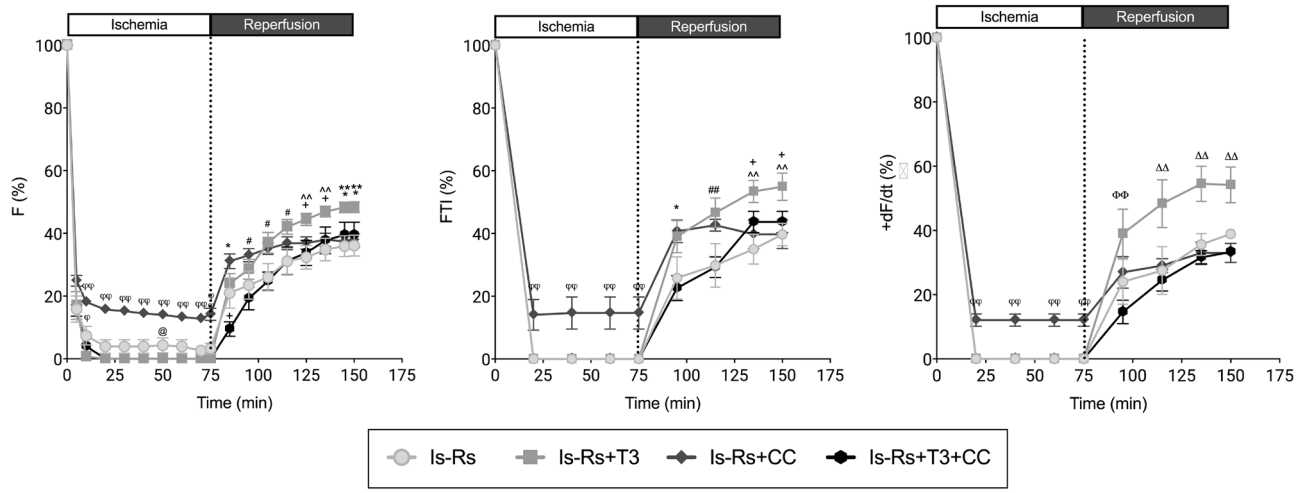

Figure 2
Systolic function parameters. Effects of acute treatment with triiodothyronine (T3) on peak developed force (F), peak rate of contraction (+dF/dt) and force-time index (FTI) in left isolated rat atria subjected to 75 min simulated ischemia–75 min simulated reperfusion (Is–Rs) in the presence or absence of compound C (CC). Values (mean ± S.E.M., $n = 8$/each condition) are expressed as a percentage of the respective basal values at the end of the 60-min stabilization period. $^{\varphi\varphi}P < 0.01$ vs Is–Rs, Is-Rs+T3, Is–Rs+T3+CC; $^{\varphi}P < 0.05$ vs Is–Rs+T3; $^{@}P < 0.05$ vs Is–Rs+T3, Is–Rs+T3+CC; $^{+}P < 0.05$ vs Is–Rs+CC, Is-Rs+T3+CC; $^{\#}P < 0.05$ vs Is–Rs, Is–Rs+T3+CC; $^{\#\#}P < 0.05$ vs Is–Rs, Is–Rs+T3+CC; $^{\wedge\wedge}P < 0.01$ vs Is–Rs; $^{**}P < 0.01$ vs Is–Rs, Is–Rs+CC; $^{*}P < 0.05$ vs Is–Rs+T3+CC; $^{\varphi\varphi}P < 0.01$ vs Is–Rs+T3+CC; $^{\Delta\Delta}P < 0.01$ vs Is–Rs, Is–Rs+CC, Is–Rs+T3+CC.

of approximately 30% on cellular viability at the end of this period (Fig. 4). Acute treatment with T3 partially prevented the impairment observed in non-treated atria, increasing cellular viability preservation after the complete experimental period. AMPK pharmacological inhibitor did not affect cellular viability percentage after simulated reperfusion period, showing no significant differences between treated and non-treated groups. However, compound C reverted the effect exerted by acute treatment with T3 in cellular viability.

AMPK phosphorylation by acute treatment with T3

The effects exerted by acute treatment with T3 in AMPK phosphorylation, both in simulated ischemia and reperfusion, were examined in the present study. Considering that AMPK α-subunit contains a serine-threonine kinase domain, which has a critical activating residue at Thr^{172} within the catalytic cleft, the phosphorylation status of this amino acid is widely used as an indicator of the activation state of this kinase

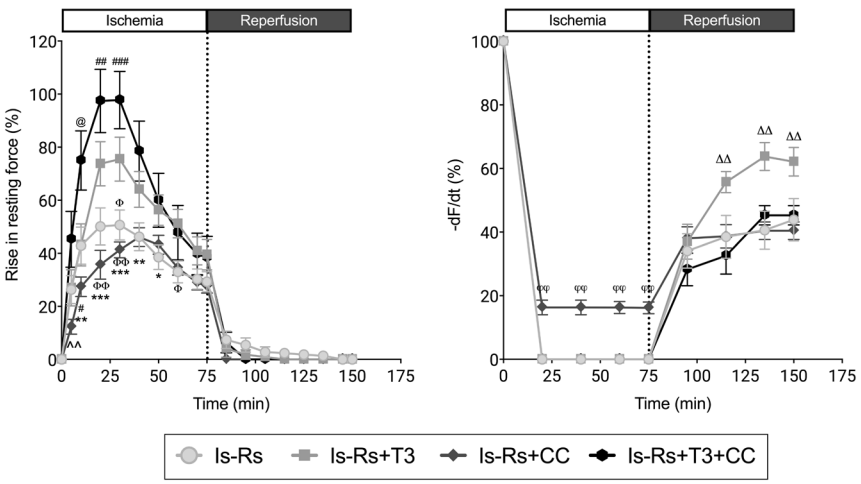

Figure 3
Diastolic function parameters. Effects of acute treatment with triiodothyronine (T3) on resting force and peak rate of relaxation (–dF/dt) in isolated left atria subjected to 75 min simulated ischemia–75 min simulated reperfusion (Is–Rs) in the presence or absence of compound C (CC). All values are expressed as mean ± S.E.M. ($n = 8$/each condition). Rise in resting force is expressed as a percentage of the peak force developed at the end of the stabilization period and –dF/dt as a percentage of the respective basal value at the end of the stabilization period. $^{\wedge\wedge}P < 0.01$ vs Is–Rs, Is–Rs+T3, Is–Rs+T3+CC; $^{*}P < 0.01$ vs Is–Rs+T3, Is–Rs+T3+CC; $^{**}P < 0.01$ vs Is–Rs+T3+CC; $^{***}P < 0.001$ vs Is–Rs+T3+CC; $^{\#}P < 0.05$ vs Is–Rs, Is–Rs+T3; $^{\#\#\#}P < 0.001$ vs Is–Rs; $^{@}P < 0.05$ vs Is–Rs, Is–Rs+T3; $^{\Phi}P < 0.05$ vs Is–Rs+T3; $^{\Phi\Phi}P < 0.01$ vs Is–Rs+T3; $^{\#\#}P < 0.01$ vs Is–Rs; $^{++}P < 0.01$ vs Is–Rs, Is–Rs+CC, $^{\varphi\varphi}P < 0.01$ vs Is–Rs, Is–Rs+T3, Is–Rs+T3+CC; $^{\Delta\Delta}P < 0.01$ vs Is–Rs, Is–Rs+CC, Is–Rs+T3+CC.

https://jme.bioscientifica.com
https://doi.org/10.1530/JME-20-0314

Table 1 Tissue ATP content.

	ESP	Control	T3	CC	T3 + CC
60 min aerobic	1040.0 ± 103.1				
20 min Is		902.2 ± 125.2	515.4 ± 92.6^^	420.5 ± 68.7^^	615.1 ± 31.0^^
75 min Is		158.5 ± 35.8***	103.2 ± 11.5***	22.83 ± 1.6***	148.3 ± 38.5***
75 min Rs		479.9 ± 36.6**#	644.8 ± 89.5**	285.8 ± 82.7**&	266.2 ± 46.0**&

Effects of 60 min aerobic stabilization period (ESP), 20 min simulated ischemia (20 min Is), 75 min simulated ischemia (75 min Is) and to 75 min simulated reperfusion (75 min Rs) on tissue ATP content of rat left atrial non-treated (control), acutely treated with triiodothyronine (T3), with compound C (CC) or co-treated with triiodothyronine and compound C (T3+CC). Values are mean ± S.E.M. (n = 6–8/experimental group).
***P < 0.001 vs ESP and respective 20 min value; **P < 0.01 vs ESP; ^^P < 0.01 vs ESP, 20 min Is; &P < 0.05 vs Is-Rs, Is-Rs+T3; #P < 0.05 vs Is-Rs+T3.

(Hawley *et al.* 1996). In this context, we explored the ratio of phosphorylated α-subunit in Thr^{172} (AMPKα-pThr^{172}) to total enzyme α-subunit (AMPKα-total) by Western blot analysis at 15-, 45- and 75-min of simulated ischemia and 15-, 45- and 75-min of simulated reperfusion.

The results showed a significant increase in the AMPKα-pThr^{172}/AMPKα-total ratio during simulated ischemia, reaching the maximum phosphorylation status at 45-min and sustaining it until the end of this experimental period. Acute treatment with T3 increased the enzyme phosphorylation ratio, reaching the maximum phosphorylation status at the end of 75-min simulated ischemia (Fig. 5 Panel A). During simulated reperfusion, AMPK remained phosphorylated for at least 45-min of simulated reperfusion, showing greater values when the atria were acutely treated with T3 (Fig. 5 Panel B). On the other hand, AMPK pharmacological inhibitor prevented the phosphorylation of the enzyme in Thr^{172} during the whole experiment either in the presence or absence of T3.

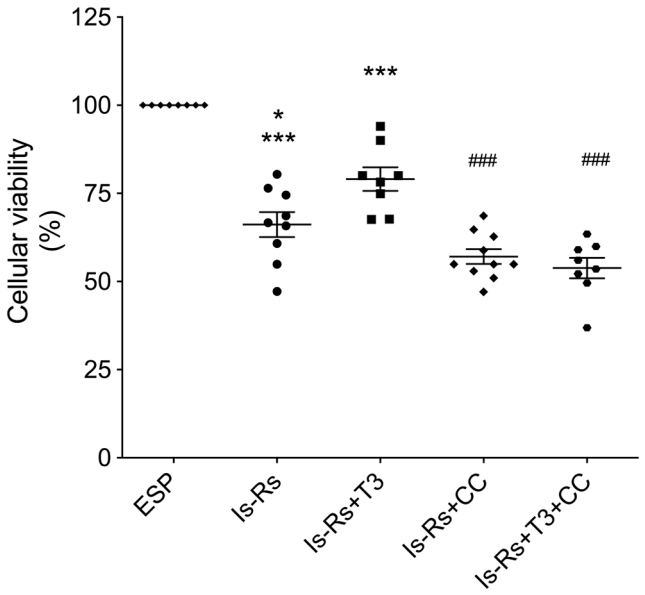

Figure 4
Cellular viability. Effects of acute treatment with triiodothyronine (T3) on cellular viability in isolated rat left atria subjected to 75 min simulated ischemia–75 min simulated reperfusion (Is–Rs) in the presence or absence of compound C (CC). Atria subjected to the 60-min aerobic stabilization period were considered 100% viable cells. Values are mean ± S.E.M. (n = 8–10/experimental group) and are expressed as percentage of the pre-ischemic value. ***P < 0.001 vs ESP; ###P < 0.001 vs ESP, Is–Rs+T3; *P < 0.05 vs Is–Rs+T3.

Myocardial and mitochondrial ultrastructure

Representative transmission electron micrographs of atria subjected to 60-min stabilization period revealed unaltered structure, showing myofibrils tightly placed alongside each other, sarcomeres arranged in a regular pattern surrounded by mitochondria with packed cristae (Fig. 6 Panel A). Electron micrographs obtained after 210-min of aerobic conditions, which corresponds to total experimental period, showed preservation of ultrastructural arrangement (Fig. 6 Panel B). Electron micrographs obtained from atria subjected to Is–Rs showed a greater deterioration of myocardial ultrastructural, frequently characterized by rounding and swelling mitochondria, altered configuration of the mitochondrial cristae, with disruption and separation of peaks and clearance of the mitochondrial matrix (Fig. 6 Panel C). Acute treatment with T3 induced mitochondrial structure preservation during simulated reperfusion (Fig. 6 Panel D), showing a reduction in the appearance of mitochondrial swelling and cristae disruption. Although compound C did not exert effects *per se* on myocardial and mitochondrial structure (Fig. 6 Panel E), it reverted the effects produced by acute treatment with T3 (Fig. 6 Panel F).

Rate of mitochondrial ATP synthesis

Present results showed that the mitochondrial rate of ATP synthesis was reduced by approximately 75% at the end of simulated reperfusion period. Mitochondria isolated from atria subjected to simulated ischemia-reperfusion and acutely treated with T3 showed a significantly lower

https://jme.bioscientifica.com
https://doi.org/10.1530/JME-20-0314

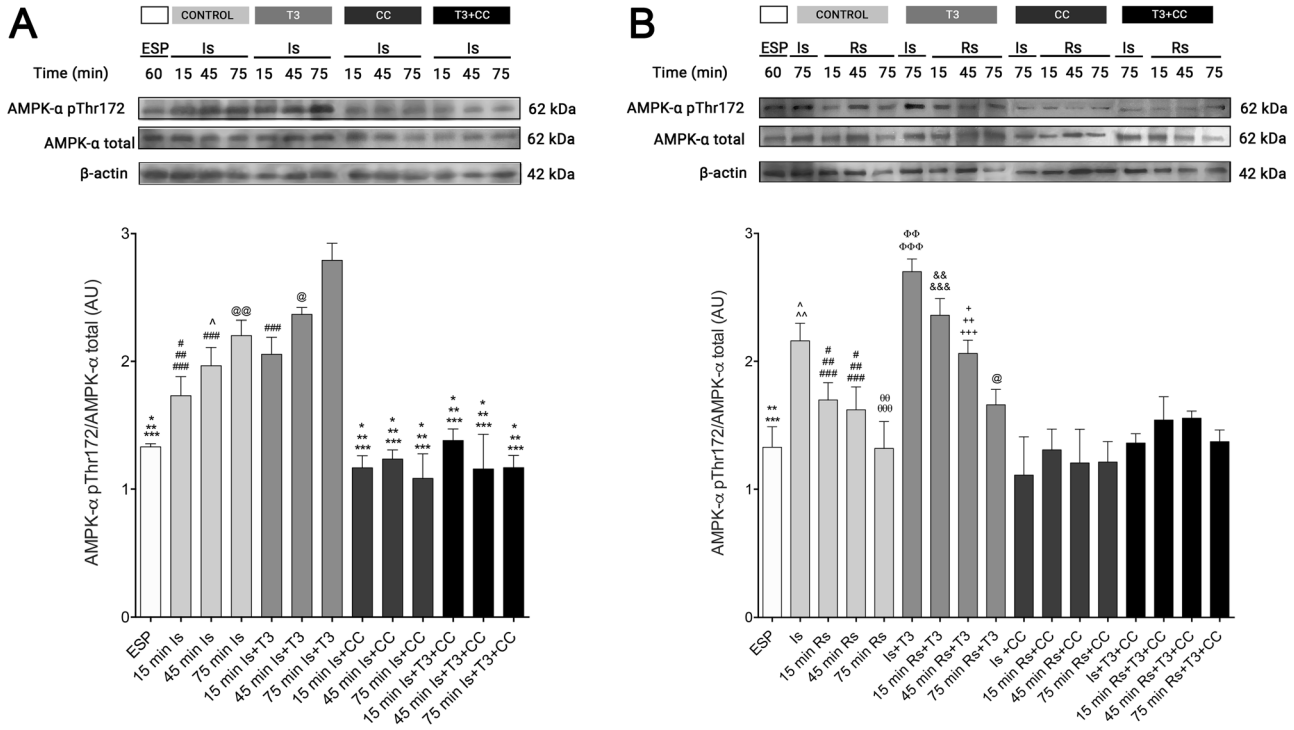

Figure 5

AMPK phosphorylation ratio during simulated ischemia and reperfusion. Immunoblot images show phosphorylated α-subunit in Thr172 (AMPKα-pThr172) and total enzyme α-subunit (AMPKα-total) expression in rat atria (A) subjected to 15-, 45- and 75-min simulated ischemia (Is) and (B) subjected to 15-, 45- and 75-min simulated reperfusion (Rs). Immunoblot bands were normalized to corresponding β-actin. The values for the AMPKα-pThr172 and AMPK-α total ratio are mean ± S.E.M. (n = 5/each condition). Results were expressed as arbitrary units (AU). T3, 60 nM triiodothyronine; CC, 10 μM compound C. Panel A: ***P < 0.001 vs 75-min Is, 15-, 45- and 75-min Is+T3; **P < 0.01 vs 45-min Is: *P < 0.05 vs 15-min Is; ###P < 0.001 vs 75-min Is+T3; ##P < 0.01 vs 45-min Is+T3: #P < 0.05 vs 75-min Is: ^P < 0,05 vs 45-min Is+T3; @@P < 0.01 vs 75-min Is+T3; @P < 0.05 vs 75-min Is+T3. Panel B: ***P < 0.001 vs Is, Is+T3, 15-min Rs+T3; **P < 0.01 vs 40-min Rs+T3; ^P < 0.05 vs 15-min Rs, 45-min Rs, 75-min Rs+T3; ^^P < 0.01 vs Is+T3, 75-min Rs, 15-min Rs +CC, 15 y 40-min Rs+T3+CC; ^^^P < 0.001 vs Is+CC, 40-min Rs+CC, Is+T3+CC, 75-min Rs+CC+T3; #P < 0.05 vs Is+CC; ##P < 0.01 vs 15-min Rs+T3; 45-min Rs+T3; ###P < 0.001 vs Is+T3; θθP < 0.01 vs 45-min Rs+T3; θθθP < 0.001 vs Is+T3; 15-min Rs+T3; Is+CC; 15, 45 y 75-min Rs+CC; Is+T3+CC; 15, 45 y 75-min Rs+T3+CC; ΦΦP < 0.01 vs 45-min Rs+T3; ΦΦΦP < 0.001 vs 75-min Rs+T3; &&&P < 0.001 vs Is+CC; 15, 45 y 75-min Rs+CC; Is+T3+CC; 75-min Rs+T3+CC; &&P < 0.01 vs 75-min Rs+T3; 15 y 40-min Rs+T3+CC; +P < 0.05 vs 15 y 40-min Rs+T3+CC; ++P < 0.01 vs 15, 45 y 75-min Rs+CC; Is+T3+CC; 75-min Rs+T3+CC; +++P < 0.001 vs Is+CC; @P < 0.05 vs Is+CC.

decrease on mitochondrial ATP synthesis capacity, showing a reduction of approximately 25% of the respective pre-ischemic value at 75-min of simulated reperfusion. This effect was partially reverted by AMPK pharmacological inhibitor, showing a reduction of approximately 50% in mitochondrial ATP production rate. However, when atria were exposed to Is–Rs in the presence of compound C, isolated mitochondria showed a significant decrease in the rate of ATP synthesis, reaching similar values when the atria were subjected to experimental conditions in the absence of AMPK inhibitor (Fig. 7).

Mitochondrial calcium retention capacity

Figure 8 shows that the number of calcium pulses required to produce the cessation of mitochondrial calcium uptake and to trigger MPTP opening was significantly higher in the mitochondria isolated from atria subjected to simulated ischemia-reperfusion and treated acutely with T3. Consequently, mitochondrial calcium retention capacity was significantly higher compared with simulated ischemic-reperfused group. When the atria were treated with both T3 and compound C, mitochondrial calcium retention capacity was significantly lower, showing no significant differences with mitochondria isolated from simulated ischemic-reperfused atria and mitochondria isolated from atria subjected to simulated ischemia-reperfusion in the presence of compound C.

Phosphorylation of GSK-3β

Since accumulating evidence suggests that the phosphorylation and inhibition of GSK-3β acts as a master switch to limit MPTP opening, the ratio of

https://jme.bioscientifica.com
https://doi.org/10.1530/JME-20-0314

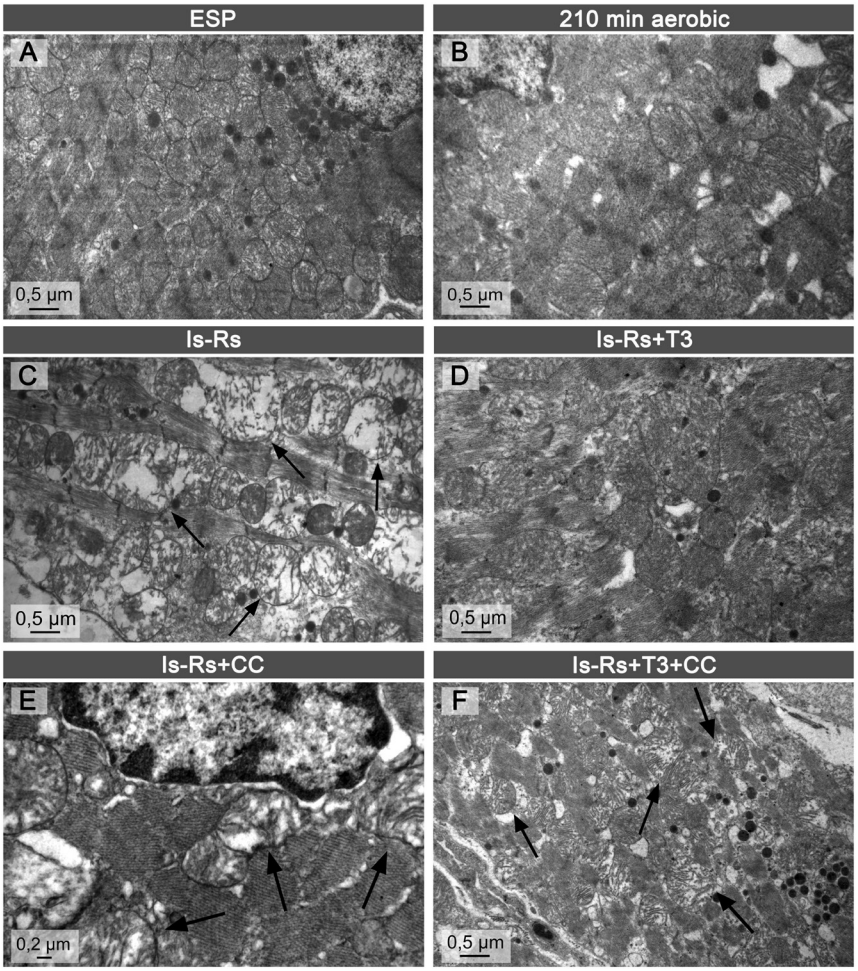

Figure 6
Electron micrographs. Representative transmission electron micrographs obtained from left atria subjected to: 60-min stabilization (A), 210-min aerobic period (B), 75-min simulated ischemia–75-min simulated reperfusion (Is–Rs) (C), Is–Rs in the presence of triiodothyronine (T3) (D), Is–Rs in the presence of compound C (CC) (E), Is–Rs in the presence of T3 and compound C (CC) (F). Black arrows show mitochondria with disruption and separation of peaks and clearance of the mitochondrial matrix.

phospho GSK-3β in Ser 9 to total enzyme was assessed by Western blot. Present results showed that acute simulated ischemia increased GSK-3β phosphorylation, which was maintained until the end of simulated reperfusion period (Fig. 9). Acute treatment with T3 significantly enhanced the enzyme phosphorylation ratio at the end of simulated reperfusion, this effect being reverted by AMPK pharmacological inhibitor, compound C.

Discussion

It is well known that thyroid hormones have profound effects on the heart and cardiovascular system (Li *et al.* 2011, Ragone *et al.* 2015). Although thyroxine and T3 are considered as the biologically active thyroid hormones, T3 is considered the most active biological form of thyroid hormone that exerts its effect on ischemic-reperfused myocardium (Pantos *et al.* 2011). To this respect, previous experimental and clinical evidence has shown that T3 can regulate important cardioprotective signaling pathways, increasing the resistance of the myocardium to ischemia (Zinman *et al.* 2006, Chen *et al.* 2008). In spite of this, the mechanism involved in T3-induced cardioprotection, especially at atrial level, is largely unknown, making it necessary to deepen the knowledge in this field in order to contribute to the future development of more effective therapeutic interventions than those existing today.

The T3 dose used in the present study has been previously established as cardioprotective in the ischemic-reperfused myocardium (Pantos *et al.* 2009, 2011). Although this dose is within the pharmacological range, it did not develop significant effects on the contractile function of the non-ischemic myocardium (data not shown). Moreover, since acute T3 administration in patients has been effective at supraphysiological doses, without producing changes in heart rate or atrial fibrillation development incidence (Ranasinghe *et al.* 2006, Kaptein *et al.* 2010), the results of the present study have a considerable clinical relevance for patients who suffer from acute myocardial infarct.

When analyzing the effects exerted by T3 on atrial myocardium contractile function, results of the present

https://jme.bioscientifica.com
https://doi.org/10.1530/JME-20-0314

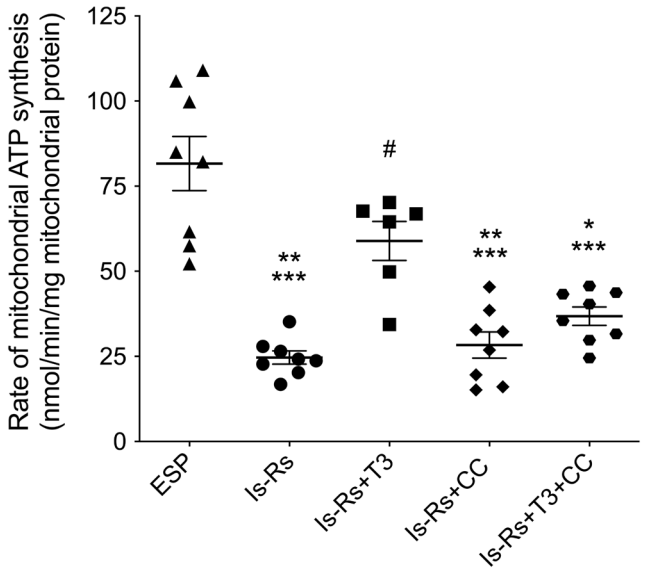

Figure 7
Rate of mitochondrial ATP synthesis. Effects of acute treatment with triiodothyronine (T3) in isolated rat left atria subjected to 75 min simulated ischemia–75 min simulated reperfusion (Is–Rs) in the presence or absence of compound C (CC) on isolated mitochondria ATP synthesis rate. Values are mean ± S.E.M. ($n = 6$–8/experimental group) and are expressed as nanomol per minute per milligram of mitochondrial protein. #$P < 0.05$ vs ESP; ***$P < 0.001$ vs ESP; **$P < 0.01$ vs Is–Rs+T3; *$P < 0.05$ vs Is–Rs+T3.

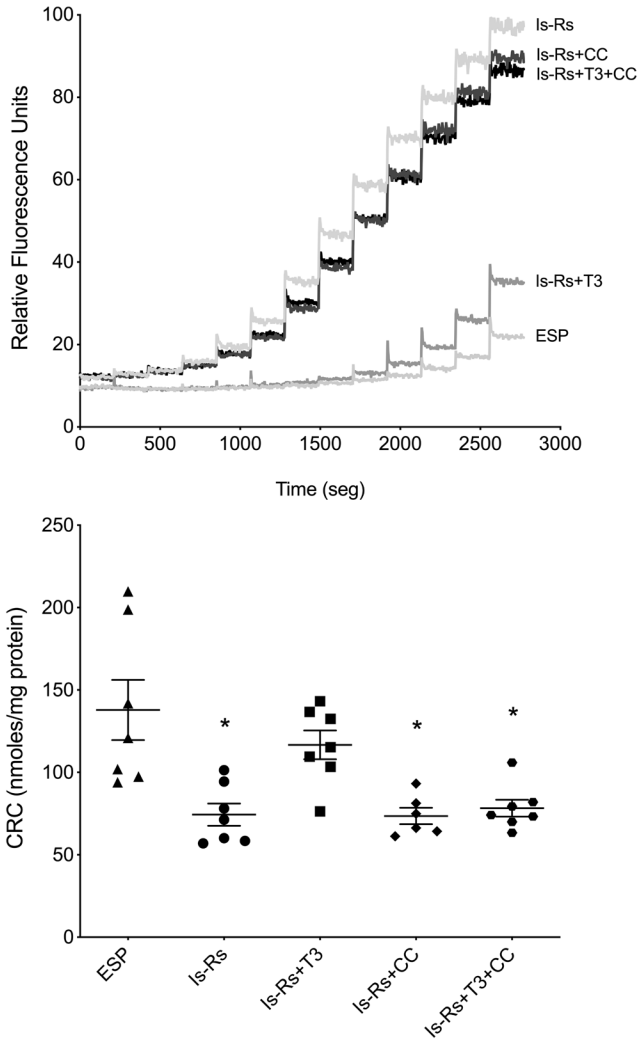

Figure 8
Mitochondrial calcium retention capacity. Typical representative trace of changes in calcium green fluorescence after calcium addition in samples of mitochondria, expressed as relative fluorescence units. Calcium retention capacity (CRC) was determined as the amount of calcium required to trigger a massive calcium release by isolated mitochondria. Values are mean ± S.E.M. ($n = 6$–7/experimental group) and are expressed as calcium nanomoles per milligram of mitochondrial protein required to trigger massive calcium release by isolated mitochondria. *$P < 0.05$ vs ESP, Is–Rs+T3.

study showed a significant increase in post-ischemic contractile recovery. In accordance with these results, it has been previously reported that T3 supplementation could markedly improve cardiac function (Pingitore *et al.* 2012, Nicolini *et al.* 2016, Ragone *et al.* 2015, Accorroni *et al.* 2016); however, T3-treated atria also showed an increase in ischemic contracture. In this concern, Kolocassides *et al.* previously reported the existence of a paradoxical relation between ischemic contracture and post-ischemic contractile function recovery (Kolocassides *et al.* 1996), providing evidence that cardioprotective interventions may accelerate ischemic contracture, although developing powerful protective post-ischemic responses, as it was observed in the present study.

Recent studies have identified that thyroid hormones could rapidly modulate distinct signaling pathways in seconds or minutes, which might contribute to the acute regulation of cardiomyocyte function in ischemic-reperfused myocardium. In this concern, present results provide significant evidence that suggests an acute treatment with T3 enhances AMPK phosphorylation during both ischemic and reperfusion periods. To this respect, the widely used AMPK pharmacological inhibitor, compound C, was able to prevent AMPK phosphorylation either in the presence or absence of T3, supporting the possible role of AMPK in ischemic-reperfused atrial myocardium in response to acute treatment with T3. In accordance to this, growing experimental evidence has suggested the existence of a possible relationship between T3 and AMPK signaling pathway in different tissues, under physiological or pathological conditions. In this concern, it has been previously reported that T3 upregulates the expression of skeletal muscle AMPK, leading to enzyme activation by phosphorylation with concomitant phosphorylation of the target proteins

https://jme.bioscientifica.com
https://doi.org/10.1530/JME-20-0314

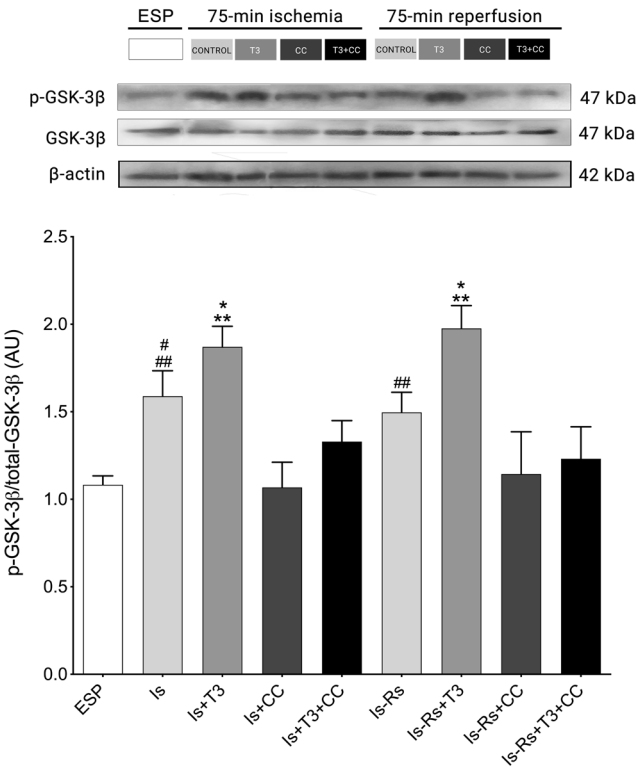

Figure 9

GSK-3β phosphorylation ratio. Immunoblot images show phosphorylated GSK-3β in Ser 9 (p-GSK-3β) and total enzyme (GSK-3β) expression in rat atria 75-min simulated ischemia (Is) and 75-min simulated reperfusion (Rs). Immunoblot bands were normalized to corresponding β-actin. The values for p-GSK-3β and total GSK-3β ratio are mean ± S.E.M. (n = 5/each condition). Results were expressed as arbitrary units (AU). $^{**}P < 0.01$ vs ESP, Is+CC, Is+T3+CC, Is-Rs+CC, Is–Rs+T3+CC; $^{\#\#}P < 0.01$ vs ESP; $^{*}P < 0.05$ vs Is–Rs; $^{\#}P < 0.05$ vs Is+CC. n = 5/each group.

(Park *et al.* 2002, Branvold *et al.* 2008, De Lange *et al.* 2008, Irrcher *et al.* 2008, Yamauchi *et al.* 2008). In addition, Takano *et al.* demonstrated that thyroid hormone exerts rapid effects in cultured cardiomyocytes growth through AMPK signaling pathway (Takano *et al.* 2013). Moreover, in heart muscle, Mourouzis *et al.* have reported that 6 weeks treatment with thyroid hormones improved the mechanical performance of the post-infarcted myocardium in rats with streptozotocin-induced diabetes, an effect which was associated at least in part with AMPK activation (Mourouzis *et al.* 2013). Although induction of AMPK signaling pathway in response to T3 has also been shown in several non-muscle tissues, as hepatocytes, adipocytes or mesenchymal stem cells (Videla *et al.* 2014, Wang *et al.* 2014, Chen *et al.* 2020), Lopez *et al.* reported that either whole-body hyperthyroidism, induced by chronic s.c. administration of T4 for a period of 3 weeks, or direct central administration of T3, decreases the activity of hypothalamic AMPK (López *et al.* 2010). In this regard, it should be considered that T3 might display differential effects over AMPK signaling pathway at central and peripheral level, contributing through this way in the regulation of the cellular energy metabolism or the whole-body central energy balance by the activation or inactivation of AMPK according to the target tissue in which T3 exerts its effects (Hardie 2010).

As compound C treatment prevented T3 enhanced recovery of contractile function, the results of the present study suggest that AMPK might be involved, at least in part, in the improved acute recovery of myocardial function after simulated ischemia when treated with T3. Although several studies have evidenced a possible relationship between T3 and AMPK signaling pathway activation, the molecular mechanisms involved in these possible effects in the ischemic-reperfused heart have not yet been elucidated. In this concern, experimental evidence has suggested that T3 should non-transcriptionally activate AMPK via intracellular Ca^{2+} mobilization. To this respect, it has been demonstrated that T3 rapidly increases intracellular Ca^{2+} levels through activation of inositol 1,4,5-triphosphate (IP_3) receptor with concomitant Ca^{2+} efflux from endoplasmic reticulum into cytoplasm or by rapid enhancement of Ca^{2+} uptake, mediating this way the activation of AMPK upstream kinase calcium/calmodulin-dependent protein kinase kinase beta (CaMKKβ) (Yamauchi *et al.* 2008, Vargas *et al.* 2013). Moreover, other experimental evidence has suggested that the induction of mitochondrial respiration by T3 is accompanied by increased generation of ROS, which might be also involved in the induction of intracellular Ca^{2+} release that activates CAMKKβ upstream activator of AMPK (Sinha *et al.* 2015). In this concern, more studies are required to elucidate the molecular pathway involved in AMPK activation in response to T3-treatment during myocardial ischemia-reperfusion.

Present results were accompanied by a greater tissue ATP content recovery and conserved mitochondrial ATP synthesis capacity after simulated reperfusion period in response to acute T3 treatment. Present data suggest that T3 might induce inotropic effects via rapid replacement and the maintenance of high energy phosphate stores within the atrial myocardium. In accordance to these results, previous investigations showed that the administration of T3 led to normal mitochondrial function recovery, reactivation of the tricarboxylic acid cycle and full aerobic metabolism after ischemia-reperfusion, being high energy phosphate stores more rapidly replaced (Novitzky & Cooper 2014, Fang *et al.* 2019). Accordingly, in experimental models of post-ischemic low T3 state,

https://jme.bioscientifica.com
https://doi.org/10.1530/JME-20-0314

thyroid hormone administration at physiological or near physiological dose rescued mitochondrial function which was related to reduced cardiomyocyte loss in the peri-infarct zone and better preserved cardiac performance (Forini *et al.* 2014). In this context, T3 may lead to both the increased synthesis of high energy stores and increased utilization of such stores, resulting in improved myocardial function recovery (Novitzky & Cooper 2014, Ragone *et al.* 2015). Although long-term effects of thyroid hormone have been recognized as a major modulator of mitochondrial metabolic efficiency, its acute effects over this organelle still remain to be more deeply identified. To this respect, present results provide evidence that intrinsic AMPK enhanced activation might be involved in the preservation of ATP tissue content and partially on mitochondrial high energy phosphate production, as the beneficial effects exerted by T3 were in part prevented by the administration of compound C. These results suggest that AMPK activation in response to acute treatment with T3 might contribute to myocardial cellular energy production and its turnover, promoting an increase in contractile function recovery. In this regard, AMPK is well known as an important regulator of diverse cellular pathways aimed to reestablish energy homeostasis at a cell-autonomous level. Once AMPK is activated, it orchestrates several metabolic pathways to maintain acceptable ATP levels required for cell survival under metabolic stress conditions. This enzyme promotes catabolic pathways, including breakdown of energy stores, glucose transport, glycolysis and fatty acid oxidation, and inhibits energy-consuming anabolic processes that are not essential for short-term cell survival (Hardie *et al.* 2012, Zaha & Young 2012). In this concern, it has been reported that T3 treatment increases the mitochondrial multienzyme pyruvate dehydrogenase complex (PDH) activity in hearts subjected to ischemia-reperfusion, improving post-ischemic contractile function in T3 treated hearts (Liu *et al.* 1998). To this respect, previous experimental data showed that atrial myocardium subjected to Is-Rs exerted an enhanced activity of PDH when AMPK was activated, but it was significantly reduced in the presence of AMPK inhibitor (Hermann *et al.* 2018). These results suggested that an increase in mitochondrial oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2 could be taking place when AMPK is activated by acute treatment with T3. This condition might result in higher ATP mitochondrial production and in high energy phosphate content restoration during reperfusion and therefore, contributing to contractile recovery when AMPK is enhanced by T3 treatment.

On the other hand, in the present study, results showed that acutely treated atria with T3 developed an increased MPTP resistance to opening calcium loading-mediated, compared with non-treated ischemic-reperfused atria, being this effect reverted by the inhibition of AMPK. To this respect, MPTP is a non-specific pore that opens in the inner mitochondrial membrane when matrix calcium is highly concentrated, especially when accompanied by oxidative stress, high inorganic phosphate and adenine nucleotide depletion (Halestrap & Richardson 2015). The loss of the selective permeability of inner mitochondrial membrane allows the indiscriminate passage of ions and metabolites until equilibrium is reached; also, the osmotic flow that accompanies this process generates the increase in the volume of the matrix. As a result of mitochondrial edema, eventually the rupture of the external mitochondrial membrane occurs and, consequently, the release of pro-apoptotic factors from the intermembrane space to the cytosol can lead to cell death by apoptosis. The opening of the calcium-induced mitochondrial transient permeability pore has been suggested to be favored in the first minutes of reperfusion since ischemia intracellular acidity prevents its opening, representing one of the main causes of reperfusion injury. In addition, opening of the MPTP not only prevents mitochondria from adequate ATP synthesis by oxidative phosphorylation but also allows hydrolysis of the ATP produced by glycolysis or remaining healthy mitochondria through reversal function of the F_oF_1-ATP synthase, resulting determinant for the extent of cardiac ischemia-reperfusion injury (Halestrap 2009). Inhibitors of MPTP opening has shown protection from reperfusion injury in a wide variety of experimental models (Piot *et al.* 2008, De Paulis *et al.* 2013, Kloner 2013, Halestrap *et al.* 2015). In this context, attenuation of MPTP opening might contribute to mitochondrial structure and function preservation, promoting a more adequate provision of ATP by oxidative phosphorylation for contractile function recovery, as it was observed in response to acute T3 treatment and that was reverted through AMPK inhibition. To this concern, it is widely accepted that mitochondrial dysfunction and particularly MPTP opening plays a major role in determining the extent of myocardial ischemia-reperfusion injury. In this regard, present results were accompanied by enhanced conservation of cellular viability when atria were acutely treated with T3, effect which was abolished when thyroid hormone and compound C were co-administrated.

Accumulating evidence has proposed that GSK-3β, a serine/threonine kinase localized in the cytosol, when phosphorylated at Ser9 and inactivated, elevates MPTP

https://jme.bioscientifica.com
https://doi.org/10.1530/JME-20-0314

opening threshold (Juhaszova *et al.* 2004), although the mechanism by which it occurs is not fully known. In the present study, results showed that acute treatment with T3 increased the ratio of the enzyme phosphorylation, effect that was prevented by compound C, suggesting the involvement of AMPK in the inhibition of GSK-3β in response to thyroid hormone treatment. To this respect, Ser9 phosphorylation and inactivation of this enzyme have been attributed to multiple kinases of different pro-survival signaling pathways (Murphy & Steenbergen 2007, Sugden *et al.* 2008). Among them, few studies have recently proposed that GSK-3β might be a downstream molecular target of AMPK (Tian *et al.* 2019). In accordance with present results, it has been suggested that enhancing AMPK activation during ischemia might protect myocardium against reperfusion injury through GSK-3β phosphorylation, contributing to MPTP opening inhibition and significantly reducing myocardial infarct size (Paiva *et al.* 2011). In this concern, present results suggest that acute treatment with T3 may improve mitochondrial function and morphology, at least in part, through activation of the AMPK/GSK-3β signaling pathway, which was shown to be involved in MPTP opening limitation.

Although the exposed acute effects of T3 might be involved in the protective effects on mitochondrial structure and function, at least partially, through AMPK enhanced intrinsic activation, it cannot be discarded that additional long-term effects might be exerted. In spite of present emerging lines of evidence, how AMPK contributes to mitochondrial structure and function preservation in response to acute T3 treatment remains to be widely investigated.

Conclusion

In conclusion, the present data provide new insight into the mechanism of T3-induced cardioprotection in the ischemic-reperfused myocardium.

The present study provides novel evidence that T3 enhances intrinsic activation of AMPK during simulated ischemia, remaining its activation increased during simulated reperfusion. The results showed that AMPK is involved, at least in part, in the protective effects exerted by T3 in the myocardium subjected to simulated ischemia-reperfusion through GSK-3β phosphorylation, contributing to MPTP opening inhibition. In this context, AMPK activation enhanced by T3 might contribute to mitochondrial structure and function preservation and thus to tissue ATP content recovery, resulting in the improvement of post-ischemic contractile recovery and cellular viability conservation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was funded by Universidad de Buenos Aires (UBACyT 20020170200163BA) and Instituto de Química y Metabolismo del Fármaco (IQUIMEFA), UBA-CONICET.

References

Accorroni A, Saponaro F & Zucchi R 2016 Tissue thyroid hormones and thyronamines. *Heart Failure Reviews* **21** 373–390. (https://doi.org/10.1007/s10741-016-9553-8)

Branvold DJ, Allred DR, Beckstead DJ, Kim HJ, Fillmore N, Condon BM, Brown JD, Sudweeks SN, Thomson DM & Winder WW 2008 Thyroid hormone effects on LKB1, MO25, phospho-AMPK, phospho-CREB, and PGC-1α in rat muscle. *Journal of Applied Physiology* **105** 1218–1227. (https://doi.org/10.1152/japplphysiol.00997.2007)

Chen YF, Kobayashi S, Chen J, Redetzke RA, Said S, Liang Q & Gerdes AM 2008 Short term triiodo-l-thyronine treatment inhibits cardiac myocyte apoptosis in border area after myocardial infarction in rats. *Journal of Molecular and Cellular Cardiology* **44** 180–187. (https://doi.org/10.1016/j.yjmcc.2007.09.009)

Chen X, Hu Y, Jiang T, Xia C, Wang Y & Gao Y 2020 Triiodothyronine potentiates BMP9-induced osteogenesis in mesenchymal stem cells through the activation of AMPK/p38 signaling. *Frontiers in Cell and Developmental Biology* **8** 725. (https://doi.org/10.3389/fcell.2020.00725)

De Lange P, Senese R, Cioffi F, Moreno M, Lombardi A, Silvestri E, Goglia F & Lanni A 2008 Rapid activation by 3,5,3-L-triiodothyronine of adenosine 5-monophosphate-activated protein kinase/acetyl-coenzyme A carboxylase and Akt/protein kinase B signaling pathways: relation to changes in fuel metabolism and myosin heavy-chain protein content in rat gastrocnemius muscle in vivo. *Endocrinology* **149** 6462–6470. (https://doi.org/10.1210/en.2008-0202)

De Paulis D, Chiari P, Teixeira G, Couture-Lepetit E, Abrial M, Argaud L, Gharib A & Ovize M 2013 Cyclosporine A at reperfusion fails to reduce infarct size in the in vivo rat heart. *Basic Research in Cardiology* **108** 379. (https://doi.org/10.1007/s00395-013-0379-4)

Di Lisa F, Semenzato M, Carpi A, Menazza S, Kaludercic N, Menabò R & Canton M 2010 Mitochondrial dysfunction in cell injury and cardiotoxicity. In *Cardiotoxicity of Non-Cardiovascular Drugs*, pp. 1–23. Chichester, UK: John Wiley & Sons, Ltd. (https://doi.org/10.1002/9780470660379.ch1)

Fang L, Xu Z, Lu J, Hong L, Qiao S, Liu L & An J 2019 Cardioprotective effects of triiodothyronine supplementation against ischemia reperfusion injury by preserving calcium cycling proteins in isolated rat hearts. *Experimental and Therapeutic Medicine* **18** 4935–4941. (https://doi.org/10.3892/etm.2019.8114)

Forini F, Kusmic C, Nicolini G, Mariani L, Zucchi R, Matteucci M, Iervasi G & Pitto L 2014 Triiodothyronine prevents cardiac ischemia/reperfusion mitochondrial impairment and cell loss by regulating

miR30a/p53 axis. *Endocrinology* **155** 4581–4590. (https://doi.org/10.1210/en.2014-1106)

Friberg L, Drvota V, Bjelak AH, Eggertsen G & Ahnve S 2001 Association between increased levels of reverse triiodothyronine and mortality after acute myocardial infarction. *American Journal of Medicine* **111** 699–703. (https://doi.org/10.1016/s0002-9343(01)00980-9)

Halestrap AP 2009 What is the mitochondrial permeability transition pore? *Journal of Molecular and Cellular Cardiology* **46** 821–831. (https://doi.org/10.1016/j.yjmcc.2009.02.021)

Halestrap AP & Richardson AP 2015 The mitochondrial permeability transition: a current perspective on its identity and role in ischaemia/reperfusion injury. *Journal of Molecular and Cellular Cardiology* **78** 129–141. (https://doi.org/10.1016/j.yjmcc.2014.08.018)

Halestrap AP, Pereira GC & Pasdois P 2015 The role of hexokinase in cardioprotection – mechanism and potential for translation. *British Journal of Pharmacology* **172** 2085–2100. (https://doi.org/10.1111/bph.12899)

Hardie DG 2010 Hot stuff: thyroid hormones and AMPK. *Cell Research* **20** 1282–1284. (https://doi.org/10.1038/cr.2010.153)

Hardie DG, Ross FA & Hawley SA 2012 AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nature Reviews: Molecular Cell Biology* **13** 251–262. (https://doi.org/10.1038/nrm3311)

Hawley SA, Davison M, Woods A, Davies SP, Beri RK, Carling D & Hardie DG 1996 Characterization of the AMP-activated protein kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. *Journal of Biological Chemistry* **271** 27879–27887. (https://doi.org/10.1074/jbc.271.44.27879)

Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Mäkelä TP, Alessi DR & Hardie DG 2003 Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *Journal of Biology* **2** 28. (https://doi.org/10.1186/1475-4924-2-28)

Hermann R, Vélez DE, Rusiecki TM, Fernández Pazos MdeL, Mestre Cordero VE, Marina Prendes MG, Perazzo Rossini JC, Savino EA & Varela A 2015 Effects of 3-methyladenine on isolated left atria subjected to simulated ischaemia-reperfusion. *Clinical and Experimental Pharmacology and Physiology* **42** 41–51. (https://doi.org/10.1111/1440-1681.12323)

Hermann R, Mestre Cordero VE, Fernández Pazos MLM, Reznik FJ, Vélez DE, Savino EA, Marina Prendes MG & Varela A 2018 Differential effects of AMP-activated protein kinase in isolated rat atria subjected to simulated ischemia–reperfusion depending on the energetic substrates available. *Pflugers Archiv* **470** 367–383. (https://doi.org/10.1007/s00424-017-2075-y)

Irrcher I, Walkinshaw DR, Sheehan TE & Hood DA 2008 Thyroid hormone (T3) rapidly activates p38 and AMPK in skeletal muscle in vivo. *Journal of Applied Physiology* **104** 178–185. (https://doi.org/10.1152/japplphysiol.00643.2007)

Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, Ziman BD, Wang S, Ytrehus K, Antos CL, *et al.* 2004 Glycogen synthase kinase-3β mediates convergence of protection signalling to inhibit the mitochondrial permeability transition pore. *Journal of Clinical Investigation* **113** 1535–1549. (https://doi.org/10.1172/JCI19906)

Kaptein EM, Sanchez A, Beale E & Chan LS 2010 Clinical review: thyroid hormone therapy for postoperative nonthyroidal illnesses: a systematic review and synthesis. *Journal of Clinical Endocrinology and Metabolism* **95** 4526–4534. (https://doi.org/10.1210/jc.2010-1052)

Kloner RA 2013 Current state of clinical translation of cardioprotective agents for acute myocardial infarction. *Circulation Research* **113** 451–463. (https://doi.org/10.1161/CIRCRESAHA.112.300627)

Kolocassides KG, Seymour AM, Galinanes M & Hearse DJ 1996 Paradoxical effect of ischemic preconditioning on ischemic contracture? NMR studies of energy metabolism and intracellular pH in the rat heart. *Journal of Molecular and Cellular Cardiology* **28** 1045–1057. (https://doi.org/10.1006/jmcc.1996.0097)

Li L, Guo CY, Yang J, Jia EZ, Zhu TB, Wang LS, Cao KJ, Ma WZ & Yang ZJ 2011 Negative association between free triiodothyronine level and international normalized ratio in euthyroid subjects with acute myocardial infarction. *Acta Pharmacologica Sinica* **32** 1351–1356. (https://doi.org/10.1038/aps.2011.118)

Liu Q, Clanachan AS & Lopaschuk GD 1998 Acute effects of triiodothyronine on glucose and fatty acid metabolism during reperfusion of ischemic rat hearts. *American Journal of Physiology* **275** E392–E399. (https://doi.org/10.1152/ajpendo.1998.275.3.E392)

López M, Varela L, Vázquez MJ, Rodríguez-Cuenca S, González CR, Velagapudi VR, Morgan DA, Schoenmakers E, Agassandian K, Lage R, *et al.* 2010 Hypothalamic AMPK and fatty acid metabolism mediate thyroid regulation of energy balance. *Nature Medicine* **16** 1001–1008. (https://doi.org/10.1038/nm.2207)

Lowry OH, Rosebrough NJ, Farr AL & Randall RJ 1951 Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193** 265–275. (https://doi.org/10.1016/0304-3894(92)87011-4)

Mourouzis I, Giagourta I, Galanopoulos G, Mantzouratou P, Kostakou E, Kokkinos AD, Tentolouris N & Pantos C 2013 Thyroid hormone improves the mechanical performance of the post-infarcted diabetic myocardium: a response associated with up-regulation of Akt/mTOR and AMPK activation. *Metabolism: Clinical and Experimental* **62** 1387–1393. (https://doi.org/10.1016/j.metabol.2013.05.008)

Murphy E & Steenbergen C 2007 Preconditioning: the mitochondrial connection. *Annual Review of Physiology* **69** 51–67. (https://doi.org/10.1146/annurev.physiol.69.031905.163645)

Nicolini G, Forini F, Kusmic C, Pitto L, Mariani L & Iervasi G 2016 Early and short-term triiodothyronine supplementation prevents adverse postischemic cardiac remodeling: role of transforming growth factor-β1 and antifibrotic miRNA signaling. *Molecular Medicine* **21** 900–911. (https://doi.org/10.2119/molmed.2015.00140)

Nishihara M, Miura T, Miki T, Tanno M, Yano T, Naitoh K, Ohori K, Hotta H, Terashima Y & Shimamoto K 2007 Modulation of the mitochondrial permeability transition pore complex in GSK-3β-mediated myocardial protection. *Journal of Molecular and Cellular Cardiology* **43** 564–570. (https://doi.org/10.1016/j.yjmcc.2007.08.010)

Novitzky D & Cooper DKC 2014 Thyroid hormone and the stunned myocardium. *Journal of Endocrinology* **223** R1–R8. (https://doi.org/10.1530/JOE-14-0389)

Obame FN, Plin-Mercier C, Assaly R, Zini R, Dubois-Randé JL, Berdeaux A & Morin D 2008 Cardioprotective effect of morphine and a blocker of glycogen synthase kinase 3β, SB216763 [3-(2,4-dichlorophenyl)-4(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione], via inhibition of the mitochondrial permeability transition pore. *Journal of Pharmacology and Experimental Therapeutics* **326** 252–258. (https://doi.org/10.1124/jpet.108.138008)

Ojamaa K, Kenessey A, Shenoy R & Klein I 2000 Thyroid hormone metabolism and cardiac gene expression after acute myocardial infarction in the rat. *American Journal of Physiology: Endocrinology and Metabolism* **279** E1319–E1324. (https://doi.org/10.1152/ajpendo.2000.279.6.E1319)

Paiva MA, Rutter-Locher Z, Goncalves LM, Providencia LA, Davidson SM, Yellon DM & Mocanu MM 2011 Enhancing AMPK activation during ischemia protects the diabetic heart against reperfusion injury. *American Journal of Physiology: Heart and Circulatory Physiology* **300** H2123–H2134. (https://doi.org/10.1152/ajpheart.00707.2010)

Pantos C, Mourouzis I, Saranteas T, Clavé G, Ligeret H, Noack-Fraissignes P, Renard PY, Massonneau M, Perimenis P, Spanou D, *et al.* 2009 Thyroid hormone improves postischaemic recovery of function while limiting apoptosis: a new therapeutic approach to support hemodynamics in the setting of ischaemia-reperfusion? *Basic Research in Cardiology* **104** 69–77. (https://doi.org/10.1007/s00395-008-0758-4)

Pantos C, Mourouzis I, Saranteas T, Brozou V, Galanopoulos G, Kostopanagiotou G & Cokkinos DV 2011 Acute T3 treatment protects the heart against ischemia-reperfusion injury via TRα1 receptor. *Molecular and Cellular Biochemistry* **353** 235–241. (https://doi.org/10.1007/s11010-011-0791-8)

Pardo AC, Rinaldi GJ & Mosca SM 2015 Mitochondrial calcium handling in normotensive and spontaneously hypertensive rats: correlation with systolic blood pressure levels. *Mitochondrion* **20** 75–81. (https://doi.org/10.1016/j.mito.2014.12.003)

Park SH, Paulsen SR, Gammon SR, Mustard KJ, Hardie DG & Winder WW 2002 Effects of thyroid state on AMP-activated protein kinase and acetyl-CoA carboxylase expression in muscle. *Journal of Applied Physiology* **93** 2081–2088. (https://doi.org/10.1152/japplphysiol.00504.2002)

Pingitore A, Chen Y, Gerdes AM & Iervasi G 2012 Acute myocardial infarction and thyroid function: new pathophysiological and therapeutic perspectives. *Annals of Medicine* **44** 745–757. (https://doi.org/10.3109/07853890.2011.573501)

Pingitore A, Nicolini G, Kusmic C, Iervasi G, Grigolini P & Forini F 2016 Cardioprotection and thyroid hormones. *Heart Failure Reviews* **21** 391–399. (https://doi.org/10.1007/s10741-016-9545-8)

Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, Elbelghiti R, Cung TT, Bonnefoy E, Angoulvant D, *et al.* 2008 Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *New England Journal of Medicine* **359** 473–481. (https://doi.org/10.1056/NEJMoa071142)

Ragone MI, Bonazzola P, Colareda GA & Consolini AE 2015 Cardioprotective effect of hyperthyroidism on the stunned rat heart during ischaemia-reperfusion: energetics and role of mitochondria. *Experimental Physiology* **100** 680–697. (https://doi.org/10.1113/EP085063)

Ranasinghe AM, Quinn DW, Pagano D, Edwards N, Faroqui M, Graham TR, Keogh BE, Mascaro J, Riddington DW, Rooney SJ, *et al.* 2006 Glucose-insulin-potassium and tri-iodothyronine individually improve hemodynamic performance and are associated with reduced troponin I release after on-pump coronary artery bypass grafting. *Circulation* **114** (Supplement) I245–I250. (https://doi.org/10.1161/CIRCULATIONAHA.105.000786)

Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA & Cantley LC 2004 The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *PNAS* **101** 3329–3335. (https://doi.org/10.1073/pnas.0308061100)

Sinha RA, Singh BK, Zhou J, Wu Y, Farah BL, Ohba K, Lesmana R, Gooding J, Bay BH & Yen PM 2015 Thyroid hormone induction of mitochondrial activity is coupled to mitophagy via ROS-AMPKULK1 signaling. *Autophagy* **11** 1341–1357. (https://doi.org/10.1080/15548627.2015.1061849)

Solaini G & Harris DA 2005 Biochemical dysfunction in heart mitochondria exposed to ischaemia and reperfusion. *Biochemical Journal* **390** 377–394. (https://doi.org/10.1042/BJ20042006)

Solem LE & Wallace KB 1993 Selective activation of the sodium-independent, cyclosporine A-sensitive calcium pore of cardiac mitochondria by doxorubicin. *Toxicology and Applied Pharmacology* **121** 50–57. (https://doi.org/10.1006/taap.1993.1128)

Sugden PH, Fuller SJ, Weiss SC & Clerk A 2008 Glycogen synthase kinase 3 (GSK3) in the heart: a point of integration in hypertrophic signalling and a therapeutic target? A critical analysis. *British Journal of Pharmacology* **153** (Supplement 1) S137–S153. (https://doi.org/10.1038/sj.bjp.0707659)

Takano APC, Diniz GP & Barreto-Chaves ML 2013 AMPK signaling pathway is rapidly activated by T3 and regulates the cardiomyocyte growth. *Molecular and Cellular Endocrinology* **376** 43–50. (https://doi.org/10.1016/j.mce.2013.05.024)

Tian X, Zhou Y, Wang Y, Zhang S, Feng J, Wang X, Guo H, Fan R, Feng N, Jia M, *et al.* 2019 Mitochondrial dysfunction and apoptosis are attenuated on-opioid receptor activation through AMPK/GSK-3β pathway after myocardial ischemia and reperfusion. *Journal of Cardiovascular Pharmacology* **73** 70–81. (https://doi.org/10.1097/FJC.0000000000000635)

Vargas R, Ortega Y, Bozo V, Andrade M, Minuzzi G, Cornejo P, Fernandez V & Videla LA 2013 Thyroid hormone activates rat liver adenosine 5-monophosphate-activated protein kinase: relation to CaMKKβ, TAK1, and LKB1 expression and energy status. *Journal of Biological Regulators and Homeostatic Agents* **27** 989–999.

Videla LA, Fernández V, Cornejo P, Vargas R, Morales P, Ceballo J, Fischer A, Escudero N & Escobar O 2014 T_3-induced liver AMP-activated protein kinase signaling: redox dependency and upregulation of downstream targets. *World Journal of Gastroenterology* **20** 17416–17425. (https://doi.org/10.3748/wjg.v20.i46.17416)

Wang CZ, Wei D, Guan MP & Xue YM 2014 Triiodothyronine regulates distribution of thyroid hormone receptors by activating AMP-activated protein kinase in 3T3-L1 adipocytes and induces uncoupling protein-1 expression. *Molecular and Cellular Biochemistry* **393** 247–254. (https://doi.org/10.1007/s11010-014-2067-6)

Yamauchi M, Kambe F, Cao X, Lu X, Kozaki Y, Oiso Y & Seo H 2008 Thyroid hormone activates adenosine 5-monophosphate-activated protein kinase via intracellular calcium mobilization and activation of calcium/calmodulin-dependent protein kinase kinase. *Molecular Endocrinology* **22** 893–903. (https://doi.org/10.1210/me.2007-0249)

Zaha VG & Young LH 2012 AMP-activated protein kinase regulation and biological actions in the heart. *Circulation Research* **111** 800–814. (https://doi.org/10.1161/CIRCRESAHA.111.255505)

Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, *et al.* 2001 Role of AMP-activated protein kinase in mechanism of metformin action. *Journal of Clinical Investigation* **108** 1167–1174. (https://doi.org/10.1172/JCI13505)

Zinman T, Shneyvays V, Tribulova N, Manoach M & Shainberg A 2006 Acute, nongenomic effect of thyroid hormones in preventing calcium overload in newborn rat cardiocytes. *Journal of Cellular Physiology* **207** 220–231. (https://doi.org/10.1002/jcp.20562)

Received in final form 2 February 2021
Accepted 22 February 2021
Accepted Manuscript published online 26 February 2021