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Early long-term L-T3 replacement rescues mitochondria and prevents ischemic cardiac remodelling in rats

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Abstract

3,5,3′-Levo-triiodothyronine (L-T3) is essential for DNA transcription, mitochondrial biogenesis and respiration, but its circulating levels rapidly decrease after myocardial infarction (MI). The main aim of our study was to test whether an early and sustained normalization of L-T3 serum levels after MI exerts myocardial protective effects through a mitochondrial preservation. Seventy-two hours after MI induced by anterior interventricular artery ligation, rats were infused with synthetic L-T3 (1.2 μg/kg/day) or saline over 4 weeks. Compared to saline, L-T3 infusion restored FT3 serum levels at euthyroid state (3.0 ± 0.2 *versus* 4.2 ± 0.3 pg/ml), improved left ventricular (LV) ejection fraction (39.5 ± 2.5 *versus* 65.5 ± 6.9%), preserved LV end-systolic wall thickening in the peri-infarct zone (6.34 ± 3.1 *versus* 33.7 ± 6.21%) and reduced LV infarct-scar size by approximately 50% (all $P < 0.05$). Moreover, L-T3 significantly increased angiogenesis and cell survival and enhanced the expression of nuclear-encoded transcription factors involved in these processes. Finally, L-T3 significantly increased the expression of factors involved in mitochondrial DNA transcription and biogenesis, such as hypoxic inducible factor-1α, mitochondrial transcription factor A and peroxisome proliferator activated receptor γ coactivator-1α, in the LV peri-infarct zone. To further explore mechanisms of L-T3 protective effects, we exposed isolated neonatal cardiomyocytes to H_2O_2 and found that L-T3 rescued mitochondrial biogenesis and function and protected against cell death *via* a mitoKATP dependent pathway. Early and sustained physiological restoration of circulating L-T3 levels after MI halves infarct scar size and prevents the progression towards heart failure. This beneficial effect is likely due to enhanced capillary formation and mitochondrial protection.

Keywords: myocardial infarction • L-triiodothyronine • remodelling • mitoKATP • angiogenesis

Introduction

During left ventricular (LV) remodelling and heart failure (HF) progression, a marked and long-lasting reduction of circulating thyroid hormone 3,5,3′-Levo-triiodothyronine (L-T3) has been found in patients with severe cardiac disease, including acute myocardial infarction (MI), by us [1, 2] and others [3–5]. Moreover, we have previously demonstrated that this condition, known as 'low T3 syndrome', represents a strong predictor of poor outcome in HF and that short-term synthetic L-T3 replacement therapy improves LV function in patients with chronic HF [1, 2]. L-T3 is a key regulator of mitochondrial biogenesis, respiration and function [6, 7]. In fact, changes in thyroid status are associated with bioenergetic remodelling of cardiac mitochondria and profound alterations in the biochemistry of cardiac muscle, with repercussions on its structure and contractility [8]. As documented by previous studies, mitochondrial dysfunctions are critical for the occurrence and progression of HF, and the expression of several

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genes involved in mitochondrial biogenesis and oxidative capacity is altered in the failing heart [9–11]. Moreover, the nuclear-mitochondrial cross-talk plays a pivotal role in signalling and cell death pathways, mitochondrial biogenesis, energy transduction in cardiomyocytes [12] and angiogenesis [13]. Therefore, L-T3 administration to HF patients might hypothetically promote angiogenesis and preserve cardiomyocytes integrity by restoring mitochondria. If that is the case, the effects of L-T3 might be particularly relevant during the ischemic cardiac remodelling, when an array of nuclear transcription factors, including hypoxic inducible factor-1α (HIF-1α), mitochondrial transcription factor A (mt-TFA) and peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α) are activated to control cell survival, angiogenesis, mitochondrial activity and biogenesis [14]. In particular, mt-TFA is a nuclear-encoded protein that promotes the transcription of mt-DNA, regulates mt-DNA copy number and mitochondrial function and preserves a mitochondrial gene expression program typical of the differentiated stage [15, 16]. On the other hand, PGC-1α acts upstream of mt-TFA, inducing mitochondrial biogenesis through its interaction with nuclear respiration factors [17] and can promote complete reversal of cardiac dysfunction [18]. The mt-TFA and PGC-1α gene down-regulation contributes to progression of several forms of cardiac failure [19]. Thus, both mt-TFA and PGC-1α myocardial expression are potential targets for L-T3 actions.

In the present study, we tested the hypothesis that early and sustained normalization of L-T3 serum levels in rats with MI exerts myocardial protective effects through mitochondrial preservation. Because an important mechanism of cardioprotection during ischemia operates through mitochondrial ATP-sensitive K^+ channels (mitoKATP) [20], we also tested the effects of L-T3 on those channels in isolated cardiomyocytes subjected to oxidative injury.

Methods

Animal procedures and experimental protocol

MI was produced in adult male Wistar rats (body weight 350–400 g) by permanent ligation of the anterior interventricular artery, as described previously [21]. Seventy-two hours after ligation, rats were randomly treated for 4 weeks with a constant subcutaneous infusion of L-T3 (1.2 μg/kg/day, $T3^+$, $n = 8$) or sterile vehicle (phosphate buffer solution, $T3^-$, $n = 8$) *via* a miniosmotic pump (Alzet, model 2ML4, Palo Alto, CA, USA), as previously described [22, 23]. A group of sham-operated rats was used as control (Sham, $n = 8$). Pilot experiments were performed to select the L-T3 dose to replace circulating levels free T3 (FT3) at euthyroid levels. (See Supporting Information.) After 4 weeks of infusion rats were killed and tissue samples of the left ventricle were obtained. Investigation was approved by the Animal Care Committee of the Italian Ministry of Health in accordance with the Italian law (DL-116, January 27, 1992) and with 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). (See Supporting Information.)

Haemodynamic recordings

Haemodynamic values were recorded in sedated animals (Zoletil 100®, 40 mg/kg im) at the fourth week of the treatment, as previously described [24].

Global and regional LV function

Transthoracic echocardiography was performed before thoracotomy, at 72 hrs after MI and at 4 weeks of infusion in sedated rats (Zoletil 100®, 40 mg/kg im) using a commercially available echocardiography system (MyLab® 30, Esaote, Genoa, Italy) equipped with a 10 MHz linear transducer, as previously described [21]. All measurements were performed by an echocardiography specialist in blinded fashion. (See Supporting Information.)

Serum thyroid hormone levels

Arterial blood samples were drawn from the femoral artery in sedated rats at the end of experimental protocol. Serum levels of total and free thyroid hormones were quantified as previously described [25].

Histological and immunohistochemical analysis

Hearts were arrested in diastole and five 2-mm-thick transverse slices were cut through the short axis of both ventricles, from the base to the apex. After paraffin embedding, each LV transverse slice was serially sliced into 4-μm-thick sections perpendicular to the long axis. For each animal we analysed four sections per LV transverse slice ($n = 20$ LV sections per animal). LV infarct scar size, tissue viability and fibrosis were estimated through nitroblue tetrazolium and Masson's trichrome staining, respectively, on fresh and formalin-fixed tissue [21]. As previously described [26], the area comprising 10% of the spared myocardium adjacent to the fibrotic tissue of transmural infarcts was identified as the *border zone*, and the viable myocardium opposite to the previously defined region was considered the *remote zone*. The myocardial capillary density (number of capillaries/mm^2) was assessed through immunohistochemical staining of CD31 (1:100; DAKO, Glostrup, Denmark), which is a specific endothelial marker [27]. Cell apoptosis in LV regions was detected by TUNEL staining (*in situ* cell death detection kit, Roche Diagnostic Corporation, Indianapolis, IN, USA), and confirmed by immunostaining of caspase-3 activated (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The apoptotic index (%) was calculated as the number of $TUNEL^+$ cardiomyocyte nuclei on total cardiomyocyte nuclei per microscopic field in each LV region, as previously described [28]. Regional capillary density and cell apoptosis were calculated using data obtained from all analysed sections for each LV transverse slice. At least six randomly selected high-power fields (40-fold of microscopic magnification) were analyzed on each LV section (24 fields for each LV transverse slice) to provide a coefficient of error < 0.1. (See Supporting Information.)

Mitochondria isolation and enzyme activities assays

Mitochondria were purified from LV fresh tissue and cultured neonatal rat cardiomyocytes (NRCM) (see below) according to the manufacturer's protocol provided with the mitochondria isolation kit (MITO-ISO1;

Sigma-Aldrich, St Louis, MO, USA), as previously described [29]. (See Supporting Information.) The activity of the cytochrome c oxidase-1 (CcO-1) of the mitochondrial respiratory chain, an essential subunit for cardiac oxidative phosphorylation [30], and of citrate synthase (CS), a representative enzyme of the Krebs cycle, were measured in purified mitochondria with commercially available kits (CYTOC-OX1, Sigma and CS0720, Sigma, respectively) according to the manufacturer's instruction, and as previously described [31, 32]. All assays were performed in duplicate. (See Supporting Information.)

Preparation of neonatal rat cardiomyocytes, caspase-3 activity and cell viability studies

NRCM were isolated and cultured as described previously [20]. Three to 5 days after isolation, cells were treated with T3 (1 μM) for 48 hrs or not subjected to any treatment. Caspase-3 activation, an early marker of mitochondrial-dependent apoptosis, was assessed through flow cytometry using caspase-3 intracellular activity assay kit (PhiPhiLux® G2D2; Calbiochem, San Diego, CA, USA) and confirmed by anti-cleaved caspase-3 monoclonal antibody (dilution 1:100; Cell Signaling, Beverly, MA, USA) and secondary antibody (Alexa Fluor 488®, dilution 1:1000; Invitrogen Corporation, Carlsbad, CA, USA). The mitochondrial membrane potential (Ψm), a marker of cell injury, was assessed using the fluorescence dye, tetramethylrhodamine ethyl ester (TMRE). H_2O_2 (100 μM) was used to induce oxidant stress on target cells, a well-known model system to study regulation of cardiomyocyte cell death associated with ischemia [20, 33]. This was applied for 30 min., after which Ψm were measured. 5-hydroxydecanoate (5HD, 500 μM), a selective inhibitor of mitochondrial K(ATP) channels (mitoKATP) [34] was added 30 min. before the addition of H_2O_2. The trypan blue study was performed as previously described [20].

mtDNA content quantification

The relative mitochondrial DNA (mtDNA) copy number, an index of mitochondrial biogenesis in cardiomyocytes, was calculated in each experimental condition by normalizing the mtDNA to 18S rRNA gene copy number as previously described [35]. Details for mRNA and mtDNA quantification and primer sequences are provided in the Supporting Information.

Quantitative real-time RT PCR

Tissue samples were stored in RNAlater (Ambion, Milan, Italy) and total RNA was isolated as previously described [36]. The gene expression of mt-TFA, PGC-1α and HIF-1α, was measured by real-time PCR (LightCycler, Roche Diagnostics, Mannheim, Germany), as previously described [21]. Sense and antisense primer sequences are listed in Table 1. (See Supporting Information.)

Western blot analysis

Protein was extracted from frozen tissue as previously described [34]. Twenty micrograms of total protein was resolved by SDS-PAGE on 15.0% gel. The membrane was probed with specific antibodies against mt-TFA (dilution 1:200, Santa Cruz Biotechnology), HIF-1α (dilution 1:1000, Santa Cruz Biotechnology) and thyroid hormone receptor type β (THR-β, dilution 1:1000, Santa Cruz Biotechnology), a key modulator of L-T3-induced cardiac angiogenesis [37], and then reprobed for β actin (dilution 1:1000, Santa Cruz Biotechnology) to verify the uniformity of protein loading. Bands were visualized by autoradiography and quantified using commercially available software.

Table 1 Oligonucleotides sequences

mRNA	Oligonucleotide sequence	GeneBanK locus
PGC-1α		
Forward (682–701)	5′cgatgaccctcctcacacca 3′	BC066868.1
Reverse (792–774)	5′ ttggcttgagcatgttgcg 3′	
HIF-1α		
Forward (1968–1990)	5′atgaccactgctaaggcatcagc 3′	NM024359.1
Reverse (2086–2064)	5′ aggttaaggctccttggatgagc 3′	
Mt-TFA		
Forward (522–542)	5′ gaaagcacaaatcaagaggag 3′	AB014089.1
Reverse (696–676)	5′ ctgcttttcatcatgagacag 3′	
β-Actin		NM031144.2
Forward (471–491)	5′ agccatgtacgtagccatcca 3′	
Reverse (551–531)	5′ tctccggagtccatcacaatg 3′	

PGC-1α: peroxisome proliferator activated receptor γ coactivator-1α; HIF-1α: hypoxic inducible factor-1α; Mt-TFA: mitochondrial transcription factor A. The numbers in parentheses indicate the position in the reported sequences.

Statistical analysis

The statistical analysis of the data were performed by using a one- and two-way analysis of variance and the Bonferroni test assuming a *P*-value less than 0.05 as the limit of significance. Data are expressed as mean ± S.E.M.

Results

Serum thyroid hormone levels

Seventy-two hours following coronary artery ligation, the untreated animals showed a marked reduction of circulating TT3 and FT3; this condition was reverted at euthyroid levels with long-term, low-dose infusion of synthetic L-T3 (Table 2). As also shown in Table 2, no changes in TT4 and FT4 levels were observed under

Table 2 Serum thyroid hormone levels

	FT3	FT4	TT3	TT4
	(pg/ml)	(pg/ml)	(ng/dl)	(μg/dl)
SHAM	4.3 ± 0.4	18.2 ± 0.9	61.5 ± 4.0	2.4 ± 0.3
$T3^-$	3.0 ± 0.2*#	17.4 ± 1.1	42 ± 2.8*#	3.1 ± 0.2
$T3^+$	4.2 ± 0.3	16.3 ± 1.5	65 ± 4.0	2.8 ± 0.2

Values are means ± S.E.M. ($n = 8$ for all groups); FT3: free fraction of T3; FT4: free fraction of T4; TT3: total T3; TT4: total T4. *$P < 0.02$ *versus* Sham; #$P < 0.05$ *versus* $T3^+$.

Table 3 Haemodynamic values

	SHAM	$T3^-$	$T3^+$
Heart rate, beats/min.	363.15 ± 52.12	320.16 ± 48.4	357.06 ± 41.29
MAP, mmHg	92 ± 10.2	80.5 ± 9.14*#	90.4 ± 12.03
LV dp/dt_{max}, mmHg/s	6162 ± 882.71	3023 ± 187.9*#	5756 ± 693.52
LVESP, mmHg	122 ± 1.45	105.2 ± 6.5*#	121.5 ± 5.5
LVEDP, mmHg	5.92 ± 1.85	17.21 ± 5.6*#	9.5 ± 3.1*

Values are means ± S.E.M. ($n = 8$ for all groups); MAP: Mean arterial pressure; LVESP: Left ventricular end-systolic pressure; LVEDP: Left ventricular end-diastolic pressure. *$P < 0.05$ *versus* SHAM; #$P < 0.05$ *versus* $T3^+$.

any experimental condition. After 4 weeks of L-T3 administration, the animals did not present cardiovascular untoward effects, such as arrhythmias or body weight loss.

Haemodynamics, LV function and remodelling

There were no significant differences in heart rate among groups (Table 3). However, L-T3 completely prevented the fall in mean arterial and LV systolic pressure and in LV dP/dtmax that occurred in $T3^-$. Moreover, $T3^+$ rats displayed less LV end diastolic pressure elevation compared to $T3^-$. Taken together, these data indicate that low-dose L-T3 preserved both systolic and diastolic functions. We then assessed global and regional LV function by echocardiography. The LV ejection fraction and internal diameter fractional shortening were significantly preserved in $T3^+$ animals compared to untreated animals (Fig. 1a and b). As shown in Fig. 1c, LV end-systolic diameter was significantly reduced in treated rats compared to $T3^-$, in the absence of significant changes of LV end-diastolic diameter. Changes in regional cardiac contractile function are shown in Fig. 1d–f. After 4 weeks of L-T3 treatment, the LV end-systolic wall thickening in the infarct border zone was preserved compared to untreated rats ($P < 0.05$). No functional changes of the LV remote regions were found in any experimental group. L-T3 infusion significantly decreased infarct-scar size by approximately 50% compared to untreated rats (Fig. 2a). As showed in Fig. 2b, the Masson trichrome staining confirmed a significant reduction in collagen fibres deposition in the myocardial perivascular and interstitial spaces of the $T3^+$-LV border zone compared to untreated heart.

Angiogenesis and apoptosis in the infarct border zone

$T3^+$ LV slices from the infarct border zone presented significantly preserved capillary density (Fig. 3a) in the presence of an increased expression of THR-β (Fig. 3b), and reduced cardiomyocyte death compared to $T3^-$LV (Fig. 4), as confirmed by immunostaining for caspase-3 activated (data not shown). No significant changes were observed in LV remote regions.

HIF-1α mitochondrial inducers and mitochondrial enzymes in the infarct border zone

mt-TFA, PGC-1α and HIF-1α are nuclear-encoded proteins that have protective [38] and neoangiogenic roles [39] in response to myocardial ischemia. As shown in Fig. 5, HIF1-α gene expression was significantly preserved in the LV border zone of $T3^+$ rats compared to untreated animals. Also myocardial expression of mt-TFA and PGC-1α was preserved in the border zone of infarcted $T3^+$ compared to $T3^-$hearts (Fig. 6a–c). The CcO-1 activity normalized to CS activity was significantly improved in the infarct border zone of $T3^+$ compared to untreated animals, yet it remained significantly lower compared to the respective remote zone (Fig. 6d).

H_2O_2-induced cell death in isolated cardiomyocytes

The results confirmed thus far show that L-T3 exerts protective effects against ischemic damage. To provide a mechanistic insight for the observed phenomenon, we suggested that the protective effects of L-T3 is through a mitoKATP-dependent pathway in rescued mitochondria. We first tested 10^{-6} and 10^{-7} M concentrations of L-T3 in NRCM. There was no protection with 10^{-7} (data not shown), however, 10^{-6} M concentration for 48 hrs resulted in significant protection against H_2O_2-induced cell death, as assessed by TMRE uptake and flow cytometry (Fig. 7a). This concentration of L-T3 was used in all subsequent studies. Pre-treatment with 5HD significantly reduced TMRE uptake in H_2O_2 stressed NRCM in the presence of L-T3. Moreover, the Trypan blue test (Fig. 7b) showed

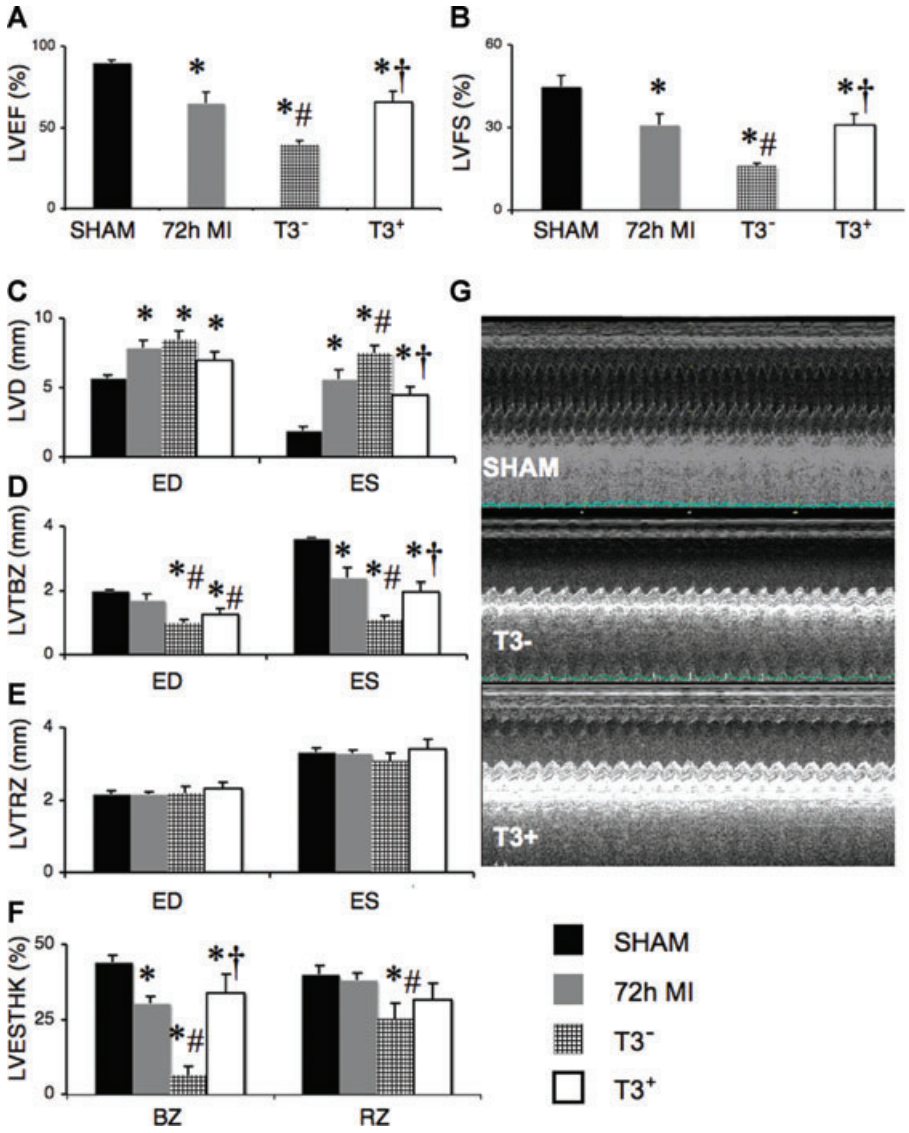

Fig. 1 Global (**A**–**C**) and regional (**D**–**F**) LV function. (**G**) Representative LV M-Mode echocardiograms for each experimental condition. MI: myocardial infarction; LVEF: LV ejection fraction; LVFS: LV fractional shortening; LVD: LV diameters; LVTBZ: LV thickness of the border zone; LVTRZ: LV thickness of the remote zone; ED: end diastolic; ES: end systolic; LVESTHK: LV end-systolic wall thickening; BZ: border zone; RZ: remote zone; LVEDV: LV end-diastolic volume; LVESV: LV end-systolic volume; ED: end-diastolic; ES: end-systolic. Values are means ± S.E.M.; $n = 8$ animals per group. $^{*}P < 0.05$ *versus* Sham; $^{\#}P < 0.05$ *versus* 72 hrs MI; $^{\dagger}P < 0.05$ *versus* $T3^{-}$.

the protective effects of L-T3 against cardiomyocyte necrosis death at the same experimental condition. The caspase-3 activity did not significantly change in cells exposed to 100 μM H_2O_2 compared to unstressed cells (data not shown).

Mitochondrial function and biogenesis in isolated cardiomyocytes

As showed in panel C of Fig. 7, L-T3 rescued CS-normalized CcO-1 activity, which is significantly reduced after H_2O_2 exposure. Moreover, L-T3 avoided a reduction of mitochondrial biogenesis in the presence of oxidant stimuli (Fig. 7d).

Discussion

The present study shows that a constant, low-dose infusion of L-T3, initiated 72 hrs after MI and prolonged over 4 weeks to restore and maintain euthyroid serum levels, markedly attenuates myocardial damage and remodelling and preserves LV systolic and diastolic function. Our data strongly suggest that physiological levels of circulating L-T3 might protect the post-MI heart, at least in part, by sustaining the expression of key nuclear factors that drive mitochondrial integrity. We have previously reported that circulating FT3 levels in patients with ischemic HF were progressively reduced in relation to disease severity [1, 40]. This alteration could be reverted by short-term L-T3 treatment in

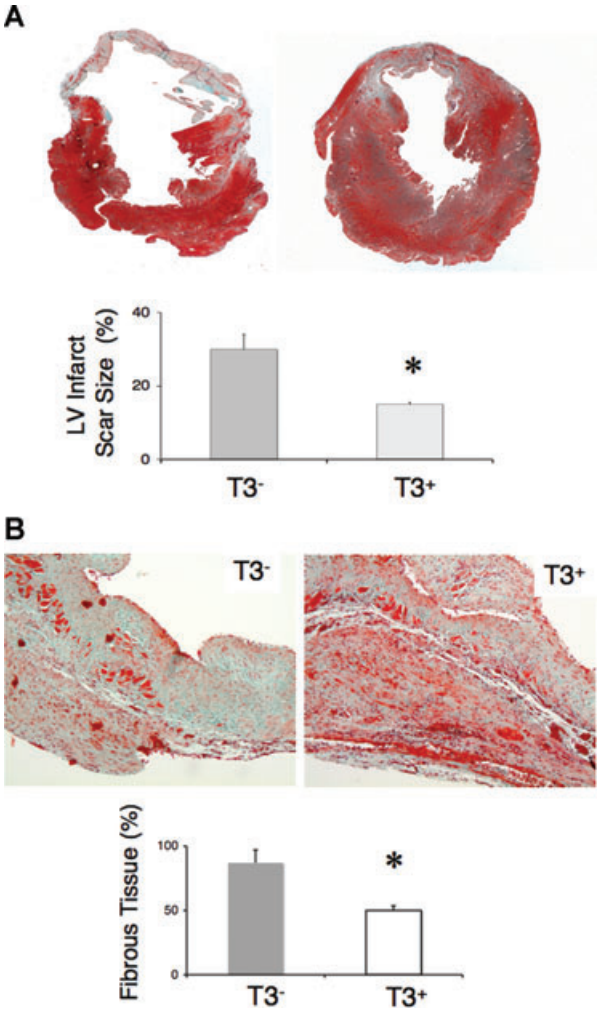

Fig. 2 (**A**) Representative LV transverse slice cut at midseptal level from $T3^+$ treated rats showed a significant reduced infarct-scar size compared with $T3^-$ animals (magnification, 10); (**B**) LV collagen accumulation revealed by Masson trichrome staining (magnification, 40). Values are means ± S.E.M.; $n = 8$ animals per group. $^*P < 0.05$ *versus* $T3^-$.

animal [41] and human [2] studies without affecting cardiac remodelling. Indeed, the effects of an early and sustained physiological L-T3 treatment on cardiac remodelling are still not well defined. Consistent with the human disease, we observed reduced FT3 serum levels in rats 72 hrs after MI [4], a pathophysiolgical feature that supports the clinical relevance of our experimental model. Because L-T3 has been shown to exert a wide spectrum of cardiovascular effects [42], we first needed to assess whether the therapeutic outcome of low-dose and long-lasting L-T3 infusion could be related to changes in heart rate and arterial blood pressure. We found that the $T3^+$ rats displayed no changes in heart rate compared to other two groups, while mean arterial pressure remained within a physiological range. Previous studies have demonstrated that post-MI LV remodelling, a major determinant of morbidity and mortality in overt HF [43], is an early process, such as the onset of post-ischemic hypothyroid state. New and more efficacious interventions aimed at preventing the initial stages of remodelling are highly needed to contrast the progression towards HF [44]. With long-term controlled L-T3 replacement, it is critical to choose the right timing and dose in order to limit cardiac remodelling and avoid the potentially adverse systemic effects (*i.e.* thyrotoxicosis). Henderson *et al.* recently showed that L-T3 replacement, initiated 1 week after MI, improved ventricular performance without reversing cardiac remodelling [22]. In a previous study, an immediate long-term, but not controlled, supplementation of thyroid hormones at high dose in post-MI improved LV function and prevented cardiac remodelling, but also induced a thyrotoxic state; in this case it cannot be excluded that the haemodynamic actions of thyroid hormones may have contributed to attenuate LV remodelling [45]. In the present study we chose to treat rats with the minimum dose sufficient to restore normal circulating L-T3 levels (as established in pilot tests). To avoid the risk of treatment in presence of unstable cardiovascular and systemic conditions frequently observed during the first 2 days after MI, we started the infusion immediately at the plateau (72 hrs) following the nadir (24–48 hrs) of L-T3 levels. Our approach proved very efficacious in that it halved the infarct-scar size. Since the myocardial healing has already begun at 72 hrs after MI in rodent heart, we investigated whether L-T3 modulates some of the major factors involved in myocardial remodelling during the healing phase. Insufficient angiogenesis is one of the causes of myocardial dysfunction and there is solid evidence of reduced myocardial capillary density in HF after MI [46]. Histological analysis of the peri-infarct myocardium revealed significantly less rarefaction of capillaries in the $T3^+$ hearts, consistent with a preserved expression of the receptor THR-β, which plays a key role in determining L-T3-induced coronary angiogenesis [37]. The improved capillary density might have in part favoured cardiomyocyte survival by enhancing oxygen supply to the border zone, the myocardial tissue survived to the ischemic insult. In fact, cardiomyocyte loss by apoptosis, a major cause of ventricular remodelling [47], was significantly reduced in the LV infarct border zone of L-T3-treated rats. On the other hand, our *in vitro* findings did not show cell death related to caspase-3 activation and confirmed previous findings recently published by others [48]. Accordingly, in our experimental model H_2O_2 rapidly damaged the plasma membrane integrity in a manner similar to the necrosis death, as assessed by trypan blue exclusion test. However, we cannot exclude that in the presence of long-term oxidative stress at lower free radicals concentrations the L-T3 protective effect against cell death might be exerted, *in vitro*, even by an inhibition of apoptosis through a deactivation of caspases, as we observed *in vivo*. We explored potential effects of L-T3 on mitochondrial integrity and nuclear-mitochondrial cross-talk. Over the past decade, convincing evidence has been provided that the nuclear transcription factors mt-TFA and HIF1-α play an important role in mediating cell survival

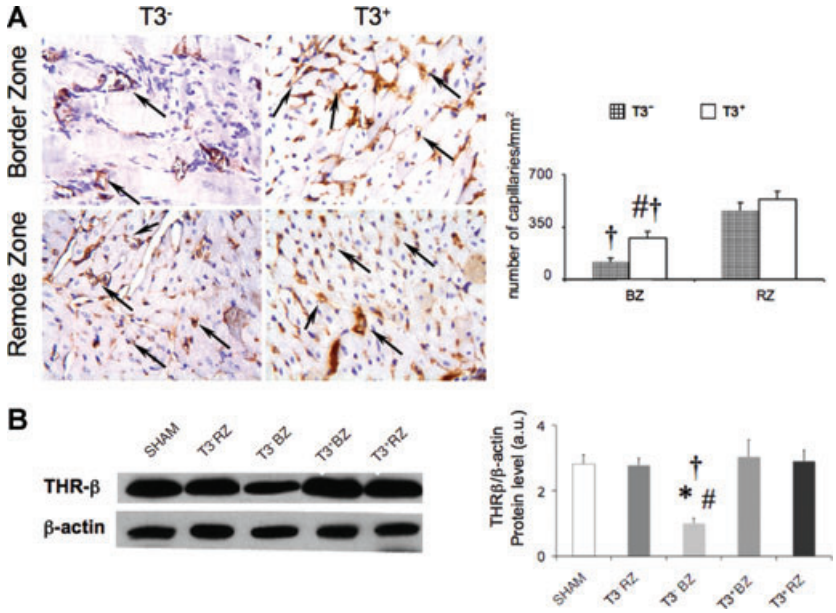

Fig. 3 (**A**) Immunohistochemical detection of endothelial cells identified by CD31 (black arrows) in border (BZ) and remote zone (RZ) of infarcted hearts to quantify LV capillary density (magnification, 400). (**B**) Western blot analysis of THR-β normalized to β-actin in BZ and RZ of infarcted hearts. Values are means ± S.E.M. ($n = 6$ microscopic fields per LV section); $n = 8$ animals per group. $^{\#}P < 0.05$ *versus* $T3^{-}$; $^{\dagger}P < 0.05$ *versus* RZ.

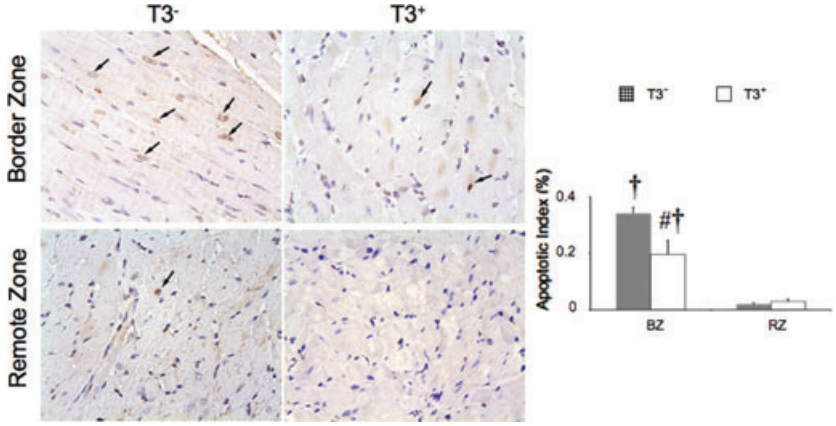

Fig. 4 TUNEL staining to quantify apoptotic cardiomyocytes (black arrows) in $T3^{+}$ and $T3^{-}$ infarcted left ventricle and haematoxylin counterstaining (magnification, 400). Values are means ± S.E.M. ($n = 6$ microscopic fields per LV section); $n = 8$ animals per group. $^{\#}P < 0.05$ *versus* $T3^{-}$; $^{\dagger}P < 0.05$ *versus* RZ.

mechanisms during myocardial ischemia [15, 38]. Their overexpression limits LV remodelling and preserves cardiac performance after MI [15, 49]. We now found that the expression of mt-TFA and HIF1-α was enhanced in the peri-infarcted myocardium of $T3^{+}$ hearts, but not in the remote zone. Mitochondrial function is regulated by the coordinated expression of nuclear and mitochondrial genes encoding mitochondrial proteins, such as subunits of respiratory chain complexes. Recently, it has been found that L-T3 modulates cardiac mitochondrial function increasing myocardial mitochondrial respiration, oxidative phosphorylation, mitochondrial protein synthesis and mtDNA content [50]. The down-regulation of CcO-I, a key enzyme of the mitochondrial respiratory chain, was significantly attenuated in the border zone of L-T3-treated hearts, consistent with a preserved expression of nuclear transcription factor PGC-1α, a powerful promoter of mitochondrial biogenesis [17] that is involved in the control of cardiac cell metabolism and signal transduction [35]. A cause-and-effect relationship between mt-TFA expression, electron transport chain activity and mtDNA copy number maintenance during the after MI remodelling process has been previously documented by others [51]. Here we demonstrated that L-T3 treatment preserved mitochondrial biogenesis and function when cardiomyocytes are exposed to oxidative stress *in vitro*. Therefore, it is likely that the beneficial effects of L-T3 in ischemic myocardium that we found *in vivo* were in part due to rescue of mitochondria function and cell metabolism during post-MI healing, which avoids a bioenergetic catastrophe culminating in cell necrosis. In terms of mechanistic investigation, since LV remodelling after MI involves side-to-side

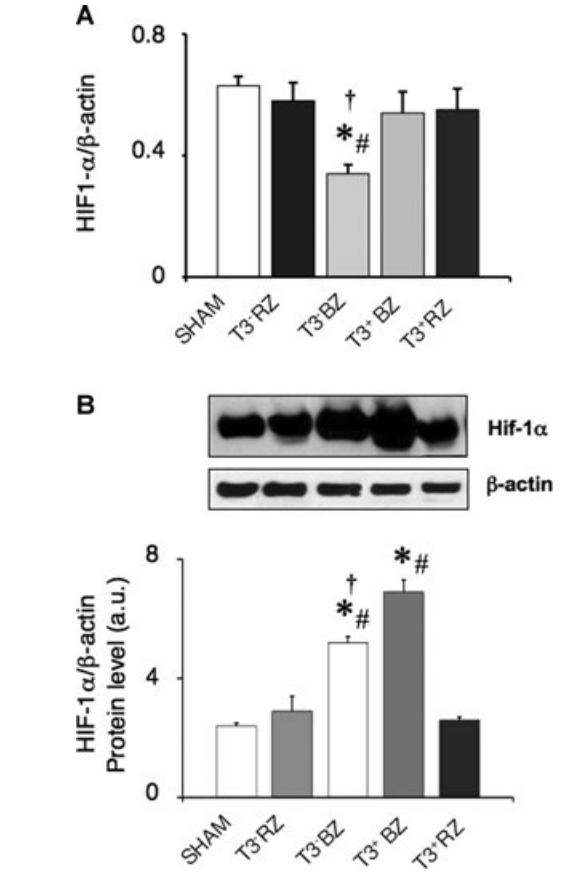

Fig. 5 Gene (**A**) and protein (**B**) expression of HIF-1α normalized to β-actin gene and protein expression in border (BZ) and remote zone (RZ) of infarcted hearts. Values are means ± S.E.M.; $n = 8$ animals per group. $^{*}P < 0.05$ *versus* Sham; $^{\#}P < 0.05$ *versus* RZ; $^{\dagger}P < 0.05$ *versus* $T3^{+}$.

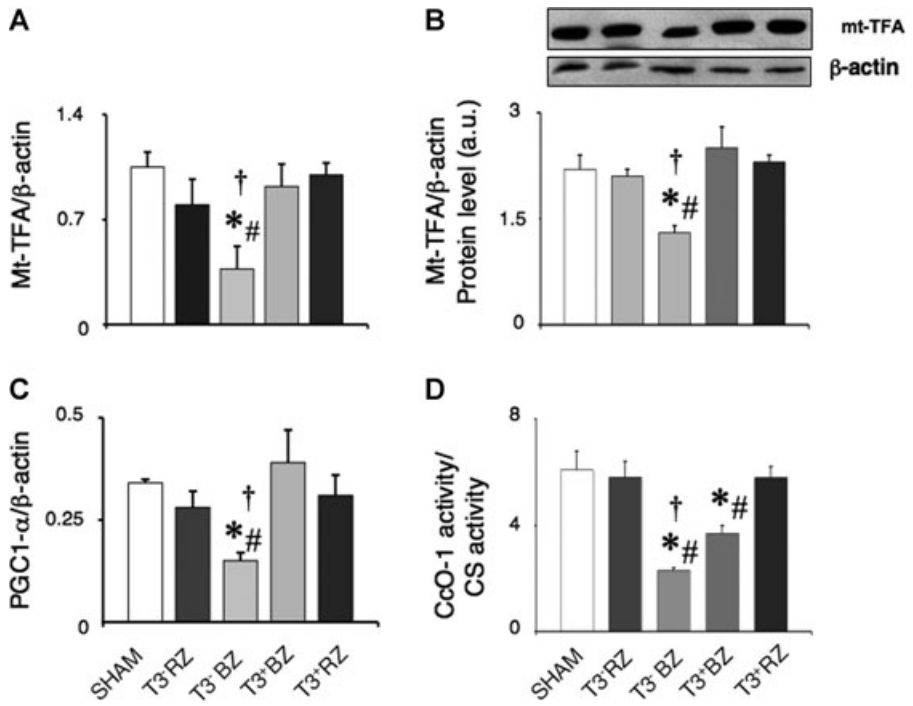

Fig. 6 Gene (**A**) and protein (**B**) expression of Mt-TFA and (**C**) gene expression of PGC-1α normalized to β-actin gene and protein expression in border (BZ) and remote zone (RZ) of infarcted hearts; (**D**) activity of the mitochondrial CcO-1 normalized to CS activity in BZ and RZ zone of infarcted hearts. Values are means ± S.E.M.; $n = 8$ animals per group. $^{*}P < 0.05$ *versus* Sham; $^{\#}P < 0.05$ *versus* RZ; $^{\dagger}P < 0.05$ *versus* $T3^{+}$.

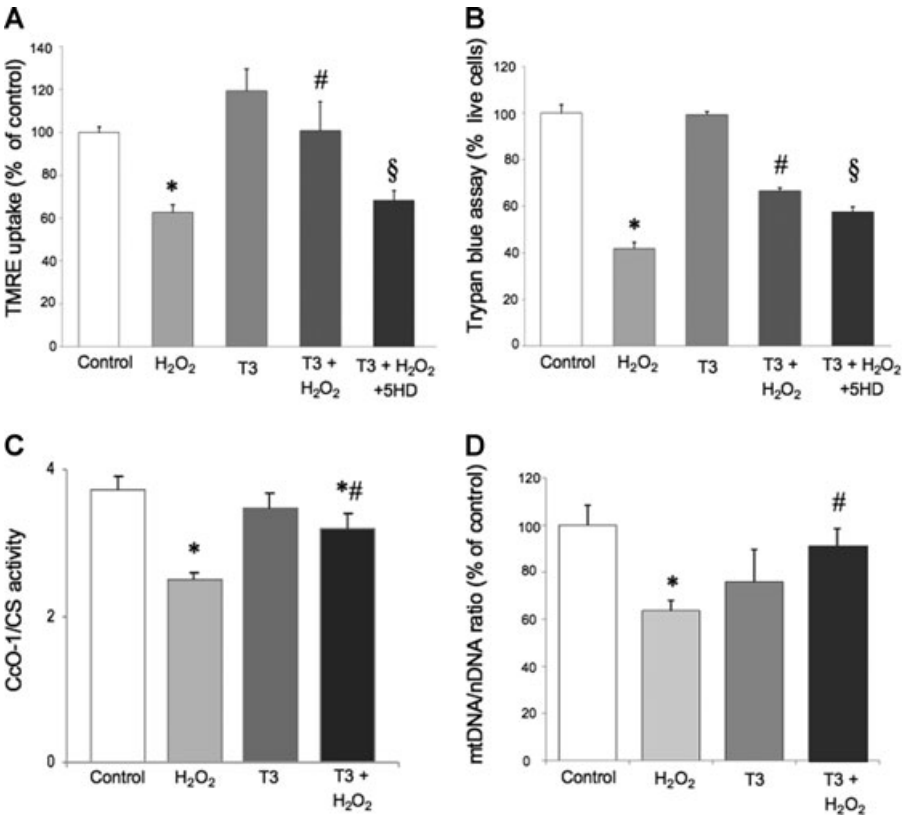

Fig. 7 *In vitro* studies in H_2O_2 stressed NRCM cultured for 48 hrs in presence or absence of L-T3: (**A**) TMRE uptake, (**B**) Trypan blue exclusion test, (**C**) activity of the mitochondrial CcO-1 normalized to CS activity and (**D**) relative mtDNA copy number. 5HD, 5- hydroxydecanoate. $^{*}P < 0.05$ *versus* Control; $^{\#}P < 0.05$ *versus* H_2O_2; $^{\dagger}P < 0.05$ *versus* $T3^{+}$ H_2O_2. Values are means ± S.E.M.; $n = 3$.

loss of cardiomyocytes mainly due to oxidative stress [52] and mitoKATP suppresses cell death by preserving mitochondrial integrity [53], it was interesting to investigate the role of L-T3 on mitoKATP function. Our results suggest, for the first time, that L-T3 exerts protective effects on cardiomyocytes through a mitoKATP-dependent pathway, as inhibitors of mitoKATP reverse the effects of L-T3. Although neonatal cardiomyocytes do not display the full phenotype of adults cells, they are routinely used in cardiac research to test mechanisms that otherwise could not be explored in whole heart or in short-lived adult cardiomyocytes [54]. Thus, we propose that L-T3 protects cardiomyocytes against oxidative stress-mediated cell death through a mitochondrial pathway and possibly by opening of the protective mitoKATP channel in rescued mitochondria. In conclusion, our study supports an early long-term and low-dose L-T3 replacement at euthyroid levels as a simple and highly efficacious therapeutic strategy to improve cardiac cell metabolism and haemodynamics, limits infarct-scar size and prevents post-MI evolution towards failure. We suppose that the favourable regional effects of L-T3 are mainly due to an early enhancement of the post-ischemic protective response during the healing phase. Although L-T3 displays multiple functions, enhanced capillary formation and mitochondrial protection seem to be major protective mechanisms responsible for these beneficial effects by using low dose of L-T3.

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Conflict of interest

The authors declare no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1 Circulating FT3 (**A**) and TT3 (**B**) levels before (Normal), 72 hours post-coronary ligation (72h MI) and after 1, 2, 3, 4 weeks of experimental T3 treatment. T3 supplementation at 0.5 or 1.2 μg/kg/day in rodents with myocardial infarction began 72 hours after coronary ligation by osmotic pump. Values are means ± SEM; n = 4 animals per group. $^{*}P < 0.05$ *versus* Normal; $^{\#}P < 0.05$ *versus* 0.5 μg/kg/day at the same experimental condition; $^{\dagger}P < 0.05$ *versus* 72 h MI.

Table S1 Oligonucleotides sequences.

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