Cardioprotective effects of triiodothyronine supplementation against ischemia reperfusion injury by preserving calcium cycling proteins in isolated rat hearts

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Abstract. Hypothyroidism is associated with profound left ventricular dysfunction. Triiodothyronine (T_3) supplementation may improve cardiac function after ischemic reperfusion (I/R) injury. In the present study, the effect of T_3 on major calcium cycling proteins and high-energy phosphate content during I/R was evaluated. Isolated perfused rat hearts were divided into 5 groups: Sham Control (Sham, n=10), Control (n=8), T_3 10 nM (T_3-10, n=10), T_3 25 nM (T_3-25, n=10) and T_3 50 nM (T_3-50, n=10). T_3 was administrated for 60 min before 30 min of ischemia and 120 min of reperfusion. The protein contents of Ca^{2+}-release channels (RyR2), Ca^{2+}-adenosine triphosphatase (SERCA2a), phospholamban (PLB), sarcolemmal Ca^{2+}-adenosine triphosphatase (PMCA) and sodium-calcium exchanger (NCX), as well as the high-energy phosphate content in heart tissues were measured by western blot analysis. The results revealed that T_3 improved the contractile recovery (left ventricular developed pressure; +dP/dt, -dP/dt) after I/R. Western blotting assays demonstrated that I/R depressed the contents of RYR2, SERCA2a and phosphorylated RYR2 and PLB; there were no effects on the contents of PLB, PMCA and NCX. T_3 reversed I/R-induced degradation of RyR2 and SERCA2a, restored the phosphorylation of RyR2 and PLB, and preserved the high-energy phosphate contents of ATP and creatine phosphate. T_3 supplementation protected the heart against I/R injury via the preservation of Ca^{2+}-cycling proteins and high-energy phosphate content.

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Abbreviations: T3, triiodothyronine; I/R, ischemic reperfusion; RyR2, Ca^{2+}-release channels; SERCA2a, Ca^{2+}-adenosine triphosphatase; PLB, phospholamban; PMCA, sarcolemmal Ca^{2+}-adenosine triphosphatase; NCX, sodium-calcium exchanger; CPB, cardiopulmonary bypass; Ca^{2+}-, calcium; SR, sarcoplasmic reticulum; RyRs, ryanodine receptors; LVP, left ventricular pressure; CF, coronary flow; MVO_2, myocardial O_2 consumption; ATP, adenosine triphosphate; CP, creatine phosphate; LVDP, Left ventricular developed pressure; CaMKII, calmodulin-dependent kinase II; PKA, protein kinase A

Key words: triiodothyronine, cardiac function, ischemic reperfusion injury, calcium cycling protein, high-energy phosphate

Introduction

Thyroid hormones have profound effects on the heart and cardiovascular system (1,2). Cardiopulmonary bypass (CPB) leads to a 'euthyroid-sick' state (3). This low triiodothyronine (T_3) state manifests as low circulating levels of T_3 with hemodynamic abnormalities (4). There is a growing body of experimental data that suggests that T_3 supplementation improves hemodynamic parameters after ischemic injury in animal models of CPB (5,6) and in isolated heart studies (7,8). Previously, studies using experimental models of ischemia reperfusion (I/R) revealed that T_3 administration improved the recovery of post-ischemic cardiac performance and reduced the pro-fibrotic process that leads to adverse cardiac remodeling (2,9-11).

Cytosolic calcium (Ca^{2+}) overload plays a major role in the development of myocardial injury during I/R. The sarcoplasmic reticulum (SR) is critical in Ca^{2+} uptake via Ca^{2+}-ATPase (SERCA2a) and Ca^{2+} release via the Ca^{2+} release channels (ryanodine receptors; RyRs). Human cardiomyocyte calcium handling and phenotype are strongly influenced by T_3. 'Low T_3 syndrome' is observed in the progression of I/R injury (12). It is possible that myocardial protection induced by T_3 supplementation involves the protection of SR function. The effects of T_3 supplementation on myocardial contractility may be attributed to their effects on intracellular Ca^{2+} homeostasis. The

authors have previously reported a marked Ca^{2+} overload in the mitochondrial compartment during I/R (13). The ability of T_3 supplementation to improve cardiac function, induce the activation of sarcolemma and SERCA2a, and decrease cytosolic (Ca^{2+}) accumulation have been reported previously (13). However, the mechanisms that underlie these cardioprotective effects of thyroid hormone remain largely unknown. In the present study, it was hypothesized that the cardiac protection of T_3 supplementation against I/R injury is associated with the preservation of Ca^{2+}-cycling proteins and high-energy phosphate contents.

Materials and methods

Animals. Adult male Sprague-Dawley rats (n=50; weight, 350-425 g) were obtained from the Animal Center of Soochow University (Suzhou, China). Rats were fed a standard diet and were housed at 25˚C with 60% humidity under a 12 h light-dark cycle. All rats were allowed access to food and water *ad libitum* for one week before experiments. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Nanjing University.

Langendorff isolated heart preparation and measurements. The methods employed have been previously described (13). Briefly, rats were anesthetized and decapitated when unresponsive to noxious stimulation. The hearts were excised and perfused in the Langendorff mode at a perfusion pressure equivalent to 80 mmHg. Perfusate and bath temperatures were maintained at 37.2±0.1˚C using a thermostatically controlled water circulator (Lauda E100; Lauda Dr R Wobser GmbH & Co., KG). Left ventricular pressure (LVP) and coronary flow (CF) were measured at a constant temperature and perfusion pressure (100 mmHg). Coronary inflow and coronary venous Na^+, K^+, Ca^{2+} and pH were measured offline with an intermittently self-calibrating analyzer system (Radiometer Copenhagen ABL 505; Radiometer, Ltd.). Coronary outflow (coronary sinus) O_2 tension was also measured online continuously with a Clark-type O_2 electrode (203B; Instech Laboratories, Inc.). Myocardial O_2 consumption (MVO_2) was calculated as [(coronary flow/g heart weight) x (arterial pO_2-venous pO_2)] x 24 μl O_2/ml at 760 mmHg; and cardiac work efficiency was calculated as [systolic-diastolic LVP x HR]/MVO_2. At the end of the experiments, the hearts were freeze-clamped and stored at -80˚C until subsequent use in western blot assays.

Experimental group establishment. Animals were randomly divided into 5 groups. The untreated sham (non-ischemic) group (Sham, n=10) was perfused for 225 min and after 15 min the equilibration/stabilization of functional parameters were employed; hearts were not subjected to ischemia. Ischemia groups underwent 15 min of stabilization and 60 min perfusion with or without T_3 administration at three different doses (10, 25 and 50 nM), followed by 30 min ischemia and 120 min reperfusion [n=10 each for the control (CTL), T_3-10, T_3-25 and T_3-50 groups]. The range of doses was selected according to a previous study (14). A three-way stopcock, located immediately above the aortic cannula, allowed the induction of global, no-flow ischemia.

Western blot assays for SR and sarcomlemmal Ca^{2+}-cycling and -regulating proteins. Western blot assays were conducted as previously described (15). Briefly, aliquots of homogenate samples (20 μg protein/lane) were solubilized in Laemmli sample buffer (cat. no. S3401; Sigma-Aldrich; Merck KGaA) and fractionated by SDS-PAGE using a 4-20% gel. After transfer to nitrocellulose membranes, membranes were blocked with 5% nonfat milk for 1.5 h at room temperature in phosphate-buffered saline and probed with primary antibodies against RyR2 (cat. no. MA3-916; 1:2,000,), SERCA2a (cat. no. 2A7-A1; 1:2,000), Phospholamban (cat. no. 2D12; PLB; 1:1,000), and sarcolemmal Ca^{2+}-adenosine triphosphatase (PMCA; cat. no. 5F10; 1:1,000). The above antibodies were purchased from Affinity Bioreagents, Inc. The sodium-calcium exchanger (cat. no. ab3516P; NCX; 1:1,000) was obtained from Sigma-Aldrich; Merck KGaA. RyR-ser2809 and PLB-Thr17 were purchased from Badrilla., Ltd., (1:1,000). Secondary antibodies (m-lgGk BP-HRP, cat. no. sc-516102, Santa Cruz Biotechnology; 1:20,000) were conjugated to horseradish peroxidase. Immunoreactive bands were visualized by enhanced chemiluminescence (SuperSignal™ west pico PLUS chemilumiluminescent substrate, cat. no. 34577; Pierce; Thermo Fisher Scientific, Inc.). The amount of protein was determined by densitometry using Kodak 1D software (version 3.4.5; Sigma-Aldrich; Thermo Fisher Scientific, Inc.) and normalized to protein load. Positive (purified proteins) and negative (blocking peptide or blot without primary antibodies) controls were used to establish the specificity of the protein signals.

Biochemical analysis. At the end of reperfusion, the hearts were freeze-clamped with aluminum tongs pre-cooled with liquid nitrogen as described previously (16), to measure myocardial ATP and creatine phosphate (CP) levels. Briefly, frozen ventricles were pulverized and mixed with 0.3 M $HClO_4$ and 0.25 mM EDTA under liquid nitrogen cooling. The extract was centrifuged at 8,000 x g for 15 min at 4˚C and the resulting supernatant was sampled to measure myocardial ATP and CP using the high pressure liquid chromatography method as previously described (16). Myocardial CP was converted to ATP via the creatine kinase enzymatic reaction.

Statistical analysis. All data are expressed as the mean ± standard deviation. Statistical analysis used SPSS 20.0 (IBM Corp.). A two-way analysis of variance was used to assess the overall difference between groups. Student Newman Keuls' post hoc test was used for multiple comparisons between the different groups. All experiments were repeated three times. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cardiac performance. Table I summarizes the changes in the indices for coronary flow, heart rate, MVO_2 and cardiac efficiency in the Sham, CTL, T_3-10, T_3-25 and T_3-50 groups before 30 min ischemia, and at 120 min of reperfusion. The heart rates of all the groups were similar prior to ischemia, but decreased after I/R. There was no significant difference among the different groups. Coronary flow was much lower

Table I. Cardiac effects in Sham, CTL, T3 10 nM, T3 25 nM and T3 50 nM before and after ischemia reperfusion.

Group	Sham	CTL	T_3 10 nM	T_3 25 nM	T_3 50 nM
HR (beat/min)					
Baseline	266±14	269±15	262±10	265±16	271±18
Reperfusion 120'	261±16	241±24	240±21	245±14	248±19
CF (ml/min)					
Baseline	12.2±0.8	11.9±0.8	12±0.7	12.2±0.9	11.9±0.7
Reperfusion 120'	11.8±1.0	5.6±0.6[a,b]	6.6±0.5[a,b]	7.9±0.6[a-c]	8.0±0.7[a-c]
MVO_2 (mmHg)					
Baseline	115±8	112±11	111±10	112±12	115±10
Reperfusion 120'	112±11	58±7[a-c]	69±7[a,b]	73±8[a-c]	75±7[a-c]
Cardiac efficiency (mmHg·beat·0.1 μl $O_2 \cdot g^{-1}$)					
Baseline	16.5±1.3	16.6±1.5	16.2±1.6	16.3±1.1	16.5±1.4
Reperfusion 120'	15.6±1.2	6.3±0.7[a,b]	7.2±0.8[a,b]	8.6±0.8[a-c]	8.7±0.8[a-c]

The HR, CF, MVO_2 and Cardiac efficiency before ischemia and after 30 min ischemia and 120 min reperfusion. Values are the mean ± standard deviation. [a]P<0.05 vs. the baseline; [b]P<0.05 vs. the Sham group. [c]P<0.05 vs. the CTL group. Sham (non-ischemia; n=10); CTL (Ischemia; n=10); T_3 10 nM (n=10); T3 25 nM (n=10); T_3 50 nM (n=10). CF, coronary flow; HR, heart rate, MVO_2, myocardial O_2 consumption; Cardiac efficiency, cardiac work efficiency; T3, Triiodothyronine.

than baseline throughout reperfusion in each of the ischemia groups, but it was increased in all of the T_3 treated groups (returned to 55% in T_3-10, 64% in T_3-25 and 67% in T_3-50) compared with the CTL group (47%) throughout reperfusion. After 120 min of reperfusion, MVO_2 and cardiac efficiency were increased in the T_3 administrated groups compared with in the CTL group, but remained lower than the baseline in all of the ischemic groups. Both parameters of MVO_2 and cardiac efficiency in the T_3-25 and T_3-50 groups were significantly increased compared with those in the CTL group (P<0.05; Table I).

Left ventricular developed pressure (LVDP) was decreased after I/R compared with the baseline in each group. LVDP was increased in the T_3 treated groups compared with in the CTL group upon reperfusion. The improvement in LVDP was significantly different in the T_3-25 and T_3-50 groups compared with the CTLgroup (P<0.05). There was no significant difference in the T_3-10 group (Fig. 1A). Hearts subjected to global ischemia for 30 min showed a marked increase in LVDP. However, a significant reduction in LVEDP was observed with T_3 25 or 50 nM treatment (P<0.05; Fig. 1B). Cardiac contractility +dP/dt and relaxation -dP/dt (Fig. 1C and D) were reduced during ischemia in all groups. Upon reperfusion, contractility increased but still remained lower than that recorded before ischemia throughout reperfusion in each group. T_3 administration at doses of 25 and 50 nM significantly improved contractile recovery in I/R injury hearts (P<0.05). This was evidenced by a greater recovery in LVDP and +dP/dt and -dP/dt, respectively, when compared with the pre-ischemic values.

Effect of T_3 supplementation on the SR Ca^{2+} regulatory proteins. There was a significant reduction in the density of RyR2 and the phosphorylation status of RyR2 at S2809 in the CTL group (P<0.05). Treatment with T_3 in I/R hearts significantly prevented the attenuation of RyR2 density and Ser-2809 (P<0.05 Fig. 2). Fig. 3 presents the protein density of SERCA2a in the control and I/R hearts that were treated with or without T_3 supplementation. Compared with the non-ischemic values, SERCA2a expression decreased significantly after I/R injury (P<0.05).

Effect of T_3 supplementation on sarcomlemmal Ca^{2+}-regulatory protein. The density of PLB was lower in all the ischemia groups; however, there was no significant difference when compared with the Sham group (Fig. 4). The phosphorylation of PLB at Thr-17 significantly decreased after 30 min of ischemia compared with the non-ischemia group (Sham group; P<0.05). T_3 treatment at all three doses before ischemia attenuated this decrease and a significant difference was detected in all T_3 dose groups compared with the ISC group (P<0.05). The immunoblots of the protein expression of sarcolemmal PMCA and NCX revealed that there were no changes in PMCA and NCX after I/R in the CTL and T_3 supplementation groups.

ATP and CP content. The ATP and CP content of transmural sections taken from the left ventricular free wall at the end of reperfusion were determined. In the CTL and T_3 groups, the ATP contents were 6.7±0.9, 9.3±1.1, 11.3±1.6 and 12.2±1.9 μmol/g, respectively; the CP contents were 8.6±1.2, 11.5±2.3, 13.7±1.8 and 14.5±1.7 μmol/g at the end of reperfusion, respectively. The contents of ATP and CP were better preserved in the T_3 (10, 25 and 50 nM) treated groups compared with in the CTL group (Fig. 5).

Discussion

A number of studies have confirmed that a cardiopulmonary bypass affects thyroid hormone metabolism to reduce circulating free T_3, which leads to a 'euthyroid-sick' state. It is not

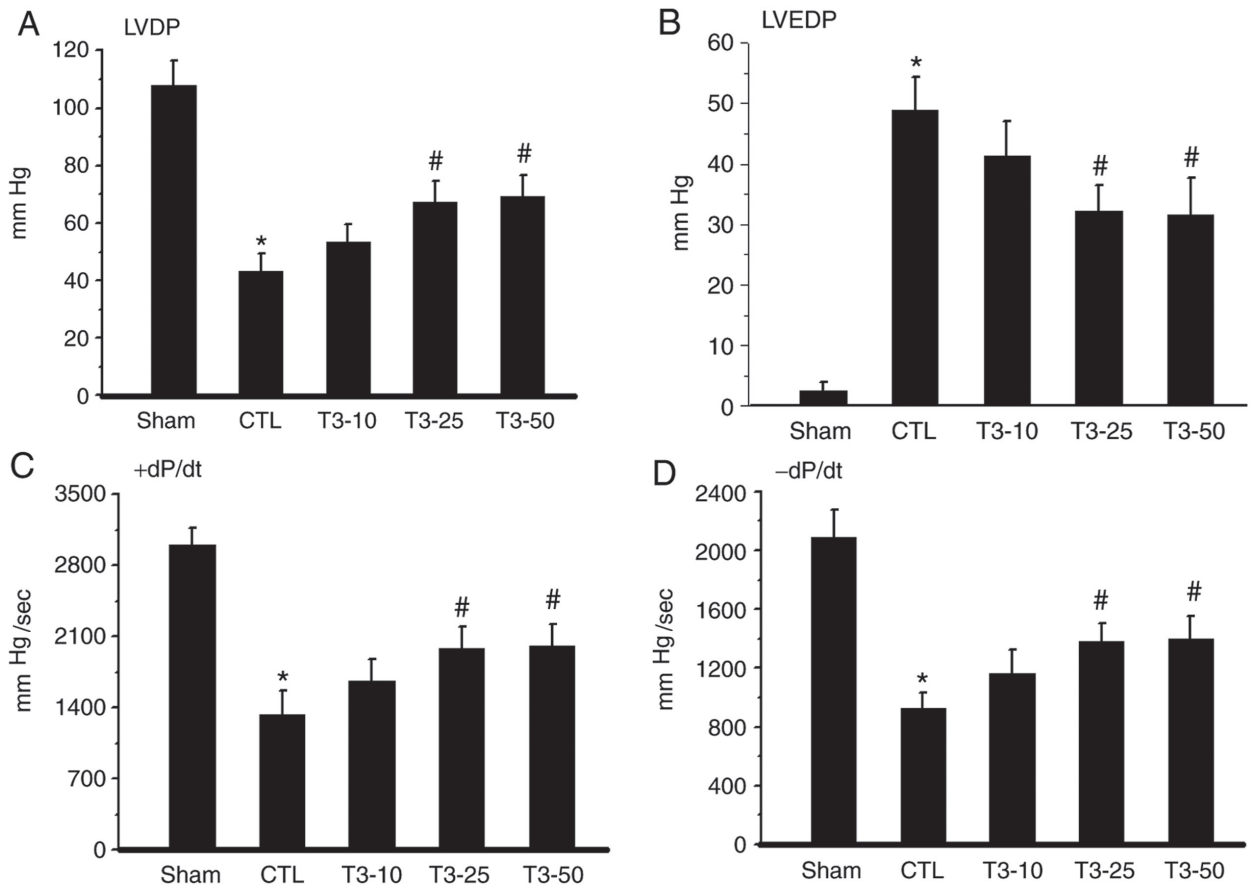

Figure 1. LVDP, LVEDP, +dP/dt and -dP/dt after 30 min ischemia and 120 min reperfusion. (A) LVDP values in the Sham, CTL, T_3-10, T3-25 and T_3-50 groups. (B) LVEDP values in Sham, CTL, T_3-10, T_3-25 and T_3-50 groups. Cardiac contractility (C) +dP/dt and relaxation (D) -dP/dt values in Sham, CTL, T_3-10, T_3-25 and T_3-50 groups. Values are presented as the mean ± standard deviation. *P<0.05 vs. the Sham group; #P<0.05 vs. the CTL group. LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; dP/dt, rate of ventricular pressure development; CTL, ischemia control group; Sham group, non-ischemic group; T_3-10, T_3 10 nM; T_3-25, T_3 25 nM; T_3-50, T_3 50 nM; T_3, Triiodothyronine.

clear how acute stress-induced hypothyroidism affects the myocardium pathophysiological change; however, previous studies (4,17) have suggested that T_3 is a positive inotrope and acts by improving the 'aerobic capacity' of the myocardium after ischemia. This then leads to an enhanced availability of myocardial high-energy phosphates for contractile work (4). The results of the present study demonstrated that the recovery of myocardial function was improved after ischemia by treatment with T_3 (at 10, 25 and 50 nM). The supplementation of thyroid hormone improved the recovery of cardiac function and persisted throughout the reperfusion interval. This is consistent with data reported by Klemperer *et al* (18), in which acute administration of T_3 to post-ischemic hearts improved the preload recruitable stroke work area, but had no effect on the preload stroke work area of the non-ischemic hearts. In the present study, T_3 at 10 nM, ten times the physiological dose, produced a measurable effect. T_3 at 25 nM had a greater effect on post-ischemic recovery, but there was no additional recovery at 50 nM. T_3 had a similar salutary effect on the recovery of the diastolic chamber, with a direct relationship between the dose of T_3 required and the severity of the injury. The observation that T_3 (50 nM) did not make additional improvements is important. It suggests the need to further define the effective therapeutic range for T_3 supplementation.

T_3 supplementation could improve cardiac functions and limit infarct size against I/R injury (10). Previous studies have clearly shown that in addition to binding to nuclear-localized receptors, T_3 can also activate signaling processes at the plasma membrane, in mitochondria or within the cytosol by targeting T_3 receptors or other proteins (2,19). Although the heart may be capable of synthesizing 1 mg of protein per g of heart tissue per h (20), the rapid post-ischemic recovery of cardiac function evident in the present study suggests that the site of action targeted by T_3 is more likely to be in the plasma membrane or cytosol. There is also strong evidence that the accumulation of cytosolic Ca^{2+} during I/R is highly correlated with the severity of injury (13). The present study using isolated hearts revealed a marked improvement in cardiac function by T_3 supplementation before I/R, which is consistent with previous studies (2,8,21). As the inhibition of calpain prevented the I/R-induced degradation of key SR Ca^{2+} cycling proteins (such as RyR2 and SERCA2a) and improved contractile function (22), the present study measured the protein contents of the Ca^{2+} release channel, Ca^{2+} uptake and other Ca^{2+}-regulating proteins. The results demonstrated that an I/R-induced depression in cardiac performance was associated with a downregulation of the major SR Ca^{2+}-cycling proteins. This downregulation could be attenuated by T_3 supplementation before ischemia.

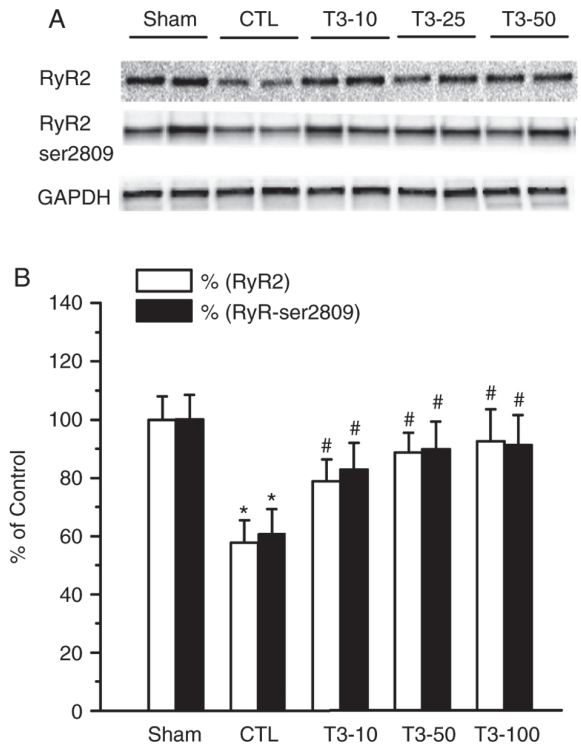

Figure 2. Western blot analysis of RyR2 and the phosphorylation of RyR2 at Ser-2809 in the homogenate of myocardial tissue from rat hearts. The data are presented as the percentage of controls. (A) Western blots presenting the levels of RyR2 and RyR2-Ser-2809 protein. Bands are quantitative immunoblots of representative samples from the hearts of the different groups run on the same gel. (B) Group results. Values (mean ± standard deviation) in the T_3 treatment groups are expressed as the percentage of the Sham group. *P<0.05 vs. time control group (Sham); #P<0.05 vs. the CTL group. RyR2, ryanodine receptor 2; CTL, ischemia control group; T_3, Triiodothyronine.

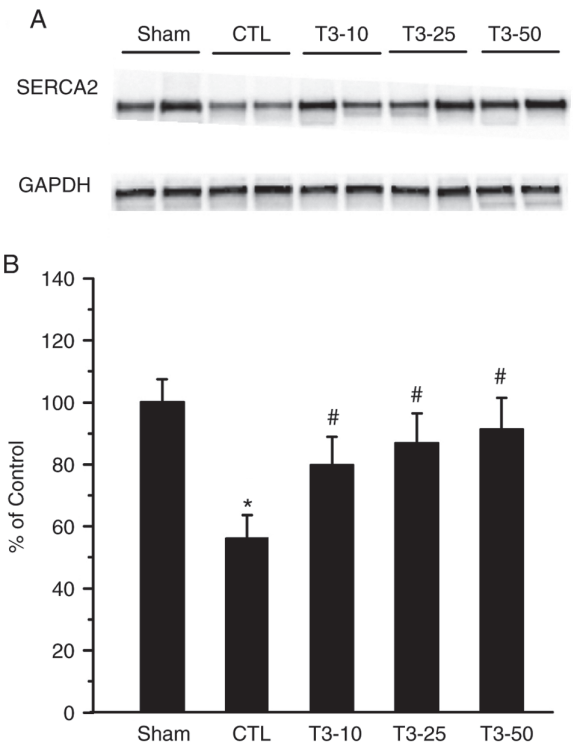

Figure 3. Western blot analysis of SERCA2 and the phosphorylation of RyR2 at Ser-2809 in the homogenate of myocardial tissue from rat hearts. The data are presented as the percentage of the Sham group. (A) Western blots presenting the levels of RyR2 and RyR2-Ser-2809 protein. Bands are quantitative immunoblots of representative samples from the hearts of different groups run on the same gel. (B) Group results. Values (mean ± standard deviation) in the T_3 treatment groups are expressed as percentage of the Sham group. *P<0.05 vs. time control group (Sham); #P<0.05 vs. the CTL group. SERCA2, Ca^{2+}-adenosine triphosphatase; RyR2, ryanodine receptor 2; CTL, ischemia control group; T_3, Triiodothyronine.

RYR2 is a protein found primarily in cardiac muscle. The RYR2 protein functions as the major component of a calcium channel located in the SR that supplies ions to the cardiac muscle during systole to initiate contraction (23). In the present study, T_3 supplementation improved cardiac function recovery in I/R hearts and was accompanied with the preservation of RyR2 protein content. To date, it has been demonstrated that Ser-2809 is one of the main sites of phosphorylation on RYR2. Phosphorylation of RYR2 by CaMKII, or protein kinase A (PKA), at Ser-2809 induced channel function changes *in vitro*. These changes include an increase in the probability of being open (24). In the present study a low level of Ser-2809 phosphorylation was observed in the CTL group, but marked Ser-2809 phosphorylation was expressed following supplementation with T_3. The results suggest that T_3 may facilitate the phosphorylation of RYR2 by CaMKII or PKA.

SERCA2 in cardiac muscle plays an important role in the muscle's overall contractility status. Improved cardiac SR function by T_3 supplementation against I/R injury may partly contribute to a higher level of the protein content of major SR Ca^{2+} uptake proteins including SERCA2a and PLB, a 52 amino acid phosphoprotein. Unphosphorylated PLB inhibits SERCA2a, but the phosphorylation of PLB prevents the inhibition of SERCA2a at either Ser-16 by PKA or Thr-17 by CaMKII, thereby increasing SERCA2a activity and the rate of Ca^{2+} uptake by the SR (25). The present results showed that the protein level of PLB was decreased in I/R hearts and SERCA2a levels were reduced even more. Therefore it is in agreement with a previous study in which the PLB/SERCA2a interaction controlled the calcium content of the SR and ultimately controlled cardiac contractility (25). A decrease in the phosphorylation of PLB, along with an increase in the PLB/SERCA2a ratio was observed (data not shown) in the CTL group, suggesting the strong inhibitory effect of PLB on SERCA2a in I/R hearts. Phosphorylation of the PKA substrate PLB is a critical determinant of Ca^{2+} re-entry into the SR and is coordinated by CaMKII and PKA. In the present study, PLB phosphorylation on Thr-17 was decreased during I/R. PLB phosphorylation was downregulated in I/R hearts, suggesting that the rate of Ca^{2+} uptake by the SR decreased due to the inhibition of SERCA2a. The present results demonstrated that T_3 supplementation improved PLB phosphorylation at Thr-17 in I/R hearts. T_3 supplementation increased the PLB/SERCA2a ratio by valid preservation of SERCA2a. The phosphorylation of PLB by T_3 reduced the inhibition of SERCA2a and therefore enhanced SR Ca^{2+} uptake in I/R hearts. T_3 supplementation inhibited ATP-dependent Ca^{2+} uptake in isolated cardiac SR vesicles (26). There is evidence that alterations in SR Ca^{2+} cycling function are components of the impaired SR Ca^{2+} uptake, Ca^{2+} release and the content of Ca^{2+}-cycling proteins (23,27); the beneficial effect of T_3 supplementation on

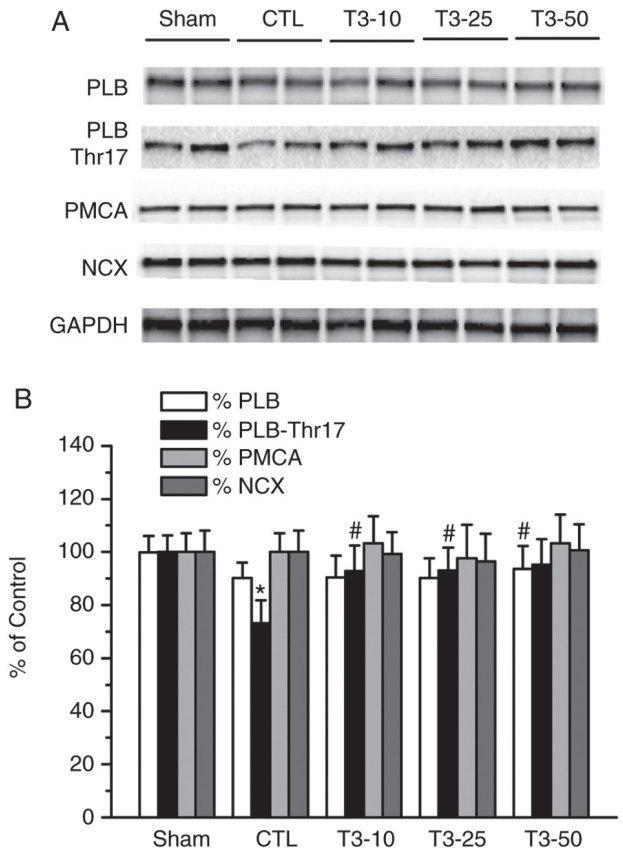

Figure 4. Western blot analysis of PLB and its phosphorylation at Thr-17 (PLB-Thr-17), PMCA and NCX in the homogenate of myocardial tissue from rat hearts. The data are presented as the percentage of controls. (A) Western blots presenting the levels of PLB, PLB-Thr-17, PMCA and NCX protein. Bands are quantitative immunoblots of representative samples from the hearts of the different groups run on the same gel. (B) Group results. Values (mean ± standard deviation) in the treatment groups are expressed as the percentage of the control group. *P<0.05 vs. time control group (Sham); #P<0.05 vs. the CTL group. PLB, phospholamban; PMCA, plasma membrane Ca^{2+}-adenosine triphosphatase; NCX, sodium-calcium exchanger; CTL, ischemia control; T_3, Triiodothyronine.

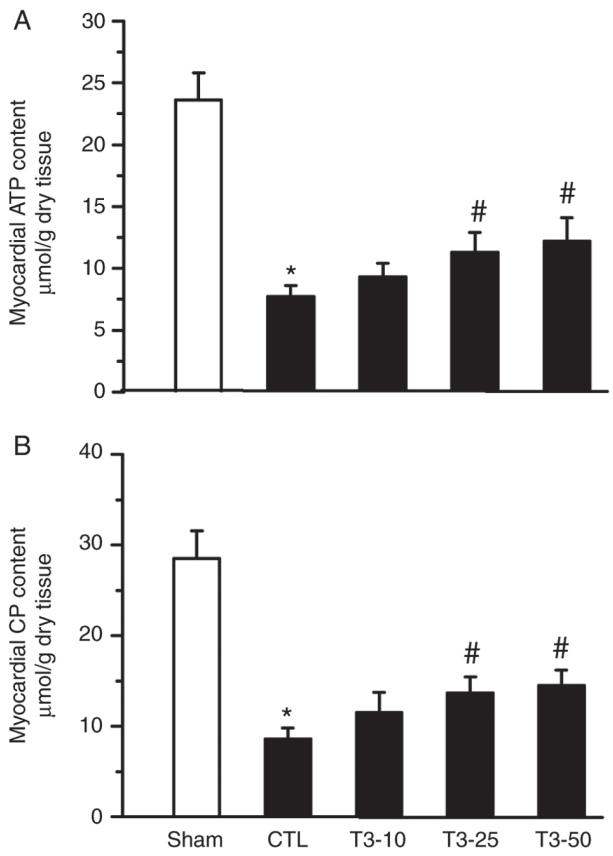

Figure 5. ATP and CP content of transmural sections taken from the left ventricular free wall of rat hearts. (A) Myocardial ATP and (B) CP contents. Values (mean ± standard deviation) in all groups are expressed as μmol/g dry tissue. *P<0.05 vs. time control group (Sham); #P<0.05 vs. the CTL group. ATP, adenosine triphosphate; CP, creatine phosphate; CTL, ischemia control; T_3, Triiodothyronine.

SR Ca^{2+} cycling function may contribute to the attenuation of I/R-induced changes in SR function and protein content.

Cytosolic Ca^{2+} regulates several cellular processes and its concentration is, in turn, finely regulated by various channels, pumps and exchangers. The NCX and the PMCA pump are two concurrent mechanisms for Ca^{2+} extrusion from the cell. There is a very large transmembrane electrochemical Ca^{2+} gradient driving the entry of the ion into cells, yet it is very important that they maintain low concentrations of Ca^{2+} for appropriate cell signaling. Thus, it is necessary for cells on pumps to remove Ca^{2+}. PMCA and NCX together are the main regulators of intracellular Ca^{2+} concentrations (25). In this manner, intracellular Ca^{2+} and SR Ca^{2+} content are regulated and maintained. The present results revealed no alterations in PMCA and NCX contents after I/R. Furthermore, T_3 supplementation did not affect the content of PMCA or NCX.

The results obtained from measuring myocardial ATP and CP in the present study suggested that T_3 induces inotropic effects via the rapid replacement and maintenance of high energy phosphate stores within the myocardium. The administration of T_3 led to the return of normal mitochondrial function, reactivation of the tricarboxylic acid cycle and full aerobic metabolism; tissue lactate levels were reduced and high energy phosphate stores were more rapidly replaced (4). T_3 may lead to both the increased synthesis of high energy stores and increased utilization of such stores, which results in improved myocardial function. This hypothesis is also supported by previous studies (2,4). Sterling *et al* (28) demonstrated the rapidly increased synthesis of ATP production secondary to mitochondrial stimulation in both euthyroid and hypothyroid rats treated with T_3.

In conclusion, the results of the present study demonstrated that T_3 supplementation improves left ventricular function after I/R in isolated rat hearts by preserving major Ca^{2+} cycling proteins. Rapid cardiac functional improvements by T_3 supplementation suggested that these effects may be mediated through T_3 binding at the plasma membrane or SR rather than at the nuclear level. Increased synthesis of myocardial high energy phosphate ATP and CP are attributed to the improvement of myocardial function. T_3-induced preservation of calcium cycling proteins potentially involves the direct inhibition of protease activities. However, the mechanisms underlying these effects require further elucidation.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Conceived and designed the experiments: LF, LL and JA. Performed the experiments: LF, ZX, JL, LH and SQ. Analyzed the data: LF, LL and JA. Contributed reagents/materials/analysis tools: LF, ZX, JL, LH and SQ. Wrote the paper: LF, LL and JA. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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