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 (T₃) supplementation may improve cardiac function after ischemic reperfusion (I/R) injury. In the present study, the effect of T₃ 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), T₃ 10 nM (T₃-10, n=10), T₃ 25 nM (T₃-25, n=10) and T₃ 50 nM (T₃-50, n=10). T₃ was administrated for 60 min before 30 min of ischemia and 120 min of reperfusion. The protein contents of Ca²⁺-release channels (RyR2), Ca²⁺-adenosine triphosphatase (SERCA2a), phospholamban (PLB), sarcoplasmal Ca²⁺-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 T₃ 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. T₃ 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. T₃ supplementation protected the heart against I/R injury via the preservation of Ca²⁺-cycling proteins and high-energy phosphate content.

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 (T₃) state manifests as low circulating levels of T₃ with hemodynamic abnormalities (4). There is a growing body of experimental data that suggests that T₃ 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 T₃ 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 (Ca²⁺) overload plays a major role in the development of myocardial injury during I/R. The sarcoplasmic reticulum (SR) is critical in Ca²⁺ uptake via Ca²⁺-ATPase (SERCA2a) and Ca²⁺ release via the Ca²⁺ release channels (ryanodine receptors; RyRs). Human cardiomyocyte calcium handling and phenotype are strongly influenced by T₃. ‘Low T₃ syndrome’ is observed in the progression of I/R injury (12). It is possible that myocardial protection induced by T₃ supplementation involves the protection of SR function. The effects of T₃ supplementation on myocardial contractility may be attributed to their effects on intracellular Ca²⁺ homeostasis. The

Key words: triiodothyronine, cardiac function, ischemic reperfusion injury, calcium cycling protein, high-energy phosphate
authors have previously reported a marked Ca\textsuperscript{2+} overload in the mitochondrial compartment during I/R (13). The ability of T\textsubscript{3} supplementation to improve cardiac function, induce the activation of sarcolemma and SERCA2a, and decrease cytosolic (Ca\textsuperscript{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\textsubscript{3} supplementation against I/R injury is associated with the preservation of Ca\textsuperscript{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 ± 0.1°C using a thermostatically controlled water circulator (Lauda E100; Laudra 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\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+} and pH were measured offline with an intermittently self-calibrating analyzer system (Radiometer Copenhagen ABL 505; Radiometer, Ltd.). Coronary outflow (coronary sinus) O\textsubscript{2} tension was also measured online continuously with a Clark-type O\textsubscript{2} electrode (203B; Instech Laboratories, Inc.). Myocardial O\textsubscript{2} consumption (MVO\textsubscript{2}) was calculated as [(coronary flow/g heart weight) x (arterial pO\textsubscript{2}-venous pO\textsubscript{2}) x 24 μl O\textsubscript{2}/ml at 760 mmHg; and cardiac work efficiency was calculated as [systolic-diastolic LVP x HR]/MVO\textsubscript{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\textsubscript{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\textsubscript{3}-10, T\textsubscript{3}-25 and T\textsubscript{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.

**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\textsubscript{2} and cardiac efficiency in the Sham, CTL, T\textsubscript{3}-10, T\textsubscript{3}-25 and T\textsubscript{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
than baseline throughout reperfusion in each of the ischemic groups, but it was increased in all of the T3 treated groups (returned to 55% in T3-10, 64% in T3-25 and 67% in T3-50) compared with the CTL group (47%) throughout reperfusion. After 120 min of reperfusion, MVO2 and cardiac efficiency were increased in the T3 administrated groups compared with in the CTL group, but remained lower than the baseline in all of the ischemic groups. Both parameters of MVO2 and cardiac efficiency in the T3-25 and T3-50 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 T3 treated groups compared with in the CTL group upon reperfusion. The improvement in LVDP was significantly different in the T3-25 and T3-50 groups compared with the CTL group (P<0.05). The improvement in LVDP was observed with T3 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. T3 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 T3 supplementation on the SR Ca2+-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 T3 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 T3 supplementation. Compared with the non-ischemic values, SERCA2a expression decreased significantly after I/R injury (P<0.05).

**Effect of T3 supplementation on sarcomlemmal Ca2+-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). T3 treatment at all three doses before ischemia attenuated this decrease and a significant difference was detected in all T3 dose groups compared with the ISC group (P<0.05). The immunoblot 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 T3 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 T3 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 T3 (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 T3, which leads to a ‘euthyroid-sick’ state. It is not

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham</th>
<th>CTL</th>
<th>T3 10 nM</th>
<th>T3 25 nM</th>
<th>T3 50 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (beat/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>266±14</td>
<td>269±15</td>
<td>262±10</td>
<td>265±16</td>
<td>271±18</td>
</tr>
<tr>
<td>Reperfusion 120'</td>
<td>261±16</td>
<td>241±24</td>
<td>240±21</td>
<td>245±14</td>
<td>248±19</td>
</tr>
<tr>
<td>CF (ml/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>12.2±0.8</td>
<td>11.9±0.8</td>
<td>12.0±0.7</td>
<td>12.2±0.9</td>
<td>11.9±0.7</td>
</tr>
<tr>
<td>Reperfusion 120'</td>
<td>11.8±1.0</td>
<td>5.6±0.6b</td>
<td>6.6±0.5ab</td>
<td>7.9±0.6c</td>
<td>8.0±0.7c</td>
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<tr>
<td>MVO2 (mmHg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>115±8</td>
<td>112±11</td>
<td>111±10</td>
<td>112±12</td>
<td>115±10</td>
</tr>
<tr>
<td>Reperfusion 120'</td>
<td>112±11</td>
<td>58±7c</td>
<td>69±7b</td>
<td>73±8c</td>
<td>75±7c</td>
</tr>
<tr>
<td>Cardiac efficiency (mmHg·beat·0.1 µl O2·g⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>16.5±1.3</td>
<td>16.6±1.5</td>
<td>16.2±1.6</td>
<td>16.3±1.1</td>
<td>16.5±1.4</td>
</tr>
<tr>
<td>Reperfusion 120'</td>
<td>15.6±1.2</td>
<td>6.3±0.7ab</td>
<td>7.2±0.8b</td>
<td>8.6±0.8c</td>
<td>8.7±0.8c</td>
</tr>
</tbody>
</table>

The HR, CF, MVO2 and Cardiac efficiency before ischemia and after 30 min ischemia and 120 min reperfusion. Values are the mean ± standard deviation. *P*<0.05 vs. the baseline; †*P*<0.05 vs. the Sham group; ‡*P*<0.05 vs. the CTL group. Sham (non-ischemia; n=10); CTL (Ischemia; n=10); T3 10 nM (n=10); T3 25 nM (n=10); T3 50 nM (n=10). CF, coronary flow; HR, heart rate; MVO2, myocardial O2 consumption; Cardiac efficiency, cardiac work efficiency; T3, 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 \(T_3\) \((10, 25 \text{ and } 50 \text{ nM})\). 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 \(\text{et al} \) \((18)\), 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, \(T_3\) \((10 \text{ nM})\), ten times the physiological dose, produced a measurable effect. \(T_3\) at \(25 \text{ nM}\) had a greater effect on post-ischemic recovery, but there was no additional recovery at \(50 \text{ nM}\). \(T_3\) 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 \(T_3\) \((50 \text{ nM})\) 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 \(1 \text{ mg}\) 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 \((22)\), 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.
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, T₃ 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 T₃. The results suggest that T₃ 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 T₃ supplementation against I/R injury may partly contribute to a higher level of the protein content of major SR Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ uptake by the SR decreased due to the inhibition of SERCA2a. The present results demonstrated that T₃ supplementation improved PLB phosphorylation at Thr-17 in I/R hearts. T₃ supplementation increased the PLB/SERCA2a ratio by valid preservation of SERCA2a. The phosphorylation of PLB by T₃ reduced the inhibition of SERCA2a and therefore enhanced SR Ca²⁺ uptake in I/R hearts. T₃ supplementation inhibited ATP-dependent Ca²⁺ uptake in isolated cardiac SR vesicles (26). There is evidence that alterations in SR Ca²⁺ cycling function are components of the impaired SR Ca²⁺ uptake, Ca²⁺ release and the content of Ca²⁺-cycling proteins (23,27); the beneficial effect of T₃ supplementation on
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.

References


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