Thyroid Hormone Diminishes Ca^{2+} Overload Induced by Hypoxia/Reoxygenation in Cardiomyocytes by Inhibiting Late Sodium Current and Reverse- $\text{Na}^+/\text{Ca}^{2+}$ Exchange Current

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Keywords

Thyroid hormone · Ventricular cardiomyocytes · Hypoxia/reoxygenation · Late sodium current · Reverse- $\text{Na}^+/\text{Ca}^{2+}$ exchange current · Action potential duration · Calcium overload

Abstract

Background and Purpose: Intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) overload occurs in myocardial ischemia and reperfusion. The augmentation of the late sodium current (I_{NaL}) causes intracellular Na^+ accumulation and subsequent $[\text{Ca}^{2+}]_i$ overload via the reverse mode of the $\text{Na}^+/\text{Ca}^{2+}$ exchange current (reverse- I_{NCX}), which can lead to arrhythmia and cardiac dysfunction. Thus, inhibition of I_{NaL} is a potential therapeutic approach for ischemic heart disease. The aim of this study was to investigate the effects of thyroid hormone on augmented I_{NaL} , reverse- I_{NCX} , altered action potential duration (APD), and $[\text{Ca}^{2+}]_i$ concentration in hypoxia/reoxygenation (H/R)-induced ventricular myocytes in vitro. **Methods:** The transient Na^+ current (I_{NaT}), I_{NaL} , reverse- I_{NCX} , and APs were recorded using a whole-cell patch-clamp technique in neonatal mouse ventricular myocytes. $[\text{Ca}^{2+}]_i$ concentration alteration were, respectively, observed by confocal microscopy and flow cytometry. **Results:** Triiodothyronine (T3) pretreatment decreased the I_{NaL} in a concentration-dependent manner. H/R injury aggravated the I_{NaL} , I_{NaT} , and reverse- I_{NCX}

in cardiomyocytes and increased the continuous accumulation of $[\text{Ca}^{2+}]_i$ ($p < 0.05$). The application of T3 prior to H/R injury significantly decreased the increased I_{NaL} , I_{NaT} , and reverse- I_{NCX} and blunted the $[\text{Ca}^{2+}]_i$ increase. Furthermore, T3 pretreatment shortened the APD induced by H/R injury. **Conclusion:** T3 inhibited H/R-increased I_{NaL} and reverse I_{NCX} augmentation, shortened the APD, and diminished $[\text{Ca}^{2+}]_i$ overload, indicating a potential therapeutic use of T3 as an I_{NaL} inhibitor to maintain Ca^{2+} homeostasis and protect cardiomyocytes against H/R injury.

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Introduction

Cardiomyocyte calcium concentration ($[\text{Ca}^{2+}]_i$) overload occurs in many pathological conditions, including hypoxia, ischemia, reperfusion, oxidative stress, cardiac hypertrophy, and heart failure [1, 2]. $[\text{Ca}^{2+}]_i$ overload causes cellular injury and myocardial apoptosis, including mitochondrial dysfunction, reduced ATP production, and activated Ca^{2+} -dependent protease [3]. The late sodium current (I_{NaL}), which is tetrodotoxin-sensitive, is increased in cardiomyocytes under many pathological conditions and can lead to Ca^{2+} overload [4, 5]. I_{NaL} also plays an important role in determining the plateau of the action potential (AP) and the AP duration (APD) under

pathological conditions. The increased I_{NaL} prolongs the APD, which could cause cardiac arrhythmias [6–9]. In addition, increased I_{NaL} causes an increase in the intracellular sodium concentration and subsequently raises $[Ca^{2+}]_i$ via the reverse mode of the Na^+/Ca^{2+} exchanger (NCX) [7]. Inhibition of I_{NaL} could reduce the intracellular $[Ca^{2+}]_i$ overload [8, 9], which is a potential therapeutic target for the treatment of cardiac diseases associated with intracellular $[Ca^{2+}]_i$ overload.

Thyroid hormone (TH) signaling is essential for heart development and cardiovascular function through both genomic and nongenomic mechanisms. The classically described cellular actions of TH are mediated by triiodothyronine (T3) by binding to specific nuclear TH receptors, which function to regulate the expression of specific cardiac genes such as voltage-activated K^+ channel genes such as $Kv4.2$, $Kv4.3$, and $Kv1.5$ [10]. T3 could increase the inward currents directly, shortening APD [13]. Previous studies suggested that T3 could rapidly and nongenomically regulate the Ca^{2+} ATPase enzyme, the Na^+ channel, and the K^+ channel and that T3 could also increase levels of sarcoplasmic reticulum Ca^{2+} and the contractility of myocytes [14]. Recent studies have demonstrated that altered thyroid status dramatically impacts cardiac electrical function. Hypothyroidism produces sinus bradycardia and prolongs cardiac repolarization, whereas hyperthyroidism is associated with accelerated heart rate and shortened APD [11, 12]. These results suggest potential protective effects of TH on cardiomyocytes. However, the underlying mechanisms are still unknown. Previous studies have found that the inhibition of I_{NaL} attenuates augmented I_{NaL} -induced $[Ca^{2+}]_i$ overload [8, 9], and no study has investigated the effects of TH on I_{NaL} . Thus, this study investigated the effects of TH on I_{NaL} , Na^+/Ca^{2+} exchange current (reverse- I_{NCX}), APD, and $[Ca^{2+}]_i$ concentration in hypoxia/reoxygenation (H/R)-induced ventricular myocytes in vitro.

Materials and Methods

Drugs and Reagents

Dulbecco's modified Eagle's medium-F12, fetal bovine serum (FBS), and D-Hank's solution were purchased from GIBCO-BRL (Grand Island, NE, USA). Trypsin, penicillin-streptomycin, and Fluo-3 AM kit were purchased from Beyotime Biotechnology (Beyotime, Shanghai, China). All other reagents, including T3, HEPES, 5-bromo-2-deoxyuridine, and collagenase type II, were purchased from Sigma Aldrich (St. Louis, MO, USA). T3 was stored in the dark at $-20^{\circ}C$. On the day of experimentation, T3 was dissolved in NaOH and added to DMEM-F12 to make a stock solution at a concentration of 20 $\mu g/L$.

Isolation of Ventricular Myocytes

The animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996), and all animal procedures were approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University (protocol number: 00013518).

Neonatal mice (1–3 days old) were purchased from the animal center of Hubei Province. Myocytes were prepared by enzymatic disaggregation of cardiac tissue as described previously [15]. In brief, the hearts were quickly excised and immersed in 0.25% trypsin at $4^{\circ}C$ overnight. The heart tissues were digested with type II collagenase (Sigma; USA) at $37^{\circ}C$, and the isolated cells were filtered and centrifuged at 1,000 rpm for 10 min. Then, the cells were cultured for 90 min at $37^{\circ}C$ for cardiomyocyte purification, and the cardiomyocytes were finally cultured at a concentration of 1×10^5 cells/mL in medium containing 10% heat-inactivated FBS (Gibco, USA), 1% penicillin-streptomycin, and 5-bromo-2-deoxyuridine to inhibit fibroblast growth. The cells were incubated at $37^{\circ}C$ in a humidified incubator with 5% CO_2 for 48 h prior to treatment.

Established H/R Model in vitro

For the experiment conducted under H/R conditions, cardiomyocytes were incubated with PBS in a tri-gas incubator (95% N_2 , 5% CO_2) at $37^{\circ}C$ for 4 h, and then the PBS was replaced with culture medium containing 10% FBS and cultured in a standard incubator (5% CO_2 , $37^{\circ}C$) for 4 h. The cells in the normal control groups were exposed to the normoxic conditions.

For T3 pretreatment, 2, 4, and 8 μL stock solutions of T3 were added into the culture medium for 24 h of pretreatment before the H/R conditions. To explore the effect of T3 pretreatment on intracellular $[Ca^{2+}]_i$ overload under H/R injury, cultured cardiomyocytes were randomly divided into 4 groups: control group, control + T3 group, H/R group, and H/R + T3 group.

Solutions

For I_{NaL} recordings, the intracellular (pipette) solution contained the following (in mmol/L): 120 CsCl, 10 TEA-Cl, 5 Na_2ATP , 5 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, and 10 HEPES (pH 7.3, adjusted with CsOH). The bath solution contained the following (in mmol/L): 135 NaCl, 0.33 NaH_2PO_4 , 5.4 CsCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 0.3 $BaCl_2$, 0.01 nifedipine, 10 HEPES, and 10 glucose (pH 7.4 adjusted with NaOH).

To record I_{NaT} , the intracellular (pipette) solution contained the following (in mmol/L): 120 CsCl, 1 $CaCl_2$, 10 TEA-Cl, 5 Na_2ATP , 5 $MgCl_2$, 10 EGTA, and 10 HEPES (pH 7.3, adjusted with CsOH). The bath solution contained the following (in mmol/L): 105 CsCl, 30 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 0.01 nifedipine, 10 HEPES, and 10 glucose (pH 7.4 adjusted with CsOH).

To record I_{NCX} , the intracellular (pipette) solution contained the following (in mmol/L): 115 CsCl, 10 NaCl, 1 $CaCl_2$, 5 $Mg-ATP$, 20 TEA-Cl, 10 EGTA, and 10 HEPES (pH 7.25, adjusted with CsOH). The bath solution contained the following (in mmol/L): 140 NaCl, 5 CsCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 5 glucose (pH 7.35, adjusted with CsOH). In addition, 1.0 mmol/L $BaCl_2$ and 1.0 $\mu mol/L$ nifedipine were added to block the potassium current and I_{CaL} , respectively.

To record AP, the intracellular (pipette) solution contained the following (in mmol/L): 110 L-aspartic acid potassium salt, 20 KCl,

8 NaCl, 2 MgCl₂, 1 CaCl₂, 10 EGTA, 10 HEPES, and 40 sucrose (pH 7.2, adjusted with KOH). The bath solution contained the following (in mmol/L): 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 0.33 NaH₂PO₄, 10 HEPES, and 10 glucose (pH 7.4, adjusted with NaOH).

Whole-Cell Patch-Clamp Technique

The conventional whole-cell patch-clamp technique using an EPC-9 amplifier (HEKA Electronic, Lambrecht, Germany) was applied to record transmembrane potentials and ion currents. The patch electrode resistance (when filled with pipette solution) was 3–5 MΩ. Cell capacitance and series resistances were electronically compensated by 60–80%. Currents were filtered at 2 kHz and digitized at 10 kHz. All experiments were performed at room temperature (22–25 °C).

I_{NaT} current–voltage (I – V) relation and activation properties were determined by applying depolarizing steps 100 ms in duration from holding potential (V_h) –120 mV in 10 mV increments, ranging from –90 to +50 mV. The maximum value of I_{NaT} was measured. For inactivation properties, I_{NaT} was determined using 100 ms conditioning pulses from a holding potential of –120 mV, ranging from –120 to –45 mV in 10 mV increments, followed by a test pulse to –10 mV for 100 ms.

I_{NaL} was recorded by a 300 ms depolarizing pulse to –20 mV from a holding potential of –90 at a frequency of 0.2 Hz. The amplitude of I_{NaL} was determined from the average current measured during a time interval of 190–210 ms after initiation of the depolarizing pulse to eliminate any contribution of I_{NaT} [2]. To record the current–voltage relationship of I_{NaL} , 300-ms depolarizing pulses to membrane potentials from –80 to +40 mV were applied at 0.5 Hz from a potential of –90 mV.

I_{NCX} was elicited by a 10 ms prepulse to +60 mV, followed by a 2 s ramp pulse protocol ranging from +60 to –120 mV (with a speed of –90 mV/s) from a holding potential of –40 mV. I_{NCX} was measured as the current sensitive to 5 mmol/L Ni²⁺ at +50 and –100 mV.

AP was evoked by 5 ms duration of 1.5 times diastolic threshold current pulses at 0.25 Hz. The AP amplitude and APD at 50 and 90% repolarization (APD₅₀ and APD₉₀) were analyzed.

Measurement of [Ca²⁺]_i Levels by Flow Cytometry and Confocal Microscopy

Ca²⁺ indicators Fluo-3/AM was employed to measure the [Ca²⁺]_i concentrations. The cardiomyocytes were seeded in circular discs and treated in the manner described above. Then washed with D-Hank's 3 times and incubated with 5 μmol/L Fluo-3/AM for 40 min at 37 °C in dark in D-Hank's solution. After loading, the cells were incubated for 20 min at room temperature to ensure the complete transformation of fluo-3 AM into fluo-3. The [Ca²⁺]_i concentration was evaluated by measuring the fluorescence intensity using flow cytometer (Becton Dickinson, San Jose, CA, USA) and the Confocal Microscopy System (Carl Zeiss, Berlin, Germany). The excitation wavelength used in the detection is 488 nm, and the emission is 525 nm. In flow cytometry experiments, at least 10,000 events were obtained.

Data Analysis

All current amplitudes were normalized to cell capacitance and expressed as pA/pF. All data were presented as the mean ± SD and were analyzed using FitMaster (v2 x 32, HEKA) and SPSS 22.0

software. Figures were plotted with Origin (version 8.0, OriginLab Co., MA, USA). Student *t* test was used to determine the difference between 2 groups of data. One-way analyses of variance were followed by the Scheffé test for multiple comparisons. Values of *p* < 0.05 were considered significant.

Current tracings were fitted using Hill or Boltzmann functions with Origin 8.0. Steady-state activation data and inactivation relationships of I_{NaT} were fitted to the Boltzmann equation as follows: $Y = 1/(1 + \exp [V_m - V_{1/2}]/k)$, where V_m was the membrane potential, $V_{1/2}$ was the half-activation or half-inactivation potential, and k was the slope factor. For the steady-state activation and inactivation curve, Y is the relative conductance (G/G_{max}) and relative current (I/I_{max}), respectively.

Results

T3 Inhibited I_{NaL} in Neonatal Mouse Ventricular Cardiomyocytes Induced by H/R Injury

Previous reports show that I_{NaL} is increased under hypoxic conditions; thus, we studied the effects of T3 on I_{NaL} after exposure to H/R injury. I_{NaL} was recorded with 300 ms voltage steps from a holding potential of –120 to –20 mV at 0.5 Hz. The recorded sodium current includes I_{NaT} in depolarization and I_{NaL} in repolarization. Mexiletine, a blocker of the late Na⁺ current as previously reported, minimally affects the I_{NaT} and the peak I_{Na} [16]. We use mexiletine to separate I_{NaL} from the sodium current in cardiomyocytes. After the application of 10 μmol/L mexiletine, I_{NaL} was almost completely blocked with current densities decreasing from 0.79 ± 0.07 pA/pF to 0.056 ± 0.014 pA/pF ($n = 10$, $p < 0.01$; Fig. 1), with no significant change in I_{NaT} , the results were consistent with the above conclusions. In addition, the increased I_{NaL} induced by H/R injury (from 0.7 ± 0.07 to 1.85 ± 0.05 pA/pF) was completely blocked by mexiletine (0.071 ± 0.014 pA/pF; $n = 10$, $p < 0.01$). These results showed that the increased recorded currents were I_{NaL} .

T3 inhibited I_{NaL} in a concentration-dependent manner; the results showed that T3 (20, 40, 80 ng/mL) decreased the I_{NaL} current density from 0.76 ± 0.07 to 0.68 ± 0.014 pA/pF ($p > 0.05$), 0.57 ± 0.031 pA/pF ($n = 10$, $p < 0.05$), and 0.49 ± 0.023 pA/pF ($n = 10$, $p < 0.01$; Fig. 1), respectively. Then, 80 ng/mL T3 was chosen for the subsequent experiments.

The I_{NaL} current/voltage (I / V) relation curves of cardiomyocytes in each group are shown in Figure 2. T3 pretreatment decreased the I_{NaL} current density of cardiomyocytes ($n = 10$, $p < 0.05$). After H/R treatment, the current density of I_{NaL} was significantly increased from 0.7 ± 0.07 pA/pF to 1.85 ± 0.05 pA/pF ($n = 10$, $p < 0.0001$). However, this increase was effectively inhibited by T3

Fig. 1. Effects of T3 on control I_{NaL} in cultured neonatal mouse cardiomyocytes. **a** The blockade of 10 $\mu\text{mol/L}$ mexiletine on control and H/R-increased I_{NaL} . **b** The peak I_{NaL} on cardiomyocytes in control group and T3 (20, 40, and 80 ng/mL) treatment group, $n = 10$ in each group. **c** Typical whole-cell I_{NaL} recorded in the control

group and presence of 20, 40, and 80 ng/mL T3. Data were expressed as mean $\pm$ SD, # $p < 0.05$, ## $p < 0.01$ vs. control. I_{NaL} , late sodium current; H/R, hypoxia/reoxygenation; T3, triiodothyronine.

pretreatment, T3 diminished the downward shift of the I/V curve in H/R-induced cardiomyocytes, and decreased the I_{NaL} to 1.16 ± 0.079 pA/pF ($n = 10$, $p < 0.0001$).

T3 Decreased I_{NaT} of Ventricular Cardiomyocytes Induced by H/R Injury

The I_{NaT} current/voltage (I/V) relation curves of cardiomyocytes in each group are shown in Figure 3. At the concentration of 80 ng/mL T3, the magnitudes of I_{NaT} were decreased from -45.093 ± 2.198 to -22.456 ± 1.58 pA/pF ($n = 10$, $p < 0.01$). During H/R injury, the current density of I_{NaT} was significantly increased from -45.093 ± 2.198 to -89.319 ± 9.08 pA/pF ($n = 10$, $p < 0.01$ vs. control). However, this increase was effectively offset by T3 pretreatment; T3 diminished the downward shift of the I/V curve in H/R induced cardiomyocytes, and the I_{NaT} was decreased to -54.403 ± 8.774 pA/pF ($n = 10$, $p < 0.05$ vs. H/R).

The affinity of T3 for the activated or inactivated states of the channel was determined by assessing the shifts in the steady-state availability curves. The steady-state activa-

tion curve was obtained from Boltzmann equation $G/G_{\text{max}} = 1/(1 + \exp[(V_{1/2} - V_m)/k])$, and the steady state inactivation curve was obtained from Boltzmann equation $I/I_{\text{max}} = 1/(1 + \exp[(V_m - V_{1/2})/k])$. The steady-state activation of I_{NaT} was examined, and the half-maximal activation potential of I_{NaT} ($V_{1/2}$) values were -20.74 ± 1.569 and -17.61 ± 1.911 mV ($n = 10$, $p < 0.01$), and the values of k were 7.61 ± 0.249 and 7.5 ± 0.80979 ($p > 0.05$) before and after application of 80 ng/mL T3, respectively (Fig. 3). The activation curve of I_{NaT} shifted toward a more positive potential with T3 pretreatment. During H/R treatment, the results showed a left shift of the activation curve of I_{NaT} with a decreased $V_{1/2}$ from -20.74 ± 1.569 to -28.82 ± 1.327 mV ($n = 10$, $p < 0.01$), and the value of k showed no significant differences between the 2 groups. However, the effect in activation potential was diminished with T3 pretreatment, with an increased value of $V_{1/2}$ (-21.51 ± 2.197 mV, $n = 10$, $p < 0.05$), and the value of k showed no significant differences between the 2 groups.

The steady-state inactivation curve of I_{NaT} was also examined, the half-maximal inactivation potential of I_{NaT}

Fig. 2. Effects of T3 on increased I_{NaL} induced by H/R injury in cultured neonatal mouse cardiomyocytes. **a** Typical whole-cell I_{NaL} recorded in each group of cardiomyocytes. **b** The current-voltage relationship for I_{NaL} in each group. **c** The peak I_{NaL} on car-

diomyocytes in each group, $n = 10$ in each group. Data were expressed as mean $\pm$ SD, # $p < 0.05$, #### $p < 0.0001$ vs. control; **** $p < 0.0001$ vs. H/R. T3, triiodothyronine; H/R, hypoxia/re-oxygenation; I_{NaL} , late sodium current.

($V_{1/2}$) value was changed from -82.21 ± 1.406 to -76.0 ± 2.577 mV ($n = 10$, $p < 0.0001$) after application of T3, and the value of k showed no significant differences between the 2 groups. The inactivation curve of I_{NaT} shifted toward a more negative potential with T3 pretreatment. During H/R treatment, the $V_{1/2}$ changed to -101.9 ± 1.678 mV, leading to a right shift of the inactivation curve, while no significant difference was observed in the k values between the 2 groups. However, the right shift in the I_{NaT} inactivation curve in H/R group was reversed by T3

pretreatment, with an increased value of $V_{1/2}$ (-86.05 ± 6.07 mV, $n = 10$, $p < 0.05$); the value of k showed no significant differences between the 2 groups (Fig. 3).

Effects of T3 on the Increased Reverse I_{NCX} Induced by H/R Injury

As shown in Figure 4, at a concentration of 80 ng/mL T3, the forward I_{NCX} current density was decreased from -0.831 ± 0.165 to -0.39 ± 0.079 pA/pF ($n = 10$, $p < 0.05$), and the reverse I_{NCX} current density was decreased

Fig. 3. Effects of T3 on I_{NaT} in cultured neonatal mouse cardiomyocytes of each group. **a** Typical whole-cell I_{NaT} recorded in each group of cardiomyocytes. **b** The current-voltage relationship for I_{NaT} in cardiomyocytes of each group. **c** The peak I_{NaT} on cardiomyocytes in each group, $n = 10$ in each group. **d** The steady-state

activation and inactivation current recordings of I_{NaT} on cardiomyocytes in each group. Data were expressed as mean $\pm$ SD, ## $p < 0.01$ vs. control; ** $p < 0.01$ vs. H/R. T3, triiodothyronine; H/R, hypoxia/reoxygenation.

Fig. 4. Effects of T3 on I_{NCX} in cultured neonatal mouse cardiomyocytes of each group. **a** Representative trace of Na^+-Ca^{2+} exchange current from cardiomyocytes conducted under conditions of control and 5 mmol/L Ni^{2+} treated. **b** Ni^{2+} -sensitive I_{NCX} obtained in each group, data were obtained by subtracting the data in trace Ni^{2+} -treated from the data in traces control, control + T3,

H/R, and H/R + T3, respectively. **c** Mean current densities of forward I_{NCX} and reverse I_{NCX} under different conditions. Data were expressed as mean $\pm$ SD, # $p < 0.05$, ### $p < 0.001$, #### $p < 0.0001$ vs. control; * $p < 0.05$, **** $p < 0.0001$ vs. H/R. I_{NCX} , Na^+/Ca^{2+} exchange; T3, triiodothyronine; H/R, hypoxia/reoxygenation.

Table 1. The effects of T3 on action potential in neonatal mouse cardiomyocytes

Treatment	Action potential amplitude, mV	APD50, ms	APD90, ms
Control	86.36 $\pm$ 2.06	18.83 $\pm$ 1.64	106.3 $\pm$ 9.85
Control + T3	85.25 $\pm$ 1.22	11.65 $\pm$ 0.98##	58.02 $\pm$ 3.97####
H/R	86.21 $\pm$ 0.95	28.03 $\pm$ 1.85##	162.6 $\pm$ 14.42##
H/R + T3	84.23 $\pm$ 1.52	15.58 $\pm$ 1.37****	101.1 $\pm$ 11.86**

Values were expressed as mean $\pm$ SD, $n = 15$.
$p < 0.01$, #### $p < 0.0001$ vs. control; ** $p < 0.01$, **** $p < 0.0001$ vs. H/R. APD 50, action potential duration at 50% repolarization; APD 90, action potential duration at 90% repolarization.
T3, triiodothyronine; APD, action potential duration; H/R, hypoxia/reoxygenation.

from 1.555 ± 0.119 to 0.94 ± 0.034 pA/pF ($n = 10$, $p < 0.001$). H/R injury increased the forward I_{NCX} to -1.264 ± 0.102 pA/pF ($n = 10$, $p < 0.05$), and the reverse I_{NCX} current density was increased from 3.829 ± 0.145 pA/pF ($n = 10$, $p < 0.0001$), compared with that of the control group. However, T3 pretreatment decreased these values to -0.832 ± 0.16 pA/pF ($n = 10$, $p < 0.05$) and 3.829 ± 0.145 pA/pF ($n = 10$, $p < 0.0001$), T3 diminished the downward shift of I/V curve induced by H/R injury.

Effects of T3 on AP of Cardiomyocytes

As shown in Table 1, the APD50 and APD90 of cardiomyocytes were shortened with T3 pretreatment, the APD50 decreased from 18.83 ± 1.64 to 11.65 ± 0.98 ms

($n = 15$, $p < 0.01$), and the APD90 decreased from 106.3 ± 9.85 to 58.02 ± 3.97 ms ($n = 15$, $p < 0.0001$), respectively, compared with the control group. After the H/R condition, the APD50 and APD90 of cardiomyocytes were increased to 28.03 ± 1.85 and 162.6 ± 14.42 ms ($n = 15$, both $p < 0.01$ vs. control). However, this increase was significantly inhibited with T3 pretreatment; the APD50 decreased to 15.58 ± 1.37 ms ($n = 15$, $p < 0.0001$), and the APD90 decreased to 101.1 ± 11.86 ms ($n = 15$, $p < 0.01$), compared with that of the control. In all groups, T3 had no effect on the AP amplitude.

T3 Blunted the $[Ca^{2+}]_i$ Increase Induced by H/R Injury

As shown in Figure 5, there was no statistical difference in Fluo-3 fluorescence intensity of flow cytometry between the control group and control + T3 group ($n = 3$, $p > 0.05$). H/R injury significantly potentiated Fluo-3 fluorescence compared with the control group, suggesting an elevation in $[Ca^{2+}]_i$ concentration. The levels of Ca^{2+} concentration in H/R group were increased by 58.1% ($n = 6$, $p < 0.05$). However, T3 pretreatment greatly blunted the increase of the $[Ca^{2+}]_i$, thus the levels of Ca^{2+} concentration in H/R+T3 group were increased by 31.2% compared with the control ($n = 6$, $p < 0.05$), which indicated that T3 can diminish H/R-induced $[Ca^{2+}]_i$ overload and maintain $[Ca^{2+}]_i$ homeostasis. The fluorescence intensity changes of the confocal microscopy were consistent with the flow cytometry.

Fig. 5. Effects of T3 on [Ca²⁺]_i concentration in cultured neonatal mouse cardiomyocytes of each group. **a** The effects of calcium overload were analyzed by flow cytometry using the fluorescent probe Fluo-3/AM. **b** Fluorescence intensity of flow cytometry in

each group. **c** The effects of [Ca²⁺]_i concentration were analyzed by the confocal microscopy using the fluorescent probe Fluo-3/AM. Data were expressed as mean ± SD, # *p* < 0.05 vs. control; * *p* < 0.05 vs. H/R. T3, triiodothyronine; H/R, hypoxia/reoxygenation.

Discussion

TH is considered a major regulator of cardiovascular function, including regulation of contractile and calcium-handling protein expression, ion channels, and sympathetic tone in cardiovascular system homeostasis [17, 18]. Recent study has shown that low-dose T3 therapy could protect post infarct cardiac tissue from dysfunction and arrhythmias without adverse effects [17], although

the mechanisms of this process remain unknown. This study investigated the cardioprotection effect of T3 in maintaining calcium homeostasis by regulating the NCX, late sodium channels and levels of [Ca²⁺]_i concentration in H/R-induced cardiomyocytes.

Calcium homeostasis is essential for maintaining cardiac function. [Ca²⁺]_i overload occurs in many pathological conditions, including hypoxia, ischemia, reperfusion, oxidative stress, cardiac hypertrophy, and heart failure

[1–2]. $[Ca^{2+}]_i$ overload leads to cellular injury, cell death, electrical activity disorders, and ventricular systolic dysfunction, which would eventually aggravate myocardial dysfunction and induce cardiac arrhythmias [5, 8, 19, 20]. Inhibiting $[Ca^{2+}]_i$ and maintaining calcium homeostasis are important factors in protecting cardiac myocytes against I/R injury. Under pathological conditions, increased I_{NaL} could cause $[Ca^{2+}]_i$ overload by increasing reverse I_{NCX} [7]. Extensive studies have reported that inhibition of NCX can prevent cardiac diseases through inhibition of I_{NaL} , which was associated with intracellular $[Ca^{2+}]_i$ overload [8, 9]. Previous studies show that I_{NaL} is increased in many pathological conditions such as hypoxia and ischemia. Past studies have reported that low T3 treatment could promote cardiac function and prevent cardiac remodeling by inhibiting I/R-induced cardiomyocyte apoptosis [21, 22]. In this study, T3 pretreatment decreased the elevation of I_{NaL} and greatly blunted the increase of the $[Ca^{2+}]_i$ concentration induced by H/R injury, and this effect was concentration-dependent. These data suggest that T3 may protect cardiomyocytes against H/R-induced injury by maintaining intracellular calcium homeostasis. T3 could rapidly, and nongenomically regulate the Ca^{2+} ATPase enzyme, the Na^+ channel, and the K^+ channel [10], suggesting that T3 contributes to Ca^{2+} homeostasis in cardiomyocytes.

NCX plays an important role in regulating $[Ca^{2+}]_i$ and maintaining Ca^{2+} homeostasis in the heart, and the expression level of NCX is high in the fetal heart but gradually decreases after birth [23]. Previous study has indicated that the reverse I_{NCX} could be suppressed under normal or hypoxic conditions by inhibiting I_{NaL} , which shows that an increased I_{NaL} causes this change [24]. Thus, the inhibition of I_{NaL} is expected to decrease the augmented reverse I_{NCX} . The present study showed that H/R-induced injury caused an increase in reverse I_{NCX} on cardiomyocytes, while pretreatment of T3 inhibited this increase. Inhibition of I_{NaL} could result in a decrease in reverse I_{NCX} , inhibition of cell shortening, and $[Ca^{2+}]_i$ overload amelioration [25]. Thus, we hypothesized that T3 could protect cardiomyocytes from H/R injury by preventing abnormal Ca^{2+} influx by decreasing reverse I_{NCX} .

In the present study, the effect of T3 on ventricular cardiomyocyte APs was also studied. A previous study reported that T3 could regulate the expression of specific cardiac genes such as voltage-activated K^+ channel genes and increase the inward currents directly, which shortened APD [13]. In this study, T3 shortened the prolonged APD50 and APD90 in H/R-induced cardiomyocytes, thus we speculate that T3 may have potential antiven-

tricular arrhythmia effects, but further researches from larger studies are needed to verify this link. In addition, the normal and H/R-augmented I_{NaT} were also inhibited by T3 pretreatment, and the increased I_{NaT} current density induced by H/R injury was significantly diminished upon T3 pretreatment. Moreover, the steady-activation curve of I_{NaT} was shifted toward a more positive voltage, and the steady-inactivation curve of I_{NaT} was shifted toward a more negative voltage, which indicated that T3 can inhibit the rate of activation and accelerate the rate of inactivation of sodium channels to keep the Na^+ channels stable.

In conclusion, T3-inhibited H/R-increased I_{NaL} and decreased the reverse I_{NCX} augmentation, shortened the APD, and diminished $[Ca^{2+}]_i$ overload, indicating a potential therapeutic use of T3 as an I_{NaL} inhibitor to maintain Ca^{2+} homeostasis and protect cardiomyocytes against H/R injury.

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Disclosure Statement

All the authors do not have any possible conflicts of interest.

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Author Contributing

B.Z. and L.L. designed the experiment. L.L. and X.L. carried out the experiments. C.Z. and H.R. conceived and coordinated the study. L.L. wrote the manuscript. All authors reviewed the results and approved the final version.

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