

Detrimental effects of thyroid hormone analog DITPA in the mouse heart: increased mortality with in vivo acute myocardial ischemia-reperfusion

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Talukder MA, Yang F, Nishijima Y, Chen CA, Xie L, Mahamud SD, Kalyanasundaram A, Bonagura JD, Periasamy M, Zweier JL. Detrimental effects of thyroid hormone analog DITPA in the mouse heart: increased mortality with in vivo acute myocardial ischemia-reperfusion. *Am J Physiol Heart Circ Physiol* 300: H702–H711, 2011. First published December 3, 2010; doi:10.1152/ajpheart.00514.2010.—There is emerging evidence that treatment with thyroid hormone (TH) can improve postischemic cardiac function. 3,5-Diiodothyropropionic acid (DITPA), a TH analog, has been proposed to be a safer therapeutic agent than TH because of its negligible effects on cardiac metabolism and heart rate. However, conflicting results have been reported for the cardiac effects of DITPA. Importantly, recent clinical trials demonstrated no symptomatic benefit in patients with DITPA despite some improved hemodynamic and metabolic parameters. To address these issues, dose-dependent effects of DITPA were investigated in mice for baseline cardiovascular effects and postischemic myocardial function and/or salvage. Mice were treated with subcutaneous DITPA at 0.937, 1.875, 3.75, or 7.5 mg·kg^{−1}·day^{−1} for 7 days, and the results were compared with untreated mice for ex vivo and/or in vivo myocardial ischemia-reperfusion (I/R). DITPA had no effects on baseline body temperature, body weight, or heart rate; however, it mildly increased blood pressure. In isolated hearts, baseline contractile function was significantly impaired in DITPA-pretreated mice; however, postischemic recovery was comparable between untreated and DITPA-treated groups. In vivo baseline cardiac parameters were significantly affected by DITPA, with increased ventricular dimensions and decreased contractile function. Importantly, DITPA-treated mice demonstrated high prevalence of fatal cardiac rhythm abnormalities during in vivo ischemia and/or reperfusion. There were no improvements in myocardial infarction and postischemic fractional shortening with DITPA. Myocardial sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA), phospholamban (PLB), and heat shock protein (HSP) levels remained unchanged with DITPA treatment. Thus DITPA administration impairs baseline cardiac parameters in mice and can be fatal during in vivo acute myocardial I/R.

cardiac function; postischemic recovery; fatal cardiac rhythm

THYROID HORMONE (TH) has profound effects on the heart and cardiovascular system. The biologically active TH, 3,5,3'-triiodothyronine (T₃), increases cardiac output through inotropic, chronotropic, and vasodilatory mechanisms (18). A TH analog, 3,5-diiodothyropropionic acid (DITPA), has been reported to have less effect on cardiac metabolism and heart rate (HR) (22), and thus it is considered to be a safer therapeutic agent than T₃. DITPA was identified as a compound that

differs chemically from thyroxine (T₄) in the absence of iodides at the 3',5' positions and in the substitution of a propionic acid side chain for the alanine side chain (34). It has been shown that DITPA exerts greater positive inotropic than chronotropic effects (32), induces angiogenesis (45), improves vasorelaxation (37), and improves calcium handling (33) in different experimental conditions. These properties of DITPA are thought to contribute to the beneficial effects of the compound in clinical application.

Circulating and cardiac T₃ levels are reduced in advanced heart disease, after acute myocardial infarction (AMI), and in patients with cardiopulmonary bypass (14, 15, 18, 24). Clinical and experimental studies have demonstrated that increased circulating levels of T₃ improved cardiac contractile function in normal myocardium as well as after acute ischemic injury to the myocardium (8, 18, 26). Recently, DITPA has been in phase II clinical trials for its efficacy as a cardiostimulant agent in stable heart failure patients; however, there was no symptomatic benefit in patients despite some improvements in hemodynamic and metabolic parameters (11). DITPA treatment was initially reported to increase baseline cardiac contractility (13, 32); however, administration of DITPA is reported to have diverse effects on postischemic/postinfarct myocardial function (for review, see Refs. 19, 22). Effects of DITPA on fractional shortening and ejection fraction have been mixed, one showing an increase (33, 45) and others no change (19). Thus it is uncertain whether and/or when a clear benefit from DITPA is achievable. It is also unknown whether the inconsistencies are related to experimental protocols or species.

TH can regulate contractile function and heart rhythm via its genomic or nongenomic actions (18, 27). In fact, most of the important regulatory contractile proteins and ion channels are TH responsive (2, 7). Sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA) is positively, while phospholamban (PLB) is negatively, regulated by TH (2). It has been reported that the effects of TH on cardiac contractility as well as rates of contraction and relaxation are mainly mediated by increases in the levels of SERCA2a and decreases in PLB in cardiomyocytes (2). DITPA has been shown to prevent downregulation of SERCA2a proteins following myocardial infarction in rabbits (33). Interestingly, short-term in vitro DITPA pretreatment has been shown to induce upregulation of genes encoding contractile proteins and SERCA2a in isolated rat cardiomyocytes (1). Recent studies have provided evidence that TH can also regulate intracellular survival signaling pathways and enhance the induction of cardioprotective molecules such as heat shock proteins (HSPs) (27). TH-induced HSP70 expression has been

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reported to increase the tolerance of the myocardium to ischemia-reperfusion (I/R) and preserve contractile function in rats (28). Mice with increased SERCA expression demonstrate protection against myocardial I/R injury (40), and conversely mice with reduced SERCA expression are susceptible to accelerated myocardial I/R injury (39). Deletion of the inducible 70-kDa HSP genes in mice is reported to impair cardiac contractile function and calcium handling (17).

While murine models are widely used and provide unique opportunity for the use of genetic modification, there are no prior reports evaluating the effects of DITPA in mice with regard to baseline cardiac function and cardioprotection. Pretreatment of a putative cardioprotective agent in animal studies is performed to know the drug effects at baseline and after specific interventions. The cardioprotective effects of T₃ or T₄ pretreatments have been reported (26, 27, 29–31). The rationale of using DITPA was to avoid the adverse sympathomimetic effects of exogenously administered TH while maintaining the beneficial molecular effects (22). The present study was thus designed to examine the dose-dependent effects of DITPA on the basal physiological and hemodynamic parameters of mice and to explore its potency in cardioprotection with preservation of postischemic myocardial function and viability.

METHODS

This study was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University and conforms with the *Guidelines for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Pub. No. 85-23, revised 1996).

Mice and DITPA treatment. Young male C57BL/6 mice (16–20 wk) were used in this study. Pretreatment with T₃ or T₄ is reported to be cardioprotective (26, 27, 29–31); however, there is no such report for DITPA pretreatment. Interestingly, 48-h DITPA or T₃ pretreatment is reported to induce upregulation of identical gene encoding contractile proteins and SERCA2a in isolated cultured rat cardiomyocytes (1). DITPA is shown to exert distinct myocardial effects on postinfarction rat hearts within 3–7 days of treatment (45). Therefore, considering these early DITPA effects (1, 45) and the well-reported dose of DITPA (3.75 mg·kg⁻¹·day⁻¹) in rabbits, rats, hamsters, and humans (19, 21, 23, 33, 34, 37), mice were initially assigned to DITPA treatment for 7 days with 3.75 mg·kg⁻¹·day⁻¹ subcutaneously for in vitro experiments. To investigate the detailed in vivo dose-dependent cardiovascular effects of DITPA at baseline and during stress conditions, four incremental doses of DITPA were included for 7-day subcutaneous treatment, 0.937, 1.875, 3.75, and 7.5 mg·kg⁻¹·day⁻¹; these doses are referred to here as DITPA-0.937, DITPA-1.875, DITPA-3.75, and DITPA-7.5, respectively. Stock solutions of DITPA (Sigma; 3.75 mg/ml) were prepared by dissolving the powder in 0.1 N NaOH and diluting with 0.9% saline. The final pH was adjusted to pH 8–9 by titration with 0.1 N HCl before injection. For TH analysis, blood samples from different sets of mice were collected on day 8, and serum TSH, total T₃ (TT₃), and free T₃ (fT₃) levels were measured by radioimmunoassay (Endocrine Diagnostic Section, Diagnostic Center for Population and Animal Health, Michigan State University, Lansing, MI).

Langendorff-perfused heart preparation. After 7-day DITPA-3.75 treatment, hearts were isolated from age-matched mice as described previously (40) in parallel with hearts from untreated mice. Briefly, mice were anesthetized with pentobarbital (50 mg/kg ip), and hearts were excised, aorta cannulated, and perfused in a Langendorff mode at a constant pressure of 80 mmHg with a modified Krebs-Henseleit buffer (KHB) equilibrated with 95% O₂-5% CO₂ at 37°C. The constituents of KHB were (in mmol/l) 120 NaCl, 5.9 KCl, 25

NaHCO₃, 1.2 MgCl₂, 2.5 CaCl₂, 0.5 EDTA, and 16.7 glucose. A fluid-filled balloon was inserted into the left ventricle (LV) across the mitral valve and connected to a pressure transducer, permitting continuous measurement of LV pressure (LVP). Hearts were immersed in a water-jacketed bath maintained at 37°C, and the LV balloon was filled with water to yield a LV diastolic pressure of 3–6 mmHg. Coronary flow was continuously monitored via a Doppler flow probe (T206, Transonic Systems, Ithaca, NY) placed in the aortic perfusion line. Aortic pressure and LVP were recorded on a PowerLab/400 multichannel data acquisition system (ADInstruments; Castle Hill, Australia). The LVP signal was digitally processed (with PowerLab Chart software version 4.2, ADInstruments) to yield diastolic and systolic pressures, LV developed pressure (LVDP), HR, rate-pressure product (RPP = LVDP × HR), and positive and negative change in pressure over time ($\pm$ dP/dt).

After 30-min equilibration, hearts underwent 30 min of global ischemia followed by 45 min of reperfusion.

Blood pressure measurements in conscious animals. Blood pressure (BP) was measured in conscious animals by the tail-cuff method (CODA-2, Kent Scientific, Torrington, CT). Briefly, each animal was acclimatized for at least three practice sessions in three consecutive days before the final BP was recorded. In each session 15 consecutive BP readings were recorded, and the average was used for systolic, diastolic, and mean BP.

Echocardiographic evaluation of LV function. In vivo cardiac dimension and contractile function were evaluated by transthoracic M-mode echocardiography under light isoflurane (1–1.5%) anesthesia as described previously (39). Briefly, with a GE Vivid7 echocardiography system and intraoperative epicardial probe (model i13L; FREQ 14 MHz), the two-dimensional short-axis view was used as a guide and LV M-mode tracings were obtained close to the papillary muscle. LV end-diastolic and end-systolic internal diameter (LVIDd and LVIDs, respectively) were measured with the American Society of Echocardiography leading-edge method (35). LV fractional shortening was calculated as FS (%) = (LVIDd – LVIDs)/LVIDd × 100. Echocardiography was performed in the same animal in the absence and/or presence of DITPA treatment, and also before and after in vivo myocardial I/R.

In vivo myocardial ischemia-reperfusion. In vivo myocardial I/R was performed as described previously (40). Briefly, mice were anesthetized with a mixture of intraperitoneal ketamine (55 mg/kg) and xylazine (15 mg/kg). After adequate anesthesia and aseptic preparations, mice were intubated and ventilated with room air by a MiniVent (type 845, Harvard Apparatus). The respiratory rate was maintained at 125–130 breaths/min with a tidal volume of 0.25 ml for a 25-g mouse. The rectal temperature of the mouse was maintained at 37°C by a thermo heating pad. After the chest was opened and the heart visualized, the left anterior descending coronary artery (LAD) was ligated 2 mm below the tip of the left auricle by a 7-0 silk ligature. Occlusion was confirmed by ST segment elevation in ECG, dramatic changes in myocardial color (red to pallor), and restricted ventricular motion. After 30-min LAD occlusion the knot was released to start reperfusion, and reperfusion was confirmed by return of the pink-red color and motion of the anterior wall of the LV. The chest was closed in layers. When mice resumed a normal breathing pattern and started walking, the ventilator was taken off and mice were transferred to a clean cage with free access to food and water.

Criteria used to determine arrhythmias during in vivo myocardial I/R. Monitoring of ECG during I/R was performed in anesthetized mice with limb lead II with needle electrodes inserted subcutaneously. ECG was recorded 5 min before (for baseline measurements) and throughout 30-min ischemia and for the first 15–20 min of reperfusion at a sampling rate of 2 KHz with PowerLab Chart 5.0 (ADInstruments). The time of 15–20 min of reperfusion was chosen because most of the mice woke up by 10–15 min of reperfusion. The acquired ECG tracings were displayed and analyzed off-line according to the Lambeth Convention guidelines for the analysis of experimental

Table 1. Effects of 7-day DITPA treatment on serum TSH and TH levels in mice

Groups	TSH, ng/ml	TT ₃ , nmol/l	fT ₃ , pmol/l
Untreated	2.48 ± 0.8	0.70 ± 0.1	14.1 ± 3.0
DITPA-0.973	2.93 ± 0.4	0.72 ± 0.1	11.8 ± 2.9
DITPA-1.85	3.16 ± 0.3	0.68 ± 0.1	16.5 ± 3.8
DITPA-3.75	2.72 ± 0.6	0.66 ± 0.2	16.9 ± 6.0
DITPA-7.5	2.72 ± 0.4	0.43 ± 0.1‡	16.4 ± 3.2

Values are means ± SD for $n = 5-14$ animals/group. TH, thyroid hormone; T₃, 3,5,3'-triiodothyronine; TT₃, total T₃; fT₃, free T₃; DITPA-0.937, DITPA-1.875, DITPA-3.75, and DITPA-7.5, 3,5-diiodothyropropionic acid (DITPA) at 0.937, 1.875, 3.75, and 7.5 mg·kg⁻¹·day⁻¹, respectively. ‡ $P < 0.001$ vs. respective untreated value.

arrhythmias (43). Premature ventricular contractions (PVCs) were identified as single premature wide QRS complexes in relation to the P wave. Ventricular tachycardia was defined as a run of three or more PVCs. Ventricular fibrillation was defined as a signal that changed beat to beat in rate and configuration or where individual QRS deflections could not easily be distinguished from one another. Atrio-ventricular (A-V) block was classically defined as a conduction defect where transfer of depolarizing signal from atria to ventricles is either partially or completely blocked. The incidences of different arrhythmias were observed for the defined period of ECG recordings during reperfusion.

Myocardial infarct size measurement. Myocardial infarction was measured at 24 h after reperfusion as previously described with slight modification (40). Mice were anesthetized, intubated, and ventilated, and the chest was opened along the previous incision line. The hearts were rapidly excised, flushed with heparinized PBS via aorta, and then infused/stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) for 5 min for the demarcation of viable and nonviable myocardium within the area at risk (AAR). With coronary reocclusion at the previous site, they were infused with 10% phthalo blue to visualize the nonrisk (nonischemic) region. The heart was frozen, serially sectioned (1 mm thick) by a heart slicer, and fixed in 10% formalin. Both sides of each myocardial slice were photographed, and AAR and area of infarction were determined by computerized planimetry with image analysis software Meta Vue, version 6.0. Infarct size was calculated as a percentage of the LV AAR.

Western blot analysis. Briefly, hearts from age-matched mice were homogenized in the lysis buffer, and protein concentrations were determined by Bio-Rad protein assay. The relative expressions of cardiac SERCA2a and PLB in cardiac homogenates were determined by immunoblotting as described previously (39, 40). Briefly, protein extracts (10 µg for SERCA2a and PLB; 40 µg for HSP70) were separated by SDS-PAGE on polyacrylamide gel and then transferred to nitrocellulose membrane with a Bio-Rad transblot apparatus. Membranes were incubated with specific antibody for SERCA2a, PLB, and HSP70 at room temperature for 1 h. The membranes were washed with TBS-T (20 mmol/l Tris-HCl, 137 mmol/l NaCl, and 0.05% Tween 20) six times (10 min each) and incubated with peroxidase-labeled secondary antibody. After extensive washing with TBS-T, antibody signals were detected with an enhanced chemiluminescence kit (Pierce).

Data analysis. All results are expressed as means ± SE. Data were analyzed either by two-tailed Student's *t*-test for paired data from the same experiment and unpaired data from different experiments or by ANOVA followed by Fisher's post hoc test. Kaplan-Meier survival analysis for in vivo myocardial I/R was performed with GraphPad Prism 4 (San Diego, CA) and log-rank test. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

Effects of DITPA on TH levels. To determine the effects of DITPA pretreatment on circulating TSH and TH levels in mice, whole serum from DITPA-pretreated mice was analyzed for TSH, TT₃, and fT₃ levels. As summarized in Table 1, compared with the untreated group there is no significant change in circulating TSH levels with any of the doses of DITPA, but DITPA at the highest dose (DITPA-7.5) significantly decreased TT₃ level without any significant effect on fT₃ level.

DITPA on cardiac function before and during ex vivo global I/R. To determine the effects of DITPA-3.75 on myocardial function, isolated hearts from both untreated and DITPA-treated mice were investigated for ex vivo baseline and post-ischemic cardiac parameters. For the ex vivo experiments, we looked for the effects of a widely used DITPA dose, 3.75 mg·kg⁻¹·day⁻¹ subcutaneously for 7 days. Table 2 summarizes the baseline functional parameters for untreated and DITPA-treated isolated hearts determined at the end of the 30-min equilibration period. There was no difference in heart weight-to-body weight ratio between untreated and DITPA-treated mice. Baseline coronary flow (normalized to the wet weight of each heart) was not significantly different between untreated and treated groups; however, DITPA-3.75 significantly impaired baseline cardiac contractile parameters in isolated hearts compared with untreated control hearts.

Figure 1 shows the time course of recovery for coronary flow (Fig. 1A), LV end-diastolic pressure (LVEDP; Fig. 1B), HR (Fig. 1C), and LVDP (Fig. 1D) in untreated and DITPA-treated hearts subjected to 30-min global ischemia and 45-min reperfusion. Upon reperfusion, coronary flow rate was comparable between untreated and DITPA-3.75 groups (Fig. 1A); however, coronary flow recovery in DITPA-treated hearts at 45 min of reperfusion was significantly higher from their respective preischemic (PI) level (13 ± 2.84 vs. 10 ± 1.63 ml·min⁻¹·g⁻¹ at PI; $P < 0.05$, $n = 8$ /group). Coronary flow remained unchanged in untreated hearts (11.8 ± 2.25 vs. 11.7 ± 1.48 ml·min⁻¹·g⁻¹ at PI; $n = 8$ /group). While a similar rise in LVEDP was seen in both groups during ischemia, upon reperfusion LVEDP increased and remained elevated above the ischemic level in both untreated and DITPA-treated hearts (Fig. 1B). Upon reperfusion, HR recovered totally in both groups (Fig. 1C); however, LVDP reduced significantly to a similar extent in both untreated and DITPA-treated hearts (Fig. 1D). Since the absolute baseline HR and LVDP were different

Table 2. Effects of DITPA-3.75 pretreatment on heart weight-to-body weight ratio and baseline hemodynamic parameters of isolated hearts

Parameters	Untreated (n)	DITPA Treated (n)
HW/BW, g%	0.50 ± 0.06 (8)	0.53 ± 0.02 (8)
Coronary flow, ml·min ⁻¹ ·g ⁻¹	11.6 ± 1.48 (8)	10 ± 1.6 (8)
HR, bpm	327 ± 28 (8)	286 ± 19 (8)†
LVDP, mmHg	106 ± 19 (8)	84 ± 10 (8)*
RPP, 10 ⁻³ mmHg/min	34 ± 5 (8)	24 ± 2.6 (8)‡
+dP/dt, mmHg/s	$3,181 \pm 576$ (8)	$2,153 \pm 457$ (8)†
-dP/dt, mmHg/s	$2,233 \pm 302$ (8)	$1,493 \pm 224$ (8)‡

Values are means ± SD for n animals. HW/BW, heart weight-to-body weight ratio; HR, heart rate; bpm, beats per minute; LVDP, left ventricular developed pressure; RPP, rate-pressure product; dP/dt, change in pressure over time. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ vs. untreated group.

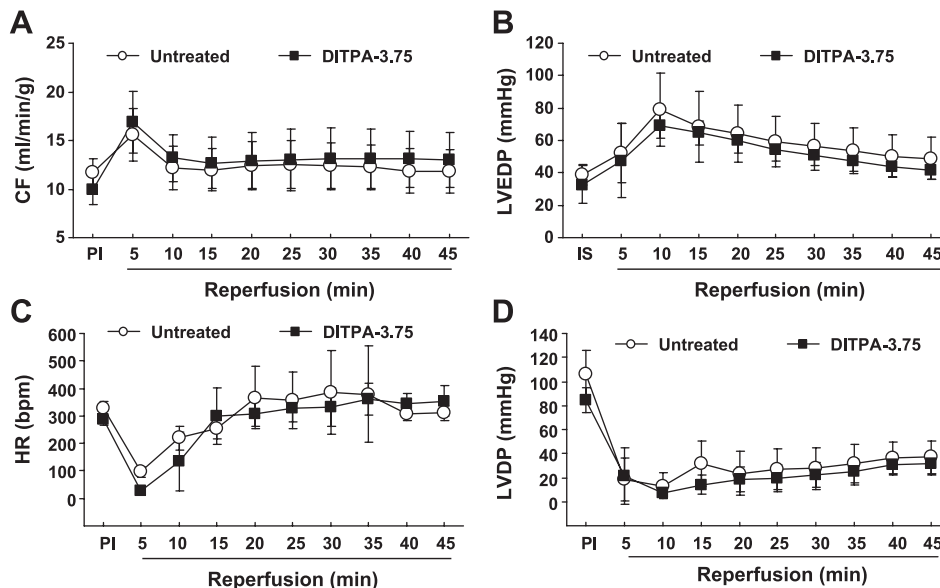


Fig. 1. Time courses for the postischemic recovery of coronary flow (CF; A), myocardial relaxation [left ventricular end-diastolic pressure (LVEDP); B], heart rate (HR; C), and myocardial contraction [left ventricular developed pressure (LVDP); D] in untreated and 3,5-diiodothyropropionic acid (DITPA)-treated [DITPA 3.75 mg·kg⁻¹·day⁻¹ (DITPA-3.75)] isolated hearts. CF is expressed as ml·min⁻¹·g heart wt⁻¹. HR is expressed as beats per minute (bpm). IS, ischemia; PI, preischemia. Values are means ± SD; n = 8/group.

between the two groups (Table 2), postischemic data were normalized to their respective PI values (100%). We observed that the time course curves were comparable between the two groups (data not illustrated). There was no difference in LVDP at 45 min of reperfusion between the two groups ($35 \pm 10.3\%$ of PI in untreated group vs. $34 \pm 7.5\%$ of PI in DITPA group; $n = 8/\text{group}$). HR in untreated hearts at 45 min of reperfusion remained unchanged compared with their PI level ($96 \pm 10\%$ of PI; $n = 8/\text{group}$); however, HR at 45 min of reperfusion in DITPA-treated hearts was significantly higher compared with their PI level ($124 \pm 22\%$ of PI; $P < 0.05$, $n = 8/\text{group}$) and also with the untreated group at 45 min of reperfusion ($124 \pm 22\%$ vs. $96 \pm 10\%$ of PI; $P < 0.05$, $n = 8/\text{group}$).

Effects of DITPA on general physiology and baseline in vivo cardiac parameters. To investigate in vivo dose-dependent effects of DITPA, we randomly assigned the animals for four different doses. Table 3 summarizes the dose-dependent effects

of DITPA treatment on general physiology and BP in age-matched animals. There was no effect of DITPA on body temperature and body weight; however, BP was mildly increased with high DITPA doses. Table 4 summarizes the effects of DITPA treatment on echocardiographic parameters of anesthetized mice. DITPA did not affect intrinsic HR; however, it caused marked abnormalities in cardiac parameters with higher doses. LVID of the hearts at both diastole and systole were significantly increased after 7-day DITPA treatment. Importantly, global contractile function of the heart determined as FS (%) was not affected by DITPA-0.937 (Table 4), but it was significantly reduced to $83 \pm 5\%$, $73 \pm 13\%$, and $63 \pm 13\%$ of respective baseline value (100%) by DITPA at 1.85, 3.75, and 7.5 mg·kg⁻¹·day⁻¹, respectively.

Effects of DITPA on myocardial infarction and function after in vivo regional I/R. To determine the effects of DITPA on postischemic myocardial salvage, dose-dependent effects of DITPA on myocardial infarction and postischemic cardiac function were evaluated in a clinically relevant model of in vivo myocardial I/R. First, in untreated mice, FS (%) 3 days before LAD ligation was taken as the preischemic (pre-MI) value, and it was compared with the subsequent postischemic (post-MI) value obtained 24 h after reperfusion. Second, in treatment groups, FS (%) after 7-day DITPA treatment was taken as the preischemic (pre-MI or DITPA) value, and it was compared with the subsequent postischemic (post-MI) value obtained 24 h after reperfusion. DITPA pretreatment at any dose had no effect on the myocardial AAR or infarct size compared with the untreated group (Fig. 2A). Table 5 summarizes the post-MI cardiac parameters compared with respective pre-MI levels. In the untreated group, post-MI FS was significantly reduced from the pre-MI level. Post-MI FS was also significantly reduced from the pre-MI level with all doses of DITPA. Since the baseline FS in untreated ($53.5 \pm 4.3\%$) and treated (Table 4, before DITPA) mice as well as the myocardial infarct size (Fig. 2A) were comparable between the groups, post-MI contractile function in untreated and treated mice was therefore compared to determine the influence of intrinsic direct effect of DITPA. Table 5 shows that DITPA treatment

Table 3. Body temperature, body weight, and blood pressure in conscious mice before (baseline) and after 7-day DITPA treatment

Groups	Before (n)	After (n)
DITPA-0.937		
Body temperature, °C	36.9 ± 0.63 (7)	36.6 ± 0.60 (7)
Body weight, g	29.3 ± 2.14 (7)	28.3 ± 2.14 (7)
Mean BP, mmHg	112 ± 5.21 (6)	111 ± 6.75 (6)
DITPA-1.875		
Body temperature, °C	36.7 ± 0.72 (10)	36.5 ± 0.81 (10)
Body weight, g	29.7 ± 1.49 (10)	29.9 ± 1.52 (10)
Mean BP, mmHg	106 ± 5.52 (6)	110 ± 4.59 (6)
DITPA-3.75		
Body temperature, °C	36.5 ± 0.53 (12)	36.6 ± 0.57 (12)
Body weight, g	30.9 ± 1.72 (12)	30.5 ± 1.93 (12)
Mean BP, mmHg	103 ± 9.01 (9)	112 ± 4.98 (9)*
DITPA-7.5		
Body temperature, °C	36.9 ± 0.51 (10)	36.8 ± 0.66 (10)
Body weight, g	28.2 ± 2.49 (10)	28.1 ± 2.53 (10)
Mean BP, mmHg	100 ± 5.14 (7)	110 ± 7.59 (7)†

Values are means ± SD for n animals. BP, blood pressure. * $P < 0.05$, † $P < 0.01$ vs. respective baseline (before) value.

Table 4. Echocardiographic parameters in anesthetized mice before (baseline) and 7 days after DITPA treatment

Parameters	DITPA-0.937		DITPA-1.875		DITPA-3.75		DITPA-7.5	
	Before	After	Before	After	Before	After	Before	After
HR, bpm	524 ± 57	552 ± 59	586 ± 78	553 ± 53	552 ± 38	577 ± 40	562 ± 55	563 ± 28
LVIDd, cm	0.35 ± 0.02	0.35 ± 0.03	0.33 ± 0.05	0.36 ± 0.02	0.33 ± 0.05	0.38 ± 0.01*	0.31 ± 0.03	0.36 ± 0.04†
LVIDs, cm	0.18 ± 0.02	0.18 ± 0.02	0.16 ± 0.03	0.20 ± 0.01*	0.15 ± 0.03	0.23 ± 0.03‡	0.15 ± 0.02	0.24 ± 0.04‡
LVPWd, cm	0.08 ± 0.02	0.08 ± 0.01	0.10 ± 0.02	0.08 ± 0.01	0.11 ± 0.02	0.09 ± 0.01	0.11 ± 0.01	0.08 ± 0.01‡
LVPWs, cm	0.14 ± 0.01	0.14 ± 0.01	0.17 ± 0.01	0.13 ± 0.02†	0.17 ± 0.02	0.14 ± 0.02	0.17 ± 0.01	0.12 ± 0.02‡
FS, %	50 ± 1.9	48 ± 1.6	51 ± 5.7	43 ± 2.2*	55 ± 4.5	40 ± 6.6†	53 ± 3.7	33 ± 7.1‡

Values are means ± SD for $n = 7$ –10 animals/group. LVIDd, left ventricular internal diameter at diastole; LVIDs, left ventricular internal diameter at systole; LVPWd, left ventricular posterior wall thickness at diastole; LVPWs, left ventricular posterior wall thickness at systole; FS, fractional shortening. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ vs. respective baseline (before) value.

did not improve, but rather further deteriorated, post-MI FS in mice treated with high doses.

Effects of DITPA on survival and incidences of arrhythmias during in vivo regional I/R. After induction of acute in vivo myocardial I/R, we did not observe any death in the untreated (0/10) or DITPA-1.875 (0/10) groups; however, posts ischemic mortality was significantly higher in DITPA-3.75 (6/20) and DITPA-7.5 (4/10) groups. The Kaplan-Meier survival rate during in vivo I/R was 70% and 60% with DITPA-3.75 and DITPA-7.5, respectively. While most of these mice died during early reperfusion (Fig. 2B), one mouse in the DITPA-7.5 group died during ischemia. The rhythm abnormalities in these mice seen on the ECG recordings were mostly conduction defects (A-V blocks). Figure 3A shows the representative ECG recordings at baseline, during LAD ligation, and at reperfusion in an untreated mouse under anesthesia. Typically, ST segment ele-

vation develops within minutes of LAD ligation and ST elevation begins to disappear within 15 min of reperfusion. Rhythm disturbances were not observed in any untreated (Fig. 3A) and DITPA-1.875 (Fig. 3B) mice; however, high-DITPA groups displayed abnormal cardiac rhythms during ischemia and/or reperfusion. Figure 3C shows ECG tracings for a DITPA-3.75 mouse that developed heart block during early reperfusion and died within 30 min of reperfusion. Figure 3D shows ECG tracings for a DITPA-7.5 mouse that developed conduction defects during early ischemia and died within 15 min of LAD ligation because of heart block. Figure 4 shows ECG tracings during I/R where a DITPA-7.5 mouse developed agonal ventricular rhythm during ischemia and died with severe heart block during early reperfusion. Of note, all of these DITPA-treated mice died with terminal heart blocks.

Effects of DITPA on cardiac proteins. To determine whether the observed cardiac effects with DITPA treatment were associated with alterations in the cardiac proteins, quantitative immunoblotting was performed for Ca^{2+} handling proteins SERCA2a and PLB, and HSP70. Figure 5A shows the expression levels of cardiac SERCA2a and PLB in untreated and DITPA-treated mice. Seven-day DITPA treatment had no significant effect on cardiac SERCA2a or PLB proteins (Fig. 5A, bar graphs). Figure 5B shows the expression levels of cardiac HSP70 in untreated and DITPA-treated mice. Seven-day DITPA treatment had no significant effect on cardiac HSP70 (Fig. 5B, bar graphs).

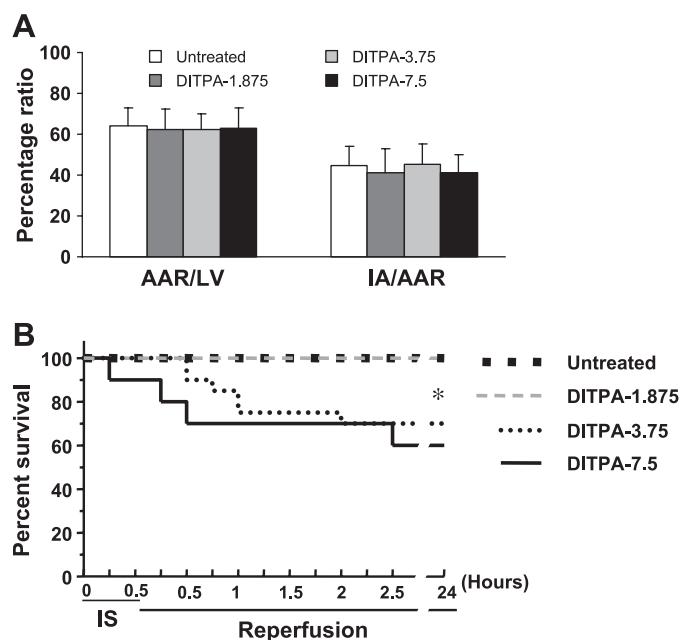


Fig. 2. Myocardial infarction and survival following 30-min left anterior descending coronary artery (LAD) ligation and 24-h reperfusion with or without DITPA treatment. A: % of area at risk (AAR) over left ventricle (LV) and infarct area (IA) over AAR are shown for different groups. Values are means ± SD; $n = 6$ –14/group. DITPA-1.875, DITPA-3.75, DITPA-7.5, 1.875, 3.75, and 7.5 $mg \cdot kg^{-1} \cdot day^{-1}$ DITPA, respectively. B: Kaplan-Meier survival curve analysis of 4 different groups showed significant differences in survival during in vivo myocardial ischemia and reperfusion (* $P < 0.05$). $n = 10$ –20/group.

DISCUSSION

The major goal of this study was to test the effects of TH analog DITPA pretreatments on cardiac function of mice under physiological and pathological conditions. We observed that DITPA pretreatments in mice resulted in 1) mildly elevated BP and impaired cardiac parameters under normal physiological conditions; 2) no improvements in myocardial infarction and post-MI cardiac function; 3) abnormal cardiac rhythms during in vivo ischemia and/or reperfusion; and 4) higher mortality during in vivo I/R. Importantly, levels of myocardial calcium handling proteins SERCA2a and PLB, and HSP70 remained unchanged after DITPA treatment. To our knowledge, this is the first study that demonstrates the potential cardiac effects of DITPA in a mouse model before and after ex vivo and in vivo I/R.

Animal studies with DITPA have shown that DITPA treatment can cause an increase (13, 21, 32, 33), a decrease (37), or no effect (19) on cardiac contractility and dP/dt. We observed that DITPA at most doses significantly impaired the cardiac

Table 5. Echocardiographic parameters in anesthetized mice before and 24 h after *in vivo* I/R

Parameters	Untreated		DITPA-1.875		DITPA-3.75		DITPA-7.5	
	Pre-MI	Post-MI	Pre-MI	Post-MI	Pre-MI	Post-MI	Pre-MI	Post-MI
HR, bpm	578 ± 53	649 ± 46	553 ± 53	563 ± 33	577 ± 40	555 ± 64	562 ± 29	562 ± 73
LVIDd, cm	0.35 ± 0.04	0.38 ± 0.04	0.36 ± 0.02	0.38 ± 0.02	0.38 ± 0.01	0.38 ± 0.02	0.36 ± 0.04	0.38 ± 0.04
LVIDs, cm	0.17 ± 0.03	0.24 ± 0.03*	0.20 ± 0.01	0.23 ± 0.02*	0.23 ± 0.03	0.26 ± 0.05*	0.24 ± 0.04	0.25 ± 0.04
LVPWd, cm	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01
LVPWs, cm	0.18 ± 0.02	0.13 ± 0.01†	0.13 ± 0.02	0.12 ± 0.01	0.14 ± 0.02	0.13 ± 0.02	0.12 ± 0.02	0.11 ± 0.01
FS, %	54 ± 4.3	36 ± 4.6‡	43 ± 2.2	39 ± 3.3*	40 ± 6.6	32 ± 8.1†	33 ± 7.1	30 ± 4.2§

Values are means ± SD for $n = 6$ or 7 animals/group before (pre-MI) or 24 h after (post-MI) ischemia-reperfusion (I/R). This data set represents those mice that survived 24-h reperfusion. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ vs. respective pre-MI value; § $P < 0.05$ vs. untreated post-MI value.

parameters, with increased *in vivo* LVID at both systole and diastole and decreased FS from the respective baseline value (Table 4). With higher doses, DITPA mildly increased BP (Table 3). Baseline contractile parameters were also impaired in *ex vivo* isolated hearts from DITPA-treated mice (Table 2). All of these cardiac effects of DITPA were independent of any

change in myocardial protein expressions (Fig. 5). DITPA treatment did not affect body weight, temperature, or HR in mice (Tables 3 and 4), and these findings are consistent with the report in rats where DITPA treatment did not alter body weight, heart weight, or HR (37). DITPA also did not increase body temperature in thyroidectomized rats compared with a

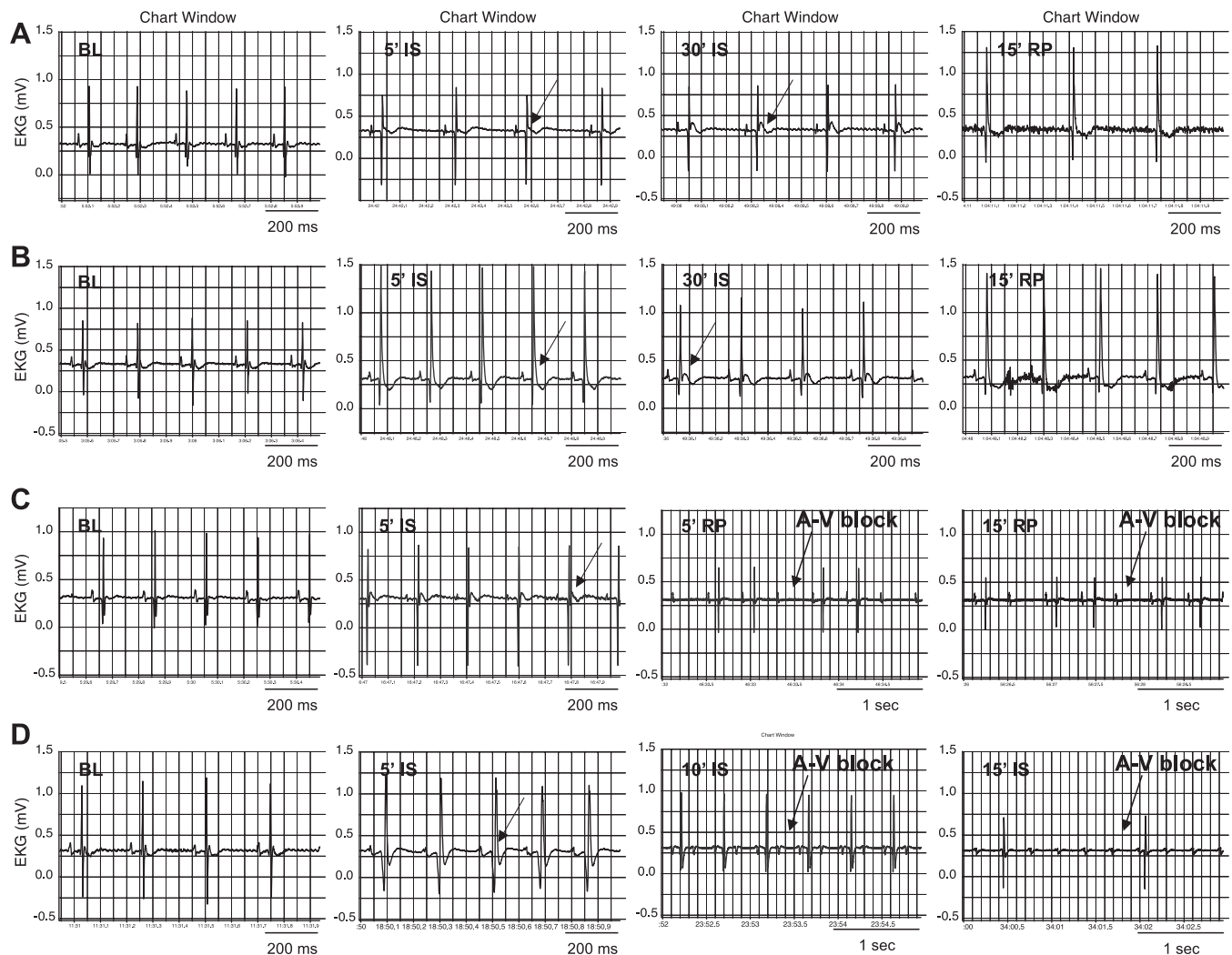


Fig. 3. Representative ECG tracings obtained before (baseline) and during *in vivo* myocardial ischemia (IS) and reperfusion (RP). A: typical ECG tracings in an untreated mouse during baseline (BL), IS with developing ST elevation (arrow), and RP with disappearing ST elevation. B: ECG tracings of a DITPA-1.875 mouse during BL, IS with developing ST elevation (arrow), and RP with disappearing ST elevation. C: ECG tracings of a DITPA-3.75 mouse showing IS with developing ST elevation (arrow) and low-voltage signals with fatal atrioventricular (A-V) blocks from 5 min of RP. D: ECG tracings of a DITPA-7.5 mouse showing ST elevation (arrow) in early IS followed by low-voltage signals and fatal A-V blocks from 10 min of IS.

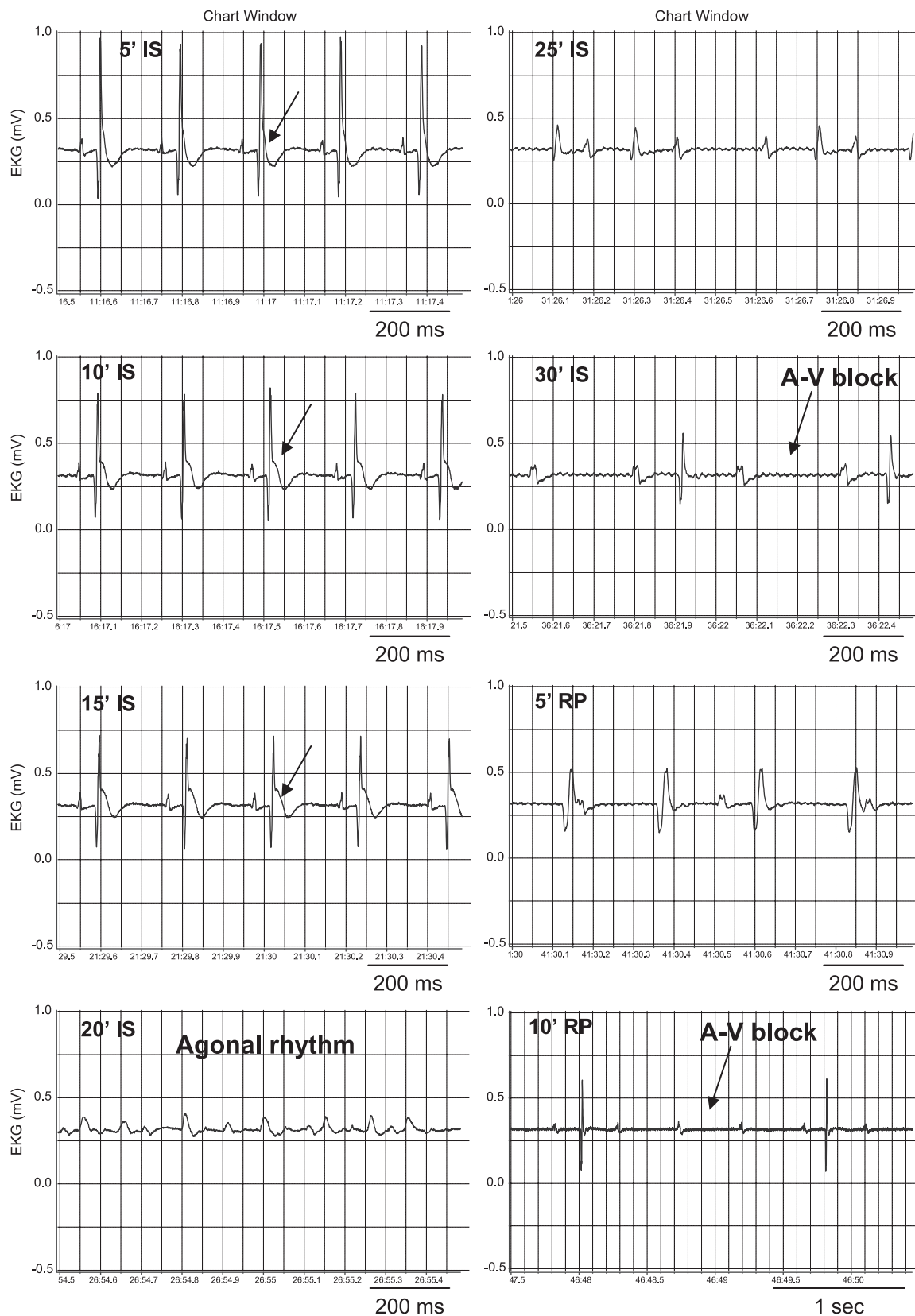


Fig. 4. Representative ECG tracings obtained from a DITPA-7.5 mouse during in vivo myocardial ischemia (IS) and reperfusion (RP). Immediately after LAD ligation, ST segment elevation developed within 5 min of IS. At 20 min of IS agonal ventricular rhythm appeared spontaneously, and it progressed to partial A-V block with low-voltage signals at 30 min of IS and finally complete A-V block developed at 10 min of RP.

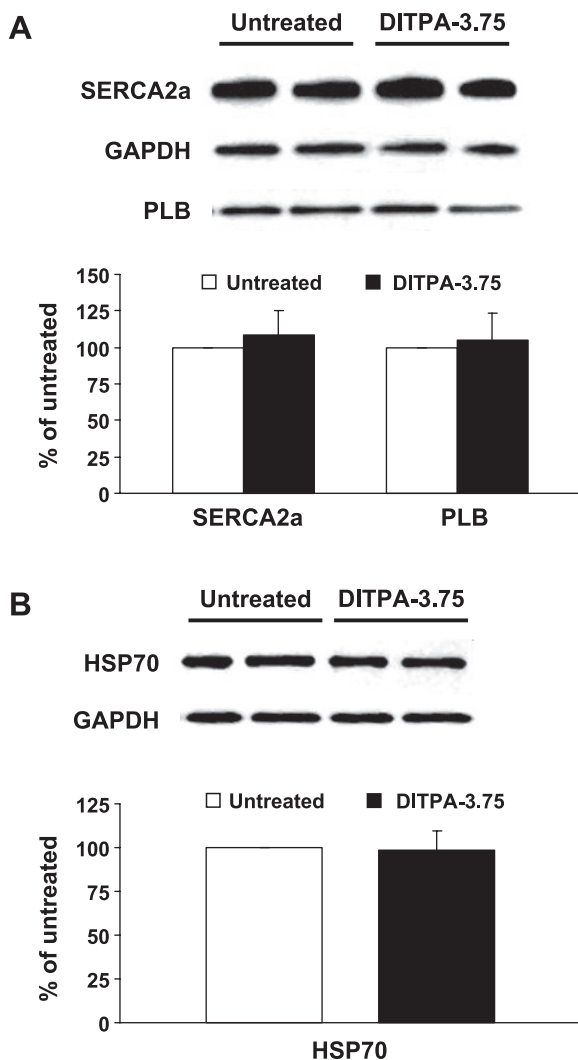


Fig. 5. Immunoblots for sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA)2a, phospholamban (PLB), and heat shock protein (HSP)70 levels in cardiac homogenates of untreated (control) and DITPA-treated mice. A: representative blots for cardiac SERCA2a, GAPDH, and PLB proteins (top) and their relative mean densitometric value (bottom). B: representative blots for cardiac HSP70 (top) and their relative mean densitometric value (bottom). y-Axis: changes in optical density expressed as % change from normalized value where untreated hearts were assigned 100%. GAPDH served as internal control for respective blot. Values are means $\pm$ SD; $n = 4$ /group.

placebo group (36). Importantly, the effects of DITPA on TH levels have been variable in different species. DITPA treatment has been shown to increase serum fT_3 level in cardiomyopathic hamsters without any effect on serum TSH level (19). In thyroidectomized rats, DITPA treatment increased serum TT_3 levels compared with placebo; however, TT_4 levels remained unchanged (36). Importantly, in clinical trials DITPA decreased serum TSH, TT_4 , or TT_3 levels in heart failure patients (20, 23). In our study, DITPA only at the maximum dose studied (DITPA-7.5) significantly decreased serum TT_3 levels without any significant changes in TSH and fT_3 levels (Table 1).

Several studies using various experimental models have shown that either acute or chronic pretreatment with T_3 before I/R improves postischemic recovery of cardiac function (for review, see Refs. 26, 27). These observations were based on the facts that plasma levels of T_3 are low during AMI, during

cardiac arrest, and immediately after coronary artery bypass graft (14, 15, 18, 24). Administration of DITPA before or after I/R is reported to have diverse effects on the postischemic/post-MI myocardial function (for review, see Refs. 19, 22). An earlier report in a rat heart failure model showed that DITPA alone did not alter hemodynamics but the combination of DITPA and captopril improved cardiac output and increased $-\text{dP}/\text{dt}$ (34). In post-MI rats, long-term DITPA treatment did not improve, but rather significantly decreased, LV dP/dt (37). In contrast, improved contractile function by DITPA alone was reported in post-MI heart failure of rabbits (33) and in post-MI rats (45). In cardiomyopathic hamsters (19), DITPA treatment did not improve $\pm \text{dP}/\text{dt}$ or ejection fraction. Importantly, while an early clinical trial reported that DITPA had no effect on systolic cardiac function but improved diastolic function in heart failure patients (23), recent clinical trials reported that the drug was poorly tolerated and the composite heart failure end point was not improved (11, 20). We observed that DITPA administration significantly and dose-dependently decreased baseline FS under normal physiological conditions (Table 4); and post-MI FS was significantly smaller in the high-DITPA group compared with untreated mice (Table 5). Interestingly, despite comparable ex vivo baseline coronary flow between untreated and DITPA-3.75 mice (Table 2), coronary flow at 45 min of reperfusion was significantly higher from their respective PI level in DITPA-treated hearts but not in untreated hearts (Fig. 1A). Of note, long-term DITPA treatment has been shown to increase in vivo myocardial blood flow in cardiomyopathic hamsters both at baseline and after maximal dilation compared with placebo, without any effect on LV function (19). Increased angiogenesis or reduced loss of arterioles has been implicated in this improved coronary flow (19). Taken together, these findings indicate that the pharmacological effects of DITPA in the heart are diverse in different species at baseline and/or under disease conditions.

TH and its analogs critically regulate cardiac performance by direct and indirect action on myocytes and by genomic and nongenomic mechanisms (3, 5). Nongenomic actions of TH include those on membrane ion channels and pumps (5, 27). The genomic action of TH is mediated through chromatin-associated nuclear TH receptors (TRs) (7, 12). It has been reported that both genomic and nongenomic actions of TH could interface at SERCA2a, where gene expression is genomic and enzyme activity is nongenomic (3, 5). Carr and Kranias (2) suggested that TH directly regulates sarcoplasmic reticulum (SR) Ca^{2+} handling proteins; thus it controls intracellular Ca^{2+} homeostasis and cardiac contraction and relaxation. However, decreased $-\text{dP}/\text{dt}$ in control rats with long-term T_3 treatment has been reported because of augmented SERCA2a enzyme activity (38). Our echocardiographic data demonstrated that DITPA treatment resulted in deleterious effects on the mouse heart under normal physiological conditions by increasing LVIDs and by decreasing global contractile function, and that these effects were evident without any alterations in cardiac SERCA2a and PLB levels. Recently, using proof-of-principle mouse models, we have clearly demonstrated that optimal SERCA function is indispensable for improving postischemic Ca^{2+} overload, myocardial contracture, and ventricular relaxation in isolated hearts, and also for reducing myocardial infarction following in vivo myocardial I/R (39–41). We observed that DITPA pretreatment did not improve postischemic ventricular relaxation (Fig. 1B) in ex vivo hearts, nor did it reduce

myocardial infarction or improve postischemic contractile function in vivo (Fig. 2A; Table 5). Thus the potential involvement of SR Ca^{2+} handling proteins is uncertain in our experiments with short-term DITPA treatment. TH has been reported to upregulate prosurvival signaling pathways by increased expression of cardiac HSP70, and this has been related to the improved postischemic contractile function in isolated rat hearts (28). However, we did not observe any change in the levels of myocardial HSP70 with DITPA or any improvements in the postischemic myocardial function in isolated hearts.

An important and perhaps clinically critical finding of this study is that despite comparable in vivo myocardial infarction, there was a prevalence of fatal heart blocks during I/R in DITPA-treated mice. ECG recordings (Figs. 3 and 4) demonstrated that myocardial conduction defects during in vivo I/R were the terminal cause of mortality. Although the exact mechanisms of action by which high DITPA evokes heart blocks cannot be assessed in our study, it is clearly evident that the high mortality rate during in vivo myocardial I/R is related to DITPA-induced defect in electrical impulse generation and conduction. With ex vivo I/R, HR at 45 min of reperfusion in DITPA-treated mice was significantly higher compared with their respective PI level as well as compared with untreated hearts. In dilated cardiomyopathic hamsters, long-term DITPA treatment is reported to increase HR compared with placebo (19). Importantly, a recent clinical trial with DITPA in heart failure patients revealed mixed primary outcome, with serious extracardiac deleterious effects and increased mortality (11). In that trial, DITPA increased HR (≥ 10 beats/min) in more patients than with placebo (61% vs. 41%) (11). Of note, DITPA treatment resulted in increased arrhythmias in heart failure patients compared with a placebo group (9% vs. 7%), and there was 4% death in the DITPA group compared with none in the placebo group (11). The composite outcome in that trial was unchanged in 48% patients, while 19% had improved and 33% had worsened outcomes. It is well known that TH exerts marked influences on cardiovascular hemodynamics and function, including effects on cardiac impulse generation and conduction (16) and on arterial BP (18). Elevation of TH concentration is usually accompanied by an increase in cardiac output mediated by the reduction of systemic vascular resistance and chronotropic and inotropic cardiac effects (18). Overt hypothyroidism is associated with diastolic hypertension (4, 9); however, the effects of overt hyperthyroidism on BP are variable (4). We observed that the highest dose of DITPA significantly lowered TT_3 levels (Table 1), and it was concurrent with impaired cardiac function and increased mortality during myocardial I/R. These unexpected and altered in vivo cardiac and hemodynamic parameters with DITPA could be of serious concern for its potential therapeutic safety and efficacy. The overall concept of myocardial protection from DITPA administration and also for amelioration of subsequent heart failure is that DITPA treatment will induce molecular changes in calcium regulation, such as increases in SERCA expression, and metabolism that can be protective and that these protective molecular changes occur without the sympathomimetic chronotropic effects of TH. Unfortunately, at least in the present model, this was not evident, and there was no cardioprotection, with adverse rather than favorable effects seen. This is a major concern in view of the ongoing clinical evaluation of this drug in patients with heart failure (11, 20). The reasons for these

diverse effects of DITPA are unknown in different species; various aspects of the signal transduction process including different cardiac contractile proteins, ion channels, TRs, second messengers, and subsequent steps that lead to functional modulation may be involved (2, 3, 5, 7, 27).

DITPA has been shown to bind with almost the same affinity to both TR- α and TR- β receptors, but the affinity is 100-fold less than the affinity of these receptors for T_3 (32). The action of TH in the heart is mediated predominantly by TR- $\alpha 1$, and TR- $\beta 1$ is expressed at low levels (10, 25). TR- $\alpha 1$ exerts a predominant effect on cardiac impulse generation and mechanical functions, and mice deficient in TR- $\alpha 1$ are reported to have lower baseline HR and prolonged QRS complex than control mice (44). Importantly, DITPA has been shown to reduce T_3 uptake in rat cardiomyocytes in a time-dependent manner ($>50\%$ in 4 h) and to interfere with plasma membrane transport of T_3 (42). However, it is unknown to what extent the inhibitory effect of DITPA on endogenous T_3 uptake and/or plasma transport in the cardiomyocytes results in receptor modulation and thus attenuation or stimulation of overall cardiac function at baseline and/or under disease conditions. A recent review by Davis et al. (6) summarized that the molecular basis for the nongenomic actions of TH analogs is linked to the plasma membrane integrin receptor. While the molecular basis of TH action in the heart continues to be explored, future studies in mouse models with deletion of specific TR isoforms would be required to address the observed multidimensional cardiovascular effects of DITPA.

In the present study, we first demonstrate that DITPA treatment induces intrinsic deleterious cardiovascular effects in mice under normal physiological conditions. We also demonstrate that DITPA treatment has a narrow safety margin, with increased risk of death during acute myocardial I/R. DITPA has also been shown to have a narrow therapeutic window in recent clinical trials (11, 20). Since the cardiac liabilities of a new drug are serious therapeutic concerns in clinical use, caution must be taken in future DITPA therapy in patients with or at risk for myocardial ischemia.

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DISCLOSURES

There are no conflicts of interests or other disclosures.

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