Changes in the Stoichiometry of Uniplex Decrease Mitochondrial Calcium Overload and Contribute to Tolerance of Cardiac Ischemia/Reperfusion Injury in Hypothyroidism

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Background: Thyroid hormone status in hypothyroidism (HT) downregulates key elements in Ca^{2+} handling within the heart, reducing contractility, impairing the basal energetic balance, and increasing the risk of cardiovascular disease. Mitochondrial Ca^{2+} transport is reduced in HT, and tolerance to reperfusion damage has been documented, but the precise mechanism is not well understood. Therefore, we aimed to determine the stoichiometry and activity of the mitochondrial Ca^{2+} uniporter or uniplex in an HT model and the relevance to the opening of the mitochondrial permeability transition pores (mPTP) during ischemia/reperfusion (I/R) injury.
Methods: An HT model was established in Wistar rats by treatment with 6-propylthiouracil for 28 days. Uniplex composition and activity were determined in cardiac mitochondria. Hearts were perfused *ex vivo* to induce I/R injury, and functional parameters related to contractility and tissue viability were evaluated.
Results: The cardiac stoichiometry between two subunits of the uniplex (MICU1/MCU) increased by 25% in animals with HT. The intramitochondrial Ca^{2+} content was reduced by 40% and was less prone to the mPTP opening. After I/R injury, ischemic contracture and the onset of ventricular fibrillation were delayed in animals with HT, concomitant with a reduction in oxidative damage and mitochondrial dysfunction.
Conclusions: Our results suggest that HT is associated with an increase in the cardiac MICU1/MCU ratio, thereby changing the stoichiometry between these subunits to increase the threshold to cytosolic Ca^{2+} and reduce mitochondrial Ca^{2+} overload. Our results also demonstrate that this HT model can be used to explore the role of mitochondrial Ca^{2+} transport in cardiac diseases due to its induced tolerance to cardiac damage.

Keywords: mitochondria, uniporter, hypothyroidism, MICU1, calcium

Introduction

HYPOTHYROIDISM (HT) INCREASES the risk for several cardiovascular pathologies, such as atherosclerosis, coronary disease, and the incidence of mortality due to heart failure (1,2). The cardiac muscle is one of the most thyroid hormone-responsive tissues (3,4), where key proteins controlling intracellular Ca^{2+} within the myocyte are regulated, such as the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), phospholamban (PLN), and the mitochondrial Ca^{2+} uniporter (MCU) are highly regulated (5,6). These alterations diminish the intra-sarcoplasmic Ca^{2+} content, impairing cell shortening (7,8). Conversely, the tolerance to oxidative damage and Ca^{2+} overload induced by ischemia/reperfusion (I/R) injury increases in HT, which preserves cardiac function (4,9,10). An increased resistance to the opening of mitochondrial permeability transition pores (mPTP) seems to be part of such protection (11,12), but the

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precise mechanism underlying this cardioprotective phenomenon is not completely understood. High mitochondrial Ca^{2+} is a prerequisite for mPTP opening (13). Mitochondrial Ca^{2+} uptake occurs through a highly selective Ca^{2+} channel known as the MCU complex or uniplex, located on the inner mitochondrial membrane (14,15). Experimental HT models are described as a reduction of Ca^{2+} transport in liver mitochondria (6,16) and suggest decreased uniplex expression (16). The uniplex comprises diverse subunits, in particular MICU1 and MICU2 (Ca^{2+} sensitivity regulators) (17). MICU1 functions as a gatekeeper at low $[Ca^{2+}]_c$, preventing high resting Ca^{2+}, and as a cooperative activator when $[Ca^{2+}]_c$ increases, ensuring rapid mitochondrial Ca^{2+} accumulation, thus stimulating different metabolic pathways (18).

The stoichiometric ratio of MICU1/MCU varies among tissues (19), resulting in a tissue-specific activity profile that matches the metabolic requirements (20). In the heart, the MICU1/MCU ratio has been reported as the lowest among those tissues (19). In addition, decreased MICU1 expression has been shown to trigger contractile impairment of the heart in a mouse model (18). Moreover, transitory silencing of MICU1, which decreases the MICU1/MCU ratio, significantly aggravates infarct size and depresses cardiac function following I/R (21). Considering this, the MICU1/MCU stoichiometry is recognized as the main regulator of mitochondrial Ca^{2+} influx (18). Here, we hypothesize that the cardioprotective effect of HT against Ca^{2+} overload is related to changes in the MICU1/MCU ratio. We present the modified stoichiometry observed in HT and the gating properties of the uniplex, which consequently reduces mitochondrial failure during I/R injury.

Materials and Methods

HT animal model

All experiments were performed in accordance with the animal care guidelines of the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85–23, revised 1996). All procedures were approved by the Animal Use and Care Committee of the Tecnologico de Monterrey-Medical School (Protocol 2012-Re-017). Male Wistar rats (300–350 g) were used for this study. HT was induced by the administration of 6-n-propyl-2-thiouracil (PTU) (0.05%) in drinking water *ad libitum* for four weeks (4,5,10,22). The control group received water without PTU. T3 and T4 were determined by radioimmunoassay using Architect CI18200 (Abbott).

Experiments with isolated heart mitochondria

Heart mitochondrial fractions were obtained according to the method described by García-Rivas *et al.* (23). The protein concentration of the mitochondrial fraction was measured by the Lowry method. Isolated mitochondria were suspended (0.600 mg/mL) in respiration buffer (RB) containing (in mM) 125 KCl, 3 KH_2PO_4, 10 HEPES, and pH 7.3. Mitochondrial O_2 consumption was determined with a Clark-type electrode (YSI, OH). State 4 respiration was first determined in the presence of 20 mM of glutamate and malate. State 3 respiration was stimulated by the addition of 200 μM of ADP. The respiratory control (RC) index was calculated by the ratio of state 3 to state 4 respiration, and ADP/O was expressed as the ratio of ADP added to that of oxygen atoms consumed during state 3 respiration. The mitochondrial membrane potential ($\Delta\psi$) was measured by fluorometry using safranin as indicator (24). Mitochondria were incubated in 1 mL of RB, 10 μM safranin, and 1 μg/mL rotenone. Cyclosporin A (CsA, 1 μM) was used as a pharmacological inhibitor of mPTP opening. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) was used as an uncoupling agent. The Ca^{2+} retention capacity (CRC) and mitochondrial swelling were measured as functional assessments of the sensitivity of the (mPTP) opening to mitochondrial Ca^{2+} overload and evaluated by monitoring the absorbance of Ca^{2+} Green-5N (CaG-5N, 0.3 μM) as Ca^{2+} indicator (24). Mitochondrial Ca^{2+} transport was determined using Fluo-3 and CaG-5N for the nanomolar and micromolar ranges, respectively. Mitochondria (1 mg/mL) in basal respiration medium exposed to the corresponding free $[Ca^{2+}]$ were calculated using CHELATOR software in 1 mM EGTA-buffered solution.

Aconitase enzyme activity as an oxidative stress marker was measured by monitoring the rate of conversion of *cis*-aconitate, an intermediate product, from L-citrate at 25°C at 240 nm using a spectrophotometer microplate reader Synergy HT (BioTek Instrument, Winooski, VT) (25). An extinction coefficient for *cis*-aconitate of 3.6 mM^{-1} was used to express the enzyme activity as the formation of nmol *cis*-aconitate/min/mg protein.

Free thiol content was measured by Ellman's reagent, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), as previously described (26). Absorbance was read using 100 μL of supernatant at 412 nm.

Isolated perfused heart experiments

After anesthesia with sodium pentobarbital (60 mg/kg) and sodium heparin (1000 U/kg), hearts were quickly excised from rats. The heart was placed in ice-cold Krebs–Henseleit (KH) buffer solution, consisting of (in mM) 118 NaCl, 4.75 KCl, 1.18 KH_2PO_4, 5.5 $MgSO_4$, 2 $CaCl_2$, 25 $NaHCO_3$, 10 glucose, and 100 nM sodium octanoate, pH 7.4. Immediately, the heart was mounted onto a Langendorff heart reperfusion system and perfused retrogradely via the aorta at a constant flux (12 mL/min) with KH solution continuously bubbled with 95% O_2 and 5% CO_2 at 37°C (23). Mechanical function was then assessed using a latex balloon inserted into the left ventricle and connected to a pressure transducer to measure the left ventricular pressure. Data Trax software (WPI, Sarasota, FL) was used for continuous recording of heart rate (HR) (bpm), left ventricular pressure, and the mechanical performance index (MPI), defined as the product of the left ventricular developed pressure × heart rate (LVDP × HR; mmHg × heart beats × min^{-1}). Hearts from control and HT groups were subjected to 20 minutes of stabilization, 20 minutes of zero-flow global ischemia, and 30 minutes of reperfusion. Ischemic contracture was assessed by measurement of the increase in left ventricular pressure during ischemia. The electrocardiograms were manually analyzed for arrhythmic events according to the Lambeth Conventions (27). Coronary effluents were collected at normoxic conditions after 3 minutes of reperfusion from control and HT hearts, and were frozen at −80°C until used. Lactate dehydrogenase (LDH) activity was determined as previously reported (28) and was used as an index of myocardial injury (10).

RNA isolation, reverse transcription, and quantitative PCR

Heart total RNA was isolated with TRIzol reagent (Invitrogen Co) according to the manufacturer's instructions.

First-strand cDNA was synthesized from 1 μg of total RNA using the ImProm-II Reverse Transcription System (Promega, Madison, WI) and 100 ng of cDNA was analyzed by quantitative PCR (qPCR) SensiFAST SYBR Lo-Rox kit (Bioline, London, United Kingdom). The housekeeping gene *HPRT* was used to normalize all data. The primer sequences to amplify fragments of *MCU*, *MICU1*, *MICU2*, *MCUb*, *EMRE*, and *MCUR1* are shown in Supplementary Table S1. Comparisons of gene expression analysis were performed using the $2^{-\Delta\Delta Ct}$ method.

Western blot assay

Mitochondrial proteins (30 μg) were resolved on 10% SDS-PAGE gels and transferred onto PVDF membranes and incubated with the anti-MCU antibody ab121499 (Abcam, Cambridge, MA) 1:2000, anti-MICU1 antibody 12514 (Cell Signaling), and subsequently probed with a secondary anti-rabbit IgG antibody conjugated with horseradish peroxidase 1:5000 (Santa Cruz) for two hours at room temperature. The blots were developed with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and quantified using a BioSpectrum 415 Image Acquisition System (UVP, Upland, CA). An anti-volatage-dependent anion channel (VDAC) antibody at 1:1000 was used as a loading control. The expression of MCU or MICU1 was normalized using the ratio of intensities of MCU and VDAC signals.

Solutions and reagents

All chemical reagents were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO), unless otherwise specified.

Data analysis

Data are expressed as mean $\pm$ standard error of the mean. Unpaired Student's *t*-test or one-way ANOVA with Bonferroni adjustment was performed when appropriate to compare experimental groups. A statistically significant difference was considered when *p* was ≤ 0.05. Data processing and statistical tests were carried out with GraphPad Prism V. 2.0 (GraphPad Software, La Jolla, CA).

Results

HT induces changes in MICU1/MCU stoichiometry in cardiac tissue

After four weeks of PTU treatment, the levels of the thyroid hormones triiodothyronine (T3) and thyroxine (T4) decreased by 43% and 44%, respectively (*p* = 0.008 and 0.004). Body weight (BW) also decreased significantly, by 27% (*p* = 0.005), in the HT group when compared with the control group. Heart weight (HW) also decreased by 26% (*p* = 0.006) in the HT group when compared with the control group (Table 1). However, the HW/BW ratio was similar for both groups. A MPI was measured in both groups, showing a 25% reduction in the HT group, but this difference from the control group was nonstatistically significant. In the HT group, the maximal rate of contraction (dp/dt) and relaxation (−dp/dt) was reduced by 23% and 34%, respectively, when compared with the control group.

The mitochondrial respiration capacity was analyzed as part of the characterization of the model (Table 2). State 3 respiration decreased by 8% in the HT group; this difference

TABLE 1. THYROID HORMONE STATUS AND GENERAL CHARACTERISTICS OF THE HYPOTHYROID MODEL

	Control, n = 10	*HT,* n = 10	p
T3 (ng/mL)	0.48 ± 0.03	0.27 ± 0.02	0.008
T4 (ng/dL)	0.74 ± 0.02	0.41 ± 0.01	0.004
BW (g)	416.8 ± 12.90	301.4 ± 2.68	0.005
HW (g)	1.16 ± 0.05	0.85 ± 0.02	0.006
HW/BW	0.0027 ± 0.001	0.0028 ± 0.0012	0.150
HR (beats/min)	262.5 ± 10.34	246.4 ± 10.49	0.305
MPI (mmHg-beats/min)	22,660 ± 3061	16,810 ± 3888	0.271
+dp/dt (mmHg/seg)	3260 ± 604	2492 ± 728	0.427
−dp/dt (mmHg/seg)	1882 ± 129	1231 ± 351	0.098

The values are given as mean ± SEM (error standard).
BW, body weight; +dp/dt, maximal rate of contraction; −dp/dt, maximal rate of relaxation; HR, heart rate; HT, hypothyroidism; HW, heart weight; MPI, mechanical performance index; T3, triiodothyronine; T4, thyroxine.

was not significant. However, the HT group displayed a 44% decrease (*p* = 0.04) in state 4 and a 40% increase (*p* = 0.02) in the RC (RC, state 3/state 4 ratio), possibly because of the lower passive permeability to protons induced in the HT model. The ADP/O ratio did not exhibit differences between the control and HT groups.

Once the HT model was established, the expression level of the uniplex subunits was analyzed (*MCU*, *MCUb*, *MICU1*, *MICU2*, *EMRE*, and *MCUR1*). Figure 1A shows the analysis of the mRNA levels of all uniplex subunits; only *MCU* and *MICU1* expression decreased by 40% and 30%, respectively (*p* $\leq$ 0.004). Figure 1B and C shows that protein expression of the MCU in the HT group decreased by 30% (*p* = 0.05) and that the MICU1 subunit decreased by 20% (*p* = 0.05) when compared with the controls. The MICU1/MCU stoichiometric ratio (Fig. 1D, E) increased by 25% in the HT group (*p* = 0.02).

Activation of Ca²⁺ transport in heart mitochondria is altered in the HT model

Changes in MICU1/MCU stoichiometry can modify the gating properties of the uniplex, thus exerting a different activation behavior. To investigate this, mitochondria isolated from rat hearts were assayed regarding the initial velocities of the Ca^{2+} transport in both nanomolar and micromolar free Ca^{2+} concentrations (free $[Ca^{2+}]$). Representative recordings in Figure 2A and B show the distinctive uptake activities in

TABLE 2. RESPIRATORY ACTIVITIES IN ISOLATED HEART MITOCHONDRIA FROM THE HYPOTHYROIDISM MODEL

	Control, n = 5	*HT,* n = 5	p
State 3, nmol O_2 mg^{-1} min^{-1}	51.29 ± 4.36	47.03 ± 4.47	0.51
State 4, nmol O_2 mg^{-1} min^{-1}	6.49 ± 0.69	4.10 ± 0.70	0.04
RC	8.92 ± 0.61	11.97 ± 0.92	0.02
ADP/O	3.34 ± 0.27	3.33 ± 0.19	0.98

The values are given as mean ± SEM (error standard). Mitochondria 0.6 mg/mL.
ADP/O, adenosine diphosphate (ADP) added to that of oxygen atoms consumed during state 3 respiration; RC, respiratory control.
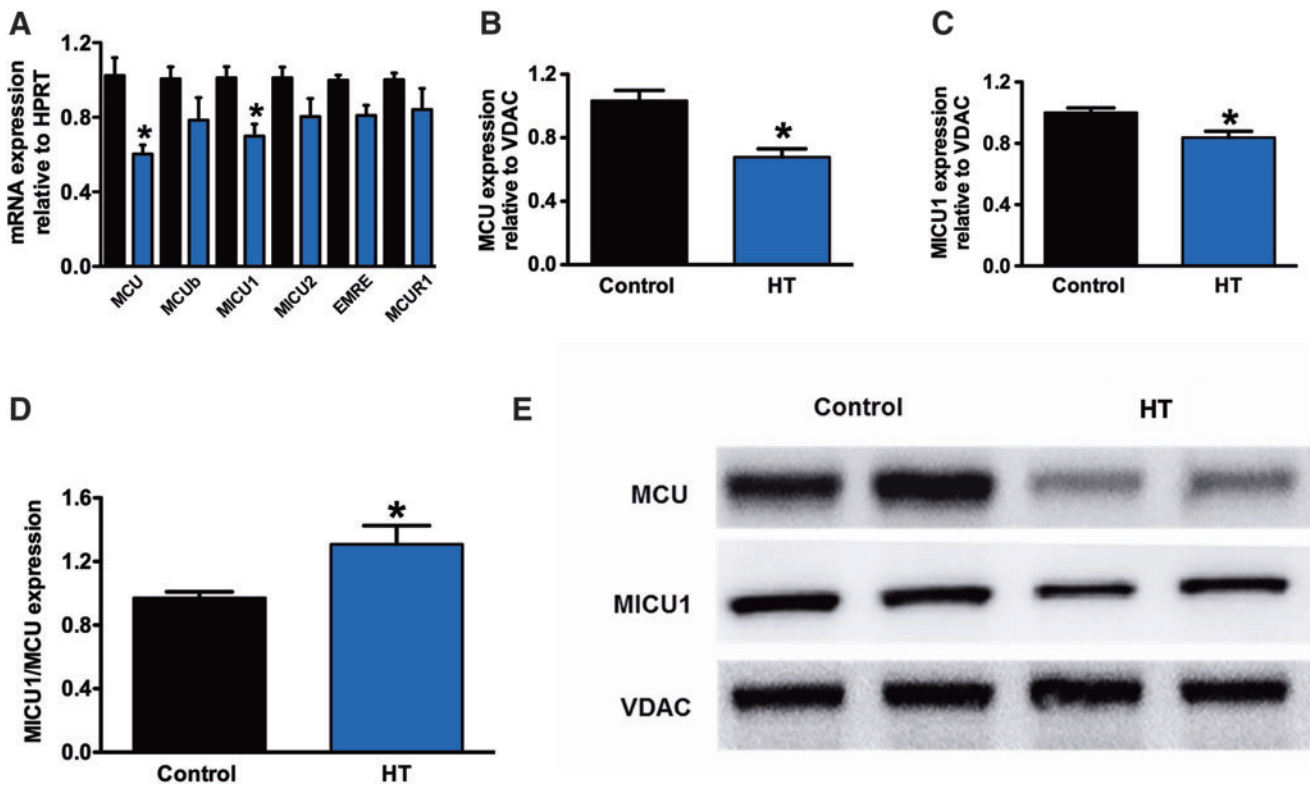
FIG. 1. HT induces changes in the expression and increases the MICU1/MCU stoichiometry in cardiac tissue. **(A)** mRNA expression of each uniplex subunit (*MCU*, *MCUb*, *MICU1*, *MICU2*, *EMRE*, and *MCUR1*) in cardiac tissue after four weeks of treatment. *MCU* and *MICU1* diminished by 40% and 30%, respectively, the rest of subunits did not show differences. **(B)** The expression at the protein level of the MCU decreased by 30% in relation to the control group. **(C)** A similar pattern showed MICU1 with a 20% reduction in expression. The values are given as mean $\pm$ SEM (error standard), *$p < 0.05$ vs. control, $n = 4$–6. **(D)** The MICU1/MCU ratio increased by 25% in the HT group. The control group is represented as the black bar and HT as the blue bar. **(E)** Western blot representative of the expression of MCU and MICU1 in both groups of rats. VDAC was used as load control, and 30 μg of protein was loaded. HT, hypothyroidism; MCU, mitochondrial Ca^{2+} uniporter; VDAC, volatage-dependent anion channel. Color images are available online.

submicromolar (700 nM) and micromolar (10 μM) free [Ca^{2+}]. Mitochondrial Ca^{2+} uptake in the HT group was 26% slower when compared with the control group in terms of nanomolar free [Ca^{2+}], while it was 42% more active under micromolar free [Ca^{2+}]. Plotting the slopes (Vi) versus free [Ca^{2+}] shows the activation curve used to estimate the half-maximal response (EC$_{50}$) with the four-parameter logistic fit. The estimated EC$_{50}$ for the control group was 3.12 ± 0.25 μM, and for the HT group, it was 4.08 ± 0.22 μM ($p = 0.006$). This shows that more Ca^{2+} is needed to activate the MCU complex in the HT model, implying that the open probability of the mPTP might be lower *in situ*. Conversely, the mitochondrial Ca^{2+} transport is more active at micromolar concentrations in the HT model.

Higher MICU1/MCU in the HT model is associated with less free [Ca^{2+}]$_m$ and delays in mPTP opening

Next, we evaluated the endogenous free [Ca^{2+}] content in isolated mitochondria from hearts previously perfused with 1 μM CGP, 2 μM Ru$_{360}$, 2 μM CsA, and 1 mM EGTA, in a basal respiration medium. The [Ca^{2+}]$_m$ was calculated using the Ca^{2+} probe Fluo-3 with the following estimation: $K_d = 310$ nM. Figure 2C shows that mitochondria from the HT model contained 35% less [Ca^{2+}]$_m$ (70 ± 1.56 nM vs. $46 \pm$

5.9 nM, $p = 0.004$). The CRC, membrane potential ($\Delta\Psi$), and mitochondrial swelling were analyzed in isolated mitochondria exposed to different extramitochondrial [Ca^{2+}]. Figure 3A shows a representative recording of the CRC, and the semiquantitative analysis shows that the control group had a CRC of 202.8 ± 37.65 nmol Ca^{2+} mg^{-1}. However, in the HT group, this capacity increased by 80% (366.2 ± 25.9 nmol Ca^{2+} mg^{-1} $p = 0.007$). Furthermore, the ability to maintain the $\Delta\Psi$ was analyzed according to the increase in the CRC. Figure 3B shows a representative trace of the $\Delta\Psi$ after Ca^{2+} overload.

Mitochondria isolated from the HT group maintained the $\Delta\Psi$ for more than 50% when compared with the control ($p = 0.03$). Finally, when mitochondrial swelling was also analyzed in both groups, mPTP opening was triggered by 400 μM of Ca^{2+}. The control group started swelling in 20 minutes, while the HT group started swelling 30 minutes after the Ca^{2+} bolus (Fig. 3C). The CRC in the control and HT groups was sensitive to CsA, a well-known inhibitor of mPTP.

The HT group showed tolerance to oxidative damage and mPTP opening after I/R injury

Basal MPI was found to be reduced in the HT group. However, the postischemic mechanical recovery function was similar in both groups after 30 minutes of ischemia (Table 3).
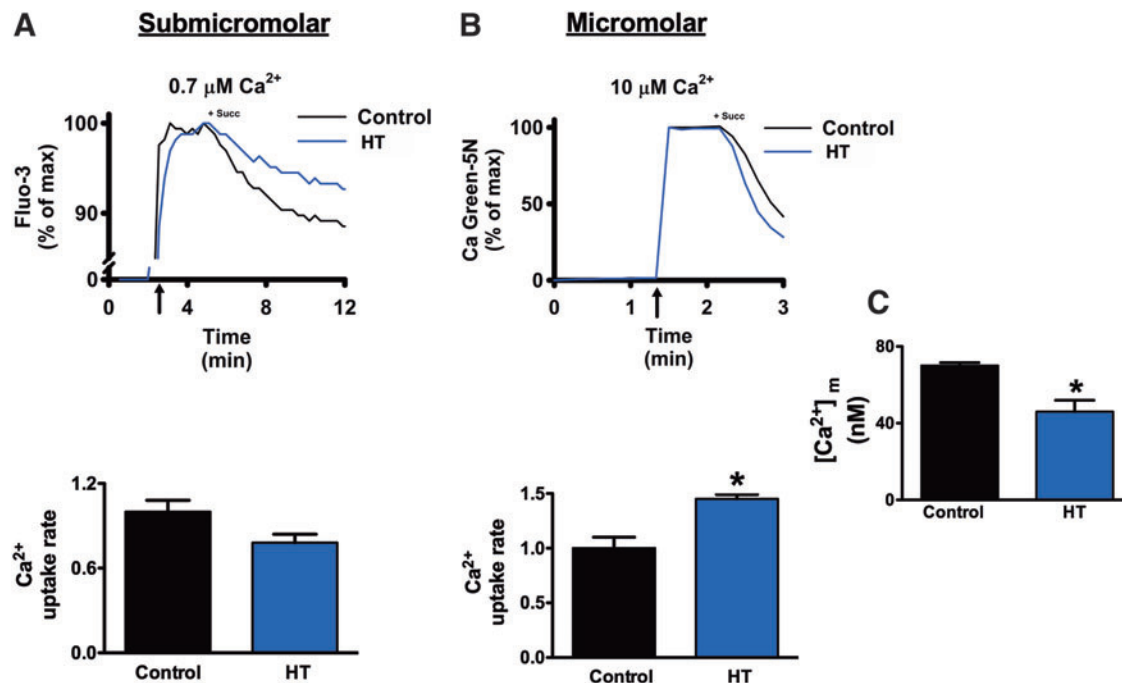
FIG. 2. Activation of Ca^{2+} transport in heart mitochondria is altered in the HT model and is associated with less free $[Ca^{2+}]_m$. **(A)** Representative trace of Ca^{2+} uptake in submicromolar (700 nM) free $[Ca^{2+}]$. Ca^{2+} transport in energized mitochondria is initiated by the addition of 10 mM succinate while recording the fluorescent extramitochondrial Ca^{2+} signal from Fluo-3. The arrow indicates the addition of Ca^{2+}. The HT group (blue line or bar) shows a 26% decrease in the rate of Ca^{2+} free transport than the control group (black line or bar). **(B)** Representative trace of Ca^{2+} uptake in micromolar (10 μM) free $[Ca^{2+}]$. The HT group shows a 42% more active concentration range in comparison with the control. Here we used CG-5N Ca^{2+} indicator, the arrow indicates the addition of Ca^{2+}. **(C)** The endogenous free Ca^{2+} intramitochondrial $[Ca^{+2}]_m$ was calculated using the Ca^{2+} probe Fluo-3 with the estimated $K_d = 310$ nM. The mitochondria from the HT model contain 35% less $[Ca^{2+}]_m$ (70 ± 1.56 nM vs. 46 ± 5.9 nM). The values are given as mean $\pm$ SEM (error standard) $*p < 0.05$, $n = 4$. Color images are available online.

Remarkably, cardiac ischemic contracture was attenuated in the HT group. Concomitantly, the onset of ventricular fibrillation occurred early in the control animals when compared with the HT group. To assess the degree of myocardial damage, we evaluated the release of LDH during reperfusion. As shown in Figure 4A, LDH release in control hearts was 2.15-fold higher than in HT hearts, which suggests that HT animals were less susceptible to I/R injury than the control animals. To explore the oxidative stress induced by I/R injury, we measured aconitase activity and reduced thiol groups as markers of oxidative damage (Fig. 4B, C). We observed a 72% aconitase activity reduction in the control group after I/R, in contrast with a 39% decrease in cardiac mitochondria in the HT group ($p < 0.01$).

We also measured reduced thiol groups, finding a 35% reduction of thiol groups in control animals, while thiols in the HT group were not modified after I/R. The semiquantitative analysis of the CRC (Fig. 4D) after reperfusion indicates that the HT group retained 270 ± 10 nmol Ca^{2+} mg^{-1}, whereas the control group only retained 150 ± 10 nmol Ca^{2+} mg^{-1}, a significant increase of 80% ($p = 0.03$). These results are consistent with the $\Delta\Psi$ and mitochondrial swelling experiments (Supplementary Fig. S1) and support the claim that HT increases resistance to mPTP opening and protects the heart from I/R injury.

Discussion

This study demonstrates that the status of thyroid hormones in HT alters mitochondrial Ca^{2+} transport in cardiac mitochondria through the changes in the expression of two uniplex subunits: MICU1 and MCU. The altered uniplex activity reduces the intramitochondrial Ca^{2+} content to increase the tolerance to mPTP opening and might be a hallmark for the reduced damage in I/R injury.

Changes in the relationship of MICU1/MCU in HT affect uniplex activity

Alvarez and colleagues demonstrated the effect of the silencing of *MICU1* on the kinetics of Ca^{2+} uptake in HeLa cells, showing that at low concentrations of extracellular Ca^{2+}, the uptake rate of Ca^{2+} into the mitochondria was greater, indicating that MICU1 behaves like a gatekeeper, preventing the overload of mitochondrial Ca^{2+} (29). However, using transgenic models, Liu *et al.* showed the effects of a complete loss of MICU1 expression in a mouse model (30). They noticed that at low Ca^{2+} concentrations (500 nM), mitochondria lacking MICU1 had a higher rate of Ca^{2+} transport when compared with the control group. However, after increasing the Ca^{2+} concentration to the micromolar range (16 μM), they noticed an inverse effect; the Ca^{2+} transport rate was lower in the mitochondria lacking MICU1 (18,30).

Our data show that the MICU1/MCU stoichiometry analysis in the HT group increased by 25%, implying that this change in the MICU1/MCU ratio might affect the dynamics of the channel. The Ca^{2+} transport rate in the HT mitochondria was lower at nanomolar concentrations (700 nM) as a result of the
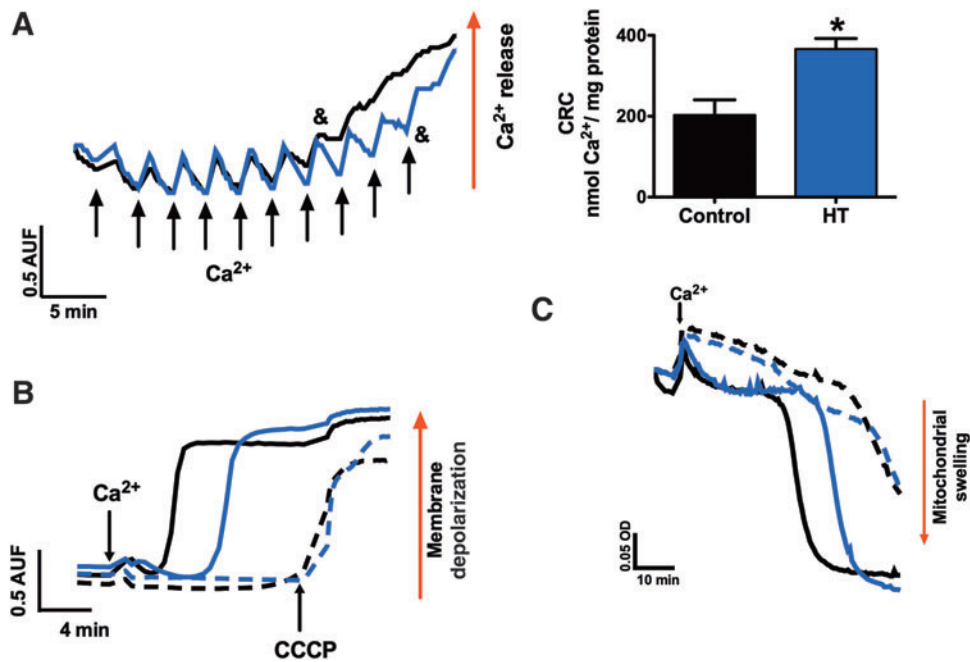
FIG. 3. The change in the regulation of the uniplex activity delays the mitochondrial membrane transition in HT. **(A)** Representative trace of CRC of heart mitochondria isolated from control and HT rats. The arrows indicate the addition of a Ca^{2+} bolus ($10\,\mu M$); we used CG-5N Ca^{2+} indicator, AFU, arbitrary fluorescence units. The HT group shows a greater CRC in comparison with the control group (see semiquantitative analysis). The $^{\&}$ indicates that Ca^{2+} cannot be transported. The values are given as mean$\pm$SEM, $*p<0.05$, $n=5$. **(B)** Representative trace of the effect of Ca^{2+} overload on mitochondrial depolarization ($\Delta\psi$) using $10\,\mu M$ of Safranin, after $100\,\mu M$ Ca^{2+} addition. The experiment was performed in the presence (dot line) or not (solid line) of $1\,\mu M$ CsA (inhibition on mPTP opening). Upon completion of the traces, $10\,\mu M$ CCCP was added as uncoupling, $n=7$. **(C)** Representative recording of mitochondrial swelling induced by $400\,\mu M$ of Ca^{2+} and $1\,\mu M$ CsA. The black color (line or bar) represents the control group; the blue (line or bar) represents the HT group. CCCP, carbonyl cyanide m-chlorophenyl hydrazone; CRC, Ca^{2+} retention capacity; CsA, cyclosporin A; mPTP, mitochondrial permeability transition pores. Color images are available online.

change in the MICU1/MCU ratio. Nevertheless, the HT group demonstrated a higher transport rate in the micromolar range, an unexpected finding since a decrease in the MCU subunit expression occurred. The change in the channel dynamics could be explained by the change in the MICU1/MCU ratio, which has been reported to vary among tissues (19). Paillard *et al.* evaluated whether protein levels of MICUs and MCU, as well as their stoichiometry, could control mitochondrial Ca^{2+} uptake in accordance with tissue-specific physiological needs (18). Our results show a behavior similar to that described by Paillard *et al.* in cardiac mitochondria overexpressing MICU1. Cardiac mitochondria in the HT group, when stimulated with a concentration in the micromolar range, showed a mitochondrial Ca^{2+} transport that resembled the representative of hepatic mitochondria (18).

Hence, with HT, both gatekeeping and cooperativity are altered in cardiac mitochondria.

A critical contribution by Robles *et al.* was the first indication of a relationship between thyroid hormone levels and possible modulations in the uniplex. Using a kinetic approach, they showed that the maximum mitochondrial Ca^{2+} transport rate (V_{max}) in the HT group diminished by 25%, while the V_{max} increased by 23% in hyperthyroidism when compared with the euthyroid group (6). In addition, after using $^{103}Ru_{360}$ (a specific MCU inhibitor) and performing Scatchard binding experiments in hepatic mitochondria, it was observed that HT decreased Ru_{360} binding by 43% when compared with the hyperthyroidism group, suggesting that the HT state reduces the MCU expression. However, this approach is not valid to determine the expression of MICU1 because Ru_{360} binds to the MCU portion at serine 259 (15), and in MICU1 knockdown cells, Ca^{2+} transport is completely sensitive to Ru_{360} (29).

Nowadays, in the molecular era of the uniplex, it is possible to investigate the expression and the role of each component in pathological conditions associated with alterations in mitochondrial Ca^{2+} transport. Here, for the first time, we observed that HT modified MICU1 expression and the MICU1/MCU ratio. It is well established that thyroid hormones generate a modulatory effect in the expression or repression of relevant genes involved in Ca^{2+} signaling in the heart (31). Our results suggest a potential regulation of MICU1 expression associated with thyroid receptor-dependent signaling. Nevertheless, the

TABLE 3. CARDIAC FUNCTION RECOVERY
AFTER ISCHEMIA/REPERFUSION

	Control, n=6	HT, n=6	p
MPI recovery (%)	67.48 ± 7.05	79.57 ± 13.08	0.434
Contracture time (minutes)	12.2 ± 2.62	21.9 ± 3.68	0.056
Onset of ventricular fibrillation (minutes)	9.5 ± 3.03	23.1 ± 3.81	0.018

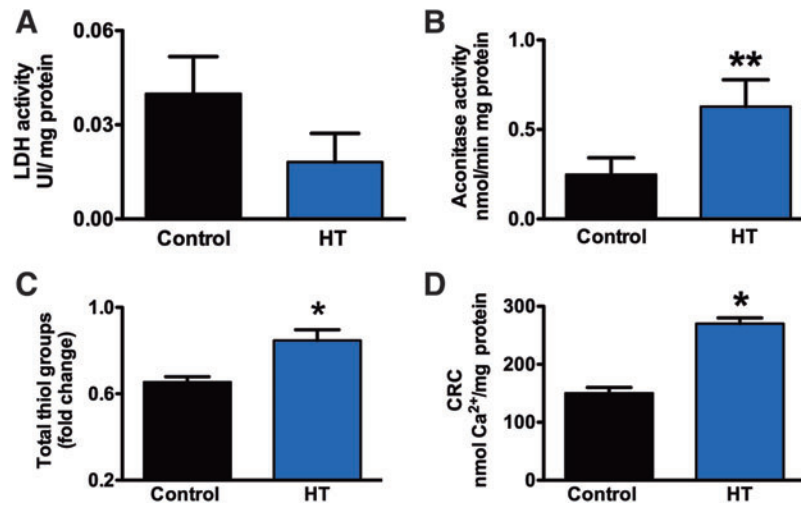The values are given as mean$\pm$SEM (error standard).

FIG. 4. Necrosis and oxidative stress markers in isolated heart mitochondria before and after I/R injury. **(A)** LDH activity of coronary effluent from control and HT hearts after I/R injury. LDH release after 20 minutes of ischemia followed by 3 minutes of reperfusion was higher by 2.15-fold in control hearts compared with hypothyroid condition, $n = 4$–5. **(B)** Changes in aconitase activity in control and HT group. A reduction of 72% was observed in control group after I/R and only a 39% was observed in the HT group. $**p < 0.001$, $n = 4$–5. **(C)** Changes in reduced thiol groups in control and HT group. The control group showed a 35% decrease after I/R injury, while the HT group only obtained a 14% decrease in reduced thiol groups $*p < 0.05$, $n = 4$–7. **(D)** Semiquantitative analysis of CRC after I/R injury. The HT group shows an increase of 80% in retaining Ca^{2+} vs. the control group, and, therefore, delay in mPTP opening protecting the heart from reperfusion injury. $*p < 0.05$ vs control. The black bar represents the control group and blue bar the HT group. I/R, ischemia/reperfusion; LDH, lactate dehydrogenase. Color images are available online.

mechanisms of regulating uniplex expression remain a fertile area for future research.

Does the increased MICU1/MCU ratio diminish damage from I/R injury?

As demonstrated in the literature, PTU treatment has a protective effect on the cardiac tissue of rats during I/R in-

jury, owing to a low concentration of thyroid hormones (4,9,10). The previous findings and those of the present study indicate that HT rats are less susceptible to damage by I/R, evident in the reduced number of fatal arrhythmias and minor tissue injuries (4,9). Elsewhere, a 45% decrease in the infarcted area, an improved contractile function, and evidence of only slight ischemic contracture following I/R injury were observed in the hearts of HT rats (4,10,32). Pantos *et al.*
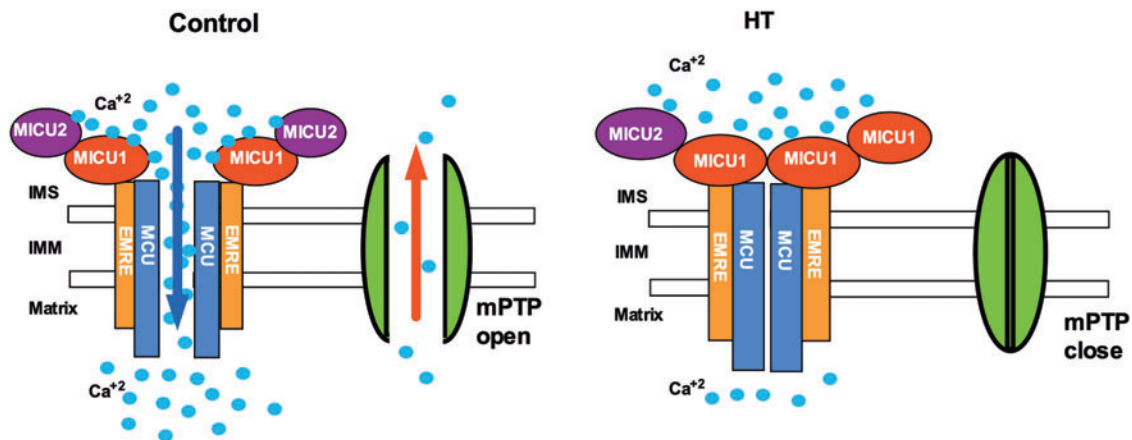

FIG. 5. A depiction of the mechanism by which changes in the stoichiometry of the MCU holocomplex contribute to tolerance of cardiac ischemia/reperfusion injury in HT. The status of the thyroid hormones in hypo changes the expression of the two uniplex subunits: mitochondrial calcium uptake 1 (MICU1) and MCU. This change increases the MICU1/MCU stoichiometry, altering the channel activity. As a result, the level of intramitochondrial Ca^{2+} is reduced due to the high cytosolic Ca^{2+} threshold imposed by the resulting gating alteration. As a consequence, Ca^{2+} overload and reactive oxygen species are triggered by ischemia/reperfusion injury, and this causes increased resistance to opening of the transition pore (mPTP) in hypo. Concomitantly, the high MICU1/MCU in HT, which impairs the balance of energy in the heart, can increase tolerance to Ca^{2+}, thereby protecting the mitochondria from ischemia/reperfusion injury. EMRE, essential MCU regulator; IMM, inner mitochondrial membrane; IMS, inner membrane space. Color images are available online.

showed that the cardioprotective effect in HT rats is associated with increased expression and activation of cardioprotective kinases (i.e., protein kinase C [PKC] and c-Jun N-terminal Kinase [JNK]) because PKC is overexpressed in the hearts of HT animals, and JNK activation is reduced in response to I/R injury (4). The overexpression of PKC has shown a protective effect due to activation of aldehyde dehydrogenase, which eliminates the by-products of peroxidation, and hence, a low reactive oxygen species concentration protecting the mitochondria from oxidative stress (33). Interestingly, PCK and JNK activation also occurs during the cardioprotection of ischemic preconditioning (34). In this regard, Korge et al. have demonstrated that PKC activation protects cardiac mitochondria by inhibiting mPTP opening (35). This has led to suggestions that PKC and JNK are part of the underlying mechanisms for tolerance of cardiac I/R injury in HT, and mPTP opening is involved in this regard.

Nevertheless, the change in MICU1/MCU stoichiometry induced by HT promotes a delay in the mPTP opening generated by Ca^{2+} overload. Mitochondria in the HT group increase their capacity to retain Ca^{2+} by 80%, maintaining their $\Delta\Psi$ for a longer time than in the control group changes that appear to be secondary to the decrease in MCU expression and the lower basal intramitochondrial Ca^{2+} content. In addition, this protective effect was observed in HT mitochondria after they underwent I/R injury. Mitochondrial Ca^{2+} overload plays an important role in the I/R process, which has been described by several groups; hence, mitochondrial Ca^{2+} influx is crucial. Our group has demonstrated that partial uniplex inhibition via Ru_{360} in ex vivo rat hearts results in preserved cardiac function after a process of ischemia. In addition, a 30% reduction in intramitochondrial Ca^{2+} in the group perfused with Ru_{360} was found (21).

Several research projects have also pointed out that a reduction in MCU subunit expression can affect mitochondrial function. Oropeza-Almazán et al. exhibited the MCUc participation in the damage induced by Ca^{2+} overload in isolated rat cardiomyocytes (36). Recently, in vivo models have been used to elucidate the role of the uniplex after I/R injury. For example, Kwong et al. showed that Ca^{2+} overload in mitochondria plays an important role in cardiac response (37). The results showed cardioprotection after I/R injury in mice without the MCU, and a similar effect was observed with Ru_{360}-mediated MCU inhibition in adult animals (23,38). Yu et al. mentioned that the MCU participates in the progression of heart failure, demonstrating the threefold increase in MCU expression in a model of heart failure, which increased intramitochondrial Ca^{2+} concentration, mitochondrial dysfunction, and therefore, the level of apoptosis in cardiomyocytes (39).

These results suggest that an increase in the MCU could induce a decrease in the stoichiometric ratio of MICU1/MCU, and therefore, the protective or gatekeeper effect of MICU1 disappears. In the HT model, Montalvo et al. showed that despite having less intra-sarcoplasmic Ca^{2+} and reduced contractility in basal conditions due to a low SERCA/PLN ratio, the acute administration of the beta adrenoceptor agonist isoproterenol did not worsen the myocyte phenotype but certainly was capable of restoring the intra-sarcoplasmic Ca^{2+} content by improving SERCA activity. This indicates that adrenergic signaling can be matched energetically by mitochondria to help with the acute contractile demand (5). Here, we demonstrated that HT increases the MICU1/MCU ratio

and the threshold to cytosolic Ca^{2+}, which in basal conditions impairs the energetic balance and contractility by reducing the mitochondrial Ca^{2+} content that activates the Ca^{2+}-sensitive dehydrogenases. However, the increased MICU1/MCU ratio in HT shows higher cooperativity in micromolar Ca^{2+} concentrations than could explain the ability to respond during the acute adrenoceptor stimulation and to match the energetic demand through the increased Ca^{2+} current. This study is the first to identify the molecular alterations in the MCU complex induced in the HT model that reduce the intramitochondrial Ca^{2+} stores, impair the heart's energetic balance and contractility, and modify the susceptibility to mPTP by altering the MCU gating components.

In conclusion, our results show that the state of thyroid hormones in HT modulates the expression of MICU1 and MCU subunits of the uniplex, increasing the stoichiometric ratio of MICU1/MCU and the threshold to cytosolic Ca^{2+}. Under a high MICU1/MCU ratio, the Ca^{2+} content is reduced in the organelle, conferring more tolerance to Ca^{2+} overload and thus delaying mPTP opening and mitochondrial dysfunction (Fig. 5). These findings help to us understand the regulation of uniplex activity in HT and provide insights for molecular therapy during mitochondrial Ca^{2+} overload and the treatment of heart failure.

Author Disclosure Statement

Funding Information

Supplementary Material

Supplementary Figure S1
Supplementary Table S1

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