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Original article

Mitochondria-mediated cardioprotection by trimetazidine in rabbit heart failure

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ABSTRACT

Trimetazidine (TMZ) is used successfully for treatment of ischemic cardiomyopathy, however its therapeutic potential in heart failure (HF) remains to be established. While the cardioprotective action of TMZ has been linked to inhibition of free fatty acid oxidation (FAO) via 3-ketoacyl CoA thiolase (3-KAT), additional mechanisms have been suggested. The aim of this study was to evaluate systematically the effects of TMZ on calcium signaling and mitochondrial function in a rabbit model of non-ischemic HF and to determine the cellular mechanisms of the cardioprotective action of TMZ. TMZ protected HF ventricular myocytes from cytosolic Ca^{2+} overload and subsequent hypercontracture, induced by electrical and ß-adrenergic (isoproterenol) stimulation. This effect was mediated by the ability of TMZ to protect HF myocytes against mitochondrial permeability transition pore (mPTP) opening via attenuation of reactive oxygen species (ROS) generation by the mitochondrial electron transport chain (ETC) and uncoupled mitochondrial nitric oxide synthase (mtNOS). The majority of ROS generated by the ETC in HF arose from enhanced complex II-mediated electron leak. TMZ inhibited the elevated electron leak at the level of mitochondrial ETC complex II and improved impaired activity of mitochondrial complex I, thereby restoring redox balance and mitochondrial membrane potential in HF. While TMZ decreased FAO by ~15%, the 3-KAT inhibitor 4-bromotiglic acid did not provide protection against palmitic acid-induced mPTP opening, indicating that TMZ effects were 3-KAT independent. Thus, the beneficial effect of TMZ in rabbit HF was not linked to FAO inhibition, but rather associated with reduced complex II- and uncoupled mtNOS-mediated oxidative stress and decreased propensity for mPTP opening.

1. Introduction

Heart failure (HF) is a hyperadrenergic state associated with high plasma levels of free fatty acids (FFA), that leads to energy depletion and decreased contractility [1,2]. Current medical therapies for HF act via suppression of neurohormonal activation (e.g. β-adrenergic antagonists, angiotensin converting enzyme inhibitors), reducing volume overload (diuretics), or improving hemodynamic symptoms (inotropic agents). Despite optimal treatment with current drugs, most patients continue to deteriorate and prognosis remains poor. Recent studies suggest that new therapies designed to decrease FFA metabolism may be particularly attractive because they could support current treatment strategies without producing negative hemodynamic effects [3–5]. Indeed, myocardial function was generally improved in HF patients treated with trimetazidine (TMZ) [6–11], an agent that optimizes energy metabolism presumably via inhibition of long-chain 3-ketoacyl CoA thiolase (3-KAT), with subsequent decrease in fatty acid oxidation (FAO) and stimulation of glucose oxidation [12]. However, direct measurement of cardiac FAO in patients with chronic non-ischemic HF revealed no changes in FFA uptake and only a 10% decrease in FAO by TMZ [8]. This finding challenges the concept that the beneficial effect of TMZ is mediated primarily by FAO inhibition [2]. Other studies [13–19] have also suggested that the cardioprotective effect of TMZ may occur via different mechanisms possibly involving regulation of mitochondrial function. The comprehensive analysis of TMZ effects on mitochondrial function in HF conditions, however, has not been performed. Therefore, the overall goal of this study was to characterize mitochondrial function and mitochondria-dependent changes in cardiac excitation–contraction coupling (ecc) in non-ischemic HF and determine the mechanisms underlying the cardioprotective effects of TMZ.

2. Materials and methods

2.1. Rabbit HF model and cardiac myocyte isolation

Single myocytes were isolated enzymatically from control and HF New Zealand white rabbits of either sex [20,21]. HF was induced by

Abbreviations: $\Delta\Psi_m$, mitochondrial membrane potential; 4-BA, 4-bromotiglic acid; $[Ca^{2+}]_i$, cytosolic calcium concentration; $[Ca^{2+}]_{em}$, extramitochondrial free calcium; CAT, carboxyatractyloside; ecc, excitation-contraction coupling; ETC, electron transport chain; FAO, fatty acid oxidation; FCCP, carbonyl cyanide 4-(trifluoromethyhoxy)-phenylhydrazone; FFA, free fatty acids; FS, field stimulation; HF, heart failure; 3-KAT, 3-ketoacyl CoA thiolase; mtNOS, mitochondrial nitric oxide synthase; NO, nitric oxide; $[NO]_{mt}$, mitochondrial nitric oxide concentration; PA, palmitic acid; mPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species; Rot, rotenone; TMRM, tetramethylrhodamine methyl ester; TMZ, trimetazidine; TTFA, 2-thenoyltrifluoroacetone.

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combined volume- and pressure-overload resulting from aortic valve insufficiency and aortic constriction [20]. Myocytes were also isolated from cyclophilin D (CypD) knock-out (KO) mice (mice with genetically deleted *Ppif* gene (*Ppif*$^{-/-}$) [22]) and their age-matched wild type C57BL/6 controls. CypD-KO mice were a kind gift from Dr. Shey-Shing Sheu, Thomas Jefferson University, Philadelphia, PA. Mouse ventricular myocytes were isolated as described before [23]. The animal sources and procedures for HF induction and cell isolation were fully approved by the Institutional Animal Care and Use Committee.

2.2. Solutions and chemicals

Normal Tyrode solution contained (in mM): 135 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, and 10 HEPES; pH 7.4. For experiments with permeabilized myocytes cells were treated with 10 μM digitonin for 1–2 min [24–27]. Digitonin was added to the "internal" solution containing (in mM): 135 KCl, 10 NaCl, 20 HEPES, 5 pyruvate, 2 glutamate, 2 malate, 0.5 KH_2PO_4, 0.5 $MgCl_2$, 15 2,3-butanedione monoxime (BDM), 5 EGTA, and 1.86 $CaCl_2$ to yield a free [Ca^{2+}] of ~100 nM. In some experiments, the $CaCl_2$ content of the internal solution was adjusted to achieve a final free [Ca^{2+}] of 2 μM or 50 μM. Trimetazidine (1-[2,3,4-trimethoxybenzyl]piperazine dihydrochloride) was obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in water as a 1 mM stock solution. Fresh TMZ stock solution was prepared on each experimental day. Unless noted otherwise, all other chemicals were purchased from Sigma-Aldrich. Spermine NONOate (Sper/NO), (4S)-N-(4-Amino-5[aminoethyl]aminopentyl)-N′-nitroguanidine (nNOS blocker I, nNOS I), and Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) were from Calbiochem-EMD Millipore (San Diego, CA).

2.3. Serum concentration of total FFA ([FFA_{tot}]) and FFA unbound to albumin ([FFA_u])

[FFA_{tot}] (FFA bound and unbound to albumin) was measured by an enzymatic, colorimetric method (assay obtained from Wako Chemicals, Richmond, VA) [28]. [FFA_u] was determined with the acrylodated intestinal fatty-acid-binding (ADIFAB) protein method (assay obtained from FFA Sciences, San Diego, CA) [29].

2.4. Measurements of cytosolic [Ca^{2+}] ([Ca^{2+}]$_i$) and cell shortening

[Ca^{2+}]$_i$ measurements were performed simultaneously with cell shortening measurements in intact Indo-1 (Molecular Probes-Life Technologies, Grand Island, NY) loaded cells using an epi-fluorescence microscopy set-up (Ionoptix, Milton, MA) [30]. Indo-1 was excited at 340 nm, with emission signals simultaneously recorded at 405 nm (F_{405}) and 485 nm (F_{485}). Changes in [Ca^{2+}]$_i$ are expressed as changes of the ratio $R = F_{405}/F_{485}$. Action potentials were triggered at 1 Hz by electrical field stimulation (FS) with a pair of platinum electrodes. The electrical stimulus was set at a voltage ~50% greater than the threshold to induce myocyte contraction. Diastolic cell length (L) and myocyte shortening (decrease in L during FS, ΔL) were measured by a video-edge detection system (IonOptix) and expressed in micrometers (μm). Fluorescent and contractile signals were analyzed with IonWizard analysis software.

2.5. Parameters of mitochondrial function

Mitochondrial NO production was measured in permeabilized cells with the fluorescent NO-sensitive dye 4,5-diaminofluorescein diacetate (DAF-2 DA, $\lambda_{exc} = 488$ nm, $\lambda_{em} = 510$ nm) loaded for 40 min at 37 °C [24,31,32]. Mitochondrial DAF-2 fluorescence intensity (F) in each experiment was normalized to the level of fluorescence recorded prior to stimulation (F_0) but after cell permeabilization. Changes in $[NO]_{mt}$ are expressed as $\Delta F/F_0$ (where $\Delta F = F - F_0$). Regions of interest (ROIs; ≤40 μm^2) were positioned over mitochondria thus representing $[NO]_{mt}$ measurements from a small number of mitochondria (≤40).

ROS production was detected in intact myocytes loaded with 10 μM 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCF DA; $\lambda_{exc} = 488$ nm, $\lambda_{em} = 510$ nm) [24,32] or 0.5 μM MitoSox Red ($\lambda_{ex} = 543$ nm, $\lambda_{em} = 555$–617 nm) for 30 min at 37 °C. Changes in CM-H_2DCF (DCF) fluorescence intensity (F) were normalized to the level of fluorescence recorded prior to stimulation (F_0), and expressed as $\Delta F/F_0$. The rate of ROS production ($d(\Delta F/F_0)/dt$) was estimated from the initial linear phase of the DCF or MitoSox Red fluorescence increase in order to minimize potential problems arising from mitochondrial dye saturation and leakage.

mPTP activity was monitored in permeabilized cells loaded with 5 μM calcein/AM ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 510$ nm) for 40 min at 37 °C [24,25,32]. Opening of mPTP resulted in the loss of mitochondria-trapped calcein (620 Da) and a decrease of fluorescence. At the end of each recording 10 μg/ml of the pore-forming antibiotic alamethicin [33] was applied to provide a control measure for maximum calcein release from mitochondria. Loss of mitochondrial calcein induced by elevating extramitochondrial [Ca^{2+}] ([Ca^{2+}]$_{em}$) was quantified as the rate of decline of fluorescence ($d(\Delta F)/dt$) calculated from the linear fit to the initial decrease of calcein fluorescence. The rate of decline was normalized (%) to the basal decline of calcein fluorescence addition (taken as 100%) before [Ca^{2+}]$_{em}$ elevation or exposure to mPTP inducers palmitic acid (PA) or carboxyatractyloside (CAT).

Calcium retention capacity (CRC) was measured in permeabilized myocytes placed in a medium containing (in mM): 150 sucrose, 50 KCl, 2 KH_2PO_4, 5 glutamate, 2 malate and 20 Tris/HCl pH 7.4. Cells were treated with 1 μM TMZ, 240 nM nNOS I, or 1 μM CsA and compared to control. After 15 min of incubation, CRC was measured by adding 1 μM Calcium Green-5N followed by repetitive 10 μM Ca^{2+} pulses applied at 1 min interval until massive release of accumulated Ca^{2+} was observed (i.e., mPTP opening) [34–36]. Measurements of [Ca^{2+}]$_{em}$ were performed confocally ($\lambda_{exc} = 488$ nm, $\lambda_{em} = 530$ nm) from ROIs outside cells. Reported results are the means of four to five independent experiments for each condition normalized to 10^6 cells.

Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were followed using the potential-sensitive dye tetramethylrodamine methyl ester (TMRM; $\lambda_{ex} = 514$ nm, $\lambda_{em} = 590$ nm) [25]. Cells were exposed to 20 nM TMRM for 15 min at 37 °C prior to experiments, and then permeabilized with digitonin [24]. All solutions contained 20 nM TMRM during recordings. The rate of the mitochondrial complex I (i.e., in the presence of 5 mM malate and 2 mM glutamate)-dependent $\Delta\Psi_m$ changes was calculated from a linear fit to TMRM fluorescence increase. The rate constant k (s^{-1}) for the mitochondrial complex II (i.e., in the presence of 5 mM succinate plus 1 μM rotenone)-dependent $\Delta\Psi_m$ recovery was calculated from the time required to reach 63% of the maximal TMRM fluorescence signal upon succinate addition.

All fluorescent indicators (DCF, MitoSox Red, calcein, Calcium Green-5N and TMRM) were obtained from Molecular Probes-Life Technologies, except for DAF-2 which was obtained from Calbiochem-EMD Millipore.

Flavin adenine dinucleotide (FAD)-linked protein autofluorescence ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 510$ nm) was measured to evaluate mitochondrial redox state [32,37,38]. Data are presented as the ratio of oxidized FAD to reduced $FADH_2$ (FAD/$FADH_2$) calculated as $(F - F_{min})/(F_{max} - F_{min})$ where F is the fluorescence intensity, and F_{min} is the fluorescence obtained after addition of 4 mM NaCN (inhibits respiration and promotes maximal FAD reduction, i.e. $FADH_2$ formation), taken as 0%. F_{max} is the fluorescence obtained after addition of 1 μM FCCP (stimulates maximal respiration, completely oxidizing the mitochondrial $FADH_2$ pool), taken as 100%.

Oxygen consumption was measured in permeabilized myocytes polarographically at 37 °C with a Clark-type oxygen electrode (Model 1302; Strathkelvin Instruments; Glasgow, Scotland) in 300 μl of MiRO5 respiration buffer containing (in mM): 110 Sucrose, 60 K-lactobionate,