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Effects of the iron chelator deferiprone and the T-type calcium channel blocker efonidipine on cardiac function and Ca^{2+} regulation in iron-overloaded thalassemic mice

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ABSTRACT

Although disturbance of cardiac Ca^{2+} regulation is involved in the pathophysiology of iron-overload cardiomyopathy, the obvious mechanisms involved in the dysregulation of iron-induced cardiac Ca^{2+} are unclear. Moreover, the roles of the iron chelator deferiprone and the T-type calcium channel blocker efonidipine on cardiac intracellular Ca^{2+} transients and Ca^{2+} regulatory proteins in thalassemic mice are still unknown. We tested the hypothesis that treatment with either deferiprone or efonidipine attenuated cardiac Ca^{2+} dysregulation and led to improved left ventricular (LV) function in iron-overloaded thalassemic mice. Wild-type (WT) mice and β-thalassemic (HT) mice were fed with either a normal diet (ND) or a high iron-diet (FE) for 90 days. Then, the FE-fed mice were treated with either deferiprone (75 mg/kg/day) or efonidipine (4 mg/kg/day) for 30 days. ND-fed HT mice had an increase in T-type calcium channels (TTCC) and an increased level of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), compared with ND-fed WT mice. Chronic iron feeding led to an increase in TTCC and expression of SERCA proteins in FE-fed WT mice. Moreover, chronic iron overload led to increased plasma non-transferrin bound iron (NTBI) and cardiac iron deposition, impaired cardiac intracellular Ca^{2+} transients including decreased intracellular Ca^{2+} transient amplitude, rising rate and decay rate, as well as impaired LV function as indicated by a decreased %LV ejection fraction (%LVEF) in both WT and HT mice. Our findings showed that treatment with either deferiprone (DFP) or efonidipine (EFO) showed similar benefits in reducing plasma NTBI and cardiac iron deposition, and improving %LVEF from 84.3 (WT) to 89.3 (DFP) and 89.2 (EFO) treatment; and from 84.2 (HT) to 88.8 (DFP) and 89.5 (EFO) treatment, however there was no improvement in the regulation of cardiac Ca^{2+} in iron-overloaded thalassemic mice. These findings provide the understanding of the effects of these drugs on the iron-overloaded heart in thalassemic mice and suggest that an alternative intervention that could improve calcium regulation under this condition is needed to improve the therapeutic outcome. Moreover, whether the benefits of the TTCC blocker is via its inhibition of the TTCC alone or together with its ability to chelate iron are still unclear and need further investigation.

Abbreviations: ATP, adenosine triphosphate; $[Ca^{2+}]_i$, intracellular calcium; CICR, calcium-induced calcium release; DFP, deferiprone; EF, ejection fraction; EFO, efonidipine; FE, iron diet; FS, fraction shortening; HRV, heart rate variability; HT, β-thalassemic type; $I_{Ca,L}$, calcium current through L-type calcium channel; LTCC, L-type calcium channel; LV, left ventricle; NCX, sodium-calcium exchanger; ND, normal diet; NTBI, non-transferrin bound iron; ROS, reactive oxygen species; RyR, ryanodine receptor; SERCA, sarcoplasmic reticulum calcium-ATPase; SR, sarcoplasmic reticulum; TDT, transfusion-dependent thalassemia; TTCC, T-type calcium channel; WT, wild-type

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1. Introduction

The secondary iron overload condition commonly occurs in transfusion-dependent thalassemic (TDT) patients and this can cause serious complications in these patients including iron overload cardiomyopathy [1,2]. Excessive body iron levels are indicated by a saturation of transferrin bound iron of more than 70% and detectable non-transferrin bound iron (NTBI) in the circulation [3]. Previous studies have demonstrated that plasma NTBI can enter into the cardiomyocytes via L-type calcium channels (LTCC) [4] and T-type calcium channels (TTCC) [5], which results in increased cardiac iron accumulation. Elevated cardiac iron leads to an increase in reactive oxygen species (ROS) formation, resulting in oxidative stress which can cause iron-overload mediated cardiac dysfunction [6].

Calcium (Ca^{2+}) is a necessary ion for the process of excitation-contraction coupling in cardiac muscle which is important in the maintenance of normal cardiac contractile function [7]. Previous studies showed that iron overload can disturb cardiac Ca^{2+} regulation by decreasing Ca^{2+} influx via LTCC into the cells [8], impairing Ca^{2+} regulatory proteins by decreasing sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) [9–11] and increasing sarcolemmal Na^{+}-Ca^{2+} exchangers (NCX) [9,12], all of which can lead to increased diastolic intracellular Ca^{2+} ($[Ca^{2+}]_i$) level, resulting in impaired cardiac contractile function under conditions of iron overload [9,11,13]. Moreover, cardiomyocytes isolated from the hearts of iron-overloaded rats exhibited an impaired level of cardiac $[Ca^{2+}]_i$ transients instigated by a decreased $[Ca^{2+}]_i$ transient amplitude, $[Ca^{2+}]_i$ transient rising rate and $[Ca^{2+}]_i$ transient decay rate, and also an increased diastolic $[Ca^{2+}]_i$ levels, thus causing cardiac dysfunction in these iron-overloaded rats [11]. However, the effects of iron overload on cardiac $[Ca^{2+}]_i$ transients and cardiac Ca^{2+} regulatory proteins in the genetically-engineered thalassemic mouse model have never been examined.

Normally, TTCC are highly expressed in the cardiomyocytes during embryonic state and their expression is suppressed after birth [14]. Hence, adult ventricular cardiomyocytes do not have TTCC under physiological conditions. However, recent studies demonstrated that cardiac TTCC are re-expressed in thalassemic cardiomyocytes [5,15] and in the hearts of iron-overloaded rats [11], and they act as a portal for iron entry into cardiomyocytes [5,11,15]. It has been shown that treatment with the TTCC blocker efonidipine (EFO), effectively prevented ferrous iron (Fe^{2+}) uptake into the thalassemic cardiomyocytes [5]. This TTCC blocker also decreased cardiac iron accumulation and exerted beneficial effects similar to the iron chelator, deferiprone (DFP), in improving left ventricular (LV) function in iron-overloaded thalassemic mice [16,17]. These benefits have been attributed to its ability to cause attenuation of cardiac mitochondrial dysfunction and a decrease in lipid peroxidation in those mice.

Despite these promising results, the effects of iron overload as well as the comparative therapeutic effects of either DFP or EFO on cardiac $[Ca^{2+}]_i$ transients and cardiac Ca^{2+} regulatory proteins in iron-overloaded thalassemic mice have never been investigated. In this study, we aimed to investigate the effects of iron overload and the roles of DFP and EFO on cardiac $[Ca^{2+}]_i$ transients and cardiac Ca^{2+} regulatory proteins in iron-overloaded thalassemic mice. We hypothesized that treatment with either DFP or EFO attenuated cardiac Ca^{2+} dysregulation and led to improved LV function in iron-overloaded thalassemic mice.

2. Materials and methods

2.1. Animal models

The animal studies were approved by the Institutional Animal Care and Use Committee at the Faculty of Medicine, Chiang Mai University (Permit number: 11/2558) and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH guide, 8th edition, 2011). Two types of adult male C57BL/6 mice aged 3-6 months old (weighing 20-25 g), including wild-type (mu$\beta^{+/+}$, WT) and heterozygous β-thalassemic type type (mu$\beta^{th-3/+}$, HT) were obtained from the Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand [15,18]. All mice were housed in an environment with controlled temperature and humidity and a constant 12-hour light/dark cycle, and were fed with food and water *ad libitum*.

2.2. Iron treatment and pharmacological intervention

WT and HT mice in the control group received a normal diet (ND group, n = 6), whereas the iron overloaded group received a high-iron diet (0.2% Ferrocene/kg of diet; FE group, n = 18) for 90 days [15]. After that, the ND group were given a normal saline solution (NSS) via oral gavage feeding once a day (a control group, $n = 6$). Mice in the FE group were randomly divided into 3 subgroups (n = 4-6 each), including: 1) FE mice treated with NSS (FE control group); 2) FE mice treated with DFP (FE-DFP group), and 3) FE mice treated with EFO (FE-EFO group). FE-DFP mice received 75 mg/kg/day of DFP (Ferriprox®, Apotex Inc., Toronto, Ontario, Canada), via oral gavage feeding, twice a day. FE-EFO mice received 4 mg/kg/day of EFO (Sigma-Aldrich, St. Louis, MO, USA), via oral gavage feeding, once a day. Each subgroup received the assigned treatment for 30 days with continuous iron-diet feeding. At the end of the experiments (i.e. 4 months after the start of iron loading and treatment), cardiac function, plasma NTBI, cardiac iron deposition, cardiac $[Ca^{2+}]_i$ transients and Ca^{2+} regulatory protein expression were examined in all mice.

2.3. Measurement of LV function by echocardiogram

Cardiac function was assessed by echocardiograph (Vivid i, GE Medical Systems, Aurora, OH, USA). Echocardiographic study was performed using a method described previously [16]. Signals from M-mode images were recorded at the papillary muscle level to determine %LV ejection fraction (%LVEF) and %LV fractional shortening (%LVFS). A decrease in %LVEF and %LVFS were considered to be an indicator of decreased LV function [9,19].

2.4. Quantification of plasma non-transferrin bound iron (NTBI)

Plasma NTBI concentration was measured using the nitrilotriacetic acid disodium salt (NTA) chelation/flow cytometry method as described previously [16,20]. Plasma samples were incubated for 30 minutes with 80-mM NTA solution at room temperature to allow formation of an Fe^{3+}-$(NTA)_2$ complex. The Fe^{3+}-$(NTA)_2$ was subsequently separated from plasma proteins by spinning the plasma mixture through a membrane filter (NanoSep®, 30-kDa cut off, polysulfone type; Pall Life Sciences, Ann Arbor, MI, USA). The Fe^{3+}-$(NTA)_2$ concentration, which represented NTBI in the ultrafitrate, was determined using chelatable fluorescent beads based on flow cytometry (Guava EasyCyte HT, Merck Millipore, Germany). Plasma NTBI concentration was calculated from a standard curve using GraphPad Prism software.

2.5. Cardiac iron deposition determination

The method of measuring cardiac iron deposition by Prussian blue staining was described in a previous study [15]. The dissected ventricular tissue was first fixed in 10% neutral buffer formalin, embedded in paraffin boxes, cut with a sliding microtome (5-μm-thick section), and then stained with Prussian blue dye solution. The tissue slides were examined under a light microscope (Olympus Corporation, Philadelphia, PA, USA) and recorded with a digital camera [15].