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HAX-1 regulates SERCA2a oxidation and degradation

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ABSTRACT

Ischemia/reperfusion injury is associated with contractile dysfunction and increased cardiomyocyte death. Overexpression of the hematopoietic lineage substrate-1-associated protein X-1 (HAX-1) has been shown to protect from cellular injury but the function of endogenous HAX-1 remains obscure due to early lethality of the knockout mouse. Herein we generated a cardiac-specific and inducible HAX-1 deficient model, which uncovered an unexpected role of HAX-1 in regulation of sarco/endoplasmic reticulum Ca-ATPase (SERCA2a) in ischemia/reperfusion injury. Although ablation of HAX-1 in the adult heart elicited no morphological alterations under non-stress conditions, it diminished contractile recovery and increased infarct size upon ischemia/reperfusion injury. These detrimental effects were associated with increased loss of SERCA2a. Enhanced SERCA2a degradation was not due to alterations in calpain and calpastatin levels or calpain activity. Conversely, HAX-1 overexpression improved contractile recovery and maintained SERCA2a levels. The regulatory effects of HAX-1 on SERCA2a degradation were observed at multiple levels, including intact hearts, isolated cardiomyocytes and sarcoplasmic reticulum microsomes. Mechanistically, HAX-1 ablation elicited increased production of reactive oxygen species at the sarco/endoplasic reticulum compartment, resulting in SERCA2a oxidation and a predisposition to its proteolysis. This effect may be mediated by NAPDH oxidase 4 (NOX4), a novel binding partner of HAX-1. Accordingly, NOX inhibition with apocynin abrogated the effects of HAX-1 ablation in hearts subjected to ischemia/reperfusion injury. Taken together, our findings reveal a role of HAX-1 in the regulation of oxidative stress and SERCA2a degradation, implicating its importance in calcium homeostasis and cell survival pathways.

1. Introduction

Ischemia reperfusion (I/R) injury and the resulting myocardial infarction are a leading cause of heart failure and death in the U.S. and worldwide [1]. One of the major characteristics of the stressed human or experimental heart is diminished contractile parameters, associated with depressed Ca^{2+}-transport by the sarco/endoplasmic reticulum (SR/ER) Ca^{2+} ATPase (SERCA2a) and its regulator phospholamban (PLN) [2,3]. The resultant reduction of SR Ca^{2+} content adversely affects Ca^{2+} homeostasis causing aberrant SR/ER, mitochondrial, and cytosolic signaling that leads to cell death. Decades of research have suggested that targeting the SERCA2a/PLN activity may restore contractile function and benefit the stressed heart. PLN exists in a complex with SERCA2a, inhibiting its function and phosphorylation of PLN during β-agonist stimulation relieves its inhibitory effects [4]. Recent evidence indicates there are several other binding partners of PLN and SERCA2a, which modulate the function of SR Ca^{2+}-transport through a much larger regulatory complex [5,6]. Among these partners, the HS-associated protein X-1 (HAX-1) has been found to directly interact with PLN [7]. In cardiomyocytes, HAX-1 also localizes to SR, where it increases inhibition of SERCA2a by PLN and depressing contractility [8].

HAX-1 is an approximately 35 kDa protein, which was originally found to form a complex with HS-1 (hematopoietic lineage cell-specific

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protein-1) in lymphocytes, and mediate lymphocyte differentiation. HAX-1 is ubiquitously expressed at the mitochondria with critical function in immune and neuronal cells [9]. Loss of HAX-1 protein as a result of human mutations causes severe neutropenia [10], through mitochondrial instability in neutrophils [9]. In the mouse, global genetic deletion of HAX-1 associates with a short life-span due to progressive loss of neuronal cells [9]. With respect to the heart, previous work has shown that HAX-1 overexpression protects from cell death and enhances recovery after ischemia/reperfusion (I/R) injury through regulation of inositol requiring enzyme-1 (IRE-1) ER stress signaling [11] and cyclophilin D dependent mitochondrial stability [12]. Interestingly, loss of SERCA2a activity by heterozygous deletion or pharmacological inhibition with thapsigargin can induce or exacerbate cell death through both of these pathways, suggesting a potential common link between them [13–15]. Furthermore, the decreases in SERCA2a protein levels and activity in human and experimental I/R [3,16–18] could serve as an upstream initiator of ER and mitochondrial stress signaling. Indeed, transgenic or viral over-expression of SERCA2a confers cardioprotection [19–21]. However, increased SERCA2a activity though PLN ablation corresponds to exacerbated injury during I/R [22], indicating a gap in our understanding of Ca^{2+} mediated cell death mechanisms.

The levels and activity of SERCA2a in cardiomyocytes can be modulated by various post-translation modifications (PTMs). Specifically, the small ubiquitin like modifier (SUMO) can be conjugated to SERCA2a, increasing expression and activity of the enzyme. Indeed, enhanced SUMOylation reverses the heart failure phenotype in small and large animal models indicating its therapeutic potential [23,24]. Additionally, tyrosine nitration and cysteine sulfonylation of SERCA2a are both associated with depressed function, whereas cysteine glutathionylation may elevate enzymatic activity. In particular, cysteine 674 is a critical site, which impacts vascular and cardiac function [25–30]. SERCA2a activity can be also modulated through proteolytic degradation, associated with increased activity of the Ca^{2+} sensitive proteases, calpain 1 and 2 after ischemia/reperfusion injury [16]. Yet, there is no clear evidence that this enzyme can serve as a specific substrate of calpain. Interestingly, previous studies suggested that HAX-1 may also down-regulate SERCA2a protein levels in HEK 293 cells [31]. However, these studies in non-muscle cell lines may not reflect findings in striated muscle.

Since global HAX-1 ablation results in early lethality, we generated a cardiac specific and inducible knockout mouse model to further characterize the role of endogenous HAX-1 in cardioprotection. Our findings demonstrate that HAX-1 deficiency results in diminished contractile recovery and increased infarct after I/R injury associated with increased SERCA2a degradation. The underlying mechanisms include increases in SERCA2a oxidation and reactive oxygen production at the ER/SR through direct interaction of HAX-1 with NOX4. Thus, a regulatory complex of SERCA2a/PLN/HAX-1/NOX4 may be a nodal point in the redox control of the heart and dysregulation may serve as a precipitating event in numerous stress pathways.

2. Materials and methods

2.1. Human myocardial tissue

The current investigation conforms to the principles outlined in the Declaration of Helsinki. Briefly, failing heart samples were acquired from seven patients (4 females, 2 males, and 1 with gender that cannot be tracked), whose ages ranged from 48 to 69 years. Cardiac dysfunction was caused by ischemic heart disease (IHD), idiopathic dilated cardiomyopathy (IDC), and congestive heart failure (CHF). The average ejection fraction of the patients was 20 ± 3%, which can be defined as heart failure with reduced ejection fraction (HFrEF). All heart samples were obtained from explanted hearts at the time of cardiac transplantation. As controls, seven non-failing hearts (5 females and 2 males in the age range of 52–61 years) were obtained from donors who had normal cardiac function and died from neurological diseases or road traffic accidents, as previously described [32].

2.2. Mouse models

The HAXiKO mouse model (C57BL/6 J: $HAX^{flox/flox}$ × αMHC-mer/cre/mer) was developed by crossing a floxed HAX-1 mouse [33] with a mouse expressing the mer/cre/mer driven by the myosin heavy chain promoter (αMHC-mer/cre/mer or CRE). Wild type (WT) mice were litter mates of the HAXiKO lacking the mer/cre/mer transgene ($HAX^{flox/flox}$). Beginning at 8 weeks of age, mice of all backgrounds were treated with tamoxifen (40 mg/kg) for 14 days to induce cre recombinase activity and this resulted in full ablation of HAX-1 in the heart. Experiments were conducted 2–4 weeks after termination of tamoxifen treatment (12–14 weeks old) and were performed according to the National Institutes of Health Publication No. 8523: Guide for the Care and Use of Laboratory Animals. To assess potential adverse effects of tamoxifen/Cre on cardiac function, we performed echocardiography experiments at baseline, after completion of the tamoxifen treatment and at two weeks post-treatment. There were no differences in ejection fraction or LV dimensions between the HAXiKOs, MerCreMer and WT mice.

2.3. Quantitative real-time PCR assay

Total RNA was extracted and purified from heart tissue with miRNeasy Mini Kit (QIAGEN). The first-strand cDNA were generated from total RNA (1 μg) with reverse transcriptase kit (Invitrogen). PCR was then performed with Bio-Rad real-time thermal cycler by using the following specific primer sequences: Human HAX-1: (Forward) 5′- CTA CAG TAA CCC GAC ACG AAG -3′, (Reverse) 5′- AAT GGG TGA GAG GTG GAA AG-3′, and Human: GAPDH (Forward) 5′-GTC AAG GCT GAG AAC GGG AA-3′, (Reverse) 5′- AAA TGA GCC CCA GCC TTC TC-3′. The values were normalized to those obtained with GAPDH.

2.4. Global ischemia/reperfusion injury ex vivo

The cellular and functional responses to ischemia/reperfusion were assessed, using an isolated perfused heart model, as previously described [11]. Briefly, hearts were mounted on a Langendorff apparatus, and perfused with Krebs–Henseleit (KH) buffer. Temperature was maintained constant at 37 °C by water-jacketed glassware for the heart chamber, buffer reservoirs, and perfusion lines. In addition, an overhead light source was used to ensure maintenance of temperature during ischemia, which was monitored by a thermometer placed close to the perfused heart in the glass chamber. A water-filled balloon made of plastic film was inserted into the left ventricle and adjusted to achieve a left ventricular end-diastolic pressure (LVEDP) of 5 to 10 mmHg. The distal end of the catheter was connected to a Heart Performance Analyzer (Micro-Med) via a pressure transducer. Hearts were paced at 400 bpm except during ischemia, and pacing was reinitiated 2 min after reperfusion. After a 20-min equilibration period, hearts were subjected to 40 min of no flow global ischemia, followed by 60 min of reperfusion. Maximum rate of contraction (+ dP/dt), and maximum rate of relaxation (− dP/dt) were monitored during this process. In some experiments, 200 μM apocynin [34,35] was added directly into KH buffer and hearts were perfused with this solution during the ischemia/reperfusion protocol.

2.5. Mouse myocyte isolation and viral infection

Mouse myocytes from were isolated as described [12]. Briefly, adult mouse hearts were excised following mouse anesthesia with sodium pentobarbital (70 mg/kg, i.p.) and cannulated on a Langendorff system. Ca-free Tyrode solution (113 nM NaCl, 4.7 mM KCl, 0.6 mM KH2PO4,