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Original article

Hypoxia and HIF-1 suppress SERCA2a expression in embryonic cardiac myocytes through two interdependent hypoxia response elements

Veli-Pekka Ronkainen [a,b], Reka Skoumal [c], Pasi Tavi [b,*]

[a] Department of Physiology, Institute of Biomedicine and Biocenter Oulu, University of Oulu, FI-90014 Oulu, Finland
[b] Department of Biotechnology and Molecular Medicine, A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, FI-70211 Kuopio, Finland
[c] Department of Pharmacology and Toxicology, Institute of Biomedicine and Biocenter Oulu, University of Oulu, FI-90014 Oulu, Finland

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ABSTRACT

Sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a) is an essential component of cardiomyocyte excitation–contraction (EC)-coupling. Suppression of SERCA2a expression induces contractile dysfunction and has been reported in various forms of ischemic cardiac disease as well as in hypobaric hypoxia. The present study investigated whether SERCA2a expression is regulated by hypoxia in embryonic mouse cardiomyocytes and explored the underlying mechanism. We show that in cultured embryonic cardiomyocytes hypoxia (1% O_2) induce time-dependent downregulation of SERCA2a expression. This mechanism manifested as specific changes in cardiac myocyte calcium signals induced by reduced expression and activity of SERCA2a. Chemical activation of hypoxia-inducible factor-1 (HIF-1) by DFO or overexpression of normoxia-stabile HIF-1α (HIF-1α/VP16) suppressed endogenous SERCA2a expression as well as the activity of the SERCA2a-promoter-luciferase reporter. Analysis of the SERCA2a promoter found two putative HIF-1 binding HRE-sites. Site-specific promoter mutagenesis revealed that co-operative HIF-1 binding to both of these hypoxia response elements on the SERCA2a promoter is required for expressional suppression. This mechanism establishes a link between oxygen supply and calcium activity in embryonic cardiac myocytes that is exploited in cardiac development, and further may offer a possible explanation for the functional depression of SERCA2a seen in ischemic and hypoxic myocardium.

1. Introduction

In mammalian cardiac muscle cells the calcium influx through L-type calcium channels during an action potential acts as a trigger for calcium-induced calcium release (CICR) from the sarcoplasmic reticulum (SR) calcium stores. This results in a massive increase in the cytosolic Ca^{2+}, which induces contraction of the cell. Following each cytosolic $[Ca^{2+}]_i$ rise Ca-ions are pumped back into the SR by the cardiac-specific sarcoplasmic reticulum calcium ATPase 2a (SERCA2a) to be released upon the next excitation. During a single contraction cycle, the main part (~92% in mouse) of the released Ca^{2+} is pumped back into the sarcoplasmic reticulum by SERCA [1]. Consequently, SERCA is one of the main regulators of cardiac contraction and relaxation, and altered SERCA activity has a tremendous impact on the contractile function of the heart [2]. Reduced SERCA2a activity is especially deleterious for heart function and has been associated with various animal models of pressure or volume overload as well as in patients with heart failure [3–5]. It has been demonstrated earlier that reduced SERCA2a mRNA expression level and function correlate with impaired relaxation of the hypertrophied and failing hearts [3,6–8]. Supporting the causal role of reduced SERCA activity and impaired function in cardiac failure, adenoviral- and lentiviral-mediated SERCA2 overexpression studies have shown that compensation of SERCA2 loss after various heart failure situations improves cardiac and isolated cardiomyocyte functions and survival [9–11].

During early embryonic development SERCA has a dual role in regulating cardiomyocyte function. SERCA is needed to pump cytosolic Ca^{2+} back into the SR, just like in adult myocytes, but in developing myocytes SERCA also has a central role in pacemaking, it refills SR stores needed for spontaneous Ca^{2+} oscillations, which pace the electrical activity of the cells [12–14]. Consequently, interference with SR function during cardiogenesis leads to severe functional and developmental impairment of the heart and embryonic lethality [15,16], suggesting that normal development requires subtle transcriptional regulation of the SR components, including SERCA2a.

Expressional regulation of SERCA2a has been studied in various animal and cell models, but the basic cellular mechanisms underlying SERCA2a expressional repression during diverse heart conditions are still largely unclear. SERCA2 promoter element analysis has revealed conservation between species and several putative cis-regulatory consensus elements in the proximal and distal promoter [17]. Promoter element deletion

* Corresponding author at: Department of Biotechnology and Molecular Medicine, A.I. Virtanen Institute for Molecular Sciences, University of Eastern Finland, P.O. Box 1627, Neulaniementie 2, FI-70211 Kuopio, Finland. Tel.: +358 40 3553272.
E-mail address: pasi.tavi@uef.fi (P. Tavi).

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studies showed that some unidentified cis-acting repressive element is located in the rabbit SERCA2 distal promoter (−1110 bp to −658 bp) [18]. This region was suggested to be one of the causes of the down-regulation of SERCA2 promoter activity in a failing heart model *in vivo* [18]. In the human SERCA2 promoter the same wide region (−1741 bp to −412 bp) also contains a negative regulatory element [17].

Suppression of SERCA2a expression has been reported in various forms of ischemic cardiac disease as well as during the early response to hypobaric hypoxia [19], suggesting that SERCA2a expression might be regulated by oxygen levels. Embryonic regulation of SERCA2a might provide an interesting model for studying hypoxia-dependent SERCA2a transcription, because mammalian development occurs in a hypoxic (1–5% O_2) uterine environment [20]. Initially, the increase in the size of the embryo leads to the development of general intra-embryonic hypoxia during mammalian embryogenesis, but upon formation of the four-chambered heart and especially due to coronary vessel formation in the developing heart, hypoxic areas are systematically restricted to smaller regions [21]. Interestingly, this phase of improved oxygenation of the heart tissue from fetal to adult mouse coincides with a several-fold increase in the expression of SERCA2a [22]. However, it is not known whether this is directly due to hypoxia-induced transcriptional regulation.

Prototypically, hypoxia-induced adaptation and remodelling includes expressional regulation of genes involved in energy metabolism, angiogenesis, and apoptosis [23]. Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that functions as a master regulator of oxygen homeostasis [23]. In developing heart HIF-1α specifically regulates the expression of transcription factors promoting cardiomyocyte differentiation, and lack of HIF-1α results in aborted heart development and early embryonic lethality [21,24]. The HIF-1 target gene promoter contains a so-called Hypoxia Response Element (HRE) and a five base pair long conserved HIF-1 Binding Sequence (5′-ACGTG-3′) [25]. HIF-1 is activated in hypoxia due to the prevention of oxygen- dependent HIF prolyl-4-hydroxylase (HIF-P4H)-mediated proline hydroxylation, which in normoxia targets the HIF-1α subunit for ubiquitination and proteosomal degradation [26–28]. Usually HIF-activation leads to an increase in target gene expression, but HIF-1 is also capable of repressing gene expression and promoting the downregulation of several genes during hypoxia [29–37]. The mechanism of the HIF-1-dependent gene expression repression is not known. Interestingly, conditional HIF-1α expression results in the downregulation of SERCA2a in mouse heart [38]. However, it is not known if this is due to the direct effect of HIF-1 on the SERCA2 gene or if this mechanism is effectively utilized in hypoxia. Therefore, the aims of the current study were to find out if SERCA2a expression and function in cardiac myocytes are sensitive to hypoxia and determine underlying mechanisms.

2. Methods

2.1. Cell isolation and culturing

Extended description of Methods is available in the Supplementary material. A previously described method was used to isolate and culture embryonic day 12.5 (E12.5) cardiomyocytes [12]. Cells plated on laminin-coated glass coverslips or 4-well plastic plates were grown for 24 h at normoxia (21% O_2) at 37 °C then subjected to 24 h hypoxia (1% O_2) or DFO (100 μM) as previously [39]. The influence of hypoxia on cell apoptosis rate and viability was monitored with the Cell Death Detection ELISA plus –apoptosis assay (Roche). Pregnant CD-1 mice from the Center for Experimental Animals at the University of Oulu were used. The experimental designs were approved by the Animal Use and Care Committee of the University of Oulu.

2.2. Confocal Ca^{2+} imaging

A previously described method was used to measure intracellular Ca^{2+} [12].

2.3. Transfections and plasmid constructs

Cells were transfected by electroporation (BTX ECM-830, Harvard Apparatus, USA) immediately after cell isolation by dissolving them in Opti-Mem (Invitrogen, USA) containing 10 μg DNA. Electroporation was performed in 2 mm gap cuvettes (BTX Harvard Apparatus, USA) using two 200 V 100 μs pulses. For constitutive HIF-1 activation, cells were transfected with a normoxia-stable HIF-1α/VP16 chimera producing plasmid (pFlag-HIF-1α(1-390)-VP16) described earlier [39]. In the control groups cells were transfected with the empty cloning vector pGL3-Basic (Promega, Madison, USA). SERCA2a-luc reporter plasmids were a generous gift from Dr. WS Simonides, described earlier by Vlasbom et al. [40]. HIF-1 binding core sequences (5′-ACGTG-3′) located −779 bp (HRE1) and −1075 bp (HRE2) upstream of the transcription start site were mutated to 5′-AATGT-3′ using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, USA) according to the manufacturer's instructions.

2.4. Reporter analysis

Twenty-four hours after transfection and plating, cells were grown for another 24 h at normoxia (21% O_2) or hypoxia (1% O_2) or in the presence of DFO (100 μM). To measure the transcriptional activity of the SERCA2a-luc reporter plasmids, the luciferase signal was measured and normalized with the Renilla-luciferase signal according to the Dual-Luciferase Reporter Assay System (Promega, Madison, USA) kit instructions.

2.5. Gene expression measurements

Total RNA from cultured cells was isolated using the E.Z.N.A. Total RNA Kit I (Omega Bio-Tek, USA). After cDNA synthesis (RevertAid First Strand cDNA Synthesis Kit, Fermentas), quantitative PCR reactions were performed with the ABI 7300 Sequence Detection System (Applied Biosystems, USA) using the TaqMan chemistry as previously [39].

2.6. Immunofluorescence labeling and microscopy

Immunolabelling of SERCA2a and HIF-1α were done as previously [39,41]. After labeling, images were taken freshly with an Olympus FV1000 confocal microscope (excitation 488 nm, emission 505–605 nm) using a 60× objective.

2.7. Western blot analysis/PLB phosphorylation

Total protein was extracted from embryonic cardiomyocytes with lysis buffer containing (in mM) of 20 Tris, (pH 7.5), 150 NaCl, 1 EDTA, 1 EGTA, 1% Triton X-100, supplemented with 1 mM β-glycerophosphate, 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4 and 50 mM NaF. One complete mini protease inhibitor cocktail tablet (Roche) was used per 10 ml lysis buffer. Equal amounts of protein (15 μg PLB and 40 μg SERCA2a) were electrophoresed on SDS-PAGE (15% PLB and 8% SERCA2a) gels and semi-dry blotted onto nitrocellulose or PVDF membranes. Membranes were labelled with antibodies against phospho-(Ser 16)-phospholamban, phospho-(Thr 17)-phospholamban, actin, SERCA2a (Santa Cruz Biotechnology, USA), phospholamban (Badrilla, UK) and α-tubulin (Sigma-Aldrich). Signals were developed by ECL.

2.8. Statistical analysis

The values are presented as mean ± SEM. Statistical significance was evaluated using unpaired Student's t-tests for comparisons of two mean values. Multiple comparisons involving more than two groups were performed using ANOVA. P-values less than 0.05 were considered statistically significant.