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The role of p38 in the regulation of Na^+–Ca^{2+} exchanger expression in adult cardiomyocytes

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Abstract

The Na^+–Ca^{2+} exchanger is crucial in the regulation of $[Ca^{2+}]_i$ in the cardiac myocyte. The exchanger is upregulated in cardiac hypertrophy and failure. This upregulation can have an effect on calcium transients and possibly contribute to diastolic dysfunction and an increased risk of arrhythmias. Here we use adenovirus mediated gene expression to examine the role of p38 MAP kinase in upregulation of the exchanger in adult cardiac myocytes. We demonstrate that p38 mediates a part of the α-adrenergic stimulated upregulation of the Na^+–Ca^{2+} exchanger gene. Overexpression of dominant-negative p38 isoforms and activated MKK3 and MKK6 in isolated adult cardiac myocytes demonstrates that p38 activation is sufficient for NCX1 promoter upregulation and that this is mediated primarily by the p38α isoform. Lastly, this work demonstrates that the p38α stimulated upregulation of the NCX1 promoter is mediated via the –80 CArG box element. This is the first time that a specific role for p38α in gene regulation has been demonstrated in isolated adult cardiomyocytes and provides an important clue to our understanding some of the factors regulating exchanger gene expression in the hypertrophic and failing heart.

Keywords: Na^+–Ca^{2+} exchanger; p38 MAP kinase; Gene expression; Hypertrophy; Signal transduction

1. Introduction

The cardiac hypertrophic response is characterized by an increase in cardiac mass, protein content and size of the individual cardiomyocyte. Initially, this results in increased myofilament synthesis and improved contractile function. But, if the pathological stimulus is prolonged or sufficiently severe, and the increase in mass is insufficient to normalize ventricular wall stress, decompensated hypertrophy or heart failure will occur. One of the hallmark features of the failing heart is a prolonged action potential and depressed contractility. In many models of heart disease and failure, the expression and activity of the calcium sequestering SR Ca^{2+}-ATPase is decreased, and/or the activity and protein level of the Na^+–Ca^{2+} exchanger (NCX1) is increased [1–4]. Increased exchanger activity leads to increased calcium extrusion and acts to preserve low diastolic calcium levels, which may compensate in part for depressed SR Ca^{2+}-ATPase function. However, this adaptation has been demonstrated to have a number of deleterious consequences.

First, increased NCX1 activity results in a higher percent of calcium being extruded from the cell and leads to a persistent unloading of sarcoplasmic reticulum Ca^{2+} stores. Second, because the exchanger is an electrogenic transporter, the increase in calcium translocation across the plasma membrane results in greater risk of an inappropriately triggered depolarization (delayed after depolarization, DAD) before the relaxation cycle has completed [3,5]. This can cause arrhythmia and sudden death, a common cause of death from heart disease. Third, lower levels of cytosolic calcium in combination with prolonged action potential duration can promote reverse mode, Ca^{2+} in Na^+ out, activity. This would not only slow the rate of relaxation and contribute to possible arrhythmogenesis, but also place the heart at risk of serious injury due to hypercontracture caused by spontaneous Ca^{2+} oscillations [6]. There is still a great deal more to be understood, but clearly, changes in exchanger expression are important to cardiac function. Therefore, we have begun to identify the

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molecular pathways that are responsible for the upregulation of the exchanger gene in cardiac hypertrophy and failure.

Several signaling pathways, including those activated by G-protein coupled receptor (GPCR) agonists, integrins, non-receptor protein tyrosine kinases, protein kinase C, intracellular calcium and calcineurin are implicated in the initiation and maintenance of hypertrophy. Activation of the mitogen-activated protein kinases (MAPKs), which transduce extracellular signals into the nucleus, is induced by hypertrophic stimuli and activation of the MAPKs leads to a hypertrophic response. p38 MAPK is of distinct interest since it has been shown to play a possible role in cardiomyocyte growth [7], pro-apoptotic [8] or anti-apoptotic [9] pathways. In vitro studies in neonatal cardiomyocytes show that activation of p38 induces expression of ANF [7,8,10] and inhibition of p38 reduced agonist-induced BNP promoter activity [11,12]. Activation of p38 has been shown to mediate a negative inotropic effect, contribute to loss of contractility and increase myocardial stiffness [13]. Until recently, the possible downstream targets of p38 that may mediate the observed negative inotropic effect were not known. Andrews et al. has also shown that p38 can mediate the downregulation of SERCA2, which results in altered $[Ca^{2+}]_i$ regulation [14]. Given its role in regulating some of the changes in gene expression seen in hypertrophy and failure, especially that of gene products which may effect calcium homeostasis, we examined whether the p38 pathway may also regulate the expression of NCX1. We previously presented preliminary evidence of a role for p38 in NCX1 expression at the Fourth International Conference on Cellular and Molecular Physiology of Sodium–Calcium Exchange [15,16]. Our findings presented here demonstrate that activation of p38α is sufficient for the upregulation of the NCX1 gene and that this is mediated via the –80 CArG element in the NCX1 promoter.

2. Experimental procedures

2.1. Adult cardiomyocyte cell culture

Adult feline cardiomyocytes were isolated via a hanging heart preparation using enzymatic digestion and cultured by the protocols approved by the Institutional Animal Care and Use Committee as described previously [17]. The cardiomyocytes were plated on to six-well culture trays that were coated with laminin at an initial plating density of 1.5×10^5 cells per well. After overnight incubation the cardiomyocytes were rinsed and maintained in serum-free media.

2.2. Adenovirus construction and cell infection

We utilized the AdEasy system to generate recombinant adenovirus plasmids [18]. The NCX1 promoter-luciferase constructs were cloned into the promoterless pAdTrack vector. The pGL-2 derived plasmid NCX-1831 was digested with *BamHI* to generate a 5.3 kb fragment that contained 1831 nt of the proximal promoter sequence, the entire first exon (H1), the first 67 nt of intron 1, the luciferase coding sequence and polyadenylated signal sequence [19,20]. This fragment was subcloned into the *BglII* site of the pAdTrack shuttle vector pAdEasy-1. This same procedure was carried out on the full-length NCX1 promoter constructs containing point mutations in the –125 GATA element, –80 CArG element, the –151 E-box element and the +106–112 novel element [20]. Homologous recombination was carried out for each of these constructs by transformation of *Escherichia coli* strain BJ5183 with the *PmeI* digested vector. The recombinant adenoviral DNA was digested with *PacI* and transfected into HEK-293 cells. Viruses were plaque purified, amplified and titers determined by the Gazes Adenoviral Core. The wild-type (wt) p38α, dominant-negative (dn) p38 and activated MKK3bE (S207, S211to E207, E211) and MKK6bE (S189E) adenoviruses were a gift of Yibin Wang. In the dn p38 constructs, the phosphorylation motif (T-G-Y) was mutated to (A-G-F) in each of the four isoforms. This renders the kinase unable to be phosphorylated by its upstream activator.

Cardiomyocytes were infected on day 1 in culture by adding titered adenovirus to the culture medium at a different multiplicity of infection (MOI). After an infection of 8 h the media was changed. When more than one adenoviral construct was used to infect cells, experiments were carried out to insure there was no competition for infection between the constructs at the MOIs used. Adult cardiomyocytes infected with MOIs of 1 or greater produced high levels (greater than 85%) of gene transfer based on GFP expression.

2.3. Mutations in the NCX1831 promoter-luciferase construct

Mutated constructs of the pGL-2 derived plasmid NCX-1831promoter-luciferase were generated using QuikChange (Stratagene, La Jolla, CA) site-directed mutagenesis. Sense and antisense oligonucleotides were designed to contain the desired mutation flanked on either side by 12 bp of wild-type NCX sequence. Then, these were used to introduce the mutations into the NCX1831 construct by polymerase chain reaction using the manufacturer's protocol. The entire promoter region of each mutant construct was sequenced using the AmpliCycle sequencing kit to ensure that they contained only the desired point mutations.

2.4. Preparation of cell lysates

Following treatment, cells were washed twice in sterile filtered cold 1 × PBS. Cells were then lyzed in 200 μl of RIPA buffer for Western blot analysis (1% NP40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.002 M EDTA, 0.05 M NaF) or 1 × Reporter Assay Buffer for luciferase assay (Promega). Protease and phosphatase inhibitors were added to these buffers (1:100 dilutions of Phosphatase Inhibitor Cocktail I and II and Protease Inhibitor Cocktail from Sigma). The cells were then incubated on ice