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Neuronal Ca^{2+} sensor-1 contributes to stress tolerance in cardiomyocytes via activation of mitochondrial detoxification pathways

Tomoe Y. Nakamura [a,*], Shu Nakao [a,1], Shigeo Wakabayashi [a,b,2]

[a] Department of Molecular Physiology, National Cerebral and Cardiovascular Center Research Institute, Fujishirodai 5-7-1, Suita, Osaka 565-8565, Japan
[b] Department of Cardiac Physiology, National Cerebral and Cardiovascular Center Research Institute, Fujishirodai 5-7-1, Suita, Osaka 565-8565, Japan

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ABSTRACT

Identification of the molecules involved in cell death/survival pathways is important for understanding the mechanisms of cell loss in cardiac disease, and thus is clinically relevant. Ca^{2+}-dependent signals are often involved in these pathways. Here, we found that neuronal Ca^{2+}-sensor-1 (NCS-1), a Ca^{2+}-binding protein, has an important role in cardiac survival during stress. Cardiomyocytes derived from NCS-1-deficient (*Ncs1*$^{-/-}$) mice were more susceptible to oxidative and metabolic stress than wild-type (WT) myocytes. Cellular ATP levels and mitochondrial respiration rates, as well as the levels of mitochondrial marker proteins, were lower in *Ncs1*$^{-/-}$ myocytes. Although oxidative stress elevated mitochondrial proton leak, which exerts a protective effect by inhibiting the production of reactive oxygen species in WT myocytes, this response was considerably diminished in *Ncs1*$^{-/-}$ cardiomyocytes, and this would be a major reason for cell death. Consistently, H_2O_2-induced loss of mitochondrial membrane potential, a critical early event in cell death, was accelerated in *Ncs1*$^{-/-}$ myocytes. Furthermore, NCS-1 was upregulated in hearts subjected to ischemia-reperfusion, and ischemia-reperfusion injury was more severe in *Ncs1*$^{-/-}$ hearts. Activation of stress-induced Ca^{2+}-dependent survival pathways, such as Akt and PGC-1α (which promotes mitochondrial biogenesis and function), was diminished in *Ncs1*$^{-/-}$ hearts. Overall, these data demonstrate that NCS-1 contributes to stress tolerance in cardiomyocytes at least in part by activating certain Ca^{2+}-dependent survival pathways that promote mitochondrial biosynthesis/function and detoxification pathways.

1. Introduction

Excessive loss of cardiomyocytes by cell death is a leading cause of impaired conditions, such as cardiac ischemia/reperfusion injury (I/R), myocardial infarction, cardiomyopathy, and congestive heart failure [1,2]. Cell loss in the heart occurs primarily by apoptosis or necrosis. Necrotic cell death caused by impaired Ca^{2+} homeostasis is a major cause of cell loss in cardiac disease. The stressed cell also responds by activating cell survival pathways, and the severity of cardiac injury ultimately depends on the balance between cell death and survival pathways. For therapeutic reasons, it is important to identify the factors regulating this balance.

Mitochondria are at the nexus of cell death and survival pathways [3–5]. As a result of mitochondrial respiration, reactive oxygen species (ROS) are produced, which may be toxic to cells. Cells respond by activating mitochondrial detoxification pathways. Dysregulated mitochondrial function can also activate cell death pathways, for example during heart failure [6]. The crucial role of mitochondria in regulating cell death and survival pathways becomes apparent when investigating stress signals (e.g. lack of growth factors, hypoxia, or oxidative stress) in cellular systems. The responsible cellular signaling pathways are the subject of much research, and the peroxisome proliferator-activated receptor gamma (PPAR-γ) co-activator (PGC)-1α is emerging as a powerful regulator of mitochondrial biogenesis and function, which is vital for cell survival [7]. Various physiological stimuli, including hypoxia and caloric restriction, regulate the expression and activity of PGC-1α. A potential role exists for intracellular Ca^{2+} signaling, since post-translational modifications of PGC-1α (e.g. phosphorylation, dephosphorylation, and acetylation) are Ca^{2+} dependent [8]. Other proteins may also promote mitochondrial function and cell survival, but these remain poorly studied.

During our studies with neuronal Ca^{2+} sensor-1 (NCS-1) knockout (*Ncs1*$^{-/-}$, KO) mice, we have observed that KO myocytes are more

Abbreviations: Akt, protein kinase B; AMPK, AMP-activated protein kinase; ATP, adenosine triphosphate; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK3β, glycogen synthase kinase 3 beta; H_2O_2, hydrogen peroxide; I/R, ischemia-reperfusion; LDH, lactate dehydrogenase; NMVM, neonatal mouse ventricular myocyte; NCS-1, neuronal calcium sensor-1; OCR, oxygen consumption rate; PGC-1α, peroxisome proliferator-activated receptor gamma-1 coactivator; ROS, reactive oxygen species; TMRE, tetramethylrhodamine ethyl ester; TTC, triphenyltetrazolium chloride.

* Corresponding author at: Department of Molecular Physiology, National Cerebral and Cardiovascular Center Research Institute, Fujishirodai 5-7-1, Suita, Osaka 565-8565, Japan.
E-mail addresses: tomoen@ri.ncvc.go.jp, tomoe.no1@gmail.com (T.Y. Nakamura).
[1] Present address: Institute of Cardiovascular Sciences, University of Manchester, 46 Grafton Street, Manchester M13 9NT, United Kingdom.
[2] Present address: Department of Pharmacology, Osaka Medical College, Daigaku-machi 2-7, Takatsuki, Osaka 569-8686, Japan.

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susceptible to stress. NCS-1 is a small (22 kDa), *N*-terminally myristoylated EF-hand Ca^{2+}-binding protein [9] that is predominantly expressed in the brain [10] and plays an important role in neuronal functions such as synaptic plasticity [11,12], learning and memory [12,13], and survival of injured neurons [14]. NCS-1 has many binding partners, including phosphatidylinositol-4-OH kinase β [15–17], various ion channels such as voltage-gated K^+ channels [18] and Ca^{2+} channels [19–21], the D2 dopamine receptor [12], and inositol 1,4,5-trisphosphate receptors [22–24]. We have shown that NCS-1 also regulates Ca^{2+} signaling in normal and diseased hearts [23]. It is unknown, however, whether NCS-1 is involved in stress tolerance in cardiomyocytes.

The aim of this study was to investigate the hypothesis that NCS-1 promotes cardiac survival during stress. We used model systems to investigate the effects of NCS-1 deficiency and overexpression on cell survival during oxidative and metabolic stress in neonatal mouse ventricular myocytes and isolated adult hearts. Using diverse assays, our data demonstrate a key role for NCS-1 in signaling through mitochondria to promote cell survival.

2. Materials and methods

2.1. Animals

This study conforms to the NIH guidelines (Guide for the care and use of laboratory animals). All animal procedures were performed according to the Animal Welfare Committee guidelines of the National Cerebral and Cardiovascular Center Research Institute and were approved by the institutional ethics review board (approval reference number: 16072). Efforts were made to minimize the number of animals and their suffering. *Ncs1*$^{-/-}$ mice (C57BL/6-NCR) were generated as previously described [23]. The WT group contained age-matched C57BL/6-NCR mice (Japan SLC, Inc.).

2.2. Primary neonatal mouse ventricular myocyte culture and treatment with stressors

One- to 3-day-old mice were anesthetized with 1–1.5% isoflurane, and hearts were excised. Ventricular myocytes were isolated, dissociated into single cells by trypsinization, and cultured in growth medium, as described previously [23,25]. After 2 days, the neonatal mouse ventricular myocytes (NMVMs) were transferred to serum-free medium and treated with H_2O_2 (100 μM for 24 h) or kept in a glucose-free medium supplemented with 2-deoxyglucose (DOG; 2 mM for 12 h).

2.3. Cardiomyocyte survival

We stained nuclei with Hoechst 33258 and measured lactate dehydrogenase (LDH) release (Roche Diagnostics, Japan).

2.4. Cellular ATP content

ATP levels were determined by Luciferase assays (Wako Pure Chemicals, Japan) and the protein content of the lysates was determined by the bicinchoninic acid (BCA) method, using bovine serum albumin as the standard (Thermo Scientific Japan, Yokohama, Japan). ATP content is expressed as nmol/mg protein.

2.5. Mitochondrial oxygen consumption rate (OCR)

We evaluated mitochondrial function by measuring the OCR of WT and KO cardiomyocytes using a Seahorse XF^{e}96 Extracellular Flux analyzer (Seahorse Biosciences, Massachusetts, USA). NMVMs were transferred at a density of 40,000 cells/well to 96-well Seahorse assay plates coated with collagen and were incubated for 48 h at 37 °C. One hour before measurements, the medium was replaced with XF assay medium supplemented with 25 mM glucose, 1 mM pyruvate, and 1% fetal bovine serum. First, basal OCR was measured in triplicate. Oligomycin (1 μM) was added to inhibit ATP synthase (non-phosphorylating respiration), followed by the addition of increasing concentrations of carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP; 0.125, 0.25, 0.5, and 1 μM). This systematic titration determined the optimal FCCP concentration (0.25 μM) that produced the maximal uncoupled respiration rate. Non-mitochondrial respiration was determined by the addition of 1 μM rotenone and 1 μM antimycin A. Using these conditions, we examined the effects of 100 and 200 μM H_2O_2 on mitochondrial basal respiration, spare respiration (FCCP-induced maximal respiration rate minus basal respiration rate), and proton leak (oligomycin-insensitive respiration rate). Measured values were compared between WT and KO myocytes. The OCR in each well was normalized to the protein content in the same plate.

2.6. Mitochondrial inner membrane potential ($\Delta\Psi_m$)

NMVMs were cultured on collagen-coated glass-bottom dishes and loaded with 100 nM tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) in modified Tyrode's solution (137 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid [HEPES]; pH 7.4) at 37 °C for 20 min. After washing, cells were exposed to 100 μM H_2O_2 over the course of the experiments. TMRE fluorescence was monitored using an inverted microscope (Olympus 1 × 81, 60×/1.42 oil immersion objective lens) attached to a confocal laser-scanning unit (Olympus Fluoview FV1000; argon laser). Time-lapse confocal imaging (excitation: 546 nm, emission: >510 nm) was performed every 5 min for 25 min. Images were acquired using the FV10-ASW imaging software (Olympus Optical Co., Tokyo, Japan) at room temperature and processed with Adobe Photoshop.

2.7. Immunoblotting

The anti-NCS-1 antibody was described previously [26]. Primary antibodies used included anti-PGC-1α and anti-catalase (Abcam), anti-AMP-activated protein kinase (AMPK; R&D Systems), phospho-AMPK (Thr174/172; SAB Signalway Antibody), and anti-alpha actinin (Sigma). Anti-Akt, anti–phospho-Akt (Thr308 and Ser473), anti-GSK3β, anti–phospho-GSK3β, anti-mTOR, anti–phospho-mTOR, and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were all obtained from Cell Signaling. The antibody cocktail for the rodent oxidative phosphorylation complex was obtained from Abcam. Protein samples were prepared from NMVMs and heart homogenates, and immunoblotting was performed as described previously [23].

2.8. Quantitative RT-PCR

Cultured NMVMs were homogenized using a QIAshredder homogenizer (Qiagen) and total RNA was extracted using the PureLink RNA Mini Kit (Life Technologies). For whole hearts, total RNA was extracted using trizol reagent (Ambion). Reverse transcription was performed using random primers (QuantiTect Reverse Transcription kit, Qiagen). Real-time semi-quantitative PCR was performed using hydrolysis probes (FastStart Essential DNA Probe Master) and a LightCycler 480 (Roche Molecular Biochemicals). The primers and probe sets for PGC-1α, AMPK, NCS-1, 18S, and L7 were from Applied Biosystems. The mRNA levels were normalized to the expression of 18S or L7, expressed relative to the WT control.

2.9. Ischemia-reperfusion

Six-week-old male WT and KO mice were anesthetized with 1–1.5% isoflurane. After intravenous injection of heparin (50 U, 2 min), hearts were excised and perfused in a Langendorff apparatus (Model IH-1 Type 844; Hugo Sachs Elektronik, March-Hugstetten, Germany). After stabilization with modified Krebs–Henseleit bicarbonate buffer for