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Full paper

A protective role of Nox1/NADPH oxidase in a mouse model with hypoxia-induced bradycardia

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ABSTRACT

Although it has been reported that endotoxin-induced expression of Nox1 in the heart contributes to apoptosis in cardiomyocytes, functional role of Nox1 at the physiological expression level has not been elucidated. The aim of this study was to clarify the role of Nox1 under a hypoxic condition using wild-type (WT, $Nox1^{+/Y}$) and Nox1-deficient ($Nox1^{-/Y}$) mice. ECG recordings from anesthetized mice revealed that $Nox1^{-/Y}$ mice were more sensitive to hypoxia, resulting in bradycardia, compared to WT mice. Atrial and ventricular electrocardiograms recorded from Langendorff-perfused hearts revealed that hypoxic perfusion more rapidly decreased heart rate in $Nox1^{-/Y}$ hearts compared with WT hearts. Sinus node recovery times measured under a hypoxic condition were prolonged more markedly in the $Nox1^{-/Y}$ hearts. Sinoatrial node dysfunction of $Nox1^{-/Y}$ hearts during hypoxia was ameriolated by the pre-treatment with the Ca^{2+} channel blocker nifedipine or the K^+ channel opener pinacidil. Spontaneous action potentials were recorded from enzymatically-isolated sinoatrial node (SAN) cells under a hypoxic condition. There was no significant difference in the elapsed times from the commencement of hypoxia to asystole between WT and $Nox1^{-/Y}$ SAN cells. These findings suggest that Nox1 may have a protective effect against hypoxia-induced SAN dysfunction.

1. Introduction

Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) oxidase is a superoxide-generating enzyme comprising multiple subunits (1). A membrane bound catalytic subunit Nox is composed of seven members (i.e., Nox1-Nox5 and Duox1 and Duox2). It was reported that Nox2 and Nox4 were abundantly expressed in cardiac tissues (2). Nox1 was detected at the marginal level in cardiomyocytes. However, a marked elevation of Nox1 mRNA was detected in cardiac tissue of lipopolysaccharide (LPS)-induced septic mice. Reduced apoptosis of cardiomyocytes was observed in Nox1-deficient ($Nox1^{-/Y}$) mice (2). Furthermore it was indicated that the deficiency in Nox1 was strongly protective against myocardial reperfusion injury (3). Functional roles of Nox1 in the heart under physiological and pathophysiological conditions have not been thoroughly examined. Therefore, we tested whether Nox1 could be related to occurrence of atrial arrhythmias by burst atrial stimulation in Langendorff-perfused hearts isolated from wild-type (WT, $Nox1^{+/Y}$) and Nox1-deficient ($Nox1^{-/Y}$) mice treated with LPS. In that study, we could not find the difference in the inducibility of atrial arrhythmias between two groups (unpublished data). However, we realized that $Nox1^{-/Y}$ hearts were vulnerable to various experimental procedures. Especially heart rate in $Nox1^{-/Y}$ mice was easily reduced compared to WT mice. These findings prompted us to examine whether there is a difference in the response of sinoatrial node to a hypoxic insult between the genotypes.

2. Materials and methods

2.1. Animals

$Nox1^{-/Y}$ mice were supplied by Kyoto Prefectural University of Medicine (4). C57BL/6N WT mice were purchased and used as

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controls because knockout mice had been back-crossed to a C57BL/6N strain. Male WT and Nox1$^{-/Y}$ mice at the age of 8–12 weeks were used in this study. All procedures, complied with the Guide for the Care and Use of Laboratory Animal published by the U.S. National Institutes of Health, were approved by the Institutional Animal Care and Use Committee of Chiba University.

2.2. In vivo experiments for ECG recordings

Mice were anesthetized with a combination of medetomidine (0.3 mg/kg), midazolam (4 mg/kg) and butorphanol (5 mg/kg). The depth of anesthesia was maintained at the level enough to prevent pedal withdrawal reflexes in forelimbs and hindlimbs, and tail pinch reflex. Spontaneous respiration was maintained during anesthesia. The surface ECG (lead II) was monitored using limb electrodes throughout the study protocol. Pure nitrogen was supplied (1.3–1.5 L/min) for anoxic ventilation to the animal through the plastic cover made with a 15-ml conical tube, whose diameter is similar to the head size of the animal. Head portion of the animal was placed in the plastic cover during the period of anoxic ventilation (30 s). Although chest movement was not observed at the end of anoxic period, respiration was spontaneously returned to normal after the cover was removed. ECG record was digitalized with an analog-to-digital converter and stored on a disk of personal computer with acquisition system (AD Instruments, Castle Hill, Australia) and analyzed to measure the longest P–P and R–R intervals in 30 s of the anoxic ventilation. Our study has indicated that the hemoglobin oxygen saturation level was less than 12% in the right ventricle of ddY mice at the end of anoxic ventilation (Matsumoto personal communication).

2.3. In vitro experiments using Langendorff-perfused heart

Mice were anesthetized with urethane (2 g/kg, i.p.) and anticoagulated with heparin (300 U/kg, i.v.). Heart were quickly excised and connected to Langendorff apparatus by inserting a perfusion cannula into the aorta. Retrograde perfusion was maintained at a constant pressure of 80 mmHg with Krebs-Henseleit (K–H) solution containing (mM): 119 NaCl, 4.8 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 10 glucose, 24.9 $NaHCO_3$. The perfusate was equilibrated with 95% O_2 + 5% CO_2. After a stabilization period of >10 min, hearts were exposed to hypoxic K–H solution, equilibrated with 95% N_2 + 5% CO_2, with or without glucose. The temperature of the solution was maintained at 36–37 °C. Atrial and ventricular electrograms were continuously recorded with bipolar electrodes attached to the walls of the left atrium and apex. A previous study from our laboratory (5) indicated that the partial pressure value of oxygen of the hypoxic perfusate, equilibrated with 95% N_2 + 5% CO_2, was less than 40 mmHg.

Isolated hearts of WT and Nox1$^{-/Y}$ mice were perfused with hypoxic K–H solution for 5 min twice at an interval of 5 min. Changes of sinus cycle length was determined from atrial electrograms during hypoxia. It was confirmed that there was no significant difference in changes of sinus cycle length between the first and second perfusion with the hypoxic solution. In a part of experiments Langendorff-perfused hearts were treated with 10 nM nifedipine or 10 μM pinacidil before and during hypoxia in order to evaluate influences of coronary vasodilators on the hypoxia-induced sinus node dysfunction. These concentrations of coronary vasodilators were selected because 10 nM nifedipine or 10 μM pinacidil were reported to increase coronary flow in isolated rat and mouse hearts (6,7).

In a part of experiments coronary effluent from isolated hearts of WT and Nox1$^{-/Y}$ mice (total K–H solution perfused through isolated hearts) were accumulated, and the volume of coronary effluent per 1 min was repeatedly measured as a coronary flowrate. WT and Nox1$^{-/Y}$ hearts were perfused with hypoxic K–H solution for 5 min twice at an interval of 5 min, 10 nM nifedipine or 10 μM pinacidil was added to the perfusate prior to the second hypoxic perfusion.

In order to evaluate the sinus node function of WT and Nox1$^{-/Y}$ hearts under hypoxia more precisely, sinus node recovery time (SNRT) was measured during control period and after introduction of hypoxic solution. For measurement of SNRT stimulating electrodes were attached to the epicardial surface of the right atrium and 20 Hz burst pacing for 15 s was performed (8). SNRT was designated as a time interval between the last paced atrial depolarization and the first sinus return cycle. SNRT was determined during control period and 5 min after introduction of hypoxic K–H solution in isolated WT and Nox1$^{-/Y}$ hearts. In this series of experiments, influences of coronary vasodilators, 10 nM nifedipine and 10 μM pinacidil, on changes of SNRT during hypoxia were also evaluated.

2.4. Patch clamp experiments using isolated sinoatrial node cells

Single sinoatrial node (SAN) cells were isolated according to the method previously described (5). Briefly, isolated hearts were perfused with an oxygenated HEPES-buffered Tyrode solution and then an oxygenated Ca^{2+}-free HEPES-buffered solution at 36 °C for 5–10 min to wash out the blood. The Ca^{2+}-free HEPES-buffered solution had the same composition as given follow, except that it contained no Ca^{2+}. After stabilization, each heart was perfused with a low enzyme-containing (collagenase; Wako Pure Chemicals, Osaka, Japan) oxygenated Ca^{2+}-free HEPES-buffered solution. The right atrium wall was cut off from the heart, and the SAN region, limited by the crista terminalis, the atrial septum and the orifices of the vena cavae, was anatomically isolated. The excised SAN tissue strip containing the primary pacemaking region was stored in Kraft-Brühe (KB) medium solution for 1–2 h at 4 °C. Single SAN cells were gently isolated from the strip by pipetting KB solution just before experiments. Then the cells were transferred to the recording bath and perfused with the normal HEPES-Tyrode solution at 36 °C. In the hypoxic condition, the glucose-free and hypoxic HEPES-Tyrode solution, equilibrated with 100% N_2, was perfused, and the bath surface was blown with 100% N_2 to keep hypoxic condition of the bath.

The composition of the HEPES-Tyrode solution was (mM): 143 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.33 NaH_2PO_4, 5.5 glucose, 5 HEPES (pH 7.4 adjusted by NaOH). KB solution contained (mM): 70 KOH, 50 L-glutamic acid, 40 KCl, 20 taurine, 20 KH_2PO_4, 3 $MgCl_2$, 10 glucose, 1 EGTA, 5 HEPES (pH 7.4 adjusted with KOH). The pipette solution contained (mM): 110 KOH, 110 L-aspartic acid, 20 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 0.1 EGTA, 5 HEPES (pH 7.4 adjusted with KOH). Nystatin (Wako Pure Chemicals, Osaka, Japan) was first dissolved in dimethyl sulfoxide (DMSO; Sigma–Aldrich Japan, Tokyo, Japan) and then added to the pipette solution at a concentration of 300–500 μg/ml just prior to use; the final concentration of DMSO was less than 0.1%.

Single SAN cells were identified by their characteristic morphology, a long and slender shape with faint striations, and spontaneous activity, as previously described (9,10) The whole-cell patch-clamp technique was used for the electrical recordings from a single SAN cell. A patch-clamp amplifier (CEZ-2400, Nihon Kohden, Tokyo, Japan) was used for current-clamp experiments, as previously described (11). Pipette electrodes (resistance 2–6 MΩ) were made by using a Narishige pipette puller (PB-7, Narishige Scientific Instruments Laboratory, Tokyo, Japan). Spontaneous action potentials and whole-cell membrane currents were recorded by nystatin-perforated patch configuration, as previously described