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Local production of $O_2^{\cdot-}$ by NAD(P)H oxidase in the sarcoplasmic reticulum of coronary arterial myocytes: cADPR-mediated Ca^{2+} regulation

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Abstract

The present study was designed to determine whether the sarcoplasmic reticulum (SR) could locally produce superoxide ($O_2^{\cdot-}$) via NAD(P)H oxidase (NOX) in coronary arterial myocytes (CAMs) and to address whether cADPR-RyR/Ca^{2+} signaling pathway regulates this local $O_2^{\cdot-}$ production from the SR. Using confocal microscopic imaging analysis in intact single CAMs, a cell-permeable indicator CM-H_2DCFDA for dynamic changes in intracellular ROS (in green color) and a highly selective ER-Tracker™ Red dye for tracking of the SR were found co-localized. A quantitative analysis based on the intensity of different spectra demonstrated a local $O_2^{\cdot-}$ production derived from the SR. M_1-receptor agonist, oxotremorine (Oxo) and a Ca^{2+} ionophore, A23187, time-dependently increased this $O_2^{\cdot-}$ production colocalized with the SR. NOX inhibitors, diphenylene iodonium (DPI) and apocynin (Apo), or superoxide dismutase (SOD) and catalase, and Nox4 (a major intracellular NOX subunit) siRNA all substantially blocked this local production of $O_2^{\cdot-}$, demonstrating an involvement of NOX. This SR-derived $O_2^{\cdot-}$ production was also abolished by the inhibitors of cyclic ADP-ribose (cADPR)-mediated Ca^{2+} signaling, such as nicotinamide (Nicot, 6 mM), ryanodine (Rya, 50 μM) or 8-Br-cADPR (30 μM). However, IP_3 antagonist, 2-APB (50 μM) had no effect. In CAMs transfected with siRNA of ADP-ribosyl cyclase or RyR, this SR $O_2^{\cdot-}$ production was attenuated. Electron spin resonance (ESR) spectromic assay in purified SR also demonstrated the production of $O_2^{\cdot-}$ that was dependent on NOX activity and Ca^{2+} concentrations. These results provide direct evidence that $O_2^{\cdot-}$ could be locally produced via NOX on the SR and that this local $O_2^{\cdot-}$ producing system is controlled by cADPR-RyR/Ca^{2+} signaling pathway.

Keywords: Redox signaling; Coronary circulation; Second messenger; Vascular smooth muscle; Nucleotides; ADP-ribose

1. Introduction

NAD(P)H oxidase (NOX) has been reported to be one of major sources of reactive oxygen species (ROS) in the vasculature[1–3]. This vascular redox regulatory enzyme is of characteristics of phagocyte NOX, which is composed of two transmembrane-bounded catalytic proteins of $gp91^{phox}$ and $p22^{phox}$, and three cytosol-associated subunits of $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$. In addition to $gp91^{phox}$ named as Nox2, some other homologues of $gp91^{phox}$ such as Nox1, Nox4 and Nox5 were identified in the vascular cells such as endothelial and smooth muscle cells [3,4]. NOX is now well accepted as an important enzyme that produces $O_2^{\cdot-}$ in the vasculature under physiological and pathological conditions to exert redox regulatory action on vascular function or to produce pathogenic responses.

In vascular smooth muscle cells (VSMCs), many studies have demonstrated that $O_2^{\cdot-}$ is accumulated when NOX is activated [1–3]. This intracellular accumulation of $O_2^{\cdot-}$ led to an assumption that a plasma membrane-bound NOX may produce and release $O_2^{\cdot-}$ into cells, which is different from the orientation of phagocyte NOX [1]. However, the topologic analysis has indicated that membrane-associated NOX should not release $O_2^{\cdot-}$ into cytosol [5]. Recent studies on subcellular localization of vascular NOX have also demonstrated that $O_2^{\cdot-}$ within VSMCs may not be derived from plasma membrane NOX (mNOX), but rather from intracellular compartmental NOXs[3,6,7]. We have recently reported that mNOX in VSMCs produced $O_2^{\cdot-}$ in autocrine or paracrine producing manner when the cells were exposed to different agonists [7]. Using purified sarcoplasmic reticulum (SR) from coronary arterial myocytes

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(CAMs), we also demonstrated that an NOX is present on the SR, and this oxidase locally activates the cyclic ADP – ribose (cADPR) – sensitive ryanodine receptors/Ca^{2+} (RyR/Ca^{2+}) release channels [3]. cADPR is a novel Ca^{2+} mobilizing second messenger, which is capable of inducing Ca^{2+} release from the SR via activation of RyR in CAMs[8–12]. Oxotremorine, a specific M_1 mAChR agonist, has been demonstrated to stimulate ADP-ribosylcyclase activity (CD38) and increase production of cADPR in vascular smooth muscle cells [13,14]. However, so far it is unknown how the SR NOX system associates with cADPR-RyR/Ca^{2+} signaling pathway in these smooth muscle cells.

The present study was designed to address these questions. First, we directly measured the dynamic changes in ROS production in intact CAMs by confocal microscopy using CM-H_2DCFDA as a cell-permeable green indicator for ROS and a highly selective ER-Tracker™ red dye for labeling of the SR. A spectrum-based analysis was used to differentiate ROS production derived from the SR or other sources when these cells were stimulated by M1-agonist. Second, we examined the role of NOX in $O_2^{\cdot-}$ production from the SR by using its inhibitors and siRNA of Nox4. To explore the mechanism regulating this SR NOX activity, we tested whether this enzyme is controlled by local Ca^{2+} level and corresponding Ca^{2+} signaling molecules. Using electron spin resonance (ESR) spectrometry, the sensitivity of the SR NOX was analyzed to further determine the Ca^{2+} regulation of NOX activity on the SR.

2. Materials and methods

2.1. Isolation and culture of CAMs

The bovine CAMs were cultured as described previously [14–16]. In brief, bovine intramyocardial coronary arteries from left anterior descending artery were dissected and rinsed with 5% FBS in medium 199 containing 25 mM HEPES with 1% penicillin, 0.3% gentamycin, and 0.3% nystatin. The arterial lumen was filled with 0.4% collagenase in medium 199. After 30 min of incubation at 37 °C, the arteries were flushed with medium199 to denude the endothelium. The strips of denuded arteries were then cut into small pieces and placed into gelatin-coated flasks with medium 199 containing 10% FBS with 1% L-glutamine, 0.1% tyrosin, and 1% penicillin–streptomycin for 3–5 days until CAMs migration to the flasks. Once growth was established, the arteries were removed and cells were continuously grown in medium 199 containing 20% FBS. The identification of CAMs was based on positive staining by an anti-α-actin antibody. All studies were performed with cells of passage of 2–4.

2.2. Confocal fluorescent microscopic detection of ROS localized around the SR in CAMs

Intracellular ROS production around the SR was monitored by their trapping with 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) and simultaneous labeling of the SR by ER-Tracker™ red dye using confocal laser scanning microscopy [7]. Subconfluent CAMs plated on the Φ35 mm cell culture plates were washed for three times with Hanks' buffered saline solution (HBSS) that contains (in mM): 137 NaCl, 5.4 KCl, 4.2 $NaHCO_3$, 3 Na_2HPO_4, 0.4 KH_2PO_4, 1.5 $CaCl_2$, 0.5 $MgCl_2$, 0.8 $MgSO_4$, 10 glucose, 10 HEPES (pH 7.4), then incubated at 37 °C with 5% CO_2 in the same HBSS buffer containing 1 μM ER-Tracker™ red dye for 15 min, followed by adding ROS detection agent CM-H_2DCFDA to a final concentration of 1 μM and continuing to incubate for another 15 min. To examine the effects of $O_2^{\cdot-}$ scavengers, NAD(P)H oxidase inhibitor, ADP-ribosyl cyclase antagonist, or the SR Ca^{2+} release channel blockers on the Oxotremorine(Oxo)-induced intracellular ROS production, CAMs loaded with both dyes were pretreated with polyethylene-glycol (PEG)-conjugated $O_2^{\cdot-}$ dismutase (PEG-SOD; 200 U/ml) [17] plus polyethylene-glycol (PEG)-conjugated catalase (CA; 200 U/ml) [18], diphenylene iodonium (DPI; 50 μM), ryanodine (Ryr; 50 μM) or 8-Br-cADPR (30 μM) for 15 min, respectively in different groups of cells and then Oxo-induced ROS production was re-determined. Confocal fluorescent microscopic images were acquired by an Olympus Fluoview System (version 4.2, FV300), which consists of an Olympus BX61WI inverted microscope with an Olympus Lumplan F1×60, 0.9 numerical aperture, and water-immersion objective. Real-time ROS generation was detected as a result of H_2DCF oxidation (Green image) by a single z-section, or 0.1-μm sections with excitation and emission wavelengths of 488 and 515 nm, and the staining of SR (Red image) was simultaneously recorded at excitation/emission of 587/615 nm. The subcellular source of ROS was determined by overlaying the ROS green image and SR organelle red image and then the merged images were analyzed off line by using co-localization function of Image Pro-Plus software. For each group of images, the intensity of different color spectrum areas was quantitated. Yellow spots were considered as ROS co-localized around the SR relative to green areas that represent intracellular global ROS or ROS from other resources. The relative fluorescence intensity to basal level before any stimuli was used as normalized fluorescence intensity to present the production of ROS.

2.3. RNA interference of Nox4, ADP-ribosyl cyclase and Ryr/Ca^{2+} receptor in CAMs

In addition to pharmacological intervention, RNA interference was also performed to silence the genes coding NOX subunits dominantly expressed in the SR, Nox4, ADP-ribosyl cyclase and ryanodine receptor 2 (RyR2) in CAMs to further demonstrate that NOX is responsible for the local $O_2^{\cdot-}$ production around the SR and cADPR/RyR Ca^{2+} signaling pathway in the regulation of SR NOX activity. Three pairs of small inhibitory RNA (siRNA) for each gene were chosen using QIAGEN siRNA design program and synthesized and double-stranded by Xeragon. These pairs of siRNA were tested for knocking down specific gene to find a most efficient pair of siRNA for our experimental protocols. A scrambled RNA or Xeragon library scrambled RNA was synthesized for negative control. The most effective pair of siRNA for targeting CD38 (NM_175798) consisted of AAGCGATCAGGCAGGCATTCA and its countpartner; for RyR2 (XM_617538) was AACTGCCAGAGCCAGTAAAAT; and for Nox4 (XM_614713) was AAGACCTGGCCAGTATATATTAT. siRNA transfection was performed according to the manufacturer's instruction in Qiagen TransMessenger kit and as we described previously [7].

2.4. Preparation of purified SR from bovine coronary arterial smooth muscle

Fresh bovine hearts were obtained from a local abattoir, and the coronary arteries were rapidly dissected, and SR-enriched microsomes (SR membrane) of these arteries were prepared as we described previously [3,10,11]. Briefly, the dissected coronary arteries (outer diameter 500–1000 μm) were cleared of surrounding fat and connective tissues. The arteries were cut open along the longitudinal axis and pinned lumen side up to a Sylgard-coated dish containing ice-cold HEPES buffered physiological saline solution (in mM: 140 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.17 MgSO4, 1.18 NaH_2PO4, 5.5 glucose, and 10 HEPES, pH 7.4). A sharp blade was used to scratch endothelial cells from the arteries. Then the segments of the arteries were cut into small (2- to 3-mm-long) pieces and homogenized with a Polytron (Brinkman) in ice-cold MOPS buffer (0.9% NaCl, 10 mM MOPS (pH 7.0), 2 M leupeptin, and 0.8 M benzamidine). The homogenate was centrifuged at 4000 *g* for 20 min at 4 °C, and the supernatant was further centrifuged at 8000 *g* for 20 min at 4 °C and then at 40,000 *g* for 30 min. The pellet, termed the crude SR membrane, was resuspended in the SR solution (0.9% NaCl, 0.3 M sucrose, and 0.1 M phenylmethylsulfonyl fluoride) [10]. The crude SR was further fractionated on a discontinuous sucrose gradient [3,19,20]. The following sucrose solutions (percent by weight) containing 10 mM HEPES, pH 7.0, were layered sequentially in a centrifuge tube (model SW28, Beckman) as follows: 4 ml of 45%, 7 ml of 40%, 12 ml of 35%, 7 ml of 30%, and 4 ml of 27%. Crude SR (30 mg) was layered on top of the gradient, and the tube was spun at 64,000 *g* overnight. A fraction from 37–40% sucrose contained the purified SR, which was collected and diluted in