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Original article

Adenosine A_2 receptor activation ameliorates mitochondrial oxidative stress upon reperfusion through the posttranslational modification of NDUFV2 subunit of complex I in the heart

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ABSTRACT

While it is well known that adenosine receptor activation protects the heart from ischemia/reperfusion injury, the precise mitochondrial mechanism responsible for the action remains unknown. This study probed the mitochondrial events associated with the cardioprotective effect of 5′-(N-ethylcarboxamido) adenosine (NECA), an adenosine A_2 receptor agonist. Isolated rat hearts were subjected to 30 min ischemia followed by 10 min of reperfusion, whereas H9c2 cells experienced 20 min ischemia and 10 min reperfusion. NECA prevented mitochondrial structural damage, decreases in respiratory control ratio (RCR), and collapse of mitochondrial membrane potential ($\Delta\Psi m$). Both the adenosine A_{2A} receptor antagonist SCH58261 and A_{2B} receptor antagonist MRS1706 inhibited the action of NECA. NECA reduced mitochondrial proteins carbonylation, H_2O_2, and superoxide generation at reperfusion, but did not change superoxide dismutase (SOD) activity. In support, the protective effects of NECA and Peg-SOD on $\Delta\Psi m$ upon reperfusion were additive, implying that NECA's protection is attributable to the reduced superoxide generation but not to the enhancement of the superoxide-scavenging capacity. NECA increased the mitochondrial Src tyrosine kinase activity and suppressed complex I activity at reperfusion in a Src-dependent manner. NECA also reduced mitochondrial superoxide through Src tyrosine kinase. Studies with liquid chromatography-mass spectrometer (LC-MS) identified Tyr118 of the NDUFV2 subunit of complex 1 as a likely site of the tyrosine phosphorylation. Furthermore, the complex I activity of cells transfected with the Y118F mutant was increased, suggesting that this site might be a negative regulator of complex I activity. In support, NECA failed to suppress complex I activity at reperfusion in cells transfected with the Y118F mutant of NDUFV2. In conclusion, NECA prevents mitochondrial oxidative stress by decreasing mitochondrial superoxide generation through inhibition of complex I via the mitochondrial Src tyrosine kinase. Phosphorylation of Tyr^{118} residue in NDUFV2 subunit may account for the inhibitory effect of NECA on complex I.

1. Introduction

Early studies have demonstrated that reperfusion itself produces reactive oxygen species (ROS) [1,2] and ROS contribute to myocardial injury at reperfusion [3]. Mitochondria are the major source of ROS in the heart [4] and mitochondrial ROS over-production at reperfusion triggers the mitochondrial permeability transition pore (mPTP) opening and cell death [5]. Ischemic preconditioning induces cardioprotection by preventing the mPTP opening through reduction of mitochondrial oxidative stress [6]. It has also been reported that reversible inhibition of the electron transport chain upon reperfusion may be cardioprotective by modulating ROS generation [7]. These reports suggest that prevention of mitochondrial oxidative stress is critical for cardioprotective interventions in the setting of ischemia/reperfusion injury.

Early studies demonstrated that adenosine A_2 receptor activation prevents myocardial reperfusion injury by reducing neutrophil superoxide generation [8,9]. A_2 receptor activation could also attenuate reperfusion injury by preventing ROS generation from cardiomyocytes [10]. However, these studies did not determine if A_2 receptors target mitochondrial ROS generation. In a later study, Xi et al. demonstrated

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that A_2 receptor activation with NECA induced cardioprotection against reperfusion injury by modulating the mPTP opening through inactivation of mitochondrial glycogen synthase kinase 3β (GSK-3β) in isolated rat hearts [11]. Similarly, NECA was also shown to inhibit the mPTP opening caused by H_2O_2 in rabbit cardiomyocytes [12]. Thus, it is conceivable that A_2 receptor activation may induce cardioprotection by alleviating mitochondrial oxidative stress, since mitochondrial ROS are the main trigger of the mPTP opening.

Complex I catalyzes oxidation of NADH by ubiquinone and is a major source of mitochondrial ROS generation [13]. Reversible S-nitrosation and inhibition of mitochondrial complex I lead to cardioprotection against reperfusion injury presumably through prevention of mitochondrial ROS generation [14,15]. Phosphorylation of complex I plays a role in cell survival and death [16] and modulates mitochondrial ROS generation [17], suggesting that posttranslational modifications of components in complex I may play a role in the regulation of mitochondrial oxidative stress and cell survival. Supporting this notion, we have recently demonstrated that ischemic preconditioning and morphine-induced pharmacological preconditioning protect the heart from ischemia/reperfusion injury by modulating mitochondrial oxidative stress through inhibition of complex I activity via mitochondrial Src tyrosine kinases [18,19]. Nevertheless, the precise mechanism by which Src tyrosine kinase suppresses complex I activity remains unknown.

The purpose of this study was to test if activation of adenosine A_2 receptors prevents mitochondrial oxidative stress by modulating complex I activity at reperfusion and to define the molecular mechanism by which A_2 receptor activation leads to the alteration of complex I activity.

2. Methods

This study conforms to the NIH Guide for the Care and Use of Laboratory Animals (NIH publication NO. 85–23, revised 1996).

2.1. Chemicals

NECA, SCH58261, MRS1706 and PP2 were purchased from Tocris Bioscience (Ellisville, MO). Antibodies including phospho-Src (Tyr^{416}), P-Tyr-100, COXIV and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-DNP antibody was purchased from Sigma-Aldrich Chemical Company. JC-1, total superoxide dismutase assay kit, GSH and GSSG assay kit and site-directed gene mutagenesis kit were purchased from Beyotime. Other fluorescence probes were purchased from Molecular Probes (Eugene, OR, USA). Complex I enzymatic activity microplate assay kit and complex I Immunocapture monoclonal antibody were purchased from Abcam.

2.2. Perfusion of isolated rat heart

Male Wistar rats (250–350 g) were anesthetized with sodium pentobarbital (100 mg kg^{-1}. i.p.). The hearts were removed rapidly and mounted on a Langendorff apparatus. The hearts were perfused with Krebs-Henseleit buffer containing (in mM) 118.5 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.8 $CaCl_2$, 24.8 $NaHCO_3$, 1.2 KH_2PO_4, and 10 glucose, which was heated to 37 °C and gassed with 95%O_2/5% CO_2. A latex balloon connected to a pressure transducer was inserted into the left ventricle through the left atrium. The left ventricular pressure and heart rate were continuously recorded with a PowerLab system. A 5-0 silk suture was placed around the left coronary artery, and the ends of the suture were passed through a small piece of soft vinyl tubing to form a snare. All hearts were allowed to stabilize for at least 20 min. Ischemia was induced by pulling the snare and then fixing it by clamping the tubing with a small hemostat. Total coronary artery flow was measured by timed collection of the perfusate dripping from the heart into a graduated cylinder.

2.3. Mitochondrial isolation

Cardiac tissues collected from the ischemic zone 10 min after the onset of reperfusion in rat hearts were used for the mitochondrial isolation. Mitochondrial and cytosolic fractions were isolated by differential centrifugation via a tissue mitochondria isolation kit (Beyotime) and according to the manufacturer's instructions.

2.4. Transmission electron microscopy (TEM)

Tissue ($< 1\ mm^3$) or isolated mitochondrial pellets from risk area were fixed in 2% glutaraldehyde containing 5 mM $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.3) for 1 h at the room temperature, and then rinsed twice with the buffer and fixed with 1% osmium tetroxide and 0.8% potassium ferricyanide for 1 h at 4 °C. After dehydration with alcohol, samples were embedded in Araldite (Electron Microscopy Sciences). Ultrathin sections of the samples were cut with Ultra 150 ultramicrotome (Pabish) and collected on 300-mesh copper grids. At least three grids were prepared for each sample and viewed with a transmission electron microscope (H-7650, Hitachi, Tokyo, Japan).

2.5. Mitochondrial respiration

Mitochondrial oxygen consumption was measured at 37 °C using an Oroboros Oxygraph-2k system. Briefly, isolated mitochondria were added to 2 ml of a mitochondrial respiration medium containing (in mM) 225 mannitol, 70 sucrose, 1 EDTA, 10 KH_2PO_4, 10 K_2HPO_4 (pH 7.4). State 2 respiration was initiated with 4 mM malate and 5 mM glutamate were added. Once the state 2 respiration was established the state 3 respiration was stared with an addition of 2.5 mM ADP. When all the added ADP was phosphorylated to ATP, the respiration rate was returned to the state 4. The respiratory control ratio (RCR) was calculated as the ratio of the respiratory rate in the state 3 to that in the state 4.

2.6. Cell culture

Rat heart tissue-derived H9c2 cardiac myoblast cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in specific temperature-controlled culture dishes with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 100 U penicillin/streptomycin at 37 °C in a humidified 5% CO_2–95% air atmosphere.

2.7. Measurement of ΔΨm

ΔΨm was measured by loading isolated mitochondria and H9c2 cells with JC-1, a cell permeable fluorescent dye. Briefly, mitochondria or H9c2 cells cultured in a specific temperature-controlled culture dish (MatTek, MA, USA) were incubated with JC-1 (100 nM) in either mitochondrial storage buffer (Beyotime) or a standard Tyrode solution containing (in mM) NaCl 140, KCl 6, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 5 and glucose 5.8 (pH 7.4) for 40 min. The fluorescence intensity of mitochondrial samples were determined with a fluorescence plate reader, while the fluorescence changes in cells were detected with an laser scanning confocal microscope (Olympus FV 1200). The green fluorescence of JC-1 monomer was excited with a 488-nm line of helium–neon laser line and imaged through a 525-nm-long path filter. Meanwhile, the red fluorescence of JC-1 aggregation was excited with a 543-nm line of helium–neon laser line and imaged through a 590-nm-long path filter. Temperature was maintained at 37 °C.

2.8. Measurement of mitochondrial ROS

Isolated mitochondria were incubated with 20 μM DCFH-DA for