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Vascular calcification abrogates the nicorandil mediated cardio-protection in ischemia reperfusion injury of rat heart

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ABSTRACT

The present study was aimed to determine the efficacy of nicorandil in treating cardiac reperfusion injury with an underlying co-morbidity of vascular calcification (VC). Adenine diet was used to induce VC in Wistar rat and the heart was isolated to induce global ischemia reperfusion (IR) by Langendorff method, with and without the nicorandil (7.5 mg/kg) pre-treatment and compared with those fed on normal diet. The adenine-treated rats displayed abnormal ECG changes and altered mitochondrial integrity compared to a normal rat heart. These hearts, when subjected to IR increased the infarct size, cardiac injury (measured by lactate dehydrogenase and creatine kinase activity in the coronary perfusate) and significantly altered the hemodynamics compared to the normal perfused heart. Nicorandil pretreatment in rat fed on normal diet enhanced the hemodynamics significantly ($P < 0.05$) along with a substantial reduction in the mitochondrial dysfunction (measured by high ADP to oxygen consumption ratio, respiratory control ratio, enzyme activities and less swelling behavior) when subjected to IR. However, this cardio-protective effect of nicorandil was absent in rat heart with underlying calcification. Our results suggest that, the protective effect of nicorandil, a known mitochondrial ATP linked K^+ channel opener, against myocardial reperfusion injury was confined to normal rat heart.

1. Introduction

Myocardial ischemia reperfusion (IR) injury is the outcome of inevitable restoration of blood flow to the ischemic heart characterized by the reduction in cardiac function and contributes to morbidity and mortality [1]. The response to IR may vary among individuals, depends on risk factors such as vascular calcification, diabetes, hypertension or hypercholesterolaemia, which increase the vulnerability of the microvasculature to IR injury [2]. Recent reports have shown that in case of patients undergoing dialysis or with chronic kidney disease (CKD), perioperative myocardial infarction is common, leads to postoperative morbidity and mortality and results in considerable impact on the length and cost of hospitalization [3].

Mitochondrial dysfunction, one of the prominent pathophysiological features of IR [4] is also reported to be involved in different calcification process of arteries [5]. The calcification nidus comprises of apoptotic bodies which are closely linked to mitochondrial dysfunction in chronic kidney diseases [6], emphasizing the role of mitochondria in the progression of the disease. Recently, researchers have shown that the heart tissue comprises of two distinct mitochondrial subpopulations namely interfibrillar mitochondria (IFM), present in the myofibrils, and subsarcolemmal mitochondria (SSM) present underneath the plasma membrane and we have shown that these subpopulations respond distinctly towards IR and corresponding cardiac conditioning [7], assume to be modulated in adenine-induced vascular calcification.

Nicorandil, a nitrite-like antianginal drug has been wildly used as an ATP-sensitive potassium-channel (K^+_{ATP}) opener and a potent activator of guanylyl cyclase [8]. It is shown to have significant protection against ischemia reperfusion injury in heart [9], kidneys [10], and brain [11]. Nicorandil administration to nephrectomized rat prevented oxidative injury, via expression of $K_{IR}6.2$ channel in the mitochondria. Similarly, by activating endogenous K_{ATP} channels, especially of mitochondrial origin, nicorandil reduces cellular damage resulting from cerebral ischemic stroke [10]. Mitochondria modulated drug mechanism to prevent

Abbreviations: VC, vascular calcification; IR, ischemia reperfusion; CKD, chronic kidney disease; SSM, subsarcolemmal mitochondria; IFM, interfibrillar mitochondria; CKD, chronic kidney disease; VSMC, vascular smooth muscle cell; RCR, respiratory control ratio; P/O ratio, ADP to oxygen consumption ratio.

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vascular calcification is shown by Kim and his co-workers [12], where they found that α-lipoic acid attenuates vascular calcification in vascular smooth muscle cell (VSMC) via recovery of mitochondrial metabolism and restoration of Gas6/Axl/Akt survival pathway.

Mounting evidences suggest that co-morbidities impede the cardiac response to multiple pro-survival signaling pathways, leaving cardiomyocytes more susceptible towards myocardial IR injury. Nicorandil, which has been successfully tested against vascular calcification as well as reperfusion injury in the rat model, is believed to target the mitochondria [13]. But a randomized controlled trials to investigate the effect of nicorandil prior to reperfusion therapy in PTCA patients by Wu and his co-workers [14] showed that even though nicorandil improve the coronary reflow, the definite clinical benefits of nicorandil were not found. Despite the cumulative data that establishes the protective effect of nicorandil against IR and VC, there is little empirical evidence for its therapeutic efficacy on VC rat heart subjected to IR and the focus of present study lies in this direction. Furthermore, the study also evaluates the contribution of cardiac mitochondrial subpopulations in understanding the mode of action of nicorandil.

2. Materials and methods

2.1. Animals

Animal experiments were conducted according to guidelines given by Committee for the purpose of conduct and supervision of experiments on animals (CPCSEA), Government of India, upon approval of the institutional animal ethical committee (IAEC) at SASTRA University, India (No. 258/SASTRA/IAEC/RPP). Eight week old male Wistar rats were purchased from the Central Animal Facility at SASTRA University, Thanjavur, India and housed in polycarbonate cages maintained at 25 ± 2 °C with 12 h light/dark cycle, at a relative humidity of 65 ± 2%. The animals were acclimatized for seven days prior to start of the study. All surgical procedures were performed under anesthesia using intra-peritoneal thiopental sodium (60 mg/kg b.wt.) and ECG was recorded using PowerLab data acquisition system (AD instruments, Australia).

2.2. Perfusion protocol

All rats were euthanized at the end of the study, and hearts were subjected to ischemia reperfusion injury using the isolated Langendorff heart system (AD instruments, Australia) except the hearts of the sham group, as per previously described protocol [7]. Briefly, hearts were excised, mounted on the Langendorff apparatus and subjected to perfusion using Krebs-Henseleit (KH) buffer (in mM): 118 NaCl, 1.2 KH_2PO_4, 4.7 KCl, 1.2 $CaCl_2$, 1.2 $MgSO_4$, 24.9 $NaHCO_3$ and 11.1 glucose, pH 7.4, equilibrated with 95% O_2 and 5% CO_2. A stabilization time of 20 min was given before global ischemia of 30 min followed by a 60 min reperfusion. The physiological parameters including end diastolic pressure (EDP), diastolic pressure (DP) and rate-pressure product were continuously monitored by inserting a balloon in the left ventricle and recorded using the Power Lab and Lab Chart-Pro module (AD instruments, Australia).

2.3. Experimental groups

The rats were randomly assigned to five groups of 6 animals each:

Group 1: Sham control; isolated rat hearts were perfused with KH buffer for 120 min as per the perfusion protocol given in Section 2.2 and the hemodynamics were recorded for the entire duration. At the end of reperfusion, hearts were flash frozen in liquid N_2 and stored at −80 °C.

Group 2: Ischemia reperfusion (IR) controls; isolated rat hearts were stabilized and subjected to 30 min ischemia followed by 60 min reperfusion. Hemodynamic changes were recorded to confirm stabilization and monitored till the end of reperfusion, to check the recovery. At the end of reperfusion, hearts were flash frozen in liquid N_2 and stored at −80 °C.

Group 3: Nicorandil treated normal animal (IR + NIC); rats were orally administered nicorandil (7.5 mg/kg b.wt.) for 28 days and the heart was subjected to IR injury as per the procedure in group 2.

Group 4: IR in vascular-calcified animal (VCIR): vascular calcification was induced as per our previously established protocol [15] using adenine diet for 28 days. The adenine diet causes renal dysfunction due to 2,8-dihydroxy adenine deposition and leads to increased circulating calcium and phosphorous levels, which promote the deposition of their products in vascular smooth muscles. The ECG was monitored after induction of calcification to evaluate the changes compared to normal rat and the hearts were isolated and subjected to IR injury as per the procedure in group 2.

Group 5: Nicorandil-treated vascular-calcified animal (VCIR + NIC); along with adenine diet as mentioned in group 4, the rats were treated concomitantly with nicorandil (7.5 mg/kg b.wt.) for 28 days orally through gavage needle. At the end of 28 days, heart was subjected to IR as per group 2 procedure.

At the end of reperfusion, all the heart tissues were frozen in liquid nitrogen and stored at −80 °C for further isolation of mitochondria and biochemical analysis.

2.4. Transmission electron microscopy of cardiac mitochondria

The heart tissue from a normal and vascular calcified rats were processed for ultrastructure imaging, using transmission electron microscopy (JOEL JEM 1400) at an accelerating voltage of 80 kV. Briefly, the tissues were fixed in 2.5% glutaraldehyde followed by 0.1% OsO_4 at 8 °C. Then the tissues were sectioned and dehydrated in graded series of acetone followed by propylene oxide treatment. The specimens were fixed in epon 812 resin mixture and ultra-sections were taken (Leica Ultracut R) for staining using uranyl acetate followed by lead citrate for imaging on copper grids.

2.5. Isolation of crude cardiac mitochondria

The crude mitochondrial fraction from rat heart was isolated as per previous protocol [16] with slight modification. Briefly, 100 mg of tissue was homogenized in ice cold isolation buffer (in mM): 220 mannitol, 70 sucrose, 20 HEPES, 2 Tris, 1 EGTA, adjusted to pH 7.2. The homogenate was subjected to centrifugation at 600 × *g* for 10 min at 4 °C. The supernatant from previous step was centrifuged at 12,000 × *g* for 10 min at 4 °C, to pellet the crude mitochondrial fraction.

2.6. Isolation of cardiac mitochondrial sub population

Rat cardiac mitochondrial subpopulations were isolated by differential centrifugation according to the method of Palmer [16]. Briefly, the crude mitochondrial pellet was subjected to two steps of 12,000 × *g* for 10 min at 4 °C to collect the sub sarcolemmal mitochondria (SSM). The homogenate pellet from the previous step (after removing the supernatant for crude mitochondrial fraction) was incubated in trypsin (0.5 mg/g), to disrupt the fibers and release the interfibrillar mitochondria (IFM). The pellet was suspended in the isolation buffer and subjected to centrifugation at 600 × *g* for 10 min at 4 °C. The supernatant was collected and centrifuged at 12,000 × *g* for 10 min at 4 °C, to pellet the IFM. The pelleted mitochondrial subpopulations were suspended in storage buffer (in mM): 25 sucrose, 75 sorbitol, 10 Tris-HCl 100 KCl, 10 K_2HPO_4, 5 $MgCl_2$ and 0.05 EDTA adjusted to pH 7.4 and stored at 4 °C until further analysis.