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Na^+/Ca^{2+} exchanger 1 inhibition abolishes ischemic tolerance induced by ischemic preconditioning in different cardiac models

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ABSTRACT

Ca^{2+}-handling disturbances play an important role in the genesis of myocardial ischemia/reperfusion (I/R) injury. Ischemic preconditioning (IPC) is a powerful strategy to induce tolerance against subsequent ischemic episodes. IPC signaling pathways may be triggered by Ca^{2+} ion. Since Na^+/Ca^{2+} exchanger 1 (NCX1) participates in modulating intracellular Ca^{2+} homeostasis, here we further defined its role in I/R and investigated its potential involvement in IPC-induced cardioprotection. In isolated ventricular cardiomyocytes, perfused rat heart and H9c2 cardiomyoblasts, I/R produced a significant cell injury, assessed by measuring extracellular lactate dehydrogenase (LDH) and, for the whole heart, also by estimating myocardial infarct size area. Characterization of cell death revealed the involvement of apoptotic processes. Interestingly, I/R challenge induced NCX1 protein upregulation. In NCX1-transfected H9c2 cells, exchanger protein upregulation was accompanied by an increase in its reverse mode activity. The effects of I/R on extracellular LDH and infarct size area were drastically reduced by 1 μM SN-6, a selective NCX1 inhibitor. Moreover, SN-6 also prevented I/R-induced increase of NCX1 reverse-mode activity and protein upregulation. These results suggested a deleterious role of NCX1 in I/R-induced cell damage.

In both isolated cardiomyocytes and perfused heart, IPC followed by I/R afforded cardioprotection, reducing extracellular LDH release and limiting ischemic area extent. Interestingly, NCX1 blockade (1 μM SN-6) completely abolished IPC protection against I/R, leading to exacerbation of cell injury, massive infarct size area and restoration of NCX1 protein expression.

These findings suggest that NCX1 is deleterious in I/R, whereas it may be beneficial in promoting IPC-induced cardioprotection.

1. Introduction

Myocardial ischemia stems from a reduced blood flow to the heart, causing oxygen and substrates supply deprivation and leading to sudden biochemical and metabolic impairments. Cell metabolism is switched to anaerobic respiration, with accumulation of toxic glycolytic products, ATP depletion, ionic derangements and inhibition of myocardial contractile function (Frank et al., 2012). Time is muscle: the quicker the perfusion is restored, the more heart tissue is saved (Kalogeris et al., 2012). At reperfusion, however, blood flow restoration paradoxically initiates a cascade of events that imposes additional stress to cardiomyocytes, giving rise to ischemia/reperfusion (I/R) injury (Sanada et al., 2011; Yellon and Hausenloy, 2007). Over the last years different approaches have been explored to limit infarct size and to improve outcomes (Ibanez et al., 2015). One of the most promising interventions is based on the ischemic conditioning, whereby short periods of subcritical ischemic stimuli confer protection from lethal injury when performed before (preconditioning) (Murry et al., 1986), during (perconditioning) (Vinten-Johansen and Shi, 2011) or after (postconditioning) (Penna et al., 2008) the index ischemia. Specifically, ischemic preconditioning (IPC) can be applied minutes or even days before the lethal ischemia, thereby producing two windows of rever-

Abbreviations: $[Ca^{2+}]_i$, Intracellular Ca^{2+} concentration; HPC, Hypoxic Preconditioning; H/R, Hypoxia/Reoxygenation; IPC, Ischemic Preconditioning; I/R, Ischemia/Reperfusion; LDH, Lactate Dehydrogenase; NCX, Na^+/Ca^{2+}exchanger; SN-6, 2-[[4-[(4Nitrophenyl) methoxy] phenyl] methyl]–4-thiazolidinecarboxylic acid ethyl ester; TTC, Triphenyltetrazolium chloride; WT, Wild Type

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sible protection, referred as early and delayed phase (Marber et al., 1993). Several studies have been performed in order to characterize the biology of IPC according to different endogenous mediators that can promote innate protective responses (Cohen and Downey, 2008; Laude et al., 2003; Ohnuma et al., 2002). In this regard, modifications of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) have been recognized as important triggers. Specifically, transient and reversible fluctuations in $[Ca^{2+}]_i$ can mimic the protective effect of IPC (Meldrum et al., 1996; Miyawaki and Ashraf, 1997; Przyklenk et al., 1997).

Dysregulation of intracellular Ca^{2+} homeostasis seems to play a critical role in the I/R-induced cell injury. Persistent elevation of $[Ca^{2+}]_i$ ultimately causes much of the damage accompanying I/R (Garcia-Dorado et al., 2012), supporting the hypothesis that timing and magnitude of Ca^{2+} response during I/R and IPC stimuli are key determinant of the cell fate.

It is well established that Na^+/Ca^{2+} exchanger 1 (NCX1) is a critical protein contributing to the Ca^{2+} homeostasis in the heart (Lytton, 2007). NCX1 catalyzes the electrogenic and reversible exchange of 3 Na^+ for 1 Ca^{2+} across the plasma membrane, participating in excitation-contraction coupling (Aronsen et al., 2013), nodal pace-maker activity (Groenke et al., 2013) and cell metabolism (Magi et al., 2013, 2012). Alteration of the exchanger activity seems to contribute to the I/R-induced cell injury, since pharmacological or genetic inhibition of NCX1 significantly limits cardiac injury induced by I/R (Imahashi et al., 2005; Li et al., 2014; Ohtsuka et al., 2004). In contrast, up to date NCX1 role in IPC is poorly investigated, although Zhang and coworkers reported that a functional NCX1 may be effectively involved in this phenomenon (Zhang et al., 2015).

Thus, in the present study we aimed to further define the role of NCX1 in I/R and its specific contribution to IPC-induced protective adaptation in different cardiac models.

2. Materials and methods

2.1. Animals

Male Wistar rats (two-month old) were used for both *ex-vivo* experiments and cardiomyocytes isolation. All the rats were bred in-house at the University Politecnica of Marche Animal Facility. Animals were housed in a 12 h light–dark cycle at room temperature (22 ± 1 °C) with free access to food and water. The animal protocol was approved by the Ethic Committee for Animal Experiments of the University Politecnica of Marche (Ref no. 721/2015-PR). All the experiments were conducted in strict accordance with the guidelines of the Italian Ministry of Health (D.L.116/92 and D.L.111/94-B) and the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the National Institutes of Health (USA). All efforts were made to minimize the number of animals used as well as their suffering.

2.2. Preparation and isolated heart perfusion

Rats were anesthetized with 4% isoflurane in 100% O_2 and then intraperitoneally injected with 1 ml of heparin (5000 IU/ml). After 10 min, the chest was opened and 1 ml of heparin (160 UI/ml) was injected into the right atrium. Then the heart was quickly excised, attached to a modified Langendorff perfusion system and retrogradely perfused with an O_2-saturated HEPES buffered solution containing (in mM): NaCl 140, KCl 4, HEPES 10, Na_2HPO_4 0.5, $MgCl_2$ 1, $CaCl_2$ 1.5, glucose 15, pH 7.4 adjusted with NaOH. Perfusion solution was constantly gassed with O_2 and the temperature was maintained at 37 °C.

2.3. Ex vivo protocols of ischemia/reperfusion (I/R) and ischemic preconditioning (IPC)

All the perfused hearts were subjected to 10 min of normoxic perfusion with HEPES solution, i.e. stabilization period. Pharmacological inhibition of NCX1 was achieved by using 2-[[4-[(4Nitrophenyl) methoxy] phenyl] methyl]–4-thiazolidinecarboxylic acid ethyl ester (SN-6) (Iwamoto et al., 2004). Seven different experimental conditions were tested. A more detailed description of the groups is provided below.

- Group 1. Control (CTR): isolated hearts were perfused with HEPES buffered solution for 150 min
- Group 2. Control +1 μM SN-6 (CTR SN-6): isolated hearts were perfused with HEPES solution enriched with 1 μM SN-6 for 150 min
- Group 3. Ischemia/reperfusion (I/R): global ischemia was induced by interrupting both oxygen and HEPES solution flows (no-flow ischemia) for 30 min, during which the hearts were submerged into HEPES solution without glucose and maintained at 37 °C to avoid reduction in myocardial temperature. Reperfusion was accomplished by restoring oxygen and HEPES solution flows for 120 min
- Group 4. Ischemia/reperfusion +1 μM SN-6 (I/R SN-6): isolated hearts were perfused with 1 μM SN-6 for 10 min. Thereafter, they were subjected to I/R as described for group 3. 1 μM SN-6 was maintained throughout the entire I/R protocol.
- Group 5. Ischemic preconditioning control (IPC): isolated hearts were subjected to a sub-lethal stimulus made up of 3 bouts of 2 min no-flow ischemia separated by 3 min of reperfusion (Bulvik et al., 2012). Then, hearts were perfused with HEPES buffered solution for 150 min
- Group 6. Ischemic preconditioning followed by ischemia/reperfusion (IPC I/R): isolated hearts were subjected to IPC protocol, as described for group 5, followed by I/R as described for group 3.
- Group 7. Ischemic preconditioning +1 μM SN-6 followed by ischemia/reperfusion (IPC SN-6 I/R): isolated hearts were subjected to IPC I/R as described in group 6, but IPC protocol was preceded by 10 min perfusion with 1 μM SN-6, which was maintained throughout the entire IPC protocol. Thereafter, I/R was induced in the absence of the NCX inhibitor.

At the end of each protocol, hearts were removed and kept at −20 °C for at least 1 h.

2.4. Evaluation of myocardial ischemic area

Measurement of the ischemic area was performed by using triphenyltetrazolium chloride (TTC) staining (Bohl et al., 2009; Csonka et al., 2010; Palfi et al., 2005). TTC stains all living tissue brick red, leaving the ischemic area unstained (white). Frozen hearts were sliced perpendicularly along the long axis from apex to base in 2-mm thick sections and then incubated for 40 min at 37 °C with 1% TTC in 0.1 M phosphate buffer, pH 7.4. Sections were then fixed in 4% formalin for 24 h, rinsed with PBS and then photographed by using a digital camera. Ischemic area was estimated by Photoshop CS6 extended software (Adobe Systems Software, Ireland) and expressed as a percentage of the total heart area.

2.5. Isolation of rat adult ventricular cardiomyocytes

Cardiomyocytes were isolated by Collagenase type II-CLS2 (Worthington Biochemical Corporation, Lakewood, NJ) digestion using a modified Langendorff perfusion system as previously described (Lariccia et al., 2011; Magi et al., 2015). Briefly, the heart was retrogradely perfused with HEPES buffered solution containing 1.5 mM $CaCl_2$. After 2 min, the solution was switched to nominally Ca^{2+}-free HEPES buffered solution for 5 min, followed by perfusion with the same solution containing 100 μM EGTA for 2 min. After that, the heart was perfused with enzyme solution (100–150 U/ml) containing 30 μM blebbistatin (Sigma, Milan, Italy) until the heart became swollen and turned lightly pale (usually 10 min). The digestion was