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Cardiac ischemic preconditioning prevents dystrophin proteolysis by MMP-2 inhibition

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ABSTRACT: Dystrophin is a membrane-associated protein responsible for structural stability of the sarcolemma in cardiac myocytes and is very sensitive to ischemic damage. The goal of our study was to determine if ischemic preconditioning could prevent dystrophin breakdown through inhibition of matrix metalloproteinase-2 (MMP-2) activity. Isolated rabbit hearts were subjected to global ischemia with or without reperfusion in order to evaluate if dystrophin is preserved by ischemic preconditioning through MMP-2 inhibition. Ischemic preconditioning significantly reduced the infarct size induced by 30 min of ischemia and 180 min of reperfusion. Importantly, it also diminished dystrophin proteolysis and attenuated MMP-2 activity after 30 min ischemia. Thus, our study shows a novel protective role of ischemic preconditioning as a mechanism of preservation of plasma membrane integrity by inhibiting MMP-2 activation.

Ischemic preconditioning is a cardioprotective strategy whereby prior brief episodes of ischaemia and reperfusion induce a state of protection against a subsequent prolonged ischemia-reperfusion injury (Murry *et al.,* 1986). This phenomenon has been the subject of intense investigation during the last years to unravel the cellular pathways activated. However, the molecular mechanisms involved are complex and not yet fully elucidated.

Dystrophin is a membrane-associated cytoskeletal protein that confers structural stability to the cell membrane of cardiac myocytes. Since ischemia-reperfusion injury may cause cell death through plasma membrane rupture, preservation of dystrophin may be a key factor to protect the cell membrane.

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Matrix metalloproteinases (MMPs) are important enzymes that mediate different aspects of cardiovascular diseases. In this regard, MMP-2 plays an important role in early ischemia-reperfusion injury (Wang *et al.,* 2002). In addition, it has been shown that ischemic preconditioning mediates the restoration of dystrophin during reperfusion, which is consistent with the protective effect of ischemic preconditioning against ischemia-reperfusion injury (Kido *et al.,* 2004). Furthermore, signaling pathways involved in the mechanism of preconditioning influence the level or activation of MMPs (Xie *et al.,* 2004), and preconditioning inhibits ischemia-induced activation and release of MMP-2 (Lalu *et al.,* 2002). For these reasons, the objective of the present study was to determine if ischemic preconditioning can prevent dystrophin breakdown through inhibition of MMP-2 activity.

Experiments were performed on male New Zealand rabbits (1.8 – 2.5 kg). All the procedures were approved by the Animal Care and Research Committee of the University of Buenos Aires (CD 2079/07) and in compliance with the

Guide for the Care and Use of Laboratory Animals published by the National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011).

Rabbits were euthanized with pentobarbital (150 mg/kg, I.V.), and each heart was rapidly excised and mounted on a Langendorff apparatus by the aortic root. Each heart was perfused with Krebs-Henseleit buffer at pH 7.2–7.4 and bubbled with a 95% O_2-5% CO_2 gas mixture at 37° C. By using a pacemaker a constant heart rate of 200 beats/min was kept constant. A latex balloon connected to a pressure transducer was placed into the left ventricle and filled with saline to achieve an end-diastolic pressure near 10 mmHg. Coronary perfusion pressure was also evaluated using a pressure transducer connected to the perfusion line and coronary flow was adjusted to obtain a coronary perfusion pressure near 70 mmHg.

Animals were randomly allocated to the following experimental groups:

- Group 1 (n=5). Myocardial infarction was induced by 30 min of global no-flow ischemia by abruptly interrupting the flow delivered by the perfusion pump and followed by 180 min of reperfusion.
- Group 2 (n=5). An ischemic preconditioning protocol of 3 cycles of ischemia-reperfusion (5 min each) before 30 min of global ischemia and 180 min of reperfusion was performed.
- Group 3 (n=5). Hearts were perfused in normoxic (Nx) conditions for 30 min.
- Group 4 (n=6). An ischemic period of 30 min of global no-flow ischemia without reperfusion was applied.
- Group 5 (n=5). An ischemic preconditioning protocol of 3 cycles of ischemia-reperfusion (5 min each) before 30 min of global ischemia was performed without reperfusion.

Infarct size was measured using triphenyltetrazolium chloride (TTC). Using this method, a reperfusion period of at least 3h is necessary to ensure an appropriate washout of certain cardiac enzymes (i.e., lactate dehydrogenase). For this reason, we measured infarct size in groups 1 and 2 as follows: after 180 min of reperfusion hearts were immediately frozen and cut into 4-mm transverse slices from the apex to base. After incubation for 20 min in 1% TTC (pH 7.4, 37°C) and then immersion in 10% formalin for 24 h sections were scanned. With this procedure viable sections stain red, whereas nonviable (infarcted) sections remain unstained, and each surface area can be easily measured (Image Pro Plus, version 4.5). Infarct size was expressed as a percentage of the total left ventricular area.

Based on previous studies (Armstrong *et al.,* 2001; Rodríguez *et al.,* 2005) that showed that dystrophin is lost during ischemia and is not recovered in early reperfusion, we selected the end of the ischemia period (30 min) to evaluate dystrophin levels. For this purpose ventricular tissue samples from groups 3-5 were used to perform Western blot as previously described (Buchholz *et al.,* 2014). Blots were incubated with mouse monoclonal anti-dystrophin (1:1,000, MANDYS-8, D8168, Sigma-Aldrich) and later incubated with goat anti-mouse antibody (1:10,000) (Millipore). Blots were developed using the enhanced chemiluminescence method (Thermo Scientific) according to the manufacturer's instructions. Relative levels of dystrophin were quantified by densitometric analysis using Image Gauge 4.0 software (Fujifilm). To verify the position of the bands, a molecular weight marker was used (Page Ruler, no. 26616, Thermo Scientific). Also, a rabbit skeletal muscle sample was used as a positive control. Dystrophin tissue levels were expressed as a percentage of control (Nx) values.

Also, ventricular tissue samples obtained from groups 3-5 were used to determine gelatinolytic activity (caused by metalloproteinases) by zymographic analysis, as previously described (Donato *et al.,* 2010). Conditioned medium from the promyelocyte U-937 cell line was used as the activity standard for pro-MMP-2. Tissue MMP-2 activity was expressed as a percentage of control (Nx) values. Data are expressed as mean ± SEM. Intergroup comparisons were carried out using one-way ANOVA followed by t-tests with the P value adjusted for multiple comparisons using the Bonferroni test. Data comparisons were not significant unless the corresponding P value was $<0.05/k$, where k is the number of comparisons.

Results of the present study showed the known protective effect of ischemic preconditioning under our experimental conditions: infarct size in the group subjected to 30 min of ischemia and 180 min of reperfusion was 41.9 ± 4.5% of the left ventricular area, whereas in the group subjected to a preconditioning protocol before 30 min of ischemia and 180 min of reperfusion was 5.3 ± 1.3%, $P<0.05$.

Regarding dystrophin, results of Western blot analysis showed that ischemia caused a significant loss of membrane dystrophin, as previously reported, whereas ischemic preconditioning prevented dystrophin proteolysis caused by 30 min of global ischemia (Fig. 1, panel A). Concerning MMP-2 activity, ischemic preconditioning attenuated MMP-2 activation that occurs after 30 min of global ischemia (without reperfusion) (Fig. 1, panel B).

The present study shows that ischemic preconditioning inhibits MMP-2 activation and prevents the proteolysis of dystrophin, suggesting a protective mechanism of ischemic preconditioning on plasma membrane integrity.

Armstrong *et al.* (2001) showed in isolated rabbit myocytes, that dystrophin and spectrin proteolysis is an early manifestation of ischemic myocardial injury and that it can be the molecular basis for membrane fragility during the transition from reversible to irreversible ischemic myocardial injury. Later, Rodríguez *et al.* (2005) using anesthetized dogs subjected to myocardial ischemia-reperfusion showed

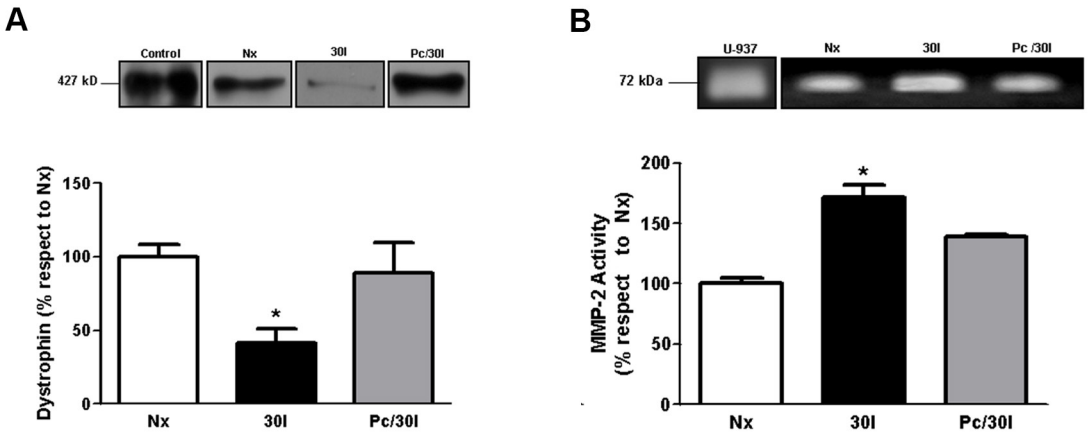

FIGURE 1. Panel A. dystrophin levels in the normoxic group (Nx), after an ischemic period of 30 min (30I), and in hearts subjected to an ischemic preconditioning protocol before 30 min of ischemia (Pc/30I). A skeletal muscle sample was used as control. Ischemic preconditioning prevented dystrophin ischemic proteolysis. Panel B. MMP-2 activity in the same experimental groups. U-937 indicates conditioned media from the promyelocyte U-937 cell line. MMP-2 activity increased during ischemia but decreased as a result of ischemic preconditioning. *P<0.05 vs. Nx and Pc/30I groups.

that dystrophin is highly sensitive to ischemia, and that its proteolysis is not reversed by reperfusion.

MMP-2 is present and can be intracellularly activated in cardiac myocytes by either proteolytic cleavage or oxidative stress, as it occurs during ischemia-reperfusion. Activated MMP-2 is able to cleave intracellular proteins, such as troponin I (Wang *et al.,* 2002), myosin light chain-1 (Sawicki *et al.,* 2005), α-actinin (Sung *et al.,* 2007) and titin (Ali *et al.,* 2010), and is involved in the mechanism of postischemic myocardial dysfunction (Singh *et al.,* 2004). We and others (D'Annunzio *et al.* 2009; Giricz *et al.,* 2006) have shown a close relationship between MMP-2 activity and infarct size. Furthermore, Lalu *et al.* (2002) showed that ischemic preconditioning reduces peroxynitrite ($ONOO^-$) formation, thus removing a major trigger for MMP-2 activation and decreasing MMP-2 activity. However, in our study dystrophin and MMP-2 measurements were performed after global ischemia, period in which oxidative stress would be minimal or absent. If there is an activation of MMP2 during ischemia mediated by $ONOO^-$ it would be quite inaccurate and other mechanisms involved in the activation of MMP-2 observed in our study should be considered. In that sense, it is possible that activation of MMP-2 during ischemia was due to phosphorylation / dephosphorylation mechanisms (Jacob-Ferreira *et al.,* 2013). Our results are in agreement with the ischemic loss of dystrophin previously reported and suggest a new possible pathway through which ischemic preconditioning may mediate infarct size reduction. In this regard, our results not only confirm previous studies suggesting that ischemic preconditioning could prevent dystrophin proteolysis (Lalu *et al.,* 2002; Kido *et al.,* 2004) but also suggests that this occurs through inhibition of MMP-2 activity. This could be possible through activation of protein kinase C (PKC) by ischemic preconditioning (Yang *et al.,* 2010), and subsequent phosphorylation and attenuation of MMP-2 activity (Sariahmetoglu *et al.,* 2012). However, this hypothesis needs still to be tested.

In conclusion, the present study shows that ischemic preconditioning inhibits MMP-2 activation, prevents dystrophin proteolysis and suggests a protective mechanism of ischemic preconditioning on plasma membrane integrity.

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