Am J Physiol Heart Circ Physiol 307: H88–H96, 2014.
First published May 2, 2014; doi:10.1152/ajpheart.00242.2013.

Dystrophin proteolysis: a potential target for MMP-2 and its prevention by ischemic preconditioning

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[1]*Institute of Cardiovascular Physiopathology, Department of Pathology, School of Medicine, and Institute of Biochemistry and Molecular Medicine, University of Buenos Aires, Buenos Aires, Argentina; and* [2]*Institute of Physiopathology and Clinical Biochemistry, Lipids and Lipoproteins Laboratory, Department of Clinical Biochemistry, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina*

Submitted 27 March 2013; accepted in final form 24 April 2014

Buchholz B, Perez V, Siachoque N, Miksztowicz V, Berg G, Rodríguez M, Donato M, Gelpi RJ. Dystrophin proteolysis: a potential target for MMP-2 and its prevention by ischemic preconditioning. *Am J Physiol Heart Circ Physiol* 307: H88–H96, 2014. First published May 2, 2014; doi:10.1152/ajpheart.00242.2013.—Dystrophin is responsible for the mechanical stabilization of the sarcolemma, and it has been shown that it is one of the most sensitive proteins to ischemic injury. However, the enzyme responsible for this proteolysis is still unknown. Isolated rabbit hearts were subjected to 30 min of global ischemia with and without reperfusion (180 min) to determine whether dystrophin is cleaved by matrix metalloproteinase (MMP)-2 during acute ischemia and whether ischemic preconditioning (PC) prevents dystrophin breakdown through MMP-2 inhibition. The activity of MMP-2 was evaluated by zymography and using doxycycline as an inhibitor. Also, to stimulate MMP-2 activity without ischemia, SIN-1 was administered in the absence and presence of doxycycline. Finally, we considered the PC effect on MMP-2 activity and dystrophin expression. The dystrophin level decreased during ischemia, reaching 21% of control values ($P < 0.05$), but the spectrin level remained unchanged. MMP-2 activity increased 71% during ischemia compared with control values ($P < 0.05$). Doxycycline administration before ischemia prevented dystrophin breakdown. In normoxic hearts, SIN-1 increased thiobarbituric acid-reactive substances by 33% ($P < 0.05$) and MMP-2 activity by 36% ($P < 0.05$) and significantly reduced the dystrophin level to 23% of control values ($P < 0.05$). PC significantly prevented dystrophin breakdown by inhibiting MMP-2 activity, and the dystrophin level reached 89% of control values ($P < 0.05$). In conclusion, MMP-2 could be responsible for the proteolysis of dystrophin. Thus, dystrophin emerges as a possible novel substrate for MMP-2 in the context of ischemic injury. Furthermore, our results demonstrate that ischemic PC prevents dystrophin breakdown most likely by inhibiting MMP-2 activity.

myocardial infarction; dystrophin; matrix metalloproteinase 2

ISCHEMIA-REPERFUSION INJURY causes cell death through different pathways, a key point being the rupture of the plasma membrane. Three major groups of structural proteins that link the extracellular and intracellular milieus and confer structural stability to the cell membrane are present in cardiac myocytes: the dystrophin-associated protein complex, the vinculin-integrin link, and the spectrin-based submembranous cytoskeleton (10). Armstrong et al. (4) demonstrated that dystrophin and spectrin breakdown may be the molecular basis for membrane fragility during the transition from reversible to irreversible ischemic myocardial injury. Their results were confirmed by Rodríguez et al. (19), who showed that, among these structural proteins, dystrophin was the most sensitive to ischemia and also that breakdown occurred during ischemia was not reversed by reperfusion.

Although it has been documented that dystrophin proteolysis occurs during ischemia (19), it remains to be proven which enzyme is responsible for this process. It has been suggested that proteolysis of dystrophin by a Ca^{2+}-activated protease, calpain I, is involved in the impairment of cardiac function (5). However, in the context of ischemia-reperfusion damage, calpain activation occurs upon reperfusion (11). It is also known that the family of matrix metalloproteinases (MMPs) is a group of enzymes important in mediating the injurious effects of cardiovascular diseases associated with enhanced oxidative stress. MMP-2, in particular, has been shown to play an important role in early ischemia-reperfusion injury (27) and could be responsible for the proteolysis of dystrophin.

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 (14). Furthermore, signaling pathways involved in the mechanism of preconditioning influence the level or activation of MMPs (28), and preconditioning inhibits ischemia-induced activation and release of MMP-2 (16).

Based on the above findings, the present work aimed to determine whether dystrophin proteolysis occurs as a consequence of activation of MMP-2 during an episode of acute ischemia. Since ischemic preconditioning inhibits MMP-2 activation, a second objective was to evaluate whether ischemic preconditioning prevents dystrophin breakdown through inhibition of MMP-2 activity.

MATERIALS AND METHODS

Ethical Approval

Experiments were performed on male New Zealand rabbits (1.8–2.5 kg). The procedures used in this study were approved by the Animal Care and Research Committee of the University of Buenos Aires (CD 2079/07) and were in compliance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (9).

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Surgical Procedures

Rabbits were euthanized with pentobarbital (150 mg/kg iv), and each heart was rapidly excised and mounted on a Langendorff apparatus by the aortic root. Each heart was perfused with Krebs-Henseleit buffer containing 118.5 mM NaCl, 4.7 mM KCl, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 10 mM glucose at pH 7.2–7.4 and bubbled with a 95% O_2-5% CO_2 gas mixture at 37°C. Two electrodes were placed and connected to a pacemaker with a constant heart rate of 200 beats/min.

A saline-filled latex balloon connected to a pressure transducer (Deltram II, Utah Medical System) was inserted into the left ventricle. The volume of the balloon was adjusted to achieve an end-diastolic pressure of 8–10 mmHg. Coronary perfusion pressure was also recorded using a pressure transducer connected to the perfusion line. Coronary flow was adjusted to obtain a coronary perfusion pressure of ~70 mmHg during the initial stabilization period. This flow level was held constant throughout the experiment.

Experimental Protocols

Experimental protocols are shown in Fig. 1. The following groups were used.

Group 1 (n = 5). Hearts were perfused under normoxic conditions (Nx) for 30 min.

Group 2 (n = 6). Myocardial infarction was induced by 30 min of global no-flow ischemia without reperfusion. Global no-flow ischemia was induced by abruptly decreasing the total coronary flow delivered by the perfusion pump.

Group 3 (n = 5). Myocardial infarction was induced by 30 min of global no-flow ischemia followed by 180 min of reperfusion.

Group 4 (n = 5). Myocardial infarction was induced by 30 min of global no-flow ischemia without reperfusion. Hearts received doxycycline (MMP inhibitor) before 30 min of ischemia. Doxycycline (50 μM) was administered for 5 min followed by 5 min of washout.

Group 5 (n = 6). Myocardial infarction was induced by 30 min of global no-flow ischemia followed by 180 min of reperfusion. Hearts received doxycycline before 30 min of ischemia. Doxycycline (50 μM) was administered for 5 min followed by 5 min of washout.

Group 6 (n = 6). A 100 μM bolus of *N*-morpholinosydnonimine hydrochloride (SIN-1) was administered for over 2 min, and hearts continued being perfused in normoxic conditions for 30 min.

Group 7 (n = 5). A 100 μM bolus of SIN-1 was administered for over 2 min, and hearts continued being perfused in normoxic conditions for 30 min. Before SIN-1 administration, we administered doxycycline (50 μM) for 5 min.

Group 8 (n = 5). An ischemic preconditioning protocol of three cycles of ischemia-reperfusion (5 min each) before 30 min of global ischemia and 180 min of reperfusion was performed.

Group 9 (n = 5). An ischemic preconditioning protocol of three cycles of ischemia-reperfusion (5 min each) before 30 min of global ischemia was performed without reperfusion.

Infarct Size Measurements

Infarct size was measured in *groups 3*, *5*, and *8*. After 3 h of reperfusion, hearts were frozen and cut into 4-mm transverse slices from the apex to base. Sections were incubated for 20 min in 1% triphenyltetrazolium chloride (pH 7.4, 37°C) and then immersed in 10% formalin. This technique stains viable sections red, whereas nonviable sections remain unstained. Sections were scanned and measured (Image Pro Plus, version 4.5). Infarct size was expressed as a percentage of the left ventricular area.

Thiobarbituric Acid-Reactive Substances Assay

Thiobarbituric acid-reactive substances (TBARS) were measured in *groups 1* and *6*. Left ventricular samples were homogenized in 5 volumes of buffer containing 140 mM KCl and 20 mM KH_2PO_4 with a protease and phosphatase inhibitor cocktail (Thermo Scientific) using a Pro-Scientific Pro 200 homogenizer. Homogenates were centrifuged at 6,539 *g* for 10 min; 0.5 ml of the supernatants were mixed with 1.5 ml of 10% trichloroacetic acid, 1 ml of 0.67% thiobarbituric acid, and 0.5 ml of distilled water. Samples were mixed, boiled for 30 min, and then centrifuged at 5,450 *g* for 10 min. TBARS were quantified at 530 nm. Results are expressed as nanomoles of malondialdehyde per milligram of protein. The extinction coefficient was 1.56×10^5 M.

Western Blot Analysis

Ventricular tissue samples from *groups 1–4*, *6*, *7*, and *9* were frozen and homogenized in buffer (pH 7.6) containing 1.2 mM Tris, 0.36 mM NaCl, 0.1% SDS, 1% Triton, 0.2 mM DTT and a protease

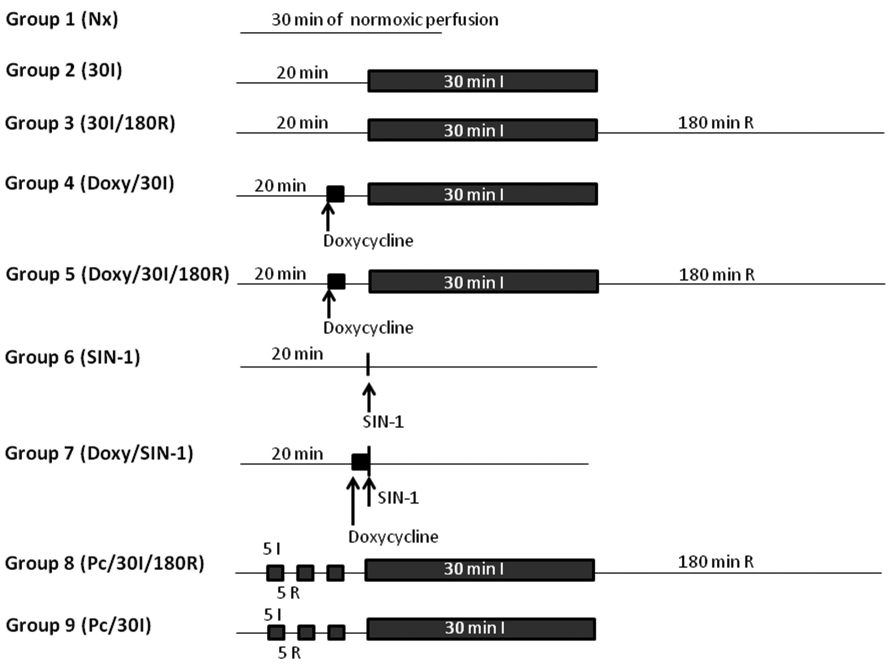

Fig. 1. Diagram showing the experimental protocols used in the present study. Nx, normoxic perfusion; 30I, 30 min of ischemia; 180R, 180 min of reperfusion; Doxy, doxycycline; SIN-1, *N*-morpholinosydnonimine hydrochloride; Pc, preconditioning.

and phosphatase inhibitor cocktail (Thermo Scientific) using a Pro-Scientific Pro 200 homogenizer. Homogenates were centrifuged at 26,156 *g* (4°C) for 20 min. The protein content in homogenates was analyzed using the Bradford protein assay (Bio-Rad). Equal amounts of protein (100 μg) were separated on 5–10% gradient SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Thermo Scientific). Given that some proteins proteolysis can occur during ischemia, the loading control was performed quantitating the total protein levels in the membrane dyed with Ponceau red (0.1%). Blots were incubated in blocking buffer containing 5% nonfat milk for 1 h at room temperature and then incubated with mouse monoclonal anti-dystrophin (1:1,000, MANDYS-8, D8168, Sigma-Aldrich), mouse monoclonal anti-spectrin (1:1,000, Millipore), or mouse monoclonal anti-β-dystroglican (1:50, Leica) overnight at 4°C. Later, blots were incubated with goat anti-mouse antibody (1:10,000) (Millipore) at room temperature for 1 h followed by rinses with Tris-buffed saline-Tween 20. Blots were developed using the enhanced chemiluminescence method (Thermo Scientific) according to the manufacturer's instructions. Relative levels of dystrophin, spectrin, and β-dystroglican were quantified by densitometric analysis using Image Gauge 4.0 software (Fujifilm). To verify the position of the bands, we used a molecular weight marker (Page Ruler, no. 26616, Thermo Scientific). Also, a sample of rabbit skeletal muscle was run as a positive control for dystrophin blots.

Zimography Analysis

Ventricular tissue samples from *groups 1*, *2*, *4*, *6*, *7*, and *9* were frozen and homogenized. Proteins were measured by Lowry's method. Metalloproteinase activity was detected by zymography. SDS-polyacrylamide gels (7.5%) were copolymerized with gelatin 0.1% (G-8150, Sigma). A constant amount of protein (2 μg) was loaded in each well under nonreducing conditions. Gels were run for 3 h in 25 mM Tris, 192 mM glycine, and 0.1% SDS at 4°C (pH 8.3) in a Mini Protean-3 (Bio-Rad). After being run, gels were rinsed with 2.5% Triton X-100 for 30 min and then incubated for 18 h in 0.15 M NaCl, 10 mM $CaCl_2$, and Tris·HCl (pH 7.4) at 37°C. After gels were stained with Coomassie blue R-250 (B-0149, Sigma) and destained with acetic acid-methanol-water (1:3:6), enzyme activity was detected as colorless bands against the blue-stained background. The gelatinolytic bands disappeared in parallel zymograms in which the development buffer contained EDTA (20 mM), confirming that the gelatinolytic activity was caused by metalloproteinases. Conditioned media from the promyelocyte U-937 cell line were used as the activity standard for pro-MMP-2. Coefficients of variation were 4.8% (intra-assay) and 8.6% (interassay). Gels were scanned, and band intensities were quantified using Sion-ImageJ software (Scion). Tissue MMP-2 activity was expressed as a percentage of control (Nx) values. To investigate the inhibitory profile of doxycycline (50 μM) on the gelatinolytic activity of MMPs, doxycycline was added to isolated hearts and also to the incubation buffer during the overnight incubation in some experiments (*groups 4*, *5*, and *7*) (6).

Immunostaining

Frozen sections (5 μm thick) were prepared using a cryostat, transferred to gelatin-covered glass slides, and fixed in 4% formaldehyde. Immunolabeling was performed using primary antibodies to dystrophin (mouse monoclonal anti-dystrophin, clone MANDYS-8, D8168, Sigma-Aldrich). Secondary antibody was biotinylated anti-rabbit IgG (Biotrend). Sections were analyzed using a Leica DM4000 B LED microscope and Leica DFC310 FX camera (Leica Microsystems) with Leica Application Suite LAS (version 4.2.0) software (Leica Microsystems).

Statistical Analysis

Data are expressed as means ± SE. 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

Figure 2*A* shows dystrophin levels in hearts from the Nx group, group subjected to 30 min of ischemia, and group subjected to 30 min of ischemia followed by 180 min of reperfusion. Dystrophin was lost during ischemia and was not recovered during reperfusion. It has been suggested that calpain may be responsible for dystrophin proteolysis. Hence, levels of spectrin, a well-known substrate of this enzyme, were also assessed (Fig. 2*B*). Our results showed that spectrin levels remained unchanged during ischemia but decreased during reperfusion, suggesting that calpain is not the enzyme responsible for dystrophin proteolysis.

Figure 3*A* shows MMP-2 activity in hearts from the Nx group, group subjected to 30 min of ischemia, and group treated with doxycycline and then subjected to 30 min of

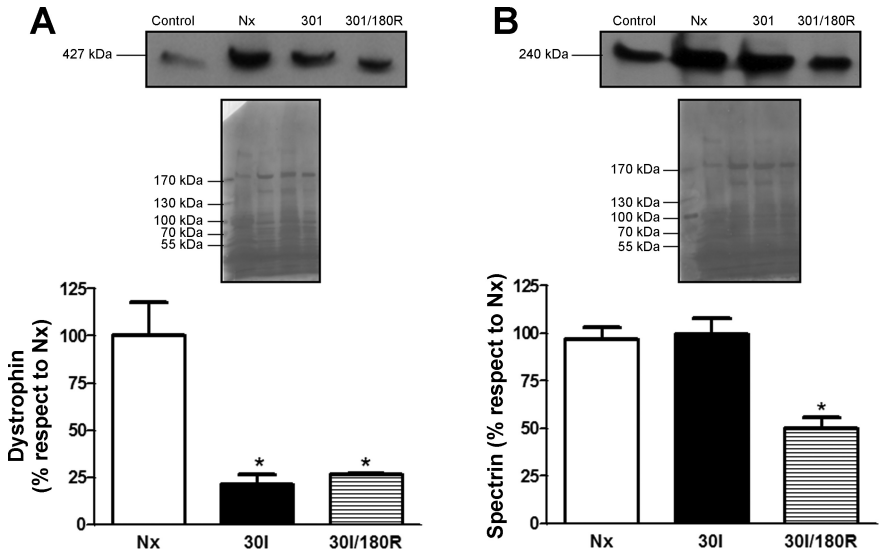

Fig. 2. Dystrophin (*A*) and spectrin (*B*) levels in hearts from the Nx group ($n = 5$), 30I group ($n = 6$), and 30I/180R group ($n = 5$). *A*: the dystrophin level decreased during ischemia and was not restored during reperfusion. $^*P < 0.05$ vs. the Nx group. *B*: conversely, spectrin proteolysis was observed during reperfusion. $^*P < 0.05$ vs. the Nx and 30I groups. There were no differences in the protein load according to membranes stained with Ponceau red. A skeletal muscle sample was used as the control.

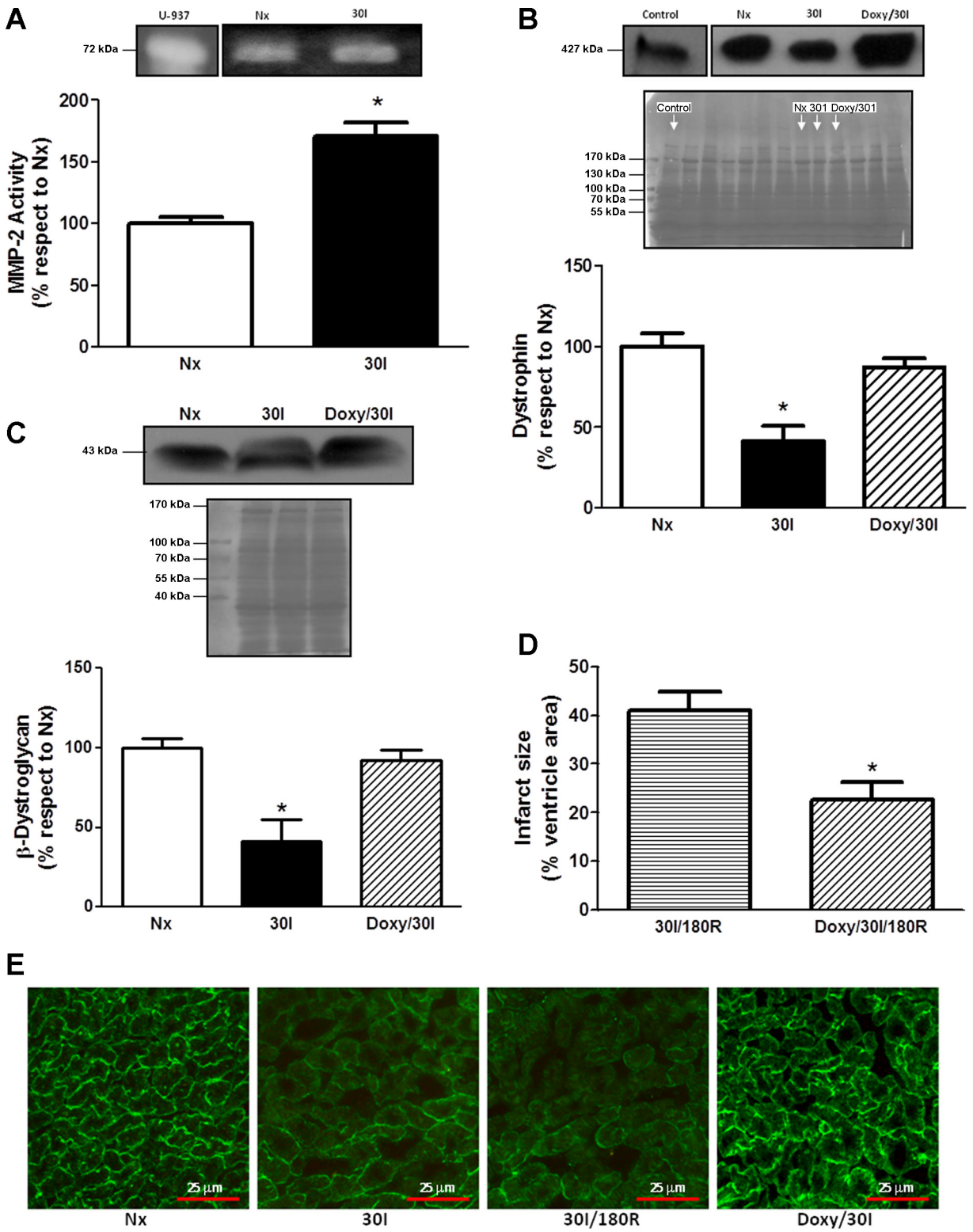

Fig. 3. Matrix metalloproteinase (MMP)-2 activity (*A*), dystrophin (*B*), and β-dystroglycan (*C*) levels in hearts from the Nx group ($n = 5$), 30I group ($n = 6$), and Doxy/30I group ($n = 5$). Ischemia increased MMP-2 activity ($P < 0.05$ vs. the Nx group), consistent with the proteolysis of dystrophin and β-dystroglycan; this effect was abolished by doxycycline ($P < 0.05$ vs. the Nx and Doxy/30I groups). There were no differences in the protein load according to membranes stained with Ponceau red. *D*: infarct size expressed as a percentage of the left ventricular area induced by 30 min of global ischemia followed by 180 min of reperfusion (30I/180R group; $n = 5$) and the protection conferred by doxycycline treatment (Doxy/30I/180R group; $n = 6$). *$P < 0.05$ vs. the 30I/180R group. *E*: representative images of the distribution of dystrophin in the myocyte membrane in hearts from the Nx, 30I, 30I/180R, and Doxy/30I groups. U-937 indicates conditioned media from the promyelocyte U-937 cell line. A skeletal muscle sample was used as the control.

ischemia. As described above, the same dose of doxycycline used on isolated hearts was also added to the zymography incubation buffer. Our results showed a significant increase in MMP-2 activity during ischemia and undetectable MMP-2 activity in doxycycline-treated hearts (data not shown). An ischemic period of 30 min produced a significant loss of dystrophin (Fig. 3*B*), whereas the administration of doxycycline to isolated hearts prevented membrane damage, preserving dystrophin. This demonstrates that inhibition of MMP-2 activity by doxycycline prevents the proteolysis of dystrophin. Figure 3*C* shows the expression of β-dystroglycan (a known substrate of MMP-2) in the same experimental groups. Ischemia significantly reduced the levels of this protein, whereas administration of doxycycline to isolated hearts prevented this detrimental effect. Figure 3*D* shows infarct sizes induced by 30 min of global ischemia and the protection conferred by doxycycline treatment. Figure 3*E* shows the distribution of dystrophin in the myocyte membrane in hearts from the Nx group, the group subjected to 30 min of ischemia and 180 min of reperfusion, and group treated with doxycycline before ischemia. An ischemic period of 30 min caused a significant loss of membrane dystrophin, and reperfusion was not able to restore it. However, treatment with doxycycline prevented the loss of membrane dystrophin caused by ischemia.

To confirm that dystrophin is a MMP-2 substrate, hearts were subjected to an increase in oxidative stress by means of SIN-1 administration, and lipid peroxidation was evaluated by measuring TBARS formation. Administration of SIN-1 significantly increased the TBARS concentration (Fig. 4*A*).

Figure 4*B* shows that MMP-2 activity increased significantly in hearts treated with SIN-1. When doxycycline was administered to isolated hearts, before the administration of SIN-1 and in the zymography incubation buffer at the same dose used in isolated hearts, undetectable MMP-2 activity was observed (data not shown). Dystrophin levels were assessed in the same experimental groups (Fig. 4*C*). The results showed that activation of MMP-2 induced by SIN-1 (without ischemia) significantly reduced dystrophin levels and that previous administration of the enzyme inhibitor prevented dystrophin proteolysis. This suggests that in those conditions, the dose used is also capable of inhibiting enzyme activity in vivo.

Ischemic preconditioning (without reperfusion) attenuated MMP-2 activity after 30 min of global ischemia (Fig. 5*A*) and prevented the proteolysis of dystrophin (Fig. 5*B*). Figure 5C shows the known effect of ischemic preconditioning on infarct size under our experimental conditions (group subjected to 30 min of ischemia and 180 min of reperfusion: 41.9 ± 4.5% vs. group subjected to preconditioning with 30 min of ischemia and 180 min of reperfusion: 5.3 ± 1.3%, $P < 0.05$). Finally, Fig. 5*D* shows the distribution of dystrophin in the myocyte membrane in hearts from the Nx group, group subjected to 30 min of ischemia, and group subjected to the ischemic preconditioning protocol. As described above, 30 min of global ischemia

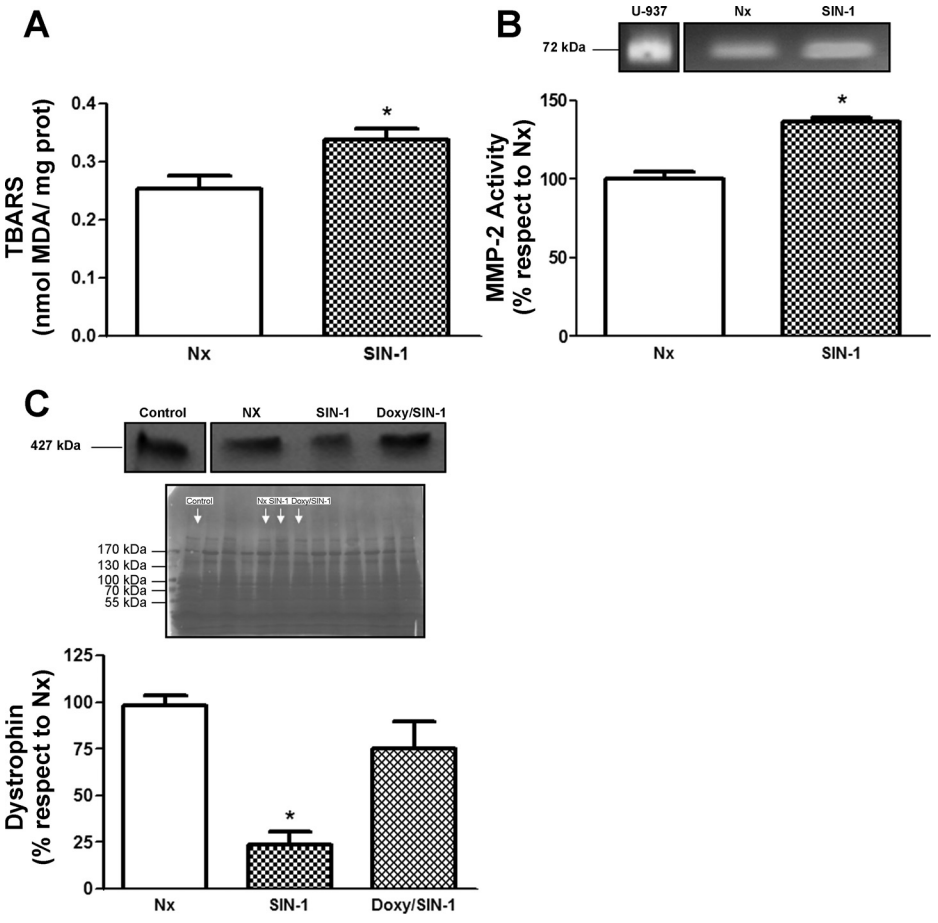

Fig. 4. *A*: concentration of thiobarbituric acid-reactive substances (TBARS) in hearts from the Nx group ($n = 5$) and SIN-1 group ($n = 6$). A significant increase in TBARS was observed after SIN-1 administration. *$P < 0.05$ vs. the Nx group. *B*: MMP-2 activity in hearts from the Nx group ($n = 5$), SIN-1 group ($n = 6$), and Doxy/SIN-1 group ($n = 5$). MMP-2 activity increased significantly in hearts treated with SIN-1. *$P < 0.05$ vs. the Nx group *C*: expression of dystrophin in the same groups. The administration of doxycycline prevented the proteolysis of dystrophin induced by SIN-1. *$P < 0.05$ vs. the Nx and Doxy/SIN-1 group. There were no differences in the protein load according to membranes stained with Ponceau red. U-937 indicates conditioned media from the promyelocyte U-937 cell line. A skeletal muscle sample was used as the control.

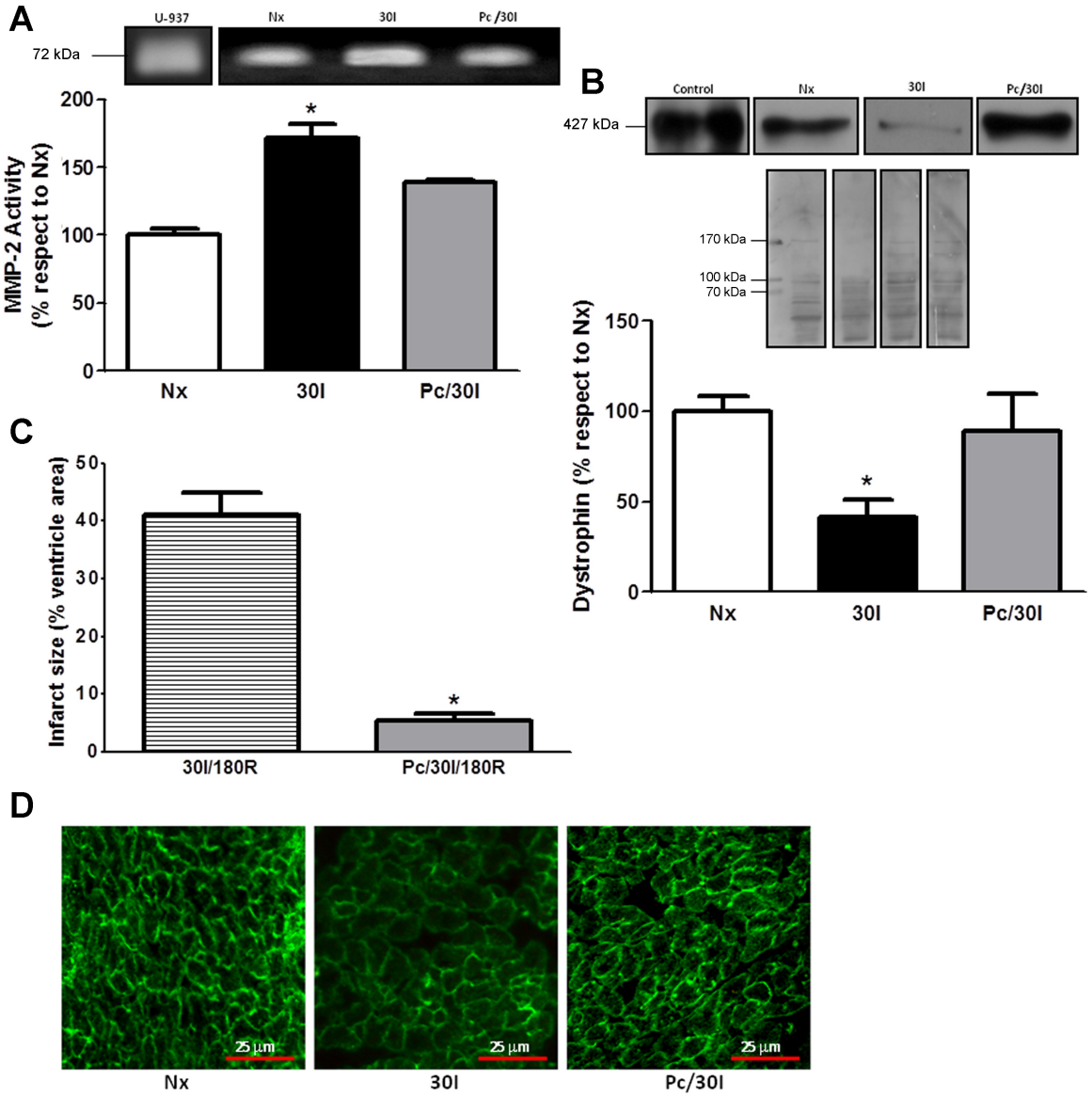

Fig. 5. MMP-2 activity (*A*) and dystrophin levels (*B*) in the Nx group ($n = 5$), 30I group ($n = 6$), and Pc/30I group ($n = 5$). MMP-2 activity increased during ischemia and decreased as a result of ischemic preconditioning. Ischemic preconditioning also prevented dystrophin proteolysis. *$P < 0.05$ vs. the Nx and Pc groups. There were no differences in the protein load according to membranes stained with Ponceau red. *C*: infarct size expressed as a percentage of the left ventricular area. Ischemic preconditioning ($n = 5$) significantly reduced the infarct size induced by 30 min of global ischemia and 180 min of reperfusion (Pc/30/180R group; $n = 5$). *$P < 0.05$ vs. the 30I/180R group. *D*: representative images of the distribution of dystrophin in the myocyte membrane in Nx, 30I, and Pc/30I hearts. U-937 indicates conditioned media from the promyelocyte U-937 cell line. A skeletal muscle sample was used as the control.

caused a significant loss of membrane dystrophin, whereas ischemic preconditioning prevented it.

DISCUSSION

The present study demonstrates that membrane dystrophin could be a new target of MMP-2 during ischemia. The administration of doxycycline, before ischemia, is capable of preventing the loss of dystrophin, suggesting that MMP-2 could be the enzyme responsible for dystrophin proteolysis. Thus, our data suggest a new role of MMP-2 in cardiac membrane destabilization during ischemia. Moreover, ischemic preconditioning inhibited MMP-2 activity, thus preventing the proteolysis of dystrophin and revealing a new protective mechanism of ischemic preconditioning.

Armstrong et al. (4) showed that dystrophin and spectrin proteolysis in isolated rabbit myocytes is an early manifestation of myocardial injury and that dystrophin proteolysis occurs during the ischemic episode. In contrast, Kyoi et al. (15), using a model of the isolated rat heart, showed that dystrophin translocates from the membrane to the myofibrils during ischemia and that protein proteolysis occurs during reperfusion. In accordance with the results by Amstrong et al. (4), other authors (19), using anesthetized dogs subjected to ischemia-reperfusion, also showed that dystrophin proteolysis occurs during ischemia and that this is one of the most sensitive proteins to ischemic injury. It is interesting to note that Amstrong et al. (4), Rodríguez et al. (19), and our laboratory used the same anti-dystrophin antibody (MANDYS-8), which binds

to the rod domain of the protein. Conversely, Kyoi et al. (15) used an antibody (MANDRA-1) that binds to the dystrophin carboxyl extreme. This difference, associated with the different species (rats, rabbits, and dogs) and experimental models could explain, at least to some extent, the controversy in the results. Nevertheless, these authors (4, 15, 19) did not evaluate the mechanism of dystrophin breakdown.

Using three different models of heart failure, Kawada et al. (13) showed that substantial activation of calpain after elevated intracellular Ca^{2+} induces the specific proteolysis of dystrophin and α-sarcoglycan. Similarly, Yoshida et al. (30) reported alterations in myocardial dystrophin in failing hearts after acute myocardial infarction in rats, suggesting that m-calpain may contribute to a decrease in dystrophin levels. Enhanced calpain activity by increased intracellular Ca^{2+} concentration results in tissue damage, as seen in myocardial infarct, stroke, and muscular dystrophy (23). However, there appears to be an overlap in the substrates and biological actions of MMP-2 and calpain in various cellular pathways (12).

It is now becoming evident that MMP-2 and calpains target a similar subset of proteins. Indeed, much of the evidence for calpain proteolysis of substrates in cardiac cells rests on the use of calpain inhibitors such as calpastatin, which was found to inhibit MMP-2 activity in vitro (3). Furthermore, in the context of acute ischemia-reperfusion injury, calpain activation does not occur during ischemia but during reperfusion, resulting in fragility of the sarcolemma and proteolysis of proteins such as spectrin and ankyrin (11). In agreement with previous studies (19, 11), our results show that spectrin remains intact during ischemia and decreases its levels during reperfusion. However, we did not directly measure calpain activity, which could be a limitation of the study. Since spectrin is a well-known calpain substrate (11), our findings clearly indicate that calpain is inactive during ischemia and only activates during reperfusion, ruling out calpain as the protease responsible for the proteolysis of dystrophin.

MMP-2 is present and can be activated in cardiac myocytes, and MMP-2 is involved in the pathogenesis of several heart diseases. MMP-2 activation can occur in the extracellular or intracellular milieu, by proteolytic cleavage or oxidative stress. One mechanism of MMP activation involves posttranslational proteolytic removal of the propeptide domain, which can be achieved by a range of endogenous proteases, such as membrane-type MMPs. However, several studies have indicated that MMP-2 activity is also regulated via nonproteolytic posttranslational modifications of the full-length zymogen form by *S*-glutathiolation, *S*-nitrosylation, and phosphorylation (2). Peroxynitrite directly activates MMP-2 via a nonproteolytic mechanism involving *S*-glutathiolation of the propeptide cysteine sulfydryl group (25). Thus, MMP-2 activity is associated with the increase in oxidative stress that occurs during ischemia-reperfusion, particularly during early reperfusion. Once activated, MMP-2 is able to cleave intracellular proteins, such as troponin I (27), myosin light chain-1 (21), titin (1), and α-actinin (24), and is involved in the mechanism of postischemic myocardial dysfunction (26). A previous study (27) of MMP-2 during ischemia-reperfusion focused on the activity of the enzyme in both perfusate and heart tissue. Despite the fact that our study did not aim to assess MMP-2 activity during reperfusion, it must be pointed out that Cheung et al. (6) demonstrated that perfusate MMP-2 peaked in the first 1–2 min of reperfusion and normalized after 20–30 min of reperfusion. In addition to the increased MMP-2 activity during ischemia, our results evidenced proteolysis of dystrophin and also β-dystroglycan, a component of the dystrophin-glycoprotein complex and a MMP target (22). It has also been shown that protein levels of β-dystroglycan are decreased during ischemia (19). Therefore, the fact that proteolysis of β-dystroglycan was observed in our study further validates the role of MMP-2 in disrupting membrane stability during ischemia. Moreover, inhibition of MMP-2 by doxycycline prevented dystrophin proteolysis and reduced infarct size. In addition, even though we did not perform a colocalization study, other authors have shown that MMP-2 colocalizes with α-actinin along the Z lines in cardiomyocytes (8, 27). Furthermore, localization of this enzyme in the myocyte sarcolemma has also been reported (7).

Taking into account that oxidative stress has been described as being responsible for MMP-2 activation (2), we subjected hearts to increased oxidative stress by administering SIN-1. Nonischemic hearts treated with SIN-1 showed increased MMP-2 activity. Evaluation of dystrophin levels in nonischemic hearts demonstrated that dystrophin levels decrease when MMP-2 activation is induced and that proteolysis of dystrophin is prevented by doxycicline. These results strongly suggest the participation of MMP-2 in the proteolysis of dystrophin. As mentioned above, unlike other MMPs, MMP-2 becomes active in the intracellular milieu and is thus able to induce dystrophin proteolysis during ischemia. The increase of oxidative stress is an important stimulus for the MMP-2 activation, and this occurs mainly at the beginning of reperfusion. In relation to this, mechanism by which MMP-2 is activated during ischemia remains to be determined, given that the main increment in cellular oxidative stress occurs during early reperfusion. Therefore, there could be another mechanism, independent of ROS, that activates MMP-2 during ischemia (20). Since hearts were perfused with a crystalloid solution, MMP-9 activity was undetectable. Our finding is in agreement with a report showing that MMP-9 becomes active during reperfusion and that neutrophils are the main source of this enzyme (17).

Our results of Western blot analysis and immunostaining are important, since they both confirmed the ischemic loss of dystrophin and suggest a new intracellular target of MMP-2 and a possible pathway through which MMPs may mediate myocardial damage.

Previous studies have shown that ischemic preconditioning prevents dystrophin proteolysis and facilitates its restoration to the sarcolemma (14, 16). Our results confirm these findings and demonstrate that this occurs through inhibition of MMP-2 activity. It is possible that ischemic preconditioning activates PKC (29), which phosphorylates MMP-2, attenuating its activity (20). However, this hypothesis should be demonstrated by further experimental evidence.

A possible limitation of our study is the different perfusion times used in the protocols. Perfusion of the hearts could mildly alter oxidative stress itself, modifying the activity of MMP-2 in a time-dependent way (18). However, the activity data of MMP-2 were accompanied by changes in dystrophin levels, which significantly decreased during ischemia when MMP-2 activity was increased. This is clearly reverted with doxycycline treatment, even when experiments were performed without ischemia in hearts treated with SIN-1. Finally, we selected only two time points to assay dystrophin expres-

sion (30 min of ischemia and 180 min of reperfusion), based on previous studies (4, 19) that demonstrated that dystrophin is lost during ischemia and is not recovered in early reperfusion. Thus, we selected the end of the ischemia period (30 min) to evaluate dystrophin levels. To evaluate infarct size using triphenyltetrazolium, a reperfusion period of at least 180 min is necessary to ensure correct washout of the cardiac enzymes (i.e., lactate dehydrogenase). Therefore, we decided to also evaluate the expression of dystrophin at the end of reperfusion, coincident with the measurement of infarct size.

In conclusion, the present study demonstrates that activation of MMP-2 could be responsible for the proteolysis of dystrophin. Thus, dystrophin emerges as a possible novel substrate for MMP-2 in the context of ischemic injury. Furthermore, our results demonstrate that ischemic preconditioning prevents dystrophin breakdown, most likely by inhibiting MMP-2 activity.

ACKNOWLEDGMENTS

The authors thank Dr. A. Jawerbaum (Centro de Estudios Farmacológicos y Botánicos, Buenos Aires, Argentina) for kindly providing the conditioned media from the promyelocyte U-937 cell line. B. Buchholz is a postdoctoral Fellow of National Council of Scientific and Technological Research of Argentina. V. Miksztowicz, M. Donato, and R. J. Gelpi are members of the National Council of Scientific and Technological Research of Argentina.

GRANTS

This work was supported by University of Buenos Aires Grant UBACYT B069 and by National Agency of Scientific and Technological Promotion Grants 05/PICT13069 and 06/PICT01071.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: B.B., V.P., N.A.S.M., V.M., M.R., and M.D. performed experiments; B.B., V.P., N.A.S.M., G.B., M.R., M.D., and R.J.G. analyzed data; B.B., V.P., N.A.S.M., G.B., M.R., M.D., and R.J.G. interpreted results of experiments; B.B., N.A.S.M., V.M., G.B., M.R., M.D., and R.J.G. edited and revised manuscript; B.B., N.A.S.M., V.M., G.B., M.R., M.D., and R.J.G. approved final version of manuscript; V.P. and M.D. prepared figures; N.A.S.M., M.R., and M.D. drafted manuscript; M.R., M.D., and R.J.G. conception and design of research.

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