

Ischemic Preconditioning Preserves Dystrophin Through Matrix Metalloproteinase-2 Inhibition

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ABSTRACT

Background

Ischemia/reperfusion injury produces cell death through different pathways, some of which induce plasma membrane rupture. In cardiac cells, dystrophin and spectrin provide cell membrane stability and link the intracellular and extracellular environments. Dystrophin breakdown causes membrane fragility. Ischemic preconditioning has been suggested to attenuate this injury, but the mechanism is still unknown.

Objective

To determine whether ischemic preconditioning prevents dystrophin breakdown through matrix metalloproteinase-2 (MMP-2) inhibition.

Methods

Isolated rabbit hearts were treated as follows: G1 (n=5): 30 min perfusion (Nx); G2 (n=6): 30 min global ischemia (GI) without reperfusion; G3: same as G2, followed by 180 min reperfusion (I/R); G4 (n=5): doxycycline (MMP inhibitor) before GI; G5 (n=6): normoxic hearts treated with SIN-1 (which stimulates ONOO production) with 30 min left ventricular function monitoring; G6 (n=5): doxycycline during 5 min, before SIN-1 administration; G7 and G8 (n=5): ischemic preconditioning (n=5) before 30 min GI with/without reperfusion, respectively.

Results

Dystrophin expression decreased during ischemia, reaching 21% of control values ($p < 0.05$); spectrin expression remained unchanged. MMP-2 activity increased 71% during ischemia compared to control values ($p < 0.05$). Doxycycline administered before ischemia prevented dystrophin breakdown. In normoxic hearts, SIN-1 increased thiobarbituric acid reactive substances (TBARS) by 33% ($p < 0.05$) and MMP-2 activity by 36% ($p < 0.05$), and reduced dystrophin expression to 23% of control values ($p < 0.05$). Ischemic preconditioning attenuated dystrophin breakdown significantly by inhibiting MMP-2 activity.

Conclusions

Activation of MMP-2 due to increased oxidative stress is responsible for dystrophin breakdown. Ischemic preconditioning attenuates dystrophin breakdown by inhibiting MMP-2 activity.

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Key words

> Ischemic Preconditioning - Dystrophin - Matrix Metalloproteinase 2

Abbreviations

I/R	Ischemia/reperfusion	PKC	Protein Kinase C
MMP-2	Matrix metalloproteinase-2	CPP	Coronary perfusion pressure
Nx	Normoxic	SIN-1	N-Morpholiniosydnonimine hydrochloride
Pc	Preconditioning	TBARS	Thiobarbituric acid reactive substances

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BACKGROUND

Peripartum cardiomyopathy (PPCM) is an uncommon cause of congestive heart failure, and due to its unpredictable outcome it is considered a distinct disease entity among cardiomyopathies. It is defined as an idiopathic cardiomyopathy presenting with heart failure towards the end of pregnancy or in the first months postpartum, without any other evident cause of heart failure. (1)

Very little is known about the incidence and prevalence of PPCM. According to different authors its estimated incidence in different geographic regions is between 1/300 to 1/1.000 in Africa and Haiti and between 1/250 to 1/4000 pregnancies in the USA. (1) The etiology of PPCM remains unclear although several mechanisms have been involved, including risk factors as age at pregnancy, multiparity, twin pregnancy, history of hypertension or preeclampsia, African people, selenium deficit and use of tocolytics.

Peripartum cardiomyopathy is considered a form of dilated cardiomyopathy (DCM) of relatively good prognosis. However, it has been associated with high maternal and fetal morbidity and mortality, ranging between 15% and 50% due to heart failure, arrhythmias and thromboembolic events. Heart transplantation (HTx) may be necessary in a few cases. (2-4)

The goal of the present study was to evaluate the clinical, hemodynamic and functional characteristics of patients with PPCM and to analyze the predictors of mortality or need for HTx.

METHODS

The experiments were performed on 35 male New Zealand rabbits, weighing 1.8 to 2.5 kg. The study procedures were approved by the Institutional Committee for the Care and Use of Laboratory Animals (Res. CD. 2079/07), in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (USA). (8)

Surgical procedure

The rabbits were sacrificed with intravenous pentobarbital (150 mg/kg). Each heart was rapidly removed and connected to an isolated perfused heart system via the aortic root according to Langendorff's technique. The hearts were perfused with Krebs-Henseleit solution buffered to a pH of 7.2-7.4 containing: NaCl 118.5 mM, KCl 4.7 mM, NaHCO₃ 24.8 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.2 mM, CaCl₂ 2.5 mM and glucose 10 mM. The temperature was kept at 37 °C and the buffer was bubbled with 95% O₂ - 5% CO₂. Two electrodes were inserted and connected to a pacemaker to achieve a constant heart rate of 200 beats per minute.

A latex balloon filled with saline solution was introduced into the left ventricle and connected to a blood pressure transducer (Deltram II, Utah Medical System). The volume of the balloon was adjusted to achieve an end-diastolic pressure of 8 to 10 mm Hg. Coronary perfusion pressure (CPP) was also recorded with a pressure transducer connected to the perfusion line. The coronary artery flow was adjusted to obtain a CPP of approximately 70 mm Hg which was kept constant throughout the experiment.

Experimental protocols (Figure 1)

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

Group 2 (n = 6): myocardial infarction was induced by subjecting the hearts to 30 min global ischemia without reperfusion. Global ischemia was achieved by the sudden and complete reduction of coronary flow provided by the perfusion pump.

Group 3 (n = 5): same as group 2, followed by 180 min reperfusion (I/R).

Group 4 (n = 5): same as group 2 with doxycycline (50 μM) treatment before the 30 min ischemic period. The drug was administered for 5 min followed by 5 min washout.

Group 5 (n = 6): N-Morpholiniosydonimine hydrochloride (SIN-1 an ONOO⁻ donor; 100 μM) administration followed by 30 min ventricular function monitoring.

Group 6 (n = 5): same as group 4 with doxycycline (50 μM) for 5 min before SIN-1.

Group 8 (n = 5): the hearts were subjected to an ischemic Pc protocol consisting in three cycles of I/R before global ischemia and 180 min reperfusion (Pc with reperfusion).

Group 8 (n = 5): same as group 7 but without reperfusion (Pc without reperfusion).

Infarct size assessment

Infarct size was measured in groups 3 and 7. After the 3 hour reperfusion period, the hearts were frozen and cut into 4-mm transverse sections from apex to base. Sections were incubated for 20 minutes in 1% of 2,3,5-Triphenyltetrazolium chloride (pH 7.4, 37° C) and then in 10% formalin. With this technique, viable areas were stained red, while non-stained areas corresponded to the infarcted region. Sections were scanned and infarct size was measured from the images (Image Pro Plus, version 4.5) and expressed as percentage of total left ventricular area.

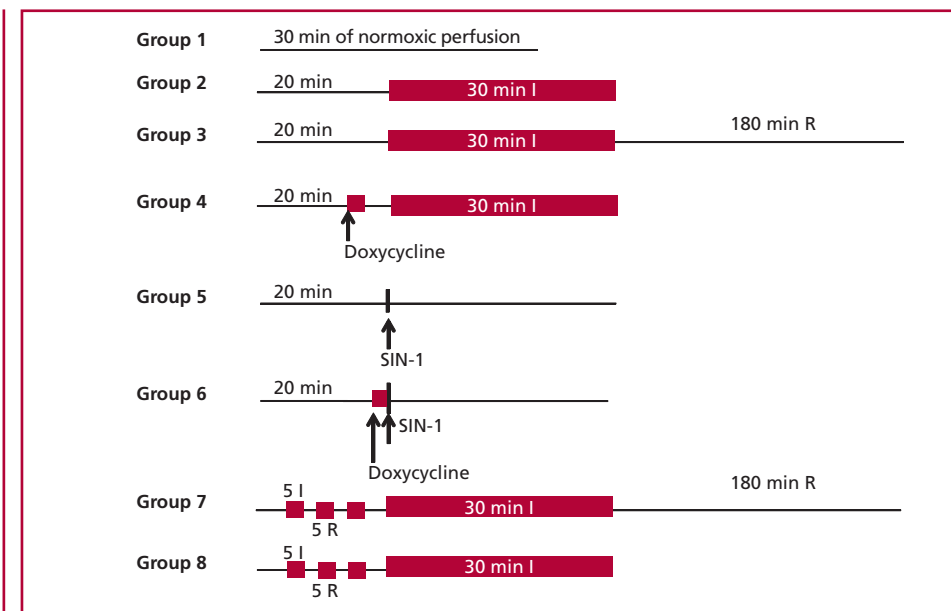
Measurement of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) were measured in groups 1 and 5. Left ventricular samples were homogenized with 5 volumes of homogenization buffer containing KCl (140 mM) and KH₂PO₄ (20 mM), pH 7.4, with a protease and phosphatase inhibitor cocktail of aprotinin (200 nM), bestatin (10 μM), E-64 (20 μM), leupeptin (100 μM), sodium fluoride (20 mM), sodium orthovanadate (100 mM), sodium pyrophosphate (100 mM) and -β-Glycerol phosphate (100 mM) (Thermo Scientific), using a Pro-Scientific Pro 200 homogenizer. Then, the homogenates were centrifuged at 3000 rpm for 10 min and 0.5 ml of the supernatant was mixed with 1.5 ml of 10% trichloroacetic acid, 1 ml of 0.67% thiobarbituric acid and 0.5 ml of distilled water. The samples were mixed and boiled for 30 min and then centrifuged at 2500 rpm for 10 min. TBARS were quantified at 530 nm. The results were expressed in nM MDA/mg protein with extinction coefficient: 1.56 × 10⁵ M.

Western blot analysis

Left ventricular samples from groups 1 to 6 and 8 were frozen and homogenized in homogenization buffer at pH 7.6 containing Tris (1.2 mM), NaCl (0.36 mM), 0.1% SDS, 1% Triton, DTT (0.2 mM) with protease and phosphatase inhibitor cocktail consisting of aprotinin (200 nM), bestatin (10 μM), E-64 (20 μM), leupeptin (100 μM), sodium fluoride (20 mM), sodium orthovanadate (100 mM), sodium pyrophosphate (100 mM) and -β-glycerol phosphate (100 mM) (Thermo Scientific), using a Pro-Scientific Pro 200 homogenizer. Then, the homogenates were centrifuged at 12 000 rpm for 20 min at 4 °C. Protein concentration was determined using the Bradford assay (Bio-Rad). Equal amounts of protein (100 μg) were separated by 5%-10% SDS-PAGE

Fig. 1. Diagram of experimental protocols.



and electrotransferred to polyvinylidene difluoride membranes (Thermo Scientific). The membranes were stained with 0.1% red Ponceau S to confirm protein loading among the groups. The membranes were incubated with monoclonal anti-dystrophin antibody (1:1.000) (Sigma-Aldrich) and with monoclonal anti-spectrin antibody for 12 hours at 4°C. Then, the membranes were incubated with a secondary antibody (1:10.000) (Millipore) at room temperature for one hour and were washed in TBST. Transferred proteins were visualized using enhanced chemiluminescence (Thermo Scientific). The relative amounts of dystrophin and spectrin were quantified by densitometric analysis with Image Gauge 4.0 software (Fujifilm)

Zimography analysis

Left ventricular samples from groups 1, 2, 4, 5, 6 and 8 were frozen, homogenized and concentrated by ultra centrifugation using Amicon Ultra Centrifugal Filters 4ml-30K (5000 G, 4°C, Millipore, MA, USA). Protein concentration was then determined using the Lowry assay. Electrophoretic separation of proteins was performed in polyacrylamide gel under non-denaturing conditions with the addition of 0.1% gelatin (Sigma-St. Louis-MO) as substrate of different metalloproteinases. After the electrophoretic run, the gel was rinsed with Triton to remove SDS and was incubated for 24 hours at 37°C in Tris buffer in the presence of Ca²⁺ as enzymatic cofactor. Finally, the gel was stained with Coomassie-Blue and the gelatinolytic bands were evaluated by densitometric analysis (identified by their molecular weight with a positive control). Results were expressed as activity of the different types of metalloproteinases.

Statistical analysis

Data were expressed as mean values \pm standard error of the mean (SEM). Inter-group comparisons were performed using one-way analysis of the variance (ANOVA) followed by t test with Bonferroni adjusted p-value for multiple comparisons. A p value $< 0.05/k$ (where k = the number of comparisons) was considered statistically significant.

RESULTS

Figure 2 (Panel A) shows dystrophin expression in Nx

hearts in the group subjected to 30 min ischemia and in the group subjected to 30 min ischemia and 180 min of reperfusion. Dystrophin breakdown was seen during ischemia with no significant changes when the hearts were reperfused. As calpain has been suggested to be responsible for dystrophin breakdown, spectrin levels, a specific substrate for calpain, were used for indirect evaluation of calpain activity (Figure 2, Panel B). Our results show that spectrin expression remained unchanged during ischemia (without calpain activity), but decreased significantly during reperfusion (increased calpain activity). Therefore, calpain cannot be responsible for dystrophin breakdown during ischemia.

Figure 3 (Panel A) shows MMP-2 activity in the group of Nx hearts and in those subjected to 30 min ischemia or treated with doxycycline. Our results showed a significant increase in MMP-2 activity during ischemia and absence of activity in the hearts treated with doxycycline. According to these results, dystrophin expression was preserved in the hearts treated with doxycycline (Figure 3, Panel B), thus demonstrating that inhibition of MMP-2 activity with doxycycline prevents dystrophin breakdown.

SIN-1, an ONOO⁻ donor, was administered in order to confirm that dystrophin is a substrate for MMP-2. Lipid peroxidation as a consequence of injury induced by increased oxidative stress was evaluated by measuring TBARS (Figure 4, Panel A). SIN-1 administration produced a significant increase in TBARS concentration. While MMP-2 activity increased significantly in aerobically-perfused hearts treated with SIN-1, the combination of SIN-1 and doxycycline prevented the activation of MMP-2 (Figure 4, Panel B). Dystrophin expression was evaluated in the same experimental groups (Figure 4, Panel C). Results showed that MMP-2 activation (without ischemia) significantly reduced dystrophin expression and that the

combination of SIN-1 and the enzyme inhibitor (doxycycline) prevented dystrophin breakdown.

Figure 5 shows infarct size in hearts subjected to 30 min ischemia followed by 180 min reperfusion and in the group subjected to Pc and reperfusion. Ischemic Pc produced a significant reduction of infarct size (31.9 ± 4.5 vs. $5.3 \pm 1.3\%$; $p < 0.05$). Interestingly, ischemic Pc (without reperfusion) attenuated MMP-2 activity after 30 min global ischemia (Figure 6, Panel A) and dystrophin breakdown (Figure 6, Panel B).

DISCUSSION

In the present study, we have demonstrated that a membrane protein, dystrophin is broken down by MMP-2 during ischemia and that doxycycline, a MMP-2 inhibitor, is capable of preventing dystrophin loss. In addition, ischemic Pc inhibits MMP-2 activity, thus preventing dystrophin breakdown. Our findings show, on one hand, a novel role of MMP-2 as a destabilizing factor of the cardiomyocyte plasma membrane, making it more vulnerable to subsequent injury and, on the other hand, a novel protective mechanism: ischemic Pc.

During acute ischemia, Armstrong et al. (2) showed that loss of dystrophin and spectrin in microsomal vesicles and membrane fractions is an early manifestation of myocardial injury, concurrent with the devel-

opment of osmotic fragility. These results were confirmed by Rodríguez et al., (3) who also demonstrated that a strong reduction or absence of dystrophin indicates severe or irreversible ischemic injury and that membrane proteins have different sensitivity to I/R injury. However, these authors did not evaluate the mechanism of dystrophin breakdown or the effect of a protective intervention as ischemic Pc.

Using three heart failure models, Kawada et al. (9) demonstrated that activation of calpains, followed by an increase of intracellular calcium, specifically induces proteolysis of dystrophin and of α -sarcoglycans. Similarly, Yoshida et al. (10) reported abnormalities in myocardial dystrophin following myocardial infarction in rats, suggesting that calpains might contribute to the reduction in the expression of dystrophin. Thus, some authors suggest that the increase in calpain activity secondary to higher concentration of intracellular calcium produces tissue injury. (11) However, there appears to be overlap in the substrates and/or biological actions of MMP-2 and calpains in various cellular pathways. (12) In fact, many tests performed to evaluate calpain substrates in cardiac cells are based on the use of calpastatin (a calpain inhibitor), that also inhibits MMP-2 activity. (13) In our study, using an in vitro model, we have reproduced the loss of dystrophin and confirmed that it is broken down only during ischemia

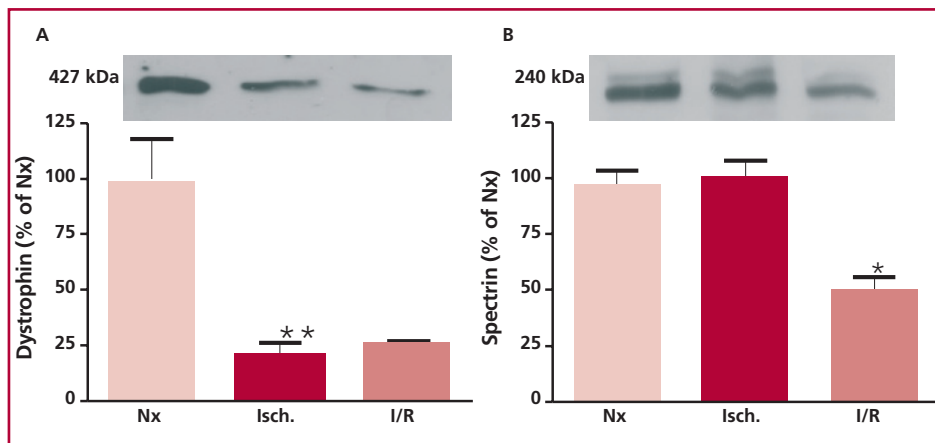


Fig. 2. Dystrophin expression (Panel A) and spectrin expression (Panel B) in the control group (Nx), in the group subjected to 30 min ischemia (Isch.) and in the group subjected to 30 min ischemia followed by 180 min reperfusion (I/R). Dystrophin expression decreased during ischemia and did not recover during reperfusion ($p < 0.05$ vs. Nx). Conversely, spectrin breakdown occurred during reperfusion ($p < 0.05$ vs. Nx and Isch.). Nx: Normoxic conditions. Isch.: Ischemia. I/R: Ischemia/reperfusion.

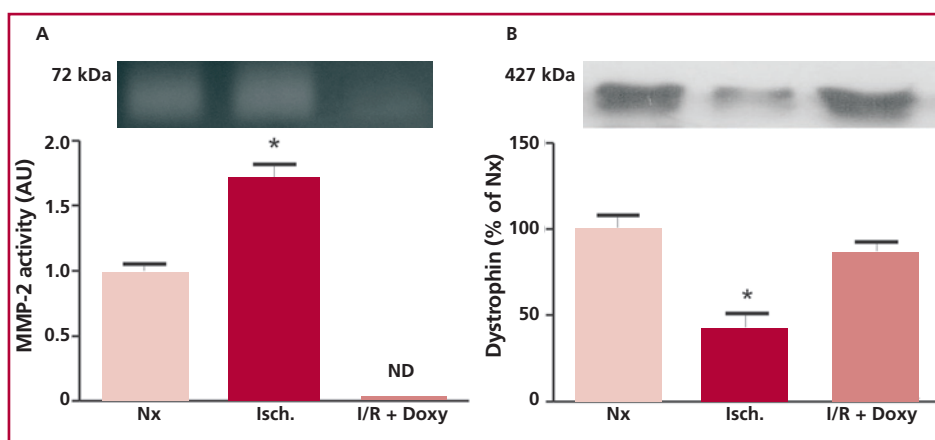


Fig. 3. MMP-2 activity (Panel A) and dystrophin expression (Panel B) in the control group (Nx), in the group subjected to 30 min of ischemia (Isch.) and in the group treated with doxycycline 30 min before ischemia (Isch. + Doxy). Ischemia increased MMP-2 activity ($p < 0.05$ vs. Nx) and dystrophin breakdown; this effect was prevented by doxycycline ($p < 0.05$ vs. Nx and Isch. + Doxy). MMP-2: Matrix metalloproteinase-2. Nx: Normoxic conditions. Isch.: Ischemia. Doxy: Doxycycline.

Fig. 4. Panel A shows TBARS concentration in the control group (Nx) and in the group treated with SIN-1. After SIN-1 administration, TBARS levels increased ($p < 0.05$ vs. Nx). Panel B shows MMP-2 concentration in control hearts (Nx), in the group treated with SIN-1 and in the group treated with SIN-1 + doxycycline. SIN-1 increased MMP-activity; this effect was prevented by doxycycline ($p < 0.05$ vs. Nx and SIN-1 + Doxy). Panel C shows dystrophin expression in the control group (Nx), in the group treated with SIN-1 and in the group treated with SIN-1 + doxycycline. SIN-1 produced dystrophin breakdown; this effect was prevented by doxycycline ($p < 0.05$ vs. Nx and SIN-1 + Doxy). TBARS: Thiobarbituric acid reactive substances. Nx: Normoxic conditions. SIN-1: N-morpholinoydnonimine hydrochloride. MMP-2: Matrixmetalloproteinase-2. Doxy: Doxycycline.

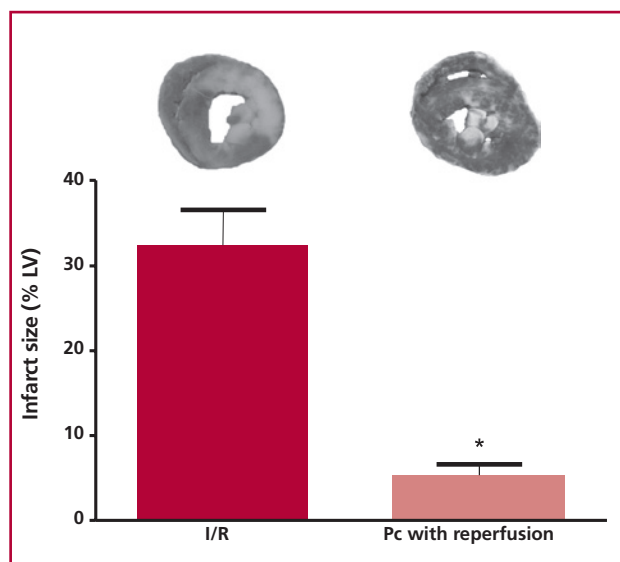
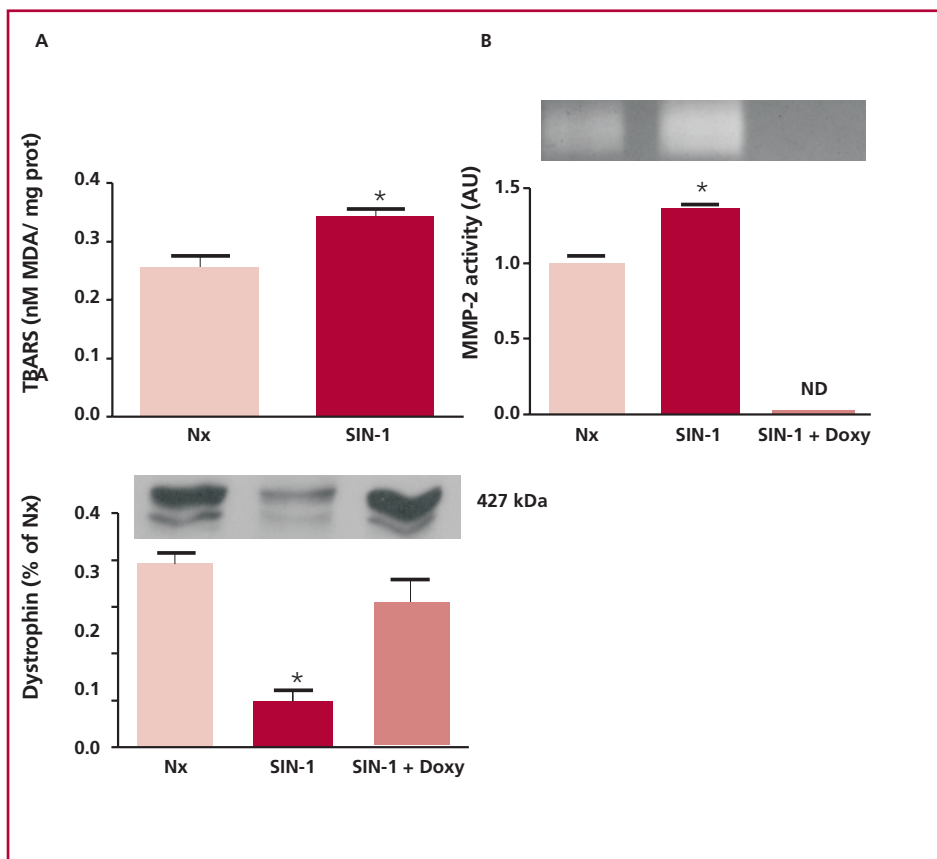


Fig. 5. Infarct size expressed as percentage of left ventricular area. Ischemic preconditioning reduced markedly infarct size produced by 30 min global ischemia ($p < 0.05$ vs. I/R). LV: Left ventricle. I/R: Ischemia/reperfusion. Pc: Preconditioning.

and that reperfusion does not modify its expression. On the contrary, spectrin levels (a specific substrate for calpain), were not modified during ischemia but decreased during reperfusion. (3, 6) This is relevant, as in the setting of acute I/R injury, the activation of

calpain does not occur during ischemia but during reperfusion, generating sarcolemmal fragility and breakdown of other proteins, as spectrin and ankirin. (6) As spectrin is a well known substrate for calpain, these facts clearly indicate that calpain is inactive during ischemia and is only active during reperfusion; thus, it cannot be held responsible for dystrophin breakdown.

MMP-2 participation in extracellular matrix remodeling is well known, and many studies have demonstrated that MMP-2 has an important intracellular role. (12) MMP-2 is present and active in cardiomyocytes and has been implicated in a variety of physiological and pathological processes in the cardiovascular system. MMP-2 is activated by proteolytic cleavage in the intracellular and extracellular compartments, (12) by oxidative stress (14, 15) or by dephosphorylation. (16) Thus, increased activity of MMP-2 is associated with greater oxidative stress occurring during I/R as has been documented in the effluent of hearts subjected to I/R. (17, 18)

Once activated, MMP-2 may cleave intracellular proteins as troponin I, (19) myosin light chain (MLC-1), (20) titin (21) and α -actinin (22) contributing to cardiac mechanical dysfunction in I/R. (17) In our study, we have found increased MMP-2 activity in cardiac tissue during ischemia. Previous studies investigating MMP-2 activity during I/R have assessed its enzymatic activity at different times during reperfusion (7) and, in general, that activity has been assayed

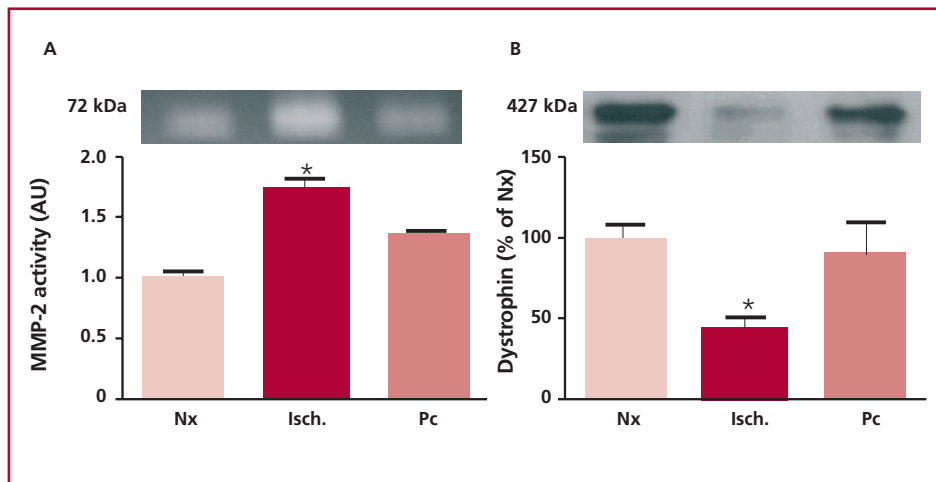


Fig. 6. MMP-2 activity (Panel A) and dystrophin expression (Panel B) in the control group (Nx), in the group subjected to 30 min ischemia and in the group subjected to ischemic preconditioning. MMP-2 activity increased during ischemia and decreased as a result of ischemic preconditioning; preconditioning also prevented dystrophin breakdown ($p < 0.05$ vs. Nx and Pc). MMP-2: Matrix metalloproteinase-2. Nx: Normoxic conditions. Isch.: Ischemia. Pc: Preconditioning.

in the perfusate and not in cardiac tissue. Although we have not evaluated MMP-2 activity during reperfusion, Cheung et al. (17) have demonstrated reduced activity as reperfusion progresses, returning to normal values after 20-30 min. Moreover, in agreement with greater MMP-2 activity during ischemia, we have observed dystrophin breakdown during this period, which was prevented by doxycycline MMP-2 inhibition. In order to confirm these findings, and considering that oxidative stress is the main mechanism for MMP-2 activation, we administered SIN-1 to the hearts to increase oxidative stress. SIN-1, an ONOO-donor, increased MMP-2 activity reducing dystrophin expression. This effect was prevented when SIN-1 was administered with doxycycline. These data confirm the role of MMP-2 in dystrophin breakdown.

Recently, Sariahmetoglu et al. (16) demonstrated that the phosphorylation status of MMP-2 contributes to I/R injury. Phosphorylated MMP-2 activity is lower, producing improved function and less cell injury. Ischemic Pc can activate protein kinase C (PKC), (23) which produces phosphorylation of different intracellular proteins participating in its protective mechanism. In addition, Pc decreases MMP-2 activity in hearts subjected to I/R. (24) The results of our study confirm that Pc attenuates the activity of this enzyme and prevents dystrophin breakdown. One limitation of our study is that we could not discriminate whether the lower activity of MMP-2 in preconditioned hearts is due to lower oxidative stress or to MMP-2 phosphorylation by PKC.

CONCLUSIONS

Activation of MMP-2 due to increased oxidative stress is responsible for dystrophin breakdown. Ischemic Pc attenuates dystrophin breakdown by inhibiting MMP-2 activity.

Our results are relevant as they confirm the ischemic loss of dystrophin, demonstrate a new intracellular target of MMP-2 and reveal a mechanism by which ischemic Pc prevents cell membrane injury.

RESUMEN

El preconditionamiento isquémico preserva la distrofina a través de la inhibición de la metaloproteínasa de la matriz de tipo 2

Introducción

La lesión por isquemia/reperusión produce la muerte celular por diferentes vías, algunas de las cuales llevan a la rotura de la membrana plasmática. En los miocitos cardíacos, la distrofina, junto con la espectrina, otorga estabilidad a la membrana celular, al mismo tiempo que asocia el medio intracelular con el extracelular. La degradación de la distrofina produce fragilidad de la membrana. Se ha sugerido que el preconditionamiento isquémico es capaz de atenuar este daño; sin embargo, el mecanismo se desconoce.

Objetivo

Determinar si el preconditionamiento isquémico previene la degradación de la distrofina a través de la inhibición de la metaloproteínasa de la matriz de tipo 2 (MMP-2).

Material y métodos

Corazones aislados de conejo se trataron de la siguiente manera: G1 (n = 5): perfundidos por 30 min (Nx); G2 (n = 6): 30 min de isquemia global (GI) sin reperusión; G3 (n = 5): se repitió el protocolo de G2 pero se reperfundió por 180 min (I/R); G4 (n = 5): los corazones se trataron con doxiciclina (inhibidor de las MMP) antes de la isquemia global; G5 (n = 6): corazones normóxicos tratados con SIN-1 (dador de ONOO-); G6 (n = 5): se administró doxiciclina durante 5 min, previo a la administración de SIN-1; G7 y G8 (n = 5): se realizó preconditionamiento previo a 30 min de isquemia con reperusión y sin reperusión, respectivamente.

Resultados

La expresión de la distrofina disminuyó durante la isquemia en un 21% respecto de los valores control ($p < 0,05$); la expresión de la espectrina se mantuvo sin cambios. La actividad de la MMP-2 aumentó en un 71% durante la isquemia en comparación con los valores control ($p < 0,05$). La administración de doxiciclina antes de la isquemia evitó la degradación de la distrofina. En corazones normóxicos, el SIN-1 aumentó las sustancias reactivas derivadas del ácido tiobarbitúrico (TBARS) en un 33% ($p < 0,05$) y la actividad de la MMP-2 en un 36% ($p < 0,05$); además, redujo significativamente la expresión de la distrofina a un 23% con respecto a los valores control. El preconditionamiento isquémico atenuó de manera significativa la degradación de la distrofina por inhibición de la actividad de la MMP-2.

Conclusiones

La activación de la MMP-2, debido a un aumento en el estrés oxidativo, es responsable de la degradación de la distrofina. El preconditionamiento isquémico atenúa la degradación de la distrofina mediante la inhibición de la actividad de la MMP-2.

Palabras clave > Precondicionamiento isquémico - Distrofina - Metaloproteinasas 2 de la matriz

Conflicts of interest

None declared.

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