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Dexmedetomidine preconditioning activates pro-survival kinases and attenuates regional ischemia/reperfusion injury in rat heart

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ABSTRACT

Pharmacological preconditioning limits myocardial infarct size after ischemia/reperfusion. Dexmedetomidine is an α_2-adrenergic receptor agonist used in anesthesia that may have cardioprotective properties against ischemia/reperfusion injury. We investigate whether dexmedetomidine administration activates cardiac survival kinases and induces cardioprotection against regional ischemia/reperfusion injury. In *in vivo* and *ex vivo* models, rat hearts were subjected to 30 min of regional ischemia followed by 120 min of reperfusion with dexmedetomidine before ischemia. The α_2-adrenergic receptor antagonist yohimbine was also given before ischemia, alone or with dexmedetomidine. Erk1/2, Akt and eNOS phosphorylations were determined before ischemia/reperfusion. Cardioprotection after regional ischemia/reperfusion was assessed from infarct size measurement and ventricular function recovery. Localization of α_2-adrenergic receptors in cardiac tissue was also assessed. Dexmedetomidine preconditioning increased levels of phosphorylated Erk1/2, Akt and eNOS forms before ischemia/reperfusion; being significantly reversed by yohimbine in both models. Dexmedetomidine preconditioning (*in vivo* model) and peri-insult protection (*ex vivo* model) significantly reduced myocardial infarction size, improved functional recovery and yohimbine abolished dexmedetomidine-induced cardioprotection in both models. The phosphatidylinositol 3-kinase inhibitor LY-294002 reversed myocardial infarction size reduction induced by dexmedetomidine preconditioning. The three isotypes of α_2-adrenergic receptors were detected in the whole cardiac tissue whereas only the subtypes 2A and 2C were observed in isolated rat adult cardiomyocytes. These results show that dexmedetomidine preconditioning and dexmedetomidine peri-insult administration produce cardioprotection against regional ischemia/reperfusion injury, which is mediated by the activation of pro-survival kinases after cardiac α_2-adrenergic receptor stimulation.

1. Introduction

Perioperative cardiac complications such as myocardial ischemia and infarction are the predominant causes of morbidity and mortality in patients undergoing noncardiac surgery [1,2]. In order to decrease the incidence of cardiovascular complications in high risk surgical patients, perioperative administration of cardioprotective drugs is recommended, but this intervention is still controversial [3,4]. A diverse variety of pharmacological agents induce intrinsic protective mechanisms against cardiac ischemia and reperfusion (I/R) injury in animal models [5,6]. This pharmacological-induced cardioprotection relies on the activation of pro-survival protein kinases in both a receptor and non-receptor mediated manner [6]. These kinases, including Akt and Erk1/2 among others, confer powerful cardioprotection when they are activated during preconditioning phase and at reperfusion [7,8].

Abbreviations: AAR, area at risk; ADCA, anterior descendent coronary artery; Akt, protein kinase B; CPP, coronary perfusion pressure; DEX, dexmedetomidine; EGF, epidermal growth factor; Erk1/2, extracellular signal-regulated kinase 1/2; eNOS, endothelial nitric oxide synthase; HR, heart rate; I/R, ischemia and reperfusion; LY, LY 294002; LV, left ventricle; LVDP, left ventricle develop pressure; LVEDP, left ventricle end-diastolic pressure; LVSP, left ventricle systolic pressure; MAP, mean arterial pressure; PI3K, phosphatidylinositol 3-kinase; Ser, serine; Thr, threonine; Tyr, tyrosine; YOH, yohimbine; $+dP/dt_{max}$, maximum rate of pressure change in the ventricle; $-dP/dt_{min}$, minimum rate of pressure change in the ventricle

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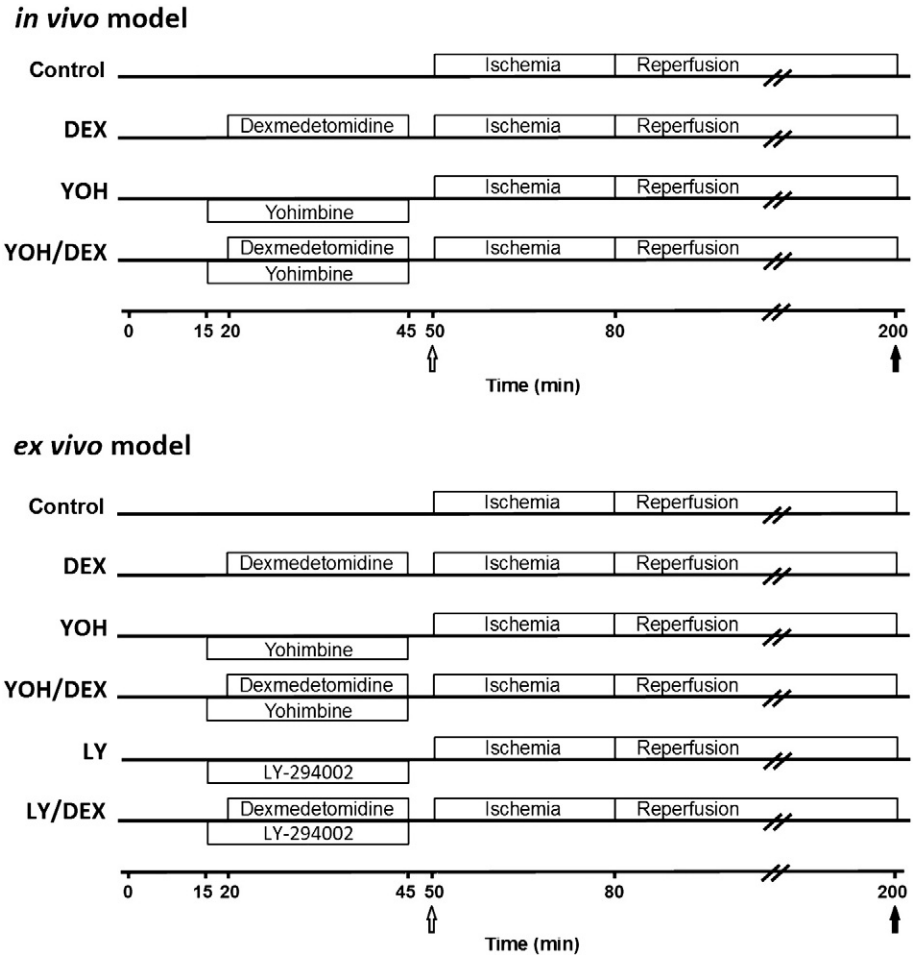

Fig. 1. Experimental protocol *in vivo* and *ex vivo* models. Rat hearts were subjected to a 30 min anterior descending coronary artery occlusion and 120 min of reperfusion. Control: no preconditioning, DEX: 10 nM dexmedetomidine preconditioning (25 min), YOH: 1 μM yohimbine preconditioning (30 min), YOH/DEX: 1 μM YOH (30 min) and 10 nM dexmedetomidine preconditioning (25 min). LY: 20 μM LY-294002 preconditioning (30 min), LY/DEX: 20 μM LY-294002 (30 min) and 10 nM dexmedetomidine preconditioning (25 min). White arrow shows the sample taking time-point for Western blot analysis. Black arrow shows the measurement time-point of recovery hemodynamic data and myocardial infarction size.

Dexmedetomidine (DEX) is a potent and highly selective α_2-adrenergic receptor agonist used in anesthesia. Clinical evidence suggests that induced autonomic nervous system modulation during perioperative administration of DEX is associated with a trend towards improved cardiac outcomes following noncardiac surgery [9,10]. Previous experiments in animals have shown benefits of DEX administration in the ischemic heart [11,12]. Okada et al. showed cardioprotective effects in global ischemia of isolated rat hearts [13]. However, perioperative myocardial ischemia and infarction are limited to a region of hypoperfused myocardium. Currently, if DEX has preconditioning action against regional I/R injury remains to be further investigated as well as whether DEX causes activation of signaling pathways associated with cardiac survival. Accordingly, we hypothesize that DEX induces heart preconditioning throughout activation of cardioprotective signaling pathways by cardiac α_2-adrenergic receptor stimulation, independently from autonomic nervous system modulation.

To test our hypothesis we study: a) the effect of DEX on phosphorylated and total levels of Erk 1/2, Akt and eNOS before regional ischemia as well as left ventricular (LV) function recovery and infarct size reduction in the area at risk (AAR) of regional ischemia after reperfusion in the *in vivo* and *ex vivo* models; b) the contribution of DEX-induced PI3K/Akt signaling pathway activation to cardioprotection in an *ex vivo* model; c) the presence and localization of α_2-adrenergic receptors in the rat heart.

2. Materials and methods

2.1. Animals and surgical procedures

This study conformed to the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH, Publication No. 85-23, revised in 1996), and was approved by the Institutional Ethics Review Committee, University of Chile. All experiments were conducted in both *in vivo* and *ex vivo* heart models of adult, male Sprague–Dawley rats (300 g). Dexmedetomidine was purchased from Hospira Chile (Santiago, Chile).

In vivo model: The rats were anesthetized with pentobarbital (80 mg/kg IP). The trachea was intubated with a 16-gauche cannula and connected to a rodent ventilator (Harvard Rodent Ventilator Model 683, Holliston, MA). Rats were ventilated with O_2 enriched room air at 40–50 breaths/min, with tidal volumes set to 1 ml/100 mg body weight. Body temperature was kept at a constant 37 °C by using an adjustable heating pad. The right carotid artery was cannulated with a 24-gauge catheter connected to a fluid-filled pressure transducer to continuously monitor systolic, diastolic and mean arterial pressure (MAP) and heart rate (HR) using a Dash 1000 General Electric monitor. An intravenous 24-gauche catheter was then installed in the left jugular vein for drug administration. Subsequently the chest was opened via a left thoracotomy through the fourth or fifth intercostal space, the ribs were gently retracted

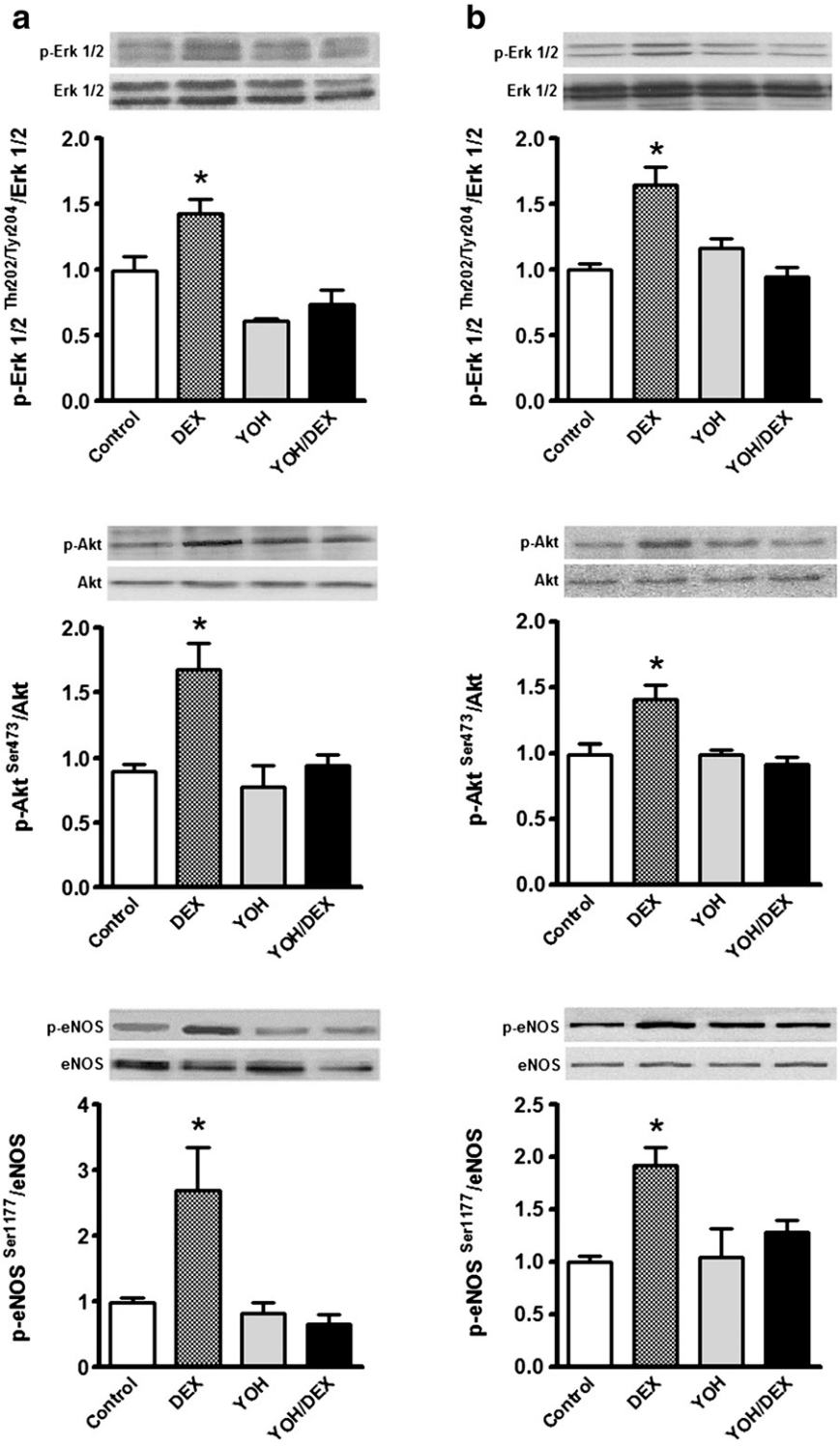

Fig. 2. Dexmedetomidine stimulates Erk 1/2, Akt and eNOS phosphorylation, which is inhibited by yohimbine. Western blots and mean value levels of p-Erk 1/2 at Thr^{202}/Tyr^{204}, p-Akt at Ser^{473} and p-eNOS at Ser^{1177} in ventricular homogenate in the Control, dexmedetomidine (DEX), yohimbine (YOH) and YOH/DEX groups before ischemia in the *in vivo* (panel a) and the *ex vivo* model (panel b). p-Erk 1/2, p-Akt and p-eNOS levels were enhanced in the DEX group compared to the other groups in both models. Values are shown as mean ± SEM, n = 8 per group in each model, $^{*}P < 0.05$ DEX vs Control, YOH and YOH/DEX. Data were analyzed by one-way ANOVA and Tukey's multiple comparison test.

and a pericardiotomy was performed to expose the heart. In order to assess temporary regional ischemia of the left ventricle, a 6-0 prolene loop was placed below the emergence of the first branch of the anterior descending coronary artery (ADCA) and the ends of the suture were threaded through polyethylene tubing (PE-50) to form a snare for reversible occlusion. After the infusion of study drugs and before the onset of ischemia heparin 100 U/kg IV was administered. Then hearts were subject to 30 min of regional ischemia followed by 120 min of reperfusion of the AAR (ADCA dependent territory). Ischemia was confirmed by cyanosis on the myocardial surface. Reperfusion was indicated by an epicardial hyperaemic response and rapid disappearance of cyanosis.

Ex vivo model: the rats were anesthetized with pentobarbital (80 mg/kg IP). Subsequently, a sternotomy was performed and

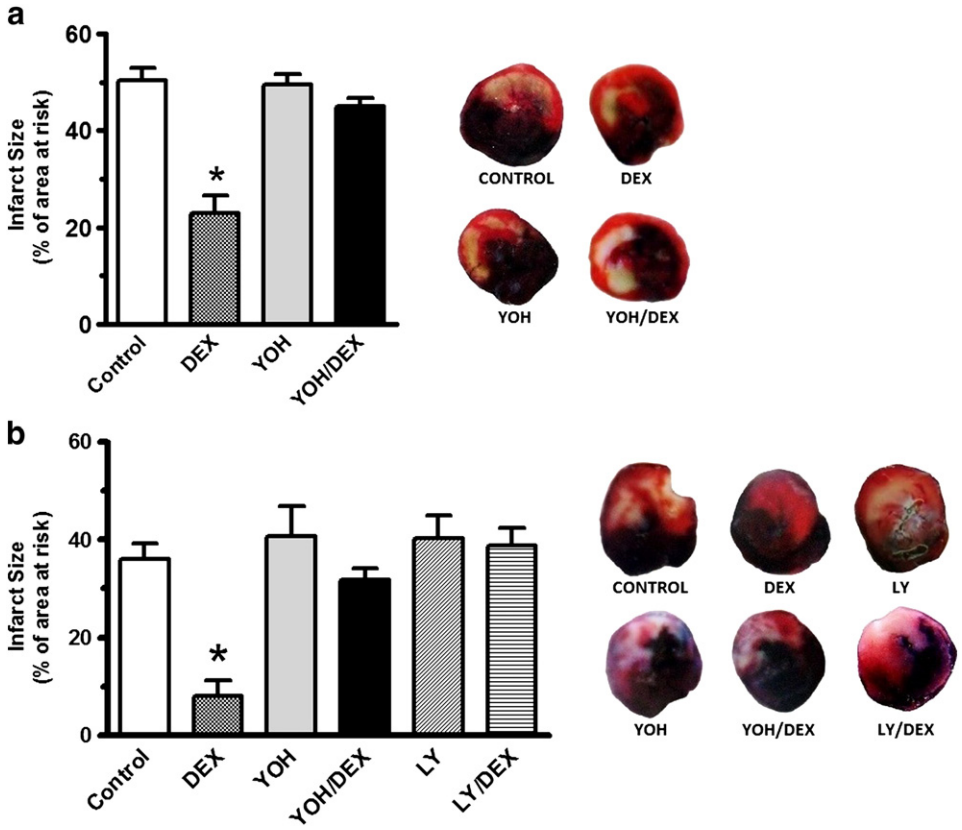

Fig. 3. Dexmedetomidine induces cardioprotection against regional ischemia and reperfusion (I/R) injury. The means of myocardial infarction size in the Control, DEX, YOH, YOH/DEX groups after I/R in the *in vivo* (panel a) and in the Control, DEX, YOH, YOH/DEX, LY, LY/DEX groups after I/R in the *ex vivo* model (panel b). One-way ANOVA revealed that DEX resulted in a significant reduction of infarct size compared to the other groups in both models. Values are shown as mean ± SEM, n = 8 per group, $^{*}P<0.05$ DEX vs. Control, YOH and YOH/DEX at the end of reperfusion. Data were analyzed by one-way ANOVA and Tukey's multiple comparison test.

heparin 100 U/kg IV was administered. The heart was rapidly excised, mounted in a temperature regulated heart chamber and perfused retrograde via the ascending aorta using a peristaltic infusion pump (Gilson Minipuls3, France) at a constant flow of 10–14 ml/min to generate an initial mean coronary (aortic) perfusion pressure (CPP) of 60–70 mm Hg with physiological modified Krebs Henseleit Buffer solution containing (in mM) NaCl (128.3), KCl (4.7), $CaCl_2$ (1.35), $NaHCO_3$ (20.2), NaH_2PO_4 (0.4), $MgSO_4$ (1.1), glucose (11.1), pH 7.4 at 37 °C when equilibrated with a mixture of 95% O_2 / 5% CO_2. Perfusate and bath temperatures were maintained at 37 °C by a thermostatically controlled water circulator (B. Braun Thermomix 1420, Germany). Then, a latex balloon inserted in the left ventricle through the mitral valve, was connected to a pressure transducer (Bridge Amp ML221 AD Instruments, Australia) and filled with normal saline to produce a left ventricle end-diastolic pressure (LVEDP) of 5–10 mm Hg. The volume of the balloon was maintained constant throughout the experiment. In order to assess temporary regional ischemia of the left ventricle, a 6-0 prolene loop was placed below the emergence of the first branch of the anterior descending coronary artery (ADCA) and the ends of the suture were threaded through polyethylene tubing (PE-50) to form a snare for reversible occlusion. After 15 min stabilization, hearts with a LVDP less than 60 mm Hg and a heart rate (HR) less than 180 bpm, were excluded from the study. The remaining hearts were subject to 30 min regional ischemia followed by 120 min reperfusion of the AAR (ADCA dependent territory).

2.2. Experimental protocols

To determine whether DEX induces survival kinases activation, cardioprotection by α_2-adrenergic receptor stimulation independently from autonomic nervous system modulation and to explore the relationship between DEX induced survival PI3K/Akt pathway activation and cardioprotection, rats were randomly assigned to one of the following groups, after 15 min stabilization period and before regional ischemia, in each model (Fig. 1).

In vivo model: a) Control group: no preconditioning (30 min administration of physiological solution); b) DEX group: preconditioning by 10 min bolus administration of 1 μg/kg DEX, followed by 15 min administration of 0.7 μg/kg/h DEX; c) yohimbine (YOH) group: preconditioning by 5 min bolus administration of 1 mg/kg YOH, followed by 20 min administration of 0.5 mg/kg/h YOH; d) YOH/DEX group: preconditioning by 5 min bolus administration of 1 mg/kg YOH, followed by 25 min administration of 0.5 mg/kg/h YOH. After 5 min into YOH , 10 min bolus administration of 1 μg/kg DEX followed by 15 min administration of 0.7 μg/kg/h DEX.

Ex vivo model: a) Control group: no preconditioning (30 min administration of buffer solution); b) DEX group: preconditioning by 25 min administration of 10 nM DEX in modified Krebs Henseleit Buffer solution, followed by 5 min washout; c) YOH group: preconditioning by 30 min administration of 1 μM YOH in buffer solution, followed by 5 min washout; d) YOH/DEX Group: preconditioning by 30 min administration of 1 μM YOH in buffer solution and after 5 min into YOH, 25 min administration of 10 nM DEX in buffer solution, followed by 5 min washout; e) LY group: preconditioning by 30 min administration of 20 μM LY-294002 in buffer solution, followed by 5 min washout; f) LY/DEX Group: preconditioning by 30 min administration of 20 μM LY-294002 in buffer solution and after 5 min into LY, 25 min administration of 10 nM DEX in buffer solution, followed by 5 min washout.

The dose of DEX in the *in vivo* and in the *ex vivo* models was used according to the manufacturer's recommendation and Okada et al. [13], respectively.

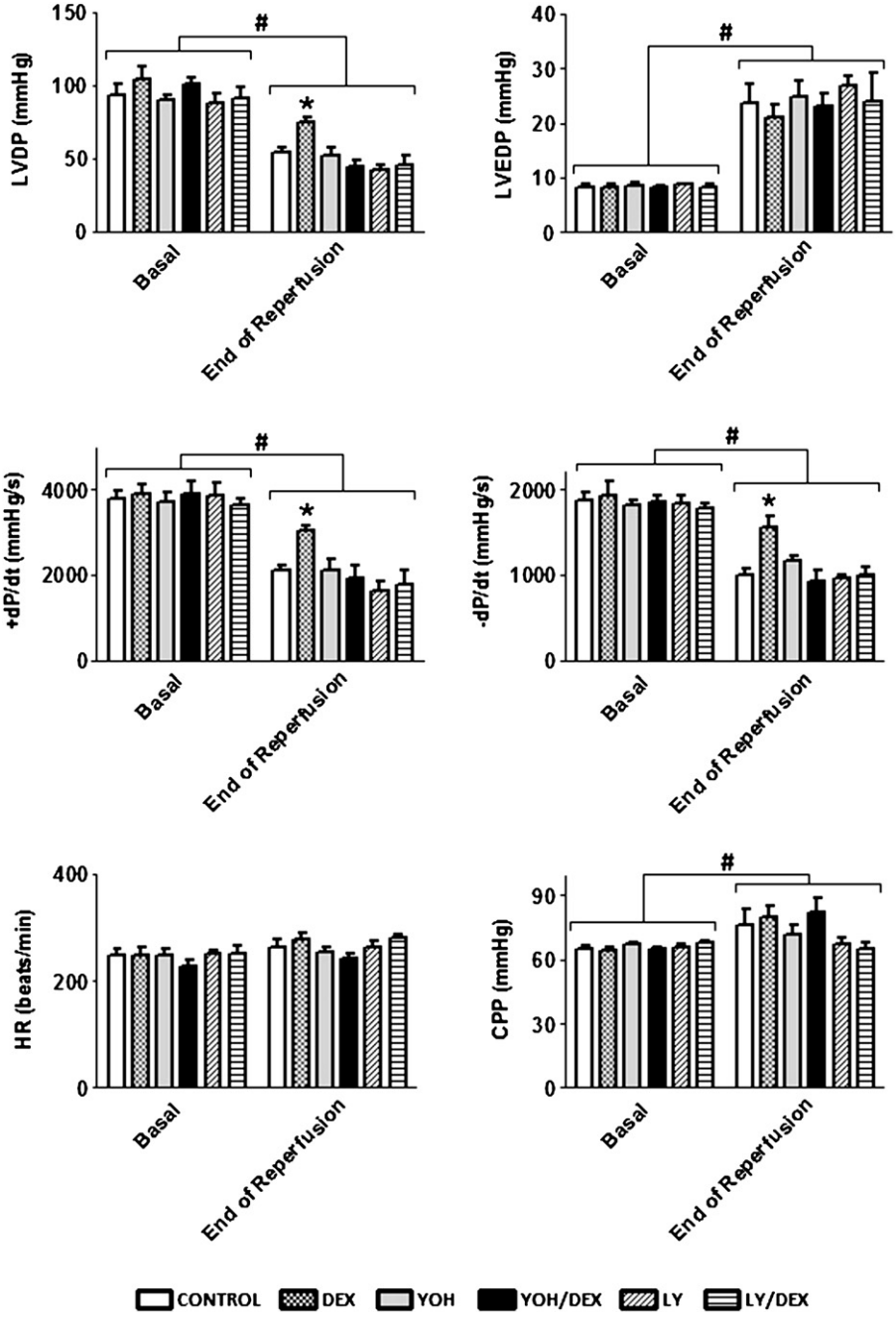

Fig. 4. Dexmedetomidine preconditioning improves LV functional recovery after I/R injury. The means of hemodynamic measures (LVDP, LVEDP, +dP/dtmax, −dP/dtmin, HR and CPP), before preconditioning and at the end of 2 h reperfusion in the *ex vivo* model. ANOVA (mixed model) revealed that compared to other groups DEX group showed improved LVDP and improved +dP/dtmax and −dP/dtmin, at the end of reperfusion. Values are expressed as mean ± SEM, n = 8 per group, $^{*}P<0.05$ DEX vs Control, YOH, YOH/DEX, LY and LY/DEX, at the end of reperfusion in *ex vivo* model. $^{\#}P<0.05$ basal vs end of reperfusion. Data were analyzed by ANOVA for repeated measurements and Bonferroni multiple comparisons test.

2.3. Measurement of infarct and risk region sizes

In both models, at the end of the reperfusion, the ADCA was again occluded and the AAR was delineated by Evans blue staining of the non-ischemic myocardium. Whether the hearts were rapidly excised from the thoracic cavity in the *in vivo* model or were unmounted from the Langendorff system, the atria and great vessels were separated from ventricles. They were sliced from the base to the tip into 2 mm slices. The slices were then counterstained with 15 ml 2,3,5-triphenyltetrazolium chloride 1% (Sigma Chemical) in phosphate buffer adjusted to pH 7.4, for 15 min at 37 °C. After overnight storage in 10% formaldehyde, slices were placed on a Plexiglas holder and covered by a glass. Two-millimeter shims in the corners hold the glass away from the bottom sheet by the desired slice thickness. Spring clamps were used to press the glass down against the shims, squashing the slices to a uniform 2 mm. Then, a digital photography of the mounted tissue was taken. For each slice, measuring the size of the AAR and infarct area was performed by planimetry using the Image J program [14]. The AAR was expressed as a percentage of the ventricles volume, and infarct size was expressed as a percentage of the AAR.

2.4. Measurement of LV function

The LV function was only determined in the *ex vivo* model. The LVEDP, left ventricle peak systolic pressure (LVSP), the maximal positive and negative peak of first derivative of LV pressure ($+dP/dt_{max}$, −dP/

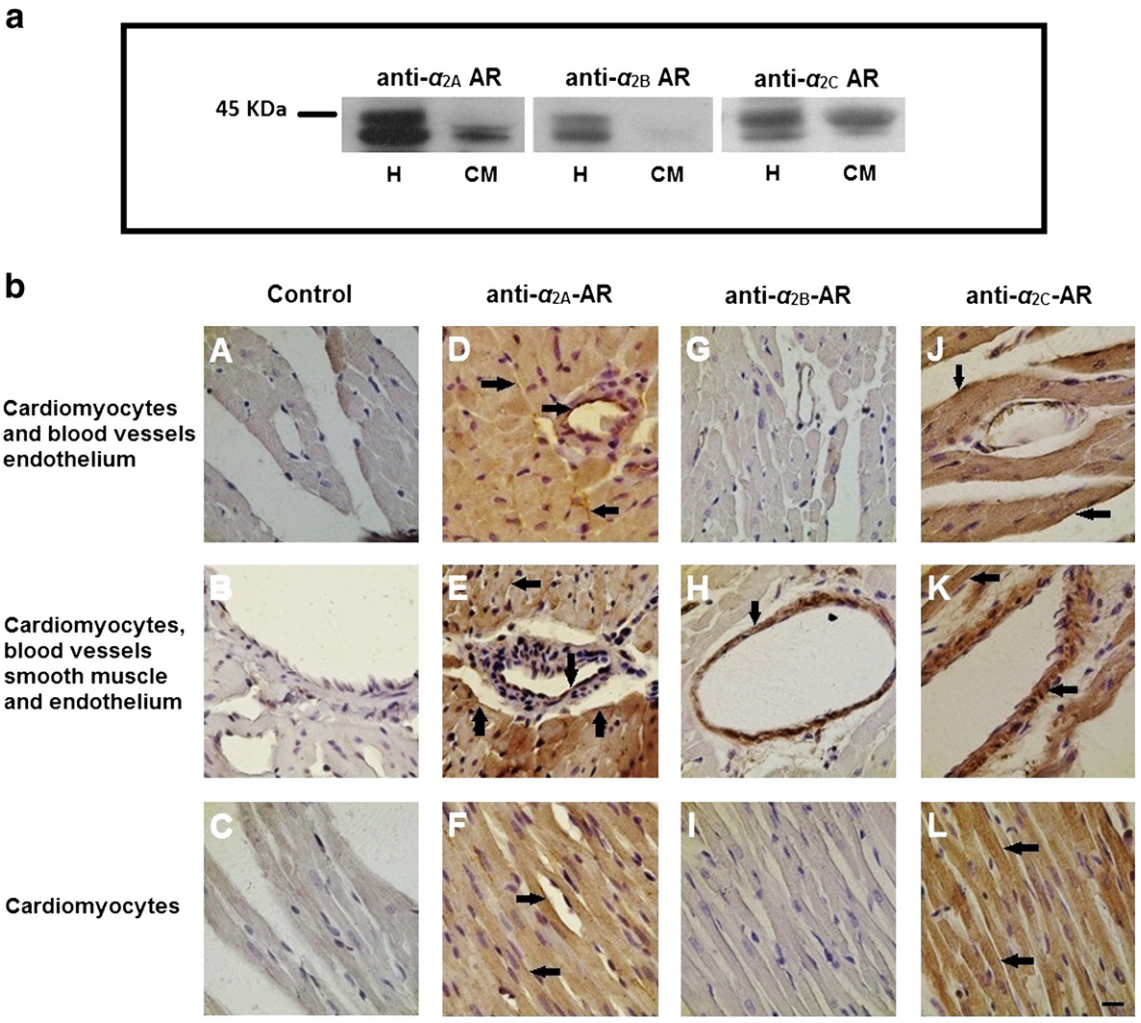

Fig. 5. Characterization of α_2-adrenergic receptors in the heart. a. Western blot showing specific α_{2A}, α_{2B} and α_{2C} adrenergic receptor subtypes expression in Sprague–Dawley rat cardiac tissues homogenate. H, whole adult hearts. CM, adult cardiomyocytes. Immunoblotting was performed as described in Materials and methods. Molecular size marker is indicated on the left. b. Ventricle sections from adult Sprague–Dawley rats were immunostained for α_{2A}-adrenergic receptors antibody (1:500) (panels D, E, F), α_{2B}-adrenergic receptors antibody 1:500 v/v (G,H,I) and α_{2C}-adrenergic receptors antibody 1:500 v/v (J, K, L). All controls (no primary antibody) were negative (A, B, C). Black arrows indicate the immunodetection of α_2-adrenergic receptors. Heart sections were processed for routine immunoperoxidase techniques and counterstained with Mayer's hematoxylin. Bar scale: 20 μm. Data showed are representative figures of 5 animals.

dt_{min}), heart rate (HR) and CPP were measured and continuously recorded throughout the entire experiment on a personal computer using PowerLab (ML866 ADInstruments, Australia). LV developed pressure (LVDP) was calculated as follow: LVDP = LVSP − LVEDP (mm Hg).

2.5. Western blot analysis

The levels of total and phosphorylated forms of Erk 1/2, Akt and eNOS in whole adult rat cardiac tissue and the presence of the α_2-adrenergic receptor subtypes in whole adult rat cardiac tissue and isolated adult rat cardiomyocytes were determined by Western blot analysis. To this end, the hearts from each animal and isolated adult cardiomyocytes were frozen in liquid nitrogen. Cardiac tissues were homogenized in iced-cold buffer containing phosphatase and protease inhibitors (in mM): Tris–HCl (50), NaCl (150), EDTA (1), Na_3V0_4 (5), NaF (20), $Na_4P_2O_7$ (10), Nonidet P-40 1%(v/v) and a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), final pH 7.4 [15]. For electrophoresis and Western blot analysis, tissue protein lysates were matched for proteins (30 μg) and separated by sodium dodecil sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) 8% and transferred to PVDF membranes (Millipore Corp., Bedford, MA). Blots were probed with antibodies raised against p-Akt^{Ser473}, total Akt, p-ERK1/2$^{Thr202/Tyr204}$, total ERK 1/2 (Cell Signaling Technology Inc. New England, NY, USA), p-eNOS^{Ser1177} and total eNOS (BD Transduction Laboratories, NJ, USA), α_{2A} adrenergic receptor (Antibodies online.com, Aachen, Germany) or α_{2B} and α_{2C} adrenergic receptors (Thermo Fisher Scientific Inc. Rockford, IL, USA). After an additional incubation period with appropriate horseradish peroxidase-linked secondary antibodies (Calbiochem, San Diego, CA), immunoreactivity was visualized by a peroxidase-based chemiluminescence detection kit, ECL plus (Amersham Biosciences, Pittsburgh, PA). The signal intensity of the bands in the immunoblots was quantified by densitometry using the software Image J [14].

2.6. Immunohistochemistry

The localization of α_2-adrenergic receptor subtypes in the adult rat heart was determined by immunohistochemistry. To this end, rat hearts were fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h, dehydrated in alcohol, clarified in xylene, embedded in paraffin wax and cut into 5 μm sections. Standard immunoperoxidase techniques were employed to detect α_{2A}, α_{2B} and α_{2C} adrenergic receptors. The corresponding antibody against the α_{2A}, α_{2B} and α_{2C} adrenergic receptors (1:500) was applied to each individual section at 4 °C overnight. Immunostaining was performed using an HRP-labelled streptavidin biotin kit (RTU-Vectastain kit)

following the manufacturer's directions. Sections were counterstained with Mayer's hematoxylin (DAKO, Carpinteria, CA) and mounted with Entellan (Merck, Santiago, Chile). Immunohistochemical controls were done by replacing the primary antibody with phosphate buffered saline. All controls were negative. All sections were examined by light microscopy (Leitz, Orthoplan) and images were captured with a Canon 1256 camera.

2.7. Statistical analysis

The sample size for these experiments was defined in 8 animals per group in each model. Levels of survival kinase phosphorylation after pharmacological preconditioning and the size of risk and infarct areas at the end of reperfusion were analyzed using one-way ANOVA. A Tukey *post-hoc* test was used to assess the activation and preconditioning effect differences among groups. Hemodynamic data at different times were analyzed with repeated-measures two-way ANOVA (mixed model). Bonferroni *post-hoc* test was used to assess the recovery differences among groups. A value of $P<0.05$ was considered significant. All data are expressed as mean ± SEM.

3. Results

3.1. Dexmedetomidine stimulates Erk 1/2, Akt, eNOS signaling pathways in an in vivo and ex vivo model of I/R injury

Fig. 2a–b shows representative Western blots and mean values of the ventricular levels of phosphorylated Erk 1/2, Akt and eNOS in Control, DEX, YOH and YOH/DEX groups, after preconditioning and immediately before ischemia in the *in vivo* and *ex vivo* models, respectively. In both models, 25 min of DEX preconditioning resulted in a significant increase in Erk, Akt and eNOS phosphorylation compared to the other three groups. YOH administration did not modify phosphorylated levels of the three survival proteins compared to the controls in both models. The DEX-induced increase in Erk, Akt and eNOS phosphorylation was blunted by the addition of YOH.

3.2. Dexmedetomidine preconditioning reduced myocardial infarction size both in vivo and ex vivo model of I/R injury

The reduction of infarct size in the *in vivo* and *ex vivo* models is shown in Fig. 3a–b, respectively. The AAR as a percent of the ventricles volume was similar among groups, in both models. DEX preconditioning significantly reduced infarct size of the AAR compared to the other groups in the *in vivo* and the *ex vivo* models. YOH administration did not modify the infarct size compared to controls in both models. However, the DEX-induced reduction in the regional infarction size was blunted by the addition of YOH in the *in vivo* (Control: 50 ± 3%, DEX: 22 ± 4%, YOH: 41 ± 2%, YOH/DEX: 32 ± 2%) and in the *ex vivo* models (Control: 36 ± 3%, DEX: 8 ± 3%, YOH: 41 ± 6%, YOH/DEX: 30 ± 3%).

3.3. Dexmedetomidine preconditioning significantly improves LV functional recovery in an ex vivo model of I/R injury

In the *ex vivo* model, the preconditioning effect with DEX was evident at the end of reperfusion from an increased recovery of LVDP, $+dP/dt_{max}$ and $-dP/dt_{min}$ compared to the control group. DEX induced improved functional ventricle recovery was abolished by YOH. LVEDP, HR and CPP were similar among groups throughout these experiments. LV performance and hemodynamic data in the *ex vivo* model are shown in Fig. 4. The effect of time and I/R process was significant in all of the measured variables, but not in the HR.

3.4. Role of PI3K signaling in dexmedetomidine preconditioning

PI3K inhibition by LY-294002 precludes the DEX induced reduction of infarct size in the *in vivo* model. In this experiment, AAR as a percent of the ventricles volume was similar among groups. Fig. 3b shows that DEX preconditioning significantly reduced infarct size of the AAR compared to the other three groups. LY-294002 did not modify infarct sizes compared to control. However, DEX-induced reduction in the regional infarct size was blunted by LY-294002 (Control: 36 ± 3%, DEX: 8 ± 3%, LY: 40 ± 4%, LY/DEX: 38 ± 4%).

DEX preconditioning effect on ventricular function was also affected by LY-294002. In these experiments, DEX increased recovery of LVDP, $+dP/dt_{max}$ and $-dP/dt_{min}$ compared to the control. DEX-induced improved functional ventricle recovery was also abolished by LY-294002. LVEDP, HR and CPP were similar among groups. LV performance and hemodynamic data in the *ex vivo* model are depicted in Fig. 4.

3.5. Detection of α_2-adrenergic receptors in the rat heart

The α_2-adrenergic receptors were studied by Western blot analysis in whole adult rat cardiac tissue and isolated adult rat cardiomyocytes. As shown in Fig. 5a, the α_2-adrenergic receptor subtypes A, B and C were observed in the whole cardiac tissue while the isotypes A and C were detected in isolated adult rat cardiomyocytes. Immunohistochemistry analysis of adult ventricular tissue revealed the presence of α_{2A}-adrenergic receptors in the endothelium of blood vessels and on the surface of cardiomyocytes (Fig. 5b). The α_{2B}-adrenergic receptors were detected in the smooth muscle cells of small arterioles and veins, and the α_{2C}-adrenergic receptors on the surface of cardiomyocytes and in the smooth muscle cells of arterioles (Fig. 5b).

4. Discussion

The main findings of this study were: a) DEX pretreatment induces Erk 1/2, Akt and eNOS activation, improves myocardial function and reduces myocardial infarction size after regional I/R of the heart in the *in vivo* and *ex vivo* models. b) The administration of the α_2-adrenergic receptor antagonist YOH blocked Erk 1/2, Akt and eNOS phosphorylation as well as DEX-induced cardioprotection in both models. c) Also inhibition of PI3K activation and subsequently eNOS phosphorylation precludes and abolishes cardioprotection induced by DEX. d) The three isotypes of α2-adrenergic receptors were detected in the whole cardiac tissue where the α_{2A} and α_{2C} adrenergic receptors were detected in isolated cardiomyocytes. Therefore, it seems likely that independently from autonomic nervous system modulation, DEX cardioprotective effects are mediated by activation of pro-survival PI3K/Akt signaling pathway after cardiac α_2-adrenergic receptor stimulation. However, while the preconditioning exists based on the finding of phosphorylation of Erk, Akt and eNOS in both models, an effective wash out period of DEX in the *in vivo* model is difficult to accomplish due to experimental limitations. Therefore, since DEX requires a complete metabolism to ensure a purely preconditioning state, a peri-insult protective effect cannot be ruled out in this model.

The present study shows that DEX preconditioning increases Erk 1/2, Akt and eNOS phosphorylation. From a molecular point of view, almost every cardioprotective strategy available activates the canonical signaling pathways associated with cell survival [5,6]. Erk, Akt and eNOS are normally activated during I/R events, but at insufficient levels to produce cardioprotection. A more powerful stimulus, either mechanical or pharmacological, is required to confer protection. Several agents have shown to confer cardioprotection by binding to their specific G-protein coupled receptor. Ligand binding at the G-protein coupled receptor results in PI3K/Akt and Ras-MEK1-2-Erk1/2 signaling cascades activation [6]. DEX is a known potent agonist of the

α_2-adrenergic receptor [16]. Therefore, it is not surprising to find the activation of these kinases with DEX preconditioning as we demonstrated here. The molecular mechanisms involved in the activation of Erk and Akt downstream of stimulation of α_2-adrenergic receptor linked to adenylil cyclase are still not completely understood. However, evidences show that some cellular responses to α_2-adrenergic receptor stimulation are mediated by Erk and PI3K/Akt signaling pathways [17–21]. Interestingly after α_2-adrenoceptor stimulation, transactivation of EGF receptor by different mechanisms depending on the investigated tissue is responsible for ERK and Akt activation and one key step in this last effect is the involvement of PI3K activation downstream of EGF transactivation [17,21]. The mechanism whereby PI3K contributes to activation of the Erk pathway is unclear and deserves further exploration in the heart. However, the regulation of the Erk signaling cascade by PI3K has been previously shown for various Gq- or Gi-coupled receptors, including α_2-adrenoreceptor and M^3 muscarinic receptor [21–23]. The identification of EGF receptor/PI3K/Akt/Erk cascade as a significant component of α_2-adrenergic receptor signaling in other tissues opens the possibility that DEX cardioprotective effects against I/R injury could be triggered by this mechanism. In the other hand, ischemic and pharmacological preconditioning induces a biphasic response in prosurvival kinase activation, with the first phase occurring immediately after preconditioning maneuvers and the second phase of activation occurring at reperfusion, after the ischemic insult [7,8,24]. Activation of PI3K/Akt/eNOS pathway is required during the preconditioning phase and at reperfusion to mediate induced-cardioprotection. Previous studies have shown that different survival pathways exhibit cross talk, with one kinase pathway interacting and influencing the activation of another [25]. In this study we explored activation of Erk1/2, Akt and eNOS before the I/R insult. Therefore, studying the phosphorylation levels of survival associated kinases after reperfusion of DEX treated hearts is warranted.

Previous investigation by Okada et al. showed DEX cardioprotective effects on global ischemia in an isolated rat heart model [13]. They found that DEX administration prior to global ischemia and reperfusion decreased coronary flow and decreased myocardial infarct size. The authors proposed that DEX-induced coronary vasoconstriction by α_2-adrenergic receptor stimulation decreased coronary flow, induced myocardial ischemia and triggered ischemic preconditioning of the heart. This study showed a reduction in coronary flow, but the referred myocardial ischemia during DEX administration was never demonstrated by the authors. Previous work in human volunteers showed coronary vasoconstriction and reduction of coronary blood flow, but a parallel reduction of myocardial oxygen demand. No ischemic episodes were reported in this trial [26]. In the present study we did not find evidence for myocardial ischemia during DEX administration. Even though, we did not determine effective coronary flow, CPP measurements were not significantly different from basal values and were similar among study groups during and after DEX infusion in our *ex vivo* model. Therefore, the theory that DEX administration produces ischemic preconditioning seems less plausible. In the other hand, since perioperative myocardial ischemic events and myocardial infarction are regional rather than global, our *in vivo* and *ex vivo* models were chosen to examine the cardioprotective effect of DEX against regional I/R injury, to explore the underlying mechanism of protection under controlled conditions and to assess the impact of neurohumoral and systemic hemodynamic effects. In this study, *in vivo* (preconditioning) and *ex vivo* (peri-insult protection) models, showed similar proportion of cardioprotective capacity of DEX after reperfusion of regional lethal ischemic insult. Prophylactic administration of DEX reduced the myocardial infarction size in the risk zone by almost 28% in both models and improved *ex vivo* recovery of myocardial function. Both DEX-induced beneficial effects on myocardial infarction size and ventricular function were abrogated by YOH. This evidence suggests that DEX cardioprotective effects are mostly mediated by a molecular response within the cardiomyocytes after α_2-adrenergic stimulation rather than exclusively neurohumoral systemic modulation. In order to confirm the importance of the α_2-adrenergic receptors in the cardioprotection induced by DEX, we also studied the cardioprotective effect of the α_2-adrenergic receptor agonist xylazine [27]. Our results show that xylazine preconditioning significantly reduced infarct size and improved hemodynamic function in the *ex vivo* model and this effect was also blunted by the addition of YOH [See Supplemental Materials].

We also study the causal relationship between DEX-induced kinase activation and its preconditioning capacity against regional I/R injury. Our findings confirm our hypothesis that DEX induces cardioprotective signaling activation by cardiac α_2-adrenergic receptor stimulation. In order to demonstrate this link, PI3K inhibition was used to investigate DEX effects on myocardial function recovery and reduction of infarct size after regional I/R in the *ex vivo* model. The results demonstrated that PI3K is essential to confer DEX-induced protection against I/R injury. However we did not inhibit Erk1/2 activation and it will be interesting to explore the role of MEK1/2–Erk1/2 signaling cascades in DEX preconditioning in future studies. In the same direction we cannot exclude the involvement of other known components related to cardioprotection. In neuronal models some potential antioxidant properties of DEX have been demonstrated [28]. Therefore, we cannot rule out protective effects of DEX on reactive oxygen species production. This particular aspect deserves further exploration in new trials.

The elucidation of DEX induced cardioprotective signaling may have important implications for the development of perioperative cardioprotective strategies. In this sense, we also explored the localization of α_2-adrenergic receptor subtypes potentially implicated in DEX preconditioning. Western blot analysis of whole cardiac tissue revealed the existence of the three α_2-adrenergic receptor subtypes which agree with previous works in rat and human heart [29–32]. However, studies addressing the localization and distribution of these receptor subtypes in rat heart are confusing [30,33,34]. Our investigation confirmed the existence of three α_2-adrenergic receptor subtypes in whole rat cardiac tissue but with a different distribution pattern for each α_2-adrenergic receptor subtype. Western blot analysis of isolated adult rat cardiomyocytes showed the presence of α_{2A} and α_{2C}-adrenergic receptor subtypes, but not α_{2B}. Our immunohistochemical studies in whole heart also showed α_{2A} and α_{2C}-adrenergic receptors in cardiomyocytes. Collectively, these evidences suggest that stimulation of cardiomyocyte α_{2A} and/or α_{2C}-adrenergic receptors by DEX could activate prosurvival kinases within these cells, protecting them against I/R injury.

The main limitations of this study were: a) Although DEX administration induces cardioprotection in the *in vivo* and *ex vivo* models, we did not observe a reduction in the levels of apoptosis in DEX-treated hearts assessed by caspase 3 levels and TUNEL (data not shown) under our experimental condition (30 min of regional ischemia followed by 120 min of reperfusion). It is possible that 2 h reperfusion is a too short period of time to show differences among groups. Even though, reperfusion appears to accelerate the timing of apoptosis in the non-salvageable cells compared with permanent occlusion, it also lowers the number of cardiomyocytes undergoing apoptosis. This fact makes that definitive identification of apoptosis in the myocardium remains complex [35–37]. We also investigated whether DEX could protect cultured neonatal rat cardiomyocytes to simulated ischemia. Our *in vitro* results show no protection with DEX (data not shown). We cannot rule out the possible influence of not studied molecular components present in the whole heart that might have enhanced or that may be required for DEX induced cardioprotection [38] and further studies should clarify this point. b) We showed that DEX preconditioning increased levels of phosphorylated Erk1/2, Akt and eNOS before I/R; being significantly reversed by YOH and demonstrated the relevance of the PI3K for DEX induced cardioprotection. However, we did not explore the underlying mechanisms involved

in Akt and Erk activation downstream cardiac α_{2A} and α_{2C}-adrenergic receptors triggered by DEX. As stated before, transactivation of EGF receptor could be involved in the activation of both kinases. The role of ERK on DEX cardioprotection was not studied. Both unsolved problems deserves further exploration. c) Although our study showed that DEX preconditioning improves ventricular performance against I/R events in the *ex vivo* model; because of experimental design limitations we could not determine the ventricular function in the *in vivo* model.

The present study shows the cardioprotective properties of DEX when administered previous to I/R events. Also, it demonstrates activation of cardiac kinases associated with cellular survival in a receptor-mediated manner. These facts support the idea that DEX is not only a useful sedative, but also plays a pivotal role in anesthetic cardioprotection. While the cardioprotective effects of perioperative recommended drugs are debated and not well established, research efforts should be addressed to elucidate unsolved issues of molecular mechanisms involved in cardioprotection of existing anesthetics. The potential benefits of DEX preconditioning and peri-insult protection in high risk cardiovascular patients undergoing surgery are enormous.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbadis.2011.12.013.

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