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Dexmedetomidine protects the heart against ischemia-reperfusion injury by an endothelial eNOS/NO dependent mechanism

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ABSTRACT

The alpha2-adrenergic receptor agonist Dexmedetomidine (Dex) is a sedative medication used by anesthesiologists. Dex protects the heart against ischemia-reperfusion (IR) and can also act as a preconditioning mimetic. The mechanisms involved in Dex-dependent cardiac preconditioning, and whether this action occurs directly or indirectly on cardiomyocytes, still remain unclear. The endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) signaling pathway and endothelial cells are known to play key roles in cardioprotection against IR injury. Therefore, the aims of this work were to evaluate whether the eNOS/NO pathway mediates the pharmacological cardiac effect of Dex, and whether endothelial cells are required in this cardioprotective action. Isolated adult rat hearts were treated with Dex (10 nM) for 25 min and the dimerization of eNOS and production of NO were measured. Hearts were then subjected to global IR (30/120 min) and the role of the eNOS/NO pathway was evaluated. Dex promoted the activation of eNOS and production of NO. Dex reduced the infarct size and improved the left ventricle function recovery, but this effect was reversed when Dex was co-administered with inhibitors of the eNOS/NO/PKG pathway. In addition, Dex was unable to reduce cell death in isolated adult rat cardiomyocytes subjected to simulated IR. Cardiomyocyte death was attenuated by co-culturing them with endothelial cells pre-treated with Dex. In summary, our results show that Dex triggers cardiac protection by activating the eNOS/NO signaling pathway. This pharmacological effect of Dex requires its interaction with the endothelium.
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Abbreviations: PTIO, (9S,10R,12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-,12epoxy 1Hdiindolo [1,2,3-fg:3′,2′,1′-kl] pyrrolo[3,4-i] [1,6] benzodiazocine-10-carboxylic acid, methyl ester KT58232phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; L-NAME, L-NG-nitroarginine methyl ester; Dex, Dexmedetomidine; TTC, triphenyltetrazolium chloride; IR, Ischemia-Reperfusion; sIR, simulated ischemia-reperfusion; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PKG, cyclic GMP dependent kinase; LVDP, left ventricle developed pressure; LVEDP, left ventricle end diastolic pressure; +dP/dtmax and −dP/dtmin, maximal positive and negative peak of first derivative of left ventricle pressure; BSA, bovine serum albumin; PI, propidium iodide; mPTP, mitochondria permeability transtition pore; TMRM, tetramethylrhodamine; ARC, adult rat cardiomyocytes; EC, endothelial cells; sGC, soluble guanylate cyclase.

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1. Introduction

Dexmedetomidine (Dex) is a lipophilic, highly selective and specific agonist of alpha2-adrenergic receptors [1,2]. In clinical settings, Dex is used for perioperative and intensive care sedation due to its analgesic and anxiolytic effects [3]. The specific stimulation of alpha2-adrenergic receptors (subtypes 2A, 2B and 2C) in the nervous system determines the analgosedative effects observed during Dex administration [4,5]. Dex also shows a biphasic blood pressure response, with an initial increase attributable to vasoconstriction after activation of alpha2B-adrenergic receptors in the vascular smooth muscle, followed by a decrease in blood pressure, after activation of alpha2A-adrenergic receptors in the central nervous system [6–8]. Furthermore, stimulation of postsynaptic alpha2-adrenergic receptors on endothelial cells produces vasodilatation [9]. The drug also reduces the release of stress hormones and catecholamines [8]. The modulation of the sympathetic nervous system theoretically maintains the balance in the input/demand relationship of myocardial oxygen. Dex is known to be protective in multiple organs and types of ischemia-reperfusion (IR) injury [10]. The mechanism by which it protects may be organ-specific. The mechanisms reported to be involved in organ protection include: (a) modulation of cell death by apoptosis [11]; (b) activation of cell survival kinases [12,13]; and, (c) modulation of the inflammatory response and oxidative stress [14]. Few studies have addressed the pharmacological mechanism involved in Dex-dependent cardioprotection [15,16]. Given the biphasic vascular effects of vasoconstriction and vasodilatation of the drug at coronary vessels, it was first proposed that Dex could mimic the effect of ischemic preconditioning [17]. Accordingly, previous work from our group showed that the cardioprotective effects of Dex are mediated by the activation of the reperfusion injury salvage kinases (RISK) [18] pathway after alpha2 adrenergic cardiac receptor stimulation [15].

Previous findings support the hypothesis that paracrine factors may also contribute to cardiomyocyte protection against IR injury. The coronary endothelium regulates coronary perfusion and cardiac function by producing vasoactive substances [19–21]. In the heart, nitric oxide (NO) is physiologically generated by endothelial nitric oxide synthase (eNOS) and neuronal endothelial nitric oxide synthase (nNOS), but the inducible nitric oxide synthase (iNOS) is an important source of NO under pathological conditions [22]. Available evidence suggests that eNOS-derived NO is a critical signaling molecule in different pharmacological cardioprotective strategies [23,24]. We showed previously that Dex induces cardioprotection and activates eNOS in cardiac tissue [15]. However, the pharmacological mechanisms involved in the potential production of endothelial NO induced by Dex and its role in cardiomyocyte protection are still not well defined. In order to study the downstream signaling involved in Dex's protective effect, we evaluated the role of the eNOS/NO pathway and the participation of the endothelium in Dex preconditioning.

2. Materials and methods

2.1. Materials

Antibodies against GAPDH, L-NG-nitroarginine methyl ester (L-NAME), 2phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), (9S,10R,12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-,12epoxy 1Hdiindolo [1,2,3-fg:3′,2′,1′-kl] pyrrolo[3,4-i] [1,6] benzodiazocine-10-carboxylic acid, methyl ester (KT5823), triphenyltetrazolium chloride, laminin, M-199 and insulin were obtained from Sigma–Aldrich (St. Louis, MO). Dexmedetomidine.HCl was acquired from Hospira, Chile. Antibody against eNOS was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Nitric oxide assay kit colorimetric was purchased from Abcam (Cambridge, MA).

2.2. Animals

The present study conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (8th Edition, 2011) and was approved by our Institutional Ethics Review Committee. Rats were obtained from the Animal Breeding Facility of the Faculty of Chemical and Pharmaceutical Sciences, University of Chile.

2.3. Ex vivo isolated rat hearts

Langendorff experiments were performed as previously described [25]. Adult male Sprague-Dawley rats (250–350 g) were anesthetized with sodium pentobarbital (80 mg/kg IP) and heparin 100 U/kg was administered. The hearts were rapidly excised and retrogradely perfused with Krebs Henseleit buffer via the aorta, 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. Left ventricle (LV) functional data were obtained inserting a latex balloon in the ventricle and connected to a pressure transducer (Bridge Amp ML221 AD Instruments, Australia). The hearts were stabilized for 20 min and then subjected to 30 min of global ischemia and 120 min of reperfusion.

2.4. Experimental protocols for ex vivo studies

To evaluate if Dex could activate eNOS and generate NO, and to test if the eNOS/NO pathway mediates the preconditioning effect of the drug, rats were randomly assigned to different experimental groups. Treatments were administered between the end of the stabilization period and before global IR: Control group: 30 min of Krebs Henseleit buffer. Dex group: 25 min of Dex (10 nM), followed by 5 min washout with Krebs Henseleit buffer. Dex was co-administered with or without L-NAME (100 nM), PTIO (100 nM) or KT5823 (1 μM), which are inhibitors of eNOS, NO and cyclic GMP dependent kinase (PKG), respectively (Fig. 1).

2.5. Determination of infarct size

The infarct size was measured through the triphenyltetrazolium chloride (TTC) technique as described previously [26]. At the end of reperfusion, the hearts were first perfused with TTC 1% and then frozen at −20 °C for 1 h. Then, hearts were cut into six slices and stored with formaldehyde 10% for 48 h before measuring the infarct area using the software imageJ.

2.6. Evaluation of LV function

The left ventricle developed pressure (LVDP), left ventricle end diastolic pressure (LVEDP) and the maximal positive and negative peak of first derivative of LV pressure (+dP/dtmax, −dP/dtmin) were measured during the whole experiment on a computer using using PowerLab (ML866 ADInstruments, Australia) as described before [15].

2.7. Nitrite assay

To measure nitrites, samples of the heart effluents from Control and Dex groups were collected during the administration period of the drug (0, 5, 10, and 20 min). A sample after the 5 min washout with Krebs Henseleit was also collected. The quantification of nitrites was determined by the Griess reaction, using the nitric oxide assay colorimetric kit ab65328 (Abcam), according to the manufacturer's instructions.

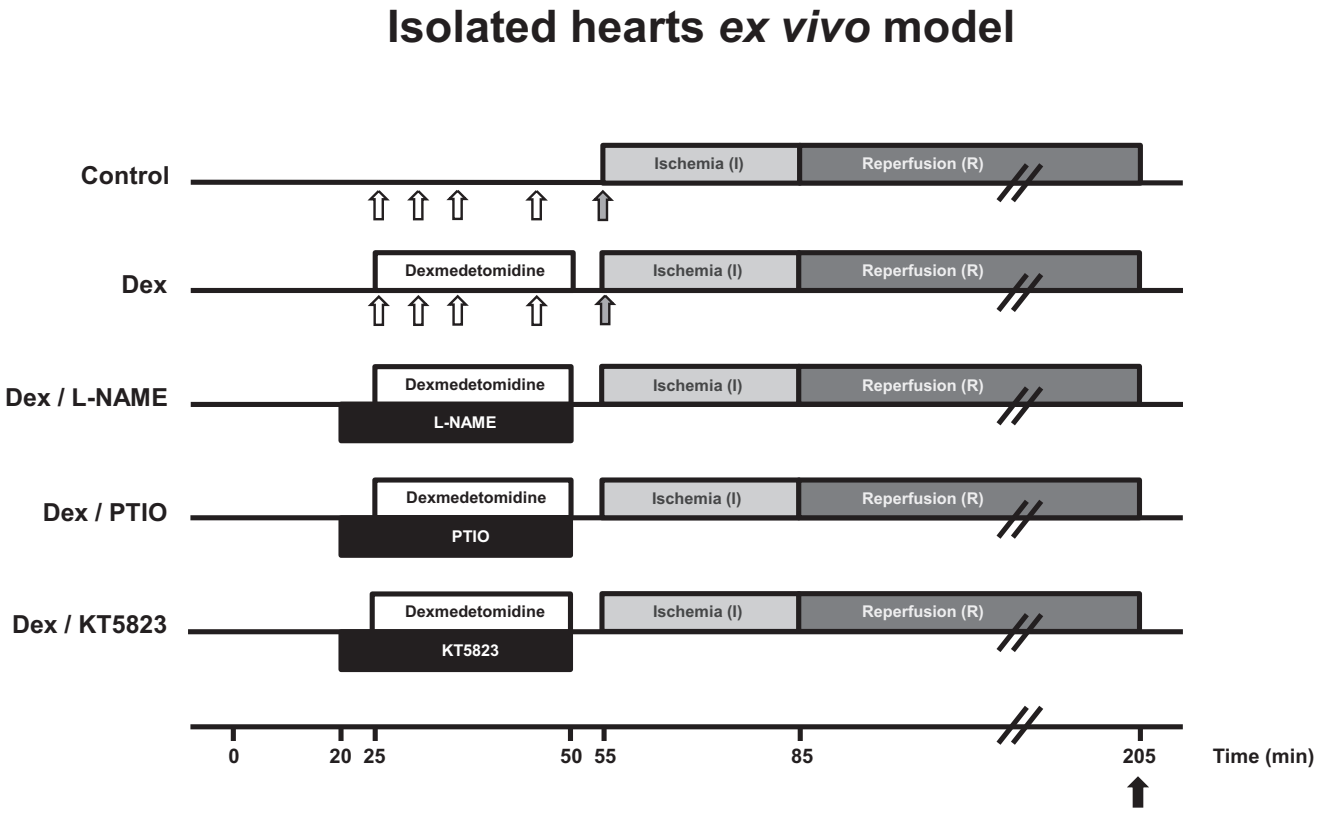

Fig. 1. Experimental protocols for Langendorff experiments**.** Isolated rat hearts were randomly assigned to the different experimental groups. Control: perfusion of Krebs Henseleit buffer for 30 min. Dex: treatment with Dex (10 nM) for 25 min. Dex/L-NAME: treatment with L-NAME (100 nM) for 30 min and co-administration of Dex (10 nM) for 25 min. Dex/PTIO: treatment with PTIO (100 nM) for 30 min and coadministration of Dex (10 nM) for 25 min. Dex/KT5823: Treatment with KT5823 (1 μM) for 30 min and co-administration of Dex (10 nM) for 25 min. After the treatments, all hearts were subjected to 30 min of global ischemia and 120 min of reperfusion. White arrows show determination of nitrites, gray arrows show evaluation of samples by Western blot and black arrows show the assessment of infarct size and LV function.

2.8. Whole heart homogenates

After the 5 min washout of Control and Dex groups, the ventricles were frozen using liquid N_2 and the tissue was homogenized in cold buffer, containing (in mM): MOPS-Tris pH 7.0 (20), sucrose (300), EDTA (2), EGTA (2) Na_3V0_4 (10), NaF (80), $Na_4P_2O_7$ (20), NP-40 1%, SDS 1%, and protease inhibitors leupeptin (2 μg/mL) and pepstatin (1 μg/mL), final pH 7.4. Samples were homogenized using a glass tissue grinder and centrifuged at 1000 × *g* for 20 min at 4 °C. Aliquots were frozen and stored at −80 °C for Western blot analysis. The protein concentration was determined by the Hartree method [27].

2.9. Western blot and eNOS dimer determination

The assessment of eNOS dimers was performed as previously described [28]. Samples were run on low temperature (LT) SDS-PAGE. Whole heart homogenates were loaded on 8% SDS-gel, pre-equilibrated at 4 °C and the electrophoresis tank was maintained in ice to keep the gel below 10 °C. After LT-SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes, and blocked with BSA 5%. Primary antibodies were used against eNOS (1:1000) and GAPDH (1:20000). The bands were quantified by densitometry using the software imageJ.

2.10. Adult rat cardiomyocyte isolation

Adult male Sprague-Dawley rats were anesthetized with sodium pentobarbital (80 mg/kg IP), and the hearts were rapidly excised and perfused with collagenase II through the aorta. The cardiomyocytes were isolated as previously described [29] and plated on laminin (15 μg/mL) coated wells. The cells were maintained with M-199 (Sigma) supplemented with 10% fetal calf serum and 1% penicillin-streptomycin and were allowed to attach for 18–24 h in a standard incubator at 37 °C with 95% O_2 and 5% CO_2.

2.11. Endothelial cells and co-culture experiments

Human umbilical vein endothelial cells (HUVECs) were maintained with EGM-2 BulletKit (Lonza Inc, Allendale, NJ) and were used between passage 3 and 9. For co-culture experiments, HUVEC cells were seeded into 6 well plate transwell inserts with 4 μm pores, according to the manufacturer's instructions (Millipore). Cells were allowed to attach for 18–24 h. Next, HUVEC cells were stimulated with or without Dex (10 nM) for 5 min and then co-cultured on plates with ARC for 15 min. Then, the transwells were removed and the primary cardiomyocytes were subjected to simulated ischemia-reperfusion (sIR).

2.12. Primary endothelial cells

Human umbilical cords were obtained immediately after delivery from full term normal pregnancies from Pontificia Universidad Católica Clinical Hospital in Santiago, Chile. Isolation of HUVEC was performed as previously described [30]. Cells were cultured up to passage 2 in M-199, supplemented with 5 mM D-glucose, 10% newborn calf serum (Gibco), 10% fetal calf serum (Gibco), 3.2 mM L-glutamine and 100 U/ml penicillin–streptomycin. The cells were cultured with the same medium containing 1% newborn calf serum

and 1% fetal calf serum for 12 h before performing the L-citrulline determination experiments.

2.13. Evaluation of L-citrulline content by HPLC

L-Citrulline content was determined in ARC and primary HUVECs by high-performance liquid chromatography (HPLC) [31]. Both cell types were pre-treated for 30 min with or without L-NAME (100 μM) and yohimbine (10 μM), which is an alpha2-adrenergic receptor antagonist. Then, cells were stimulated with or without Dex (10 nM) for 20 min in HEPES buffer, containing (in mM): HEPES (50), NaCl (100), KCl (5), $CaCl_2$ (2.5), $MgCl_2$ (1) supplemented with L-arginine (100 μM). Then, ARC and HUVEC were homogenized and the protein concentration was determined by a modified Lowry's method. L-Citrulline content was assessed using an analytical column (C18: 4.6 mm × 250 mm) (HiQsil; KYA Tech, Japan) coupled to an HPLC system (PU2089s; Jasco, Tokyo, Japan) and the fluorescence at excitation and emission wavelengths (340 and 455 nm, respectively) were determined as previously described [30].

2.14. Simulated ischemia-reperfusion

Adult rat cardiomyocytes (ARC) were subjected to in vitro sIR using a standard method [32]. The M-199 medium was replaced with a buffer that simulates the alterations during ischemia, containing (in mM): NaCl (128), $NaHCO_3$ (2.2), KCl (14.8), $MgSO_4$ (1.2), K_2HPO_4 (1.2), $CaCl_2$ (1), Na-lactate (10) (pH 6.4). The cells were placed in a hypoxic chamber containing 95% N_2 and 5% CO_2 for 3 h. Then, cardiomyocytes were reperfused with normoxic buffer for 1 h and placed in a standard incubator at 37 °C with 95% O_2 and 5% CO_2. Control cardiomyocytes were incubated with normoxic buffer, containing (in mM): NaCl (118), $NaHCO_3$ (22), KCl (2.6), $MgSO_4$ (1.2), K_2HPO_4 (1.2), $CaCl_2$ (1), glucose (10) (pH 7.4).

2.15. mPTP opening assessment

Mitochondrial permeability transition pore (mPTP) opening experiments were performed as previously described [33]. ARC were incubated with (3 μM) tetramethylrhodamine (TMRM) (a lipophilic cation that localizes to the mitochondria) for 20 min. TMRM was prepared in methanol, before being diluted into Tyrode's buffer, containing (in mM): NaCl (137), KCl (5.4), $MgCl_2$ (0.4), $CaCl_2$ (1), glucose (10), HEPES (10). Next, cells were treated with or without Dex (10 nM) for 5 min. Laser illumination of the fluorophore using a confocal microscope with a 543 nm laser induces mitochondrial oxidative stress, and mPTP opening. The mPTP opening is associated with a release of the TMRM red dye from the mitochondria to the cytosol, where the TMRM fluorescence is dequenched, resulting in an increase in cellular fluorescence. Pore opening was measured as the "half-time to maximal intensity" (sec).

2.16. Cell death evaluation

ARC subjected to sIR were stained with propidium iodide (PI) and the percentage of PI positive cells was assessed using a fluorescence microscope as previously described [32].

2.17. Statistical analysis

Results are shown as representative images or as mean ± SEM of at least three independent experiments. Data was analyzed by *t* test, one or two-way ANOVA with either Bonferroni or Tukey's post-test. Differences were considered significant at $P < 0.05$.

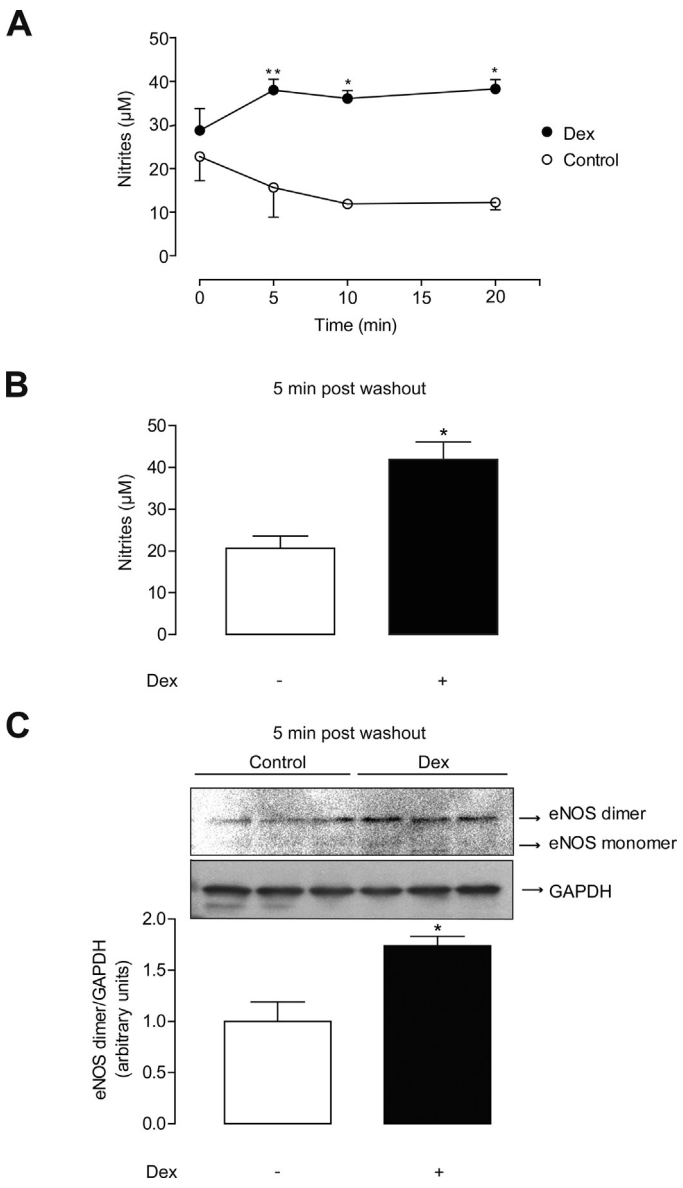

Fig. 2. Dex promotes eNOS dimerization and nitrite production in *ex vivo* hearts. Isolated adult rat hearts were treated with or without Dex (10 nM) for 25 min. (A) Nitrites were assessed by the Griess reaction during perfusion with Krebs Henseleit buffer (white circles) or Dex (10 nM) (black circles) at 0, 5, 10 and 20 min ($N = 3$–6). (B) Nitrites were also measured at the end of the washout period ($N = 4$–6). (C) Western blot of whole heart lysates showing the formation of eNOS dimers in response to Dex (10 nM) after the 5 min washout period (upper panel). Densitometric quantification of eNOS dimers vs. GAPDH (lower panel) ($N = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control. Data were analyzed by paired *t* test with two tails (A and B) and unpaired one-tailed *t* test followed by Mann–Whitney post-test (C). Bar graphs represent mean ± SEM.

3. Results

3.1. Dex activates eNOS and promotes the generation of NO in isolated adult rat hearts

To study the role of the eNOS-NO pathway in the cardiac preconditioning effect of Dex, we first evaluated whether Dex could activate eNOS and stimulate the production of NO in isolated adult rat hearts. Treatment with 10 nM Dex increased the generation of nitrites, a stable marker for NO [34], after 5, 10 and 20 min of administration (Fig. 2A), and the increase was also maintained after 5 min washout (Fig. 2B). To confirm our results, the dimerization of eNOS (the active form of the enzyme [35,36]), was evaluated by West-

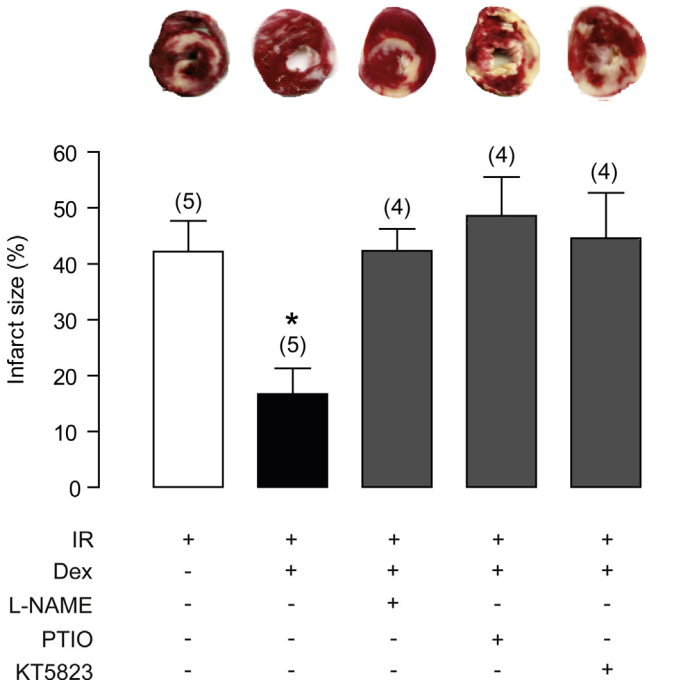

Fig. 3. Dex reduces myocardial infarct size by an eNOS-NO-PKG dependent mechanism in isolated adult rat hearts subjected to global IR. Representative images of heart slices (upper panel). Mean percentage of the infarct size of groups IR, Dex, Dex/L-NAME, Dex/PTIO and Dex/KT5823 after 30 min of global ischemia and 120 min of reperfusion. Hearts were stained with TTC 1% at the end of reperfusion. Bar graphs represent mean ± SEM. The number of experiments is shown for each bar (lower panel). $^{*}P < 0.05$ vs. IR, Dex/L-NAME, Dex/PTIO and Dex/KT5823. Data were analyzed by one-way ANOVA, followed by Tukey's post-test.

ern blot analysis using low temperature SDS-PAGE. Fig. 2C shows that perfusion of hearts with Dex (10 nM) increases the formation of eNOS dimer compared to the control. Thus, the results show that the preconditioning effect of Dex promotes the activation of eNOS and generation of NO in the heart.

3.2. Dex attenuates IR injury through the eNOS/NO/PKG pathway in isolated adult rat hearts

To assess if the eNOS/NO pathway plays a role in the cardioprotective effect of Dex, isolated adult rat hearts were preconditioned by perfusion with Dex (10 nM) for 25 min, followed by 5 min washout with Krebs-Henseleit buffer, before being subjected to 30 min of global ischemia and 120 min of reperfusion. Dex reduced the infarct size (16 ± 10%) compared to untreated hearts (42 ± 12%, $P < 0.05$, $N = 5$). This protection was eliminated when Dex was co-administered with the NOS inhibitor L-NAME (100 nM) or the NO scavenger PTIO (100 nM), (infarct sizes 42 ± 7% and 48 ± 13%, respectively, $N = 4$, n.s. with respect to untreated hearts) (Fig. 3). In order to further explore the pathway downstream of eNOS in Dex preconditioning, we evaluated the role of PKG. To test this, Dex (10 nM) was co-administered with the PKG inhibitor KT5823 (1 μM) and the protection was also abrogated (44 ± 16, $N = 4$, n.s. with respect to untreated hearts) (Fig. 3).

3.3. Dex improves LV function recovery through the eNOS/NO/PKG pathway in isolated adult rat hearts

To confirm our results, LV function was measured. The results show that Dex (10 nM) improves the recovery of LVDP, LVEDP, +dP/dtmax, and -dP/dtmin at the end of reperfusion (Fig 4. A–D), whereas the co-administration of Dex with the eNOS-NO-PKG inhibitors prevents the recovery of LVDP, LVEDP, and +dP/dtmax

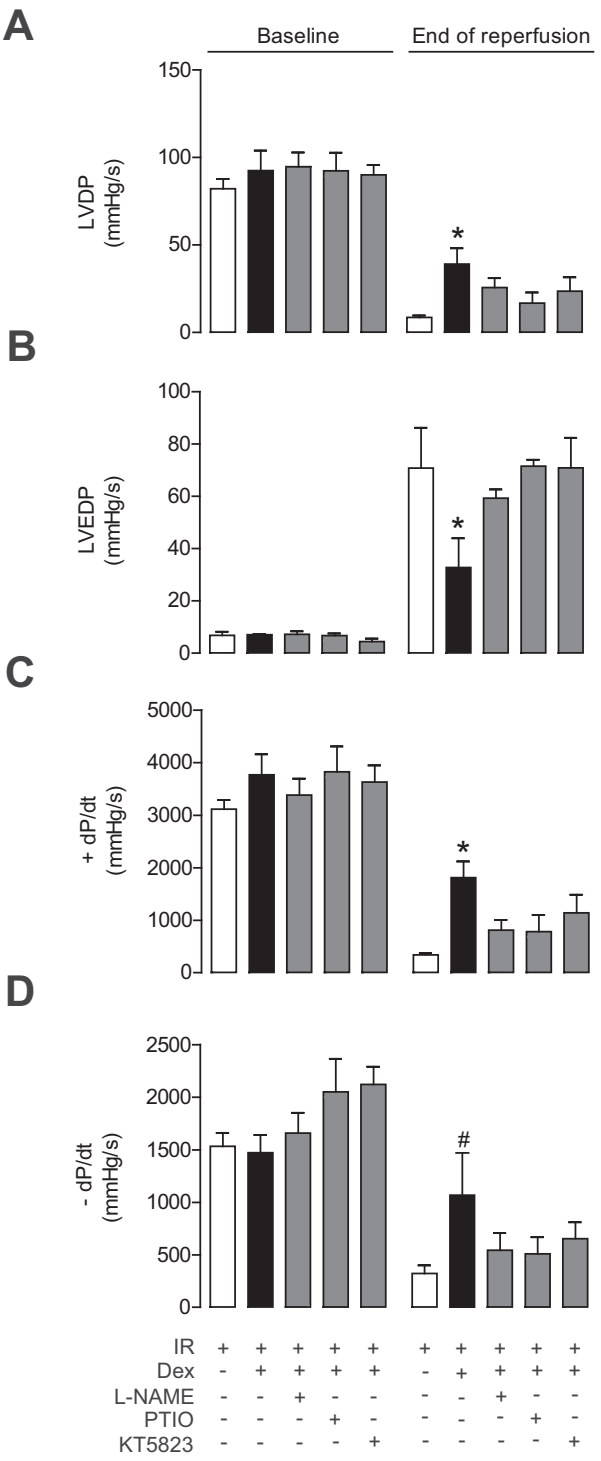

Fig. 4. Dex improves hemodynamic parameters through an eNOS-NO-PKG dependent mechanism in isolated adult rat hearts after global IR. Means of LV function recovery: (A) LVDP, (B) LVEDP, (C) +dP/dtmax, and (D) -dP/dtmin of groups I/R, Dex, Dex/L-NAME, Dex/PTIO and Dex/KT5823 at baseline conditions and at the end of reperfusion ($N = 4$–5). Bar graphs represent mean ± SEM. $^{*}P < 0.05$ vs. IR, Dex/L-NAME, Dex/PTIO and Dex/KT5823. $^{\#}P < 0.05$ vs. IR. Data were analyzed by two-way ANOVA, followed by Bonferroni's post-test.

(Fig 4A–C). Taken together, these results suggest that Dex protects against global IR injury through the eNOS/NO/PKG pathway.

3.4. Dex does not protect adult rat cardiomyocytes subjected to sIR

To investigate whether Dex triggers protective responses directly on isolated cardiomyocytes, we first assessed if the drug was able to delay the formation of the mPTP, a key factor for the onset of necrosis or apoptosis in IR [37–39]. To do this, ARC were loaded with TMRM (3 μM) for 20 min and then treated them with or without Dex (10 nM) for 5 min. mPTP opening was measured by confocal microscopy. Dex was unable to delay the mPTP opening compared to the untreated control (Fig. 5A). Next, we tested the effect of Dex on cell death. To evaluate this, ARC were subjected to 3 h of simulated ischemia and 1 h reperfusion and then stained with the vital dye propidium iodide (PI); we determined the percentage of PI^+ cells using fluorescence microscopy. Insulin (10 nM) was used as a positive control for protection in this model. Our results show that sIR generated 63 ± 8% of cell death compared to normoxic conditions (18.7 ± 0.3%) and Dex did not protect against cell death generated by sIR (65 ± 8%, n.s. vs. sIR, $N = 3$) (Fig. 5B). These results suggest that the cardioprotective actions of Dex on the heart are not via a direct effect on cardiomyocytes.

3.5. Dex requires the endothelium to protect the cardiomyocyte against IR injury

We hypothesized that the presence of endothelial cells was necessary for Dex to exert its protective effect on cardiomyocytes. To this end, ARC were co-cultured with HUVEC cells for 15 min in the presence or absence of Dex, before removing the cardiomyocytes and subjecting them to sIR. The results show that co-culture of cardiomyocytes with untreated endothelial cells was not protective (64 ± 10%), but when HUVEC cells were pre-treated for 5 min with Dex (10 mM) prior to co-culture, the cell death of cardiomyocytes was reduced to 39 ± 6% (Fig. 6). These results indicate that Dex requires the presence of the endothelium to protect the myocardium against IR injury. In order to complement these findings, we evaluate eNOS activation in response to Dex in ARC and primary HUVECs. To test this, ARC and primary HUVECs were stimulated with Dex (10 nM) co-administered with or without L-NAME (100 μM) or yohimbine (10 μM) for 20 min and L-citrulline content was measured by HPLC. The results show that Dex triggers eNOS activation through an alpha2-adrenergic receptor dependent mechanism only in the endothelium, but not in cardiomyocytes (Supplementary Fig. 2A, B).

4. Discussion

This work shows that Dex activates eNOS and generates NO. In addition, we show for the first time that the cardiac preconditioning-mimicking effect of Dex is lost when the eNOS/NO pathway is inhibited. Moreover, we showed that Dex requires the endothelium to reduce cardiomyocyte death. Taken together, these novel findings suggest that Dex protects the myocardium through an eNOS/NO-dependent mechanism, and that cardiomyocyte protection of Dex is achieved indirectly via endothelial cells.

Dex is known to be protective against IR in various organs including the kidneys, lung, brain and liver [14,40–42]. The cardiac preconditioning effect of Dex has also been extensively demonstrated [15–17,43,44]. However, the exact mechanisms by which Dex attenuates IR injury are still being elucidated. Dex can protect against renal, lung and brain IR through the regulation of the JAK/STAT, TLR4/MyD88/MAPK and RISK pathways, respectively [14,41,45]. Regarding the heart, Okada *et al.* described that Dex protected the myocardium against global IR. They discuss that vasoconstriction elicited by Dex may be triggering ischemic preconditioning [17]. Furthermore, our previous studies have shown that Dex activated the RISK pathway in the myocardium [15]. This pathway has been shown by Yellon's group to be central to the myocardial protection both as a consequence of preconditioning and against IR injury [18].

In order to further study the downstream signaling involved in the Dex protective effect, we studied the role of the eNOS/NO signaling pathway. Our results show that Dex activated eNOS and generated NO, which is in agreement with our previous work showing that the drug promoted the phosphorylation of eNOS in the whole heart [15] and other studies that show that Dex can stimulate the production of NO in HUVECs [9,46]. Despite this background, there are no studies showing that the preconditioning effect of Dex depends on this pathway. Our study shows that Dex cannot protect the heart when eNOS and NO are inhibited.

The understanding of the pharmacological mechanism of Dex is essential to ensure its safe use. Dex is an alpha2-adrenergic receptor agonist used as a sedative in anesthesia [47]. Nevertheless, its cardioprotective effects still remain to be fully elucidated. In this context, Dex has shown detrimental effects in the myocardium when used as a post-conditioning rather than a pre-conditioning agent [48]. This observation lead Cai *et al.* to hypothesize that the protective effect of Dex depends on timing [10]. Our study may support this idea, since NO is known to be cardioprotective against IR injury [49,50]. However, when NO bioavailability increases during pro-oxidative conditions, such as reperfusion, it can react with superoxide and generate peroxynitrite, which is toxic to the myocardium [51–54]. Even though exploring this hypothesis is beyond the scope of this work, it may suggest that Dex should be administered as a preconditioning agent during the perioperative period rather than once the myocardial infarction has been developed.

NO can potentially protect against IR injury through both PKG-independent and PKG-dependent mechanisms [54,55], however, we found that PKG is necessary for protection by Dex. In the PKG-dependent pathway, NO activates soluble guanylate cyclase (sGC), which increases cyclic GMP to activate PKG. Interestingly, the cardioprotective effects of sGC activation have been shown to require PKG in cardiomyocytes [56]. This supports our hypothesis that Dex stimulates production of NO in the endothelium, which then may diffuse into cardiomyocytes to activate PKG downstream of sGC. However, further studies are required to thoroughly address this point.

PKG has previously been shown to be cardioprotective in IR [57,58]. The PKG pathway is known to cause vasodilatation [59], which could potentially improve reperfusion of the ischemic tissues. Interestingly, Okada *et al.* found that the coronary flow in isolated rat hearts actually decreased during perfusion with Dex, although it returned to control values soon after the return to normal perfusate and was the same as control values during reperfusion [17]. Alternatively, PKG may protect through mechanisms independent of vasodilatation. For example, evidence shows that PKG can protect cells from IR injury by activating signaling cascades that delay mPTP opening [60–62].

Our previous work showed the existence of the three alpha2-adrenergic receptor subtypes (A–C) in rat cardiac tissue, but with a different distribution pattern for each alpha2-adrenergic receptor subtype. Immunohistochemistry analysis of adult ventricular tissue revealed the presence of alpha2A-adrenergic receptors in the endothelium of blood vessels and on the surface of cardiomyocytes. The alpha2B-adrenergic receptors were detected in the smooth muscle cells of small blood vessels, and the alpha2C-adrenergic receptors on the surface of cardiomyocytes and in the smooth muscle cells of arterioles [15]. Therefore, the evidence suggested that stimulation of cardiomyocyte alpha2A and/or alpha2C-adrenergic receptors by Dex could activate pro-survival kinases and pro-

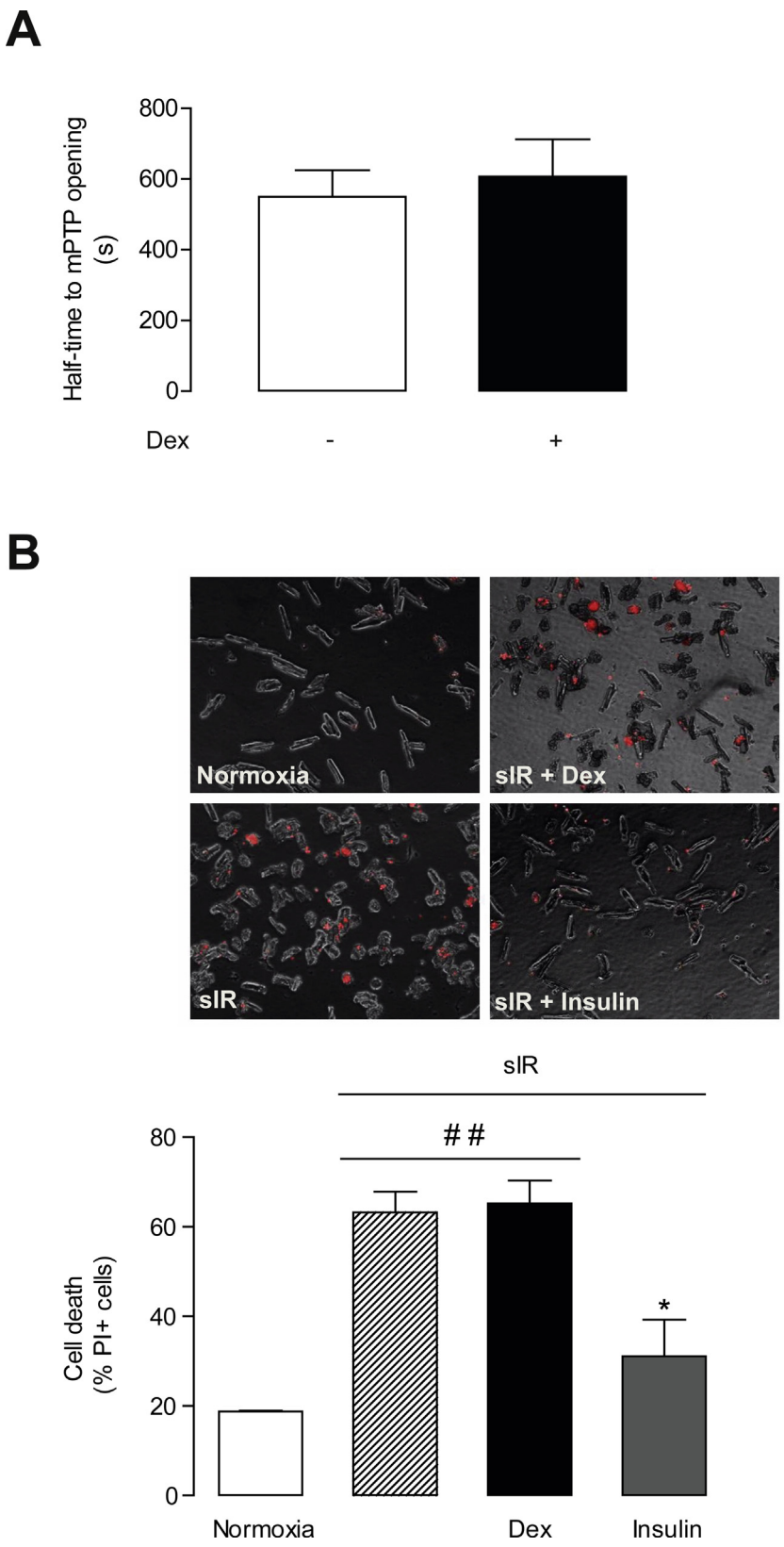

Fig. 5. Dex does not delay mPTP opening or reduce cell death in cardiomyocytes. (A) ARC were incubated with TMRM (3 μM) for 20 min and treated with or without Dex (10 nM) for 5 min and the opening of mPTP was assessed by confocal microscopy ($N = 3$). (B) Representative images of ARC treated with or without Dex (10 nM) for 15 min and then subjected to 3 h of simulated ischemia and 1 h of reperfusion. Insulin (10 nM) was used as a positive control (left). Cell death was assessed by quantifying the percentage of PI+ cells (right) ($N = 3$). Images were taken with a Nikon Eclipse TE200 fluorescent microscope (10× magnification). $^{\#\#}P < 0.001$ vs. normoxia. $^{*}P < 0.05$ vs. sIR and sIR + Dex. Data were analyzed using one-way ANOVA followed by Tukey's post-test.

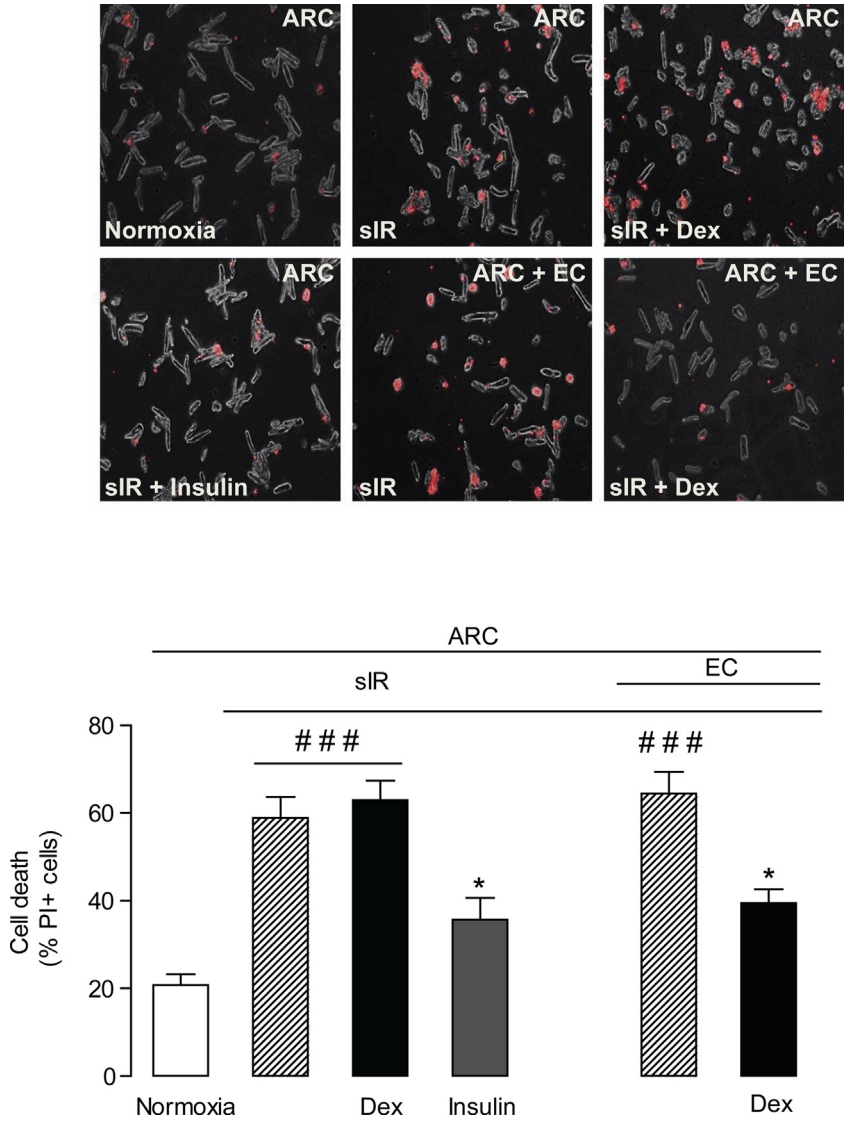

Fig. 6. Dex requires the endothelium to reduce cardiomyocyte death after sIR. (A) Representative images of ARC co-cultured for 15 min with HUVECs (EC) pre-treated with or without Dex 10 nM for 5 min. Co-cultured transwells were then removed and cardiomyocytes were subjected to sIR (upper panel). Quantification of the percentage of PI^+ cells (lower panel) ($N = 4$ independent experiments). Images were taken with a Nikon Eclipse TE200 fluorescent microscope (10× magnification). $^{###}P < 0.0001$ vs. normoxia. $^{*}P < 0.05$ vs. sIR, sIR + Co-culture 15 min and sIR + Dex. Data were analyzed by one-way ANOVA followed by Tukey's post-test.

mote the production of NO within these cells. Thus, we initially hypothesized that Dex could directly exert its preconditioning effect on the cardiomyocyte. Interestingly, our results suggest that Dex requires the presence of the endothelium to reduce cardiomyocyte death triggered by IR. Conventionally described, alpha2-adrenergic receptors are associated with Gi-proteins and decrease of cAMP levels [63]. However, besides Gi-protein coupling, it has also been reported Erk1/2, Akt and PI3K activation after transactivation of epidermal growth factor receptor (EGFR) in porcine veins, CaCo2 and PC12 cells [64–66]. Also, in our previous work, we showed activation of Erk1/2, Akt and eNOS in an alpha2-adrenergic receptor-mediated mechanism in the rat heart. Moreover, Wong et al. showed that Dex could trigger relaxation or contraction in small arteries. Relaxation depended on NO, endothelium, and Gi-protein, mediated by alpha2A-adrenergic receptors, and contraction was mediated by alpha2B and alpha1-adrenergic receptors and involved the action of prostanoids [7]. Furthermore, Porter et al. showed that Dex increased actin cytoskeleton organization and inhibited isoproterenol-stimulated cAMP accumulation in the fetal rat heart. These effects were mediated by stimulation of fully functional alpha2A and alpha2C-adrenergic receptor subtypes present in the fetal cardiomyocyte, where they may have a role in cardiac development [67]. This may imply the possibility of different functions or effects depending on the alpha2-adrenergic receptor subtype involved or the cell type in which it is expressed. This hypothesis is supported by our current results showing that Dex promotes the alpha2-receptor mediated activation of eNOS in primary HUVECs, but not in ARC (see Supplementary Figure 2A,B). However, more studies are required to clarify the underlying mechanisms triggered by the alpha2-adrenergic receptor activation by Dex in the endothelium and the cardiomyocyte.

In complement to the aforementioned results, cardiomyocytes represent roughly 75% of heart volume, but they are estimated to be less than 40% of the total number of cardiac cells [19,68]. Cardiomyocytes are outnumbered by endothelial cells (3:1) and the distance from the capillary endothelium and the closest cardiomyocyte is 1 μm [19]. This generates an ideal context for the interaction between these two cell types. Furthermore, the endothelium is required for protection against IR injury [24]. Moreover, the presence of endothelial cells is necessary for isoflurane to protect

cardiomyocytes subjected to IR [23]. Thus, our findings agree with the concept that the endothelium is important to achieve pharmacological protection against IR and that conditioning strategies should aim for the whole heart rather than the cardiomyocytes [68].

In conclusion, our results suggest that the eNOS/NO pathway mediates the pharmacological cardioprotective effect of Dex and that this protection requires the presence of a functioning endothelium.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2015.11.004.

References

[1] R. Virtanen, J.M. Savola, V. Saano, L. Nyman, Characterization of the selectivity, specificity and potency of medetomidine as an alpha 2-adrenoceptor agonist, Eur. J. Pharmacol. 150 (1988) 9–14.
[2] S. Masuki, F.A. Dinenno, M.J. Joyner, J.H. Eisenach, Selective alpha2-adrenergic properties of dexmedetomidine over clonidine in the human forearm, J. Appl. Physiol. 99 (2005) 587–592.
[3] N. Bhana, K.L. Goa, K.J. McClellan, Dexmedetomidine, Drugs 59 (2000) 263–268, discussion 9–70.
[4] D.B. Bylund, Heterogeneity of alpha-2 adrenergic receptors, Pharmacol. Biochem. Behav. 22 (1985) 835–843.
[5] D.B. Bylund, J.W. Regan, J.E. Faber, J.P. Hieble, C.R. Triggle, R.R. Ruffolo, Jr: Vascular alpha-adrenoceptors: from the gene to the human, Can. J. Physiol. Pharmacol. 73 (1995) 533–543.
[6] B.C. Bloor, D.S. Ward, J.P. Belleville, M. Maze, Effects of intravenous dexmedetomidine in humans. II. Hemodynamic changes, Anesthesiology 77 (1992) 1134–1142.
[7] E.S. Wong, R.Y. Man, P.M. Vanhoutte, K.F. Ng, Dexmedetomidine induces both relaxations and contractions, via different {alpha}2-adrenoceptor subtypes, in the isolated mesenteric artery and aorta of the rat, J. Pharmacol. Exp. Ther. 335 (2010) 659–664.
[8] A. Snapir, J. Posti, E. Kentala, J. Koskenvuo, J. Sundell, H. Tuunanen, et al., Effects of low and high plasma concentrations of dexmedetomidine on myocardial perfusion and cardiac function in healthy male subjects, Anesthesiology 105 (2006) 902–910, quiz 1069-70.
[9] M.S. Joshi, T.B. Ferguson Jr., F.K. Johnson, R.A. Johnson, S. Parthasarathy, J.R. Lancaster Jr., Receptor-mediated activation of nitric oxide synthesis by arginine in endothelial cells, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 9982–9987.
[10] Y. Cai, H. Xu, J. Yan, L. Zhang, Y. Lu, Molecular targets and mechanism of action of dexmedetomidine in treatment of ischemia/reperfusion injury, Mol. Med. Rep. 9 (2014) 1542–1550.
[11] K. Engelhard, C. Werner, E. Eberspacher, M. Bachl, M. Blobner, E. Hildt, et al., The effect of the alpha 2-agonist dexmedetomidine and the *N*-methyl-*D*-aspartate antagonist S(+)-ketamine on the expression of apoptosis-regulating proteins after incomplete cerebral ischemia and reperfusion in rats, Anesth. Analg. 96 (2003) 524–531, table of contents.
[12] S. Dahmani, A. Paris, V. Jannier, L. Hein, D. Rouelle, J. Scholz, et al., Dexmedetomidine increases hippocampal phosphorylated extracellular signal-regulated protein kinase 1 and 2 content by an alpha 2-adrenoceptor-independent mechanism: evidence for the involvement of imidazoline I1 receptors, Anesthesiology 108 (2008) 457–466.
[13] O. Eser, H. Fidan, O. Sahin, M. Cosar, M. Yaman, H. Mollaoglu, et al., The influence of dexmedetomidine on ischemic rat hippocampus, Brain Res. 1218 (2008) 250–256.
[14] Y.M. Zhu, C.C. Wang, L. Chen, L.B. Qian, L.L. Ma, J. Yu, et al., Both PI3K/Akt and ERK1/2 pathways participate in the protection by dexmedetomidine against transient focal cerebral ischemia/reperfusion injury in rats, Brain Res. 1494 (2013) 1–8.
[15] M. Ibacache, G. Sanchez, Z. Pedrozo, F. Galvez, C. Humeres, G. Echevarria, et al., Dexmedetomidine preconditioning activates pro-survival kinases and attenuates regional ischemia/reperfusion injury in rat heart, Biochim. Biophys. Acta 1822 (2012) 537–545.
[16] C. Jiang, M. Xia, M. Wang, S. Chen, Dexmedetomidine preconditioning protects isolated rat hearts against ischemia/reperfusion injuries and its mechanism, Zhejiang da xue xue bao Yi xue ban 42 (2013) 326–330.
[17] H. Okada, T. Kurita, T. Mochizuki, K. Morita, S. Sato, The cardioprotective effect of dexmedetomidine on global ischaemia in isolated rat hearts, Resuscitation 74 (2007) 538–545.
[18] D.J. Hausenloy, D.M. Yellon, Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection, Heart Fail. Rev. 12 (2007) 217–234.
[19] D.L. Brutsaert, Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity, Physiol. Rev. 83 (2003) 59–115.
[20] D.L. Brutsaert, P. Fransen, L.J. Andries, G.W. De Keulenaer, S.U. Sys, Cardiac endothelium and myocardial function, Cardiovasc. Res. 38 (1998) 281–290.
[21] P.C. Hsieh, M.E. Davis, L.K. Lisowski, R.T. Lee, Endothelial-cardiomyocyte interactions in cardiac development and repair, Annu. Rev. Physiol. 68 (2006) 51–66.
[22] M. Zhang, A.M. Shah, ROS signalling between endothelial cells and cardiac cells, Cardiovasc. Res. 102 (2014) 249–257.
[23] T.M. Leucker, M. Bienengraeber, M. Muravyeva, I. Baotic, D. Weihrauch, A.K. Brzezinska, et al., Endothelial-cardiomyocyte crosstalk enhances pharmacological cardioprotection, J. Molecular Cellular Cardiol. 51 (2011) 803–811.
[24] T.M. Leucker, Z.D. Ge, J. Procknow, Y. Liu, Y. Shi, M. Bienengraeber, et al., Impairment of endothelial-myocardial interaction increases the susceptibility of cardiomyocytes to ischemia/reperfusion injury, PLoS One 8 (2013) e70088.
[25] R.M. Bell, M.M. Mocanu, D.M. Yellon, Retrograde heart perfusion: the Langendorff technique of isolated heart perfusion, J. Mol. Cell. Cardiol. 50 (2011) 940–950.
[26] M. Vila-Petroff, M.A. Salas, M. Said, C.A. Valverde, L. Sapia, E. Portiansky, et al., CaMKII inhibition protects against necrosis and apoptosis in irreversible ischemia-reperfusion injury, Cardiovasc. Res. 73 (2007) 689–698.
[27] E.F. Hartree, Determination of protein: a modification of the lowry method that gives a linear photometric response, Anal. Biochem. 48 (1972) 422–427.
[28] B.H. Chung, S. Kim, J.D. Kim, J.J. Lee, Y.Y. Baek, D. Jeoung, et al., Syringaresinol causes vasorelaxation by elevating nitric oxide production through the phosphorylation and dimerization of endothelial nitric oxide synthase, Exp. Mol. Med. 44 (2012) 191–201.
[29] D.J. Hausenloy, D.M. Yellon, S. Mani-Babu, M.R. Duchen, Preconditioning protects by inhibiting the mitochondrial permeability transition, Am. J. Physiol. Heart Circ. Physiol. 287 (2004) H841–H849.
[30] F. Pardo, L. Silva, T. Saez, R. Salsoso, J. Gutierrez, C. Sanhueza, et al., Human supraphysiological gestational weight gain and fetoplacental vascular dysfunction, Int. J. Obes. 39 (2015) 1264–1273.
[31] G. Wu, C.J. Meininger, Analysis of citrulline, arginine, and methylarginines using high-performance liquid chromatography, Methods In Enzymol. 440 (2008) 177–189.
[32] J.M. Vicencio, D.M. Yellon, V. Sivaraman, D. Das, C. Boi-Doku, S. Arjun, et al., Plasma exosomes protect the myocardium from ischemia-reperfusion injury, J. Am. Coll. Cardiol. 65 (2015) 1525–1536.
[33] S.M. Davidson, D. Hausenloy, M.R. Duchen, D.M. Yellon, Signalling via the reperfusion injury signalling kinase (RISK) pathway links closure of the mitochondrial permeability transition pore to cardioprotection, Int. J. Biochem. Cell Biol. 38 (2006) 414–419.
[34] M. Kelm, Nitric oxide metabolism and breakdown, Biochim. Biophys. Acta 1411 (1999) 273–289.
[35] T.S. Schmidt, N.J. Alp, Mechanisms for the role of tetrahydrobiopterin in endothelial function and vascular disease, Clin. Sci. 113 (2007) 47–63.
[36] R. Rafikov, F.V. Fonseca, S. Kumar, D. Pardo, C. Darragh, S. Elms, et al., eNOS activation and NO function: structural motifs responsible for the posttranslational control of endothelial nitric oxide synthase activity, J. Endocrinol. 210 (2011) 271–284.
[37] M. Crompton, The mitochondrial permeability transition pore and its role in cell death, Biochem. J. 341 (1999) 233–249, Pt. 2.
[38] A.P. Halestrap, S.J. Clarke, S.A. Javadov, Mitochondrial permeability transition pore opening during myocardial reperfusion–a target for cardioprotection, Cardiovasc. Res. 61 (2004) 372–385.
[39] S.B. Ong, P. Samangouei, S.B. Kalkhoran, D.J. Hausenloy, The mitochondrial permeability transition pore and its role in myocardial ischemia reperfusion injury, J. Mol. Cell. Cardiol. 78 (2015) 23–34.
[40] J. Lempiainen, P. Finckenberg, E.E. Mervaala, M. Storvik, J. Kaivola, K. Lindstedt, et al., Dexmedetomidine preconditioning ameliorates kidney ischemia-reperfusion injury, Pharmacol. Res. Perspect. 2 (2014), e00045.
[41] L. Jiang, L. Li, J. Shen, Z. Qi, L. Guo, Effect of dexmedetomidine on lung ischemiareperfusion injury, Mol. Med. Rep. 9 (2014) 419–426.
[42] A. Kucuk, F. Yaylak, A. Cavunt-Bayraktar, M. Tosun, M. Arslan, F.M. Comu, et al., The protective effects of dexmedetomidine on hepatic ischemia reperfusion injury, Bratisl. Lek. Listy 115 (2014) 680–684.
[43] O. Yoshitomi, S. Cho, T. Hara, I. Shibata, T. Maekawa, H. Ureshino, et al., Direct protective effects of dexmedetomidine against myocardial ischemia-reperfusion injury in anesthetized pigs, Shock 38 (2012) 92–97.
[44] H. Kocoglu, K. Karaaslan, E. Gonca, O. Bozdogan, N. Gulcu, Preconditionin effects of dexmedetomidine on myocardial ischemia/reperfusion injury in rats, Curr. Ther. Res. Clin. Exp. 69 (2008) 150–158.
[45] Y. Si, H. Bao, L. Han, H. Shi, Y. Zhang, L. Xu, et al., Dexmedetomidine protects against renal ischemia and reperfusion injury by inhibiting the JAK/STAT signaling activation, J. Transl. Med. 11 (2013) 141.

[46] H.J. Kim, J.T. Sohn, Y.S. Jeong, M.S. Cho, H.J. Kim, K.C. Chang, et al., Direct effect of dexmedetomidine on rat isolated aorta involves endothelial nitric oxide synthesis and activation of the lipoxygenase pathway, Clin. Exp. Pharmacol. Physiol. 36 (2009) 406–412.
[47] S. Naaz, E. Ozair, Dexmedetomidine in current anaesthesia practice- a review, J. Clinical Diagnostic Res.: JCDR 8 (2014) GE01–GE04.
[48] S. Mimuro, T. Katoh, A. Suzuki, S. Yu, Y.U. Adachi, M. Uraoka, et al., Deterioration of myocardial injury due to dexmedetomidine administration after myocardial ischaemia, Resuscitation 81 (2010) 1714–1717.
[49] R. Schulz, M. Kelm, G. Heusch, Nitric oxide in myocardial ischemia/reperfusion injury, Cardiovasc. Res. 61 (2004) 402–413.
[50] L. Phillips, A.H. Toledo, F. Lopez-Neblina, R. Anaya-Prado, L.H. Toledo-Pereyra, Nitric oxide mechanism of protection in ischemia and reperfusion injury, J. Invest. Surg. 22 (2009) 46–55.
[51] J. Zaman, S. Jeddi, A. Ghasemi, The effects of ischemic postconditioning on myocardial function and nitric oxide metabolites following ischemia-reperfusion in hyperthyroid rats, Korean J. Physiol. Pharmacol. 18 (2014) 481–487.
[52] Q. Fan, X.C. Yang, Y. Liu, L.F. Wang, S.H. Liu, Y.G. Ge, et al., Postconditioning attenuates myocardial injury by reducing nitro-oxidative stress in vivo in rats and in humans, Clin. Sci. 120 (2011) 251–261.
[53] P. Ferdinandy, R. Schulz, Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning, Br. J. Pharmacol. 138 (2003) 532–543.
[54] C. Farah, C. Reboul, No better way to protect the heart duringIschemia-reperfusion: to be in the right place at the right time, Front. Pediatr. 3 (2015) 6.
[55] M.V. Cohen, X.M. Yang, Y. Liu, N.V. Solenkova, J.M. Downey, Cardioprotective PKG-independent NO signaling at reperfusion, Am. j.physiol. Heart circulatory physiology. 299 (2010) H2028–36.
[56] C. Methner, R. Lukowski, K. Grube, F. Loga, R.A. Smith, M.P. Murphy, et al., Protection through postconditioning or a mitochondria-targeted S-nitrosothiol is unaffected by cardiomyocyte-selective ablation of protein kinase G, Basic Res. Cardiol. 108 (2013), 337.
[57] D.S. Burley, P. Ferdinandy, G.F. Baxter, Cyclic GMP and protein kinase-G in myocardial ischaemia-reperfusion: opportunities and obstacles for survival signaling, Br. J. Pharmacol. 152 (2007) 855–869.
[58] A. Das, A. Smolenski, S.M. Lohmann, R.C. Kukreja, Cyclic GMP-dependent protein kinase Ialpha attenuates necrosis and apoptosis following ischemia/reoxygenation in adult cardiomyocyte, J. Biol. Chem. 281 (2006) 38644–38652.
[59] M.T. Gewaltig, G. Kojda, Vasoprotection by nitric oxide: mechanisms and therapeutic potential, Cardiovasc. Res. 55 (2002) 250–260.
[60] A. Das, L. Xi, R.C. Kukreja, Protein kinase G-dependent cardioprotective mechanism of phosphodiesterase-5 inhibition involves phosphorylation of ERK and GSK3beta, J. Biol. Chem. 283 (2008) 29572–29585.
[61] A. Das, F.N. Salloum, L. Xi, Y.J. Rao, R.C. Kukreja, ERK phosphorylation mediates sildenafil-induced myocardial protection against ischemia-reperfusion injury in mice, Am. J. Physiol. Heart Circ. Physiol. 296 (2009) H1236–H1243.
[62] S.B. Ong, R.K. Dongworth, H.A. Cabrera-Fuentes, D.J. Hausenloy, Role of the MPTP in conditioning the heart - translatability and mechanism, Br. J. Pharmacol. 172 (2015) 2074–2084.
[63] Z.P. Khan, C.N. Ferguson, R.M. Jones, alpha-2 and imidazoline receptor agonists: their pharmacology and therapeutic role, Anaesthesia 54 (1999) 146–165.
[64] B. Buffin-Meyer, P.A. Crassous, C. Delage, C. Denis, S. Schaak, H. Paris, EGF receptor transactivation and PI3-kinase mediate stimulation of ERK by alpha(2A)-adrenoreceptor in intestinal epithelial cells: a role in wound healing, Eur. J. Pharmacol. 574 (2007) 85–93.
[65] R.E. Roberts, Alpha 2 adrenoceptor-mediated vasoconstriction in porcine palmar lateral vein: role of phosphatidylinositol 3-kinase and EGF receptor transactivation, Br. J. Pharmacol. 138 (2003) 107–116.
[66] G. Karkoulias, O. Mastrogianni, A. Lymperopoulos, H. Paris, C. Flordellis, alpha(2)-Adrenergic receptors activate MAPK and Akt through a pathway involving arachidonic acid metabolism by cytochrome P450-dependent epoxygenase, matrix metalloproteinase activation and subtype-specific transactivation of EGFR, Cell. Signal. 18 (2006) 729–739.
[67] A.C. Porter, S.P. Svensson, W.D. Stamer, J.J. Bahl, J.G. Richman, J.W. Regan, Alpha-2 adrenergic receptors stimulate actin organization in developing fetal rat cardiac myocytes, Life Sci. 72 (2003) 1455–1466.
[68] R.M. Bell, D.M. Yellon, Conditioning the whole heart–not just the cardiomyocyte, J. Molecular Cell. Cardiol. 53 (2012) 24–32.