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

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Abstract

The alpha2-adrenergic receptor agonist Dexmedetomidine (Dex) is a sedative medication used by intensive care units and anesthesiologists. Previous reports have shown that Dex protects the heart against ischemia-reperfusion (IR) and can also act as a preconditioning mimetic. However, the biochemical/pharmacological 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)/cyclic GMP dependent kinase (PKG) 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/PKG 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 measured. , Hearts were then subjected to 30 min of global ischemia and 120 min of reperfusion and the role of the eNOS/NO/PKG pathway was evaluated. Dexmedetomidine promoted the activation of eNOS and production of NO. Furthermore, Dexmedetomidine reduced the infarct size and improved the left ventricle function recovery, but this effect was reversed when Dexmedetomidine was co-administered with inhibitors of the eNOS/NO/PKG pathway. In addition, Dexmedetomidine was unable to reduce cell death in isolated adult rat cardiomyocytes subjected to simulated IR (sIR). However, cell death of cardiomyocytes was attenuated by co-culturing them with endothelial cells that were pre-treaed with Dex. In summary, our results show that Dexmedetomidine triggers cardiac protection by activating the eNOS/NO/PKG signaling pathway. This pharmacological effect of Dexmedetomidine requires its interaction with the endothelium.

Keywords: Dexmedetomidine, α2-adrenergic receptor, heart, endothelium, preconditioning, ischemia-reperfusion.

Chemical compounds: Dexmedetomidine, hydrochloride (PubChem CID: 6918081), L-NAME hydrochloride (PubChem CID: 135193), PTIO (PubChem CID: 2733513), KT5823 (PubChem CID: 108152).

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, 2C) in the nervous system determines the analgosedative effects observed during Dex administration [4, 5]. Dex also demonstrates a biphasic blood pressure response, with an initial increase attributable to vasoconstriction after activation of alpha2/betaadrenergic 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-specifc. The biochemical and pharmacological 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 RISK pathway after α2-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 [18-20]. 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 [21]. Available evidence suggests that eNOS-derived NO is a critical signaling molecule in different pharmacological cardioprotective strategies [22, 23]. We showed previously that Dex induces cardioprotection and activates eNOS in cardiac tissue [15]. However, the biochemical and 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/PKG 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 (8^{th} 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 [24]. 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/PKG 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) (Fig. 1).

2.5. Determination of infarct size

The infarct size was measured through the triphenyltetrazolium chloride (TTC) technique as described previously [25]. At the end of reperfusion, the hearts were perfused with TTC 1% and 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.

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 x 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 [26].

2.9. Western blot and eNOS dimer determination

The assessment of eNOS dimers was performed as previously described [27]. 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 LTSDS-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 [28] and plated on laminin (15 µg/mL) coated wells. The cells were maintained with M199 (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

HUVEC cells 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 adult rat cardiomyocytes for 15 min. Then, the transwells were removed and the primary cardiomyocytes were subjected to simulated ischemia/reperfusion.

2.12. Simulated ischemia-reperfusion (sIR)

Adult rat cardiomyocytes were subjected to *in vitro* sIR using a standard method [29]. 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).

2.13. mPTP opening assessment

Mitochondrial permeability transition pore (mPTP) opening experiments were performed as previously described [30]. Adult rat cardiomyocytes 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).

2.14. Cell death evaluation

Adult rat cardiomyocytes 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 [29].

2.15. 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 $P<0.05$.

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 [31], 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 [32, 33]), was evaluated by Western blot analyses using LT-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, before they 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 NOS 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 4A-C). Taken together, these results suggest that Dex protects against global I/R 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 mitochondrial permeability transition pore (mPTP) , a key factor for the onset of necrosis or apoptosis in IR [34-36]. To do this, adult rat cardiomyocytes 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, adult rat cardiomyocytes 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= ?) (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 I/R injury

We hypothesized that the presence of endothelial cells was necessary for Dex to exert its protective effect on cardiomyocytes. To this end, adult rat cardiomyocytes 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 nM) prior to co-culture, the cell death of cardiomyocytes was reduced to 39 ± 6 % (Fig. 6). These indicate that Dex requires the presence of the endothelium to protect the myocardium against IR injury.

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/PKG 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/PKG-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, 37-39]. The cardiac preconditioning effect of Dex has also been extensively demonstrated [15-17,40,41]. However, the exact biochemical mechanisms by which Dex attenuates IR injury are still being elucidated. Dex can protect against renal, lung and brain I/R through the regulation of the JAK/STAT, TLR4/MyD88/MAPK and RISK pathways,

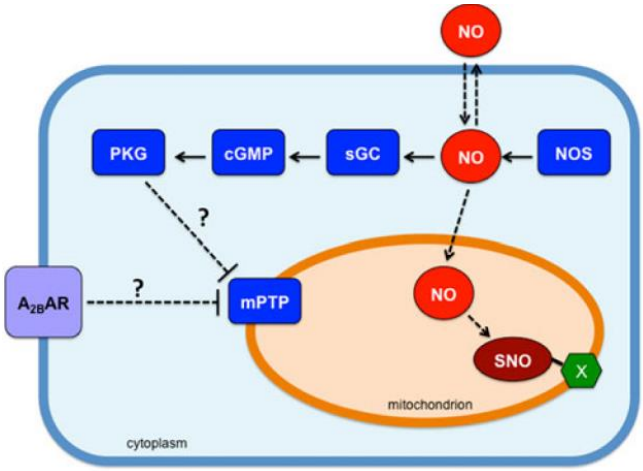
respectively [14, 38, 42]. Regarding the heart, Okada *et al* described that Dex protected the myocardium against global IR. They discuss that vasoconstriction elicited by Dex may be triggering ischemic preconditioning [17]. Furthermore our previous studies have shown that Dex activated the RISK pathway 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 ischaemia-reperfusion injury (ref).

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 HUVEC cells [9, 43]. 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 [44]. 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 [45]. This observation lead Cai *et al* to hypothesize that the protective effect of Dex depend on timing [10]. Our study may support this idea, since NO is

known to be cardioprotective against IR injury [46, 47]. 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 [48-51]. 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 [51, 54], 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 [Methner et al Basic Res Cardiol. 2013 Mar;108(2):337]. This supports our hypothesis that Dex stimulates production of NO in the endothelium, which then diffuses into cardiomyocytes to activate PKG downstream of sGC.



PKG has previously been shown to be cardioprotective in IR [52, 53]. The PKG pathway is known to cause vasodilatation [55], which could potentially improve reperfusion of the ischemic tissues. Interestingly, Okada *et al* found that the coronary flow in isolated rats 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 [56-58].

Our previous study showed that the alpha2?–adrenergic receptors are expressed in the endothelium, but cardiomyocytes also express subtypes 2A and 2C [ref]. Thus, we initially hypothesized that Dex could directly exert its preconditioning effect on the cardiomyocyte. Interestingly, our results suggested that Dex requires the presence of the endothelium to reduce cardiomyocyte death triggered by IR. In this context, cardiomyocytes represent roughly 75% of heart volume, but they are estimated to be less than 40% of the total number of cardiac cells [19, 59]. 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 [23]. Moreover, the presence of endothelial cells is necessary for isoflurane to protect cardiomyocytes subjected to IR [22]. 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 [59].

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

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Conflicts of interests

None.

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Figure legends

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.

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 *t* test. Bar graphs represent

mean ± SEM.

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.

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. 5. Dex does not delay mPTP opening or reduce cell death in cardiomyocytes**.** (A) Adult rat cardiomyocytes 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 adult rat cardiomyocytes 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). $^{\#\#}$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 adult rat cardiomyocytes (ARC) co-cultured for 15 min with HUVEC cells (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). $^{\#\#\#}$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.