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The novel H_2S-donor 4-carboxyphenyl isothiocyanate promotes cardioprotective effects against ischemia/reperfusion injury through activation of mitoK_{ATP} channels and reduction of oxidative stress

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ABSTRACT

The endogenous gasotransmitter hydrogen sulphide (H_2S) is an important regulator of the cardiovascular system, particularly of myocardial function. Moreover, H_2S exhibits cardioprotective activity against ischemia/reperfusion (I/R) or hypoxic injury, and is considered an important mediator of "ischemic preconditioning", through activation of mitochondrial potassium channels, reduction of oxidative stress, activation of the endogenous "anti-oxidant machinery" and limitation of inflammatory responses. Accordingly, H_2S-donors, i.e. pro-drugs able to generate exogenous H_2S, are viewed as promising therapeutic agents for a number of cardiovascular diseases. The novel H_2S-donor 4-carboxy phenylisothiocyanate (4CPI), whose vasorelaxing effects were recently reported, was tested here in different experimental models of myocardial I/R.

In Langendorff-perfused rat hearts subjected to I/R, 4CPI significantly improved the post-ischemic recovery of myocardial functional parameters and limited tissue injury. These effects were antagonized by 5-hydroxydecanoic acid (a blocker of mitoK_{ATP} channels). Moreover, 4CPI inhibited the formation of reactive oxygen species. We found the whole battery of H_2S-producing enzymes to be present in myocardial tissue: cystathionine γ-lyase (CSE), cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (MPST). Notably, 4CPI down-regulated the post-ischemic expression of CSE.

In Langendorff-perfused mouse hearts, 4CPI reduced the post-ischemic release of norepinephrine and the incidence of ventricular arrhythmias. In both rat and mouse hearts, 4CPI did not affect the degranulation of resident mast cells.

In isolated rat cardiac mitochondria, 4CPI partially depolarized the mitochondrial membrane potential; this effect was antagonized by ATP (i.e., the physiological inhibitor of K_{ATP} channels). Moreover, 4CPI abrogated calcium uptake in the mitochondrial matrix.

Finally, in an in vivo model of acute myocardial infarction in rats, 4CPI significantly decreased I/R-induced tissue injury.

In conclusion, H_2S-donors, and in particular isothiocyanate-based H2S-releasing drugs like 4CPI, can actually be considered a suitable pharmacological option in anti-ischemic therapy.

1. Introduction

The gasotransmitter hydrogen sulphide (H_2S) is a pleiotropic and ubiquitous mediator which influences almost all the functions of the mammalian body [1]. Among these, the regulation of

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the cardiovascular system is an important role of this gasotransmitter [2,3]. H_2S is biosynthesized by cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) [4], and by the cooperation between cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (MPST) [4–6]. The pattern of distribution and localization of these enzymes is quite complex and they may coexist in cardiovascular tissues [7–9].

H_2S exhibits cardioprotective activity against ischemia/reperfusion (I/R) or hypoxic injury and is considered an important mediator of "ischemic preconditioning" (IPC), a self-defence cardioprotective mechanism against myocardial I/R injury. The mechanisms of action accounting for this cardioprotective activity are heterogeneous and not yet completely understood. Mitochondrial ATP-sensitive potassium channels ($mitoK_{ATP}$) are well-known effectors of ischemic preconditioning [10]. Their activation by H_2S is likely to be a relevant cardioprotective mechanism [11], since the anti-ischemic effects of H_2S are largely antagonized by blockers of mitochondrial potassium channel [12]. Other mechanisms have also been proposed to explain the cardioprotective activity of H_2S. Antiapoptotic responses play a role in cardioprotection against I/R injury, and are due to the H_2S-induced triggering of pathways of intracellular signalling, such as PI3K/Akt, PKC and ERK 1/2, and the Nrf-2-mediated antioxidant machinery [13]. Even the inhibition of type-5 phosphodiesterase by H_2S plays a potential role. In fact, the intracellular rise of cGMP, and the consequent cGMP-dependent protein kinase activation, trigger downstream effectors of ischemic preconditioning [14], upregulate CSE levels and promote further H_2S production [15].

Exacerbation of I/R-induced tissue injury can be also due to an intense inflammatory response, triggered by the degranulation of resident heart mast cells and the release of cytokines, growth factors, chemokines, and other pro-inflammatory mediators [16]. Remarkably, cardiac mast cells contain renin and the release of this proteolytic enzyme from cardiac mast cells contributes to the I/R-associated heart injury [17].

Noteworthy, H_2S was reported to inhibit antigen-induced degranulation of rat basophile leukemic RBL-2H3 cells (a well known mast-cell like model) and murine bone marrow-derived mast cells, suggesting an inhibitory role of this gasotransmitter in mast cell-mediated inflammatory responses [18,19]. Therefore, a possible H_2S-mediated inhibition of the degranulation of resident heart mast cells may be a further (and poorly investigated) mechanism contributing, at least in part, to the overall cardioprotective activity.

Given the intriguing biological activity of H_2S in the cardiovascular function, natural and synthetic H_2S-donors, i.e. pro-drugs able to generate exogenous H_2S, are viewed as promising cardioprotective agents [20–22]. 4-carboxy phenyl-isothiocyanate (4CPI, Fig. 1) is a H_2S-releasing compound, known to evoke vasorelaxing responses in isolated rat aorta and to increase coronary flow in isolated rat hearts [23]. Moreover, 4CPI was shown to cause membrane hyperpolarization in human aortic smooth muscle cells through the activation of Kv7 potassium channels, which play a role in H_2S-induced vasodilation [24]. Noteworthy, Kv7 channels (in particular, Kv7.4) have been recently recognized in heart mitochondria, where they appear to play a cardioprotective role [25].

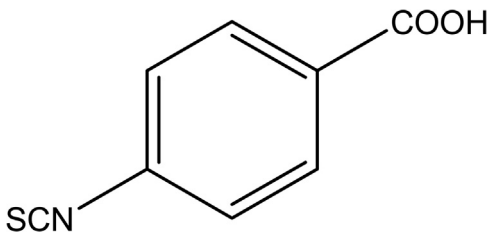

Fig. 1. Chemical structure of 4CPI.

Although the vascular effects of the novel H_2S-donor 4CPI have been elucidated, its potential cardioprotective activity is yet to be evaluated. Hence, the aim of this study was to investigate the effects of 4CPI in different experimental models of I/R in isolated hearts and of acute myocardial infarction in vivo, by evaluating well-known markers of I/R-induced myocardial injury (i.e., reduced myocardial contractility, ventricular arrhythmias, cell death, oxidative stress). The involvement of some relevant mechanisms of action, such as activation of mitochondrial ion channels and/or inhibition of degranulation of resident heart mast cells, was also tested in isolated cardiac mitochondria and isolated hearts, respectively.

2. Materials and methods

2.1. Substances

4CPI (Fluorochem Ltd, Hadfield, UK) was dissolved (10^{-2} M) in dimethylsulfoxide (DMSO), and further diluted in bi-distilled water. 5-hydroxy decanoic acid (5HD; Sigma–Aldrich, Milano, Italy) was dissolved in bidistilled water. Tetraphenylphosphonium chloride (TPP^+Cl^-, Sigma–Aldrich, Milano, Italy) was dissolved in bi-distilled water and 2,3,5-triphenyltetrazolium chloride (TTC, Sigma–Aldrich. Milano, Italy) was dissolved (1%, p/w) in phosphate buffer (pH 7.4). Olygomicin, 2,4-dinitrophenol (DNP), carbonyl cyanide m-chlorophenylhydrazone (CCCP) and valinomycin were purchased from Sigma–Aldrich (Milano, Italy), dissolved in pure DMSO (10 mM) and further diluted in bi-distilled water.

2.2. Pharmacological procedures

All the experimental procedures were carried out following the guidelines of the European Community Council Law 2010/63 and have been approved by the Committee for animal experimentation of the University of Pisa. All procedures on mice were approved by Weill Cornell Medicine Institutional Animal Care and Use Committee.

2.3. Langendorff-perfused rat hearts

Male Wistar rats (260–350 g) were treated with an i.p. injection of different increasing doses of 4CPI 0.072 mg/kg, 0.24 mg/Kg, 0.72 mg/kg and 2.4 mg/kg or their vehicle (DMSO). After 2 h, all the animals were anaesthetized with sodium pentobarbital (100 mg/kg i.p.) and heparinized (100 UI i.p.) to prevent blood clotting. When required from experimental procedure, 5HD (10 mg/kg i.p.) was administrated 20 min before 4CPI (0.24 mg/kg)-treatment.

After opening the chest, hearts were quickly excised and placed in a 4 °C Krebs solution (composition mM: $NaHCO_3$ 25.0, NaCl 118.1, KCl 4.8, $MgSO_4$ 1.2, $CaCl_2 \times 2H_2O$ 1.6, KH_2PO_4 1.2, glucose 11.5), equilibrated with 95% O_2 and 5% CO_2, to stop contraction and reduce oxygen consumption. Rapidly, the ascending aorta was cannulated and the hearts were mounted on a Langendorff apparatus, and then perfused with Krebs solution (thermostated at 37 °C and continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2) at constant pressure (70–80 mmHg). The above procedure was completed within 2 min. A water-filled latex balloon connected to a pressure transducer (Bentley Trantec, mod 800, UgoBasile, Comerio, Italy) was introduced into the left ventricle via the mitral valve and the volume was adjusted to achieve a stable left ventricular end-diastolic pressure of 5–10 mmHg during initial equilibration. After 30 min of equilibration, hearts were subjected to 30 min of global ischemia (no flow). Thereafter, hearts were reperfused for 120 min. Functional parameters were continuously recorded during the whole experiment. At the end of reperfusion hearts were

removed from the Langendorff apparatus and left ventricles were isolated and submitted to morphometric, histological and biochemical assays.

2.3.1. Recording of functional parameters

Reduction of heart contractility after I/R has been used as a suitable indicator of I/R-induced injury. Therefore, to test potential cardioprotective effects of 4CPI, we recorded prospective improvements of inotropic parameters in hearts submitted to I/R.

Heart rate (HR), left ventricular developed pressure (LVDP) and the rate of rise of the left ventricular pressure (dP/dt) were continuously monitored by a computerized Biopac system (California, USA). RPP was calculated as Rate x Pressure Product. Post-ischemic dP/dt and RPP recorded during reperfusion were expressed as a% of the corresponding values recorded in the pre-ischemic period. Hearts showing severe arrhythmia or unstable LVDP and HR values, during the pre-ischemic phase, were discarded.

2.3.2. Beta-hexosaminidase assay in coronary effluent

Measurement of mast cell-derived beta-hexosaminidase (β-hexo) is widely used as a suitable marker of mast cell degranulation induced by I/R. Thus, to test potential inhibitory effects of 4CPI on mast cell degranulation, we investigated whether any reduction in mast cell-derived β-hexo occurred in 4CPI-treated hearts submitted to I/R.

At the beginning of reperfusion, 5 ml of coronary effluent were collected and used to measure β-hexo concentration. 50 μl of perfusate were placed into a 96-well plate, then 50 μl of β-hexo substrate (p-nitrophenyl-N-acetyl-D-glucosaminide 1.3 mg/ml dissolved in citrate buffer 0.1 M, pH4.5) were added and incubated for 60 min at 37 °C. The reaction was stopped with 100 μl of TRIS buffer (pH = 9 at 4 °C). Optical density was read at 405 nm on a plate reader (EnSpire, Perkin Elmer). β-hexo release was expressed as arbitrary units of absorbance.

2.3.3. Morphometric analysis of the ischemic area

The size of ventricular tissue exhibiting I/R-induced cell death is widely used as a suitable indicator of I/R-induced injury. Therefore, to test potential cardioprotective effects of 4CPI, we measured the possible reduction of the size of injured areas in 4CPI-treated hearts submitted to I/R.

The left ventricle was cut in 2 mm-wide slices which were immersed in a 1% aqueous solution of TTC for 20 min and then in a 10% aqueous solution of formaldehyde. After 24 h, ventricular slices were photographed and analyzed to highlight necrotic areas due to the ischemic process (visible as a white or light pink color) and the healthy areas (visible as a strong red due to the TTC reaction).

2.3.4. Determination of CSE, CBS and MPST expression

The expression of CSE, CBS and MPST was determined in heart tissue, in order to evaluate the influence of I/R on the H_2S biosynthesizing machinery. Moreover, the effects of 4CPI on the expression of these enzymes was evaluated in hearts (with and without I/R), in order to investigate the mechanisms of action of this compound. As above described, hearts were isolated from rats pretreated with vehicle or 4CPI and submitted to the I/R cycle. Moreover, CSE, CBS and MPST expression was also detected in the hearts of a "sham group": in this group, hearts were isolated from rats pretreated with vehicle or 4CPI, mounted in a Langendorff apparatus and perfused for 150 min, but not submitted to I/R.

Following an I/R cycle or a sham 150 min perfusion time, left ventricular tissue from rat hearts were homogenized in lysis buffer. Denatured proteins (40 μg) were separated on 10% sodium-dodecylsulfate polyacrylamide gels and transferred to polyvinylidene fluoride membrane (PVDF). Membranes were blocked in phosphate-buffered saline containing 0.1%v/v Tween-20 (PBST) and 3% w/v non-fat dry milk for 30 min, followed by overnight incubation at 4 °C with rabbit polyclonal anti-MPST (1:500, Novus Biologicals, Italy), mouse monoclonal anti-CSE (1:1000, Proteintech, UK), rabbit polyclonal anti-CBS (1:1000, Santa Cruz Biotechnology, Germany) or rabbit polyclonal anti-GAPDH (1:5000, Sigma–Aldrich, Italy). Membranes were extensively washed in PBST prior to incubation with horseradish-peroxidase conjugated secondary antibody for 2 h at room temperature. Following incubation, membranes were washed and chemiluminescence was detected by using ImageQuant-400 (GE-Healthcare, USA). Target protein band intensity was normalized in consideration of housekeeping protein GAPDH intensity.

2.3.5. Detection of oxidative stress

Increased production of reactive oxygen species (ROS) was considered as a suitable indicator of I/R-induced oxidative stress, i.e. an additional marker of myocardial injury. Thus, to further test the potential cardioprotective effects of 4CPI, we recorded a possible reduction of ROS production in 4CPI-treated hearts submitted to I/R.

For histological labeling and qualitative image acquisition, left ventricles from rats were isolated and readily included in Optimal Cutting Temperature (OCT, Sakura, Japan) without fixation. Cryosection 20 μm thick were obtained using a manual cryostat (Leica, Germany) and collected onto glass slides. Ventricle sections were then incubated in 10 μM dihydroethidium (DHE) solution at 37 °C for 30 min. Sections were then rinsed 3 × 5 min in phosphate buffered saline (PBS) and mounted for confocal imaging with Vectashield (Vector, Burlingame). Confocal images were obtained for each section using a 16× oil objective (NA 0.50) on a TCS-SP2 confocal microscope (Leica, Germany). Each image was acquired as the maximum intensity projection of a 10 μm confocal stack. In order to quantify ROS+ areas in the different experimental group, were acquired six fields of each section of the left ventricle analyzed. It was sampled three sections for each left ventricle of three animals per group.

2.4. Langendorff-perfused mouse hearts

Wild-type C57BL/6J mice (male, 10-to12-week old, Jackson Laboratory, Bar Harbor, ME) were injected i.p. with 4CPI 0.24 mg/kg 2 h before the experiment. Mice were injected i.p. with heparin (100 UI), anaesthetized with CO_2 and euthanized by cervical dislocation.Hearts were isolated and transferred to a Langendorff apparatus (Radnoti, Monrovia, CA, USA). The aorta was cannulated with a flanged 20-gauge stainless-steel needle. The heart was perfused through the aorta in a retrograde mode at a constant pressure of 100 cm H_2O with modified Krebs-Henseleit (KH) buffer containing (mM): NaCl, 120; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 11; Pyruvate acid, 2; EDTA, 0.5. The perfusion fluid was bubbled with 95%O_2 and 5% CO_2 at 37 °C to give a pH of 7.4. After equilibration, all hearts were subjected to 30-min ischemia (glucose- and pyruvic acid-free KH buffer, 95% N_2 + 5% CO_2 and sodium dithionite) followed by 30-min reoxygenation (reperfusion) with KH buffer. Coronary flow was measured by timed collections of the effluent every 5 min; samples were assayed for β-hexo, renin and norepinephrine (NE).

2.4.1. Evaluation of ventricular arrhythmias

Surface ECG was recorded from leads attached to the left ventricle and the right atrium and analyzed using Power Laboratory/8SP (AD Instruments, Colorado Springs, CO, USA). Reperfusion arrhythmias were recorded and quantified according to the Lambeth Conventions.

2.4.2. Determination of norepinephrine release

Increased release of norepinephrine (NE) from heart sympathetic nerve endings was considered a suitable indicator of I/R-induced adrenergic activation. Thus, to further test potential cardioprotective effects of 4CPI, we investigated a possible reduction of NE release in 4CPI-treated hearts submitted to I/R.

Norepinephrine (NE) was assayed in the coronary perfusate by high-performance liquid chromatography coupled to electrochemical detection [26]. Perchloric acid and EDTA were added to samples to achieve final concentration of 0.01 N and 0.025%, respectively. NE present in the effluent was adsorbed on acid-washed alumina adjusted at pH 8.6 with Tris 2% EDTA buffer (in agitation for 45 min) and then extracted in 150 μl of 0.1 N perchloric acid. These final samples were injected onto a 3 μm ODS reverse-phase column (3.2 × 100 mm, Bioanalytical System, West Lafayette, IN) with an applied potential of 0.65 V. The mobile phase consisted of monochloroacetic acid (75 mM), Na_2EDTA (0.5 mM), sodium actysulfate (0.5 mM) and acetonitrile (15%) at pH 3.0. Flow rate was 1.0 mg/min. Dihydroxybenzylamine (DHBA) was added to each sample as an internal standard before alumina extraction and used for recovery calculation. The recovery of NE was 77% and the detection limit was approximately 0.2 pmol.

2.4.3. Determination of β-hexo release

The determination of mast cell-derived β-hexo has been used as a suitable marker of mast cell degranulation induced by I/R. Therefore, to test the potential inhibitory effects of 4CPI on mast cell degranulation, we ascertained whether the release of mast cell-derived β-hexo was reduced in 4CPI-treated mouse hearts submitted to I/R. Sample of coronary effluent were concentrated 8- to 10-fold by centrifugal filtration (EMDMillipore), then the same procedure described above was carried out.

2.4.4. Determination of renin release

Determination of mast cell-derived renin has been used as an additional suitable marker of mast cell degranulation induced by I/R. Thus, as a further test of potential inhibitory effects of 4CPI on mast cell degranulation, we evaluated a possible reduction of mast cell-derived renin in 4CPI-treated hearts submitted to I/R.

Renin activity was measured in samples collected from coronary effluent using a GammaCoat Plasma Renin Activity 125I Radioimmunoassay kit (DiaSorin, Stillwater, MN, USA) according to the manufacturer's instructions. Briefly, 200 μl each sample of coronary effluent were incubated with porcine angiotensinogen (0.1 mg/ml), maleate buffer and PMSF (part of the DiaSorin kit). The reaction for angiotensin I production was conducted for 1.5 h at 37 °C. Results were normalized to total heart weight. The detection limit was approximately 0.01 pmol.

2.5. Acute myocardial infarction

The cardioprotective effects of 4CPI were evaluated in vivo, in an experimental model more closely resembling the clinical condition of acute myocardial infarction.

The experimental protocol for coronary occlusion-reperfusion was performed as described in [27], with minor modifications. Two hours before the experimental procedure, rats received an i.p. injection (about 0.3 ml) of 4CPI (0.24 mg/kg) or vehicle (DMSO). Then, rats were anaesthetized with sodium pentobarbital (70 mg/ kg, i.p.). The trachea was intubated and connected to a rodent ventilator (mod. 7025 UgoBasile, Comerio, Italy) for artificial ventilation with room air (stroke volume, 1 ml/100 g body weight; 70 strokes/min). Electrocardiogram (ECG) was continuously measured by lead II (Mindray, PM5000, 2 Biological Instruments, Varese, Italy). The chest was opened by a left thoracotomy. A 6-0 surgical needle was passed around the left anterior descending coronary artery (LAD), located between the base of the pulmonary artery and left atrium. The ends of the suture were passed through a polypropylene tube (PE50) to form a snare, allowing reversible artery occlusion. The acute infarction protocol consisted of 30-min occlusion/120-min reperfusion; successful occlusion was confirmed by observing regional cyanosis downstream of the ligature, and by ST elevation in the ECG recording. A group of vehicle-pretreated animals was submitted to an IPC procedure, achieved by 2 cycles of 5-min occlusion/10 min reperfusion, followed by 30-min coronary occlusion and 120-min reperfusion. Each experimental group was composed of 6–10 animals. At the end of reperfusion, rats were euthanized by an overdose of sodium pentobarbital, hearts were quickly excised, mounted on a Langendorff apparatus (Radnoti, California, USA) and perfused for 100 with Krebs solution at 37 °C to cleanse coronary blood vessels. Then, the atria and right ventricle were removed from the hearts. The left ventricular tissue was dried, frozen at −20 °C, and cut into 4–5 transverse slices from apex to base of equal thickness (about 2 mm). The slices were then incubated in a 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution in a phosphate buffer (pH 7.4) at 37 °C for 20 min. TTC reacts with NADH in the presence of dehydrogenase enzymes, to form a formazan derivative, which stain the viable cells with intense red color. The slices were then fixed overnight in 10% formaldehyde and finally photographed. Red-stained viable tissue was easily distinguished from the infarcted white-unstained necrotic tissue.

2.6. Isolated cardiac mitochondria

To investigate a possible mitochondrial involvement, the effects of 4CPI on membrane potential and calcium uptake were tested in isolated heart mitochondria.

Mitochondria were isolated by differential centrifugation from hearts excised from a different group of rats. These rats were not pretreated with either 4CPI or vehicle. These hearts were not submitted to any I/R protocol. Male Wistar rats (260–350 g) were killed by pentobarbital overdose, hearts were removed immediately and placed in an ice-cold isolation buffer (composition mM: sucrose 250, Tris 5, EGTA 1, pH 7.4 adjusted with HCl). The atria were removed and the ventricular tissue was finely minced with surgical scissors (about 2 mm pieces) and homogenized using an Ultra-Turrax homogenizer (20 ml of isolation buffer per heart, IKA1-Werke GmbH & Co., Staufen-Germany). Three homogenization cycles (each of 20 s) were performed on ice, and then the suspension was centrifuged at 1075*g* for 3 min at 4 °C (EuroClone, Speed Master 14 R centrifuge, Milano, Italy). The resulting supernatant was centrifuged at 11,950*g* for 10 min at 4 °C. The pellet containing the mitochondrial fraction was further resuspended in the isolation buffer (without EGTA) and centrifuged at 11,950*g* for 10 min at 4 °C, this step was repeated once more. The final mitochondrial pellet was resuspended in a minimal volume of 400 μl of the isolation buffer (without EGTA) and stored on ice throughout the experiments, which were performed within 2 h. Mitochondrial protein concentrations were determined using the Bradford reaction. Previous experiments (data not shown) confirmed the reliability of the isolation procedures by measurement of the mitochondrial respiratory function with an ATP bioluminescence assay, in agreement with the method of Drew and Leeuwenburgh, as previously described [28].

2.6.1. Mitochondrial membrane potential

Mitochondrial membrane potential (Ψm) was measured potentiometrically with tetraphenylphosphonium (TPP^+)-sensitive mini-electrodes, coupled with a reference electrode (WPI, FL, USA), using a data acquisition software (Biopac Inc. California, USA), as previously described [28]. Briefly, electrodes were calibrated before each experiment using known concentrations of TPP^+Cl^-. Mito-

chondria (1 mg protein/ml) were suspended under gently stirring in the incubation medium (composition mM: KCl 120, K_2HPO_4 5, Hepes 10, succinic acid 10, $MgCl_2$ 2, EGTA 1, plus TPP^+Cl^- 10 μM, pH 7.4 adjusted with KOH). The value of the potential was calculated by the following Nernst-derived experimental equation: where Ψm is the mitochondrial membrane potential (mV),

$$\Delta\psi = 60 \times Log \frac{V_0 \cdot \frac{[TPP^+]_0}{[TPP^+]_t} - V_t - K_0 P}{V_m P + K_i P}$$

V_0 is the volume of the incubation medium before the mitochondria addition, V_t is the volume of the incubation medium after the mitochondria addition, V_m is the volume of the mitochondrial matrix (ml/mg protein), $[TPP^+]_0$ and $[TPP^+]_t$ are concentrations of TPP^+ recorded before the addition of mitochondria and at time t, respectively, P is the protein concentration (mg), K_0 and K_i are apparent external and internal partition coefficients of TPP^+, and were estimated as 14.3 ml/mg and 7.9 ml/mg, respectively. The volume of mitochondria was taken as 1 ml/mg of protein. Mitochondria showing a basal level of membrane potential >−170 mV were discarded. Changes of Ψm were continuously recorded (in mV) before and after the addition in the incubation medium of cumulative increasing concentrations of 4CPI (10–300 μM). When required, ATP (200 μM), physiological blocker of K_{ATP} channels, or XE991 (10 μM, blocker of Kv7 channels) were incubated in the medium 2 min before the mitochondria addition. Effects of the addition of the corresponding vehicles were evaluated. Each concentration-response curve was obtained with mitochondria isolated from the hearts of 6–10 different animals.

2.6.2. Mitochondrial calcium uptake

Mitochondrial calcium uptake was measured by potentiometric technique, as described [28]. In particular, the changes of calcium concentration in the medium (i.e. extra-mitochondrial calcium) were continuously measured with a calcium-selective mini-electrode, coupled with a reference electrode (WPI, FL, USA), using a data acquisition software (Biopac Inc. California, USA). The selectivity of the electrode for calcium over other cations, such as magnesium, potassium and sodium, is $>10^5$. In order to correlate the potentiometric measurements (in mV) with the corresponding concentrations of calcium ions in the solution, calibration curves were generated before each experiment, using known concentrations of $CaCl_2$. Mitochondria (1 mg protein/ml) were added, under gently stirring, to the incubation medium (composition mM: KCl 120, K_2HPO_4 5, Hepes10, succinic acid 10, $MgCl_2$ 2, plus $CaCl_2$ 100 μM, pH 7.4 adjusted with KOH) containing the vehicle (DMSO 1%) or 4CPI (100 μM or 300 μM). After the addition of mitochondria, the maximal decrease of the concentration of calcium in the medium, related to its accumulation in the mitochondrial matrix, was measured. Each result was obtained with mitochondria isolated from the hearts of 6 different animals.

2.7. Data analysis

Data were expressed as means ± standard errors and were statistically analyzed by ANOVA and Student's *t*-test (software: GraphPadPrism 4.0). P values lower than 0.05 were considered as indicative of significant differences.

3. Results

3.1. Effects on isolated rat heart subjected to I/R injury

I/R caused marked functional damage to isolated hearts of vehicle-treated rats, with a significant reduction of myocardial contractility. Indeed, during reperfusion, the RPP value was always less than 50% of the corresponding pre-ischemic one. Consistently, dP/dt values were markedly reduced (Fig. 2A–B). The functional impairment induced by I/R was associated with a high degree of tissue injury detected by morphometric analysis (Fig. 2C).

I/R caused less damage in isolated hearts of rats treated with 4CPI (0.072-0.72 mg/kg), as indicated by an improved recovery of both functional and morphometric parameters (Fig. 2A-C). In contrast, at the highest dose tested (2.4 mg/kg), 4CPI failed to produce cardioprotective effects, as an evident ischemic injury was observed at this dose (Fig. 2A–C).

Finally, pre-treatment of animals with 5HD (10 mg/kg i.p.), a selective blocker of mito-KATP channels, almost completely abolished the cardioprotective effects of 4CPI, administered at the selected dose 0.24 mg/kg (Fig. 2A–C).

DHE staining of control non-ischemic samples of myocardial tissue showed a very low level of ROS production. In contrast, hearts of vehicle-treated animals submitted to I/R revealed a high density of DHE-stained nuclei, indicating a significant level of oxidative stress and ROS production. Treatment with 4CPI 0.24 mg/kg significantly reduced the levels of DHE staining in myocardial samples after I/R injury (Fig. 3A–B).

No release of β-hexo, a marker of mast cell degranulation, was detectable in the coronary perfusate of control non-ischemic hearts. showed High levels of β-hexo were instead detected in the coronary perfusate of rat hearts subjected to I/R. No changes in β-hexo release were observed after 4CPI treatment (0.072, 0.24 and 0.72 mg/kg). In contrast, at the highest dose (2.4 mg/kg) 4CPI caused a significant increase in β-hexo release (Fig. 3C).

Western blot analysis showed a basal expression of CSE, CBS and MPST in myocardial tissue (sham group). I/R did not significantly modify the expression of the three enzymes, although there was a slight not significant increase in CSE (Fig. 4A). In hearts subjected to I/R, treatment with 4CPI significantly reduced CSE protein levels, as also demonstrated by densitometry analysis (Fig. 4B). In contrast, no significant changes occurred in either CBS or MPST protein expression following 4CPI administration (Fig. 4C–D). Notably, 4CPI did not change levels of CBS, MPST and CSE in "sham group" hearts (data not shown).

3.2. Effects on isolated mouse hearts subjected to I/R

When Langendorff-perfused murine hearts were subjected to 30 min of ischemia, followed by 30 min reoxygenation, a large increase in renin, β-hexo and norepinephrine (NE) overflow was observed during the first 5 min of reperfusion, which was accompanied by ventricular tachycardia/fibrillation (VT/VF). Pre-treatment with 4CPI 0.24 mg/kg significantly diminished NE release, as well as the duration of VT/VF, whereas renin and β-hexo release from resident mast cells was not modified (Fig. 5A-D).

3.3. 3 In vivo model of acute myocardial infarction

LAD ligature led, in vehicle-treated rats, to wide I/R-induced tissue injury (Ai/AVL = 39 ± 2%). IPC significantly reduced I/R-induced tissue injury (Ai/AVL = 17 ± 6%);pretreatment with 4CPI 0.24 mg/kg also significantly reduced the size of I/R-injured areas (Ai/AVL = 25 ± 3; Fig. 6A–B).

3.4. Effects on mitochondrial membrane potential

Membrane potential of isolated heart mitochondria was lower than −170 mV. Cumulatively increasing concentrations of 4CPI (10–300 μM) were added to a mitochondrial suspension (1 mg/ml), and only at the highest concentration 4CPI elicited a significant depolarization of mitochondrial membrane potential of about 40 mV. The effect of 4CPI was significantly antagonized by ATP

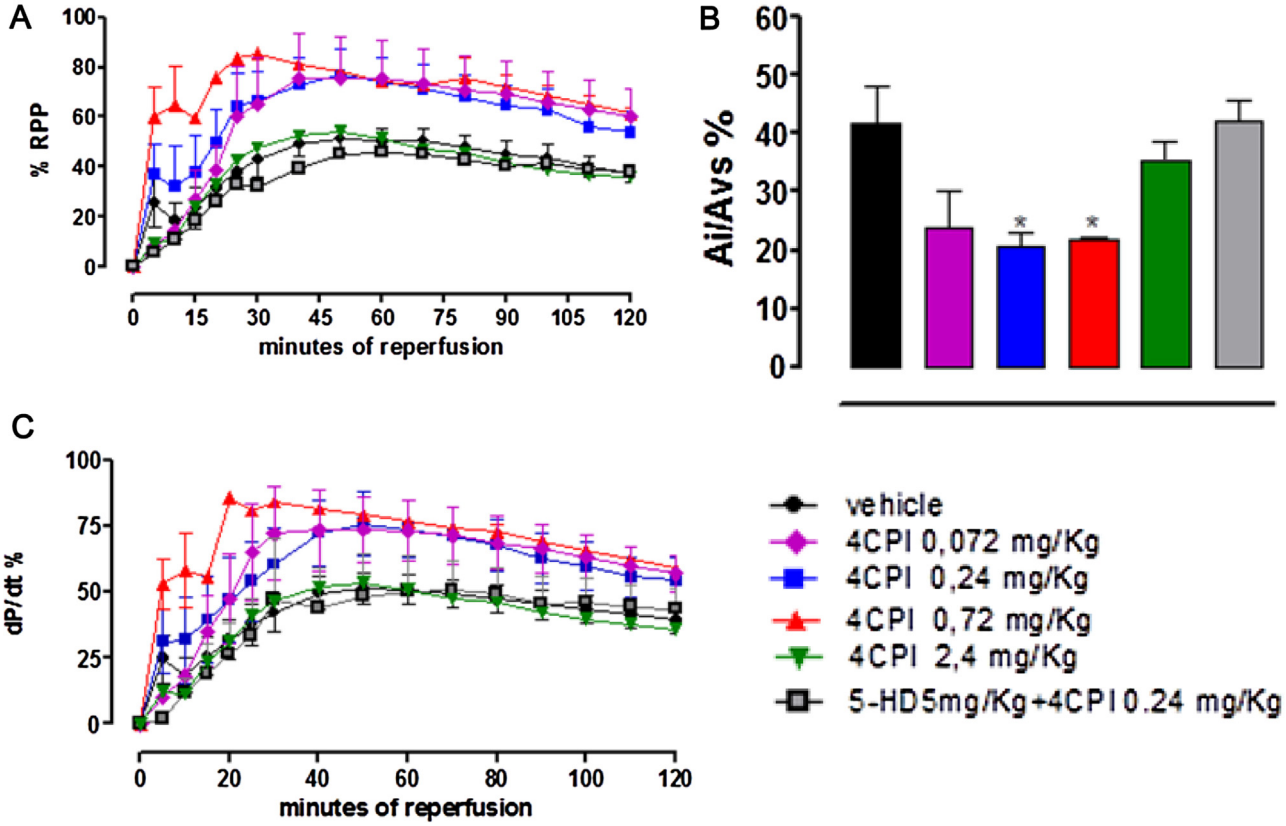

Fig. 2. Post-ischemic functional parameters of RPP (A) and dP/dt (B) recorded during reperfusion in Langendorff rat hearts pre-treated with vehicle (black), with increasing doses of 4CPI (0.072 mg/Kg, magenta;0.24 mg/Kg, blue; 0.72 mg/Kg, red;2.4 mg/Kg, green) or with 4CPI 0.24 mg/kg plus 5HD 5 mg/kg (gray). Two way ANOVA indicated that the curves obtained in hearts of rats pre-treated with 4CPI (0.072, 0.24 and 0.72 mg/kg) show significant differences ($P < 0.01$) vs vehicle. (C) The corresponding data emerging from the morphometric analysis of the areas of I/R-induced tissue injury (Ai/Avs) are also shown; asterisks indicate significant differences (* = $P < 0.05$) vs vehicle. In all the panels, the different pharmacological pre-treatments are indicated by different colors of lines and histograms: vehicle (black); 4CPI 0.072 mg/Kg, magenta; 4CPI 0.24 mg/Kg, blue; 4CPI 0.72 mg/Kg, red; 4CPI 2.4 mg/Kg, green; 4CPI 0.24 mg/kg plus 5HD 5 mg/Kg, gray.

(200 μM), the physiological mitoK_{ATP} blocker (Fig. 7A). In contrast, XE991, a Kv7 potassium channel blocker, did not influence the effects of 4CPI (data not shown).

3.5. *Effects on mitochondrial calcium uptake*

Cardiac mitochondria (1 mg/ml), exposed to 100 μM $CaCl_2$, rapidly and almost fully accumulated this cation, leading to an almost complete reduction of calcium concentration in the extra-mitochondrial buffer (82 ± 13 μM). 4CPI 100 μM and 300 μM significantly reduced calcium accumulation in a concentration-dependent mode (Fig. 7B).

4. Discussion

Heart diseases, such as myocardial ischemia and infarction, are major causes of mortality, and the identification of novel pharmacological tools to limit I/R-induced cardiac injury remains a challenging issue. The discovery of H_2S as the third gasotransmitter and the understanding of its pivotal role in regulating cardiovascular function, and in mediating cardioprotection, paved the way to new exciting perspectives in this field of research [29]. In particular, H_2S-releasing drugs, such as GYY4137, have shown significant protective effects in experimental model of myocardial I/R [30], suggesting that H_2S-donors can actually be viewed as a promising class of anti-ischemic drugs.

In this paper, we evaluated the cardioprotective effects of 4CPI and investigated the mechanisms of action. The H_2S-releasing properties of 4CPI were clearly characterized in previous studies by usual amperometric and spectrometric measurements, and further confirmed by gas chromatography, coupled with mass spectrophotometry [23].

In an *ex-vivo* experimental model of myocardial I/R (Langendorff-perfused rat hearts), i.p. pre-administration of 4CPI, at doses of 0.072, 0.24 and 0.72 mg/Kg, led to significant recovery of RPP and dP/dt during reperfusion and to an evident limitation of tissue injury, although no clear dose-dependency was observed. Accordingly, as an H_2S-donor, 4CPI affords significant cardioprotection against I/R-mediated myocardial tissue injury. Notably, at high doses (2.4 mg/kg) 4CPI failed to show cardioprotective effects: indeed, both functional and morphometric parameters recorded after I/R in hearts of rats pre-treated with this dose of 4CPI were almost equivalent to those recorded in vehicle-treated animals. Such a "bi-modal" effect of 4CPI can be reasonably attributed to an excessive formation of H_2S and the consequent induction of toxic effects, typical of the higher concentration of all gasotransmitters [31,32]. This hypothesis is supported by the determination of mast cell-derived β-hexo. This marker of mast cell degranulation could not be detected in the perfusate of non-ischemic rat hearts, but was clearly released in hearts of vehicle- treated animals, subjected to I/R. Pre-treatment with cardioprotective doses of 4CPI (0.072, 0.24 and 0.72 mg/kg) did not reduce the release of β-hexo, suggesting that inhibition of

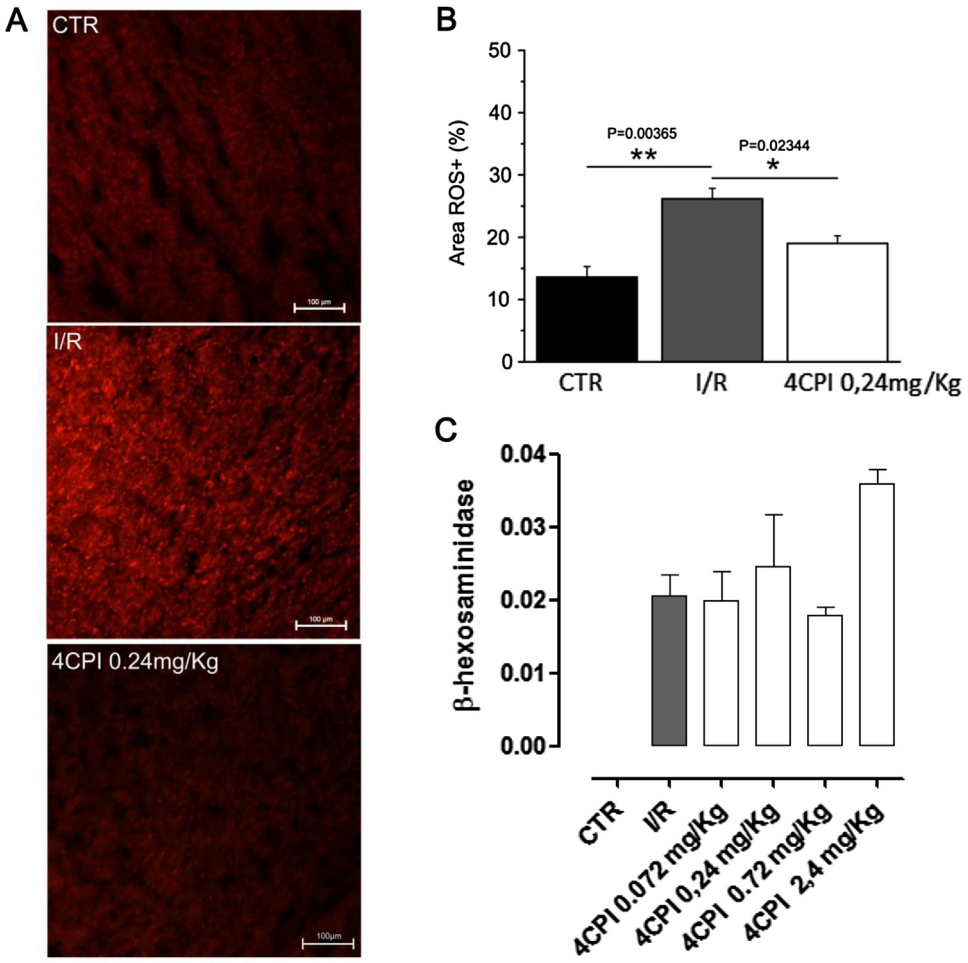

Fig. 3. Representative pictures (A) and relative quantification of the ROS-positive areas (B) of the DHE-positive nuclei observed by fluorescence microscopy in ventricular tissue. Non ischemic control (CTR) hearts were isolated from vehicle-treated rats and were not subjected to I/R. Hearts subjected to I/R were isolated from vehicle-treated rats (I/R) or from 4CPI-treated rats (0.24 mg/kg). In panel C, β-hexo release in the reperfusion overflow of Langendorff hearts of vehicle- (I/R) or 4CPI-treated rats are also shown. In the control pre-ischemic overflow (CTR) no release of β-hexo was detected. Asterisks indicate significant differences (* = P < 0.05; ** = P < 0.01).

mast cell degranulation does not play a relevant role in the cardioprotection afforded by 4CPI. In contrast, a significant increase of β-hexo release was observed in the perfusate from heart of animals pre-treated with the highest dose of 4CPI (2.4 mg/kg), suggesting a possible triggering of toxic responses.

Based on the above results, 0.24 mg/kg was selected as an effective and safe dose for further characterizing the mechanism of action of 4CPI.

During myocardial ischemia, excessive NE release from sympathetic nerve endings is a major cause of arrhythmias and increase in metabolic demand, which largely contribute to morbidity and mortality following myocardial infarction. This NE release is reinforced by renin release from mast cells and activation of a local renin-angiotensin cascade [33]. We evaluated possible effects of 4CPI on arrhythmias and release of NE, renin and β-hexo in mouse hearts subjected to I/R. In this model, I/R was associated with severe ventricular arrhythmias and a massive release of NE, renin and β-hexo. As already observed in rat hearts, pre-treatment of mice with 4CPI (0.24 mg/kg) led to clear cardioprotective effects. Indeed, the H_2S-donor caused a significant reduction of post-ischemic arrhythmias, and a significant reduction of NE release. Yet, renin and β-hexo release was not influenced. These results suggest that the reduction of NE release and consequent attenuation of ventricular arrhythmias may be attributed to an H_2S-induced stabilization of sympathetic nerve endings. Indeed, H_2S donors, such as GYY4137, have been recently reported to inhibit electrically-evoked NE release from isolated bovine iris-ciliary bodies [34]. In contrast, as already observed in the rat hearts, inhibition of the release of mast cell products does not seem to play a significant role.

H_2S easily reacts with many reactive species, such as superoxide radical anion, hydrogen peroxide, peroxynitrite and hypochlorite, leading to their neutralization and reduction of oxidative stress [35]. However, the anti-oxidant activity of H_2S is due to further and more complex mechanisms, such as the activation of powerful endogenous mechanisms, such as the Nrf2-mediated "anti-oxidant" machinery [36], possibly involved also in the cardioprotective effects of H_2S [13]. Indeed, in experimental models of cardiac I/R, H_2S-donors such as GYY4137 were found to strongly inhibit oxidative stress in myocardial tissue [30]. Consistently, in our study, I/R caused a dramatic increase in DHE-stained nuclei in rat hearts, indicating a very high level of ROS production. Importantly, 4CPI (0.24 mg/kg) completely abolished such an increase, suggesting that a reduction of oxidative stress may be an additional protective effects of 4CPI against I/R injury.

CSE is likely to be the main source of endogenous H_2S in the heart, but conflicting results concerning the influence of I/R on CSE expression have been reported [37]. Ischemic events in

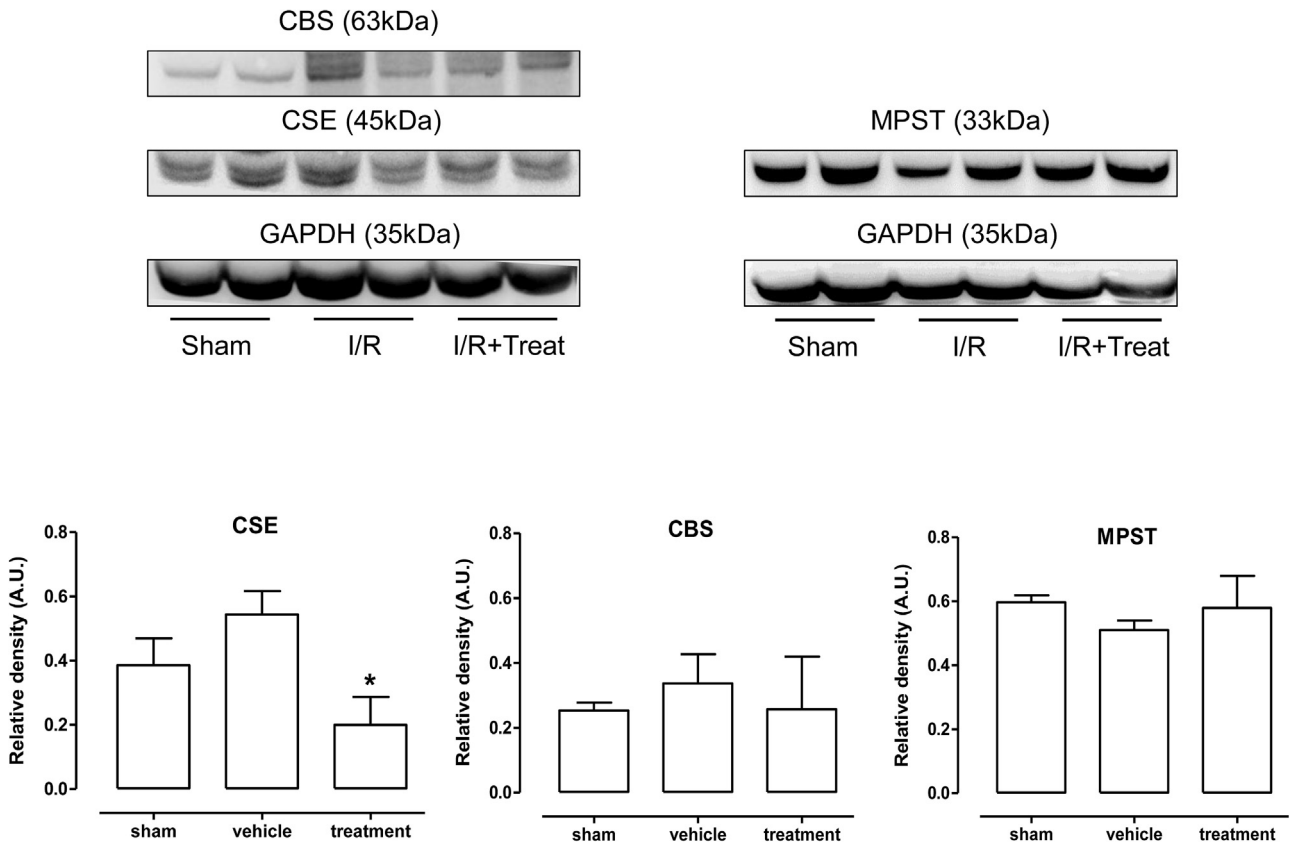

Fig. 4. (A) Western Blots show the basal expression of CSE, CBS and MPST in ventricular tissue of hearts of the "sham group" (sham), not subjected to I/R. Moreover, the expression of the three enzymes in ventricular tissue of hearts subjected to I/R, isolated from vehicle-treated or 4CPI-treated rats (0.24 mg/kg) is also shown. The corresponding quantitative analysis for CSE (B), CBS (C) and MPST (D) expression is also shown. Data are expressed as arbitrary units of densitometry. Asterisks indicate significant differences vs vehicle (** = P < 0.01). The original uncropped images of Western Blot experiments are available as Supplementary material.

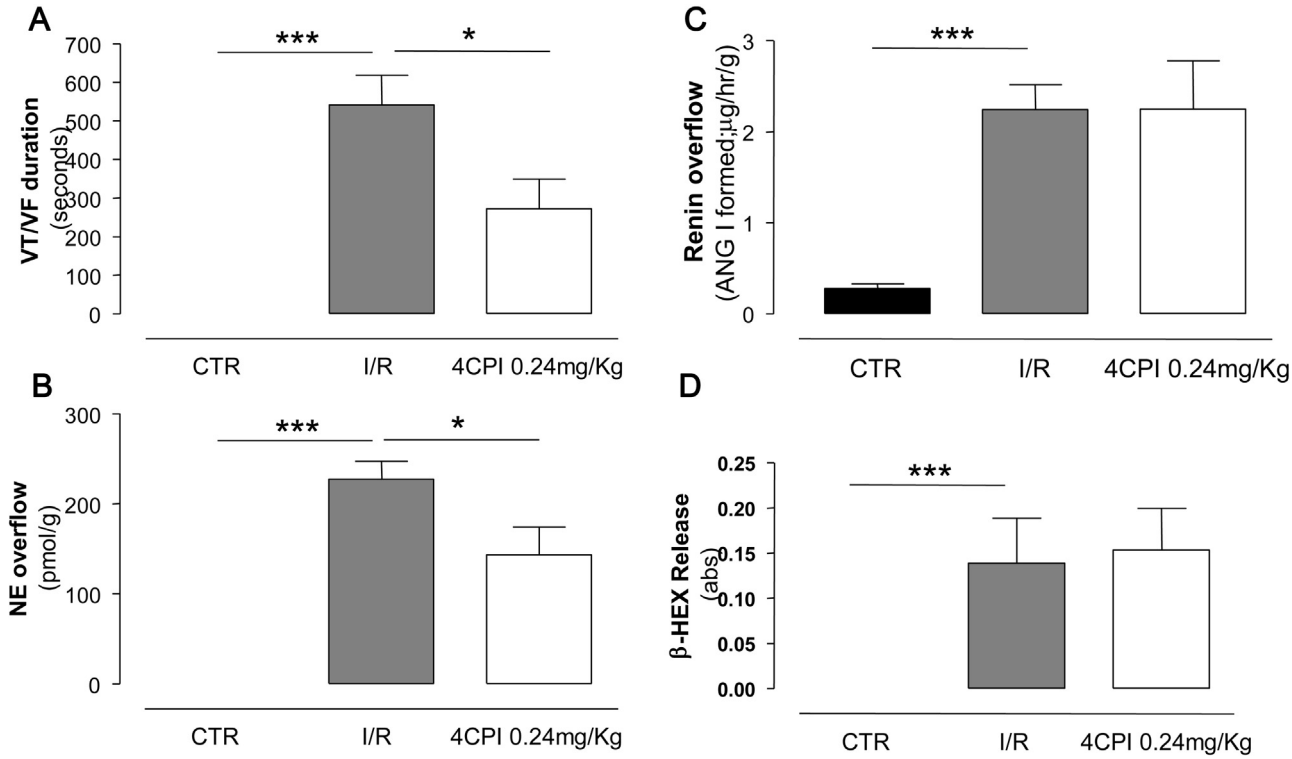

Fig. 5. Panel A reports the duration (seconds) of ventricular arrhythmias recorded during reperfusion after following ischemia. Release of NE (B), renin (C) and β-hexo (D) in the reperfusion overflow of Langendorff hearts of vehicle- (I/R) or 4CPI-treated rats (0.24 mg/kg) are also shown. In the control pre-ischemic overflow (CTR) no release of NE and β-hexo, and a very modest release of renin were detected. Asterisks indicate significant differences (* = P < 0.05; *** = P < 0.001).

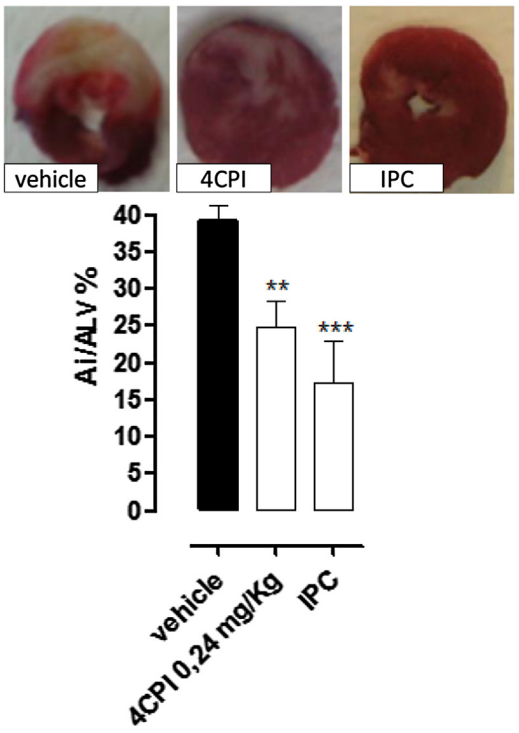

Fig. 6. Morphometric quantification of I/R-induced tissue injury observed in ventricular slices of rat hearts, after acute myocardial infarction in vivo. Infarction was created in vehicle-treated rats (vehicle) or in 4CPI-treated rats (0.24 mg/kg). In some vehicle-treated rats, acute myocardial infarction was preceded by ischemic preconditioning (IPC). Asterisks indicate significant differences vs vehicle (** = $P < 0.01$; *** = $P < 0.001$). Images of representative ventricular slices are also shown. Pale areas indicate I/R-injured tissue, while healthy tissue is colored in red.

myocardium have been associated with an enhancement of CSE expression, suggesting a possible cardiac defensive mechanism directed at increasing H_2S levels and limiting ischemic damage [38]. In our study, Western blotting showed the basal presence of all three H_2S-biosynthesizing enzymes in rat ventricular tissue: CSE, CBS and MPST. I/R did not significantly alter the expression of these enzymes; however, a slight not significant increase of CSE expression was observed, suggesting the triggering of a self-defence mechanism. Pre-treatment with 4CPI (0.24 mg/kg) caused a dramatic inhibition of CSE expression in hearts subjected to I/R, while it did not cause significant effects on CBS and MPST. In contrast, 4CPI did not influence the expression of CSE in hearts not subjected to I/R. Thus, we can speculate that since 4CPI directly increases H_2S level, an increase in CSE expression as a self-defence mechanism against myocardial ischemic injury may become superfluous.

As reported above, the activation of mitochondrial K_{ATP} channels is likely to be involved in the cardioprotective effects evoked by H_2S [11,12]. In our study, the mitoK_{ATP}-blocker 5HD completely abolished the effects of 4CPI, strongly suggesting that mitoK_{ATP} may be a main target of the anti-ischemic effects of this novel H_2S-donor. To solidify this hypothesis, we investigated the effects of 4CPI on isolated rat heart mitochondria. As previously observed with activators of mitochondrial potassium channels, 4CPI caused a partial depolarization of the mitochondrial membrane potential [28]. This effect was inhibited by ATP, thus suggesting the involvement of ATP-sensitive channels. In contrast, XE991 did not cause any significant influence, indicating that the activation of mitochondrial Kv7 channel does not contribute significantly to cardioprotection. Mitochondrial calcium accumulation is a key event in promoting cell death after I/R, and the inhibition of calcium uptake into the mitochondrial matrix is considered a pivotal mechanism of action of the anti-ischemic effects of mitochondrial potassium channel activators [10]. Consistently, 4CPI strongly prevented calcium accumulation into the mitochondrial matrix, further indicating that the mitoK_{ATP} channel is likely to be a relevant pharmacological target of this novel H_2S-donor.

Finally, the cardioprotective effects of 4CPI were tested in vivo, in an experimental model of acute myocardial infarction in rats, more closely resembling the clinical pattern of myocardial infarction. Also in this model, 4CPI (0.24 mg/kg) exhibited cardioprotective effects, which were comparable to those evoked by IPC, i.e. an endogenous and powerful self-defence mechanism. These results strongly suggest that isothiocyanate-based H_2S-releasing drugs, such as 4CPI, can trigger a "pharmacological pre-conditioning" and could represent a suitable pharmacological option in anti-ischemic therapy.

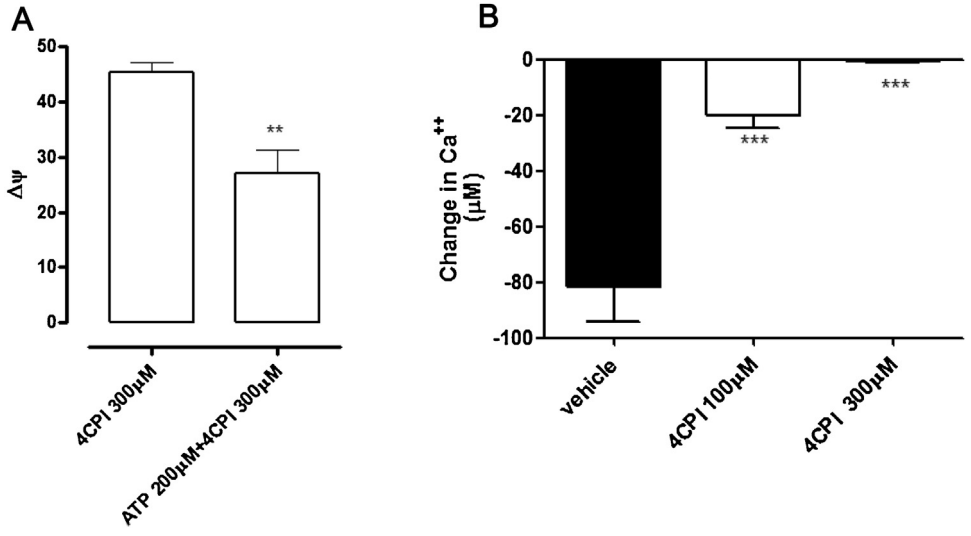

Fig. 7. (A) Change in mitochondrial membrane potential (Δy in mV) upon incubation of mitochondria with 4CPI (300 μM), in the absence or presence of ATP (200 μM). Asterisks indicate significant statistical differences (** = $P < 0.01$). B) Changes in extra mitochondrial Ca^{2+} concentration (in μM) upon addition of mitochondria to a calcium-rich solution (100 μM), in the absence (vehicle) or presence of 4CPI (100 or 300 μM). Asterisks indicate significant statistical differences vs vehicle (*** = $P < 0.001$).

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.2016.09.006.

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