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Catestatin Reduces Myocardial Ischaemia/Reperfusion Injury: Involvement of PI3K/Akt, PKCs, Mitochondrial K_{ATP} Channels and ROS Signalling

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Short title: Cardioprotection by Catestatin

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Abstract

Aims**:* Catestatin (CST) limits myocardial ischaemia/reperfusion (I/R) injury with unknown mechanisms. Clearly phosphoinositide-3-kinase (PI3K), protein-kinase-C (PKC) isoforms, including intra-mitochondrial PKCε, mitochondrial-K_{ATP} (mitoK_{ATP}) channels and subsequent reactive-oxygen-species (ROS)-signalling play important roles in postconditioning cardioprotection, preventing mitochondrial permeability transition pore (mPTP) opening. Therefore, we studied the role of these extra- and intra-mitochondrial factors in CST-induced protection. ***Methods and Results**:* Isolated rat hearts and H9c2 cells underwent I/R and oxidative stress, respectively. In isolated hearts CST (75nM, CST-Post) given in early-reperfusion significantly reduced infarct-size, limited post-ischaemic contracture, and improved recovery of developed left ventricular pressure. PI3K inhibitor, LY-294002 (LY), large spectrum PKC inhibitor, Chelerythrine (CHE), specific PKCε inhibitor (εV1-2), mitoK_{ATP} channel blocker, 5-Hydroxydecanoate (5HD) or ROS scavenger, 2-mercaptopropionylglycine (MPG) abolished the infarct-sparing effect of CST. Notably the CST-induced contracture limitation was maintained during co-infusion of 5HD, MPG or εV1-2, but it was lost during co-infusion of LY or CHE. In H9c2 cells challenged with H_2O_2, mitochondrial-depolarization (an index of mPTP-opening studied with JC1-probe) was drastically limited by CST (75nM). ***Conclusions: Our results suggest that the protective signalling pathway activated by CST includes mitoK_{ATP} channels, ROS-signalling and prevention of mPTP opening, with a central role for upstream PI3K/Akt and PKCs. In fact, all inhibitors completely abolished CST-infarct-sparing effect. Since CST-anti-contracture effect cannot be explained by intra-mitochondrial mechanisms (PKCε activation and mitoK_{ATP} channel opening) or ROS-signalling, it is proposed that these downstream signals are part of a reverberant loop which re-activates upstream PKCs, which therefore play a pivotal role in CST-induced protection.

Key words: Catestatin**;** Chromogranin A; Cardioprotection; Ischaemia/reperfusion; Postconditioning.

1. Introduction

Rapid reperfusion is the gold-standard therapy for acute myocardial infarction (AMI). However reperfusion induces myocardial damages, which can be prevented by timing treatments in early reperfusion phase [7,13,21,34,53]. Reperfusion injury includes all forms of cell death as well as contractile dysfunction of surviving cells [7,13,34,53]. It has been shown that several peptides are able to induce cardioprotection when infused in the early reperfusion; that is *pharmacological postconditioning* [13,21,38,39]. These peptides, acting on their specific receptors, can trigger both pharmacological pre- and postconditioning *via* pro-survival intrinsic signalling pathways, which include in rodents the so called Reperfusion Injury Salvage Kinase (RISK) and Survivor Activating Factor Enhancement (SAFE) pathways [18,48].

Survival kinases, such as phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt/PKB) and protein kinase C (PKC) may act on downstream mitochondrial targets to open ATP sensitive potassium (mitoK_{ATP}) channels and to affect cellular survival, reducing both necrosis and apoptosis [7,13,41,56]. The mitoK_{ATP} channels *via* multiple mechanisms, including the *partial depolarization* of the mitochondrial membrane, may favour reactive oxygen species (ROS)-signalling, which may lead to a re-activation of a pool of PKCs [15,35,53]. In particular, in concert with mitoK_{ATP} channels, the intramitochondrial protein kinase Cε (PKCε) activation may take part to the so-called *"memory-associated protection"*. Nevertheless, the signal transduction pathways for protection from lethal and non-lethal reperfusion injury are still under intense debate [2,7,9,15,18,34].

Recently, several biologically active peptides derived from chromogranin A (CgA), including Vasostatin-1 (VS-1) and Catestatin (CST), have received attention as regulators

of cardiovascular function [2,11,12,19,22,29,43]. For instance, the N-terminal VS-1 induced a preconditioning-like effect *via* adenosine/nitric oxide (NO) protective signalling, reducing the extension of myocardial infarction in the rat heart [10].

The C-terminal CgA-derived peptide CST is a non-competitive antagonist of nicotine-evoked catecholamine secretion [17,29,32] and it induces vasodilatation through both inhibition of catecholamine release and increased circulating levels of histamine [22,23]. CST plasma levels are decreased not only in hypertensive patients but also in their still-normotensive offspring [27-29,32]; accordingly, exogenous CST rescues arterial hypertension of CgA knockout mice [28]. CST also caused vasodilatation in human subjects with a NO-dependent mechanism [16]. Recently, on the isolated rat heart, CST was found to elicit a vasorelaxant influence on coronary arteries pre-contracted by endothelin-1, together with negative inotropic and lusitropic actions, counteracting the positive effects elicited by the β-adrenoceptor activator, isoproterenol [3]. CST inhibition of β-adrenoceptor activation in cardiomyocytes [3] is "presumably mediated by the relaxing effect of the endothelial cell-derived NO-release mediated by Akt/PKB signalling to endothelial NO synthase" [19]. Importantly, in AMI patients an initial reduction with a subsequent increase in CST plasma levels has been recently reported [55].

We have studied the cardioprotective effect of CST infused during the early reperfusion as *pharmacological postconditioning* (CST-Post) [36]. CST-Post reduced post-ischaemic myocardial contracture and significantly improved post-ischaemic recovery of contractility. Using isolated cardiac cells, we also reported that the protection observed is primarily due to a direct effect on the cardiomyocytes and does not necessarily depend on the endothelial effects of CST [36]. Since in multiple models [39,41,42,51,52,56], including isolated cells [48,51,56], an involvement of PI3K/Akt, PKCs, mitoK_{ATP} channel and ROS-signalling have been observed in ischaemic

postconditioning, we hypothesized that the CST-induced cardioprotective response in reperfusion may involve potential signalling proteins (*e.g.* PI3K/Akt and PKCs) and potential downstream targets and mediators (*e.g.* PKCε and mitoK$_{ATP}$ channel activation, and ROS production). To explore the role of these elements in the CST-Post-induced cardioprotection, we used Langendorff rat hearts reperfused in the presence of inhibitors of either PI3K/Akt, PKCs, PKCε or mitoK$_{ATP}$ channels or a ROS scavenger. Moreover, to corroborate the involvement of mitochondria in the CST-induced cardioprotection, we also studied CST protective role in cardiac cell line in the presence of oxidative stress, using a specific probe for the evaluation of intra-mitochondrial potential variation.

2. Methods

2.1 Animals

Male Wistar rats were used in accordance with the Italian law (DL-116, Jan. 27, 1992) and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The project was approved by the Italian Ministry of Health, Rome, and by the ethics review board of the University of Turin. Adult rats were anaesthetized by i.p. injection of urethane (1 g/kg) and killed by decapitation.

2.2 Isolated heart perfusion

The methods were similar to those previously described [1,33,35,36,39-41]. In brief, after anaesthesia chest was opened and heart excised. Hearts were immediately constant-flow perfused with Krebs–Henseleit buffer solution (127 mM NaCl, 17.7 mM $NaHCO_3$, 5.1 mM KCl, 1.5 mM $CaCl_2$, 1.26 mM $MgCl_2$, 11 mM D-glucose (Sigma-Aldrich Corp., St. Louis, MO, USA) gassed with 95% O_2 and 5% CO_2. The hearts were instrumented to record coronary pressure and left

ventricular pressure (LVP), electrically paced at 280 b.p.m. and kept in a temperature-controlled chamber (37°C) [1, 33,35,36,39-41].

2.2.1 Cardiac function and Infarct-size studies

After 40 min stabilization, hearts were subjected to 30-min, normothermic, zero-flow global ischaemia, followed by 120-min reperfusion (Group 1, I/R).

In a second Group (CST-Post group), CST (75nM) [36] was infused for 20-min at the beginning of reperfusion. Then the action of CST-Post was studied in the presence of specific inhibitors.

In Group 3 and 4, the large spectrum PKC inhibitor, chelerythrine (CHE, 5µM; CST-Post+CHE Group) or the selective inhibitor of PI3K/Akt, LY-294002 (LY, 50µM; CST-Post+LY Group) were used to test the involvement of these pivotal kinases in cardioprotection scenario [35,42].

In Group 5 a specific PKCε inhibitor, εV1-2, was used to test the involvement of this kinase (εV1-2 1µM; CST-Post+εV1-2 Group) [44].

In Group 6, the mitoK$_{ATP}$ channel blocker, 5-hydroxydecanoate (5HD, 100µM; CST-Post+5HD Group) [41 and references therein] was used to test whether the action of CST-Post could include mitoK$_{ATP}$ channel activation.

In Group 7, to confirm ROS involvement, we used a ROS scavenger, 2-mercaptopropionylglycine (MPG, 300µM; CST-Post+MPG Group) which is a sulfhydryl donor specific for mitochondrial activity [30,39 and references therein].

Perfusion with inhibitors started 5-min before ischaemia and continued during the early 20-min of reperfusion in the presence of CST-Post (n= 8 for each group). The concentrations of inhibitors were chosen on the basis of data found in the literature [14,35,39,41-44 and references therein].

In preliminary experiments these doses of inhibitors did not affect basal cardiac performance. CST concentration was chosen on the basis of dose-response curve on infarct-size limitation [36].

2.2.2 Additional experiments

To determine the effects of the antagonists themselves, control hearts were infused 5-min before the global ischaemia and during the initial 20-min of reperfusion with one only of the antagonists (CHE, LY, εV1-2, 5HD or MPG, Groups 8-12, n= 6 for each group).

Moreover, in order to evaluate the effective role of CST in cardioprotection, in additional experiments, hearts were perfused with heat-inactivated CST [3] at the identical concentration of active CST (75nM), for 20-min at the beginning of reperfusion.

At the end of the experiments all the hearts were removed from the apparatus to evaluate the extension of infarct-size.

2.3 Assessment of cardiac function

A polyvinyl-chloride balloon was placed into the left ventricle (LV) and connected to the electromanometer filled with saline to obtain an end diastolic LVP of 5 mmHg. Such a volume of LV was maintained throughout the experiment [33,35,36,39-41].

Coronary perfusion pressure and LVP were monitored to assess the preparation conditions. LVP was analyzed offline with LabView software (National Instruments). Care was taken to measure LVP in a period (15–20 s) without arrhythmias.

2.4 Assessment of myocardial injury

Infarct areas were assessed at the end of the experiment as previously described [32,34,35,38-40]. In brief, immediately after reperfusion hearts were rapidly removed from the perfusion

apparatus and the LV dissected into 2–3 mm circumferential slices. Following 20 min of incubation at 37°C in 0.1% solution of nitro-blue tetrazolium in phosphate buffer, unstained necrotic tissue was carefully separated from stained viable tissue by an independent observer, who was unaware of the protocols. The necrotic mass was expressed as a percentage of total left ventricular mass. In fact, only the LV had a fixed volume and pre-load; therefore only the LV mass was considered as risk area, though in this model the whole heart underwent normothermic ischaemia.

2.5 Oxidative stress and mitochondrial potential in H9c2

2.5.1 Cell culture and drug treatment.

The cardiac myoblast H9C2 cell line (ATCC, CRL-1446) was maintained in DMEM supplemented with 15% heat-inactivated FCS, 100 U/mL penicillin and 100 µg/mL streptomycin sulphate in a humidified incubator at 37ºC under 5% CO_2/air prior to use. H9c2 cells were seeded in quadruplicate in 24-well plates and the experimental groups were: *i)* control: CTRL, absence of any additional treatment; *ii)* H_2O_2: H9c2 were exposed to 50µM H_2O_2 for 2 hours [20]; *iii)* CST: cells were exposed to CST 75nM for 3 hours; *iv)* CST+H_2O_2: cells were exposed to CST for 1 hour, then CST plus H_2O_2 were continued for other 2 hours.

2.5.2 Cell survival

The cell viability was determined by a colorimetric MTT assay, as described previously [54].

2.5.3 Measurement of mitochondrial potential

The quantification of mitochondrial potential variation was obtained with JC-1 during fluorimetric analysis [45]. In brief, JC-1 (10µM) was added to cultured cells [46] in control and after the

treatment with H_2O_2 (50μM) [20] with or without CST (75 nM). The ratio between red and green JC-1 florescence was taken as an index of mitochondrial membrane potential [25].

2.6 Drugs

CST was a generous gift of Dr. Sushil K. Mahata (University of California and Veterans Affairs San Diego Healthcare System, San Diego, California; USA). CHE, MPG, LY, and 5HD were purchased from Sigma (St. Louis, MO, USA); εV1-2 from AnaSpec Inc. (Fremont, California, USA).

2.7 Statistical analysis

All data are expressed as means±SEM. One-way ANOVA and Newman–Keuls multiple comparison tests (for post-ANOVA comparisons) have been used to compare infarct-size. Functional data were compared with repeated measures ANOVA (time/group). A *t* test with Bonferroni correction was also used to compare the last-time points of functional data. Differences with $p<0.05$ were regarded as statistically significant.

3. Results

The baseline values of the considered parameters at the end of stabilization period were in the range of those usually used in this model. Specifically, a coronary perfusion pressure (CPP) of about 80 mmHg was obtained adjusting the coronary flow (CF) to 9±1 ml/min/g, and an end-diastolic LVP (LVEDP) of about 5 mmHg was achieved adjusting the volume of the balloon placed into the LV. Basal values of CPP and LVEDP did not change significantly throughout the stabilization period. At the end of stabilization, the hearts developed a LVP (dLVP) of about 80 mmHg.

3.1 CST-Post improved post-ischaemic cardiac function in a PI3K/Akt and PKCs dependent manner

3.1.1 Systolic function

In Fig 1, panel A, developed LVP data are reported as percent variation with respect to baseline level. In Fig 1, panel B, dLVP data are reported as area under the curve (AUC) during reperfusion.

The hearts of the I/R group showed a marked limitation of dLVP recovery during reperfusion; in fact, dLVP was 34±10% of baseline level at the end of reperfusion (dLVP decreased from 81±4 to 28±6 mmHg; $p<0.001$, this *p* value is not reported in Fig 1A).

As can be seen in Fig 1A and 1B, CST-Post markedly improved the dLVP recovery during reperfusion ($p<0.01$ *vs.* I/R group); in particular, at the end of reperfusion dLVP was 111±13% of baseline levels. The co-infusion of antagonists of upstream signals, such as LY or CHE (inhibitors of PI3K/Akt and PKCs, respectively), along with CST abolished the cardioprotective effects of CST-Post (Fig 1A and 1B). However, the co-infusion of CST with antagonists of downstream signals, such as εV1-2, 5HD or MPG (inhibitors of PKCε, mitoK_{ATP} channels or ROS scavenger, respectively), partially reduced the protective effect of CST-Post. Statistical analysis yielded similar results whether are compared the last time points (120^{th} min of reperfusion, Fig 1A) or the AUC (Fig 1B).

3.1.2 Diastolic function

In Fig 1, panel C, the levels of LVEDP are reported in mmHg throughout the entire experiment duration. In Fig 1, panel D, these data are reported as area under the curve (AUC) during reperfusion. Contracture can be defined as an increase in LVEDP of 4 mmHg above the baseline level [5,33].

I/R induced a marked increase of LVEDP, which reached 54±14 mmHg at the end of reperfusion ($p<0.001$ *vs.* baseline level; this p value is not reported in Fig 1). CST-Post significantly limited the development of contracture occurring during reperfusion; at the end of reperfusion, in fact, LVEDP was 23±7 mmHg ($p<0.01$ *vs.* I/R). Co-infusion with upstream antagonists (CHE or LY) abolished the protective effect of CST against contracture (p = NS *vs.* I/R last time point, Fig 1C). However, in the presence of these inhibitors (LY or CHE) the contracture development was somewhat slower than in I/R; in fact AUCs were smaller than I/R, though bigger than CST-Post (Fig 1D).

Yet the co-infusion with either εV1-2, 5HD or MPG (downstream antagonists) did not abolish the contracture limitation induced by CST-Post ($p<0.01$ *vs.* I/R last time point; p = NS *vs.* CST-Post last time point, Fig 1C). Similar results were shown by analysis of AUCs (Fig 1D).

The cardiac function parameters observed in hearts perfused with only inhibitors (groups 8-12) or inactivated-CST during reperfusion were similar to those observed in I/R group (data not shown).

3.2 CST-Post limited infarct-size through PKC and mitoK_{ATP} channel involvement

The risk area, *i.e.*, left ventricular mass, was similar in all groups. Infarct-size (Fig 2A), expressed as a percentage of LV mass, was 67±6% in I/R. CST-Post reduced infarct-size to 37±4% of LV mass ($p<0.01$ *vs.* I/R). The co-infusion with either the upstream blockers (CHE or LY), or the downstream antagonists (εV1-2, 5HD or MPG) abolished the protective effect of CST against infarct size (see Fig. 2A).

Risk area and infarct-size assessed in hearts perfused with only inhibitors (groups 8-12) were similar to those observed in I/R control group (Fig.2B). This lack of effects on I/R injury has been already reported [14,35,39,41-44].

The infusion of inactivated CST had no protective effects against infarct size (64±4%, p= NS *vs.* I/R, p<0.01 *vs.* CST-Post).

3.3 CST protected the cultured cells from oxidative stress

Figure 3 shows the protective effects of CST against oxidative stress in the H9c2 cells. Oxidative stress with H_2O_2 significantly reduced the survival of H9c2 cell (p<0.05 *vs.* Control group), and the co-treatment with CST abolished this effect (Fig 3A).

3.3.1 CST avoided mitochondrial membrane potential dissipation due to oxidative stress

As expected, H_2O_2 drastically reduced the mitochondrial potential (p<0.01 *vs.* CTLR and CST groups), while the co-treatment (CST+H_2O_2) significantly limited this reduction (p<0.05 *vs.* H_2O_2 and CTLR groups) (Fig 3B). Of note, CST *per se* induced a partial, non-significant, depolarization of the mitochondrial membrane.

4. Discussion

The C-terminal fragment of Chromogranin A, CST, is known as an inhibitor of catecholamine secretion and a regulator of cardiovascular functions [19,23,28]. The cardiac activity of CST is achieved through signalling pathways expected for receptor-orphan peptides with membrane-interacting properties [4]. Here, using classic antagonists [14,35,39,41-44], we demonstrated for the first time that the cardioprotective effect of CST-Post depends on the activation of PI3k/Akt, PKCs and of mitoK_{ATP} channels, which may include a ROS signalling. The prevention of mPTP opening in myocytes challenged with oxidative stress reinforces the idea that CST protective effects converge on mitochondria.

Most studies with cardioprotective agents have addressed either the post-ischaemic mechanical recovery or the reduction of the infarct-size. Thus it remains unclear whether the mechanical

recovery is attributable to the reduction of infarct-size only and/or to a direct effect of the cardioprotective agent on post-ischaemic mechanical function of myocardium. Here, we show that CST-Post significantly reduces both the extension of infarct-size and the myocardial contracture development drastically improving post-ischaemic systolic function during reperfusion (see Fig 1). Importantly, the distinct inhibitors targeting different elements of the protective pathway allow dissecting the effect on mechanical function and cell death. In fact, in the present study, all the antagonists used abrogate CST-Post protection against *infarct-size* development (Fig 2). However, the post-ischaemic *contractile function* was differently affected by antagonists of upstream and downstream targets of CST in the protective signalling pathway (Fig 1 and Fig 4).

CST-cardioprotective effects against infarct size were abolished by either upstream antagonists, namely the broad range inhibitor of PKCs, CHE, or the PI3K/Akt antagonist, LY, as well as by the downstream target antagonists, namely the specific PKCε antagonist, εV1-2, the mitoK_{ATP} channel blocker, 5HD, or the ROS scavenger, MPG. This supports the viewpoint that the activation of pathways both outside (PI3K/Akt and pool of PKCs) and inside the mitochondria (PKCε activation, mitoK_{ATP} channel opening and subsequent ROS diffusion) are required for CST-induced protection against infarct size.

With respect to *contracture development,* we observed that its limitation by CST can only be slowed down in the presence of CHE and LY, but can be still reduced in the presence of the downstream target inhibitors (εV1-2, 5HD and MPG.) In other words, while CHE and LY were able to limit CST-induced contracture protection, εV1-2, 5HD or MPG (antagonists of mitochondrial factors) were not able to limit the protective effect of CST against contracture development. This suggests that the CST-induced limitation of contracture is mitochondrial-independent. Possible

mechanisms include CST-induced phospholamban phosphorylation, as shown by Angelone *et al* [4].

It may be stressed that PKCε-dependent cardioprotection is mainly due to its mitochondrial activity. In fact, under I/R conditions mitochondrial translocation of PKCε occurs rapidly, with a corresponding decline in cytosolic PKCε levels [9]. Moreover, a number of cardioprotective stimuli enhance mitochondrial translocation of PKCε, which may mediate protection *via* the phosphorylation of intramitochondrial proteins [9 and references therein]. Therefore, it is not a surprise that the specific PKCε inhibitor, εV1-2, affects mitochondrial-dependent protection.

Although several authors report that the ROS scavenger, MPG, given in reperfusion does not affect infarct size [13,14,26,34,39], it is currently unknown why the addition of MPG does not elicit any protection, but may limit cardioprotective efficacy of various protective interventions [13,14,26,34,39]. The most likely explanation is that the ROS signal is carried by specific radical species and/or in specific intracellular compartment [26,40]. MPG, which concentrates 500-fold in mitochondria and is highly effective against some mitochondrial ROS [26,30], evidently, is able to scavenge the few radical species that signal the protection, but is not effective against I/R injury due to massive ROS production [13,14,26,30,34,39].

We suggest that the activation of the PKCs pool is a pivotal step in CST-Post cardioprotection; in fact the co-infusion with CHE abolishes all CTS-Post-elicited protective effects (Figs 1 and 2). Likely, the mitoK_{ATP} channel opening with subsequent ROS formation signalling is implicated in the CST-Post induced protection [13,15,31]. In fact ROS can activate a redundant pool of kinases involved in cardioprotection [7,13,18,35,41], including PKCs, thus eliciting a positive feed-back loop [47], which may provide a sort of cardioprotection memory (*reverberant loop*, Fig 4) [15]. In fact εV1-2 and MPG, similarly to 5HD, only partially block the CST protective effects. Hence, we can argue that the above described memory effect is necessary for limiting the infarct, but less

important for post-ischaemic contracture limitation. In fact, contracture limitation persists in εV1-2, 5HD or MPG treated hearts. ROS, likely produced after mitoK$_{ATP}$ channels opening, may act as double edge swords; that is, they may act as second messengers to trigger the pathway of protective kinases, being at the same time involved in mediating part of myocardial dysfunction [7,34,38].

While the PKCs pool plays a pivotal role in the protective effects (see for instance the cardioprotective effect of the large spectrum PKC activator, 4 beta-phorbol 12-myristate 13-acetate (PMA) [26], and our data with the large spectrum PKC inhibitor, CHE), the PKCε iso-enzyme is only part of the reverberant loop. In fact, as said, the specific PKCε inhibitor, εV1-2, does not counteract the CST-induced contracture limitation.

Theoretically, the improved *systolic function* might be due to the limitation of infarct-size and contracture, as well as to a direct CST induced anti-stunning effect. However, the long-lasting post-ischaemic systolic recovery in CST-Post experiments could not be explained by the inotropic effect of CST, which is known to exert transient (a few minutes) positive [6] and prolonged negative inotropic effects in normoxia [4,6]. Therefore, the CST-induced improvement of post-ischaemic systolic function can be attributed to both the limitation of infarct-size and the prevention of contracture [36].

CST exerted direct protective effect against simulated I/R in isolated adult cardiomyocytes [36]. Of note, here, we show CST-protection on myocardial cell line, in which CST may induce a partial mitochondrial depolarization. Since the latter is considered part of the protective response of the targeted mitochondria [15,35,53], it is intriguing that CST may protect these cells against oxidative injury and excessive mitochondrial depolarization (Fig 3). Nevertheless, a cross-talk between endothelium and myocardium may play a role in the peptide induced protection [40],

since the CST inotropic and anti-adrenergic effects have been attributed to a PI3K/Akt-dependent NO release from endothelial cells [4,6].

It has been reported that under certain conditions CST may exert deleterious effects and does not activate Akt [8]. Our results demonstrate that the CST-elicited cardioprotection is PI3K/Akt-dependent, being abolished by the co-infusion of CST plus LY. It can be argued that the use of different models of ischaemia (*e.g.,* global *vs.* regional ischaemia) and/or the dose/duration of treatment may be the cause of these conflicting results. In fact, depending on the conditions used, several substances may be protective or deleterious [13,35,37], as we and others have observed for other mediators such as platelet-activating factor [24,35,37] and Angeli's salt, which are protective in pre- but not in postconditioning [32,33,37,38 and references therein].

In conclusion, our study demonstrates that CST-Post significantly reduces infarct-size and improves post-ischaemic cardiac function in isolated constant-flow perfused rat hearts; these effects being mediated *via* PI3K/Akt, PKCs and mitoK$_{ATP}$ channel activation, thus favouring ROS signalling. By inhibiting alternatively PKCε only or various PKC isoforms, as well as signal elements of either upstream (PI3K/Akt) or downstream (mitoK$_{ATP}$ channel opening and ROS formation) protective pathway, we have identified distinct components implicated in CST protection against infarct size and post-ischaemic contractile dysfunction.

Clinical considerations

The reported delayed increase of CST levels after infarction [55] may be implicated in the compensatory response against myocardial injury. Pharmacological postconditioning, which, however, target the first few minutes of reperfusion, may be clinically useful at the time of angioplasty, thrombolysis or cardiac surgery.

Actually, CST is known to counteract exaggerated β-adrenergic activity, which is relevant part of

the neuroendocrine scenario of heart failure [1-4], and to dilate human vessels *in vivo* [16], thus exerting positive effects on cardiac afterload. In line with these evidences and since β-blockade is largely used as post-ischaemic treatment, the present work suggests that exogenous CST may provide new tool for pharmacological postconditioning.

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Figure Legends

Fig 1. Cardiac performance.

A *Percent variation of developed LVP (dLVP)* during the 30-min ischaemia (delimited by two vertical dashed lines) and the 120-min reperfusion; data are presented as percent changes with respect to baseline level for each group.

B *Area under the curve (AUC):* dLVP recovery during the 120 min of reperfusion (a.u.: arbitrary units).

C *Left ventricular end-diastolic pressure (LVEDP)* during the 30-min ischaemia (delimited by two vertical dashed lines) and the 120-min reperfusion; data are expressed in mmHg.

D *Area under the curve (AUC):* LVEDP recovery during the 120 min of reperfusion (a.u.: arbitrary units).

*p<0.01 *vs.* all groups; #p<0.05 *vs.* I/R and CST-Post-CHE; °p<0.05 *vs.* CST-Post; §p < 0.05; NS: not significant.

Fig 2. Infarct-size.

The amount of necrotic tissue is expressed as percent of the left ventricle (% IS/LV), which is considered the risk area. **A** Effects of CST-Post with and without antagonists. **B** Effects of antagonists only (for comparative purpose are also reported I/R and CST-Post groups).

**p<0.01 with respect to I/R; NS: not significant.

Fig 3. Oxidative stress and mitochondrial potential in H9c2.

A Effects of H_2O_2 on the cell survival with and without CST; **B** Measurement of mitochondrial potential with JC-1 in H9c2 in the presence and in the absence of H_2O_2 with and without CST. Data presented are mean values±SEM of four experiments.

*p<0.05 *vs.* all groups; **p<0.01 *vs.* all groups; #p<0.05 *vs.* Control and H_2O_2.

Fig 4. Hypothetical scheme outlining the 'reverberant' pathway between PKCε and mitoK_{ATP} channels, at the time of reperfusion, in response to CST-Post.

The hypothetical reverberant protective pathway may converge on the pivotal PKCs. This may explain why the inhibition of the main pathway using CHE, the inhibitor of PKCs, abolishes all the protective effects, whereas blockade of mitoK_{ATP} channels with 5HD and PKCε with εV1-2, as well as the ROS scavenger MPG can limit only part of the cardioprotective effects. For further explanations, see text.

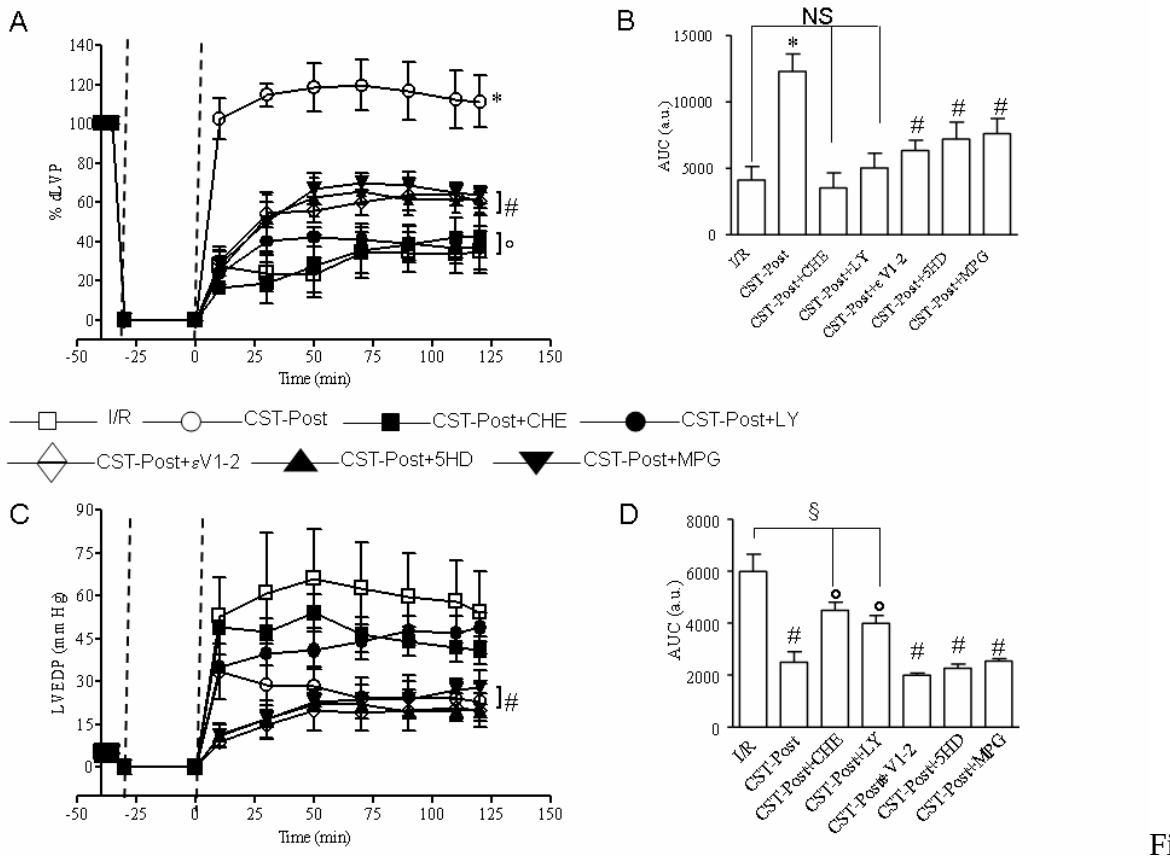
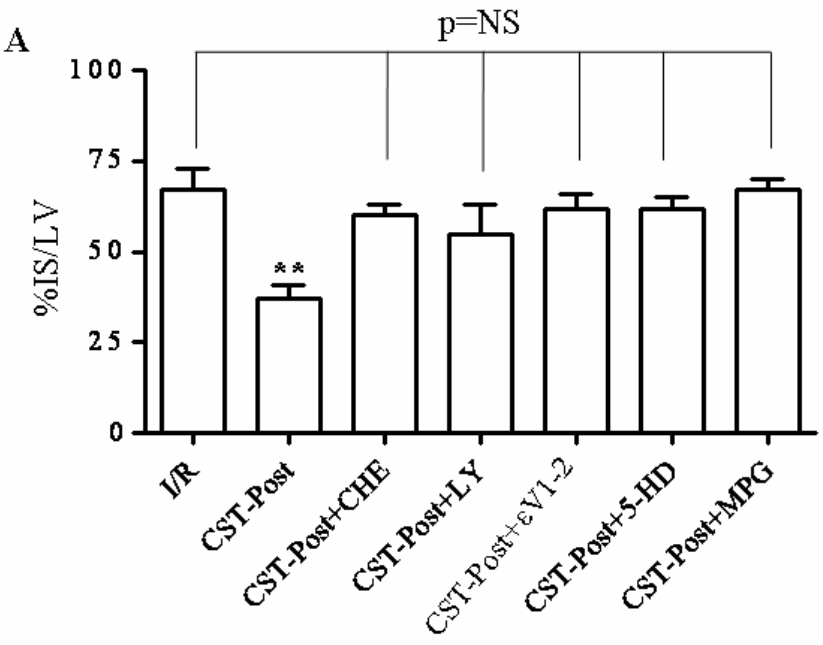

A
B
NS
C
D
§
% dLVP
Time (min)
AUC (a.u.)
LVEDP (mm Hg)
I/R
CST-Post
CST-Post+CHE
CST-Post+LY
CST-Post+εV1-2
CST-Post+5HD
CST-Post+MPG

Fig.1

A


p=NS
100
75
50
25
0
%IS/LV
**
I/R
CST-Post
CST-Post+CHE
CST-Post+LY
CST-Post+εV1-2
CST-Post+5-HD
CST-Post+MPG

B

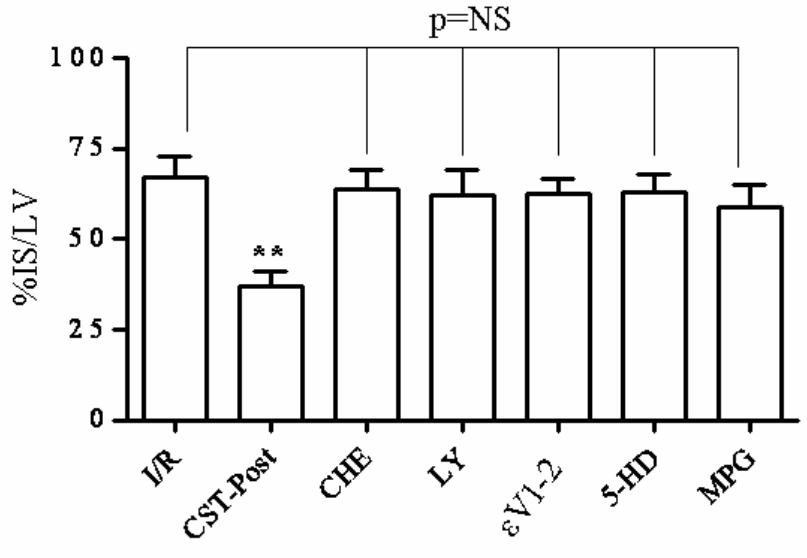

p=NS
100
75
50
25
0
%IS/LV
**
I/R
CST-Post
CHE
LY
εV1-2
5-HD
MPG

Fig.2

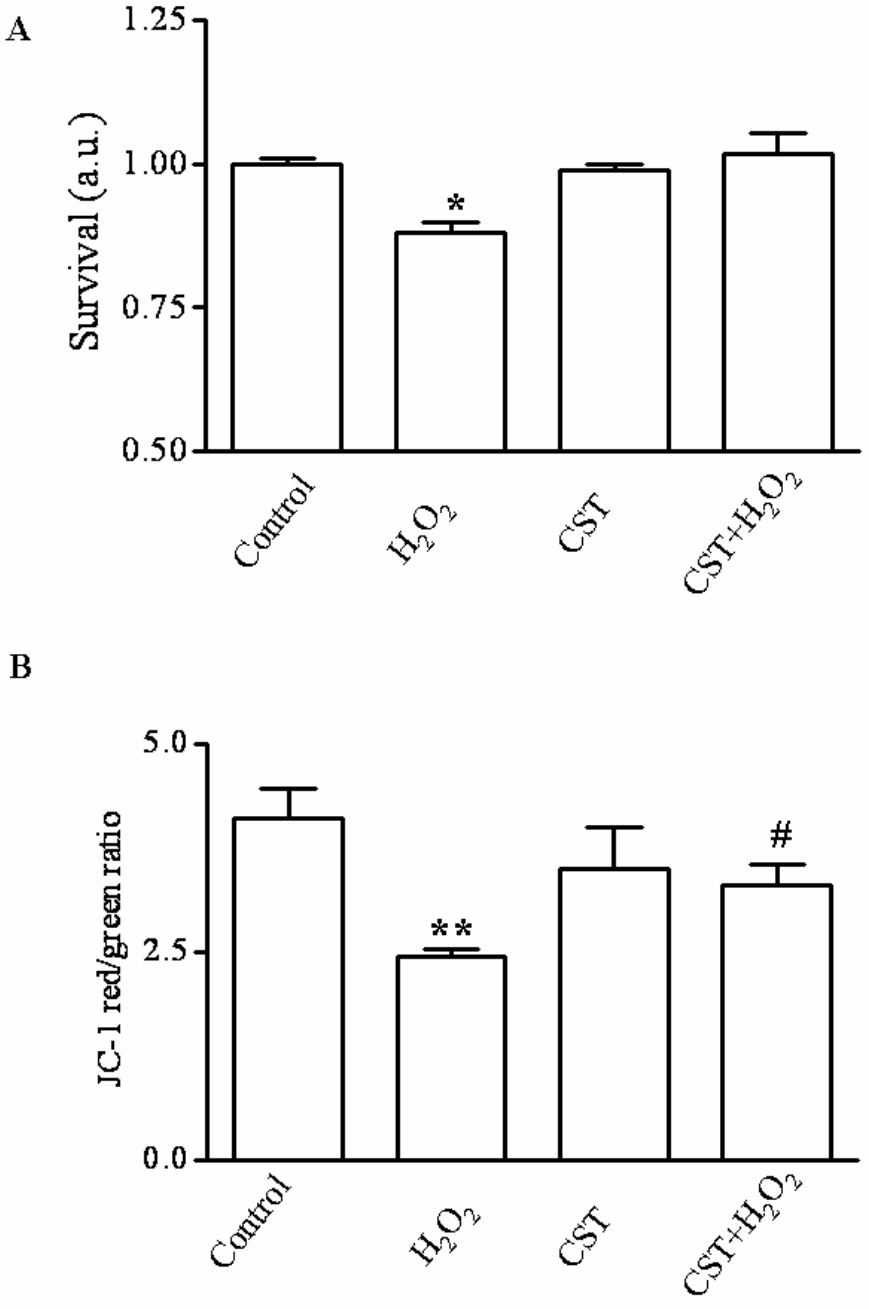

A
1.25
1.00
0.75
0.50
Survival (a.u.)
*
Control
H_2O_2
CST
CST+H_2O_2
B
5.0
2.5
0.0
JC-1 red/green ratio
**
#
Control
H_2O_2
CST
CST+H_2O_2

Fig.3

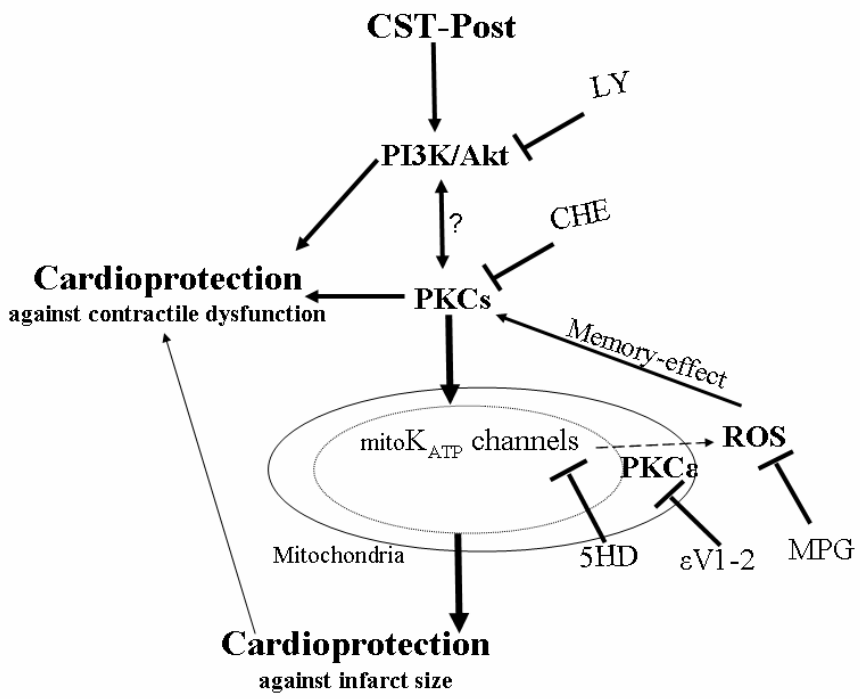

CST-Post
LY
PI3K/Akt
?
CHE
Cardioprotection
against contractile dysfunction
PKCs
Memory-effect
mitoK$_{ATP}$ channels
ROS
PKCε
Mitochondria
5HD
εV1-2
MPG
Cardioprotection
against infarct size

Fig.4