Elsevier Editorial System(tm) for Peptides
Manuscript Draft

Manuscript Number:

Title: Chromofungin, CgA47-66-derived peptide, produces basal cardiac effects and postconditioning cardioprotective action during ischemia/reperfusion injury

Article Type: Research Paper

Keywords: Chromogranins; Chromofungin; Nitric Oxide; cardioprotection; intracellular pathways

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To the Editor-in-Chief

**Dear Editor,**

We would like to submit our manuscript titled: "Chromofungin, CgA47-66-derived peptide, produces basal cardiac effects and postconditioning cardioprotective action during ischemia/reperfusion injury", by Filice et al., for consideration for publication in *Peptides*.

We would like to emphasize some points of interest of this study.

Endogenous chromogranin A (CgA)-derived peptides are secreted by nervous, endocrine and immune cells. Chromofungin (Chr: CgA47-66) is one of these peptides that display antimicrobial activities and activate neutrophils, with important implications in inflammation and innate immunity. The aim of the present paper is to examine the cardioprotection effect of Chr on rat hearts. The study was performed by using the isolated and Langendorff perfused rat hearts, ELISA assay and real-time PCR. We found that, under basal conditions, increasing doses (11–165 nM) of Chr induced negative inotropic effects without changing coronary pressure. This action was mediated by AKT, eNOS and cGMP. We also found that Chr protected the heart as a postconditioning (PostC) agent against ischemia/reperfusion (I/R) damages, reducing infarct size and LDH level. Cardioprotection involved IP3K, RISK pathway, MitoKATP and miRNA21.
We suggest that Chr directly affects heart performance, protects against I/R myocardial injuries through the activation of prosurvival kinases. Results may propose Chr as a new physiological neuroendocrine modulator able to prevent heart dysfunctions, also encouraging the clarification of its clinical potential.

The manuscript, or part of it, neither has been published (except in form of thesis) nor is currently under consideration for publication by any other Journal.

The submitting authors declare that the co-authors have read the manuscript and approved its submission to *Peptides.*

Thank you for your attention.

Sincerely yours
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## Highlights

1) The antifungal and antimicrobial Chromofungin depresses rat cardiac performance

2) Negative inotropism is mediated by the AKT/eNOS/cGMP/PKG pathway

3) Chromofungin acts as a postconditioning agent against ischemia/reperfusion damage

4) Chromofungin-dependent cardioprotection involves IP3K, the RISK pathway, MitoKATP and miRNA21

Short title: Chromofungin and cardiac physiology

# Chromofungin, $CgA_{47-66}$-derived peptide, produces basal cardiac effects and postconditioning cardioprotective action during ischemia/reperfusion injury

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This research was supported by “Ministero dell’Università e Ricerca Scientifica e Tecnologica” (ex 60% M.C.C, T.A), “Istituto Nazionale di Ricerche Cardiovascolari (INRC)” (M.C.C, T.A.), “Dottorato di Ricerca in Scienze della Vita” (P.C., F.S., T.A., M.C.C.).

**Disclosure Statement**: The authors have nothing to disclose.

**Keywords:** Chromogranins; Chromofungin; Nitric Oxide; cardioprotection; intracellular pathways

## Abstract

Endogenous chromogranin A (CgA)-derived peptides are secreted by nervous, endocrine and immune cells. Chromofungin (Chr: CgA47-66) is one of these peptides that display antimicrobial activities and activate neutrophils, with important implications in inflammation and innate immunity. The aim of the present study is to examine the effects of Chr on isolated and Langendorff perfused rat hearts.

The study was performed by using the isolated and Langendorff perfused rat hearts, Elisa assay and real-time PCR.

We found that, under basal conditions, increasing doses (11–165 nM) of Chr induced negative inotropic effects without changing coronary pressure. This action was mediated by the AKT/eNOS/cGMP/PKG pathway. We also found that Chr acted as a postconditioning (PostC) agent against ischemia/reperfusion (I/R) damages, reducing infarct size and LDH level. Cardioprotection involved PI3K, RISK pathway, MitoKATP and miRNA21.

We suggest that Chr directly affects heart performance, protects against I/R myocardial injuries through the activation of prosurvival kinases. Results may propose Chr as a new physiological neuroendocrine modulator able to prevent heart dysfunctions, also encouraging the clarification of its clinical potential.

## 1. Introduction

Chromogranin A (CgA), the index member of the granin family of proteins, is precursor of several biologically active peptides (Metz-Boutigue et al., 1993). They are generated by proteolytic cleavage not only insecretory vesicles of chromaffin cells, the main site for CgA synthesis and release, but also in extramedullary sites, including the heart [13, 26]. CgA-derived peptides include the N-terminal Vasostatin-1 (CgA1-76; VS-1), Catestatin (CgA352-372; CTS), and the C-terminal Serpinin (Serp) [7, 5, 3, 35]. All of them elicit direct effects on the rat heart. VS-1 and CTS involve a nitric oxide (NO)-dependent signaling to depress basal inotropism and lusitropism [5, 33]. They are also able to counteract beta-adrenergic and endothelinergic stimulation, acting as cardiac stabilizers against excitatory cascades [7, 3]. Moreover, by recruiting the Reperfusion-Injury-Salvage-Kinases (RISK)-pathway, both peptides elicit cardioprotection acting as pharmacological pre-conditioning (VS-1) and post-conditioning (CTS) agents [5, 33]. Recently, the cardiac relevance of CgA and its fragments has been further supported by the characterization of the cardiostimulatory, beta1-adrenergic-like properties of Serp and the cardiodepressant activity of the full lenght CgA [35, 26].

In 2001, Lugardon and coworkers identified a natural CgA-derived peptide corresponding to the N-terminal VS-1-derived sequence 47-66, and named chromofungin (Chr) because of its antifungal activity [22]. It is generated during infections by cleavage by *Staphylococcus aureus* protease Glu-C [23]. Chr acts as an immediate protective shield against pathogens [24], being able to inhibit microbial cell metabolism [4], and to penetrate the cell membrane, thus inducing extracellular calcium entry by a CaM-regulated iPLA2 pathway [39].

Interestingly, a cardioactive role was also suggested for Chr. As shown on the frog heart bioassay, a fragment corresponding to the Chr sequence, was found to depress myocardial contractility by eliciting a direct negative inotropic effect [36]. These observations represent an intriguing starting point for investigating the heart sensitivity to Chr.

Based on these premises, by using the rat heart as a prototypic mammalian heart, we investigate whether Chr affects the basal cardiac performance and is able to protect the myocardium against I/R damage. To corroborate the results on cardioprotection, the mechanism of action on RISK pathway activated during I/R were evaluated.

## 2. Materials and Methods

**2.1-Animals**. Adult Wistar rats (250–300 g body weight; Harlan, Italy), fed a standard diet and water ad libitum, were used. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (publication No. 85–23, revised 1996) and are in accordance with the Italian law (DL 116, January 27, 1992). The scientific project was supervised and approved by the local ethical committee.

**2.1.1-Drugs and chemicals.** Chr (CgA47–76) was synthesized at the INSERM U1121, Biomaterials and Bioengineering, Strasbourg, France, as previously described [22]. It was dissolved in water before use. Selective eNOS inhibitor, N(5)-(1-Imino-3-butenyl)-L-ornithine(L-NIO); soluble guanylate cyclase (sGC) inhibitor [1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one](ODQ); protein kinase G (PKG) inhibitor, KT5823; PD98059 (PD), a specific inhibitor of ERK1/2, Wortmannin (WT), a potent phosphatidylinositol 3-kinase (PI3K) inhibitor, 5-hydroxydecanoate (5HD), a mitoKchannels blocker, were purchased from Sigma Chemical Co. (St. Louis, MO). All drug-containing solutions were freshly prepared before experimentation.

**2.2-Perfusion technique.** Rats were anesthetized by ethyl carbamate (2 g/kg body weight, i.p). Hearts were rapidly excised, placed in ice-cold perfusion buffer, cannulated via the aorta and perfused in the Langendorff mode at a constant flow-rate of 12 ml/min and temperature of 37°C. The perfusion solution was a modified Krebs-Henseleit solution (KHs) (pH 7.4) gassed with 95 % O2 and 5 % CO2 containing (in mmol/l): 113.0 mM NaCl; 4.7 mM KCl; 1.2 mM MgSO4; 25.0 mM NaHCO3; 1.2 mM KH2PO4; 1.8 mM CaCl2; 11,0 mM glucose 1.1 mM mannitol, Na-pyruvate 5 [7].
Left ventricular pressure (LVP) was measured by means of a latex water-filled balloon inserted into the left ventricle via the left atrium (adjusted to obtain left ventricular end-diastolic pressure (LVEDP) of 5–8 mmHg) and connected to a pressure transducer (BLPR gauge, WRI, Inc. USA). The maximal values of the first derivative of LVP, (+(LVdP/dt)max, mmHg $s^{-1}$), which indicates the maximal rate of left ventricular contraction, the time to peak tension of isometric twitch (TTP, s), the maximal rate of left ventricular pressure decline of LVP (-(LVdP/dt)max, mmHg $s^{-1}$)), the half time relaxation (HTR, s), which is the time required for intraventricular pressure to fall from the peak to 50%, Tau (τ, s), which is the relaxation time constant; the T/-t ratio obtained by +(LVdP/dt)max/-(LVdP/dt)max, as indexes of contraction and relaxation, and LVEDP were used to assess cardiac function. Mean Coronary Pressure (CP, mmHg) was calculated as the average of values obtained

during several cardiac cycles, while Heart Rate (HR) changes (beats/min) were used to estimate chronotropism.

**2.3-Chr stimulated preparations.** Preliminary experiments (data not shown) obtained by repetitive exposure of each heart to a single concentration of Chr (65 nmol/L) revealed absence of desensitization. Thus, concentration response curves were obtained by perfusing cardiac preparations with KHs enriched with increasing concentrations of Chr (1 nmol/L-165 nmol/L) for 10 min. To evaluate the possible effects of CHR on chronotropism, in this set of experiments hearts were unpaced.

**2.4-Chr-dependent mechanism of action.** To investigate the involvement of the NO pathway in the mechanism of action of Chr, hearts were stabilized for 20 min with KHs and perfused for 10 min with Chr (65nM); then hearts were washed out with KHs and, after returning to control conditions, perfused with KHs containing Wortmannin (WT) (100nM) or L-NIO (10μM) or ODQ (10μM) or KT5823 (0.1μM). Subsequently, cardiac preparations were exposed to KHs enriched with Chr plus each of the above inhibitors (n=6 for each group).

**2.5-Ischemia/Reperfusion.** Each heart was allowed to stabilize for 40 min; at this time, baseline parameters were recorded. After stabilization, hearts were randomly assigned to one of the groups described below and then subjected to 30-min of global, no-flow ischemia followed by 120-min of reperfusion (I/R). Although under basal conditions Chr did not modify HR, to avoid interference by HR during I/R maneuvers, hearts were paced according to Pagliaro and co-workers [25].

**2.6-Cardiac function and Infarct size**

1) Group 1 (Sham group n=6), hearts were stabilized and perfused for 120 min.

2) Group 2 (I/R group, n=6), hearts were stabilized and subjected to I/R protocol only.

3) Group 3 (postC-Chr group, n=6), Chr (65 nmol/L) was infused for 20-min at the beginning of 120-min reperfusion.

4) Group 4 (postC-Chr + inhibitors group, n=6 for each group), hearts were perfused with Chr plus one of the following inhibitors: WT (100nM; Chr + WT), or PD (10nM; Chr + PD), or ODQ (0.1μM; Chr + ODQ), or (5HD (10μM; Chr + 5HD); perfusion with inhibitors started 5-min before ischemia and continued during the early 20-min of reperfusion in the presence of Chr (65 nmol/L).

Chr concentration was chosen on the basis of preliminary dose-response curves as the dose that induced the highest infarct size reduction (data not shown).

Cardiac performance before and after ischemia was evaluated by analyzing LVP recovery, as an index

of contractile activity, and LVEDP as an index of contracture, defined as an increase in LVEDP of 4 mmHg above the baseline level [25].

**2.7-Assessment of myocardial injury.** To obtain infarct areas, hearts were rapidly removed from the perfusion apparatus at the end of reperfusion, and the left ventricle was dissected into 2- to 3-mm circumferential slices. After 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 not aware of the nature of the intervention. The weights of the necrotic and non necrotic tissues were then determined, and the necrotic mass was expressed as a percentage of total left ventricular mass, including septum [25].

**2.8-Cyclic Guanosine Monophosphate (cGMP).** At the end of perfusion, for cGMP determination frozen ventricles (200–300mg) were treated with 6% (m/V) trichloroacetic acid at 0°C and centrifuged at 1000g for 10 min. Supernatants were extracted three times with 3 ml of diethyl ether saturated with water, and the aqueous phases were collected and stored at -80°C. the cGMP determination was obtained by using cGMP: Biotrack Enzyme Immunoassay (EIA) System, (GE Healthcare, Milan, Italy).

**2.9-RNA preparation and quantitative real-time polymerase chain reaction for miRNA expression.** Hearts were homogenized prior to RNA extraction. Total RNA was extracted with Trizol, according to the manufacturer. RNA purity and integrity were confirmed by spectroscopy and gel electrophoresis before use. The single-tube TaqMan miRNA assays (Applied Biosystems, Life Technologies) was used to detect and quantify mature miR-(21) according to the manufacturer, by the use of iQ5 multicolor detection system (Bio-Rad, Berkeley, CA, USA). miRNA expression was normalized on RNU6.

**2.10-Lactate Dehydrogenase**. Since in isolated rat hearts, ischemic PostC is known to reduce the production of lactate dehydrogenase (LDH) during reperfusion [31], the release of this enzyme was examined in each experimental group. Samples of coronary effluent were withdrawn with a catheter inserted into the right ventricle via the pulmonary artery. Samples were taken during reperfusion. LDH release was measured as previously described [31]. Data were expressed as cumulative values for the entire reperfusion period.

**2.11-Statistics.** Statistical analysis was performed by using the GraphPad Prism Software® (version 5.0; Graph Pad Software, San Diego, CA, USA). Data were expressed as the means±SEM. Since each heart represents its own control, the statistical significance of differences within-group was assessed using the ANOVA test ($p < 0.05$).

Comparison between groups was made by using a one-way analysis of variance (ANOVA) followed by the Dunnett's Multiple Comparison test for post-hoc t-tests. Differences were considered to be statistically significant for $p < 0.05$.

## 3. Results

### *3.1-Basal cardiac parameters*

Cardiac parameters, obtained after 20 min equilibration, are in Table 1. Endurance and stability of the preparations were analyzed by measuring the performance variables every 10 min, revealing that each heart was stable up to 180 min.

### *3.2-Chr effects on myocardial contractility and relaxation*

A preliminary work on the frog heart bioassay revealed that the peptide CgA47-66, corresponding to Chr is able to negatively modulate myocardial contractility [36]. Here we evaluated whether exogenous Chr directly affects the basal performance of the mammalian heart. By exposing Langendorff perfused rat cardiac preparations to increasing concentrations (1nmol/L-165nmol/L) of Chr for 10 min, we found that the peptide depressed myocardial contractility and relaxation. From 11 nM, the negative inotropic (decrease of LVP and +(LVdP/dt)max) and lusitropic (reduction of -(LVdP/dt)max and increased T/-t) effects were significant and were elicited without influencing CP and HR (Fig. 1).

### *3.3-Mechanisms of action elicited by Chr*

In mammals, Nitric Oxide (NO) is crucially involved in the beat-to-beat, medium- and long-term modulation of the cardiac function [6]. To evaluate whether the cardiac effects elicited by Chr involve NO generation, Chr-dependent negative inotropism and lusitropism were analysed in the presence of specific inhibitors of the NO pathway. Exposure to the peptide induced a decrease of LVP, +(LVdP/dt)max and -(LVdP/dt)max which was abolished by co-treatment with WT, a specific inhibitors of PI3K, or L-NIO, an selective inhibitor of eNOS, or ODQ, a specific inhibitor of CG, or KT5823, a specific inhibitor of PKG (Fig. 2a). By ELISA assay, the increased cGMP levels were observed in cardiac extracts after exposure to Chr (65 nmol/L) (Fig. 2b)

### *3.4-Chr effects on post-ischemic cardiac function*

The possibility that Chr elicits cardioprotection was investigated by comparing the effects induced by I/R manoeuvres with those elicited by the peptide administered after I/R (PostC) (Fig. 3a). Both systolic and diastolic functions were analysed. Although under basal condition, Chr did not change HR, hearts undergoing I/R protocols were paced to avoid chronotropic influences.

Systolic function is represented by the level of inotropic activity (i.e., LVP recovery). Hearts of the I/R group presented a limited LVP recovery; in fact, at the end of reperfusion, LVP was 11±1.7 mmHg (baseline values: 87.75±9.3 mmHg). Chr markedly improved LVP recovery during reperfusion, being

LVP at the end of reperfusion 51±4 mmHg (baseline values, 87.75±9.3 mmHg) (Fig. 3b), obtaining a recovery of performance of ~73% with respect to the control. Diastolic function is represented by the level of contracture (i.e., LVEDP 4 mmHg or more above baseline level) [2]. I/R markedly increased LVEDP (from 7.1±0.7 mmHg in the baseline to 38±10 mmHg at the end of reperfusion). During reperfusion, Chr abolished contracture development; in fact, LVEDP at the end of reperfusion was 5±0.5 mmHg (Fig. 3ab). Total infarct size was expressed as a percentage of LV mass. LV mass was similar in all groups (LV weight was 930±9 mg; range 500-1200 mg). Infarct size was 65±5% in I/R and 35±3% in the heart perfused with Chr (Fig. 3c).

LDH release in the I/R group was 1320±170 U/g (units per g of wet heart), while it was significantly reduced after reperfusion with Chr (820±140 U/g) (Fig. 3d).

### *3.5-Chr influence on cardioprotective pathways*

The Chr-dependent improvement of post-ischemic LVP was abolished when hearts were co-treated with Chr plus the inhibitor of PI3K (WT), or of CG (ODQ), ERK1/2 (PD), or mitoKATP channels (5HD). Also the improvement induced by Chr on contracture (LVEDP) and on infarct size were abolished by co-treatment with the above inhibitors (Fig. 4). In hearts perfused only with inhibitors, the recovery of systolic function, the development of contracture and the infarct size were similar to those in I/R group (data not shown). The cardioprotection determines also an increase of cGMP intracardiac level (Fig. 5a). miRNA-21 is an interesting candidate to inhibit apoptosis in the heart and to induce cardioprotection [19]. To verify the involvement of miRNA-21 in Chr-dependent cardioprotection we measured the level in I/R and PostC-Chr hearts. Results showed that miRNA-21 levels in I/R hearts were reduced with respect to Sham. In PostC-Chr, miRNA-21 levels were statistically increased with respect to the I/R group (fig. 5b).

## 4. Discussion

We here demonstrated that Chr (CgA47-66) directly affects the performance of the isolated and Langendorff perfused rat heart by dose-dependently inducing negative inotropism and lusitropism and involving the AKT/NOS/cGMP/PKG pathway. Chr also protected against I/R injury, acting as a Post conditioning agent through activation of RISK pathways and mitoKATP channels.

### *4.1-Chr as a cardioinhibitory peptide*

On the isolated and Langendorff-perfused rat heart, we observed that exogenous Chr elicited negative inotropism and lusitropism starting from 11 nM. Under unstimulated conditions, it significantly reduced LVP, and +(LVdP/dt)max (indexes of inotropism), and (LVdP/dt)max and T/-t (indexes of lusitropism), without affecting HR and CP. These effects were obtained at Chr concentrations close to the physiological range of its precursor, CgA, in human serum (normal levels: 0.5–4 nM; neuroendocrine tumors and last stages of chronic heart failure: 100 nM) [16]. In our study, we reported for the first time the direct effects induced by Chr on the mammalian heart. With respect to the data reported on the frog heart [36], we demonstrated in the rat that Chr reduced not only contractility (~40% rat vs ~18% in frog), but also relaxation without changing heart rate. The negative inotropic and lusitropic effects induced by Chr (~40%) resemble the cardiodepression elicited on the rat heart by human recombinant (hrVS1) (~20%) which includes the vasostatin-1 domain and Chr sequence [7]. This highlights the contractile properties of the CgA N-terminal domain. Cerra and co-workers [8] reported that the negative inotropism and lusitropism of the N-terminal domain are confined to the CGA1-64 region, since ratCGA65-76 failed to modify the basal inotropism. A previous study reported that by endogenous proteolytic degradation of vasostatin-1, the two fragments CGA1-64 and CGA65-76 are produced and secreted with catecholamines during stress situations [23]. At the same time, it was reported that ratCGA1-64 induces coronary vasodilatation [8]. This effect is in contrast with the increase of coronary activity induced on the rat heart by human recombinant VS-1, containing Chr [7]. Here we found that Chr did not change rat coronary reactivity. Accordingly, although VS-1 and Chr elicit similar effects on myocardial contractility and relaxation, sequence-specific vascular activities may account for the observed differences in coronary responses. Of note, a strong structure-function relationship has been demonstrated for CgA-derived peptides suggesting that different fragments may display different cardioactive potency. In addition Chr is not completely included in CgA1-64 and we have previously shown by using the helical wheel prediction of secondary structure that sequence CgA53-66 presents a longer hydrophobic domain than CgA53-64 [16].

Our study revealed that, as for full length CgA [26] and other CgA-derived inhibitory fragments, Vasostatin 1 and Catestatin [5, 7, 3], the effects induced by Chr involve the AKT/NOS-NO/cGMP signal transduction pathway. In fact, Chr exposure increased AKT and eNOS phosphorylation, thus activating the NO-generating cascade. This promotes cGMP production by soluble Guanylate Cyclase (sGC), with final depressant effects on contractility and relaxation. As demonstrated on the mammalian heart, activation of this pathway decreased L-type Ca2+ currents [1] and troponin I phosphorylation [18], thus contributing to myocardial contractile performance inhibition. It also attenuates sarcoplasmic reticulum $Ca^{2+}$ reuptake by inhibiting phospholamban phosphorylation [12]. In recent years, great attention has been focused on cardiac relaxation (lusitropism), being this a crucial component of the cardiac cycle. A proper relaxation (i.e. restoring the diastolic ventricular pressure after each contraction) allows the ventricle to be adequately filled with blood, with impact on the subsequent contraction. Notably, we observed that Chr negatively influenced lusitropism. This is particularly important since an impaired relaxation contributes to cardiac dysfunction, such as in heart failure with normal ejection fraction [20].

### *4.2-Chr-dependent cardioprotection*

We found that Chr, given in the early reperfusion, limited the I/R-dependent myocardial damage. This protection, similar to that obtained by ischemic PostC maneuvers [37], was indicated by a significant reduction of both infarct size and LDH release, and by a marked improvement of the post-ischemic contractile function expressed as a decrease of contracture development. A reduced contracture is the goal of cardioprotective protocols, due to its inverse relation with the I/R-dependent myocardial damage [27]. Our results extended to Chr the cardioprotective properties of other CgA fragments, such as VS-1 and CTS [5], paving the way for analyzing the applicative potential of this peptide as pharmacological PostC agent.

*4.2.1-Chr-dependent mechanism of action in cardioprotection*- Like Pre- and Post-conditioning maneuvers, many substances protect the heart by recruiting pro-survival intrinsic signaling cascades involving PI3K/Akt, PKCε and ERK1/2, which may converge on GSK-3β, a substrate of multiple pro-survival protein kinases that in rodents include the RISK pathway [17, 27], and require the opening of mitoKATP channels [29]. Here we observed that this cascade is activated in hearts exposed to Chr in the early reperfusion. In fact, inhibition of the RISK upstream kinases (PI3K and ERK1/2) abolished the recovery of the systolic performance induced by Chr. Our results agree with previous data obtained by our and other laboratory which showed that these kinases are recruited by pharmacological PostC elicited by many protective substances, including CST, GLP2 etc. [2, 29, 34]. During reperfusion, both PI3K-Akt and ERK1/2 are activated and converge on GSK3β, inducing its

phosphorylation/inactivation with a final control on mitoKATP channels, one of the terminal elements of PostC protection [14]. Our results suggest that, in addition to RISK, the efficacy of Chr PostC can be attributed to the control of mitochondria function. We also observed that Chr-dependent cardioprotection is accompanied by increase of intracellular cGMP, an effect which disappears in hearts co-treated with specific inhibitor of CG. The importance of cGMP in cardioprotection is well known [30]. In fact, PostC depends on GC activation via either NOS-dependent or NOS-independent pathways [30, 32].

Of note, Chr-dependent cardioprotection is accompanied by an increased miRNA21 expression. miRNA are small noncoding RNAs that mediate post-transcriptional gene silencing [38]. They are involved in cardiac physiopathology and their deregulated expression is linked to the developement of cardiovascular disorders [10]. In particular, it was demonstrated that miRNA21 overexpression reduces infarct size, and this is associated with pro-apoptotic genes inhibition and anti-apoptotic genes increase [11, 9].

### *5. Conclusion*

We demonstrated that the antibacterial and antifungal Chr modulates the heart by eliciting NO-dependent negative inotropism and lusitropism. The peptide elicits post-conditioning protection on the myocardium against I/R injury by activating RISK pathway and by modulating miR21 expression. Our data are of physiological relevance since they contribute to clarify the role of naturally occurring CgA-derived peptides in cardiac stabilization against stress responses. They are also of interest in an applicative context since left ventricular functional alterations consequent to I/R are major determinants of mortality and prognosis, and the specific targets observed playing a significant negative effect in exacerbation myocyte death [15, 28, 21]. Accordingly, in the search of novel endocrine modulators that target and attenuate reperfusion-induced cell death, to improve the classic reperfusion strategy aimed to limit myocardial infarction, our results on Chr induced cardioprotection deserve further attention.

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**Figure legends**

**Fig. 1.** Concentration-response curves of increasing concentrations (1 nmol/L-165 nmol/L) of Chr on LVP, +(LVdP/dt)max, -(LVdP/dt)max, T/-t, HR, and CP on the isolated and Langendorff perfused rat heart. For abbreviations and basal values, see Results. Percentage changes were evaluated as means±SEM of 8 experiments for each group. Significance of difference from control values (one-way ANOVA): *p<0.05.

**Fig. 2. (a).** Effects of Chr alone and in the presence of WT (100 nM), L-NIO (10μM), ODQ (10μM), and KT5823 (0.1μM) on LVP, +(LVdP/dt)max, and -(LVdP/dt)max on the isolated and Langendorff perfused rat heart. **(b).** cGMP concentrations in heart extracts. Percentage changes were evaluated as means±SEM of 6 experiments for each group. Significance of difference (one-way ANOVA) from control values: *p<0.05.

**Fig. 3. (a).** Protocol groups. **(b).** LVP and LVEDP variations. Data are expressed as changes of LVP and LVEDP values (mmHg) from the stabilization to the end of the 120-min of reperfusion with respect to the baseline values for each group. Vertical lines indicate ischemic administration. Comparison between groups followed by Dunnett's Multiple Comparison test: §<0.05; **(c).** Infarct size. The amount of necrotic tissue measured after 30-min global ischaemia and 120-min reperfusion is expressed as percentage of left ventricle (% IS/LV): *p<0.05 with respect to I/R. Significance of differences from control values of Chr *vs* I/R (one-way ANOVA): *p<0.05; **(d)** Effects of Chr on LDH release. Values are expressed as means±SEM of absolute data (U/wet wt, units per g of wet heart). Significance of difference from control values (one-way ANOVA): *p<0.05; Changes were evaluated as means±SEM of 8 experiments for each group.

**Fig. 4.** Infarct size, LVP, LVEDP and LDH variations of I/R, Post-Chr and Post-Chr plus inhibitors groups (WT; ODQ; PD; 5HD). Data are expressed as changes of IF (%), of LVP and LVEDP values (mmHg), of LDH (U/wet wt, units per g of wet heart) from the stabilization to the end of the 120-min of reperfusion with respect to the baseline values for each group. Percentage changes were evaluated as means±SEM of 6 experiments for each group. Significance of difference (one-way ANOVA): *p<0.05 Comparison between I/R groups *vs* PostC-Chr; §p<0.05 Comparison between groups followed by Dunnett's Multiple Comparison test.

**Fig. 5. (a).** cGMP concentrations in sham, I/R and PostC Chr heart extracts. **(b).** Real-Time PCR of miR-21 expression in Sham, I/R, and PostC Chr-treated hearts. Percentage changes were evaluated as means±SEM of 7 experiments for each group. Significance of difference (one-way ANOVA) from control values of I/R *vs* Sham: *p<0.05. Comparison between I/R groups *vs* PostC-Chr; §p<0.05 Comparison between groups followed by Dunnett's Multiple Comparison test.

Figure1

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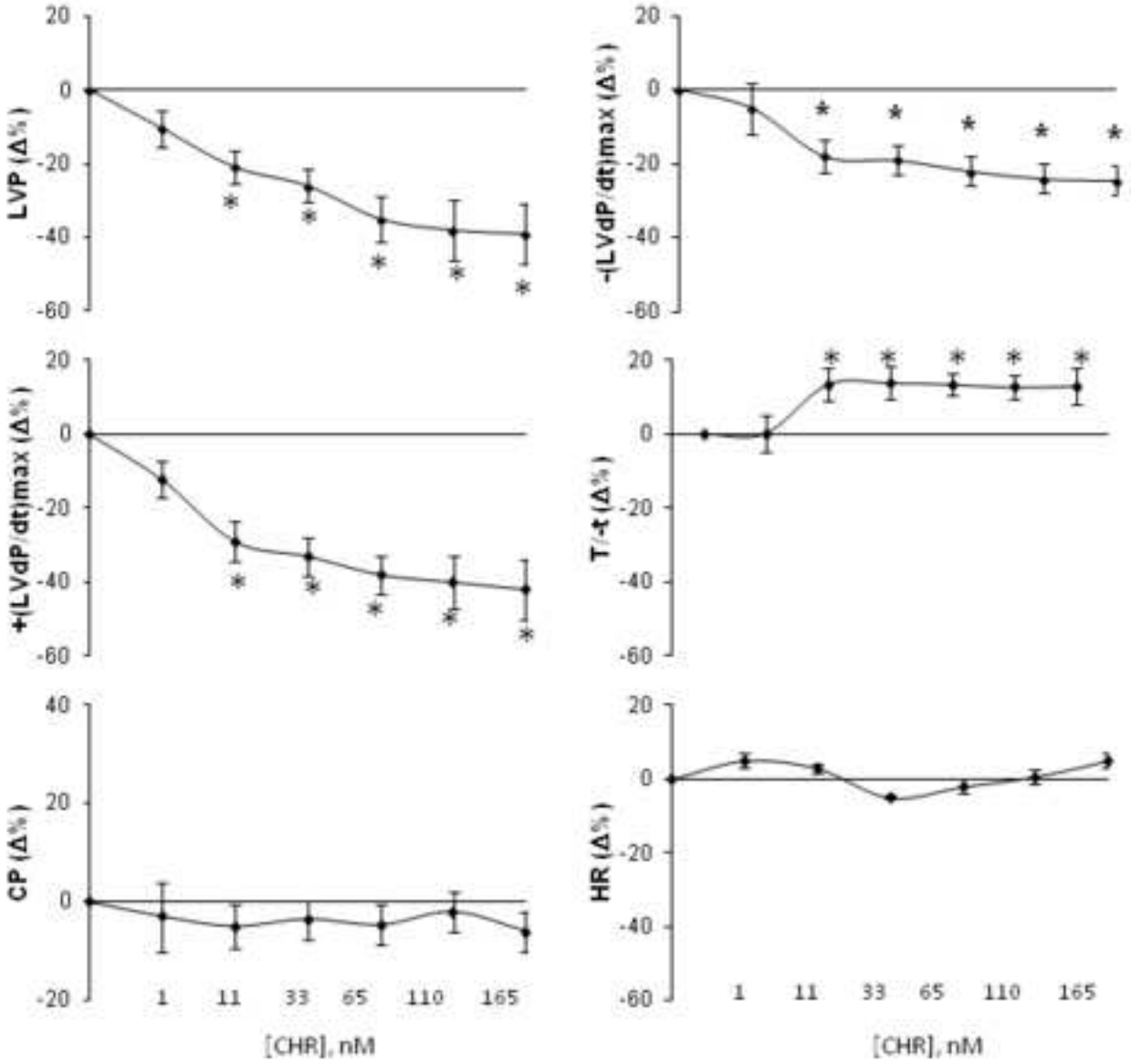


Fig. 1

Figure2
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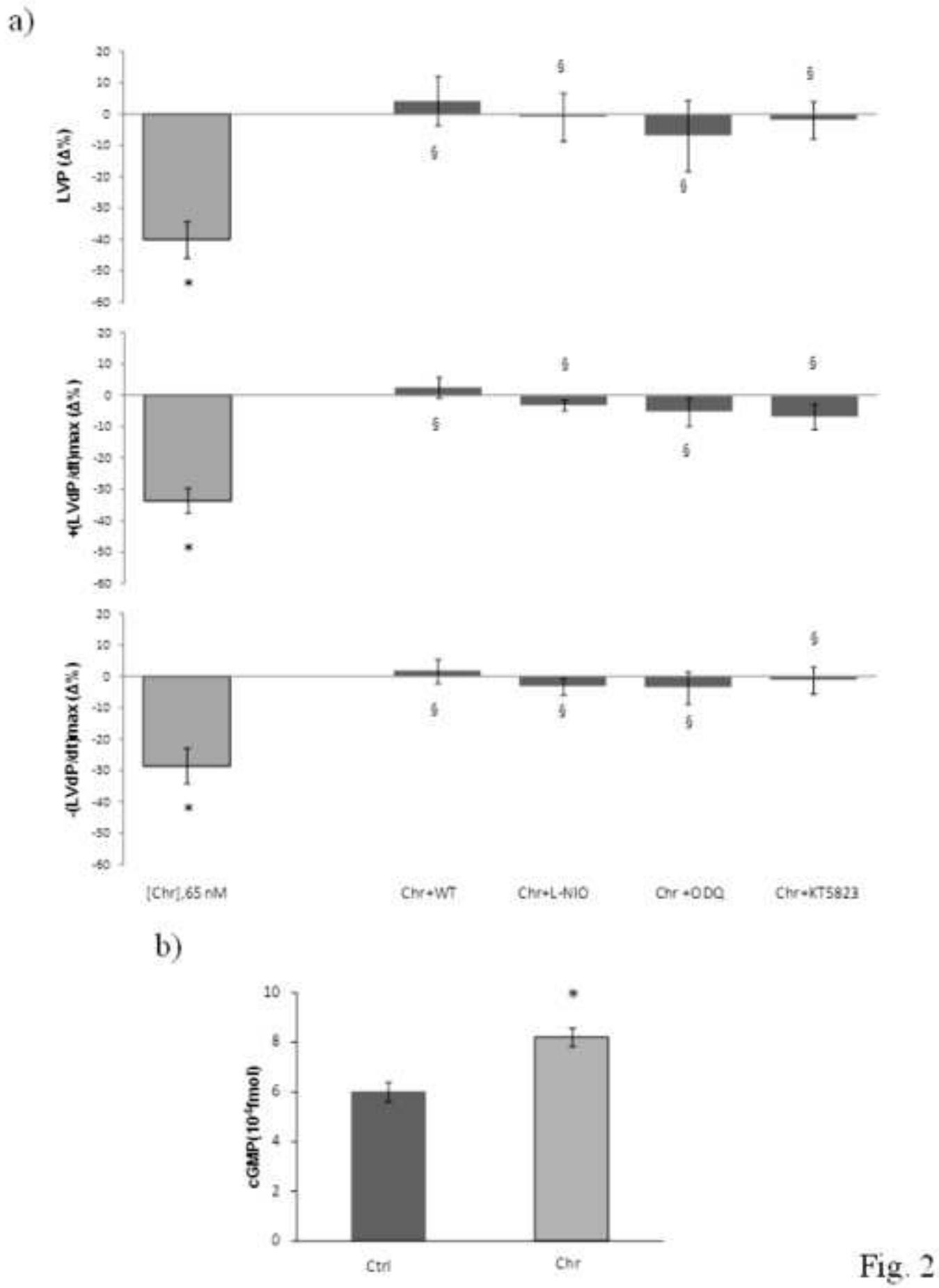


Fig. 2

Figure3

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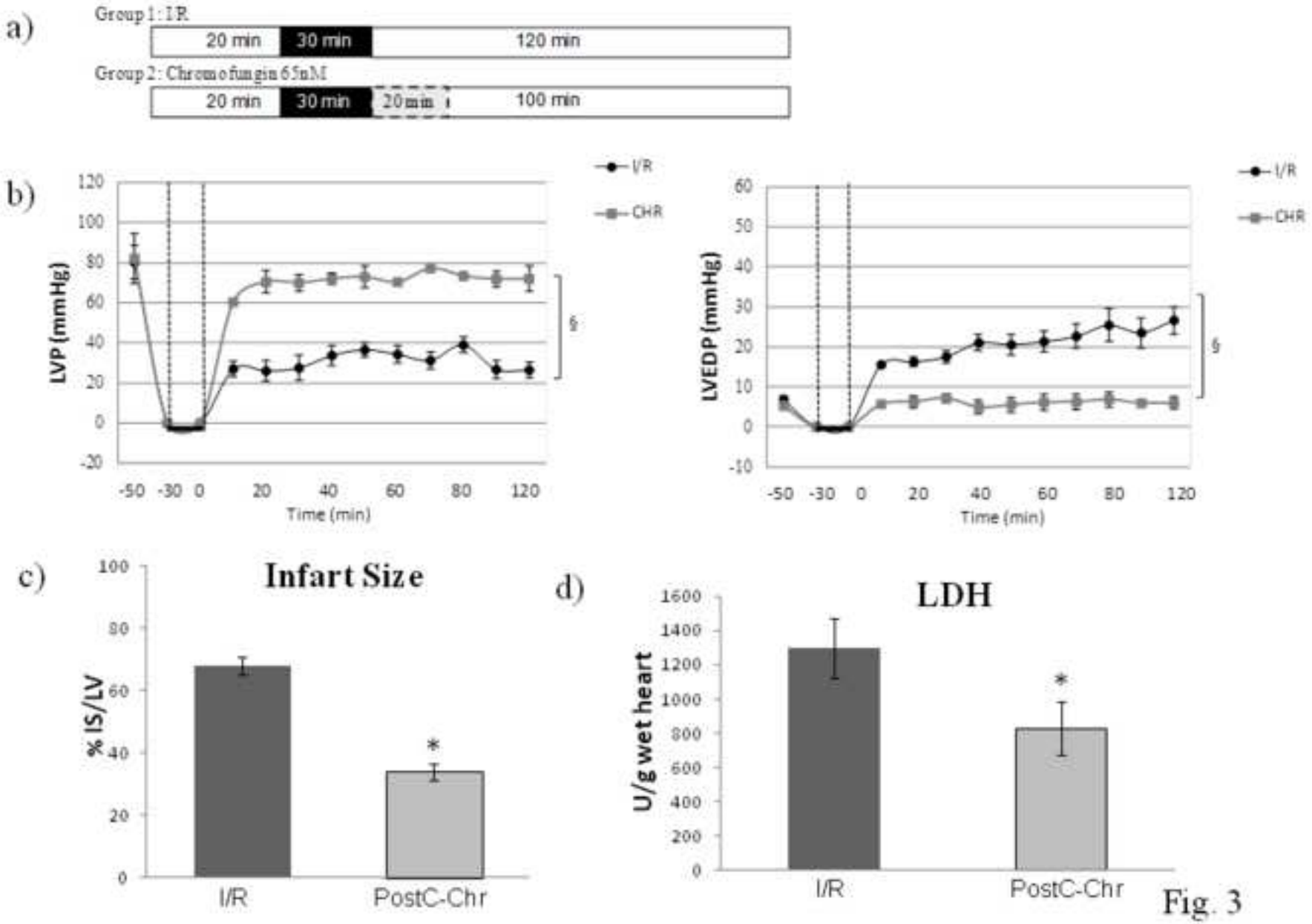


Fig. 3

Figure4

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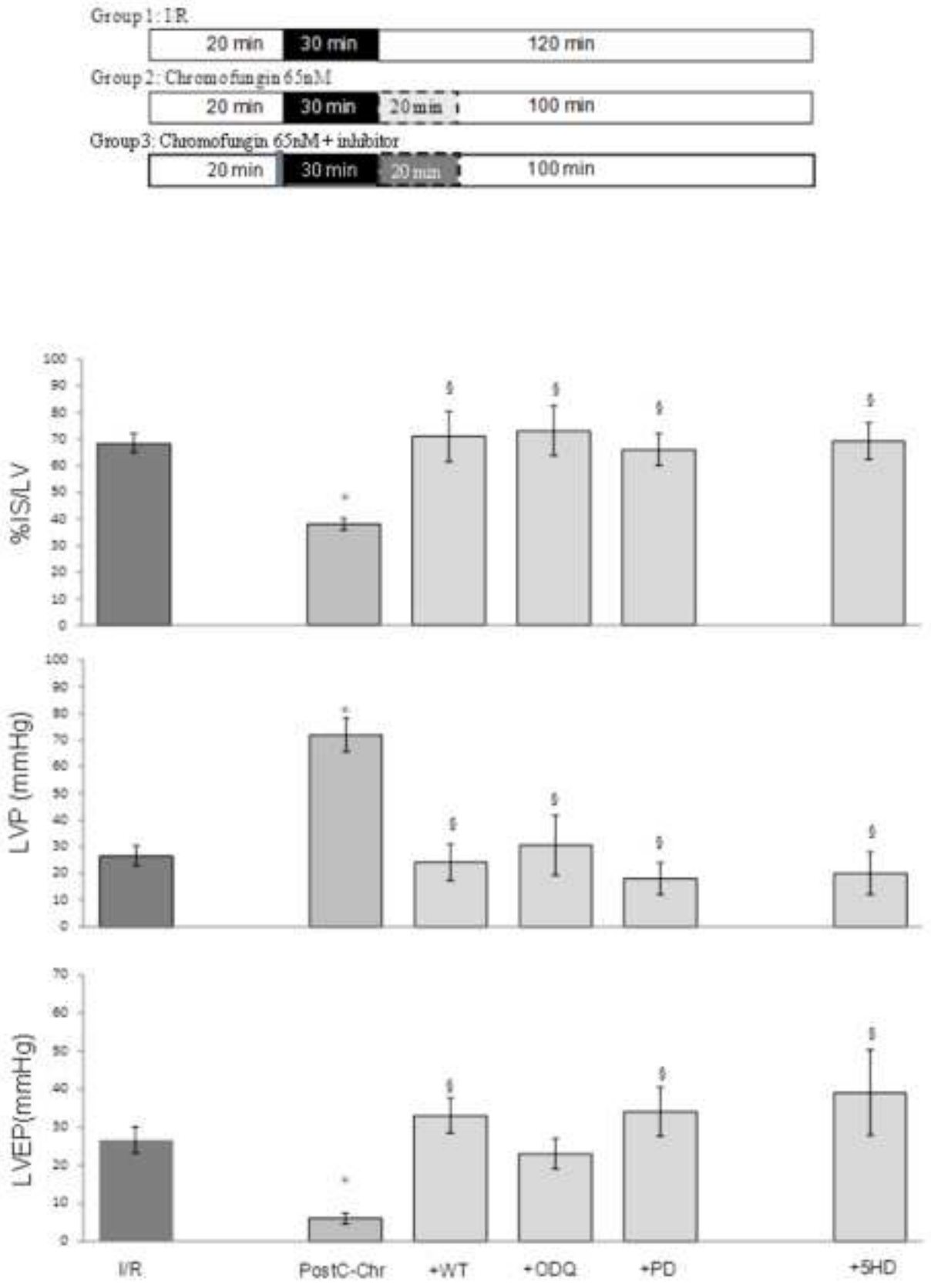


Fig. 4

Figure5
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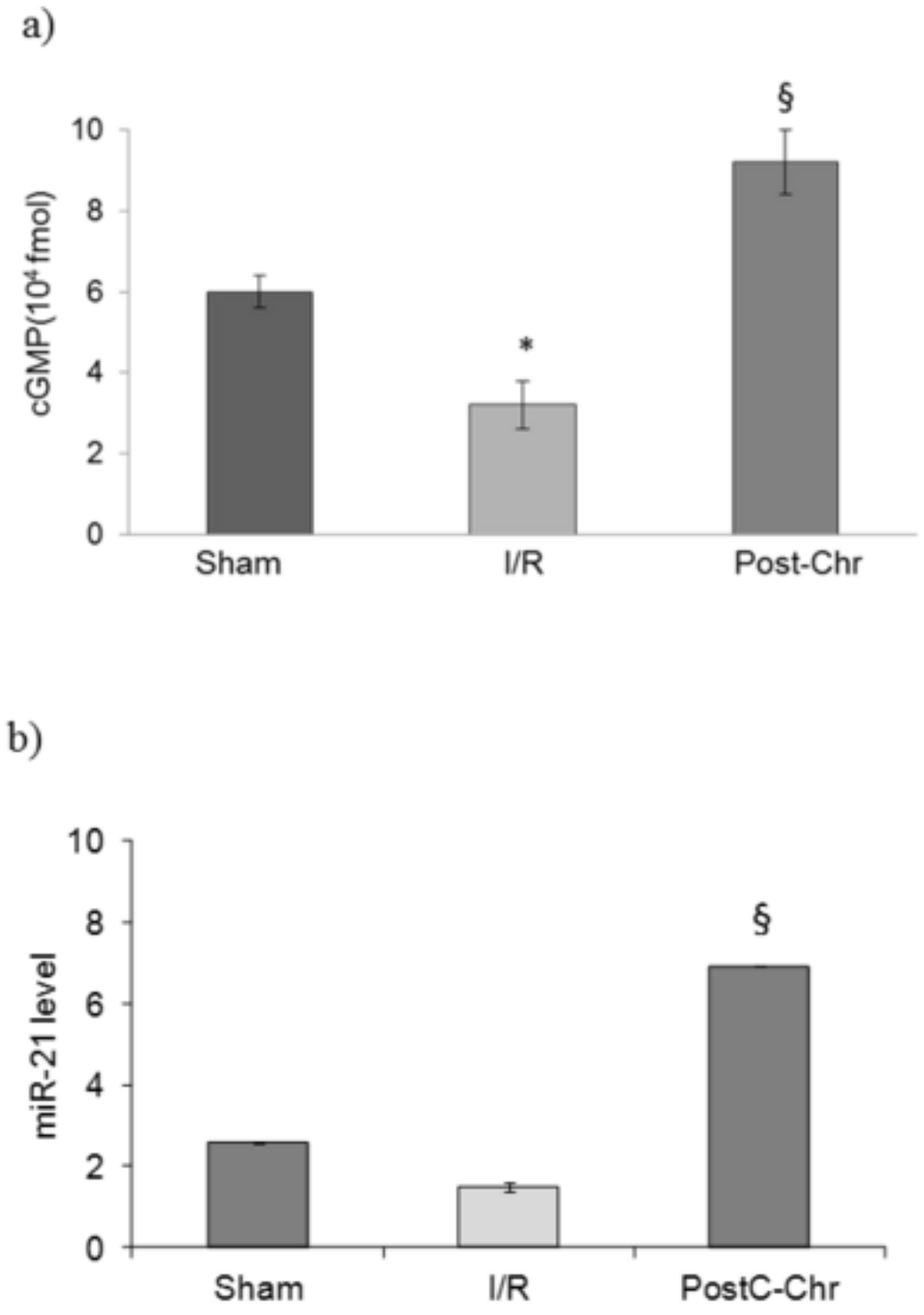


Fig. 5

**Table1**

| LVP (mmHg) | HR (beats $min^{-1}$) | EDVP (mmHg) | $+(LVdP/dt)_{max}$ (mmHg $s^{-1}$) | $-(LVdP/dt)_{max}$ (mmHg $s^{-1}$) | CP (mmHg) | Pressure perfusion (mmHg) |
|---|---|---|---|---|---|---|
| 81 ± 4 | 271 ± 8 | 5-8 | 2502 ± 131 | -1641 ± 66 | 59 ± 3 | 100 |

**Table 1.** Basal cardiac parameters. For abbreviation see Material and Methods.