Published in final edited form as:

J Endocrinol. 2015 December ; 227(3): 167–178. doi:10.1530/JOE-15-0199.

pGlu-serpinin protects the normotensive and hypertensive heart from ischemic injury

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Abstract

Serpinin peptides derive from proteolytic cleavage of Chromogranin-A at C-terminus. Serpinin and the more potent pyroglutaminated-Serpinin (pGlu-Serp) are positive cardiac beta-adrenergic-like modulators, acting through β1-AR/AC/cAMP/PKA pathway. Since in some conditions this pathway and/or other pro-survival pathways, activated by other Chromogranin-A fragments, may cross-talk and may be protective, here we explored whether pGlu-Serp cardioprotects against ischemia/reperfusion injury under normotensive and hypertensive conditions. In the latter condition cardioprotection is often blunted because of the limitations on pro-survival Reperfusion-Injury-Salvage-Kinases (RISK) pathway activation. The effects of pGlu-Serp were evaluated on infarct size (IS) and cardiac function by using the isolated and Langendorff perfused heart of normotensive (WKY) and spontaneously hypertensive (SHR) rats exposed to ischemic pre-conditioning (PreC) and post-conditioning (PostC). In both WKY and SHR rat, pGlu-Serp induced mild cardioprotection in both PreC and in PostC. pGlu-Serp administered at the reperfusion (Serp-PostC) significantly reduced IS, being more protective in SHR than in WKY. Conversely, developed Left Ventricular Pressure (LVDevP) post-ischemic recovery was greater in WKY than in SHR. pGlu-Serp-PostC reduced contracture in both strains. Co-infusion with specific RISK inhibitors (PI3K/AkT, MitoK_{ATP} channels, and PKC) blocked the pGlu-Serp-PostC protective effects. To show direct effect on cardiomyocytes, we pre-treated H9c2 with pGlu-Serp which were thus protected against hypoxia/reoxygenation. These results suggest pGlu-Serp as a potential modulatory agent implicated in the protective processes which can limit infarct size and overcome the hypertension-induced failure of PostC.

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Conflict of interest: Authors have nothing to disclose

Keywords

Serpinin; Chromogranin A-derived peptides; hypertension; rat heart

INTRODUCTION

Chromogranin-A (CgA) is a multifunctional protein and a major marker of the sympatho-adrenal neuroendocrine activity (SAN) (Zhang et al., 2011), with important implications on cardiovascular homeostasis (Angelone et al., 2012a). Following stimulus- and differential cell-type-specific or tissue-specific proteolytic processing at dibasic sites, CgA generates several biologically active peptides (Helle et al., 2007) that *via* direct and/or indirect SAN interactions exert relevant modulations on endocrine, cardiovascular, metabolic and immune systems (Bartolomucci et al., 2011). With reference to cardio-circulatory homeostasis, full-length CgA (Pasqua et al., 2013) and three CgA-derived peptides, Vasostatin 1 (hCGgA1-76), catestatin (hCgA352-372) and the recently discovered serpinin (see Fig.1) have revealed interesting influences on heart function and its endocrine/paracrine control (Angelone et al., 2012a). Serpinins are generated by the proteolytic cleavage of the penultimate and the last pair of basic residues at CgA C-terminus (CgA403-428) (Koshiumizu et al., 2010; Koshimizu et al., 2011). Three forms of naturally occurring serpinin have been found in rat heart, i.e. serpinin (Ala26Leu), pyroglutaminated serpinin (pGlu-Serp) and a C-terminal extended form, serpinin-RRG (Ala29Gly) (Tota et al., 2012). Unlike the other two CgA-derived cardioactive peptides VS-1 and CST, which are NO-dependent negative anti-β adrenergic inotropes, serpinin and the more potent pGlu-Serp have been shown to act as positive beta-adrenergic-like inotropes through a NO-independent β1-Adrenergic Receptor/Adenylate Cyclase/cAMP/PKA pathway (Tota et al., 2012). The serpinin-PKA pathway also induces phosphorylation of ERK1/2 and GSK3β (Tota et al., 2012). These proteins mediate prostaglandin E2 and prostanoid receptor signaling in neonatal ventricular myocytes and are components of the protective RISK (*reperfusion injury signaling kinase*) pathway implicated in myocardial protection against ischemia-reperfusion (I/R) injury in rodents (Hausenloy & Yellon, 2004). Conceivably, they could also be involved in the serpinin-induced anti-apoptotic effects against reactive oxygen species (ROS) reported in cultured cerebral neurons (Koshimizu et al., 2011). Intriguingly, activation of ERK and PI3K/PKB/Akt is associated with cardioprotection by beta-adrenergic preconditioning (Salie et al., 2012). In fact, peptides, such as Growth hormone-releasing hormone (GHRH), which induce cardioprotection during either pre or post ischemic phases, activate ERK and PI3K/PKB/Akt pathways reducing apoptosis through beta-adrenergic stimulation (Granata et al., 2009; Penna et al., 2013a). Therefore a cross-talk between beta-adrenergic-PKA and RISK pathways exists. On the basis of these observations Tota et al. (2012) hypothesized a cardioprotective action of serpinins. To verify this hypothesis, in the present work we evaluated whether serpinin can be cardioprotective against I/R injury. Using isolated Langendorff perfused hearts of both normotensive (WKY) and spontaneously hypertensive rats (SHR), we studied the effects of pGlu-Serp on infarct size (IS) and cardiac function, i.e., developed left ventricular pressure and postischemic contracture (left ventricle end diastolic pressure) in ischemic conditioning administered before (i.e. Preconditioning) or onset (e.g. Postconditioning) reperfusion. Preconditioning (PreC) and Postconditioning

(PostC) are pharmacologically and/or mechanically modified reperfusion interventions that counteract reperfusion injury, reducing IS *via* mechanisms such as activation of the RISK pathway (Ferdinandy et al., 2014; Zhao et al., 2003). It is acknowledged that, although PreC has been successfully used in the clinical setting, its application is limited by the inability to predict ischemia. Accordingly, interventions at the time of reperfusion are now considered the golden standard for cardioprotection (Zhao et al., 2003). However, the therapeutic scheme may be complicated by comorbidities, such as hypertension. This risk factor determines important alterations in cellular signaling pathways with consequences on the development of I/R damage and the efficacy of cardioprotective interventions (Ferdinandy et al., 2014). Notably, in the rat heart, PostC cardioprotective effects are blunted in the presence of hypertension (Penna et al., 2010a).

Materials and Methods

Animals

Experiments were conducted according to Italian law (D.L. 26/2014), the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (2011), and the Directive 2010/63/EU of the European Parliament on the protection of animals used for Scientific research. The project was approved by the Italian Ministry of Health, Rome, and by the ethics review board. Experiments were conducted in age-matched male Wistar Kyoto (WKY) and Spontaneously hypertensive (SHR) rats (250-300g) (Janvier, St Berthevin Cedex-France). All animals were identically housed under controlled light and temperature conditions with free access to standard rat chow and tap water. Tail cuff method was used for daily measures of systolic pressure. Tail cuff was connected to a pneumatic pulse transducer and a programmed electro-sphygmomanometer (BP-2000 series II; blood pressure analysis system, Visitech System).

Isolated Langendorff heart perfusion

Before dissection, rats were anesthetized by i.p. injection of ethyl carbamate (2 g/kg rat). Hearts were rapidly excised and immediately transferred in ice-cold buffered Krebs-Henseleit solution (KHs) for immediate aorta cannulation through a glass cannula (Angelone et al., 2012b). Perfusion was performed at constant flow by using a Krebs-Henseleit buffer (KHS) containing (in millimolar) KCl 4.7, NaCl 113, $NaHCO_3$ 25, $CaCl_2$ 1.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, mannitol 1.1, glucose 11, Na-pyruvate 5 (pH 7.4; 37°C; 95% O_2-5% CO_2). A water-filled latex balloon was connected to a pressure transducer (BLPR; WRI, Inc., Saratota, FL) and inserted into the LV through the mitral valve, to record cardiac mechanical parameters. Another pressure transducer was located above the aorta to measure coronary pressure (CP). The developed left ventricular pressure (LVDevP; mmHg, index of contractile activity) and the left ventricular end diastolic pressure (LVEDP; mmHg, index of contracture) were measured to evaluate inotropism (Angelone et al., 2012b).

Cardiac performance was recorded by using PowerLab data acquisition system. Parameters were analyzed by using Chart software (ADInstruments, Oxford-UK), as described in Angelone et al., (2012b).

Experimental protocols

After 40 min of stabilization, WKY and SHR heart were subjected to ischemia at zero flow for 30 min, and subsequently, to 120 min of reperfusion (I/R Groups hereafter named Control)

WKY Experiments (Groups 1-4)—In order to have a reference group for I/R (Control) and pGlu-Serp effects, hearts from normotensive WKY animals (n=6 for each group) were divided in three groups (WKY_ Control, WKY_pGlu-Serp-Pre and WKY_pGlu-Serp-Post; Group 1, Group 2 and Group 3, respectively). In WKY_ Control, the hearts were subjected to ischemia for 30min and subsequent 120 min reperfusion. In WKY_pGlu-Serp-Pre, pGlu-Serp (75 nM) was infused for 20 min before 30 min of global ischemia and 120 min reperfusion (Penna et al. 2005). In WKY_pGlu-Serp-Post, pGlu-Serp (75 nM) was infused for 20 min at the beginning of 120-min reperfusion (Penna et al., 2012). In Group 4 (WKY_Sham, n=6), WKY hearts underwent only to 190 min of perfusion.

SHR Experiments (Groups 5-8)—SHR hearts were divided, after the stabilization, in four groups: hearts which underwent only to 190 min perfusion (Group 5 SHR_Sham, n=6); SHR_ Control (Group 6, n=6) in which hearts were subjected to ischemia for 30min and subsequent 120 min reperfusion; SHR_ pGlu-Serp-Pre (Group 7, n=6) and SHR_pGlu-Serp-Post (Group 8, n=6), in which pGlu-Serp (75 nM) was infused for 20 min either before or after 30 min of global ischemia and subsequent reperfusion, similar to WKY groups and 120 min reperfusion (Penna et al., 2012). Experiments were performed by using a peptide concentration (75 nM) able to elicit significant cardiac effects (Tota et al., 2012).

RISK pathway (Groups 9-14)—The action of pGlu-Serp-Post in WKY and SHR hearts was studied in the presence of specific inhibitors of pivotal elements in cardioprotection, such as PI3K/Akt, PKC and the mitochondrial potassium ATP ($MitoK_{ATP}$) channel (n=4 for each groups). Inhibitors were infused 5 min before and 20 min after ischemia (Penna et al., 2012).

Groups 9-10: inhibition of PI3K by Wortmannin (WT, 10^{-7}M; SHR_pGlu-Serp-Post+WT or WKY_pGlu-Serp-Post+WT, Groups 9 and 10, respectively) (Penna et al., 2012).

Groups 11-12: inhibition of PKC by chelerythrine (Chel, 5μM; SHR_pGlu-Serp-Post+CHE or WKY_pGlu-Serp-Post+CHE Group 11 and 12 respectively) (Perrelli et al., 2013).

Groups 13-14: inhibition of $MitoK_{ATP}$ channels by 5-hydroxydecanoate (5HD, 10μM; SHR_pGlu-Serp-Post+5HD or WKY_pGlu-Serp-Post+5HD Group 13 and 14 respectively).

Inhibitors alone at these concentrations did not affect I/R damages (Cappello et al., 2007). In all experiments, antagonist concentration was selected on the basis of previous reports (Penna et al., 2012; Perrelli et al., 2013).

Infarct size

Infarct area was assessed at the end of 120 min reperfusion as previously described (Pagliaro et al., 2003; Penna et al., 2009) and the necrotic mass was expressed as a percentage of total

LV mass which was considered as risk area. 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 not aware of the nature of the intervention.

H9c2 experiments

To verify whether or not pGlu-Serp may induce a direct protection on cardiac cells, we studied pGlu-Serp protective effects on H9c2 rat embryonic cardiac myoblasts (ATCC) cells. Commercially available H9c2 cells were grown in Dulbecco's modified Eagle's medium F-12 HAM (F-12 HAM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (100IU/ml)/streptomycin (100 μg/ml) (Wisent Inc, Quebec, Canada) at 37°C, 5% CO_2. Cells were seeded at a density of 5.0×103 cell/well in 96-well plates, incubated overnight, starved for 24h serum free and treated or not with pGlu-Serp (75 nM) for 24 hours in 1%FBS fresh medium. After the pretreatment, simulated ischemia/reperfusion was achieved by culturing the cells in 1% FBS in F-12 HAM in a hypoxic chamber, followed by reoxygenation (3 h) using 1% FBS DMEM (see below) in a hypoxic chamber (INVIVO2 200, Belsar, Varese, Italy) (Penna et al., 2013b).

Normoxic and hypoxic experimental conditions—Cell survival was studied under Normoxia: i.e. standard conditions (normoxic: 21% O_2, 5% CO_2). The effects of Hypoxia/Reoxygenation (H/R) were studied by exposing the cells to hypoxic mixture (3% O_2, 5% CO_2) for 72 h and subsequent reoxygenation (normoxic: 21% O_2, 5% CO_2) for 3h. The experimental groups are: Groups A-B: cells in F-12 HAM in normoxia (H9c2_N) or treated with pGlu-Serp (75 nM) in normoxia (H9c2_N+pGlu-Serp); Groups C-D: cells subjected to H/R without pGlu-Serp (H9c2_H) or cell pretreated with pGlu-Serp and then subjected to H/R (H9c2_H+pGlu-Serp). Group E: cells after hypoxia (Boccalini et al., 2015) are treated with pGlu-Serp (75 nM) for all reoxygenation period (H9c2_H+ pGlu-Serp-Post).

Cell survival—At the end of experiments cell survival was assessed by using the cell viability test 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit (Sigma-Aldrich, St Louis, MO-USA) (Penna et al., 2013b). The absorbance was measured at 570 nm using a microplate reader and the results were expressed as percentage of control.

Drugs

pGlu-Serp (pGlu23Leu) was custom synthesized by Phoenix Pharmaceuticals, Inc., (Burlingame, CA, USA). Chelerythrine (Chel), 5-Hydroxydecanoate (5HD) and Wortmannin (WT) were purchased from Sigma Aldrich (St. Louis, MO-USA). All drug-containing solutions were freshly prepared before experimentation.

Statistics

All data are expressed as means±SEM. One-way ANOVA and Bonferroni multiple comparison test (for post-ANOVA comparisons) have been used when appropriate (Graphpad Prism). A p value <0.05 was considered statistically significant.

Results

Blood pressure (BP), measured before each experiment was: WKY: Systolic BP = 118±5 mmHg and Diastolic BP=88±3 mmHg; SHR: Systolic BP=201±5 mmHg and Diastolic BP=151±4 mmHg. Heart weights were: WKY: 1.53±0.03 g; SHR: 2.73±0.05 g ($p<0.05$).

Serp-Pre Limits I/R Injury in WKY and SHR hearts

Systolic function in the post-ischemic phase is represented by the value of LVDevP recovery (i.e., inotropic activity). As shown in Fig.2, in groups treated with pGlu-Serp before ischemia (WKY-pGlu-Serp-Pre and SHR-PGlu-Serp-Pre), at the end of reperfusion LVDevP resulted significantly higher in SHR_PGlu-Serp-Pre (142.5±9.5 mmHg) as compared to SHR_ Control (26.1±6.5 mmHg). In contrast, in WKY_pGlu-Serp-Pre LVDevP was significantly different from that of WKY_ Control (Fig 2a) at the first time and the last time of reperfusion, 0-40 min and 100-120 min respectively. Diastolic function in the post-ischemic phase is represented by the LVEDP value (i.e., contracture state). In our experiments, contracture was evaluated only after ischemia, although during ischemia an increase of contracture was observed, as reported by Dunay et al. (2015). pGlu-Serp infusion before ischemia significantly reduced LVEDP in SHR_pGlu-Serp-Pre group compared to the respective Control counterpart ($p<0.05$ vs. SHR_Control). In contrast, with respect to Control, in WKY_pGlu-Serp-Pre, a significant reduction of LVEDP was observed starting from 90 min of reperfusion ($p<0.05$ vs. WKY- Control) (Fig. 2b). In addition, at the end of reperfusion we did not observe significant modifications in coronary pressure, compared with the values observed during the stabilization period (data not shown).

H9c2 pre-treatment with pGlu-Serp protects against H/R induced cell death

H9c2 rat embryonic cardiac myoblasts, pre-treated for 24-hrs with pGlu-Serp, were subsequently exposed to H/R (72-hrs 3% O_2 and 3-hrs of reoxygenation) and Post-hypoxic vitality determined with MTT assay. The analysis revealed that pre-treatment with pGlu-Serp protects against H/R. In fact, survival was 75±2% in untreated cells with respect to 89±4% in pGlu-Serp pre-treated ($p<0.05$ vs. H9c2_H) (Fig. 3). Non significant differences were obtained in H9c2_H+pGlu-Serp-Post group (79±6%) with respect to H9c2_H group (p=NS vs. H9c2_H).

PGlu-Serp-Post limits I/R Injury in WKY and SHR hearts

Systolic function: as shown in Fig.4a, pGlu-Serp-Post significantly increased LVDevP in both WKY (75±4 mmHg) and SHR (68.5±4 mmHg) with respect to their Control counterparts (18.5±4 mmHg and 21.8±7 mmHg, respectively). Diastolic function: as documented by LVEDP values, pGlu-Serp-Post significantly reduced contracture in both WKY and SHR hearts ($p<0.05$) with respect to their corresponding Control counterparts (Fig. 4b). In addition, at the end of reperfusion we did not observe significant modifications in coronary pressure, compared with the values observed during the stabilization period (data not shown).

Infarct size in WKY and SHR hearts

The risk area was similar in untreated and pGlu-Serp treated hearts (data not shown). Using WKY hearts (Fig. 5a) as a normal counterpart of SHR hearts (Fig. 5b) the effects of pGlu-Serp-Pre was evaluated on IS (expressed as a percentage of risk area). IS in Control group of SHR hearts was larger (78±3% of risk area) compared to WKY group (69.7±11% of risk area). pGlu-Serp-Pre significantly reduced IS in both WKY and SHR hearts, the reduction being 51±2.5% and 50±3.7%, respectively ($p<0.05$ vs. WKY_ Control and $p<0.05$ vs. SHR_ Control). Similarly, pGlu-Serp-Post induced a significant protection in both normotensive and hypertensive hearts, the IS being in WKY_ Control and SHR_ Control 40±3.6% and 33±2.5%, respectively ($p<0.05$ vs. WKY_ Control and $p<0.05$ vs. SHR_ Control) (Fig. 5a,b). These results were paralleled by the profile of LDH release in WKY-pGlu-Serp-Pre and -Post, as well as in the SHR-pGlu-Serp-Pre and -Post, respectively (data not shown).

The systolic recovery of pGlu-Serp-Pre and pGlu-Serp-Post rats was compared by two way ANOVA, followed by Bonferroni multiple comparison test. Results showed that the systolic function in WKY-pGlu-Serp-Post was significantly higher than in WKY-pGlu-Serp-Pre (Interaction between pre and post treatment=4.72%, $p=0.0146$). In contrast, it is higher in SHR-pGlu-Serp-Pre than SHR-pGlu-Serp-Post (Interaction between pre and post treatment=9.22%, $p=0.0001$).

Compared to that exerted by pGlu-Serp-Pre, the cardioprotective influence of pGlu-Serp-Post on Infarct Size appeared more relevant with respect to I/R alone under both normotensive and hypertensive conditions. Therefore, we evaluated in the latter some of the putative mechanisms involved.

pGlu-Serp-Post cardioprotective pathways and anti-apoptotic properties

Inhibitors (Fig. 6): in both WKY and SHR hearts, co-infusion of pGlu-Serp with either WT (PI3K inhibitor), or Chel (PKC inhibitor) blocked the pGlu-Serp-Post-elicited protective effects on LVDevP and LVEDP (Fig. 6 a,b). Similarly, in both WKY and SHR hearts, infusion with 5HD, an inhibitor of MitoK_{ATP} channel, abolished the protective effect of pGlu-Serp-Post on LVDevP and LVEDP (Fig. 6 a,b). The effects of the different inhibitors on IS were similar in both WKY and SHR hearts (Fig. 7 a,b). Inhibitors alone induced similar effects on the systolic function, the development of contracture, and IS as those observed in I/R group (data not shown).

DISCUSSION

In this work we used pre- and post-conditioning protocols in normotensive and hypertensive rat heart models to validate, with an unbiased evidence, the hypothesis that pGlu-Serp, the CgA-derived C-terminal peptide, is a cardioprotective modulator. The spontaneous (genetically) hypertensive SHR rat represents one of the most used animal models for studying cardiac vulnerability (e.g. myocardial ischemia and oxidative stress) associated with the hypertensive condition (Wu & Juurlink, 2002). Although its pathogenesis is complex, human hypertension also displays signs of increased oxidative stress associated

with a decreased antioxidant activity and a reduced ability to scavenge oxygen derived free radicals (McIntyre et al., 1999).

We here demonstrated that, in both WKY and SHR hearts, pGlu-Serp induces cardioprotection in PreC and, especially, in PostC providing also evidence for a hypertension-induced defect in the protective efficacy of PostC which was overcame by pGlu-Serp administration at reperfusion. Our results, together with the findings on pGlu-Serp effects in *in vitro* cardiomyocytes, strongly support the pGlu-Serp role in I/R cardioprotection with a direct action on myocardial cells.

Cardioprotective profile of pGlu-Serp

In all experiments in which the Langendorff perfused WKY and SHR hearts were subjected to I/R there was a good correlation among systolic function, contracture development, and IS. This is noteworthy in view of the recognized importance of evaluating myocardial protection as a whole, including not only the improvement of mechanical recovery during reperfusion, but also the limitation of IS and contracture (Penna et al., 2009; Skyschally et al., 2009). The Langendorff perfused heart remains the most widely used technique to probe pathophysiology of ischaemia/reperfusion and disease states and it represents a stalwart tool in the study of the impact upon the physiology of the heart by pharmacological inhibitors and their impact upon intracellular signalling and adaption to clinically relevant stressful stimuli (Bell et al., 2011).

We found that pGlu-Serp differently elicited protection if administered either in PreC or in PostC. Interestingly, in PreC pGlu-Serp appeared more active under the hypertensive condition on the systolic recovery, while its influence on contracture is similar in SHR and WKY. Opposite to this behavior, pGlu-Serp-PostC cardioprotective effects appear more potent in SHR than in WKY. This is shown by the greater post-ischemic recovery of LVDevP observed in WKY than in SHR. In both strains, pGlu-Serp-Post reduces contracture. Notably, the striking PostC protection elicited by the peptide is documented by the significant IS reduction observed in both WKY and SHR. Of note, Penna et al. (2010a) reported that, while ischemic PostC elicits cardioprotection by reducing IS in normotensive WKY, it is not protective in SHR. In line with these data, we found a larger IS in SHR than in WKY hearts. The protection exerted by pGlu-Serp on the ischemic rat heart is in line with the *in vitro* report of Koshimizu et al. (2011) on a cellular cerebral model exposed to oxidative stress. Of note, survival observed in hypoxic H9c2 pre-treated with pGlu-Serp suggested that the cardioprotection elicited by the peptide occurs through a direct action on cardiomyocytes. However, in the absence of more specific observations, the possibility that the effect of pGlu-Serp increasing cell survival following hypoxia/reoxygenation by a reduction of cell death or by other mechanisms, including an increase on H9c2 proliferation, cannot be excluded. We observed also a non-significant protection in H9c2 exposed to pGlu-Serp at the beginning of re-oxygenation. We do not have a definitive explanation for this apparent discrepancy. It may be due to several reasons, including the fact that H9c2 are proliferating cells with signal transduction pathway that might affect their response to cardioprotective stimuli (Lecour et al., 2014). We should also consider that, while in unstressed models (i.e. in pre-treatment) serpinin triggers protective intracellular

mechanisms in both the heart and the isolated cells, in stressed models the protective cascades can be activated after ischemia/reperfusion in *ex vivo* heart, but not in cells subjected to hypoxia/reoxygenation, which is a different damaging stimulus. All these features could explain the different behavior showed by pGlu-Serp on H9c2 and on the *ex vivo* heart. Therefore, these preliminary results on cell line, confirm that one of the primary targets of pGlu-Serp are the cardiomyocytes, but opened a perspective for a future follow-up study to further ascertain the differences in post-ischemic/hypoxic conditions.

We have previously shown cardioprotective effects for the two cardioactive CgA-derived peptides, VS-1 and CST, the former exerting PreC-like protective effects (Cappello et al., 2007) and the latter acting as PostC-like protective agent (Tota et al., 2014). Yet, we were unable to show PreC effects for CST and PostC effects for VS-1. Therefore, compared to VS-1 and CST, pGlu-Serp at the same concentrations of the other two peptides, appears to possess a wider spectrum of cardioprotection. These concentrations are indeed below those of plasma CgA found in coronary syndromes and heart failure (Angelone et al., 2012a). This expands the modulatory role exerted by CgA in the control of the murine heart under physiological and physiopathological conditions (Angelone et al., 2012a; Tota et al., 2014). As reported by Pasqua et al (2013) intracardiac CgA is proteolytically processed to generate short fragments (i.e. VS-1) after exposure to stress challenges, such as adrenergic and endothelinergic stimulation (Pasqua et al., 2013). This suggests that under physiopathologic conditions CgA-derived peptides may induce beneficial effects on the heart. Whether and to which extent the I/R-induced stress may enhance the intracardiac proteolytic generation of pGlu-Serp is an issue under study in our lab.

Antiapoptotic signaling mechanisms & mitochondrial preservation

To obtain a mechanistic insight on the peptide cardioprotection, we exposed the heart of pGlu-Serp-PostC group to pharmacological inhibitors of either RISK or apoptotic pathways. The finding that pGlu-Serp-PostC protective effects were abolished by co-infusion with specific RISK inhibitors (PI3K/AkT, MitoK_{ATP} channels, and PKC) suggests the implication of this pathway. In our whole heart model, the protective effect is abolished by blocking PKC or mitoK_{ATP} channels with CHE or 5-HD, respectively. It is known that mitoK_{ATP} channel opening, redox signaling and PKC activation are involved in pro-survival signaling cascade (Gao et al., 2013; Thuc et al., 2010). Intriguingly, the free radical scavenger NAC attenuated cardioprotection by isoproterenol when administered during both trigger and mediation phases of preconditioning (Salie et al., 2012). Of note, an anti-apototic effect correlated with mitok$_{ATP}$ channel-redox signaling and PKC activation has also been reported for other CgA fragments (Perrelli et al., 2013; Tota et al., 2014). These data reinforce the possibility that a link exists between an upstream β1-like pGlu-Serp stimulus and downstream protective cascades. Although further work is needed to define this issue, it is pertinent to consider that Serpinin signals through β1-Adrenergic Receptor(AR)/ Adenylate Cyclase/cAMP/PKA pathway with consequent activation of ERK1/2 and GSK3β which are components of the I/R injury-elicited RISK cardioprotective cascade (Salie et al., 2012; Hausenloy et al., 2011).

Conclusions and Perspectives

This new evidence on the cardioprotective profile of the CgA-derived pGlu-Serp widens the spectrum of the SAN activities exerted by CgA and its derived peptides on cardiovascular homeostasis. It also reveals a distinct and relevant influence of pGlu-Serp in PostC. Although indispensable to salvage infarcted myocardium, reperfusion can pose risk to it, since may paradoxically lead to cardiomyocyte death, a phenomenon termed lethal reperfusion-induced cell injury (Hausenloy et al., 2011). Our data strongly suggest pGlu-Serp as a potential therapeutic agent to be tested in mechanistically oriented studies aimed to deepen the cardioprotective processes under ischemic conditions.

Acknowledgements

Thanks are given to Dr. Stefano Gentile for his technical contribution to preconditioning protocols.

Fundings

This work was supported by the MIUR of Italy (Programma di Ricerca Scientifica di Interesse Nazionale), “Programma ARUE - POR Calabria - FSE 2007/2013 Asse IV” and in part by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Institutes of Health, USA.

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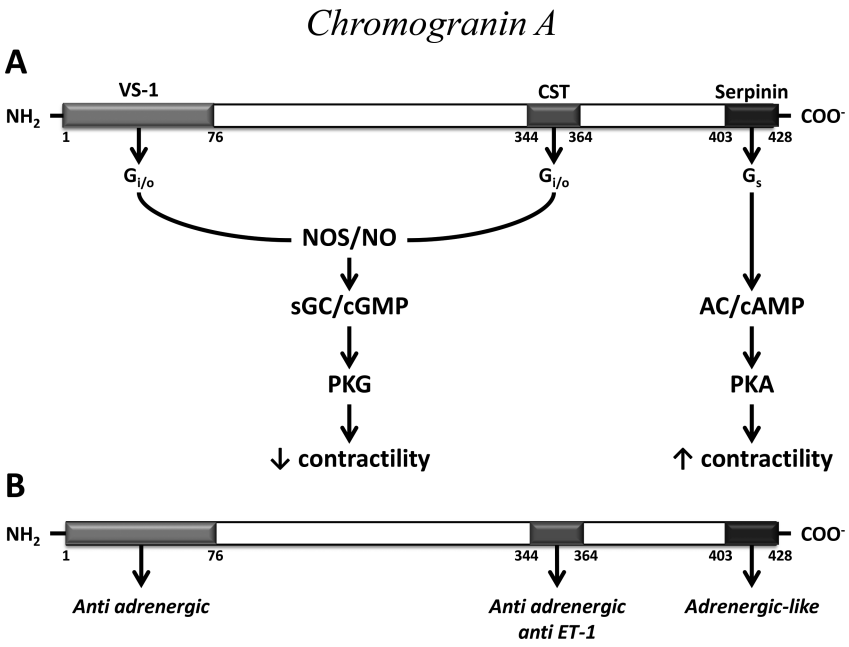

Fig 1.

Synopsis of the cardiac modulation elicited by CgA and and its derived fragments VS-1, CST and Serpinin. In both A and B the white bar represents the precursor CgA and the N- and C-terminal fragments VS-1, CST, and Serpinin which are indicated as dark boxes. The involvement of G proteins and of the NO-cGMP-PKG pathway in the reduction of contractility induced by VS-1 and CST, and of the cAMP-PKA cascade in the increased contractility induced by Serpinin are reported in A. B illustrates the effects induced by VS-1 and CST under sympatho-adrenergic stimuli, and the adrenergic-like effect induced by Serpinin.

Abbreviations. NOS: Nitric Oxide Synthase; NO: Nitric Oxide; sGC: soluble Guanylate Cyclase; cGMP: cyclic GMP; PKG: cGMP-dependent Protein Kinase; AC: Adenylate Cyclase; cAMP: cyclic AMP; PKA: cAMP-dependent Protein Kinase.

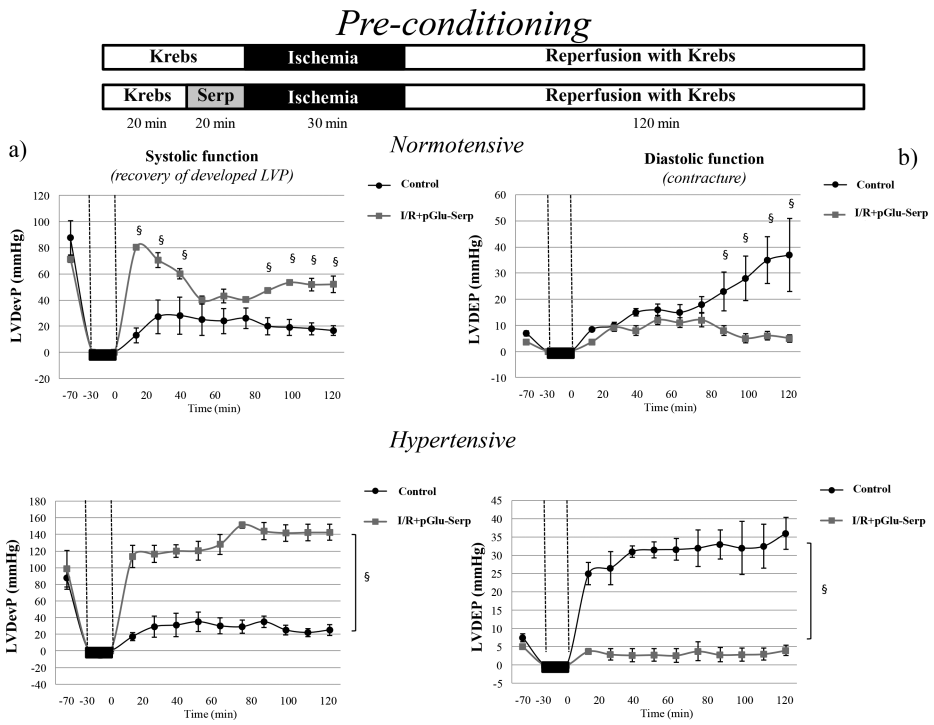

Fig 2. pGlu-Serp-PreC protocols

LVDevP (A) and LVEDP (B) variations in WKY and SHR rats. Data are expressed as changes of LVDevP 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 (n=6 for each group). Vertical lines indicate ischemic administration. Comparison between groups followed by Bonferroni Multiple Comparison test: §<0.05

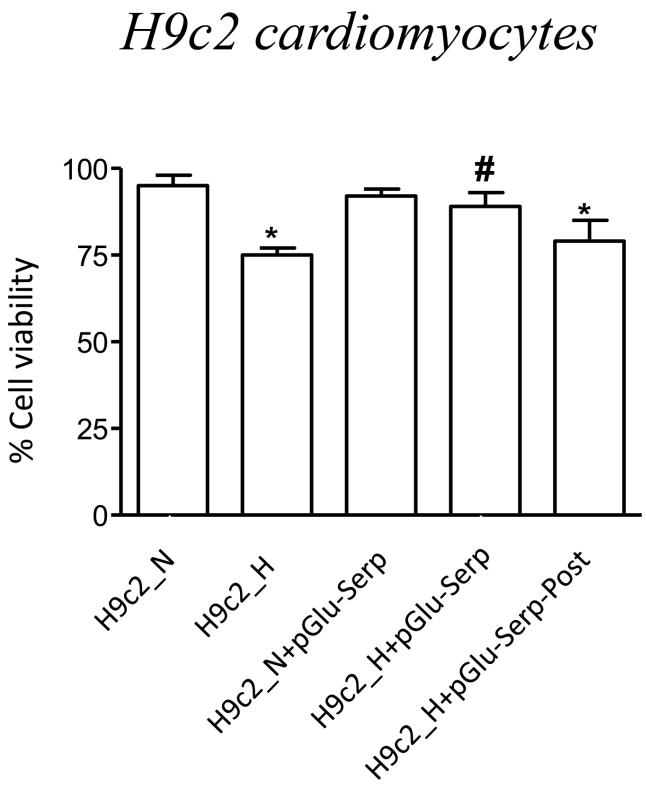

Fig 3. Cell survival in hypoxia(H)/reoxigenation protocol (72-hrs 1% O2 and 3-hrs of reoxygenation)

Post-hypoxic vitality was determined by MTT assay. Group A (H9c2_N: Normoxia) and B (H9c2_H: Hypoxia): cells subjected to H/R without pGlu-Serp; Group C (H9c2_N+ pGlu-Serp) and D (H9c2_H+ pGlu-Serp): cells pre-treated for 24-hrs with pGlu-Serp were subsequently exposed to H/R; Group E (H9c2_H+ pGlu-Serp-Post): after hypoxia cells were treated with pGlu-Serp (75 nM) throughout reoxygenation. n=5; * $p<0.05$ vs Group A, # $p<0.05$ vs Group B. Comparison between groups followed by Bonferroni Multiple Comparison test

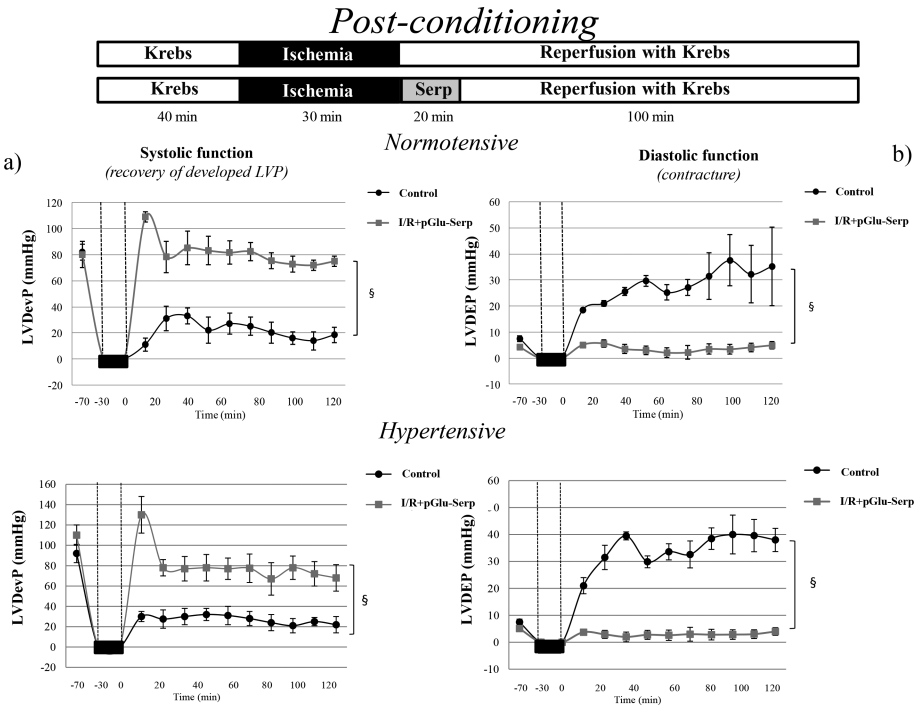

Fig 4. pGlu-Serp-PostC protocols

LVDevP (A) and LVEDP (B) variations in WKY and SHR rats. Data are expressed as changes of LVDevP 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 (n=6 for each group). Vertical lines indicate ischemic administration. Comparison between groups followed by Bonferroni Multiple Comparison test: §<0.05

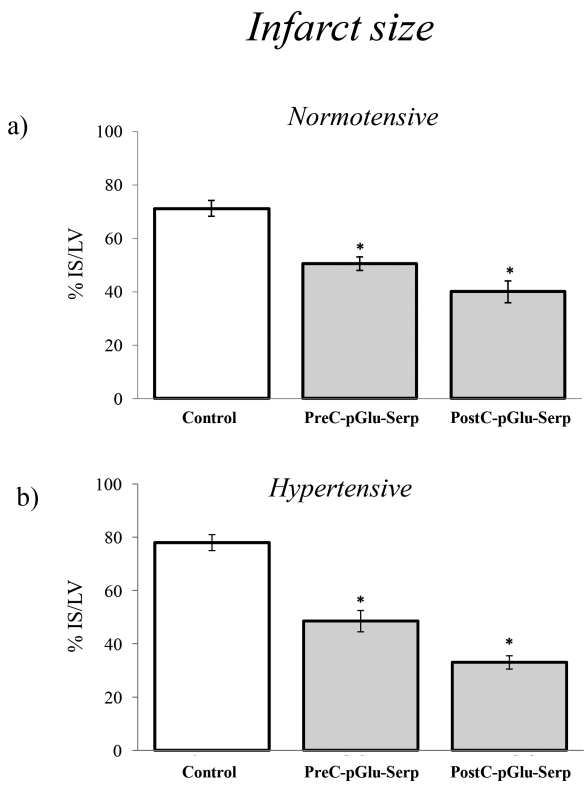

Fig 5. Infarct size

The amount of necrotic tissue measured after 30-min global ischaemia and 120-min reperfusion is expressed as percent of the left ventricle (% IS/LV) in both WKY and SHR hearts (n=6 for each group). **p<0.001 with respect to I/R and each antagonist group. Comparison between groups followed by Bonferroni Multiple Comparison test.

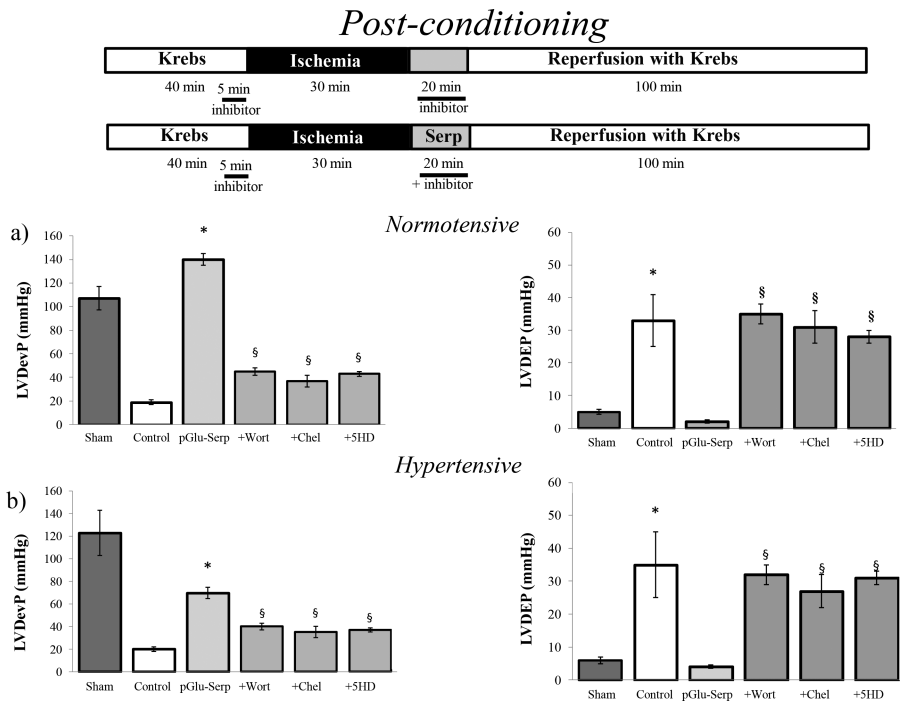

Fig 6. RISK pathway signalling in postconditioning

LVDevP and LVEDP variations of sham, I/R, and pGlu-Serp-PostC plus inhibitors groups (WT:100 nM, an PI3K inhibitor; Chel:100 nM, a protein kinase C epsilon inhibitor; 5HD:10 μM, a mitoKATP channels blocker). Data are expressed as changes of LVDevP 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 (n=4 for each group). Significance of difference (one-way ANOVA): *p<0.05 Comparison between I/R groups *vs* pGlu-Serp-PostC; §p<0.05 Comparison between groups followed by Bonferroni Multiple Comparison test.

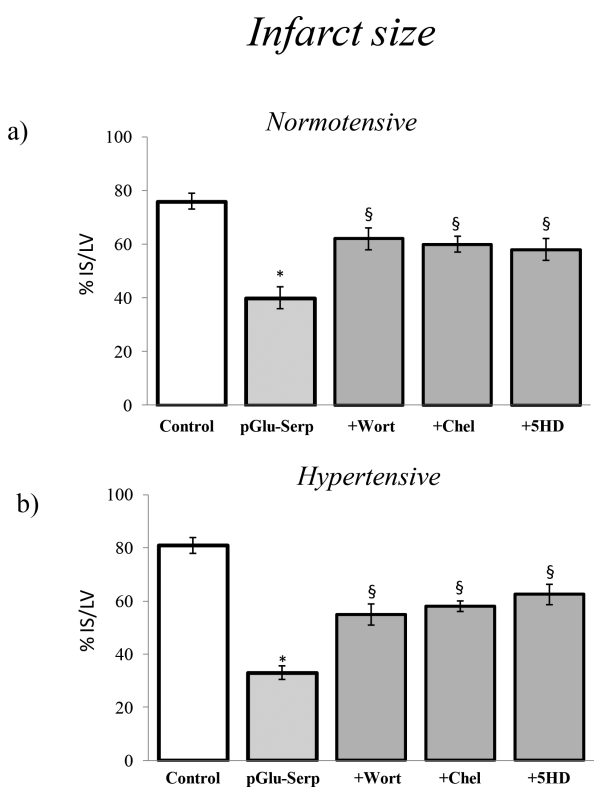

Fig 7. RISK pathway signalling in postconditioning

Infart Size (IS) variations of sham, I/R, and pGlu-Serp-PostC plus inhibitors groups (WT: 100 nM, an PI3K inhibitor; Chel:100 nM, a protein kinase C epsilon inhibitor; 5HD:10 µM, a mitoKATP channels blocker). Data are expressed as changes of IF (%) from the stabilization to the end of the 120-min of reperfusion with respect to the baseline values for each group (n=4 for each group). Significance of difference (one-way ANOVA): *p<0.05 Comparison between I/R groups *vs* pGlu-Serp-PostC; §p<0.05 Comparison between groups followed by Bonferroni Multiple Comparison test