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

T Pasqua*, B Tota*, C Penna¹, A Corti², M C Cerra, Y P Loh³ and T Angelone

Laboratory of Cardiovascular Pathophysiology, Department of Biology, Ecology, and E.S., University of Calabria, 87036 Arcavacata di Rende (CS), Italy

¹Department of Clinical and Biological Sciences, University of Turin, Turin, Italy

²Tumor Biology and Vascular Targeting Unit, Division of Molecular Oncology, San Raffaele Scientific Institute, Milan, Italy

³Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, USA

*T Pasqua and B Tota contributed equally to this work

Correspondence should be addressed to Y P Loh or T Angelone

Emails

lohp@mail.nih.gov or tommaso.angelone@unical.it

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 β -adrenergic-like modulators, acting through β 1-AR/AC/cAMP/PKA pathway. Because 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 (Wistar Kyoto, 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 PostC. pGlu-Serp administered at the reperfusion (Serp-PostC) significantly reduced IS, being more protective in SHR than in WKY. Conversely, left ventricular developed 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 cells 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 that can limit infarct size and overcome the hypertension-induced failure of PostC.

Key Words

- ▶ serpinin
- ▶ Chromogranin A-derived peptides
- ▶ hypertension
- ▶ rat heart

Journal of Endocrinology
(2015) 227, 167–178

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 cardiocirculatory homeostasis, full-length CgA (Pasqua *et al.* 2013) and three CgA-derived peptides, vasostatin 1 (VS-1) (hCgA1-76), catestatin (CST) (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) (Koshimizu *et al.* 2010, 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 β -adrenergic-like inotropes through a NO-independent β 1-adrenergic receptor/adenylate cyclase/cAMP/PKA pathway (Tota *et al.* 2012). The serpinin-PKA

pathway also induces phosphorylation of ERK1/2 and GSK3 β (Tota *et al.* 2012). These proteins mediate prostaglandin E₂ and prostanoid receptor signaling in neonatal ventricular myocytes and are components of the protective reperfusion injury signaling kinase (RISK) 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 β -adrenergic preconditioning (Salie *et al.* 2012). In fact, peptides, such as growth hormone-releasing hormone (GHRH), that induce cardioprotection during either pre- or post-ischemic phases activate ERK and PI3K/PKB/Akt pathways reducing apoptosis through β -adrenergic stimulation (Granata *et al.* 2009, Penna *et al.* 2013a). Therefore, a cross-talk between β -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 (Wistar Kyoto, WKY) and spontaneously hypertensive (SHR) rats, we studied the effects of pGlu-Serp on infarct size (IS) and cardiac function, i.e., developed left ventricular pressure and post-ischemic contracture (left ventricle end diastolic pressure) in ischemic conditioning administered before (i.e., preconditioning) or onset (i.e., 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 (Zhao *et al.* 2003, Ferdinandy *et al.* 2014). 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,b).

Figure 1

Synopsis of the cardiac modulation elicited by CgA 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. (A) 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. (B) Illustrates the effects induced by VS-1 and CST under sympatho-adrenergic stimuli and the adrenergic-like effect induced by serpinin. NOS, nitric oxide synthase; NO, nitric oxide; sGC, soluble guanylate cyclase; cGMP, cyclic GMP; PKG, cGMP-dependent protein kinase; AC, adenylate cyclase; PKA, cAMP-dependent protein kinase.

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 SHR rats (250–300 g) (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. The tail cuff method was used for daily measures of systolic pressure. The tail cuff was connected to a pneumatic pulse transducer and a programmed electro-sphygmomanometer (BP-2000 series II; blood pressure analysis system, Visitech System, Apex, NC, USA).

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 for immediate aorta cannulation through a glass cannula (Angelone *et al.* 2012b). Perfusion was performed at constant flow by using a Krebs-Henseleit buffer containing (in millimolar) KCl 4.7, NaCl 113, NaHCO₃ 25, CaCl₂ 1.8, MgSO₄ 1.2, KH₂PO₄ 1.2, mannitol 1.1, glucose 11 and Na-pyruvate 5 (pH 7.4; 37 °C; 95% O₂–5% CO₂). A water-filled latex balloon was connected to a pressure transducer (BLPR, WRI, Inc., Sarasota, FL, USA) and inserted into the left ventricle (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 the 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 hearts 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)

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 30 min and a subsequent 120 min of 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 190 min of perfusion.

SHR experiments (groups 5–8)

SHR hearts were divided, after the stabilization, into four groups: SHR_Sham (group 5, $n=6$) in which hearts underwent only 190 min perfusion; SHR_Control (group 6, $n=6$) in which hearts were subjected to ischemia for 30 min and a 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 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, 100 nM; 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, 5 μ M; SHR_pGlu-Serp-Post+CHE or WKY_pGlu-Serp-Post+CHE, groups 11 and 12 respectively) (Perrelli *et al.* 2013).

Groups 13–14: inhibition of MitoK_{ATP} channels by 5-hydroxydecanoate (5HD, 10 μ M; SHR_pGlu-Serp-Post+5HD or WKY_pGlu-Serp-Post+5HD, groups 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 (100 IU/ml)/streptomycin (100 μ g/ml) (Wisent, Inc., St Bruno, Quebec, Canada) at 37 °C, 5% CO₂. Cells were seeded at a density of 5.0×10^3 cell/well in 96-well plates, incubated overnight, starved for 24 h serum free and treated or not with pGlu-Serp (75 nM) for 24 h 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% O₂, 5% CO₂). The effects of hypoxia/reoxygenation (H/R) were studied by exposing the cells to a hypoxic mixture (3% O₂, 5% CO₂) for 72 h and subsequent reoxygenation (normoxic: 21% O₂, 5% CO₂) for 3 h. The experimental groups are groups A

and 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 and 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); and 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 the 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) (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). Chel, 5HD and WT were purchased from Sigma-Aldrich. All drug-containing solutions were freshly prepared before experimentation.

Statistical analysis

All data are expressed as means $\pm$ S.E.M. One-way ANOVA and Bonferroni multiple comparison test (for post-ANOVA comparisons) have been used when appropriate (GraphPad Prism, San Diego, CA, USA). A *P* value <0.05 was considered statistically significant.

Results

Blood pressure (BP), measured before each experiment was as follows: 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 and 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), LVDevP resulted significantly higher in SHR_pGlu-Serp-Pre (142.5 ± 9.5 mmHg) at the end of reperfusion

Figure 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

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 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

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: $\$ < 0.05$.

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 h with pGlu-Serp, were subsequently exposed to H/R (72 h

3% O₂ and 3 h of reoxygenation) and post-hypoxic vitality was 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). Nonsignificant differences were obtained in the H9c2_H+pGlu-Serp-Post group (79%±6%) with respect to the 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 the control group of SHR hearts was larger (78±3% of risk area) compared to the 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 and B). These results were paralleled by the profile of lactate dehydrogenase (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 the 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 IS 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 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. 6A and B). Similarly, in both WKY and SHR hearts, infusion with 5HD, an inhibitor of the MitoK_{ATP} channel, abolished the

Figure 3

Cell survival in hypoxia(H)/re-oxygenation protocol (72 h 1% O₂ and 3 h of re-oxygenation). 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; groups C (H9c2_N+ pGlu-Serp) and D (H9c2_H+ pGlu-Serp): cells pre-treated for 24 h 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 re-oxygenation. $n=5$; * $P<0.05$ vs group A, # $P<0.05$ vs group B. Comparison between groups followed by Bonferroni multiple comparison test.

Figure 4

pGlu-Serp-PostC protocols: LVDevP (A) and LVDEP (B) variations in WKY and SHR rats. Data are expressed as changes of LVDevP and LVDEP values (mmHg) from the stabilization to the end of the 120 min of reperfusion

protective effect of pGlu-Serp-Post on LVDevP and LVDEP (Fig. 6A and B). The effects of the different inhibitors on IS were similar in both WKY and SHR hearts (Fig. 7A and 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

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: $\S < 0.05$.

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 demonstrated that, in both WKY and SHR hearts, pGlu-Serp induces cardioprotection in PreC and, especially, in PostC, also providing evidence for a

Figure 5

Infarct size. The amount of necrotic tissue measured after 30 min global ischemia and 120 min reperfusion is expressed as percent of the left ventricle (% IS/LV) in both WKY (A) and SHR (B) 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.

hypertension-induced defect in the protective efficacy of PostC, which was overcome 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 ischemia/reperfusion and disease states, and it represents a stalwart tool in the study of the impact on the physiology of the heart by pharmacological inhibitors and their impact on intracellular signaling 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, although 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 nonsignificant 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 cells are proliferating cells with signal transduction pathways 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, whereas 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 of these features could explain the different behavior showed by pGlu-Serp on H9c2 and on the *ex vivo* heart. Therefore, these preliminary results on

Figure 6

RISK pathway signaling in post-conditioning in normotensive (A) and hypertensive (B) hearts. LVDevP and LVDEP variations of sham, I/R and pGlu-Serp-PostC plus inhibitors groups (WT:100 nM, an PI3K inhibitor; Chel: 5 μ M, a protein kinase C epsilon inhibitor; 5HD:10 μ M, a MitoK_{ATP} channels blocker). Data are expressed as changes of LVDevP and LVDEP 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.

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 a 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 as 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

Figure 7

RISK pathway signaling in post-conditioning in normotensive (A) and hypertensive (B) hearts. Infarct size (IS) variations of sham, I/R and pGlu-Serp-PostC plus inhibitors groups (WT:100 nM, an PI3K inhibitor; Chel: 5 μ M, a protein kinase C epsilon inhibitor; 5HD:10 μ M, a MitoK_{ATP} channels blocker). Data are expressed as changes of IS (%) 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.

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.

Anti-apoptotic signaling mechanisms and 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 (Thuc *et al.* 2010, Gao *et al.* 2013). Intriguingly, the free radical scavenger N-acetylcysteine (NAC) attenuated cardioprotection by isoproterenol when administered during both trigger and mediation phases of pre-conditioning (Salie *et al.* 2012). Of note, an anti-apoptotic 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 β 1-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 β 1-adrenergic receptor/adenylate cyclase/cAMP/PKA pathway with consequent activation of ERK1/2 and GSK3 β 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 a risk to it, because it 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.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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.

Acknowledgements

Thanks are given to Dr Stefano Gentile for his technical contribution to pre-conditioning protocols.

References

- Angelone T, Mazza R & Cerra MC 2012a Chromogranin-A: a multifaceted cardiovascular role in health and disease. *Current Medicinal Chemistry* **24** 4042–4050. (doi:10.2174/092986712802430009)
- Angelone T, Quintieri AM, Pasqua T, Gentile S, Tota B, Mahata SK & Cerra MC 2012b Phosphodiesterase type-2 and NO-dependent S-Nitrosylation mediate the cardioinhibition of the anti-hypertensive Catestatin. *American Journal of Physiology. Heart and Circulatory Physiology* **302** H431–H442. (doi:10.1152/ajpheart.00491.2011)
- Bartolomucci A, Possenti R, Mahata SK, Fischer-Colbrie R, Loh YP & Salton SR 2011 The extended granin family: structure, function, and biomedical implications. *Endocrine Reviews* **6** 755–797. (doi:10.1210/er.2010-0027)
- Bell RM, Mocanu MM & Yellon DM 2011 Retrograde heart perfusion: the Langendorff technique of isolated heart perfusion. *Journal of Molecular and Cellular Cardiology* **50** 940–950. (doi:10.1016/j.yjmcc.2011.02.018)
- Boccalini G, Sassoli C, Formigli L, Bani D & Nistri S 2015 Relaxin protects cardiac muscle cells from hypoxia/reoxygenation injury: involvement of the Notch-1 pathway. *FASEB Journal* **29** 239–249. (doi:10.1096/fj.14-254854)
- Cappello S, Angelone T, Tota B, Pagliaro P, Penna C, Rastaldo R, Corti A, Losano G & Cerra MC 2007 Human recombinant chromogranin A-derived vasostatin-1 mimics preconditioning via an adenosine/nitric oxide signaling mechanism. *American Journal of Physiology. Heart and Circulatory Physiology* **293** H719–H727. (doi:10.1152/ajpheart.01352.2006)
- Dunay GA, Paragi P, Sára L, Ács N, Balázs B, Ágoston V, Répás C, Ivanics T & Miklós Z 2015 Depressed calcium cycling contributes to lower ischemia tolerance in hearts of estrogen-deficient rats. *Menopause* **22** 773–782. (doi:10.1097/GME.0000000000000377)
- Ferdinandy P, Hausenloy DJ, Heusch G, Baxter GF & Schulz R 2014 Interaction of risk factors, comorbidities, and comedications with ischemia/reperfusion injury and cardioprotection by preconditioning, postconditioning, and remote conditioning. *Pharmacological Reviews* **66** 1142–1174. (doi:10.1124/pr.113.008300)
- Gao J, Wang A, Zhang M, Li H, Wang K, Han Y, Wang Z, Shi C & Wang W 2013 RNAi targeting of CCR2 gene expression induces apoptosis and inhibits the proliferation, migration, and invasion of PC-3M cells. *Oncology Research* **21** 73–82. (doi:10.3727/096504013X13775486749173)
- Granata R, Trovato L, Gallo MP, Destefanis S, Settanni F, Scarlatti F, Brero A, Ramella R, Volante M, Isgaard J *et al.* 2009 Growth hormone-releasing hormone promotes survival of cardiac myocytes *in vitro* and protects against ischaemia-reperfusion injury in rat heart. *Cardiovascular Research* **83** 303–312. (doi:10.1093/cvr/cvp090)
- Hausenloy DJ & Yellon DM 2004 New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. *Cardiovascular Research* **61** 448–460. (doi:10.1016/j.cardiores.2003.09.024)
- Hausenloy DJ, Lecour S & Yellon DM 2011 Reperfusion injury salvage kinase and survivor activating factor enhancement pro-survival signaling pathways in ischemic postconditioning: two sides of the same coin. *Antioxidants & Redox Signaling* **5** 893–907. (doi:10.1089/ars.2010.3360)
- Helle KB, Corti A, Metz-Boutigue MH & Tota B 2007 The endocrine role for chromogranin A: a prohormone for peptides with regulatory properties. *Cellular and Molecular Life Sciences* **22** 2863–2886. (doi:10.1007/s00018-007-7254-0)
- Koshimizu H, Kim T, Cawley NX & Loh YP 2010 Reprint of Chromogranin A: a new proposal for trafficking, processing and induction of granule biogenesis. *Regulatory Peptides* **1** 95–101. (doi:10.1016/j.regpep.2010.09.006)
- Koshimizu H, Cawley NX, Yergy AL & Loh YP 2011 Role of pGlu-serpinin, a novel chromogranin A-derived peptide in inhibition of cell death. *Journal of Molecular Neuroscience* **2** 294–303. (doi:10.1007/s12031-011-9521-7)
- Lecour S, Bøtker HE, Condorelli G, Davidson SM, Garcia-Dorado D, Engel FB, Ferdinandy P, Heusch G, Madonna R, Ovize M *et al.* 2014 ESC working group cellular biology of the heart: position paper: improving the preclinical assessment of novel cardioprotective therapies. *Cardiovascular Research* **104** 399–411. (doi:10.1093/cvr/cvu225)
- McIntyre M, Bohr DF & Dominiczak AF 1999 Endothelial function in hypertension: the role of superoxide anion. *Hypertension* **34** 539–545. (doi:10.1161/01.HYP.34.4.539)
- Pagliaro P, Mancardi D, Rastaldo R, Penna C, Gattullo D, Miranda KM, Feelisch M, Wink DA, Kass DA & Paolocci N 2003 Nitroxyl affords thiol-sensitive myocardial protective effects akin to early preconditioning. *Free Radical Biology & Medicine* **34** 33–43. (doi:10.1016/S0891-5849(02)01179-6)
- Pasqua T, Corti A, Gentile S, Pochini L, Bianco M, Metz-Boutigue MH, Cerra MC, Tota B & Angelone T 2013 Full-length human chromogranin-A cardioactivity: myocardial, coronary, and stimulus-induced processing evidence in normotensive and hypertensive male rat hearts. *Endocrinology* **9** 3353–3365. (doi:10.1210/en.2012-2210)
- Penna C, Alloatti G, HD S, Gattullo D, Berta G, Mognetti B, Losano G & Pagliaro P 2005 Platelet-activating factor induces cardioprotection in isolated rat heart akin to ischemic preconditioning: role of phosphoinositide 3-kinase and protein kinase C activation. *American Journal of Physiology. Heart and Circulatory Physiology* **288** H2512–H2520. (doi:10.1152/ajpheart.00599.2004)
- Penna C, Tullio F, Merlino A, Moro F, Raimondo S, Rastaldo R, Perrelli MG, Mancardi D & Pagliaro P 2009 Postconditioning cardioprotection against infarct size and post-ischemic systolic dysfunction is influenced by gender. *Basic Research in Cardiology* **104** 390–402. (doi:10.1007/s00395-008-0762-8)
- Penna C, Tullio F, Moro F, Folino A, Merlino A & Pagliaro P 2010a Effects of a protocol of ischemic postconditioning and/or captopril in hearts of normotensive and hypertensive rats. *Basic Research in Cardiology* **105** 181–192. (doi:10.1007/s00395-009-0075-6)
- Penna C, Alloatti G, Gallo MP, Cerra MC, Levi R, Tullio F, Bassino E, Dolgetta S, Mahata SK, Tota B *et al.* 2010b Catestatin improves post-ischemic left ventricular function and decreases ischemia/reperfusion injury in heart. *Cellular and Molecular Neurobiology* **30** 1171–1179. (doi:10.1007/s10571-010-9598-5)
- Penna C, Pasqua T, Perrelli MG, Pagliaro P, Cerra MC & Angelone T 2012 Postconditioning with glucagon like peptide-2 reduces ischemia/reperfusion injury in isolated rat hearts: role of survival kinases and mitochondrial KATP channels. *Basic Research in Cardiology* **107** 272–283. (doi:10.1007/s00395-012-0272-6)
- Penna C, Settanni F, Tullio F, Trovato L, Pagliaro P, Alloatti G, Ghigo E & Granata R 2013a GH-releasing hormone induces cardioprotection in isolated male rat heart via activation of RISK and SAFE pathways. *Endocrinology* **154** 1624–1635. (doi:10.1210/en.2012-2064)
- Penna C, Perrelli MG, Karam JP, Angotti C, Muscari C, Montero-Menei CN & Pagliaro P 2013b Pharmacologically active microcarriers influence VEGF-A effects on mesenchymal stem cell survival. *Journal of Cellular and Molecular Medicine* **17** 192–204. (doi:10.1111/j.1582-4934.2012.01662.x)
- Perrelli MG, Tullio F, Angotti C, Cerra MC, Angelone T, Tota B, Alloatti G, Penna C & Pagliaro P 2013 Catestatin reduces myocardial ischaemia/

- reperfusion injury: involvement of PI3K/Akt, PKCs, mitochondrial KATP channels and ROS signalling. *Pflügers Archiv* **465** 1031–1040. (doi:10.1007/s00424-013-1217-0)
- Salie R, Moolman JA & Lochner A 2012 The mechanism of β -adrenergic preconditioning: roles for adenosine and ROS during triggering and mediation. *Basic Research in Cardiology* **107** 281. (doi:10.1007/s00395-012-0281-5)
- Skyschally A, van Caster P, Iliodromitis EK, Schulz R, Kremastinos DT & Heusch G 2009 Ischemic postconditioning: experimental models and protocol algorithms. *Basic Research in Cardiology* **104** 469–483. (doi:10.1007/s00395-009-0040-4)
- Thuc LC, Teshima Y, Takahashi N, Nagano-Torigoe Y, Ezaki K, Yufu K, Nakagawa M, Hara M & Saikawa T 2010 Mitochondrial K (ATP) channels-derived reactive oxygen species activate pro-survival pathway in pravastatin-induced cardioprotection. *Apoptosis* **15** 669–678. (doi:10.1007/s10495-010-0473-0)
- Tota B, Gentile S, Pasqua T, Koshimizu H, Cawley NX, Cerra MC, Loh YP & Angelone T 2012 The novel Chromogranin A-derived serpinin and pyroglutaminated serpinin peptides are positive cardiac β -adrenergic-like inotropes. *FASEB Journal* **26** 2888–2898. (doi:10.1096/fj.11-201111)
- Tota B, Angelone T & Cerra MC 2014 The surging role of Chromogranin A in cardiovascular homeostasis. *Frontiers in Chemistry* **2** 64–76. (doi:10.3389/fchem.2014.00064)
- Wu L & Juurlink BH 2002 Increased methylglyoxal and oxidative stress in hypertensive rat vascular smooth muscle cells. *Hypertension* **39** 809–814. (doi:10.1161/hy0302.105207)
- Zhang K, Chen Y, Wen G, Mahata M, Rao F, Fung MM, Vaingankar S, Biswas N, Gayen JR, Friese RS *et al.* 2011 Catecholamine storage vesicles: role of core protein genetic polymorphisms in hypertension. *Current Hypertension Reports* **1** 36–45. (doi:10.1007/s11906-010-0170-y)
- Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA & Vinten-Johansen J 2003 Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *American Journal of Physiology. Heart and Circulatory Physiology* **285** H579–H588. (doi:10.1152/ajpheart.01064.2002)

Received in final form 16 September 2015

Accepted 23 September 2015

Accepted Preprint published online 23 September 2015