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Human Catestatin Peptides Differentially Regulate Infarct Size in the Ischemic-Reperfused Rat Heart

Bhawanjit K. Brar[1,2], **Erik Helgeland**[3], **Sushil K. Mahata**[1,2], **Kuixing Zhang**[2], **Daniel T. O'Connor**[1,2], **Karen B. Helle**[3], and **Anne K. Jonassen**[3]

[1] Department of Medicine, UCSD, La Jolla, California, USA

[2] VA San Diego Healthcare System, San Diego, California, USA

[3] Department of Biomedicine, University of Bergen, Norway

Abstract

In acute myocardial infarction increased plasma levels of chromogranin A is correlated with decreased survival. At the human chromogranin A gene locus there are two naturally occurring amino acid substitution variants within the catestatin region, i.e. Gly^{364} Ser and Pro^{370}Leu, displaying differential potencies towards inhibition of nicotinic cholinergic agonist-evoked catecholamine secretion from sympathochromaffin cells and different degrees of processing from the prohormone. Here, we examine whether two of the variants and the wild type catestatin may affect the development of infarct size during ischemic reperfusion in the Langendorff rat heart model. The hearts were subjected to regional ischemia followed by reperfusion in the presence or absence of synthetic variants of human catestatin. Compared to the Gly^{364}Ser variant both the wild type and the Pro^{370}Leu variant increased infarct size while decreasing the cardiac levels of phosphorylated Akt and two of its downstream targets, FoxO1 and BAD. In conclusion, these findings suggest that, in contrast to the Gly^{364}Ser variant, the wild type catestatin and the Pro^{370}Leu variant (allele frequency ~0.3%) increased myocardial infarct size via a mechanism involving dephosphorylation of Akt and the two downstream targets during ischemic reperfusion in the isolated rat heart.

Keywords

infarction; reperfusion; ischemia; catestatin; chromogranin A

INTRODUCTION

Ischemia/reperfusion (I/R) injury causes an inflammatory response as a consequence of oxidative damage, which then triggers stress-signaling processes resulting in death of cardiac myocytes and increasing the susceptibility to cardiac dysfunction [1]. In the clinical situation an acute myocardial infarction (MI) results from a regional occlusion of the

Corresponding authors: AK Jonassen, Department of Biomedicine, University of Bergen, Norway, Anne.Jonassen@biomed.uib.no and Bhawanjit K. Brar, Department of Medicine, UCSD, La Jolla, and. VA San Diego Healthcare System, San Diego, California, USA. bervy@yahoo.com..

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coronary supply of oxygenated blood. It is well established that post-ischemic reperfusion may worsen the myocardial injury if the heart is not preconditioned or post-conditioned by brief periods of coronary occlusions before or after the ischemic insult [2]. However, as the onset of infarction is usually unpredictable, ischemic preconditioning is of little practical use. A large component of the ischemic damage to the myocardium takes place during the first minutes of reperfusion when the oxygen overflow amplifies the injury and/or cause additional damage [3]. Cardioprotection after MI is best achieved by pharmacological interventions during the post-ischemic reperfusion [4]. Accordingly, a period of global reperfusion after a regional ischemic insult offers an important window for cardioprotective therapy in the clinical setting. In this context, the regionally ischemic and reperfused *ex vivo* rat heart is a well-established model for evaluation of pharmacological interventions during post-ischemic reperfusion of relevance for the clinical situation [5,6,7]. Taking into account that plasma chromogranin A (CGA) is significantly elevated in patients after MI [8] and that after acute MI, CGA is predictive of mortality [8-12], a question arises whether elevated plasma levels of CGA are beneficial or detrimental to the ailing heart. At the human CHGA gene locus there are 2 naturally occurring amino acid substituted variants within the Cts region: Gly^{364} Ser (GS-Cts) and Pro^{370} Leu (PL-Cts) in addition to one outside this region (Arg^{374} Gln) [13,14]. As these variants also display differential potencies towards inhibition of the basal cardiac performance and isoproterenol induced inotropism and lucitropism [15], we hypothesize that these Cts isoforms may also differ in protective potencies against the post-ischemic injury of the myocardium during reperfusion after a period of regional ischemia in the *ex vivo* rat heart model.

The human chromogranin A (CGA_{1-439}) was originally identified in the adrenal medulla where it is co-stored and exocytotically co-released with catecholamines [16-18]. CGA is not only expressed throughout the neuroendocrine system [19,20], but is also expressed in rat and human heart tissue [21-23]. Moreover, in human heart CGA is elevated in biopsies taken from patients with dilated or hypertrophic cardiomyopathy [23]. CGA is proteolytically processed to give rise to several peptides of biological importance, including the dysglycemic hormone pancreastatin [24], the vasodilator N-terminal vasostatin-I (CGA_{1-76}) [25] and the catecholamine release inhibitory peptide catestatin (Cts, human $CGA_{352-372}$, bovine $CGA_{344-364}$) [26]. Four N-terminal CGA peptides have been extracted from the rat heart: i.e. CGA_{4-113}, CGA_{1-119}, CGA_{1-124} and CGA_{1-135} [22]. Cts is a novel cardiosuppressive peptide in the isolated rat and frog hearts [15,27] and mediates this action via activation of endothelial nitric oxide production including Akt dephosphorylation and subsequent inhibition of isoproterenol and endothelin signaling. We and others [5,6,7,28] have shown a correlation between Akt phosphorylation and increased cytoprotection induced by pharmacological intervention at early ischemic reperfusion. The Akt signaling pathway is already a therapeutic target against I/R injury due to its pivotal role in cell survival [29]. Akt functions as a “survival” kinase by phosphorylating a number of apoptosis regulatory molecules acting in parallel, such as the forkhead transcription factor (FoxO1) [30-33] and the cytosolic protein BAD [34,35]. When phosphorylated by Akt these proteins are retained in the cytoplasm through interaction with 14-3-3 proteins and thus become functionally inactivated. If Akt does not inactivate forkheads and BAD in this manner, the forkheads translocate to the nucleus and initiate transcription of pro-apoptotic proteins [30], while BAD translocates to the mitochondria with subsequent heterodimerization with Bcl-xl or Bcl-2 to promote cell death [34,35].

Given that CGA is expressed in the heart, that circulating levels of CGA are increased following CHF and MI in humans , and that these sources of CGA are processed into Cts variants to a variable degree [36-38], we have reasoned that Cts isoforms might also differ in their influence on the pathophysiology of cardiac I/R injury. When these isoforms are present during the post-ischemic reperfusion period following a regional ischemic injury in

the *ex vivo* rat heart model, they may modulate the Akt-regulated phosphorylation of BAD [34,35] and possibly also FoxO1 [30-33].

MATERIALS AND METHODS

All experiments were approved by the Norwegian State Commission for Laboratory Animals and carried out in accordance with the European Communities Council Directive of 1986 (86/609/EEC).

The *ex vivo* Langendorff perfused heart model

Male Wistar rats (250-350 g) fed with a standard diet were heparinized (200 IU) and anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). The hearts were excised and rapidly mounted onto a Langendorff perfusion system as described elsewhere [5,39]. A water-filled latex balloon, connected to a hydrostatic physiological pressure transducer (SP844, Memscap, Norway) and coupled to a high performance data acquisition system (PowerLab 8/30, Chart Pro software-MLS250), was inserted into the left ventricle (LV) through an incision in the left atrium and inflated to set an end diastolic pressure (LVDP) of 5-10 mm Hg-[5,39].

Coronary flow (CF) was measured by timed collection of effluent over 1 min at each sampling point. A 3-0 silk suture was passed around the main branch of the left coronary artery, and the ends were threaded through a small vinyl tube to form a snare. Regional ischemia was achieved by pulling the snare and was confirmed by a substantial fall in both LVDP and CF. All hearts underwent 20 min of stabilization [5,39,40], 30 min of regional ischemia (RI), and then 120 min of reperfusion during which Cts isoforms were present for comparison of effects upon reoxygenation (Fig. 1).

Four groups of hearts were included: 1) control (in Krebs-Henseleit buffer, KHB), 2) GS-Cts, 3) PL-Cts or 4) WT-Cts (all in 100 nM in KHB from the onset of reperfusion). The control group was exposed to the identical protocol as for the hearts treated with the Cts-isoforms, both in regards to infarct size and protein status. The ligation was retied at the end of the experimental protocol, and Evans blue dye (EBD) 0.2% (w/v) was infused to demarcate the risk zone (RZ). The hearts were frozen at −20°C and thereafter cut into 2-mm tick slices from the apex to the atrioventricular groove. The slices were then stained with 1% triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) at 37 °C for 20 min, before fixation in 10% (v/v) formalin solution to enhance the contrast of the stain. The slices were then compressed to a uniform thickness by placing them between two glass plates separated by a 2-mm spacer. The area of the left ventricle, the infarcted area (TTC negative) and the risk zone (blue) were traced on an acetate transparency, and the infarct size was determined using a computerized planimetry program. The infarct size/risk ratio (%) was determined by expressing the infarcted (TTC positive) area in percent of the risk zone (RZ; EBD negative). The measurement of ischemic risk zone and infarct size was performed in a blinded fashion.

Preparation of neonatal mouse cardiomyocyte cultures

The isolation procedure for cardiac myocytes from hearts of C57BL/6 mice was based on the method of Simpson [41] with additional modifications [42] using sequential digestion in collagenase type II (Worthington Biochem Inc., Lakewood, NJ). The cardiomyocyte cell suspension was transferred to 24-well, (1-cm diameter) 2% (wt/vol) gelatin-coated plates at a density of 10^5 cells/well for protein extraction. After 24 h the cell medium was replaced with DMEM supplemented with 1% (vol/vol) fetal bovine serum for an additional 24 h. Within 2 d a confluent monolayer of spontaneously beating myocytes was formed.

Preparation of adult mouse cardiomyocyte cultures

Adult mouse ventricular cardiomyocytes were prepared by excising hearts from three-month-old mice (anesthetized with 0.25% (wt/vol) Avertin and anticoagulated with heparin 250 U/mouse i.p.) and placed in ice-cold DMEM containing 4% (vol/vol) fetal bovine serum (FBS). Then the hearts were mounted onto a modified Langendorff apparatus and perfused at a rate of 3.5 ml/min for 1 min with prewarmed 37°C Ca^{2+}-free Joklik's medium supplemented with 10 mM HEPES, 30 mM taurine, 2 mM DL-carnitine, and 2 mM creatine (pH 7.36–7.4) (Sigma). The hearts were then perfused and digested with 0.75 mg/ml collagenase type 2 in 0.1% (wt/vol) BSA for 9–15 min (20 μM $CaCl_2$) in supplemented Joklik's media. The hearts were excised and digested for a further 3–6 min in the collagenase solution and washed thoroughly in 70 μm^2 nylon mesh with supplemented Joklik's medium containing 10% (w/v) BSA and 20 μM $CaCl_2$ (wash solution). $CaCl_2$ was added gradually until a concentration of 2 mM was reached. The cardiomyocytes were then plated in DMEM medium (Gibco BRL, Grand Island, NY) containing 4% (vol/vol) FBS at a density of 1×10^4 cells/well on 10 μg/ml prelaminated (Sigma) coated plates for 1 h (6 cm^2). The media were replaced with DMEM (serum free) before experimentation.

Peptides

The human WT-Cts; $CGA_{352-372}$ (S^{352}SMKLSFRARGYGFRGPGPQL^{372}), or its two variants Gly^{364}Ser (SSMKLSFRARAYS^{364}FRGPGPQL) and Pro^{370}Leu (SSMKLSFRARAYGFRGPGL^{370}QL), were synthesized by the solid-phase method and purified as previously described [36,43-45].

Signalling analyses

Rat heart tissue was isolated and snap-frozen. Later the tissue were thawed and homogenized in 1 ml of ice cold heart lysis buffer: 0.2 M sucrose, 10 mM Tris maleate (pH 7.0) buffer, 2 mM EDTA (pH 8.0), 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM henylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml aprotinin. Cytosolic fractions were isolated and the protein content determined by the Bradford colorimetric assay (BIO-RAD, CA, USA). Cytosolic protein (25 μg) was subjected to immunoblot analysis for phosphorylated Akt-Ser473 (P-Akt-Ser^{473}), Akt-$Threonine^{308}$ (P-Akt-Thr^{308}), total Akt (T-Akt) and P-FoxO1-Ser^{256} using an antibody that detects a doublet including P-FoxO1-Ser^{256} (all from Cell Signaling Technology, CA, USA). P-FoxO1 levels were normalized to actin (Santa Cruz Biotechnology, CA, USA).

Immunoreactive Cts in the myocardium

Protein extracts were retained for immunoblot analysis of catestatin-containing CGA using a specific rabbit-polyclonal antibody that was commercially generated against the human catestatin domain ($hCGA_{352-372}$) by Strategic Biosolutions, Windham, Maine, USA [46]. The antibody detects the Cts sequence within the full-length and processed fragments of CGA and cross-reacts with mouse and human Cts and CGA. The primary antibody and dilutions were routinely characterized for specificity towards intact CGA and free WT-Cts peptide (data not shown).

To investigate the expression of immunoreactive Cts, samples containing 40 μg of total cytosolic protein were taken from baseline control hearts, the non-ischemic area (NI) and risk zone (RZ) area of hearts subjected to I/R. Extracts from neonatal and adult mouse cardiac myocytes were prepared as described elsewhere [47]. Cytosolic protein from these cells were separated on NuPAGE® Novex 4-12% Bis-Tris Midi gels (Invitrogen, CA) using 2-(N-morpholino)ethanesulfonic acid (MES) buffer (Invitrogen, CA) using the XCell SureLock® Mini-Cell. After separation, the proteins were transferred on to 0.2 μm

nitrocellulose membranes and XCell II™ Blot Module Kit (Invitrogen, CA) and the membranes were immunoblotted with a 1:5000 dilution of the primary anti-human Cts antibody. The apparent molecular weight of CGA in heart extracts was compared to CGA in 5 μg of mouse adrenal extract protein. Membranes were then washed in phosphate buffer saline (PBS) supplemented with 0.05% Tween-20 and incubated with 1:2000 dilution of peroxidase conjugated anti- rabbit IgG (Bio-Rad Laboratories, Hercules, CA) followed by detection using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Additional immunoblots were conducted on adult and neonatal cell extracts. To clearly identify the molecular weight forms of Cts-immunoreactive proteins in heart vs. the adrenal, 20 and 40 μg aliquots of heart extract were run for comparison with 5 or 40 μg samples of adrenal extract.

P-BAD immunoprecipitation

Protein (100 μg) from cell lysate was pre-cleared by incubation with 20 μl of a 50% slurry of protein G plus (Santa Cruz) with agitation (20 min, 4 °C). Following centrifugation (10,000 rpm, 10 min, 4 °C), the cell lysates were incubated with agitation (18 h, 4 °C) with an anti-Phospho-BAD-Ser136 (P-BAD-Ser^{136}) antibody (1:100). Subsequently, 20 μl of a 50% slurry of protein G plus was incubated with the lysate for 3 h, with agitation, at 4 °C. P-Bad immunoreactive proteins were pelleted by centrifugation (10,000 rpm, 10 min, 4 °C) and washed three times with ice-cold heart lysis buffer. Thirty μl of 2X Laemmli sample buffer was added to the pellet which was then heated and subjected to SDS-PAGE. BAD levels were determined by immunoblot analysis using a P-BAD-Ser^{136} specific antibody [5].

Statistics

Values are presented as mean ± SEM. For protein analysis, the volumes of total and phosphorylated proteins were quantified using Quantity One (Bio-Rad), normalized to actin or total protein. Fold increase was calculated from the control of non-ischemic hearts. Comparisons of coronary flow (CF), heart rate (HR) and left ventricular developed pressure (LVDP) between groups were performed with repeated-measures by the general linear model and within-group differences were tested by the paired Student's t-test. Infarct size, immunoblots and hemodynamic results were tested for group differences by ANOVA (one-way analysis of variance) combined with Fisher's post-hoc test. *$p \leq 0.05$ was considered statistically significant.

RESULTS

Endogenous cardiac CGA is affected by ischemia-reperfusion

Using an antibody directed against human Cts [46], two major immunoreactive, Cts-containing forms were detected in the control hearts with apparent molecular weights of ~55 kDa and ~27 kDa, respectively (Fig. 2A). Higher molecular weight bands, including a faint band of 78 kDa, were detected in the control hearts. In the NI and RZ samples one form of ~ 58 kDa was observed in addition to the predominance of the ~27 kDa form (Fig. 2A). In the mouse adrenal gland used as a positive control the ~55 kDa form dominated over the ~58 kDa and the ~27 kDa forms (Fig. 2A, right lane and Fig. 2B, left lane). On the other hand, in the *ex vivo* rat heart tissue (HE) and in the isolated adult (ACM) and neonatal mouse cardiac myocytes (NCM), the ~27 kDa form of Cts-immunoreactivity dominated (Fig. 2B). In HE, five forms of Cts-immunoreactive peptides were observed (78, 58, 55, 53 and 27 kDa, respectively). In the adrenal extract at 1/8 dilution (5 μg protein, Fig.2C), only two major forms of Cts positive fragments of 55 and 53 kDa were detected. At this dilution the 27 kDa band was no longer visible.

Effect of Cts variants on infarct size and hemodynamic variables

The marked declines in the ~55 kDa Cts-immunoreactive band in the perfused hearts suggested a considerable degree of CGA processing, presumably generating Cts-containing fragments also smaller than the predominant ~27 kDa form. We therefore examined whether WT-Cts and the two human variants could modulate infarct size following simulated I/R stress. Administration of WT-Cts, PL-Cts or GS-Cts from the onset of reperfusion resulted in comparable infarct sizes for the control *versus* the GS-Cts groups and for the WT-Cts *versus* the PL-Cts groups, respectively (Fig. 3), however, with significantly higher degrees of infarct size for the latter than for the former. The average area at risk (RZ)/left ventricular mass ratio was 50% for all groups. A significant reduction in LVDP and CF after 5 min of regional ischemia (RI, Table 1) confirmed that all groups obtained similar and expected degrees of ischemia relative to the baseline values at stabilization. On the other hand, there was no difference between groups with in regard to LVDP, CF and HR at 20 min of stabilization or at 60 and 120 min of reperfusion (Table 1). However, there was a significant fall in both LVDP and CF in all groups as compared to the corresponding stabilization value at both 60 and 120 min of ischemic reperfusion.

Effect of reperfusion treatment with Cts on Akt-meditated signaling

To probe the mechanism by which WT-Cts and PL-Cts increased infarct size as compared to GS-Cts, signaling analysis were conducted on cytosolic fractions of heart tissue. Both WT-Cts and PL-Cts significantly decreased the levels of phosphorylated the Akt-Ser^{473} isoform compared to the effects of GS-Cts and control hearts (Fig. 4A). The diminished phosphorylation of this isoform of Akt corresponded to the increased infarct size in the same groups relative to the GS-Cts treated and control groups. In addition, phosphorylated levels of the other Akt isoform, Akt-Thr^{308}, was also significantly decreased in the PL-Cts hearts (Fig. 4B). Consistent with decreased levels of P-Akt, we also observed modifications in the phosphorylation of P-FoxO1-Ser^{236} (Fig. 5) and P-BAD (Fig. 6). However, there was no difference between the control and the GS-Cts group in folds of P-FoxO1 and P-BAD, while the PL-Cts and WT-Cts groups were significantly lower than the GS-Cts group in this respect.

Correlation between infarct size and reduction of Akt phosphorylation

A 2-D plot was drawn to compare the effects of the different Cts- variant peptides with the % infarct size (Fig. 3) and Akt phosphorylation at Ser^{473} (Fig. 4). As shown in Fig. 7, the greatest % increase of infarct size correlated with the most marked decrease in Akt-Ser^{473} phosphorylation, as evident for the PL-Cts and WT-Cts peptides.

DISCUSSION

Cts-containing fragments from cardiac CgA in the rat heart are indeed generated in response to regional ischemia. Atrial myocardial secretory granules are established as a source of CGA and CGB in the rat [21], while both CGA [23] and CGB [48] are produced by the human and rat ventricular myocytes, respectively. Putative candidates for proteolytic enzymes are members of the prohormone converting enzyme reported to be differentially expressed in heart [37] and assumed to be involved in processing of atrial CGA to Cts peptides. Cathepsin L, co-localized with CGA in chromaffin granules, has also been reported to generate Cts and other active peptides [38]. If present within the atrial granules, this enzyme might also contribute to processing of cardiac CGA into Cts-containing fragments. In the extracellular space the plasmin system is a likely candidate for processing of CGA into to Cts [36]. The potential of these and other enzymes in processing of CGA in the I/R hearts of the rat remains a challenge for future studies.

It is well established that I/R injury culminates with hypercontracture of the cardiomyocytes, causing sarcolemmal rupture and cell death [49]. Opening of the mitochondrial permeability transition pore (MPTP) and the calcium overload are crucial players in the final stage of this detrimental process [50]. Most of the damage takes place during the first minutes of reperfusion and this period is therefore critical for cardioprotection. Inhibition of MPTP is mediated via the reperfusion injury salvation kinase (RISK) pathway, involving NO-dependent and –independent cascades activated by G protein coupled receptors. [6]. Here, Akt is pivotal for three subsequent downstream cascades terminating on inhibition of MPTP opening. While the e-NOS – cGMP-PKC cascade leads cardioprotection via opening of the mitochondrial potassium ATP channel and subsequent activation of reactive oxygen species [51], two other NO-independent cascades are also activated by Akt, namely by down regulation of BAD [5,6] and by phosphorylation of GSK-3β [52] .

In the normoxic rat heart *ex vivo* Cts isoforms have been shown to vary in their activation of the endothelial NO cascade [15]. The present study has therefore aimed at an evaluation of the cardioprotective potentials of the Cts isoforms when present throughout the reperfusion following the ischemic insult. Unexpectedly, the human WT-Cts and its naturally occurring variant PL-Cts significantly enhance infarct size in the *ex vivo* rat heart when present from onset of reperfusion. In contrast, the most frequent variant, GS-Cts, was without effect on infarct size relative to control. Moreover, the enhanced infarct sizes in the WT-Cts and PL-Cts groups correlate with blunted Akt phosphorylation, implicating minimal cardioprotective potentials of these two isoforms in the *ex vivo* rat heart.

Detection of Cts-containing CGA forms during I/R in the rat heart

Using the antibody specifically directed towards full length human WT-Cts [46], we show that immunoreactive Cts is present in rat as a ~55 kDa form which predominates in the control hearts. However, this form decreased markedly relative to the ~27 kDa form in response to treated hearts. This suggests a pronounced degree of proteolytic processing of the larger form of immunoreactive Cts, presumably leading to release of Cts-containing peptides also smaller sized than the ~27 kDa form. In a previous study N-terminal cleavage of normal rat heart CGA into betagranin-like peptides ($CGA_{4\text{-}113}$, $CGA_{1\text{-}119}$, $CGA_{1\text{-}124}$, and $CGA_{1\text{-}135}$) has been demonstrated [22], consequently resulting in a range of betagranin-free forms of variable sizes. The present detection of the ~ 55 kDa bands as the predominant immunoreactive Cts in control hearts may correspond to such a partly processed, betagranin-free rat CGA while the ~ 58 kDa form in the NI and RZ samples may be another, probably induced by the ischemic stress. It is likely that the slight differences in apparent molecular weight of the Cts containing bands between adrenal and heart tissues represent variations in post-translational modifications in these two tissues.

Effect of Cts variants on infarct size during ischemia-reperfusion

The concentration of Cts peptides (100 nM) was selected on the basis of the IC_{50} value for WT-Cts inhibition of the nicotinic cholinergic receptor-mediated catecholamine release in bovine adrenal chromaffin cells [26] and previous studies in the isolated rat [15] and frog heart [27]. In the present study the local concentration of Cts in the coronary effluent was not determined, either under basal conditions or in the reperfusion period following ischemia. Of note, circulating concentration of CGA in patients 3 days after a MI reached 36 ng/ml or ~0.78 nM [16], i.e. close to the normal value. On the other hand, in CHF patients in the New York Heart failure class IV plasma CGA may reach 11 nM [9]. Thus, in the present experiments the rat hearts were perfused with 10-100 fold higher concentrations of Cts peptide than the concentrations of the parent CGA in plasma of CHF or MI patients [8-10].

The allele frequency for the variant GS-Cts accounts for no more that 3-4% of the total, predicting a ~6-8% population prevalence of heterozygotes, while that of the PL-Cts variant the frequency (~0.3%) is much lower [45]. Our present data shows that the infarct size with the GS-Cts peptide was not different from the control (Fig. 3). Hence, the rank order for *increase in* infarct size was PL-Cts = WT-Cts>> GS-Cts = control, i.e. similar to the rank order for *decrease in* Akt phosphorylation (Figs. 4A and 6). Analogously, the rank order of potency for the inhibition of nicotinic stimulated catecholamine secretion from chromaffin cells is PL-Cts>WT-Cts>GS-Cts [14]. Although a receptor for Cts in the heart has yet to be identified, the inotropic and lusitropic negative effects of WT-Cts under normoxic conditions in the rat heart involved an endothelial Gi/o protein → nitric oxide → cGMP signaling pathway and a non-competitive activation of beta-2-adrenergic receptors [15]. Importantly, the eNOS-NO-cGMP-PKG cascade mediates specific cardioprotection via the RISK pathway [6]. Stimulation of beta-2-adrenergic receptors also acts through Akt to phosphorylate eNOS to generate NO [15]. Furthermore, these authors suggest that the negative inotropism and lusitropism found after infusion of 110 nM of WT-Cts is caused by reduced P-PLN (phospholamban) levels that can influence basal Ca^{2+} handling and contractility via the SR Ca^{2+} pump (SERCA2a) and the SR Ca^{2+} release via ryanodine receptor 2a, resulting in altered Ca^{2+} transients with consequences for inotropy and lusitropy.

WT-Cts and PL-Cts suppress the pro-survival Akt signaling pathway in the I/R hearts

The PL-Cts and WT-Cts treated hearts had considerably larger infarct sizes (Fig. 3) and lower pro-survival P-Akt levels compared to the GS-Cts treated and control hearts (Fig. 4). Thus, the levels of phosphorylated Akt were inversely correlated to infarct size in the peptide treated groups (Fig. 7). The reduction in P-Akt with WT-Cts presently observed is consistent with recent findings of reduced phosphorylation of Akt in the normoxic rat heart [15]. The possibility that WT-Cts might induce cell death also in normoxic conditions was not supported using cultures of cardiac myocytes (data not shown). Relevant in this context is the important contributions of AKT and ERK ½ to the RISK-signaling pathway, conveying cardioprotection during ischemic-reperfusion [5,6,28]. Both in the RISK cascade [6] and in the β_2-ARs-Gi/o protein-eNOS-NO-cGMP-PKG dependent cGMP signaling pathway, e-NOS is downstream to Akt [15]. If the β_2-ARs-Gi/o protein-eNOS-NO-cGMP-PKG pathway stimulated by WT-Cts [15] also depended on Akt phosphorylation [15], one would have expected the total Akt phosphorylation from EC and the myocardium to be elevated in presence of WT-Cts. However, Cts infusion dephosphorylated both AKT and ERK ½ under basal conditions [15] and failed to alter ERK1/2 phosphorylation during ischemic-reperfusion in our study (data not shown). Hence, it is most likely that a Cts-induced dephosphorylation of Akt, independent of the NO-dependent pathway, may play the most important role under the present conditions.

Akt inhibition by WT-Cts and PL-Cts variants also inhibits downstream FoxO1 and BAD

Akt plays a critical role in controlling survival and apoptosis in a variety of cells and tissues [29-33]. Because WT-Cts and the human variant PL-Cts inhibited Akt activity as a result of ischemic reperfusion, these peptides would also be expected to inhibit the phosphorylation of downstream anti-apoptotic targets of this kinase. Our data show that both WT-Cts and PL-Cts target to the FoxO1 forkhead transcription factor and the pro-apoptotic protein BAD. Thus, the inhibition of Akt, FoxO1 and BAD pathways by both PL-Cts and WT-Cts compared to GS-Cts may relate to the observed differences between these Cts peptides with respect to infarct size.

In the normoxic heart [15] the negative inotropy and lusitropy was associated with reduced P-PLN (phospholamban) levels that could influence basal Ca^{2+} handling and contractility

via the SR Ca^{2+} pump (SERCA2a) and the SR Ca^{2+} release via ryanodine receptor 2a [15]. However, there is today no data suggesting that Cts works directly on any particular subcellular compartment. Therefore, the effects of Cts were examined in the cytosolic fraction only. On the other hand, the reduced phosphorylation of AKT in this fraction of neonatal and adult mice cardiomyocytes suggests that the enhanced infarct size in response to WT-Cts and PL-Cts in the rat heart most likely is linked to a reduced inhibition of MPTP as the final stage in the RISK cascade.

Blood pressure is lower in individuals with the GS-Cts variant than in those with WT-and PL-Cts [13,45]. Also, GS-Cts heterozygotes display increased baroreceptor slope, increased cardiac parasympathetic index, and decreased cardiac sympathetic index [53]. Thus, the present findings suggests that in addition to having favorable blood pressure-lowering effects, persons who carry the most common variant, i.e. GS-Cts, may be at diminished risk during I/R injury in comparison to normal individuals or PL-Cts carriers due to marked differences in suppression of the Akt signaling pathway as presently demonstrated. Importantly, under basal conditions WT-Cts exerted maximal negative inotropic and lucitropic effects 5 min after administration, remaining stable for 15 min and then graduallydecreasing with time [15]. On the other hand, after ischemia the cardiac function of the isolated rat hearts with a RZ of 50% would remain depressed at the start of reperfusion. Moreover, during ischemic reperfusion LVDP and CF were not significantly different between the groups (Table 1). It is therefore unlikely that these factors could have contributed significantly towards the WT-Cts and PL-Cts mediated increases on infarct size (Fig. 3). Intriguingly, the deleterious response to WT-Cts in the ischemic rat heart is in contrast to the protective effect of another CGA peptide, the N-terminal vasostatin-I against the development of cardiac infarction when administered as a mimetic of ischemic preconditioning [54].

Concluding remarks

Consistent with previous findings in rat [22] and human [23] hearts, our study has demonstrated CGA in the rat heart and mouse cardiocytes. Importantly, our data makes it evident that the endogenous cardiac CGA was modified in response to the I/R injury, resulting in an enhanced processing into immunoreactive Cts-containing fragments such as the 27 kDa band prominent not only in the rat heart, but also in the mouse cardiomyocytes.

The cardiovascular actions previously demonstrated for the two CGA derived peptides vasostatin-I and Cts under normoxic conditions have now been extended to post-ischemic reperfusion injury. However, in contrast to beneficial effects of Vasostatin-I during preconditioning [54], the present data imply that WT-Ct may enhance the infarct size when present at high 100 nM concentration during the reperfusion phase following a period of regional ischemia. We have also demonstrated that three isoforms of the human Cts differently affect recovery from regional ischemic injury in the rat heart *ex vivo.* This fact points to significant differences in primary structure for activation of the signaling cascades implicit in the cardiac responses.

The negative inotropic effect of WT-Cts via activation of eNOS in the normoxic rat heart model is reported to be sensitive to inhibition of pertussis toxin, implicating a Gαi/o subunit in the signaling pathway [15]. In want of a surface receptor for Cts outside the sympathoadrenal system, receptor-independent cell activation via a Gαi/o subunit has been proposed for the cationic Cts in rat mast cells and cardiac tissue [55]. Alternatively, the cationic Cts may penetrate through pores in the cell membranes, as postulated for the antimicrobial properties [56] and conformation plasticity [57] of the biologically active cateslytin domain of the bovine WT-Cts ($bCGA_{344-358}$). Of note, while PL-Cts accounts for a substitution of Pro^{370} for Leu, i.e. outside the cateslytin region, GS-Cts comprises a

substitution of Gly^{364} to Ser within the cationic cluster of the active domain. This substitution site corresponds to Gly^{356} of the bovine Cts, previously shown to be one of the crucial sites for inhibition of desensitization of CA release [43]. Hence, it seems likely that the substitution Gly^{364}Ser in human Cts has rendered this isoform inactive compared to WT-Cts and PL-Cts with respect to infarct size and Akt phosphorylation during I/R reperfusion.

The quantitative aspects of the cardiac synthesis and constitutive release of CGA have yet to be established. So far, our data have made it evident that when the human WT-Cts is present at a high 100 nM concentrations during reperfusion after the regional ischemic insult in the rat heart *ex vivo*, the infarct size was increased relative to the peptide-free control. Moreover, as the two human variants, PL-Cts and GS-Cts varied markedly with respect post-ischemic infarct size, and only PL-Cts caused reduced phosphorylation of the pro-survival Akt kinase and the downstream signaling mediator FoxO1 and BAD, these findings are consistent with a mechanistic link between infarct size and reduced phosphorylation of Akt also via the NO-independent, FoxO1 and BAD cascades. This aspect certainly calls for future studies.

A possible limitation of this study could be our use of regional rather than global ischemia prior to the post-ischemic reperfusion in presence of the Cts isoforms. In view of the reported vasodilatory actions of Cts in the normoxic rat heart [15] we had expected to see a vasodilatory action following 120 min reperfusion in presence of Cts, manifested by increased coronary flow and potentially decreased infarct size. However, as both WT-Cts and PL-Cts were deleterious, this is unlikely to be due to vasodilator properties of these two isoforms, in contrast to GS-Cts containing a substitution in the biologically active cateslytin domain of this peptide [43,56]. In the vast majority of clinical events with MI the ischemic injury is regional and not global. Therefore, our findings suggest that ischemic reperfusion injury following regional ischemia in the *ex vivo* rat model is not alleviated by the presence of free Cts isoforms.

Elevated plasma CGA levels in the acute and subacute phase after MI and later stages of CHF signify a poor prognosis in humans [8-10]. Assuming an increased degradation also of the circulating CGA during phases of regional ischemic in humans, as presently demonstrated in the *ex vivo* rat model, the GS-Cts variant may be of considerable advantage during the recovery phase after an ischemic insult.

Acknowledgments

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Abbreviations

Akt	a phosphatidylinositol 3′-kinase dependent Ser/Thr kinase
AU	arbitrary units
BAD-Bcl-2	protein
Cts	human $CgA_{352\text{-}372}$
CGA	chromogranin A
CGB	chromogranin B
CF	coronary flow

CHF	chronic heart failure
CHGA	chromogranin A gene locus
EBD	Evans blue dye
FoxO1	forkhead transcription factor
GS-Cts	Gly^{364}Ser-catestatin
HR	heart rate
IA	infarct myocardial area
I/R	ischemia/reperfusion
KHB	Krebs-Henseleit buffer
LVDP	left ventricular developed pressure
MI	myocardial infaction
PKB	protein kinaseB
PL-Cts	Pro^{370}Leu-catestatin
P-Akt-Ser^{473}	phosphorylated Akt-Ser^{473}
P-Akt-Thr^{308}	Akt-$Threonine^{308}$
P-FoxO1-Ser^{256}	Phospho-FoxO1-Ser^{256}
RI	regional ischemia
NI	non-infarct myocardial zone
RZ	risk zone
T-Akt	total-Akt
TTC	triphenyltetrazolium chloride
WT-Cts	$hCGA_{352-372}$

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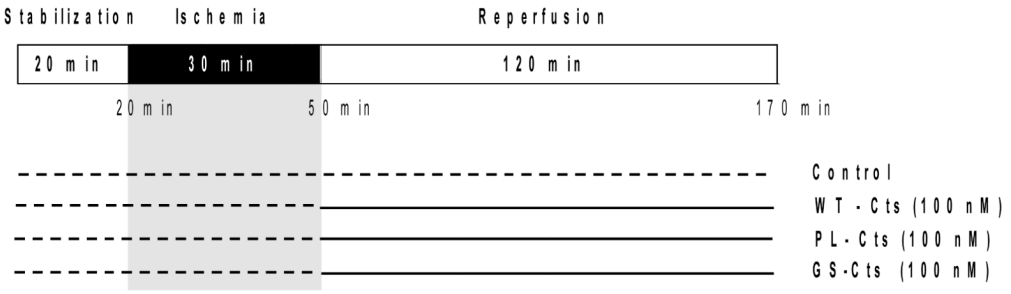

Figure 1. Experimental protocol for the Langendorff perfused rat heart

The isolated heart was subjected to 20 min of stabilization, 30 min of regional ischemia and 120 min of reperfusion. Cts peptides were administrated to the hearts at onset of reperfusion.

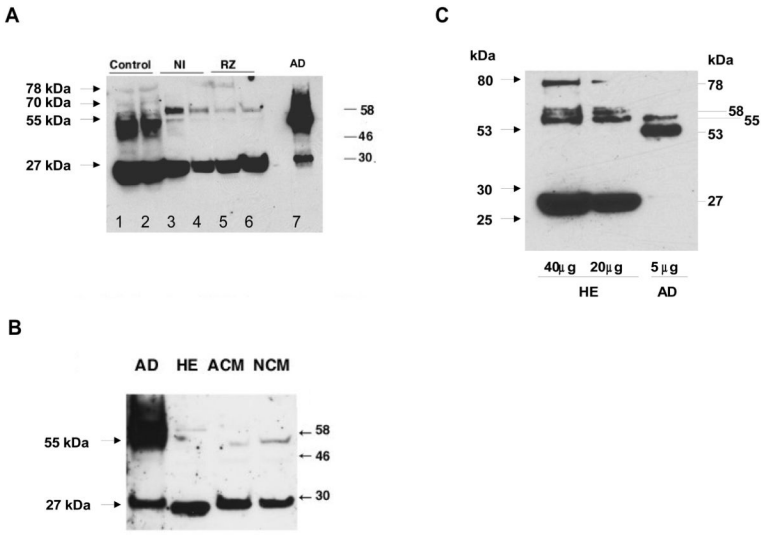

Figure 2. Expression and processing of CGA during the I/R experiment

A) Two Cts-containing CGA size forms of ~55 kDa and ~27 kDa, respectively, were apparent in the non-perfused hearts (control, left lanes 1 and 2). Both forms were also expressed in the mouse adrenal (AD, 40μg) as positive control (lane 7) Tissue from the non-ischemic area (NI, lanes 3 and 4) and risk zone (RZ, lanes 5 and 6) revealing markedly diminished levels of the ~55 kDa form. **B)** Both the ~55 and ~27 kDa Cts immunoreactive peptides were found in mouse adrenal (AD, 40μg) while in the isolated rat heart (HE) and in isolated adult (ACM) and neonatal mouse cardiac myocytes (NCM) the ~27 kDa form accounted for the predominant Cts immunoreactivity. **C)** To clearly demonstrate the MW forms of Cts-immunoreactive peptides in heart extract (HE) and adrenal (AD) we separated 40 and 20 μg of HE against 5μg of adrenal extract (AD). In HE, five forms of Cts-immunoreactive peptides were observed (78, 58, 55, 53 and 27 kDa). In adrenal, two major forms of 55 and 53 kDa were detected with 1 μg of protein. Positions of molecular weight markers (in kDa) are indicated at right sides of **A)** and **C)** and at the left side of **B)**.

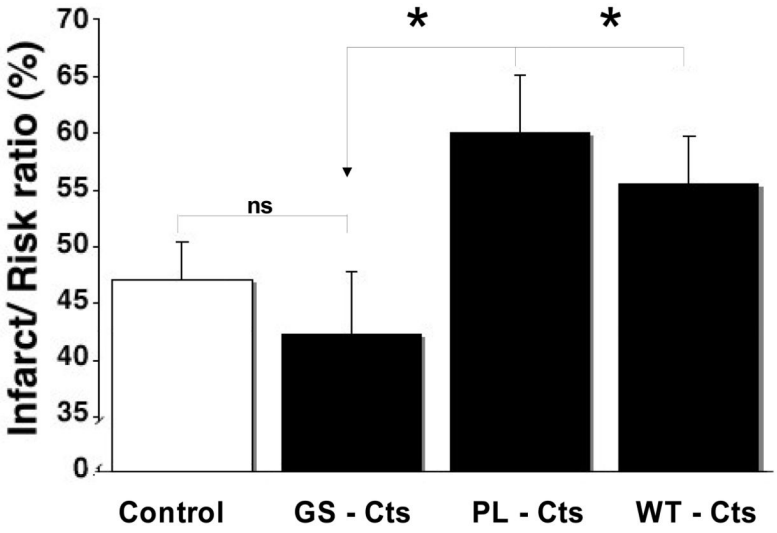

Figure 3. Effect of reperfusion treatment with Cts variants on infarct size after I/R

Rat hearts were subject to ischemia and perfused with Cts peptides (100 nM) or control at reperfusion. The infarct size given as the infarct/risk ration in % are shown for the control (n=5), GS-Cts, (n=10), PL-Cts (n=6) or WT-Cts (n=5). Bars represent means ± SEM. * p≤ 0.05 for differences from CS-Cts.

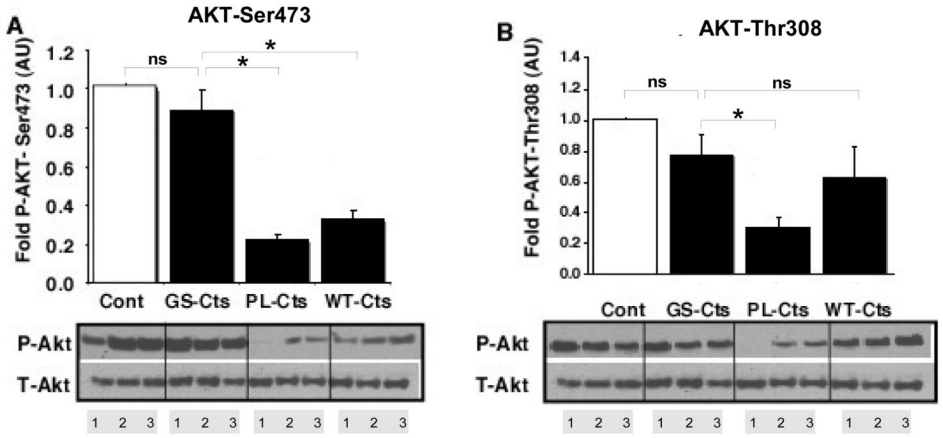

Figure 4. Effects of Cts variants on Akt phosphorylation after I/R

The phosphorylation status of **A)** Akt-Ser473 and **B)** Akt-Thr308 were normalized to total Akt. Densitometric scans are represented in arbitrary units (AU) relative to the control (Cont = 1). The bars represent mean ± SEM of 3 hearts (lanes 1,2 and 3). *p≤ 0.05 for differences between PL-Cts and WT-Cts versus GS-Cts.

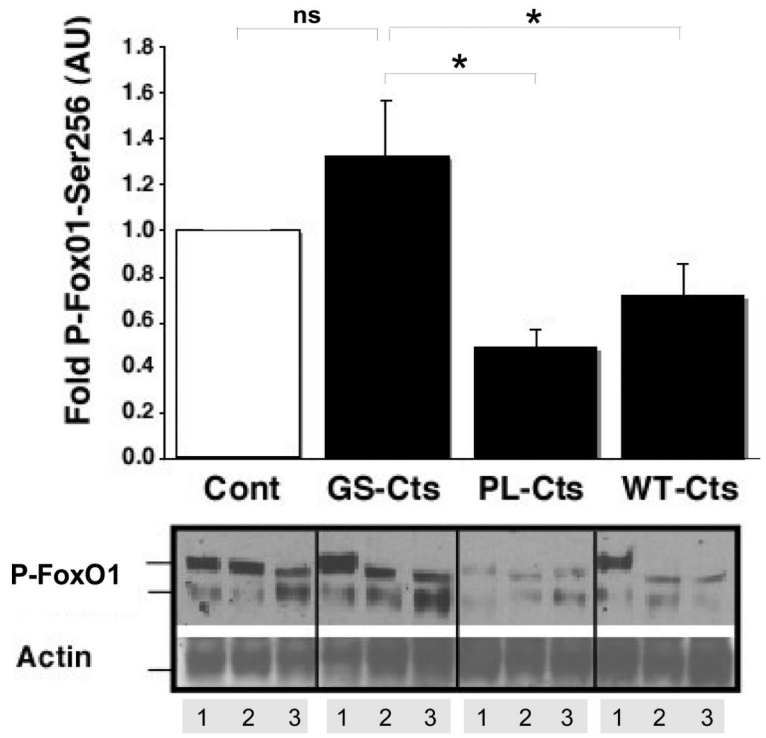

Figure 5. Effects of Cts variants on signaling downstream from Akt

Levels of protein phosphorylation were normalized to actin and expressed in arbitrary units (AU) relative to control hearts (Cont = 1). The double bands of immunoreactive FoxO1 are indicated. The bars represent the sum of immunostaining in both bands as means ± SEM of three hearts (lanes 1-3). *p≤ 0.05 for differences between PL-Cts and WT-Cts versus GS-Cts.

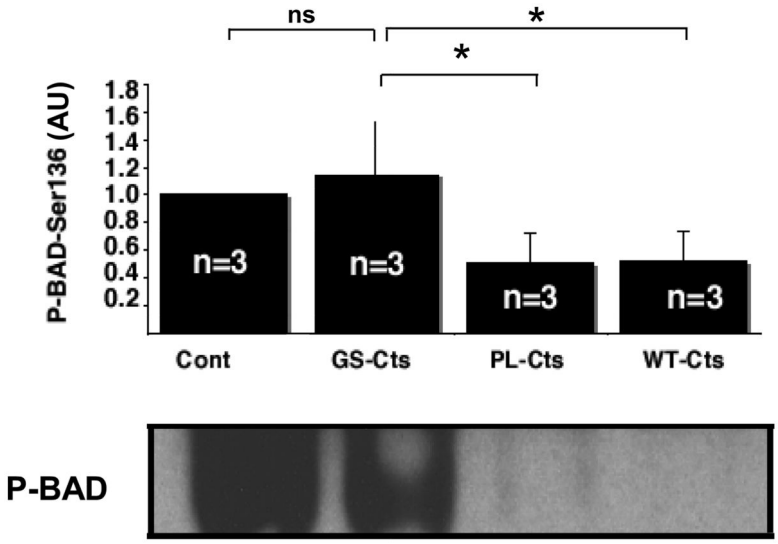

Figure 6. Effects of Cts variants on BAD phosphosphorylation

Levels of BAD phosphorylation expressed in arbitrary units (AU) relative to control hearts (Cont = 1). The bars represent mean ± SEM of 3 hearts. *p≤ 0.05 for differences between PL-Cts and WT-Cts versus GS-Cts.

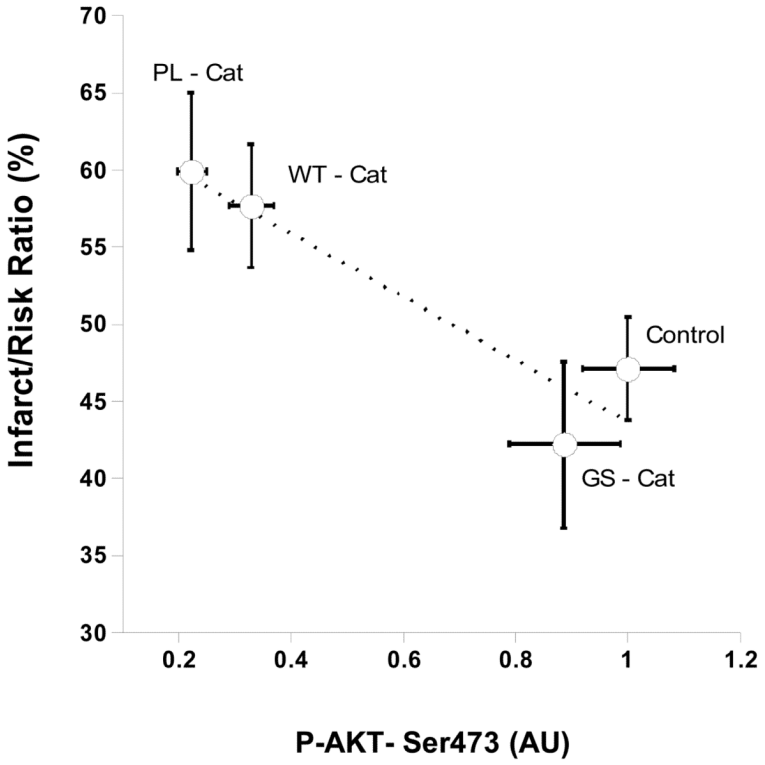

Figure 7. Correlation between infarct size (%) and Akt-Ser^{473} phosphorylation

A 2-D plot was constructed using the Kalidograph® programme for the data for AktSer^{473} phosphorylation (Fig.4) versus the % infarct size (Fig.3). The correlation value (r^2=0.877) indicates that the infarct size is inversely correlated with Akt-Ser^{473} phosphorylation for the Cts peptides examined.

Table 1

Cardiac hemodynamics in the isolated rat heart during ischemic-reperfusion.

	GROUP	Stabilization 20 min	RI 5 min	Reperfusion 60 min	Reperfusion 120 min
LVDP (mmHg)	**Control**	128 ± 24	62 ± 16 *	70 ± 8 *	65 ± 6 *
	WT-Cts	133 ± 10	67 ± 20 *	80 ± 6 *	66 ± 8 *
	PL-Cts	131 ± 14	68 ± 8 *	74 ± 9 *	71 ± 9 *
	GS-Cts	137 ± 7	71 ± 11 *	69 ± 5*	60 ± 5 *
CF (ml/min)	**Control**	14 ± 1	8 ± 1 *	9 ± 1 *	7 ± 1 *
	WT-Cts	13 ± 1	7 ± 1*	10 ± 1*	9 ± 2 *
	PL-Cts	13 ± 1	8 ± 1 *	9 ± 1 *	7 ± 1 *
	GS-Cts	12 ± 1	8 ± 1 *	8 ± 1 *	7 ± 1 *
HR (beats/min)	**Control**	300 ± 14	259 ± 22	261 ± 21	254 ± 22
	WT-Cts	295 ± 9	248 ± 43	285 ± 45	265 ± 16
	PL-Cts	270 ± 12	226 ± 22	252 ± 16	242 ± 18
	GS-Cts	271 ± 8	233 ± 20	254 ± 8	245 ± 9

RI - regional Ischemia; LVDP - Left Ventricular Developed Pressure; CF - Coronary Flow; HR - heart rate. Values represent mean ± SEM. Control (Krebs-Heinsleit buffer, KHB) and peptides (100nM) in KHB. n≥5.

* $p<0.05$ vs. corresponding stabilization value.