Cardioprotection against ischemia/reperfusion injury and chromogranin A derived peptides

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Summary

Chromogranin A (CgA) is produced by cells of the sympathoadrenal system, as well as by human ventricular myocardium. In the clinical setting CgA has been mainly used as a marker of neuroendocrine tumors, but in the last decade much data have been published on the role of CgA and its derived peptides, particularly catestatin and vasostatin, in the regulation of cardiovascular function and cardiovascular diseases, including heart failure and hypertension. CgA-derived peptides, namely catestatin and vasostatin, may exert negative inotropic and lusitropic effects on mammalian hearts. As such CgA and its derived peptides may be regarded as a complex feedback system able to modulate the exaggerated release of catecholamines. This system may be also regarded as an attempt for compensatory cardioprotective response against myocardial injury in pre and postischemic scenario. In fact, while vasostatin can trigger cardioprotective effects akin ischemic preconditioning (protection is triggered before ischemia), catestatin is a potent cardioprotective agent in the early post-ischemic phase, *i.e.* it is a postconditioning agent (protection is triggered at the onset of reperfusion). Admittedly, the exact mechanism of cardioprotection of this system is far from being fully understood. Nevertheless, both vasostatin and catestatin have shown to be able to activate multiple cardioprotective pathways. In particular, both catestatin and vasostatin may induce nitric oxide dependent pathway, which may play a pivotal role in cardioprotection against ischemia/reperfusion injury. Here, we describe the cardiac effects of catestatin and vasostatin, the mechanisms of myocardial injury and protection and the role of CgA derived peptides in cardioprotection.

Key words: Chromogranin-A, Catestatin, Vasostatin, Cardioprotection, Preconditioning Postconditioning

Introduction

Pharmacological strategies that have consistently provided cardioprotection, in laboratory experiments and even in patients, are those involving peptides, like atrial natriuretic peptides (ANPs) and other drugs stimulating cyclic guanosyn monophopsphate (cGMP) synthesis [1]. Among emerging peptides in the cardiovascular system chromogranin A (CgA) derived peptides are occupying a role of paramount importance. CgA is a key player in neuroendocrine regulation of cardiac function. In fact, CgA is ubiquitously distributed in nervous system, immune system and diffuse neuroendocrine system of both vertebrates and invertebrates [2-6].

Yet, CgA is the major soluble product within the secretory granules of chromaffin cells of the adrenal medulla where it is costored and coreleased with catecholamines [7]. In particular, in mammalian myocardium, CgA is costored with ANPs within the atrial myoendocrine cells [8]. Moreover, human ventricular myocardium produces and releases CgA. In these cells CgA is co-localized with brain natriuretic peptide (BNP). Importantly, there is strong correlation between BNP and CgA circulating levels in heart failure patients. Therefore CgA may be a potential therapeutic target in heart failure [6] and CgA derived peptides may play a role in regulating cardiovascular function.

Chromogranin A derived peptides

Human CgA gene consists of eight exons separated by seven introns and has been localized into chromosome 14q32. The mature human CgA protein (439 amino acids) has many pairs of basic residues that are potential cleavage sites for endoproteases that also coexist in the secretory granules (*e.g.* prohormone convertases PC1/3 and PC2) [9]. CgA proteolytic processing gives rise to several peptides of biological importance, including serpinins [10] pancreastatin [11], vasostatin-1 (VS-1) [12], and catestatin (human CgA352-372, bovine CgA344-364) [13-18] (For more details, see reviews in this forum).

Whether CgA is proteolytically cleaved in human myocardium is not unequivocally ascertained; recent findings suggest that proteolytic processing could, indeed, occur in myocardial tissue. In

fact, N-terminal fragments containing the VS-1 domain has been detected in the rat heart [19]. Intriguingly, murine cardiomyocytes are also able to secrete catestatin (CST), which may establish a paracrine function in cardiac tissue [20]. Nevertheless, several cleavage products of CgA have been identified to date and have been shown to exert important biological functions. For instance, vasostatin (human CgA 1–76) is a vasodilatator agent; serpinin (human CgA411–436) is an antiapoptotic agent, pancreastatin (human CgA250–301) is a dysglycemic hormone, and CST (human CgA352–372) is a catecholamine release-inhibitory peptide. Recently, it has been reported that high doses of VS-1 may exert *in vivo* an adrenoceptor-dependent initial vasoconstriction, which masks the persistent adrenoceptor-independent vasodilatation [21]. Notably, it has been suggested that the relation between CgA expression and blood pressure is U-shaped, thus suggesting that both CgA deficiency and excess could results in an increase in catecholamine release [22].

Very recently Meng *et al.* [23] have suggested that CST modulates the cardiac hyperthophic response to high blood pressure. These authors observed that among hypertensive patients, the ratio of CST to norepinephrine was lower in patients with than in those without left ventricular hypertrophy.

Clearly, both CgA and its derived peptides have a role in the pathogenesis of hypertension, being a complex system able to modulate sympathoadrenal tone and cardiovascular functions. Importantly, among CgA derived peptides, both vasostatin-1 and CST have been proposed as cardioprotective agents [24,25].Therefore we will focus our attention on these two peptides.

Vasostatin-1

Vasostatins (VS-1 and VS-2) are N-terminal CgA-derived peptides (See reviews in this forum). In particular, vasostatin (VS-1) is the highly conserved CgA1–76, which was named vasostatin because of its vaso-suppressive effects on isolated and pre-contracted human vessels [12,26]. VS-1 contains three amphipathic domains, *i.e.* one in the positively charged CgA47–66 and the other two in the negatively charged CgA1–40 [27]. Clearly, VS-1 and its smaller fragments act as

multifunctional regulatory peptides displaying several proprieties. These include antifungal, antibacterial and vasodilatory properties [28-30]. Importantly, as above mentioned, VS-1 acts as cardio-suppressive agents on isolated beating heart preparations obtained from rats, frogs and eels [31]. The cardiotropic and vasoactive properties of CgA-derived vasostatins suggest that these peptides play a role as regulator of the cardiovascular function, particularly under conditions of sympathetic overstimulation [32-34].

Catestatin

Catestatin, one C-terminal CgA derived fragment, is a peptide with different variants within a species and with different sequencing in the different species. The different variants and sequencing will be considered in other reviews of this forum. The human CST (sequence: SSMKLSFRARAYGFRGPGPQL) is a cationic and hydrophobic peptide. Recent studies have documented that lower plasma level of CST is a risk factor for development of hypertension in humans and animals [35]. It has been also documented that a naturally occurring human variant of CST alters autonomic function and blood pressure, and that arterial hypertension of CgA knockout mouse is rescued by exogenous injection of catestatin. Importantly, CST is a nicotin cholinergic anatagonist, antagonizes catecholamine secretion, vasodilates and is a negative myocardial inotrope [9,36]. Thus, also CST may play a crucial role in regulating the cardiovascular functions.

The CgA-derived peptides vasostatin-1 and catestatin as regulatory of cardiac functions

Cardiac inotropic and lusitropic effects by vasostatin-1

Increasing physio-pharmacological evidences indicate that peptides containing the N-terminal domain of CgA (*i.e.* VS-1) contribute to the autocrine/paracrine cardiac regulation. In all species tested their major actions consist of negative inotropism and in the counteraction of the β-adrenergic mediated positive inotropism, typically induced by isoproterenol (Iso) [6,33,37-39]. In the rat heart, these peptides also induce negative lusitropism. VS-1 also counteracts endothelin-1 (ET-1)-induced positive inotropic effects and ET-1-dependent coronary constriction [24] without

affecting calcium transients on isolated ventricular cells [24]. So that it suggests a noncompetitive antagonistic action indirectly via endothelium-derived nitric oxide [40].

Intracellular mechanisms

The negative inotropic and lusitropic effects by VS-1 have been extensively studied by Tota's group [9,36-41] and Alloatti's group [40,42] and will be the topic of other reviews of the present Forum. Here we will briefly describe the intracellular mechanism of these contractile effects that are relevant for the cardioprotective mechanisms.

Clearly, VS-1 interacts with multiple intracellular effectors. In fact in the rat heart we have show that the NOS-NO-cGMP-PKG system plays a key role in mediating specific intracardiac signaling involved in the control of the contractile performance [24]. In particular, we have shown that the reduction of left ventricular pressure, rate-pressure product, and $+dP/dt_{max}$ induced by VS-1 was abolished by either scavenging NO with hemoglobin (Hb) or blocking NOS activity with NOS antagonists as well as by inhibiting soluble Guanylyl Cyclase (sGC) or cGMP-dependent protein kinase (PKG). These data are all consistent with an NO-dependent mechanism underlying the VS-1induced negative inotropic action in the rat heart. However, the mechanism by which the peptide interacts with these intracellular effectors (*i.e.*, via either a still unknown receptor or lipophilic interaction with the membrane) is still under intense investigation [41]. Recently, a heparan sulfate proteoglycans (HSPGs) interaction and caveolae endocytosis of VS-1 has been proposed as mechanism that is coupled with a phosphatidylinositol 3-kinase (PI3K)-dependent eNOS phosphorylation [40].

It has been demonstrated that, in rat ventricular myocytes, NO by targeting sGC, and thus PKG, negatively affects contractility. This negative effect was mediated by reducing L-type Ca^{2+} current and by phosphorylating troponin-I, thus reducing the affinity of troponin-C for Ca^{2+} [43-45]. We proposed that both L-type Ca^{2+} current reduction and PKG-mediated myofilament desensitization to Ca^{2+} may account for VS-1 induced negative inotropy. Moreover, activation of PKG induced the phosphorylation of α1-subunit of the PTx-sensitive Gi/o proteins, and this potentiated the

inhibition operated on L-type Ca^{2+} current [43]. In addition, this PKG-induced action on Gi/o proteins, at same time, negatively affected adenylyl cyclase, causing a decrease of cAMP levels, and stimulated eNOS-dependent NO production [46,47]. Interestingly, in our experiments we observed that the VS-1-induced negative inotropic effect was blocked not only by inhibition of the NOS-NO-cGMP-PKG system but also by the impairment of Gi/o proteins by PTx [24]. Intriguingly, it has been also reported that cytoskeleton integrity is necessary for the VS-induced negative inotropy and lusitropy in the rat heart. These results are of importance because the cytoskeleton alterations in cytoskeleton dynamics play a pivotal role in both physiological and physiopathological cardiac mechanics in the case of systolic and diastolic dysfunctions [41]. Recently, experiments conducted on rat isolated cardiomyocytes and bovine aortic endothelial cells suggested that the negative inotropism induced by VS-1 in rat papillary muscle is probably due to an endothelial PI3K-dependent NO-release, rather than to a direct action on cardiomyocytes [39]. Taken together, these data strongly suggest that in the inotropic effects a cross-talk between endothelium and myocardium may play an important role.

Cardiac inotropic and lusitropic effects by catestatin

The cardiotropic actions of wild-type-CST, including the beta-adrenergic and endotelin-1 (ET-1) antagonistic effects, support an important role of this peptide as an autocrine-paracrine modulator of cardiac function, particularly when the heart is targeted by either adrenergic or ET-1 stimuli. In the frog heart CST reduced contractility by inhibiting phosphorylation of phospholamban. Moreover, the CST effect was abrogated by pretreatment with either NOS or sGC inhibitors, or an ET(B) receptor antagonist. CST also non-competitively inhibited the positive inotropic action of isoproterenol [9]. In the rat heart slightly different effects were achieved by different variants of CST (see other reviews of this Forum). Nevertheless, wild-type-CST increased heart rate and coronary pressure and decreased left ventricular pressure, rate pressure-product and both positive and negative dP/dt. Wild-type-CST not only inhibited phospholamban phosphorylation, but also the inotropic and lusitropic effects of wild-type-CST were abolished by chemical inhibition of β_2-

adrenergic receptors, Gi/o protein, nitric oxide or cGMP, indicating involvement of this pathway [36]. CST displays a transient positive inotropic effect, which is abolished by the H_1 histamine receptor antagonist mepyramine [48].

Intracellular mechanisms

The NO synthase (NOS)-NO-cGMP-dependent protein kinase (PKG) cascade plays a key role in the control of contractile depression by CST. In particular studies highlight the pivotal involvement of a Ca^{2+}-independent/PI3K- and Akt-dependent NO release in the cardiodepressant effects of CST [36,40]. It has also been reported that, the release of NO derives mainly from endothelial cells *via* Akt-dependent phosphorylation of eNOS [1,40]. In fact, CST dose-dependently reduced the effect of β-adrenergic stimulation. This effect was not mediated by a direct action on cardiac cells, but likely involved a PI3K-dependent NO release from endothelial cells. When inotropic effects of CST were evaluated in the presence of selective antagonists it has been confirmed a role for both α- and β-adrenergic receptors, as well as for Gi/o proteins and the NO pathway [36].

Therefore both VS-1 and CST cardiac effects consider a cross-talk between endocardia/endothelium and cardiomyocytes. This cross-talk is of paramount importance in cardioprotection.

Before to consider the cardioprotective effects of CgA derived peptides we will briefly consider the *"Ischemia/reperfusion injury"* and will consider with more details the *"Cardioprotective strategies and pathways"*.

Ischemia/reperfusion injury (Fig. 1)

Coronary heart disease is one of the leading worldwide causes of death; in particular the acute myocardial infarction is a major cause of such mortality. The only way to salvage myocardium in patients with acute myocardial infarction is the rapid restoration of blood flow which reduces

infarct size. However, the rapid reperfusion induces additional lethal injuries that are not present at the end of the ischemic period, that is *reperfusion injury* [49-51]. Reperfusion injury is due to complex biochemical and mechanical mechanisms involving extracellular and intracellular processes. In the myocardium reperfusion injury includes cellular death, which can occur for necrosis autophagy and apoptosis. It has been proposed that necrosis can be caused mainly by ischemia as well as by reperfusion, whereas the apoptosis is typically induced by reperfusion [52]. Autophagy may be both deleterious and beneficial, depending on a number of circumstances [for reviews see 53]. Reperfusion injury also includes myocardial stunning, endothelial dysfunction and no-reflow phenomenon [51].

Causes of reperfusion injury (751 words 5148 Char) (530 3644)

The mechanisms of reperfusion-induced cell death are not completely understood, but it is well established that myocardial damages during reperfusion among others can be due to the liberation of ROS, to the cellular/mitochondrial overload of Ca^{2+}, to the activation of mitochondrial permeability transition pore (mPTP), to the reduced availability of NO and to the activation of the NFkB.

Oxidative stress related to the generation of *reactive oxygen species* (ROS) plays an important role [51,54] and contributes to the onset and maintenance of post-ischemic inflammation [52]. During reperfusion, the superoxide anion (O_2^-) production increases, which along with other ROS strongly oxidize the myocardial fibers, thus favoring apoptosis [51-58]. It reacts with the NO forming peroxynitrite ($ONOO^-$). Thus, $ONOO^-$ is a sign of a reduced availability of NO [59,60] and participates with O_2^- to the lesion of myocardium [61-63]. Peroxynitrite dependent damages may be reduced in the presence of a sufficient quantity of NO which can react, in a so-called *secondary reaction*, with $ONOO^-$ leading to protein nitrosylation and damage reduction [64]. Reperfusion injury is also due to the *cellular Ca^{2+} overload*. In fact, the Ca^{2+} overload, which starts during ischemia, is further increased during reperfusion. Altered cytosolic Ca^{2+} handling may induce structural fragility and excessive contractile activation during ischemia and upon reperfusion, as

also evidenced from a progressive increase of ventricular diastolic pressure and band necrosis [57,65,66]. Increasing cellular osmolarity, the overload of Ca^{2+} favors the swelling (explosive swelling) of myocardiocytes. Mitochondria undergo rapid changes in matrix Ca^{2+} concentration upon cell stimulation. Within mitochondria Ca^{2+} overload can promotes the expression/release of proapoptotic elements, such as the release of caspase cofactors [58]. Ca^{2+} overload is also considered one of the conditions responsible of the opening of mPTP. Although, mitochondrial transition pore (mPTP) opening is strongly inhibited by acidosis during ischemia, it is also favored by ATP depletion, oxidative stress and high intra-mitochondrial Ca^{2+} concentrations, conditions all occurring during myocardial reperfusion [67]. In fact, the opening of mPTP results in cessation of ATP production and cell necrosis and/or to the release of cytochrome c (Cyt c) and cell apoptosis. It is likely that a large number of cells are killed by these mechanisms during reperfusion [68-71].

In the reperfusion the role of nuclear factor kappa B (NFκB) is also important. Its activation is induced from several agents included hydrogen peroxide and contributes to the exacerbation of the myocardium lesions sustaining inflammatory reactions [49,72-75]. Also the reduced NO availability determined by I/R favors the activation of the transcription of the genes that codify for molecules of cellular adhesion [73,76]. The NO deficiency can also cause vasoconstriction and formation of micro-thrombi into the lumen of the small vessels [77,78]. These mechanisms, combined to the adhesion of the leucocytes to the endothelium, can lead to the so-called "*no-reflow phenomenon*" [79].

In summary, reperfusion injury are due to several mechanisms that include Ca^{2+} overload, ROS generation, reduced availability of NO, mPTP opening and to the activation of the NFκB, which lead to the augmented expression of molecules of cellular adhesion, leukocyte infiltration and no-reflow phenomenon. Therefore, the mPTP play a central role being primed by ischemia to open upon reperfusion, so that leading to reperfusion-induced cell necrosis.

Effects of reperfusion injuries

Among the outcomes of *reperfusion injury* are included *a)* endothelial and vascular dysfunction and the sequels of impaired coronary flow, which may concur to the "no-reflow phenomenon"; *b)* metabolic and contractile dysfunction; *c)* arrhythmias; and *d)* cellular death, by apoptosis, swelling and contraction band necrosis.

Cardioprotective strategies and pathways

Preconditioning and vasostatin-1 (w 1157 c 8120) (901 6256)

An initial impulse to the concept of cardioprotection was given by the seminal studies of Braunwald, Maroko and colleagues in the early seventies [80,81]. However only in 1986 when the ***ischemic preconditioning*** (IP) phenomenon was described by Murry *et al*. [82] research in the field increased exponentially. These authors reported that 4 cycles of 5-min ischemia/5-min reperfusion prior to a 40-min coronary occlusion decreased infarction by 75% from that seen in dogs with only a 40-min coronary occlusion. Thus the brief periods of ischemia had preconditioned the myocardium to make it more resistant to the stress of a longer ischemic interval. In all animal models IP consists of brief periods (a few minutes) of ischemia, separated from one another by brief periods (a few minutes) of reperfusion just prior to a prolonged period of ischemia followed by reperfusion (see Fig. 1). Preconditioning limits the severity of the ischemia/reperfusion injury. In fact, after IP, the extent of the area of a subsequent infarction is reduced by 30–80% versus matched controls. Preconditioning also reduces ischemia/reperfusion arrhythmias and may reduce contractile dysfunction.

Downey's group was the first to report that IP is triggered by adenosine released during the brief periods of preconditioning ischemia [83]. Subsequently we and other investigators have identified many of IP's signaling steps. It is now clear that cardioprotection by ischemic preconditioning is triggered by autacoids such as adenosine, bradykinin, opioids and platelet activating factor, produced as a response to the cycles of brief ischemia/reperfusion [84-92]. Therefore, it seems that cardioprotective substances have their receptors coupled to signal transduction pathways that ultimately inhibit the formation of mPTP during the reperfusion phase following the infarcting

ischemia (Fig. 2) [93-97]. Actually, it is becoming clear that the protective pathway is complex and can be divided at least into a pre-ischemic trigger phase and a mediator phase in early reperfusion.

Cardioprotection by IP requires a complex signaling cascade, which includes the opening of mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}) [98-105]. Intriguingly preconditioning can be completely blocked by free radical scavengers, such as N-acetyl-cysteine (NAC) or mercaptopropionyl glycine (MPG) [98,100]. These results confirmed that redox signaling is involved in triggering cardioprotection by preconditioning.

In the signaling cascade "trigger pathway" protein kinase C (PKC) plays a pivotal role. In fact PKC activation may occur quite directly through activation of phospholipase C (PLC) for the action of adenosine receptors. Other substances via their specific receptor may activate a more complex pathway that includes the activation of PI3K, PKB/Akt, nitric oxide synthase (NOS), guanylyl cyclase and PKG, opening of mitoK_{ATP} channels, and finally production and release of ROS which target PKC [106] (Fig. 2). When this pathway is activated the heart displays a protected phenotype which persists for a couple of hours even after the triggering agonists have been washed out.

In the mediation phase, it seems that the activated PKC modifies another adenosine receptor subtype, the A_{2b} receptor [105,108], which becomes ready to be activated in the reperfusion period. The subsequent downstream signaling events trace somehow those seen in the trigger pathway. These signaling are thought to ultimately prevent formation of mPTP [71,109].

The reperfusion signaling pathways include the *Reperfusion Injury Salvage Kinase* (RISK) pathway and the more recently described *Survivor Activating Factor Enhancement* (SAFE) pathway, two apparently distinct signal cascades which may actually interact to convey IP and Postconditioning (PostC) cardioprotection [110].

Several studies have demonstrated that activation of Akt plays a role of paramount importance in cardioprotection. Akt leads to the cardioprotective effects of G protein–coupled receptors

[111,112], glycoprotein 130–linked receptors and receptors of tyrosine kinases [113,114]. These receptors activate PI3K which results in an increase of phosphatidylinositol triphosphate (PIP3) levels [113-115]. PIP3 favors Akt translocation to the plasma membrane. Akt is subsequently activated through phosphorylation at Thr308 by phosphoinositide-dependent kinase 1 (PDK1) and by phosphorylation at Ser473 *via* both TORC2 mechanism and the intrinsic catalytic activity of Akt [116-119].

The possibility that Akt is also phosphorylated by signal transducer and activator of transcription 3 (STAT3) or janus kinase (JAK) has been put forward by Goodman *et al.* [120] and by Gross *et al.* [121]. Recently, it has been proposed that STAT3 is part of the protective SAFE pathway which includes JAK/STAT signal transduction pathway [122,123]. In the context of cardioprotection STAT3 has been identified in cardiomyocyte mitochondria [124] and its presence was confirmed in isolated mitochondria [125,126].

STAT3 is activated by phosphorylation during ischemia, and a further increase in its phosphorylation occurs during reperfusion [121,127-133]. Such a phosphorylation of STAT3 reduces cardiomyocyte death and attenuates adverse cardiac remodeling after I/R injury [134]. STAT3 phosphorylation and DNA-binding are also induced by IP [135]. Ischemic postconditioning also induces STAT3 phosphorylation [120,136,137].

We have previously shown that NO and its derivative nitroxyl (HNO) play an important role in the cardioprotective mechanism of preconditioning triggering [138]. It has been proposed that both an NO-cGMP- PKG signaling plays a role in the cardioprotective effect of ischemic postconditioning, as well as a NO-dependent PKG independent mechanism. The latter include the intervention of $ONOO^-$ and other reactive nitrogen species, which in concert with ROS can activate PKC [51].

According to results of Cohen and Downey group [139], the cardioprotective effect of ischemic preconditioning involves activation of PKC *via* a direct mechanism linked to adenosine type 1 receptor, which is independent of the NO-cGMP pathway [88,140]. Therefore, other mechanisms couple indirectly to PKC *via* the activation of a NO-cGMP-PKG pathway [140-143].

Vasostatin-1

Regarding N-terminal CgA-derived VS-1, it has been reported that VS-1 is able to counteract the effects of adrenergic stimulation [37] via an endothelial and endocardial release of NO, thus contributing to protection against excessive excitatory sympathetic challenges [39, 42]. Therefore, we wondered whether VS-1 may trigger preconditioning-like effects. Recently, we showed that the VS-1 (human recombinant CgA1–78) protects against the extension of myocardial infarction in the rat, inducing a pre-conditioning-like effect via adenosine/nitric oxide signaling if administered at low concentration before ischemia/reperfusion [24]. The protective signaling activated by VS-1 converges on PKC. Using the model of isolated rat heart, we found that the reduction of the left ventricular pressure (LVP), the maximal values of the first derivative of LVP (dP/dt_{max}) and of the rate-pressure product elicited by VS-1 at 33 nM is abolished by blocking Gi/o proteins with pertussis toxin, scavenging NO with hemoglobin, and blocking NOS activity with NG-monomethyl-L-arginine or N5-(iminoethyl)-L-ornithine, soluble guanylate cyclase with 1H-[1,2,4]oxadiazole-[4,4-a]quinoxalin-1-one, and PKG with KT5823. Data confirmed the involvement of the Gi/o proteins/NO-cGMP-PKG pathway in the VS-1-dependent negative inotropic effects. When given before 30-min of ischemia, VS-1significantly reduced the size of the infarct from about 65% to about 30% of the left ventricular mass. This protective effect was abolished by either NOS inhibition or PKC blockade and was attenuated, but not suppressed, by the blockade of A_1 receptors. These results suggest that VS-1 activity triggers two different pathways: one of these pathways is mediated by A_1 receptors, and the other is mediated by NO release, and both converge on PKC. Therefore, similarly to preconditioning ischemia, VS-1 may be considered a stimulus strong enough to trigger the two pathways, which may converge on PKC. We concluded that, since VS-1 does not present membrane receptor [40], possibly it interferes with other membrane receptors [24]. Importantly, between VS-1 infusion and 30-min ischemia there was a wash-out period which allowed the recovery from inotropic effect. Therefore

cardioprotection was not due to a reduction of oxygen consumption because of cardiac inotropism depression.

These data emphasize the potential importance of the release of CgA as an attempt of the cardiovascular system to protect itself against I/R damages and, eventually, against sympathetic overstimulation. Actually, increased plasma levels of CgA are present in patients after myocardial infarction [39]. Importantly, very recently, in acute myocardial infarction (AMI) patients an initial reduction with a subsequent increase in CST plasma levels has been reported [144]. The increased levels of CST and its precursor, CgA, are more supportive of the potential importance of the release of CgA and derivatives as tentative to protect the heart against I/R injury. Since the majority of I/R damages occur in the early reperfusion, we wondered whether a supplementation of VS-1 or CST in the early reperfusion phase may protect the heart against reperfusion injury. In our laboratory we observed that VS-1 does not protect when given in post-ischemic phase (unpublished observations). On the contrary CST only given in reperfusion resulted highly protective against I/R damages [25]. Before to consider CST protective effects in reperfusion, let us consider the postconditioning phenomenon as protective tool against reperfusion injury.

Postconditioning and Catestatin (w 560 c 4181) (428 3225)

Preconditioning is a robust cardioprotective intervention that salvages ischemic myocardium in experimental animals and in humans. However, preconditioning must be applied before an ischemic event to be protective. Although emergency angioplasty, thrombolysis, or revascularization surgery can effect reperfusion with documented salvage of myocardium, these procedures contribute to the injury (reperfusion injury). Therefore, interventions are needed that can supplement the reperfusion strategy and attenuate reperfusion injury in the heart.

The concept of cardioprotective reperfusion pathways was first proposed in the late 1990s when it was discovery that a variety of growth factors and drugs are capable of limiting myocardial infarct size if administered at the onset of myocardial reperfusion [51,92]. However, only when Zhao *et al.* [145] reported a most improbable observation that several brief coronary occlusions after a 60-

min occlusion significantly reduced infarct size, researchers renewed they interest on the opportunity to limit reperfusion injury. ***Postconditioning*** (PostC) has a great clinical appeal. In clinical practice, postconditioning is a promising adjunctive technique to reperfusion since it can improve postinfarction outcome, limit left ventricle dilatation and attenuate contractile dysfunction. However, ischemic/mechanical PostC cannot be applied to all patients with acute myocardial infarction. This makes pharmacological postconditioning an intriguing clinical objective.

In the original description of ischemic PostC in 2003 [145], the infarct-limiting effects were attributed to the prevention of myocardial reperfusion injury, attenuated apoptotic cell death, less oxidative stress, preserved endothelial function, reduced calcium overload, less myocardial inflammation and edema. However, it was soon clear that an actual signal transduction pathway was involved in the cardioprotective effects induced by ischemic PostC [146-149]. The novel aspect, therefore, was the opportunity of recruiting endogenous pro-survival signaling pathways to protect the heart against lethal myocardial reperfusion injury with both intermittent ischemia and drugs.

The main pathways (RISK and SAFE) involved in preconditioning are also involved in postconditioning (Fig. 3). However, the mechanism of cardioprotection involved in postconditioning are not exactly the same of those involved in preconditioning (for review see 109).

RISK cascade considers the intervention of several enzymes starting from PI3K-Akt and MEK-ERK-1/2 as above described. These cascades converge on mitochondria and sarcoplasmatic reticulum to reduce cytoplasmatic and mitochondrial calcium overload and to limit damages.

In mice with a deletion of STAT3 within cardiomyocytes, infarct size reduction by a postconditioning stimulus of 3 cycles 10 s ischemia and reperfusion each was abolished [132]. Also, postconditioning with exogenous tumor necrosis factor α did not protect isolated hearts from cardiomyocyte-specific STAT3 knockout mice [132,150]. However, whether or not specifically

mitochondrial STAT3 contributes to the cardioprotection by postconditioning must be confirmed in further studies [151].

Catestatin

Regarding *pharmacological postconditioning with CST*, in a recent study [25], we demonstrated the possibility to protect the heart infusing CST at beginning of reperfusion. We perfused the post-ischemic isolated rat heart with a CST concentration of 75 nM. In this model CST decreased the infarct size, limited the contracture and improved the post-ischemic systolic function. Moreover, we found that CST was protective in a model of isolated cardiomyocytes exposed to simulated ischemia, increasing cell viability rate of about 65%. The CST concentration we used in the isolated heart is within the same range of concentrations of the precursor CgA, detected in plasma of patients suffering IMA (about 1 nM) or cardiac heart failure (about 10 nM) [152,153]. It is also similar to the peptide concentration (IC50 ~ 100 nM) which depresses myocardial inotropism in normal perfused hearts [36], and appears slightly lower than the IC50 value for CST-induced inhibition of the nicotinic cholinergic receptor-mediated catecholamine release in bovine adrenal chromaffin cells [17]. CST applied in the reperfusion is protective especially in terms of improvement of post-ischemic cardiac function. Since protection was observed in both isolated heart and isolated cardiomyocytes, we suggested that the protective effect was primarily due to a direct effect on the myocardium and did not necessarily depend on the antiadrenergic and/or endothelial effects of CST [25]. However, endothelial effects could be additive.

In ongoing experiments we are studying the main mechanism for CST cardioprotection [154]. We have shown that the CST given in early reperfusion facilitates the phosphorylation of Akt, PKCε and GSK3β which may regulate mitochondrial function [49,110,139,155]. The mechanisms seem similar to those described in ischemic PostC. However, the protective pathways partially diverge, as mitoK_{ATP} channel blockade (5-hydroxydecanoate, 5-HD) or ROS scavenger does not avoid CST-dependent contracture limitation, whereas PKC inhibition abolishes infarct size, antiapoptotic activity, contracture limitation and systolic function recovery. Since 5HD attenuates the PKCε

activation due to CST, a reverberant circuit (PKCε-dependent mitoK_{ATP} channel activation ROS formation and subsequent PKCε re-activation, Fig. 3) has been hypothesized [70]. We also observed that the anti-infarct effect of CST is abolished by scavenging ROS with a sulfhydryl donor specific for mitochondrial activity [156], namely N-(2-mercaptopropionyl)glycine (MPG, 300 μM); whereas the contracture limitation is not affected by MPG.

Contrary to what was seen for VS-1, catestatin was unable to induce preconditioning-like effects (unpublished observations).

Why is vasostatin-1 a preconditioning and catestatin a postconditioning agent?

Several substances may be protective or deleterious when certain conditions have changed [51,139], as we and others have observed for other mediators such as PAF (platelet-activating factor) [142,154,157] and Angeli's salt, which are two protective agents when given in pre- but not in postconditioning phase [138,158,159]. On the contrary, we and other authors [160,161] have shown that Apelin is a postconditioning agent, but it is not protective when given as preconditioning mimetic.

Actually, it is unknown why VS-1 is a preconditioning and CST a postconditioning agent. We can only propose a hypothesis: since the preconditioning effect of VS-1 is attenuated by the blockade of adenosine type 1 receptors, and since the receptor involved in the protection against reperfusion injury are the A_{2b} receptors, we can argue that the type of receptors influenced by the two CgA derived peptides are different. However, this is a hypothesis and as such needs to be verified.

CgA derived peptides, ischemia/reperfusion and comorbidities

Since ischemic heart disease in humans is a complex disorder caused by or associated with known cardiovascular risk factors (e.g. smoking, aging, hypertension, myocardial hypertrophy and/or metabolic syndrome), which may be "confounders" in the outcome of cardioprotection, it is worthwhile to consider CgA and derived peptides in the presence of some of these comorbidities/confounders.

The role of CgA and its derived peptides, namely VS-1 and CST, in clinical scenario as players of pathophysiological mechanisms and/or as markers of cardiovascular diseases will be considered by other Reviews of this Forum. Here we briefly consider these peptides in the context of cardiovascular diseases and cardioprotection.

CgA plasma levels correlate with the severity of cardiac dysfunction and are a predictive factor for mortality in patients with chronic heart failure [162]. Moreover high levels of CgA were correlated to long-term mortality in patients with acute myocardial infarction [163]. Elevated circulating levels have also been reported in patients with tumors and with kidney and liver failure [164-166]. Whether the increased levels contribute to the development of diseases or represent an attempt of protection against injury is not clear at the moment.

Of note, CgA knockout mice show hypertension and cardiac enlargement [167]. In this in *vivo* studies conducted in CgA knockout mice the authors have shown that the injection of CST rescue animals from elevated blood pressure. Moreover, studies on individuals with primary and secondary hypertension showed that CgA levels and catecholamine storage in the adrenal medulla were reduced as compared with controls [168-170]. These data seem promising in considering CST as a possible treatment in systemic arterial hypertension, at least in patients with increased levels of circulating catecholamines. Actually, CST is a potent vasodilator also in human and is an anti-hypertensive agent [167-170]. Importantly, in patients with acute myocardial infarction an initial reduction with a subsequent increase in CST plasma levels has been recently reported [143].

We observed a non-significant trend toward reduction of infarct size with ischemic PostC in spontaneously hypertensive rats [171]. In the same model we have preliminary evidence that CST given in early reperfusion is more effective than ischemic PostC in inducing protection against infarct size [172], supporting the idea that the addition of CST plays a beneficial role in the presence of hypertension. The delayed increased levels of CST after infarction [143] are in keeping with an attempt of compensatory cardioprotective response against myocardial injury, which misses the early phase of reperfusion, when the majority of damages occur [51,92].

Vasostatins appear to function as endocrine/paracrine cardiac stabilizers, particularly in the presence of intense adrenergic stimuli, e.g., under stress responses. The “anti-adrenergic” action of vasostatins points to a counter-regulatory role of these CgA fragments in cardiovascular homeostasis, which may protect the heart against overstimulation at several levels [8,36]. An important role of vasostatins in normal and abnormal cardiac function is also supported by the finding that elevated levels of circulating CgA in patients with chronic heart failure represent an independent prognostic indicator of mortality and depend on the severity of the disease [162].

To the best of our knowledge, no other CgA derived peptides have been tested in the context of ischemia/reperfusion, cardioprotection and comorbidities. However, it would be worthwhile to test some of these peptides in these contexts. An interesting peptide may be the CgA dysglycaemic fragment pancreastatin. In fact, it has been suggested that pancreastatin, may be a component of a “zero steady-state error” counter-regulatory homeostatic mechanism [173]. Interestingly pancreastatin may be increased in hypertension, is involved in metabolic syndrome and affects both carbohydrate and lipid metabolism playing an important role in intermediary metabolism and disease [174].

Clearly more study is needed to identify which CgA derived peptides and interventions (pre or postconditioning with peptides) may be protective in the presence of comorbidities.

Conclusions

Interestingly VS-1 is a preconditioning inducer and CST a postconditioning agent. Delayed increased levels of CST after infarction [143] are in keeping with an attempt of compensatory cardioprotective response against myocardial injury. Early interventions such as pharmacological postconditioning, which target the first few minutes of reperfusion, may be clinically useful at the time of angioplasty, thrombolysis or cardiac surgery. Conceivably, CgA and its derived peptides influence may be multifunctional, being achieved not only *via* the nervous and sympathoadrenal systems, but also via direct timely protective mechanisms on cardiomyocytes. Importantly, both VS-1 [41,175] and CST [39] positively influence endothelial function, and this may be of pivotal

importance in organ protection. Our studies on cardioprotection also provide insights into the importance of the stimulus-secretion coupling of CgA and its spatio-temporal processing as an attempt of the cardiovascular system to protect itself against I/R damages and associated patho-physiological processes. All together, our results suggest that CgA derived peptides might represent a class of compounds dedicated to reduce cardiac reperfusion injury in a time dependent fashion. Future research on the mechanisms of action of the CgA-derived peptides, on their efficacy in the presence of comorbidities and on the elucidation of their receptors will also be important in developing these peptides as potential therapeutics against ischemia/reperfusion injury.

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LEGENDS

Figure 1. Flowchart depicting the main mechanisms of myocardial ischemia/reperfusion injury. In the hearts reactive oxygen species (ROS) production slightly increases during the initial part of ischemia until the O_2 is exhausted. Then sharply increases in reperfusion. Formation of mitochondrial permeability transition pores (mPTP) had been limited during ischaemia by the low pH despite increased cellular levels of ROS, Ca^{2+} and Pi overload. However, as pH returns to its baseline level and ROS formation increases prolonged opening occurs. The limited damages occurring during ischaemia are exacerbated by the prolonged mPTP opening which mediates irreversible cell damages in reperfusion. The

opening effect, besides Ca^{2+} overload, is also due to indirect effects, such as phospholipase A2 (PLA2) and calpain activations and consequent arachidonic acid release after membrane phospholipids degradation. A part mitochondrial membrane depolarization also inorganic phosphate (Pi) and lower levels of nitric oxide (NO) contribute to mPTP opening. Pore opening leads to cell-death through the release of pro-apoptotic factors as cytochrome c (Cyt c) and via ROS-induced ROS release (RIRR).

Figure 2. Scheme depicting the principal factors involved in cardioprotective pathways triggered by preconditioning. Activation of cell-surface receptors in response to an ischaemic conditioning stimulus recruits cGMP/PKG, RISK pathways. These signal transduction pathways, together with acidosis, activated at the time of reperfusion will crosstalk and will terminate on mitochondria to activate protective pathways. Akt: Serine/threonine protein kinase; cGMP/PKG: Cyclic guanosin monophosphate/protein kinase G; eNOS: Endothelial NO synthase; ERK1/2: Extracellular regulated kinase 1/2; GSK3β: Glycogen synthase kinase 3 β; MEK: Mitogen-activated protein kinase kinase; mPTP: Mitochondrial permeability transition pore; mitoKATP: mitochondrial ATP-dependent Kþchannels; NO: Nitric oxide; P70S6K: p70 ribosomal S6 protein kinase; PLC: phospholipase C; PI3K: Phosphoinositide 3-kinase; PKG: Protein kinase G; RISK: Reperfusion injury salvage kinases; ROS, reactive oxygen species; VS-1: vasostatin-1.

Figure 3. Scheme depicting the main elements of Reperfusion Injury Salvage Kinases (RISK) and Survivor Activating Factor Enhancement (SAFE) pathways. These two protective pathways are activate in the early reperfusion phase of both pre and postconditioning. SR, sarcoplasmatic reticulum; PLN, phospholamban. See text for further explanation

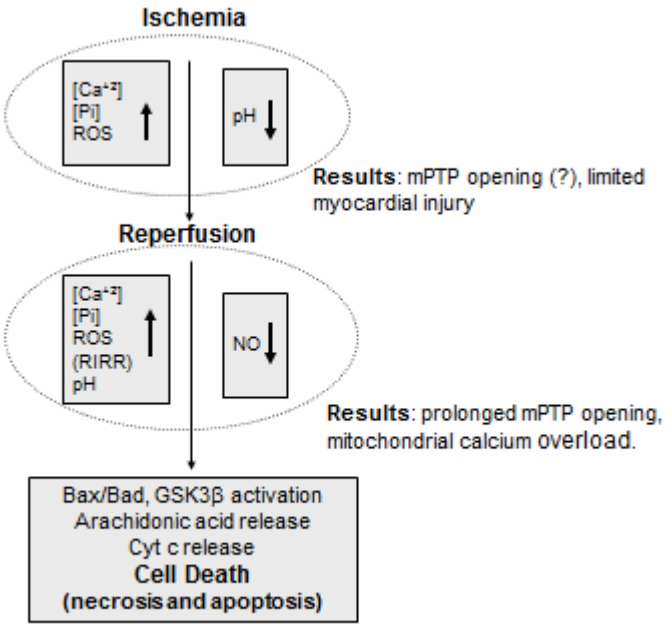
Ischemia
[Ca+2]
[Pi]
ROS
pH
Results: mPTP opening (?), limited myocardial injury
Reperfusion
[Ca+2]
[Pi]
ROS
(RIRR)
pH
NO
Results: prolonged mPTP opening, mitochondrial calcium overload.
Bax/Bad, GSK3β activation
Arachidonic acid release
Cyt c release
Cell Death
(necrosis and apoptosis)

Fig.1

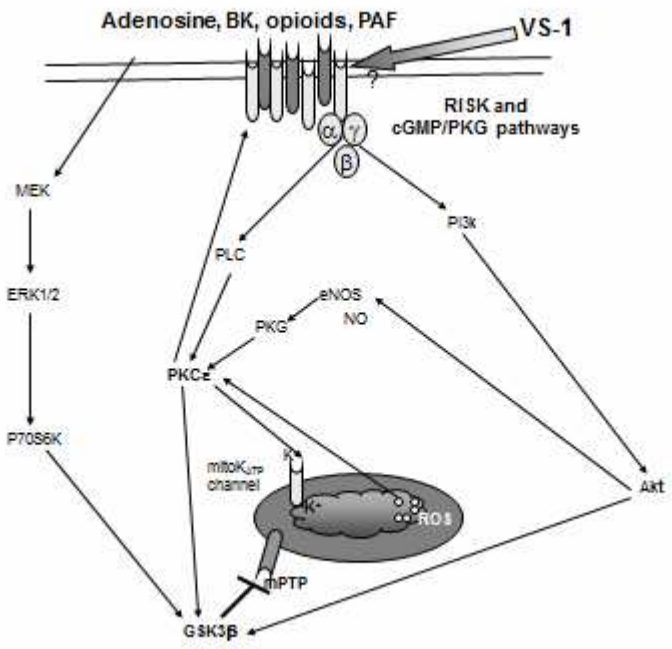
Adenosine, BK, opioids, PAF
VS-1
?
RISK and
cGMP/PKG pathways
α
γ
β
MEK
PI3k
PLC
ERK1/2
eNOS
NO
PKG
PKCε
P70S6K
mitoK$_{ATP}$
channel
K+
Akt
ROS
mPTP
GSK3β

Fig.2

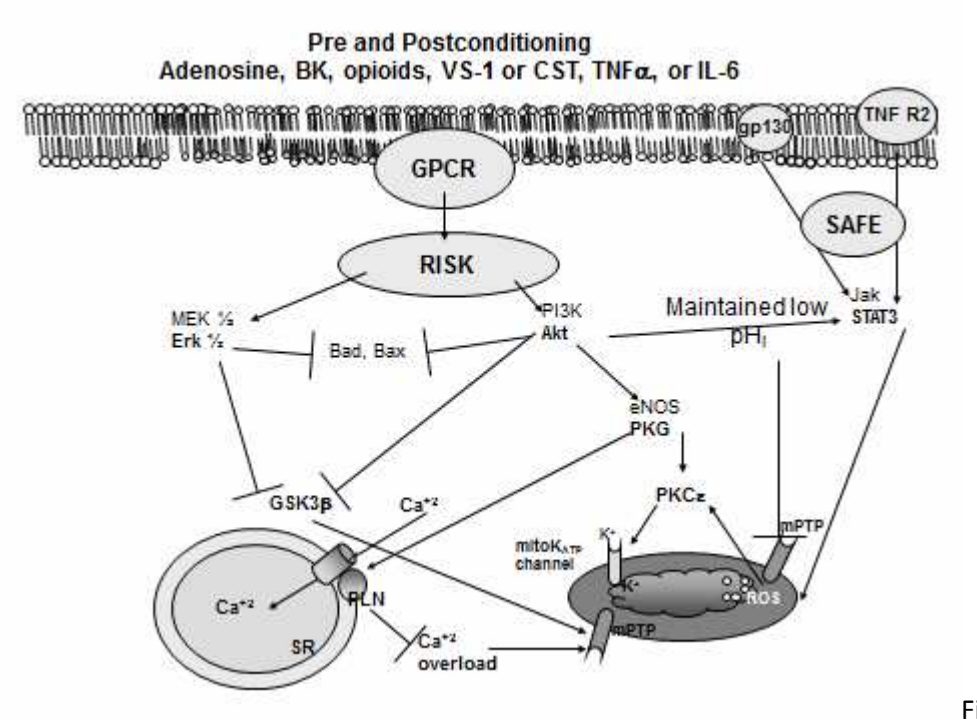
Pre and Postconditioning
Adenosine, BK, opioids, VS-1 or CST, TNFα, or IL-6
TNF R2
gp130
GPCR
SAFE
RISK
MEK ½
Erk ½
PI3K
Akt
Bad, Bax
Maintained low
pHi
Jak
STAT3
eNOS
PKG
GSK3β
Ca+2
PKCε
mPTP
mitoKATP
channel
K+
ROS
Ca+2
PLN
SR
Ca+2
overload
mPTP

Fig.3