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REVIEW

Chromogranin A and its fragments in cardiovascular, immunometabolic, and cancer regulationSushil K. Mahata^{1,2} and Angelo Corti³¹VA San Diego Healthcare System, San Diego, California. ²Metabolic Physiology & Ultrastructural Biology Laboratory, Department of Medicine, University of California, San Diego, La Jolla, California. ³IRCCS San Raffaele Scientific Institute, San Raffaele Vita-Salute University, Milan, Italy

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Chromogranin A (CgA)—the index member of the chromogranin/secretogranin secretory protein family—is ubiquitously distributed in endocrine, neuroendocrine, and immune cells. Elevated levels of CgA-related polypeptides, consisting of full-length molecules and fragments, are detected in the blood of patients suffering from neuroendocrine tumors, heart failure, renal failure, hypertension, rheumatoid arthritis, and inflammatory bowel disease. Full-length CgA and various CgA-derived peptides, including vasostatin-1, pancreastatin, catestatin, and serpinin, are expressed at different relative levels in normal and pathological conditions and exert diverse, and sometime opposite, biological functions. For example, CgA is overexpressed in genetic hypertension, whereas catestatin is diminished. In rodents, the administration of catestatin decreases hypertension, cardiac contractility, obesity, atherosclerosis, and inflammation, and it improves insulin sensitivity. By contrast, pancreastatin is elevated in diabetic patients, and the administration of this peptide to obese mice decreases insulin sensitivity and increases inflammation. CgA and the N-terminal fragment of vasostatin-1 can enhance the endothelial barrier function, exert antiangiogenic effects, and inhibit tumor growth in animal models, whereas CgA fragments lacking the CgA C-terminal region promote angiogenesis and tumor growth. Overall, the CgA system, consisting of full-length CgA and its fragments, is emerging as an important and complex player in cardiovascular, immunometabolic, and cancer regulation.

Keywords: pancreastatin; vasostatin; catestatin; cardiovascular diseases; immunometabolism; cancer

Introduction

In September 1965, Banks and Helle reported the release of proteins from the stimulated adrenal medulla.¹ In July 1967, Blaschko's group named splanchnic nerve-stimulated release of soluble proteins "chromogranins;"² and in September 1967, his group coined the term *chromogranin A* (CgA) for the major component of these proteins.³ Subsequently, it has been reported that patients suffering from neuroendocrine tumors, heart failure (HF), renal failure, hypertension, rheumatoid arthritis, and inflammatory bowel disease display elevated levels of circulating CgA-related polypeptides consisting of full-length molecules and fragments (Table 1). The human chromo-

granin A gene (*CHGA*; protein abbreviation hCgA) encodes a 439-amino-acid mature protein of approximately 48–52 kDa with a coiled-coil structure.^{4–8} Structurally, hCgA has nine dibasic sites and is proteolytically cleaved by prohormone convertases,^{9–11} cathepsin L,¹² plasmin,^{13–15} and kallikrein¹⁶ to generate biologically active peptides, including (1) the dysglycemic peptide pancreastatin (PST: hCgA_{250–301}),^{17,18} (2) WE14 (hCgA_{324–337}), which acts as the antigen for highly diabetogenic CD4⁺ T cell clones;^{19–21} (3) the vasodilating, antiadrenergic, and antiangiogenic peptide vasostatin-1 (hCgA_{1–76});^{22–26} (4) the antiadrenergic, antihypertensive, antibacterial, proangiogenic, antiobesogenic, and antiinflammatory peptide catestatin

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Table 1. Plasma CgA levels in health and diseases

Diseases/ abnormalities	Diseases (nM)	Controls (nM)	References
Cardiovascular diseases			
Controls		2.64	O'Connor ⁶⁰
Hypertension (primary)	4.04		
Hypertension (primary)	4.05		O'Connor ⁶¹
Hypertension (secondary)	3.93		
Controls		3.29	Cryer ²⁰⁷
Cardiac arrest	3.23		
Controls		1.46	Ceconi <i>et al.</i> ⁵²
CHF NYHA I	2.25		
CHF NYHA II	3		
CHF NYHA III	5.7		
CHF NYHA IV	11.4		
Controls		0.49	Omland <i>et al.</i> ⁵³
Myocardial infarction	0.61		
Controls		1.31	Pieroni <i>et al.</i> ⁴⁸
DCM	3.14		
HCM	3.07		
Controls		1.0	Larsen <i>et al.</i> ⁷¹
CHF NYHA II and III	2.1		
Controls		0.98	Tombetti ²⁰⁸
Takayasu arteritis	2.36		
Renal diseases			
Controls		0.57	O'Connor ²⁰⁹
Renal failure	7.99		
Controls		1.16	Spadaro ²¹⁰
Chronic renal failure	28.41		
Liver diseases			
Controls		0.57	O'Connor ²⁰⁹
Liver dysfunction	2.04		
Controls		1.16	Spadaro ²¹⁰
Liver cirrhosis	5.09		
Lung diseases			
Controls		2.51	Sobol ²¹¹
COPD	3.45		
Inflammatory bowel disease			
Controls		1.16	Spadaro ²¹⁰
IBD	4.46		
Rheumatoid arthritis			
Controls		1.13	Di Comite ²⁰³
Rheumatoid arthritis	5.79		
Sepsis			
Controls		4.86	Hsu ²¹²
Severe sepsis	9.89		
Controls		1.76	Zhang ²¹³
Severe sepsis	3.34		

Continued

Diseases/ abnormalities	Diseases (nM)	Controls (nM)	References
Controls		<4.0	Rosjo ²¹⁴
Sepsis survivors	9.1		
Sepsis nonsurvivors	14.0		
Meniere's disease			
Controls		0.67	Teggi ²¹⁵
Meniere's disease	1.46		
Erdheim–Chester disease			
Controls		0.72	Ferrero ²¹⁶
Erdheim–Chester disease	3.13		
Tumors/cancer			
Controls		2.64	O'Connor ⁶¹
Pheochromocytoma	32.99		
Controls		1.17	O'Connor ²¹⁷
Pheochromocytoma	15.04		
Carcinoid tumor	723.68		
Pancreatic islet tumor	101.39		
Thyroid carcinoma	11.53		
Parathyroid adenoma	4.46		
Controls		2.51	Sobol ²¹¹
Small cell lung carcinoma	16.66		
Large cell lung carcinoma	3.74		
Adenocarcinoma	3.68		
Controls		0.57	Hsiao ²¹⁸
Pheochromocytoma (familial)	2.41		
Pheochromocytome (sporadic)	3.76		
Controls		3.22	Cryer ²⁰⁷
Pheochromocytoma	6.81		
Controls		0.29	Blind ²¹⁹
Medullary thyroid carcinoma	16.48		
Controls		1.16	Spadaro ²¹⁰
Hepatocellular carcinoma	15.04		
Controls		1.19	Lee <i>et al.</i> ²²⁰
Pancreatic ductal adenocarcinoma	2.87		

(CST: hCgA_{352–372});^{27–41} and (5) the proadrenergic peptide serpinin (hCgA_{411–436}).^{42,43} Several of these CgA-derived peptides exhibit opposing (i.e., with regard to each other) regulatory effects. For example, cardiac contractility in rodents is controlled by vasostatin-1 and CST, which are antiadrenergic,^{36,44} as well as by serpinin, which is proadrenergic.⁴³ Likewise, angiogenesis is controlled by vasostatin-1 functioning in an antiangiogenic manner^{26,41} and

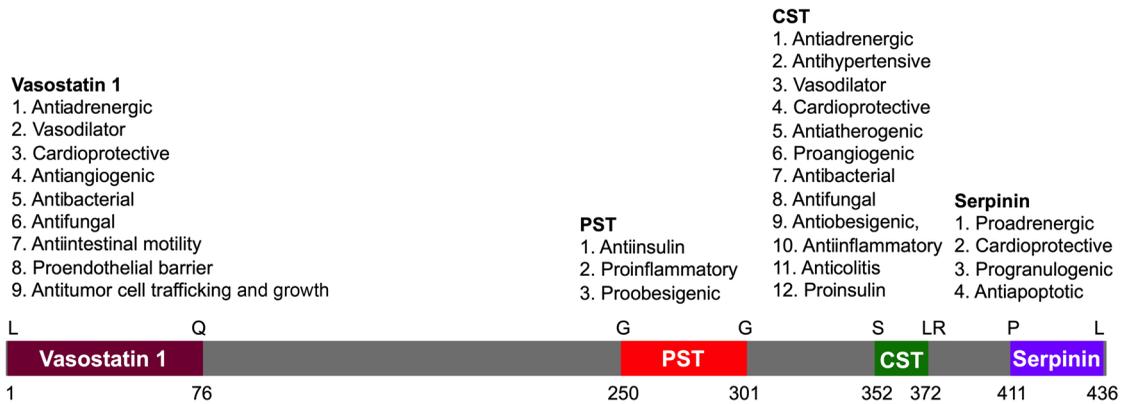


Figure 1. Schematic diagram showing the major peptide domains of CgA protein. Relative locations of vasostatin-1, pancreastatin (PST), catestatin (CST), and serpinin domains along with the first and last amino acids for each peptide domain are shown except for CST, where an arginine at position 373 is shown in addition to the last amino acid. Major functions of each of the peptide domains are shown above the peptide domain.

CST functioning in a proangiogenic manner^{35,41} (Fig. 1). CgA gene (*Chga*) knockout (KO) was generated by targeted deletion of exon 1 and approximately 1.5 kbp of its proximal promoter, resulting in complete elimination of *Chga* expression. *Chga*-KO mice provide an important model to study the roles of individual CgA-derived peptides through analysis of phenotypes after supplementation.^{33,40,45–47} Here, we focus on how full-length CgA and its peptides, including PST, vasostatin-1, CST, and serpinin, are important modulators of cardiovascular functions, immunometabolism, and cancer.

Cardiovascular functions

Circulating CgA in patients with cardiovascular diseases

Several studies have addressed the role of circulating CgA as a biomarker in patients with cardiovascular diseases using CgA-ELISA systems that were unable to discriminate between the full-length CgA and its fragments. Using these assays, increased levels of immunoreactive CgA-related polypeptides have been detected in the blood of patients with myocardial infarction, HF, decompensated and hypertrophic heart, and acute coronary syndromes, with important prognostic implications in several cases.^{48–55} Studies in patients with dilated and hypertrophic cardiomyopathy revealed that plasma CgA (detected by a homemade ELISA that was unable to discriminate the full-length CgA and

its fragments) strongly correlates with circulating B-type natriuretic peptide (BNP), an important diagnostic and prognostic marker of HF.⁴⁸ A later study performed in patients with acute destabilized HF showed that CgA adds independent prognostic information to N-terminal proBNP, another known prognostic marker of the disease, thus showing an improved prognostic ability of the combination of the two markers.⁵⁶ However, the prognostic value of CgA in acute and chronic myocardial failure is still a matter of debate.⁵⁷ Indeed, results of a trial conducted on a large group of patients with chronic HF (GISSI, *Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico*) showed CgA association with all-cause mortality or cardiovascular morbidity in univariate, but not multivariate, regression analysis adjusted for known risk factors.⁵⁴ Given the highly heterogeneous nature of the CgA antigen owing to differential post-translational modifications and proteolytic processing, and considering that CgA and its fragments can work as cardioregulatory hormones in certain cases with opposite functions (discussed below), the detection of specific CgA-related polypeptides with specific immunoassays is necessary to fully assess the prognostic value of the CgA system in cardiovascular diseases. This concept is well exemplified by the results of a recent study showing that serum levels of the hCgA_{1–113} fragment (known as vasostatin-2) are reduced in patients with ischemic chronic HF and are negatively, and independently,

associated with major adverse cardiac effects.⁵⁸ The role of CgA and its fragments on cardiovascular functions will be discussed below.

CgA in hypertension

O'Connor's group was the first to report that circulating levels of CgA are associated with hypertension. In 1984, they reported a normal plasma CgA level of 2.64 nM, which was increased by ~12-fold (32.99 nM) in pheochromocytoma patients,⁵⁹ as well as ~1.5-fold in hypertensive patients (4.04 nM).⁶⁰ His group also reported that plasma CgA levels are increased in both essential (primary) hypertension (4.05 versus 2.64 nM) and secondary hypertension (3.93 versus 2.64 nM).⁶¹ Since blocking sympathetic outflow by guanabenz resulted in reduced plasma CgA, it was suggested that plasma CgA is, at least in part, regulated by sympathetic nerve, and essential hypertension could represent an increased sympathoadrenal activity.⁶¹ Subsequently, increased CgA content was reported both in the adrenal gland^{62,63} and plasma.⁶³

CgA in HF

Characterized by defective ventricular filling or the ejection of blood, HF is caused by structural and functional defects in myocardium. The major pathogenic mechanisms involved in HF include increased hemodynamic overload, ischemia-associated dysfunction, ventricular remodeling, abnormal myocyte calcium handling, and excessive neurohumoral stimulation.⁶⁴ Longstanding hypertension is one of the major causes of HF; and most patients with HF show a history of hypertension.^{65,66} Augmented sympathetic nerve activity is also recognized as a major contributor to the complex pathophysiology of HF.^{67,68} Since sympathoexcitation has been reported to increase plasma CgA, studies were undertaken to evaluate the possible association of CgA with HF.^{61,69} A study was conducted with 103 healthy subjects and 160 consecutive elective patients (83.1% men; 16.9% women) with chronic heart failure (CHF), among which patients were classified in accordance with New York Heart Association (NYHA): NYHA I, NYHA II, NYHA III, and NYHA IV, to find out whether plasma level of CgA is increased in CHF subjects. Because of a nonnormal distribution, only median values were presented, which revealed that the increase in CgA in plasma was positively correlated with the severity of the dis-

eases (normal subjects, 1.46 nM; NYHA I, 2.25 nM; NYHA II, 3 nM; NYHA III, 5.7 nM; and NYHA IV, 11.14 nM).⁵² Of note, although CgA did not correlate with CHF-activated hormones, such as catecholamines, vasopressin, endothelins, and components of the renin-angiotensin-aldosterone system, it did correlate with the soluble tumor necrosis factor-alpha (TNF- α) receptors and, to a lower extent, with TNF- α itself.⁷⁰ Another study of 20 healthy volunteers and 25 men with stable CHF (NYHA II and NYHA III) also showed elevated plasma CgA (2.1 versus 1.0 nM controls);⁷¹ this study also confirmed a positive correlation between CgA and TNF- α , as well as between CgA and N-terminal atrial natriuretic peptide. Since plasma catecholamine levels were comparable, the results suggested that inflammatory responses contribute more to elevated plasma CgA in CHF than to activation of the sympathoadrenergic system.

To find out whether plasma CgA could serve as a prognostic marker for myocardial infarction, Omland's group measured plasma CgA in 119 patients (88 men; 31 women) on day 3 after the onset of myocardial infarction.⁵³ They found significantly higher plasma CgA levels in patients with symptoms and signs of HF (0.61 versus 0.49 nM); the mortality rate was significantly higher in patients with plasma CgA of >0.49 nM. They concluded that plasma CgA levels are associated with long-term mortality after myocardial infarction.⁵³ In a subsequent study in 217 patients with acute myocardial infarction complicated with HF or ventricular dysfunction, plasma CgA emerged as a strong and independent predictor of death and hospitalization.⁴⁹ In another study in 137 patients (94% men; 6% women) admitted to a single center with acute decompensated HF, CgA was found to exhibit comparable prognostic accuracy to N-terminal probrain natriuretic peptide (NT-proBNP) and provided further prognostic information after adjustment for other risk factors, including estimated glomerular filtration rate and NT-proBNP.⁵⁶

One of the largest studies evaluating the prognostic value of CgA in 1233 chronic HF patients (GISSI: 86.4% men and 13.6% women) found that plasma CgA concentrations are increased in proportion to disease severity.⁵⁴ The estimated probability of death was calculated to be the highest with CgA concentrations >33 U/L and lowest with CgA

concentrations <16 U/L. Univariate analyses found a correlation between baseline CgA levels with the two coprimary outcome measures in GISSI-HF: mortality and mortality of hospitalization for cardiovascular disease during follow-up. The study found significant correlations between plasma CgA levels and BNP and hs-C-reactive protein levels. However, multivariate analyses did not find significant association between elevated CgA levels and mortality or CgA levels and either mortality or cardiovascular morbidity during follow up. Thus, by these data, it was concluded that plasma CgA levels in patients with chronic, stable HF do not provide incremental prognostic information over that obtained from physical examination, routine biochemical analysis, and contemporary HF biomarkers.⁵⁴ By contrast, the larger study of acute coronary syndromes in a single coronary care unit of a Scandinavian teaching hospital (1268 patients; 70% men and 30% women) found CgA measured on day 1 to be an independent predictor of long-term mortality and HF hospitalizations, as well as providing incremental prognostic information above evaluation of conventional cardiovascular risk factors.⁵⁰ Of note, recurrent myocardial infarction was found not to be associated with plasma CgA levels after adjustments for conventional cardiovascular risk markers.⁵⁰

Full-length CgA and vasostatins

A growing body of evidence suggests that full-length CgA (hCgA₁₋₄₃₉) and its N-terminal fragments hCgA₁₋₇₆ and hCgA₁₋₁₁₃ are important players in the regulation of the cardiovascular system.^{72,73} These fragments were named vasostatin-1 and vasostatin-2, respectively, because of their ability to suppress vasoconstriction in isolated human conduit vessels, originally observed.^{22,74-76} After their discovery, a wide range of other biological effects were assigned to these polypeptides. For example, vasostatin-1 has been implicated in the homeostatic regulation of plasma calcium, vascular tension, endothelial barrier integrity, innate immunity, inflammation, pain relief, intestinal motility, intestinal barrier, and cardiac functions.^{55,74,76-78} Full-length CgA and vasostatin-1 can modulate the adhesion of cardiomyocytes, smooth muscle cells, and fibroblasts, and to proteins involved in the extracellular matrix

and endothelial cell-cell adhesion.⁷⁴ Interestingly, in certain cases, full-length CgA and fragments may exert opposite effects; for example, while vasostatin-1 can enhance the adhesion of fibroblasts to extracellular matrix proteins, full-length CgA inhibits cell adhesion.²⁵

Studies performed with isolated and perfused rat hearts have shown that recombinant hCgA₁₋₄₃₉ and vasostatin-1 can depress myocardial contractility and relaxation, counteract the β -adrenergic-induced positive inotropism, and modulate coronary tone mainly via a nitric oxide-dependent mechanism.^{72,73} Thus, these polypeptides can work as cardioregulatory hormones.

Vasostatin-2, which includes the vasostatin-1 (hCgA₁₋₇₆) and the vasoconstrictive-inhibitory factor sequence (hCgA₇₉₋₁₁₃, known to act as a vasoregulatory molecule when liberated by cleavage⁷⁹), increases coronary pressure in Langendorff-perfused rat hearts without affecting inotropism, but can counteract the cardiostimulatory effects of isoproterenol.⁴⁴ Using a rat model of myocardial infarction, other investigators have shown that vasostatin-2 can improve cardiac function and reduce remodeling, fibrosis, and inflammation, suggesting that the reduced levels of this fragment observed in patients with ischemic CHF may have a pathogenic role.⁵⁸

CgA and vasostatin-1 can exert cardioprotective effects against ischemia/reperfusion (I/R) injury.^{72,73} In this setting, vasostatin-1 can act as a preconditioning inducer.⁸⁰ Remarkably, hemodynamic and excitatory stimulation of rat hearts induce intracardiac CgA proteolytic processing to generate fragments containing the homologous vasostatin-1 sequence.⁸¹ A recent study has shown that full-length human CgA and vasostatin-1 can also prevent cardiotoxic events induced by doxorubicin, a widely used anticancer drug whose clinical application is hampered by dose-limiting cardiotoxicity.⁸² In particular, experimental evidence obtained in a rat model showed that low-dose hCgA₁₋₄₃₉ can protect the heart from inflammation, apoptosis, fibrosis, and ischemic injury caused by doxorubicin.⁸² Notably, this study also showed that pharmacologically active doses of hCgA₁₋₄₃₉ do not impair the antitumor activity of doxorubicin in various murine models of solid tumors. Doxorubicin could also reduce both intracardiac expression and plasma levels of CgA.⁸² It appears,

therefore, that doxorubicin can reduce the levels of this endogenous cardioprotective agent and that systemic administration of exogenous hCgA₁₋₄₃₉ can restore cardioprotection. Interestingly, exogenous vasostatin-1 could also exert cardioprotective effects on doxorubicin-induced cardiotoxicity in the same rat model.⁸² However, in this case, a higher dose, compared with that necessary for hCgA₁₋₄₃₉, was required to achieve cardioprotection, suggesting that the full-length molecule is more potent. Monitoring CgA and vasostatin-1 levels in cancer patients before and after doxorubicin therapy, the administration of low-dose full-length CgA to patients with low CgA levels might represent a novel approach to prevent doxorubicin-induced cardiotoxicity, thereby meriting further investigation.

Catestatin

Although initially identified as a physiological brake on catecholamine secretion,²⁷⁻³² CST is now established as a pleiotropic hormone that reduces blood pressure,^{33,83-87} positively regulates baroreflex sensitivity (BRS)⁴⁶ and heart rate variability (HRV),⁸⁸ and provides cardioprotection.^{36,39,89-91}

Vasodilatory and hypotensive effects of CST

The hypotensive effects of CST (bovine CgA₃₄₄₋₃₆₄) were first identified in pithed rats.⁸³ Intravenous injection of bCST resulted in the reduction of pressor responses to the activation of sympathetic outflow by electrical stimulation. bCST also blunted neuropeptide Y agonist-induced hypertension. The vasodepressor effects of bCST appeared to be mediated by massive release of histamine (21-fold increase) from mast cells, which is consistent with the vasodilatory effects of histamine.⁹² Using primary pleural and peritoneal cell populations containing 5% of mast cells, it was reported that bCST acts directly on mast cells to release histamine. The potency and efficacy of bCST were better evaluated when compared with the wasp venom peptide mastoparan.⁹³ In human mast cell line LAD2, hCST induced migration of peripheral blood-derived mast cells to migrate, and caused degranulation and release of leukotriene C₄ and prostaglandins D₂ and E₂.⁹⁴ hCST also caused increased intracellular Ca²⁺ mobilization and induced the production of proinflammatory cytokines/chemokines, such as granulocyte-macrophage colony-stimulating fac-

tor, chemokine C-C motif ligand 2 (CCL2, also known as MCP1), chemokine C-C motif ligand 3 (CCL3, also known as MIP1A), and chemokine C-C motif ligand 4 (CCL4, also known as MIP1B).⁹⁴ The vasodilatory effect of bCST was subsequently tested on human dorsal hand vein (to avoid systemic counterregulation) using hCST after pharmacologic vasoconstriction with phenylephrine, which showed dose-dependent vasodilation, especially in female subjects;⁸⁶ histamine-induced vasodilation is believed to be mediated through increased production of nitric oxide. In rodents, the hypotensive effects of hCST were reported both in monogenic (hypertensive and hyperadrenergic *Chga*-KO mice) and polygenic (blood pressure high mice) models of hypertension. In the monogenic model, hCST exerted its hypotensive effects by inhibiting the secretion of catecholamines³³ (Fig. 2).

Effects of CST on BRS and HRV

Consistent with diminished BRS in hypertensive subjects, hypertensive *Chga*-KO mice also display diminished BRS.⁴⁶ Intravenous administration of hCST increased BRS in hypertensive *Chga*-KO mice, where both the reflex bradycardia caused by phenylephrine-induced hypertension and the reflex tachycardia caused by sodium nitroprusside-induced hypotension were abolished or attenuated.⁴⁶ These findings indicate that hCST resets the entire autonomic nervous reflex arc to restore normal cardiovascular function.⁴⁶ Like BRS, hypertensive subjects display decreased HRV; and hypertensive *Chga*-KO mice also exhibit decreased HRV.⁸⁸ Intraperitoneal administration of hCST decreased total power (TP: ms²), which comprises low-frequency power (LF: 0.4–1.5 Hz), high-frequency power (HF: 1.5–4.0 Hz), and frequencies outside of low LF and HF power components, in the frequency domain of HRV spectra. hCST also improved each of the time domain parameters, such as mean difference in R–R between consecutive beats in ms (N–N), standard deviation of N–N in ms (SDNN), coefficient of variation of N–N (CVNN), % of consecutive beats more than 7 ms different in R–R (NN7%), reflecting cardiac parasympathetic tone, and root mean square of standard deviation of N–N in ms (RMSSD), which were decreased in *Chga*-KO mice.⁸⁸ Thus, hCST improves both BRS and HRV in *Chga*-KO mice, which represents a monogenic model of rodent hypertension (Fig. 2).

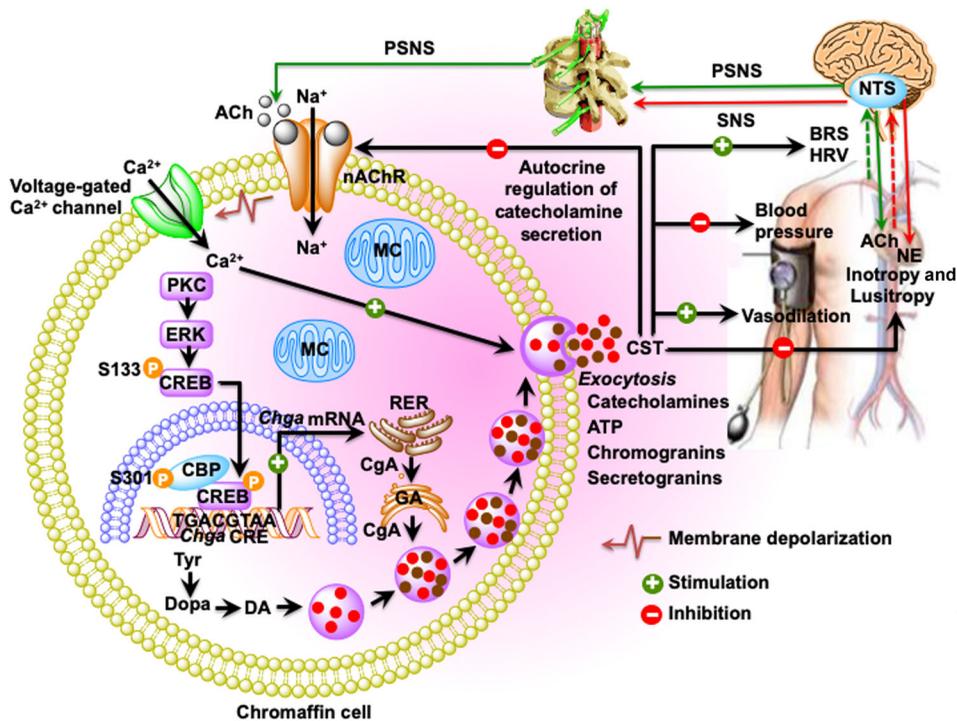


Figure 2. Schematic diagram showing the inhibition of catecholamine secretion and regulation of cardiovascular functions by CST. Acetylcholine released from preganglionic sympathetic endings at the splanchnic–adrenal synapse binds to nicotinic acetylcholine receptors and induces influx of Na^+ inside chromaffin cells resulting in the depolarization of the cell membrane and opening of voltage-gated Ca^{2+} channels. Increased cytosolic Ca^{2+} concentration stimulates the transcription of *Chga* and induces exocytotic release of the secretory cocktail containing catecholamines, CST, ATP, chromogranins, and neuropeptides. Exocytotically released CST inhibits further release of catecholamines by an autocrine/paracrine mechanism; decreases blood pressure and improves BRS and HRV by inhibiting catecholamine secretion; causes vasodilation by stimulating massive release of histamine; and decreases inotropy and lusitropy by activating the β_2 -AR- $\text{G}_{i/o}$ -protein-PI-3K-AKT-NO-cGMP signaling pathway. Ach, acetylcholine; BRS, baroreflex sensitivity; CA, catecholamine; CBP, CREB binding protein; CgA, chromogranin A protein; CREB, cAMP-responsive element-binding protein; DA, dopamine; ERK, extracellular signal-regulated kinase; GA, Golgi apparatus; HRV, heart rate variability; MC, mitochondria; NE, norepinephrine; NTS, nucleus tractus solitarius; PKC, protein kinase C; PSNS, peripheral sympathetic nervous system; RER, rough endoplasmic reticulum; SNS, sympathetic nervous system; Tyr, tyrosine.

Central effects of CST

CgA is ubiquitously expressed in mouse,^{30,95} rat,^{96–98} and sheep⁹⁹ central nervous systems. More precisely, CgA is expressed in the cell bodies of most anatomically defined inhibitory, excitatory, and aminergic cell groups of the brain and spinal cord. Barosensitive, bulbospinal neurons in the rostral ventrolateral medulla (RVLM) provide the major tonic excitatory drive to the sympathetic neurons that maintain arterial pressure.¹⁰⁰ Microinjection of a pharmacological dose of CST (rat CgA_{367–387}; 50 nL, 1 mM dissolved in PBS) into the RVLM of vagotomized normotensive rat resulted in sympathoactivation of up to 22% with

an increase in arterial blood pressure of up to 35 mmHg. rCST also increased sympathetic BRS with concomitant attenuation of somatosympathetic reflex, implicating rCST as a major player in the regulation of adaptive reflexes. The caudal ventrolateral medulla (CVLM), residing caudal to the RVLM, is the major source of the tonic GABAergic inhibition of presympathetic RVLM neurons. CVLM neurons inhibit immediate changes in blood pressure via the baroreflex and tonically blunt the activity of the presympathetic RVLM neurons by baroreceptor-independent mechanisms. The later effects play an important role in determining the long-term level of sympathetic vasomotor tone

and blood pressure.¹⁰⁰ Microinjection of rCST into the CVLM neurons of vagotomized normotensive rats caused excitation of CVLM neurons resulting in decreased mean arterial blood pressure (by ~23%) and attenuation of sympathetic baroreflex (by ~64%).¹⁰¹ By contrast, in spontaneously hypertensive (SHR) rats, infusion of hCST into the pyramidal neurons of the central amygdala exerted profound decrease in arterial blood pressure (by 65 mmHg).¹⁰² Like CVLM, central amygdaloid neurons are also GABAergic, send projections to RVLM, and inhibit excitatory outputs from RVLM with consequent decreases in arterial blood pressure. It thus appears that centrally CST acts as an excitatory peptide as opposed to its inhibitory action in the periphery.

The role of CST in cardioprotection

The first indication that CST acts as a cardioprotective peptide came from a Langendorff-perfused rat heart preparation study, where hCST inhibited both inotropy and lusitropy under basal and stimulated (β -adrenergic and endothelin) conditions.³⁶ These cardioprotective effects were shown to be mediated by the activation of the β_2 -AR-G_{i/o} protein-NO-cGMP pathways. In rat papillary muscles and isolated cardiomyocytes, hCST exerted antiadrenergic effects through the endothelial PI3K-AKT-eNOS pathway.¹⁰³ It was later shown that hCST provides cardioprotection by stimulating phosphodiesterase type-2 and NO-dependent S-nitrosylation.³⁹ SHR rats mimic human CHF and display decreased Frank-Starling response.¹⁰⁴ In both normotensive Wistar-Kyoto (WKY) and SHR rats, hCST caused a significant increase of the Frank-Starling response as evidenced by hCST-induced increase in left ventricular pressure (LVP) and the maximal rate of LV contraction (LV dP/dt max) with greater effects in SHR rats. hCST also resulted in decreased myocardial relaxation of both WKY and SHR rats as evidenced by CST-induced decrease in the maximal rate of LV relaxation (LV dP/dt min). A positive correlation was reported between hCST-induced improvement in myocardial mechanical response and protein S-nitrosylation. Thus, aged HF patients suffering from ventricular dilation or diminished autonomic heart modulation coupled with increased afterload and impaired Frank-Starling response could receive benefits from hCST.

hCST decreased I/R-induced infarct size by ~41%, which is comparable to the cardioprotection (~44% decrease in infarct size) achieved through a postconditioning mechanism.¹⁰⁵ Although data for attenuated I/R injury for hCST may seem promising, the data are based on experimental animal models that may not reflect human disease and previous clinical studies in the field have all had negative outcomes. Exogenous hCST (75 nM) at the onset of reperfusion resulted in attenuated postischemic rise of diastolic LVP (an index of contracture) and improved postischemic recovery of developed LVP. In addition, in isolated cardiomyocytes, hCST caused profound increase (by 65%) in cell viability after simulated I/R.¹⁰⁵ Subsequently, it was shown that cardioprotection by hCST in early reperfusion stage was achieved by the activation of mitochondrial ATP-sensitive K⁺ (mitoK_{ATP}) channels, ROS signaling, and the prevention of mitochondrial permeability transition pore opening via upstream PI-3K-AKT and PKC signaling¹⁰⁶ (Fig. 3). In isolated rat cardiomyocytes undergoing simulated I/R, hCST increased cell survival 65% and decreased cell contracture of I/R cardiomyocytes. The maintenance of mitochondrial membrane potential in I/R cardiomyocytes and increased phosphorylation of AKT at S473, GSK3 β at S9, PLB at T17, and eNOS at S1179 have been shown to play important roles in cardioprotection induced by hCST¹⁰⁷ (Fig. 3).

Effects of human variants of CST on rodent cardioprotection

Five single nucleotide polymorphisms have been discovered in the CST expressing region of *CHGA* in different human populations, namely, Gly364Ser (rs9658667), Pro370Leu (rs965868), Arg374Gln (rs9658669),³² Tyr363Tyr (rs9658666), and Gly367Val (rs200576557).^{108,109} The cardioprotective actions of WT-CST, Gly364Ser-CST, and Pro370Leu-CST were tested in isolated, Langendorff-perfused rat heart preparation, demonstrating a dose-dependent (11–200 nM) decrease in LVP (index of contractile activity), rate pressure product (index of cardiac work), and both positive and negative LV dP/dt (index of maximal rate of left ventricular contraction and relaxation, respectively), and increased coronary pressure by WT-CST. Gly364Ser-CST was ineffective on basal cardiac performance, and Pro370Leu-CST induced only negative inotropic activity. Human CST

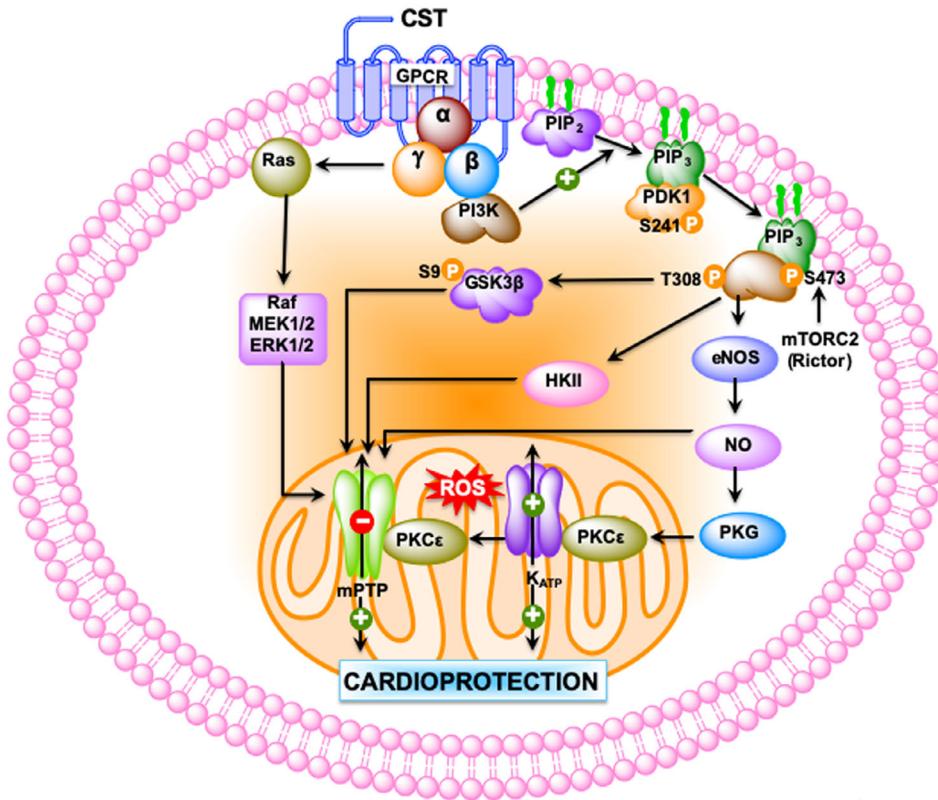


Figure 3. Schematic diagram showing the signaling pathways used by CST to provide cardioprotection. CST-induced cardioprotection begins by the activation of G protein-coupled or cytokine receptors and the consequent recruitment of signaling pathways: (1) the reperfusion injury salvage kinase (RISK) pathway, including PI-3K-AKT, ERK1/2, and the downstream target glycogen synthase kinase 3 beta (GSK-3 β); and (2) the PKG pathways. These salvage pathways activate downstream mediators, such as endothelial nitric oxide synthase (eNOS), GSK-3 β , hexokinase II (HKII), protein kinase C-epsilon (PKC ϵ), the mitochondrial ATP-dependent potassium channel (KATP) with consequent inhibition of mitochondrial permeability transition pore (mPTP). PDK1, pyruvate dehydrogenase kinase 1; ROS, reactive oxygen species.

variants also differed in the potencies with which they counteracted the positive inotropic and lusitropic effects of β -adrenergic stimulation by ISO, with WT-CST > Gly364Ser-CST > Pro370Leu-CST (against ISO-induced positive inotropism) and Gly364Ser-CST > WT-CST > Pro370Leu-CST (against ISO-induced positive lusitropism).³⁶

Effects of Gly364Ser-CST in BRS in humans

In humans, CST 364Ser variant caused profound changes in parasympathetic and sympathetic activity in individuals harboring the variant allele, which include an \sim 47% and \sim 44% increase in baroreceptor slope during upward and downward deflections, respectively, an \sim 2.4-fold increase in cardiac parasympathetic index, and an \sim 26% decrease

in cardiac sympathetic index in comparison with wild-type individuals.¹¹⁰ It has been proposed that heightened baroreflex control of circulation may increase the longevity of 364S carriers. However, in response to cold stress, the blood pressure response was significantly less in the Gly/Ser heterozygotes when compared with the WT homozygotes.¹¹⁰

Serpinin

In Langendorff-perfused rat heart and papillary muscle preparations, both serpinin and pGlu-serpinin cause dose-dependent (11–165 nM) increase in cardiac contractility and relaxation, with pGlu-serpinin being most potent and involving the β 1 adrenoceptor-adenylate cyclase-cAMP-PKA pathway.⁴³ This β 1-adrenergic agonist-like activity of serpinin is in sharp contrast with the

β 2-adrenoceptor depressive effects of vasostatin-1 and CST. It thus appears that serpinin is either mimicking intracardiac sympathetic neurotransmitters and/or circulating catecholamines.⁴³ Like CST, pGlu-serpinin also provides cardioprotection in both pre- and postconditioning mechanisms in WKY and SHR rats. While in the preconditioning mechanism, pGlu-serpinin caused ~50% decrease in I/R-induced infarct size, it caused an ~33% decrease in infarct size in a postconditioning mechanism of cardioprotection.¹¹¹ How CST (β 1-adrenergic antagonist) and pGlu-serpinin (β 1-adrenergic agonist) exerted comparable effects on cardioprotection by a postconditioning mechanism is yet to be addressed.

Immunometabolic functions of catestatin

Effects of CST on metabolism

The role of CST on metabolism came from a study in *Chga*-KO mice, which are deficient in CST and PST and display obesity on a normal chow diet.⁴⁰ Chronic hCST treatment was shown to decrease plasma triglyceride levels in *Chga*-KO mice, which is believed to be due to increased lipolysis as evident from increased plasma glycerol and non-esterified fatty acids.⁴⁰ This effect was mediated through inhibition of α_2 -adrenergic receptor (α_2 -AR) signaling. In *Chga*-KO mice, hCST showed tissue-specific effects on [¹⁴C]-palmitate incorporation into lipids: decreased incorporation in adipose tissue, but increased incorporation in liver. By contrast, hCST-induced oxidation of palmitate was comparable between adipose tissue and liver.⁴⁰ Based on the above findings, it was concluded that hCST inhibits the expansion of adipose tissue and promotes fatty acid uptake in liver for β -oxidation. Although hCST augmented expression of fatty acid oxidation genes, including carnitine palmitoyltransferase 1 α (*Cpt1a*), peroxisome proliferator-activated receptor- α (*Ppara*), acyl-CoA oxidase 1 (*Acox1*), and uncoupling protein 2 (*Ucp2*), it exerted no effect on the expression of lipogenic genes, such as sterol regulatory element-binding protein 1 (*Srebp1*) and peroxisome proliferator-activated receptor- γ (*Pparg*).⁴⁰ In view of the above findings, it was concluded that hCST stimulates the incorporation of fatty acids into triglycerides, but not *de novo* lipogenesis.⁴⁰ It thus appears that hCST mobilizes fat from adipose to liver for β -oxidation. In diet-induced obese

and insulin-resistant mice, hCST caused substantial decreases in hepatic triglyceride, NEFA, and ceramides concomitant with decreases in plasma TG and NEFA levels.¹¹²

Effects on CST on immunometabolism

The term *immunometabolism* was coined in 2011 to describe the multifaceted interactions between the metabolic and immune systems.¹¹³ Both the innate (macrophages, eosinophils, neutrophils, and innate lymphoid type 1 and 2 cells)^{114–124} and adaptive (CD8⁺ T cells, CD3⁺CD4⁺ T helper type 1 and type 2 cells, CD3⁺CD4⁺FOXP3⁺ T regulatory cells, invariant natural killer T cells, and B lymphocytes)^{125–132} immune cells control metabolism. The above studies were conducted in adipose tissues. The liver not only integrates nutrient, hormonal, and environmental signals to regulate glucose and lipid metabolism, but it also plays a key role as part of the immune system. It appears from the literature that cytokines, galectin-3, and eicosanoids (LTB4) released from immune cells affect hepatocyte insulin signaling resulting in insulin resistance.^{133–135} TNF- α , an inflammatory cytokine released from immune cells in the liver, potentiates steatosis by augmenting lipogenic activity and increasing biosynthesis of ceramides in liver.¹³⁶ Increased intrahepatocyte ceramide levels in turn cause insulin resistance by inhibiting AKT signaling pathways.¹³⁷ An intrahepatocyte proinflammatory program has also been shown to promote insulin resistance.^{138,139} The first report on the role of CST on immunometabolism came from a recent study in diet-induced obesity (DIO) and CST gene-KO mice, where hCST was shown to improve insulin sensitivity by decreasing intrahepatic ceramide levels and inhibiting infiltration of monocyte-derived macrophages (Ly6C⁺ proinflammatory macrophages) in liver. Chronic treatment of DIO mice with hCST resulted not only in the reduction of expression of proinflammatory genes (*Tnfa*, *Adgre1* (F4/80), *Itgam*, *Itgax*, *Ifng*, *Nos2*, and *Ccl2*), but also increased the expression of anti-inflammatory genes (*Il10*, *MglI*, *Il4*, *Arg1*, and *Mrc1*) in both Kupffer cells (resident macrophages) and recruited monocyte-derived macrophages (LY6C⁺ macs) (Fig. 4). In addition, hCST caused significant reduction in proinflammatory cytokines, including IL-1 β and TNF- α in plasma of DIO mice¹¹² (Fig. 4). In human monocyte-derived THP-1

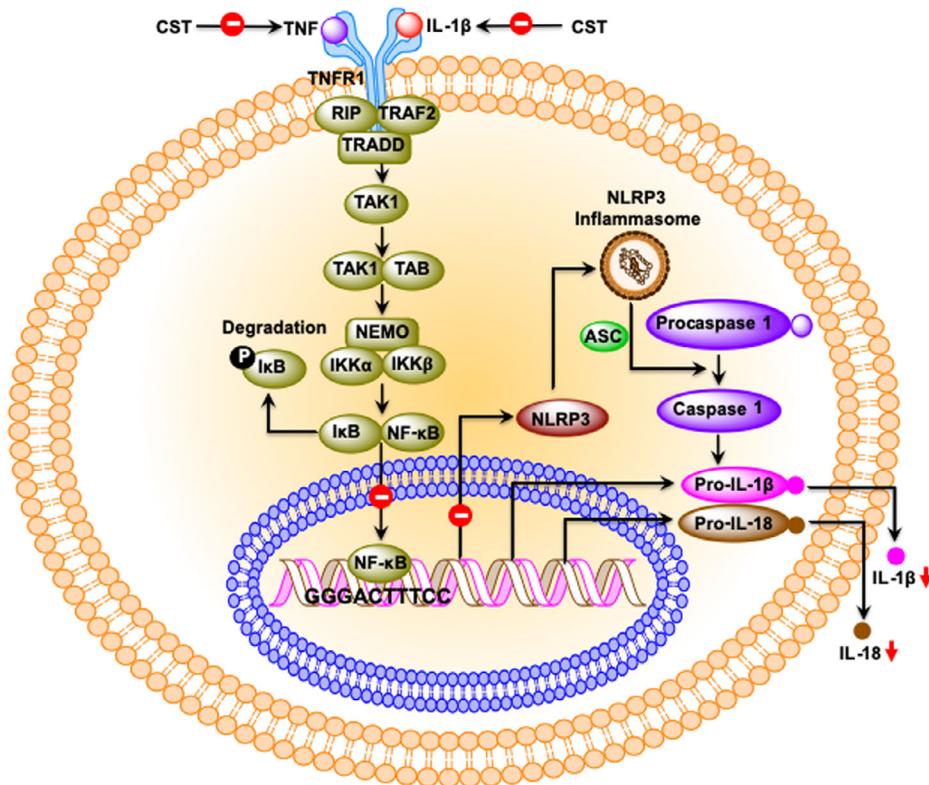


Figure 4. Schematic diagram showing the putative signaling pathways for the anti-inflammatory actions of CST. Since CST decreases plasma TNF- α levels, it is expected that less TNF- α will be available for binding to its receptor (TNFR1), resulting in the attenuation of the I κ B–NF- κ B signaling pathway leading to the translocation of fewer NF- κ B to the nucleus. This reduced NF- κ B signaling will affect the formation of NLRP3 inflammasome resulting in less cleavage procaspase 1 to active caspase 1 leading to less conversion of pro-IL-1 β and pro-IL-18 to IL-1 β and IL-18 and secretion of IL-1 β and IL-18.

cells, hCST also increased the expression of the anti-inflammatory gene *Mrc1*.¹⁴⁰

Immunometabolic functions of PST

Effects of PST on glucose-stimulated insulin secretion

PST has been reported to inhibit glucose-stimulated insulin secretion (GSIS) *in vivo* in mice, rats, dogs, and pigs, as well as *in vitro* from isolated rat islets.¹⁴¹ In the perfused rat pancreas model, PST (porcine CgA_{240–288}) was shown to inhibit unstimulated and stimulated insulin secretion.^{142–145} Consistent with the inhibitory role of PST in GSIS, GSIS was found to be ~1.7-fold higher in PST-deficient *Chga*-KO mice at 7 and 15 min after the administration of glucose.⁴⁵ Although PST is reported to inhibit glucagon secretion induced by low glucose,¹⁴⁶ it had no effect on somatostatin secretion.¹⁴⁷ Besides the

inhibition of GSIS, PST (hCgA_{273–301}) also inhibits insulin-stimulated glucose transport in primary rat and mouse adipocytes,^{45,148,149} differentiated 3T3-L1 adipocytes,^{150,151} and primary hepatocytes.^{45,152}

Effects of PST on hepatic glucose metabolism

pPST has been shown to inhibit insulin-stimulated glycogen synthesis in primary hepatocytes¹⁵³ and activates glycogenolysis in the rat liver, which implicates a direct anti-insulin effect on liver metabolism.^{154,155} pPST decreases the phosphorylation of insulin receptor substrate 2 at tyrosine residues through the activation of conventional PKC, resulting in the activation of gluconeogenesis. Consistent with the anti-insulin action, hPST also increases the production of NO with subsequent attenuated phosphorylation of protein kinase B (AKT), forkhead box protein O1 (FOXO1), and reduced matured sterol regulatory element-binding

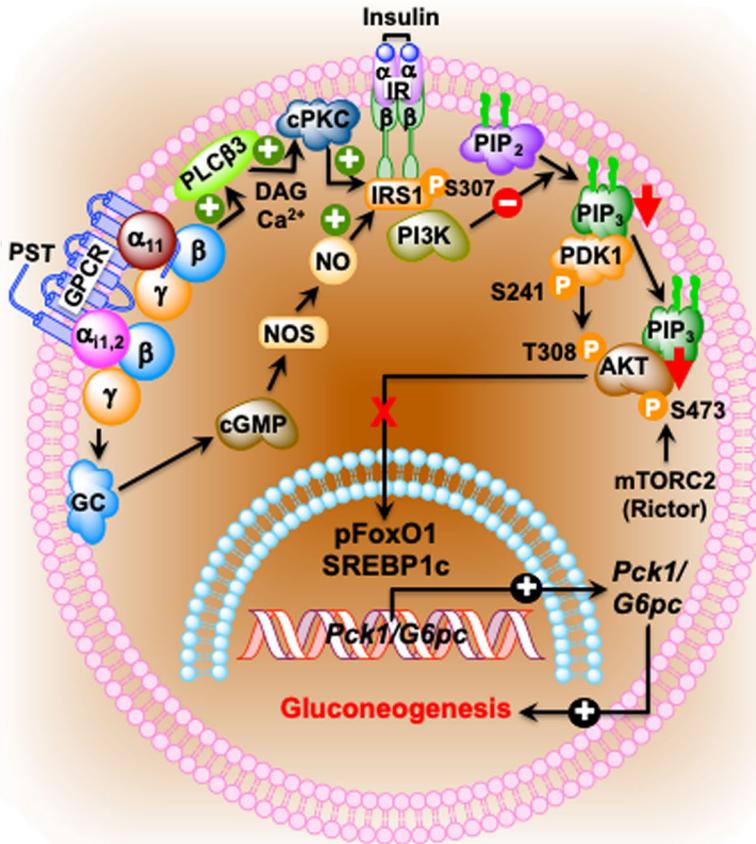


Figure 5. Schematic diagram showing the PST inhibition of gluconeogenesis in hepatocytes. PST initiates a GTP-binding protein-linked signaling cascade, which results in the activation of diacylglycerol (DAG) and calcium-dependent conventional PKC (cPKC), thereby attenuating IRS–PI3K–PDK1–AKT signaling pathway. In addition, the stimulation of the cGMP–NOS pathway also dampens this signaling pathway by nitrosylation of IRS. Suppression of this pathway by PST allows FoxO1 and SREBP1c to stimulate the expression of gluconeogenic genes, *Pck1* (also known as *Pepck*) and *G6pc* (also known as *G6Pase*), and thus prevents insulin action.

transcription factor 1c (SREBP1c),⁴⁵ thereby promoting gluconeogenesis (Fig. 5).

Effects of PST on lipid metabolism

In addition to pronounced effects on glucose metabolism, PST also modulates lipid metabolism. PST (rat CgA_{263–314}) decreases insulin-stimulated synthesis of lipids in rat adipocytes.¹⁵⁶ This finding is consistent with the hPST-dependent increased expression of hepatic lipogenic genes in *Chga*-KO mice, including *Srebp1c*, peroxisome proliferator-activated receptor- γ (*Pparg*), and glycerol-3-phosphate acetyltransferase (*Gpat*).⁴⁵ rPST has also been shown to stimulate the release of glycerol and free fatty acids from rat adipocytes, which is com-

pletely inhibited by insulin.¹⁵⁶ In humans, hPST augments free fatty acid efflux into the circulation, resulting in an overall spillover of ~ 4.5 -fold, which is consistent with the reported lipolytic action of rPST,¹⁵⁶ confirming the anti-insulin effects of PST.

Effects of PST on inflammation and insulin resistance

The hallmarks of insulin resistance in DIO mice include obesity, hyperinsulinemia, and increased inflammation.^{133,157–161} Therefore, the suppression of inflammation in DIO mice has been shown to improve insulin sensitivity.^{162–164} hPST treatment of *Chga*-KO-DIO mice resulted in increased expression of the proinflammatory genes, including

interleukin-1-beta (*Il1b*), TNF- α (*Tnfa*), interleukin-6 (*Il6*), chemokine C-C motif ligand 2 (*Ccl2*), and nitric oxide synthase 2a (*Nos2*), thereby underscoring the proinflammatory nature of PST. Furthermore, the treatment of *Chga*-KO-DIO mice with hPST caused significant reduction in the expression of *Arg1* and *Il10*. Consistent with gene expression data, *Chga*-KO-DIO mice show decreased plasma levels of IL-12p70, IFN- γ , and chemokine C-C motif ligand 3-like 1 (CCL3L1), IL-6, and chemokine C-X-C motif ligand 1 (CXCL1) compared with WT-DIO mice.

Regulation of angiogenesis and tumor growth

Circulating CgA in patients with neuroendocrine and nonneuroendocrine tumors

Experimental evidence suggests that physiological levels of circulating CgA and its fragments contribute to regulate the vascular physiology in normal and neoplastic tissues.¹⁶⁵ CgA is exocytotically released in circulation by the neuroendocrine system to reach, in healthy subjects, about 0.2–1 nM levels.¹⁶⁵ This fact implies that tumors with mature and functional blood vessels, either neuroendocrine or nonneuroendocrine, are exposed to physiological amounts of blood-borne CgA mainly derived from the diffuse neuroendocrine system. Furthermore, although many patients show normal levels of plasma CgA, some patients may have higher levels for a variety of reasons. For example, patients treated with proton pump inhibitors—a class of drugs known to induce gastric enterochromaffin-like cell hyperplasia and CgA production—may have increased levels of circulating CgA.^{166–169} Increased levels of immunoreactive CgA have been also detected in the blood of patients with renal failure, HF, hypertension, atrophic gastritis, inflammatory bowel disease, rheumatoid arthritis, sepsis, and other inflammatory diseases.⁵⁵ Thus, in patients with nonneuroendocrine neoplasms, the tumor vasculature is exposed to variable levels of CgA derived from the diffuse neuroendocrine system.

The situation is even more complex in the case of neuroendocrine tumors or tumors with neuroendocrine differentiation, as in both these cases the tumor vasculature can be exposed not only to CgA molecules released by neuroendocrine sys-

tem into the blood, but also to CgA released in tumor microenvironments (and then into the blood) by neoplastic cells. Indeed, cancer cells in neuroendocrine tumors (including pheochromocytomas, carcinoid tumors of the intestine, lung and stomach, parathyroid and medullary thyroid carcinomas, neural tumors, anterior pituitary tumors, small cell lung cancer, pancreaticoduodenal tumors, and many others) can express and secrete CgA in relatively large amounts.¹⁶⁷ Also, tumors presenting the focal expression of CgA consequent to neuroendocrine differentiation (such as breast and prostate cancer, non-small cell lung cancer, and gastric and colonic adenocarcinomas) may also release CgA.^{7,167} Thus, the tumor vasculature, in these cases, might be affected by CgA present in the blood and by tumor-derived CgA in very complex ways.

In this regard, it is important to highlight the fact that detection of full-length CgA and fragments using specific ELISA systems based on selective antibodies has shown that CgA, either systemically or locally released, consists of variable mixtures of intact protein and fragments, including full-length CgA (hCgA_{1–439}), the vasostatin-1 fragment hCgA_{1–76}, fragments lacking part or the entire C-terminal region, such as hCgA_{1–373}, and other shorter peptides.^{41,170} For example, hCgA_{1–373}, which is either not or minimally present in the blood of healthy subjects, is increased in peripheral blood and bone marrow plasma of patients with multiple myeloma, while full-length CgA is decreased in these patients.¹⁷⁰ A central issue is whether changes in the circulating levels of full-length CgA and its fragments (absolute or relative), which may occur in cancer patients, are epiphenomena of the presence of the tumors or are contribute to tumor pathophysiology. In the following sections, we will discuss the experimental evidence suggesting that CgA and its fragments contribute to regulate tumor growth and responses to therapy.

Role of full-length CgA and vasostatin-1 in tumor angiogenesis and growth

It is well known that the unbalanced production of anti- and proangiogenic factors in tumors can lead to vascular abnormalities that contribute to tumor cell proliferation, invasion, trafficking, and metastasis formation.^{171–173} CgA may be one of the factors that contributes to regulate tumor vascular biology.^{76,165} For example, recent studies have

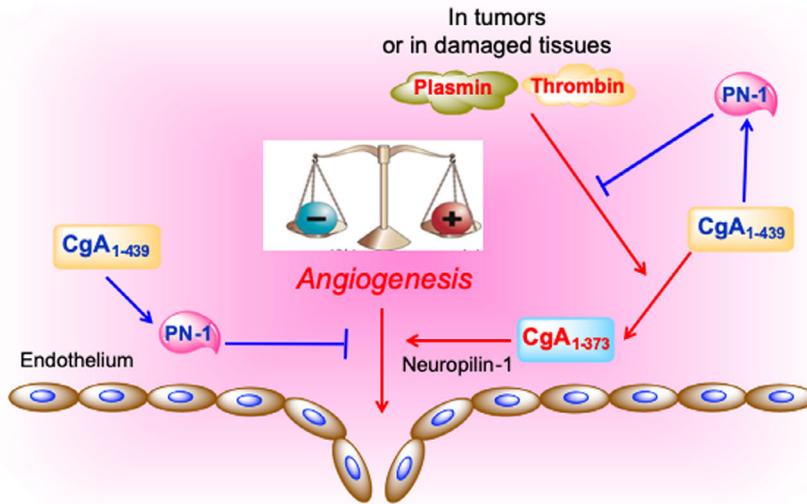


Figure 6. Hypothetical model of the CgA-dependent angiogenic switch. According to this model, in normal conditions, full-length CgA₁₋₄₃₉ exerts antiangiogenic effects by inducing protease nexin-1 (PN-1), an antiangiogenic protein. Since PN-1 is also a potent inhibitor of plasmin and thrombin, this protein can also prevent CgA cleavage by these enzymes and preserve its antiangiogenic activity. In damaged tissues or tumors, the balance of protease/antiprotease activity is altered leading to CgA cleavage and formation of fragments, for example, CgA₁₋₃₇₃, capable of interacting with neuropilin-1 on endothelial cells (via its PGPQLR sequence) and stimulating angiogenesis (see text).

shown that full-length CgA (hCgA₁₋₄₃₉) can function as an antiangiogenic factor, with a functional antiangiogenic site in the C-terminal region 410–439 and a latent (or less active) site in the N-terminal region 1–76 (vasostatin-1), which requires proteolytic cleavage for full activation.⁴¹ Full-length CgA and vasostatin-1 can inhibit endothelial cell migration, motility, invasion, and capillary-like structure formation induced by vascular endothelial growth factor (VEGF) or fibroblast growth factor-2 (FGF2), two important proangiogenic factors.^{41,174} Vasostatin-1 can also inhibit the nuclear translocation of hypoxia inducible factor-1 α , a master regulator of angiogenesis, in endothelial cells.¹⁷⁵ These proteins can enhance the endothelial barrier function and reduce vascular leakage and trafficking of tumor cells through the endothelium.^{176,177}

Studies performed in mice have shown that endogenous murine CgA or exogenous recombinant hCgA₁₋₄₃₉, but not the fragments lacking the C-terminal region, can inhibit tumor growth in models of nonneuroendocrine solid-tumors, including fibrosarcoma, mammary adenocarcinoma, Lewis lung carcinoma, and primary and metastatic melanoma.¹⁷⁸ The inhibition of tumor growth is associated with reduction of microves-

sel density and blood flow in neoplastic tissues. Also, low-dose vasostatin-1 can delay tumor growth and reduce microvessel density in murine models of solid tumors.⁴¹ In these models, neutralization of endogenous murine CgA with antibodies against its C-terminal region enhances tumor growth rate, whereas systemic administration of low amounts of hCgA₁₋₄₃₉ reduces tumor growth.¹⁷⁸ It appears, therefore, that circulating full-length CgA and vasostatin-1 can work as physiological inhibitors of tumor growth.

Mechanistically, full-length CgA induces the production of protease nexin-1 in endothelial cells, a serine protease inhibitor (serpin) that can inhibit endothelial cell migration, capillary tube formation, and angiogenesis, independently of its serpin activity^{179,180} Given that the neutralization of protease nexin-1 with specific antibodies can suppress the antiangiogenic and antitumor effects of recombinant hCgA₁₋₄₃₉, it is possible that the induction of protease nexin-1 by CgA, as observed in animal models, is an important mechanism underlying its antiangiogenic and antitumor activities (Fig. 6). Furthermore, considering that protease nexin-1 is also a potent inhibitor of plasmin and thrombin,¹⁸¹ two proteolytic enzymes often present in tumors,

and that CgA can be cleaved by these enzymes,^{41,170} this inhibitor might also serve to prevent the local cleavage of CgA, thereby preserving its antiangiogenic activity (Fig. 6). In view of the inhibitory activity of circulating full-length CgA on angiogenesis and tumor growth, and considering that cleavage of its C-terminal region markedly reduces its activity, it is reasonable to hypothesize that pathophysiological changes of circulating levels of full-length CgA and/or its fragmentation may contribute to the regulation of disease progression in patients with non-neuroendocrine tumors.

CgA typically affects tumor growth with a U-shaped dose–response curve.¹⁷⁸ This implies that a marked increase of its concentration may paradoxically result in a reduced effect. For this reason, the effects of CgA on neuroendocrine tumors could be different from those observed in murine models of nonneuroendocrine tumors (and difficult to predict), considering the high concentration that CgA can reach in the tumor microenvironment because of the secretory activity of neuroendocrine cancer cells. Furthermore, one cannot exclude the possibility that the tumor-derived CgA might be fragmented in a different manner, compared with CgA released in the blood by the neuroendocrine system. Thus, although CgA is a widely used serological and histopathological marker for neuroendocrine tumor diagnosis and follow-up, development of appropriate models is necessary to address the functional role of CgA and its fragments in patients with such tumors.

Role of CgA_{1–373} in tumor angiogenesis and growth

Recent studies have shown that a human CgA fragment encompassing residues 1–373 (hCgA_{1–373}; thus lacking the C-terminal region) promotes angiogenesis by inducing the release of FGF2 from endothelial cells.^{35,41,182} Notably, hCgA_{1–373}, hCgA_{1–76}, and hCgA_{1–439} can counterbalance the activity of each other in angiogenesis assays,¹⁸³ suggesting that these proteins may provide a balance of pro- and anti-angiogenic activities tightly regulated by proteolysis. The transition from anti- to pro-angiogenic forms, which can be induced by limited digestion of CgA with plasmin or thrombin,^{41,183} occurs in the bone marrow of multi-

ple myeloma patients and correlates with increased tumor microvessel density.¹⁷⁰

Studies aimed at elucidating the proangiogenic mechanisms of hCgA_{1–373} and its role in tumor progression have shown that cleavage of the R₃₇₃R₃₇₄ dibasic site of CgA leads to the exposure of the PGPQLR₃₇₃ epitope (100% conserved in mouse and human CgA), and that this is crucial for tumor progression in various murine models of nonneuroendocrine tumors.¹⁸⁴ Indeed, blockade of the PGPQLR site of murine CgA with specific antibodies, unable to recognize full-length CgA, inhibits tumor growth in various experimental models.¹⁸⁴ Blockade of the PGPQLR site with antibodies induces changes in the tumor vascular bed and blood flow in murine fibrosarcomas, suggesting that circulating CgA might work as an “off/on” switch for tumor angiogenesis, when cleaved at the PGPQLR site. Thus, fragmentation of circulating CgA in tumors by local proteases may contribute to “switch on” angiogenesis, and changes in the balance of protease/antiprotease molecules in tumor tissues might represent major mechanisms for regulating the CgA activity (Fig. 6).

Mechanistic studies have shown that hCgA_{1–373}, but not hCgA_{1–439}, can bind neuropilin-1 with good affinity ($K_d = 3.49 \pm 0.73$ nM), suggesting that this protein is an important receptor for hCgA_{1–373}. In particular, the PGPQLR site of hCgA_{1–373}, but not that of hCgA_{1–439}, can bind the VEGF binding-site of neuropilin-1.¹⁸⁴ It appears, therefore, that the PGPQLR binding site is cryptic in the full-length molecule or that this region undergoes profound conformational changes upon cleavage of CgA at the R₃₇₃R₃₇₄ dibasic site. No binding to neuropilin-1 occurs with hCgA_{1–372}, a fragment lacking R₃₇₃, suggesting that the C-terminal arginine of PGPQLR site (R₃₇₃) is crucial for binding¹⁸⁴ (Fig. 6). Considering that the interaction between fragmented CgA and neuropilin-1 is important for tumor growth, the PGPQLR site may be a potential therapeutic target.

Neuropilin-1 is an important coreceptor of various protein factors that regulate vascular permeability and angiogenesis, such as semaphorins and VEGF. The C-terminal sequence of VEGF (CDKPRR), which contains an R/K-X-X-R/K C-end rule motif (CendR), can recognize a pocket in the b1 domain of neuropilin-1, a site that can also accommodate other peptides containing the CendR motif;^{185,186} in addition, the PGPQLR

site of hCgA₁₋₃₇₃ can interact with this pocket of neuropilin-1.¹⁸⁴ Of note, while both hCgA₁₋₃₇₃ and VEGF can bind neuropilin-1 with similar affinity, only VEGF can bind neuropilin-2.¹⁸⁴

It is possible that hCgA₁₋₃₇₃ engages other coreceptors for signaling. The hCgA₃₅₂₋₃₇₂ sequence (catestatin), for example, is known to bind the acetylcholine nicotinic receptor (nAChRs).^{85,187,188} Considering that this class of receptors is expressed on endothelial cells and known to regulate angiogenesis,¹⁸⁹⁻¹⁹² nAChRs might be potential targets. This view is supported by the observation that mecamylamine and α -bungarotoxin, two nAChRs antagonists, can inhibit the proangiogenic activity of hCgA₁₋₃₇₃ in a human umbilical vein endothelial cell (HUVEC) spheroid assay.¹⁸⁴ Thus, these receptors, in addition to neuropilin-1, might play a role in the proangiogenic activity of hCgA₁₋₃₇₃.

The R₃₇₃ residue of hCgA₁₋₃₇₃ can be rapidly removed by a plasma carboxypeptidase,¹⁸⁴ resulting in the loss of neuropilin-1 binding and proangiogenic activity and gain of antiangiogenic activity. Interestingly, subnanomolar concentrations of hCgA₁₋₃₇₂ (antiangiogenic) can inhibit the activity of subnanomolar levels of hCgA₁₋₃₇₃ (proangiogenic), but not that of VEGF and FGF2.¹⁸⁴ Thus, the transition of hCgA₁₋₃₇₃ to hCgA₁₋₃₇₂ may be a specific counter-regulatory mechanism that serves to limit the activity of hCgA₁₋₃₇₃ at the site of production in tumors. Another potential counter-regulatory mechanism induced by an excess of hCgA₁₋₃₇₃ could be related to its biphasic behavior, as suggested by its bell-shaped dose-response curve observed in angiogenesis assays.^{41,178,184} The mechanisms underlying this behavior are unknown.

In summary, the cleavage of the R₃₇₃R₃₇₄ site of CgA in tumors and the subsequent removal of R₃₇₃ in plasma may provide an important on/off switch for spatiotemporal regulation of angiogenesis in tumors and normal tissues. The interaction of the PGPQLR site with neuropilin-1 might be, therefore, an important target for angiogenesis inhibition and tumor growth suppression.

Role of full-length CgA and vasostatin-1 in tumor cell trafficking

Cancer progression involves the seeding of malignant cells in circulation and the colonization of distant organs. Circulating neoplastic cells can

also reinvade the tumor of origin in a process referred to as *tumor self-seeding*.¹⁷⁷ A study performed in murine models of solid tumors has shown that a modest increase of circulating CgA, as obtained in mice after the administration of omeprazole (a proton pump inhibitor) or low-dose recombinant hCgA₁₋₄₃₉, can reduce tumor self-seeding and dissemination.¹⁷⁷ In particular, the study showed that hCgA₁₋₄₃₉ can inhibit shedding of cancer cells in circulation, tumor reinvagination by circulating cells, and lung colonization in murine models of mouse mammary adenocarcinomas and melanomas. Mechanistic studies showed that hCgA₁₋₄₃₉ can reduce endothelial cell gap formation and vascular leakage induced by tumor-derived factors in tumors, as well as the transendothelial migration of cancer cells.^{176,177} Thus, circulating full-length CgA, besides reducing angiogenesis in tumors, can enhance endothelial barrier function in both tumors and normal tissues thereby inhibiting the trafficking of tumor cells via tumor-to-blood and blood-to-tumor/normal tissue routes (i.e., metastasis and tumor self-seeding processes).^{176,177} Similar effects have been reported for vasostatin-1.¹⁷⁷

A role for circulating CgA in cancer cell trafficking has also been investigated in chronic lymphocytic leukemia (CLL), a hematological cancer characterized by the progressive accumulation of CD5⁺ leukemic B cells in peripheral blood, lymphoid tissues, and bone marrow, and by the continuous trafficking of these cells among these tissue compartments.¹⁹³ Physiologically relevant doses of recombinant hCgA₁₋₄₃₉ have been shown to reduce the bone marrow/blood ratio of leukemic cells in the E μ -TCL1 murine transgenic CCL model, and decrease bone marrow, kidney, and lung infiltration in *Rag2*^{-/-} γ c (*Il2rg*)^{-/-} mice engrafted with human MEC1 CLL cells.¹⁷⁰ This treatment also reduced the loss of body weight associated with disease progression and improved animal motility. hCgA₁₋₄₃₉ was found to enhance the integrity of the endothelial barrier and to reduce the transendothelial migration of MEC1 cells with a bimodal dose-response curve. Vasostatin-1, but not hCgA₁₋₃₇₃, could inhibit CLL progression in the xenograft model, suggesting that the N-terminal domain contains a site activated by proteolytic cleavage, and that the C-terminal region is crucial for CgA activity.¹⁸³ It is therefore possible that CgA and

its fragments contribute to regulate leukemic cell trafficking and tissue infiltration in patients with CLL.

Full-length CgA and response to anticancer therapy

The efficacy of chemotherapy in cancer patients is often limited by inadequate and uneven penetration of drugs in tumor tissues.^{194,195} This phenomenon depends on the abnormal vasculature and altered tissue composition typically present in solid tumors, which may cause irregular blood flow, heterogeneous permeability, increased interstitial fluid pressure, vascular compression and, consequently, uneven drug penetration.^{195–197} We have shown that NGR-TNF, a derivative of TNF- α that targets tumor blood vessels and enhances vascular permeability, improves drug diffusion into neoplastic tissues in various animal models and patients.^{197–200} A recent study in patients with primary lymphomas of the central nervous system has shown that this drug can break the blood–brain barrier in tumor and peritumoral areas and enhance the response to chemioimmunotherapy.²⁰¹

Studies performed in murine lymphoma and melanoma models have shown that pathophysiologically relevant doses of hCgA_{1–439} are sufficient to reduce the NGR-TNF-induced penetration of doxorubicin in tumors by enhancing the endothelial barrier function.¹⁷⁷ *In vitro* studies, performed with human endothelial cell monolayers, showed that hCgA_{1–439} can inhibit the disassembly of VE-cadherin dependent adherence junctions and permeability to drugs induced by NGR-TNF. Similar effects in animal models were observed with vasostatin-1.¹⁷⁷ *In vitro*, this fragment can inhibit various effects exerted by TNF- α on endothelial cells, such as p38-MAP kinase phosphorylation, VE-cadherin redistribution, gap formation, VEGF- and thrombin-induced endothelial cell permeability, and paracellular transport of macromolecules.^{174,176,202,203} Thus, both full-length CgA and vasostatin-1 are efficient inhibitors of TNF- α -induced alteration of the endothelial barrier function and permeability.

It is therefore possible that increased levels of circulating CgA and its fragments, as may occur in the subpopulation of cancer patients with non-neuroendocrine tumors, can reduce drug delivery to tumor cells, particularly when they are combined

with NGR-TNF. Of note, daily treatment of mice with omeprazole, a proton pump inhibitor commonly used in the treatment of acid peptic disorders, increased the circulating levels of CgA by twofold and inhibited the penetration of doxorubicin induced by NGR-TNF in tumors.¹⁷⁷ Thus, even a modest enhancement of CgA levels is sufficient to inhibit the synergism of NGR-TNF with chemotherapy. Considering the wide use of proton pump inhibitors in patients, these findings may have important clinical implications. Therefore, monitoring CgA and its fragments might help to select patients who respond better to this combination therapy. Discontinuation of administration of proton pump inhibitors to patients and/or its replacement with H₂ receptor antagonists might increase the response rate in patients with elevated levels of circulating CgA or vasostatin-1.

Conclusions

The CgA system, comprising full-length CgA and its fragments, is emerging as an important player in cardiovascular, immunometabolic, and cancer regulation. A major gap of knowledge in this field is related to the receptor mechanisms of the diverse biological functions of the CgA-related polypeptides, with few exceptions regarding the direct interaction of human CgA with the integrin α_v/β_6 in the regulation of keratinocyte adhesion,²⁰⁴ CST with nicotinic acetylcholine receptors in the inhibition of catecholamine secretion by chromaffin cells,^{27,31,187,188,205,206} and hCgA_{1–373} with neuropilin-1 in angiogenesis and tumor growth stimulation.

In this regard, it is important to highlight the fact that most of the information on the biological functions of CgA and its fragments has been obtained with experiments performed with human-derived molecules in mouse or rat *in vivo* models, with only few exceptions. Although the regions corresponding to the main bioactive fragments of CgA show high degree of sequence similarity among different species, the sequences are not identical, introducing the possibility that different receptor mechanisms are brought into play. For example, while human CgA contains an RGD site that can interact with high selectivity and affinity with the integrin α_v/β_6 , this site is changed with QGD in mouse and rat CgA, with loss of integrin binding.²⁰⁴

Furthermore, most experiments with full-length CgA have been performed with hCgA_{1–439} produced by recombinant DNA technology in bacterial cells, and thus lacking potentially important posttranslational modifications. These points are potential important limitations of studies performed thus far and may explain some of the controversial results obtained in some cases. Further experiments with syngeneic polypeptides and animal models are necessary to fully elucidate the activity of the CgA system in normal and pathological conditions.

Nevertheless, data have highlighted the complexity of the CgA system in cardiovascular, immunometabolic, and cancer regulation and suggest that qualitative and quantitative characterization of CgA and all its fragments, in normal subjects and diseased patients, are necessary to fully assess the biological role of this system and its potential diagnostic/prognostic value.

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Competing interests

The authors declare no competing interests.

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