

Myocardial production of chromogranin A in human heart: a new regulatory peptide of cardiac function

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Aims High chromogranin-A (CgA) levels were observed in patients with heart failure but its source remained uncertain. We evaluated whether CgA is produced by myocardium and might affect myocardial function.

Methods and results We measured plasma CgA levels and performed immunohistochemistry with anti-CgA antibodies on myocardial biopsies in 40 patients with dilated cardiomyopathy (DCM) and 20 patients with hypertrophic cardiomyopathy (HCM). Surgical myocardial specimens from DCM and HCM patients were used for PCR and ELISA. The presence of CgA-derived fragments in plasma was evaluated by gel-filtration HPLC and their effects on cardiac performance assessed in isolated perfused rat heart. All patients showed increased CgA plasma levels (DCM 153.7 ± 158.5 ng/mL; HCM 150.2 ± 86.7 ng/mL vs. 64.1 ± 17.9 ng/mL of controls) with a positive correlation between CgA and left ventricular end-diastolic pressure (DCM, $R = 0.86$; HCM, $R = 0.83$), and plasma brain natriuretic peptide (BNP) levels (DCM, $R = 0.88$; HCM, $R = 0.85$) ($P < 0.001$). Immunohistochemistry showed cytoplasmic expression of CgA and colocalization with BNP in all patients, but not in controls. PCR detected CgA mRNA in both pathologic and normal myocardium, while ELISA showed a significant amount of CgA only in pathologic myocardium (>0.5 μ g/g of tissue); gel-filtration HPLC of plasma samples identified immunoreactive N-terminal CgA fragments containing vasostatin-1. Administration of vasostatin-1 to perfused rat heart produced negative inotropic and lusitropic effects counteracting isoproterenol actions.

Conclusion We demonstrate for the first time that CgA is produced by human myocardium and exerts negative inotropic and lusitropic effects on mammalian heart. CgA may represent a key player in neuroendocrine regulation of cardiac function and a potential therapeutic target in heart failure.

Chromogranin A (CgA) is a 49 kDa acid protein co-stored with amine, nucleotides, calcium, and other peptide hormones in secretory granules of several endocrine and neuronal cells and released in the extracellular environment by exocytosis.^{1,2} Elevated circulating levels have been described in patients with neuroendocrine tumours³ and renal⁴ or liver⁵ failure. There is growing evidence that CgA plays an intracellular role in secretory vesicle biogenesis and an extracellular function as a prohormone.⁶ Tissue-specific proteolytic processing can give rise to various peptides with different biological functions.⁷ With regard to the cardiovascular system, the N-terminal fragments of CgA, named vasostatins, are released by sympathetic nerve terminals and inhibit arterial vasoconstriction in conduit and resistance vessels, including coronary arteries.⁸ In addition it has been recently observed that vasostatins exert a negative

inotropic effect on isolated frog and eel hearts and counteract the actions of β -adrenergic drugs.^{9,10}

Serum CgA levels correlate with severity of cardiac dysfunction and are a predictive factor for mortality in patients with chronic heart failure.¹¹ Similarly high levels of CgA were related to long-term mortality in patients with myocardial infarction.¹² However, no correlation between CgA and circulating levels of norepinephrine, epinephrine, aldosterone, or plasma renin activity was observed in both studies and the source of CgA in these patients was not identified.

In the present study we examined whether CgA is produced and released by human ventricular myocardium and may be involved in the modulation of cardiac function in patients with systolic and diastolic heart failure.

Methods

Patient population

We studied 60 consecutive patients (mean age 54 ± 12 years, 34 M/26 F) with idiopathic dilated cardiomyopathy (DCM, $n = 40$) and

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hypertrophic cardiomyopathy (HCM, $n = 20$). Patients with abnormal renal and hepatic function were excluded, given the increased CgA levels observed in kidney and liver failure. Similarly, given the CgA increase in myocardial ischaemia,¹² patients with ischaemic cardiomyopathy were not included in the study to eliminate possible confounding factors in the evaluation of myocardial production of CgA; in addition, endomyocardial biopsy in patients with an established ischaemic aetiology of cardiac dysfunction was not considered ethically correct.

Cardiac catheterization and endomyocardial biopsy

As our institution is a tertiary referral centre dedicated to the study of heart muscle diseases, all patients were submitted to coronary and LV angiography, measurement of LV end-diastolic pressure (LVEDP), and LV endomyocardial biopsy. Endomyocardial biopsies were processed for histology and immunohistochemistry. Surgical samples obtained from DCM patients undergoing heart transplantation and HCM patients submitted to surgical septal reduction were frozen in liquid nitrogen and stored at -80°C for reverse transcriptase-polymerase chain reaction (RT-PCR) and ELISA. All invasive studies were performed after obtaining written informed consent from the patient and approved by the Ethics Committee of our Institution.

Measurement of CgA and brain natriuretic peptide circulating levels

Circulating levels of CgA were measured on plasma samples using a sandwich ELISA based on the anti-CgA monoclonal antibody (B4E11)

and rabbit polyclonal anti-CgA antiserum, as previously described.¹² Brain natriuretic peptide (BNP) plasma levels were also measured in all samples, with a specific immunoradiometric assay (Shionoria BNP kit, Shionogi and Co. Ltd., Japan). As controls, we used plasma samples obtained from 60 age- and sex-matched healthy subjects (mean age 54 ± 11 years, 34 M/26 F) selected among blood donors, with no history of cardiovascular disease, normal electrocardiogram, and 2D-echocardiogram (Table 1).

Histology and immunohistochemistry

Multiple $5 \mu\text{m}$ -thick paraffin sections were cut and stained with haematoxylin-eosin, Miller's elastic Van Gieson, Masson's trichrome and examined by light microscopy. Immunohistochemistry for CgA was performed using three different mouse anti-human CgA monoclonal antibodies B4E11 and 5A8, directed against the N-terminal domain (vasostatin-1)^{13,14} and a commercial monoclonal antibody (DAK-A3, Dako) directed against the C-terminal domain (amino acids 210–439). To quantify the immunostaining, CgA-positive myocytes were counted in 20 random high-power fields (HPF, $400\times$) in each specimen, and the percentage of positive cells on total count of myocytes was calculated.

Paraffin sections of myocardial samples obtained at necropsy from 40 normal hearts (mean age 53 ± 10 years, 23 M/17 F) (no evidence of coronary, valvular, and myocardial disease at gross and histological evaluation, no clinical and pathological evidence of neuroendocrine tumour) represented normal controls. Pancreas sections were used as positive controls (Table 1).

Table 1 Characteristics of different controls used in different tests performed in the study

Methodology	Population	<i>n</i>	Age (years)	M/F	Biological material	Clinical features
Assessment of CgA plasma levels	Cases	60	54 ± 12	34/26	Plasma	Heart failure patients 40 DCM, 20 HCM
	Controls	60	54 ± 12	34/26	Plasma	Age- and sex-matched healthy blood donors with no cardiovascular diseases or risk factors
	<i>P</i>	–	0.994	1	–	–
Immunohistochemistry and immunofluorescence	Cases	60	54 ± 12	34/26	Formalin-fixed and paraffin-embedded endomyocardial biopsies	Heart failure patients 40 DCM, 20 HCM
	Controls	40	53 ± 10	23/17	Formalin-fixed and paraffin-embedded myocardial samples obtained at necropsy	Deceased patients with normal hearts (normal valves, coronary arteries, and myocardial tissue at gross pathology and histology) and no clinical and/or pathological evidence of neuroendocrine tumours
	<i>P</i>	–	0.316	n.s.	–	–
Molecular biology and ELISA	Cases	10 ^a	59 ± 9	4/6	Frozen surgical myocardial samples obtained at cardiac surgery	10 out of 60 heart failure patients (five DCM and five HCM) submitted to cardiac surgery for cardiac transplantation or septal myectomy
	Controls	10	60 ± 9	4/6	Frozen surgical myocardial samples obtained at cardiac surgery	10 patients with mitral valve stenosis undergoing surgical valve replacement, with normal coronary arteries, normal systolic function, and normal circulating CgA plasma levels
	<i>P</i>	–	0.788	1	–	–

^aTen patients (five DCM and five HCM) among the 60 included in the study, submitted to cardiac surgery for cardiac transplantation or septal myectomy. DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy.

Co-localization of BNP and CgA was performed using the polyclonal rabbit anti-human BNP antibody (US Biological, USA; 1:1500). Myocytes were labelled by alpha-sarcomeric actin antibody (clone 5C5, Sigma; 1:50) and nuclei were imaged with DAPI. Pancreatic cells were labelled with guinea pig anti-human insulin polyclonal antibody (Biogenesis, USA). IgG antibodies conjugated with fluorescein isothiocyanate, cytochrome C5, or tetramethylrhodamine isothiocyanate were used as secondary antibodies. The sections were examined by confocal microscopy.

RT-PCR and ELISA on myocardial tissue

RT-PCR and ELISA were performed on frozen surgical myocardial samples (500–600 mg) obtained from surgical myectomy or at cardiac transplantation from 10 patients (five HCM, five DCM, mean age 59 ± 9 years, four male/six female). Because of the large amount of frozen myocardial tissue required, as normal controls we used papillary muscle specimens, obtained at cardiac surgery from patients (mean age 60 ± 9 years, 4 M/6 F) with mitral stenosis but normal diastolic and systolic function, normal coronary arteries, and normal CgA plasma levels (Table 1).

RT-PCR

Total RNA was extracted from surgical biopsies using a commercial kit (Total RNA Extraction, Promega). The following primers, ATGCGCTCCGCCGCTGTCTGGC (sense primer) and ATCGGTGACTCATTCTTCTGCTGATGTGC (anti-sense) designed to amplify the N-terminal region of CgA corresponding to vasostatin-1, were used for PCR reactions. pRSNeo-CgA plasmid, coding for human CgA was used as positive control, whereas the integrity of the cDNA was verified using human β -actin primers. The identity of amplified bands was confirmed by sequence analysis.

ELISA

Myocardium tissue extracts (heat-stable fractions) and pheochromocytoma tissue extract (heat-stable fraction) as positive control, were prepared as described previously.¹⁵ We performed sandwich ELISAs based on four different monoclonal antibodies (B4E11, 5A8, A11, 7D1), directed against residues 34–46, 53–57, 68–70, 81–90 of human CgA, respectively,^{13,16} in the capture step and a rabbit-polyclonal anti-CgA antiserum in the detection step. Each ELISA was carried out as described.¹¹

Gel-filtration HPLC of plasma samples

To characterize the structure of circulating CgA in patients with DCM or HCM and to assess the extent of proteolytic processing in the N-terminal region, we analysed patients' plasma samples by gel-filtration HPLC. Fractions were detected by sandwich ELISA using mAb B4E11 in the capture step and biotinylated 5A8 in the detection step. Gel-filtration HPLC was carried out as described previously.¹³

Physiology studies on rat-perfused heart

Animals

Male Wistar rats (Charles River Laboratories, Italy S.p.A.) weighing 250–350 g were housed three per cage in a ventilated cage rack system under standard conditions. Animals had food and water access *ad libitum*. Animal care, sacrifice, and experiments were performed following the European Community guiding principles in the care and use of animals and the project was supervised by the Local Ethics Committee.

Isolated heart preparation

Hearts of anaesthetized rats were rapidly excised, aorta was immediately cannulated with a glass cannula, and connected with the Langendorff apparatus to start perfusion at a constant flow-rate of 12 mL/min, as described previously.¹⁷

A water-filled latex balloon, connected to a BLPR gauge (WRI Inc., USA), was inserted through the mitral valve into the LV to allow isovolumic contractions and to continuously record mechanical parameters. Coronary pressure was also recorded using another pressure transducer. The perfusion solution consisted of modified non-re-circulating KHS.¹⁸ All drug-containing solutions were freshly prepared before the experiments. Isoproterenol hydrochloride (ISO) was purchased from Sigma Chemical Company (St Louis, MO, USA).

Haemodynamic parameters were assessed using a PowerLab data acquisition system and analysed using a Chart software (both purchased by ADInstruments, Basile, Italy).

Basal conditions

Cardiac performance was evaluated by analysing heart rate, left ventricular pressure (LVP) (as an index of contractile activity), rate-pressure product (RPP) (as an index of cardiac work),¹⁹ $+(LV\ dP/dt)_{max}$ (maximal rate of LV pressure contraction), time to peak tension of the isometric twitch, $-(LV\ dP/dt)_{max}$ (maximal rate of LV pressure decline), half-time relaxation (HTR), and T/t ratio obtained by $+(LV\ dP/dt)_{max}/-(LV\ dP/dt)_{max}$.¹⁹ The mean coronary pressure was calculated as average of values obtained during several cardiac cycles.

Vasostatin-1-stimulated preparations

Recombinant Ser-Thr-Ala-hCGA₁₋₇₈ (hrSTA-CGA1-78, vasostatin-1) and Ser-Thr-Ala-hCGA₁₋₁₁₅ (hrSTA-CGA-1-115, vasostatin-2), were obtained and purified as described.^{13,14,16} The products were homogeneous by SDS-PAGE under reducing and non-reducing conditions and gel-filtration HPLC, and showed a single component of expected molecular weight by mass spectrometry analysis. Endotoxin content of STS-CgA178 and STA-CgA1-115 was 0.015 and 0.075 U/ μ g, respectively, as measured by the quantitative chromogenic Limulus Amebocyte Lysate (LAL) test (BioWhittaker).

Preliminary experiments (data not shown) obtained by the repetitive exposure of each heart to one concentration of vasostatin-1 revealed absence of desensitization. Thus, concentration-response curves were obtained by perfusing the cardiac preparation with KHS enriched with increasing concentrations of vasostatin-1 (11–165 nM) for 10 min.

ISO-stimulated preparations

The hearts were stabilized for 20 min with KHS and were perfused with 5 nM ISO for 10 min and then washed-out with KHS. After returning to control conditions, each heart was perfused with KHS containing a single concentration of vasostatin-1 (from 11 to 165 nM) plus 5 nM ISO for other 10 min.

Statistical analysis

Normal distribution of data was verified with Kolmogorov-Smirnov test. Data showing a normal distribution are presented as mean \pm SD. Categorical data are presented as proportion of cases or percentages. Comparisons between pairs of Gaussian variables were performed with Student *t*-test. Comparisons between non-Gaussian variables were performed with Mann-Whitney or Wilcoxon signed-rank test, as appropriate. Comparisons of proportions between groups were performed with χ^2 test or Fisher's exact test, as appropriate. For plasma studies patient to control matching was also performed using paired *t*-test.

Since each heart represented its own control, the statistical significance was assessed using the repeated measures ANOVA test with administered vasostatin-1 as within group factor, followed by Duncan's test. A value of two-tailed $P < 0.05$ was considered as statistically significant. The SPSS Statistical Software, version 11 was used for the analysis.

Results

Clinical and echocardiographic features of patient population are reported in *Table 2*. No patient experienced syncope, documented sustained ventricular tachycardia, or cardiac arrest. No patient was carrier of a pace-maker or an implantable cardioverter defibrillator. In all cases cardiac catheterization showed normal coronary arteries with increased LVEDP (DCM, 22.35 ± 6.8 mmHg; HCM, 23.5 ± 6.9 mmHg).

CgA and BNP plasma measurement

Plasma levels of CgA were higher than controls in all patients (DCM = 153.7 ± 158.5 ng/mL and HCM = 150.2 ± 86.7 ng/mL vs. 64.1 ± 17.9 ng/mL of controls; $P < 0.001$). According to previous studies, BNP levels were also increased in all patients (DCM = 321.95 ± 248.1 pg/mL and HCM = 179.65 ± 99.4 pg/mL vs. 10.4 ± 4.2 pg/mL of controls; $P < 0.001$). In both

DCM and HCM, CgA levels showed a significant correlation with NYHA class (Spearman coefficient 0.88 and 0.76, respectively, $P < 0.001$) and LVEDP (Pearson coefficient 0.86 and 0.83, respectively, $P < 0.001$) (*Figures 1* and *2*). Interestingly, a similar correlation was observed between CgA and BNP plasma levels (Pearson coefficient 0.88 in DCM and 0.85 in HCM, $P < 0.001$) (*Figures 1* and *2*).

Neither the additional factors including age, sex, LV size, and ejection fraction nor the presence of atrial fibrillation or LV outflow gradient in HCM was significantly associated with CgA plasma levels. However, DCM patients with the worse contractile function and two HCM patients with initial remodelling towards a dilated phase showed the highest CgA values.

Histology, immunohistochemistry, and confocal microscopy studies

In patients with DCM a moderate to severe myocyte degeneration and vacuolization associated with interstitial and replacement fibrosis in the absence of inflammatory infiltrates was observed. Severely hypertrophied cardiomyocytes often in total disarray and frequently interrupted in short-runs because of interstitial and replacement fibrosis, were observed in HCM patients.

Immunohistochemistry with B4E11 and 5A8 anti-CgA antibodies against the N-terminal domain showed the presence of a diffuse granular cytoplasmic positivity of myocardial cells in all DCM and HCM patients, while no positive staining was observed in the same biopsies using the DAK-3A antibody against the C-terminal region. Conversely, all three monoclonal antibodies showed a positive staining on human pancreas sections. No CgA-positive staining with any of the three antibodies was observed in myocardial tissue from all normal controls (*Figure 3*), nor in formalin-fixed sections of the papillary muscles used as controls for RT-PCR and ELISA studies (not shown). The mean percentage of CgA-positive myocytes was $66 \pm 27\%$ (range 45–79%) in DCM and $63 \pm 22\%$ (range 34–78%) in HCM with a significant correlation with plasma levels (Pearson coefficient 0.75 and 0.69, $P < 0.001$ in DCM and HCM, respectively). Confocal microscopy studies showed the co-localization of CgA-positive and BNP-positive granules in the cytoplasm of myocytes in both DCM and HCM patients (*Figure 4*). These results suggest that CgA is expressed in myocardial tissue from DCM and HCM patients. The observation that BAE11, 5A8, and DAK-3A epitopes are differentially recognized by antibodies in heart and pancreas sections points to differential post-translational modifications in these tissues.

RT-PCR and ELISA on myocardial tissue

RT-PCR showed the presence of CgA mRNA in myocardial tissue from both patients and normal control myocardium (*Figure 5A*). The identity of these bands was confirmed by sequence analysis. Moreover, the integrity of our cDNA preparation was checked by PCR with β -actin primers, all bands corresponding to the expected size.

All CgA-ELISAs, but not control ELISA, detected CgA in heat-stable fraction of pheochromocytoma tissue extract, known to contain a large amount of CgA¹³ (*Figure 5B*). These assays detected CgA antigen also in the heat-stable fraction of HCM and DCM myocardial extracts but not in normal control myocardium (assay detection limit $0.06 \mu\text{g/g}$)

Table 2 Characteristics of patient population

	DCM (n = 40)	HCM (n = 20)	P
Age (years)	59.1 ± 7.4	45.0 ± 14.2	<0.01
Sex (M/F)	22/18	12/8	0.787
NYHA class, n (%)			
I	9 (23)	7 (35)	0.360
II	13 (32)	5 (25)	0.766
III	12 (30)	5 (25)	0.769
IV	6 (15)	3 (15)	1.000
Atrial fibrillation, n (%)	12 (30)	5 (25)	0.769
Maximal wall thickness (mm)	9.4 ± 1.7	24.3 ± 5.1	<0.001
LV end-diastolic diameter (mm)	64.2 ± 4.8	39.5 ± 3.8	<0.001
LV ejection fraction (%)	33.0 ± 6.4	67.6 ± 3.8	<0.001
Left atrium (mm)	42.7 ± 3.2	43.3 ± 6.6	0.456
LV outflow tract gradient, n (%)	0	4 (20)	<0.01
Restrictive filling pattern, n (%)	4 (10)	5 (25)	0.145
Mitral regurgitation, n (%)	30 (75)	8 (40)	0.011
Mild	15 (50)	6 (75)	0.145
Moderate	12 (40)	2 (25)	0.112
Severe	3 (10)	0	0.544
Drugs, n (%)			
ACE-inhibitors	30 (75)	2 (10)	<0.001
ARBs	20 (50)	0	<0.001
Diuretics	30 (75)	2 (10)	<0.001
Beta-blockers	28 (70)	15 (75)	0.769
Calcium-channel blockers	0	10 (50)	<0.001
Digoxin	16 (40)	0	<0.001
Amiodarone	12 (30)	10 (50)	0.002
LV end-diastolic pressure (mmHg)	22.35 ± 6.8	23.5 ± 6.9	0.265
BNP (pg/mL)	321.95 ± 248.1	179.65 ± 99.4	0.006
CgA (ng/mL)	153.7 ± 158.5	150.2 ± 86.7	0.163

DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; LV, left ventricle; ARBs, angiotensin-receptor blockers; BNP, brain natriuretic peptide; CgA, chromogranin A.

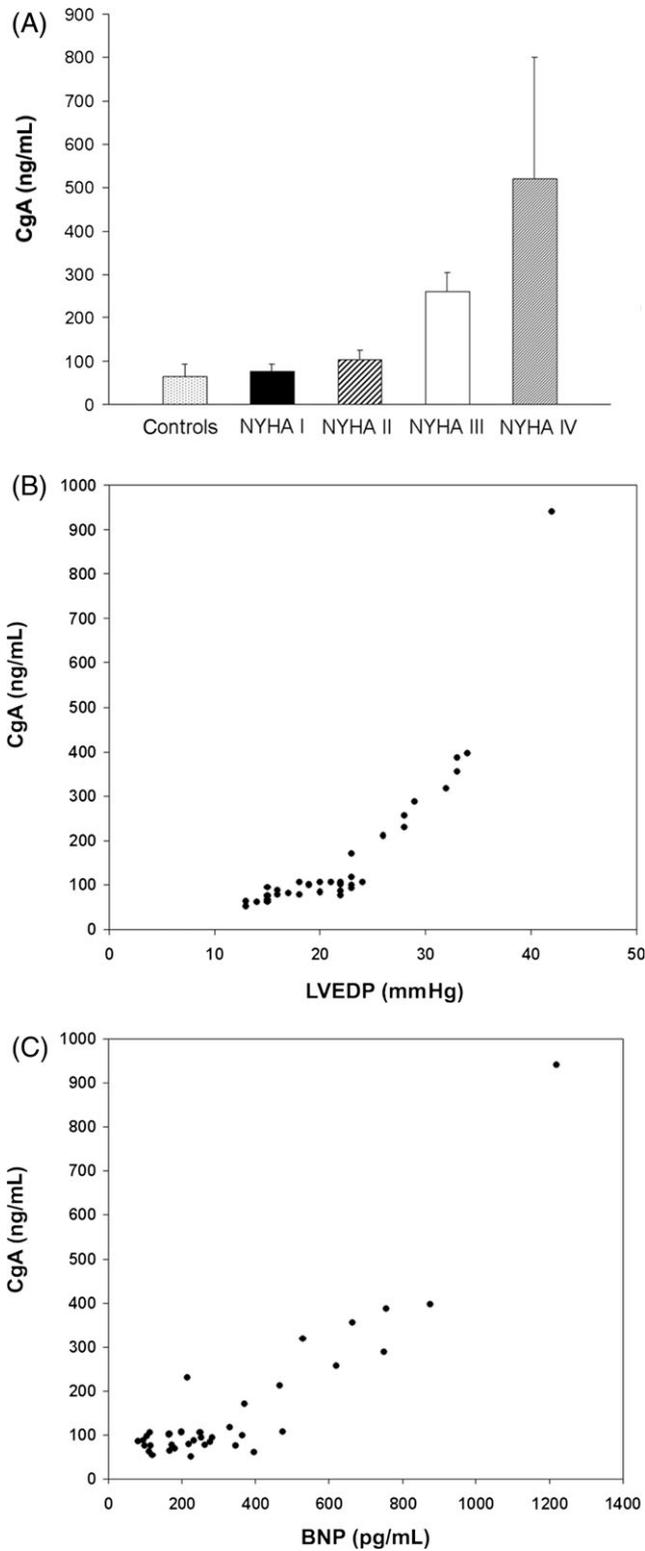


Figure 1 Relationship between chromogranin A (CgA) plasma levels, NYHA class, left ventricular end-diastolic pressure (LVEDP), and plasma brain natriuretic peptide (BNP) in patients with DCM. Patients with more severe clinical manifestations of heart failure present higher circulating levels of CgA correlating with NYHA class (Spearman coefficient 0.88; $P < 0.001$) (A), LVEDP (Pearson coefficient 0.86; $P < 0.001$) (B), and BNP (Pearson coefficient 0.88; $P < 0.001$) (C).

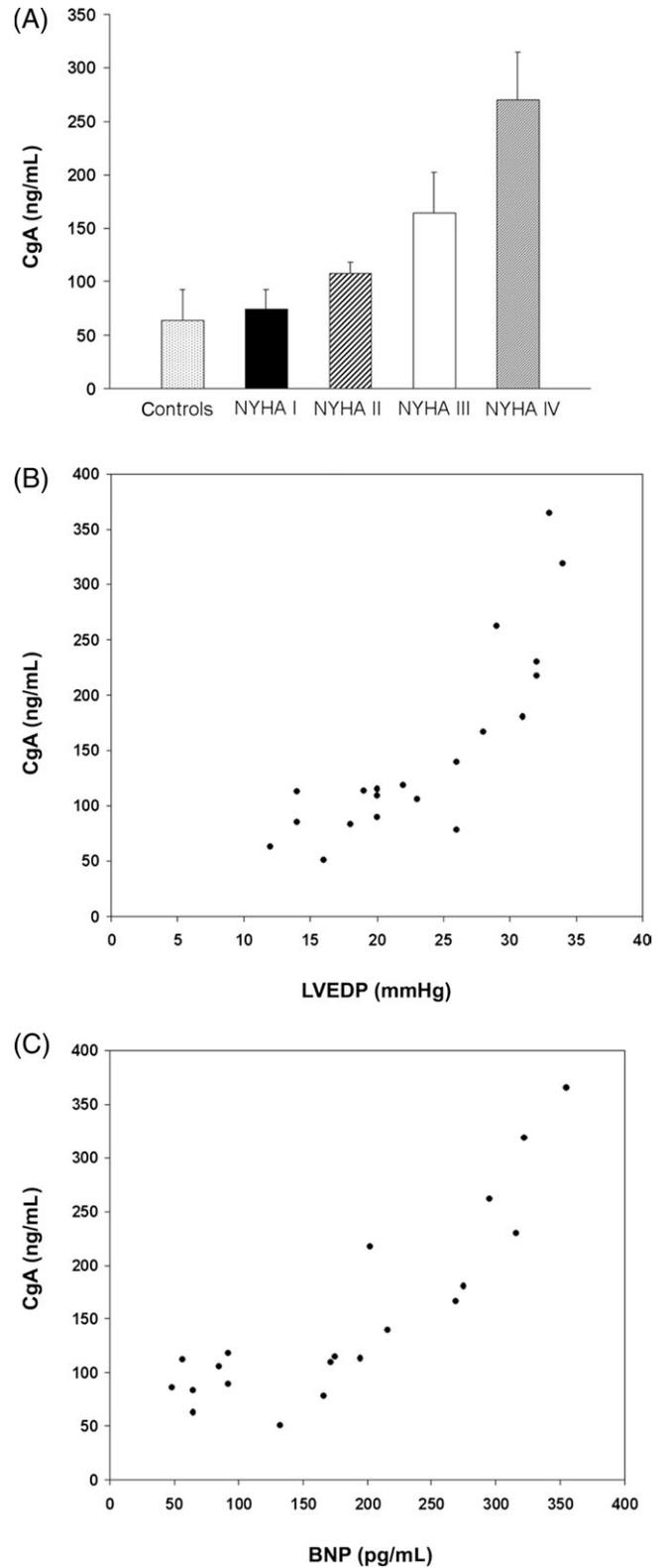


Figure 2 Relationship between CgA plasma levels, NYHA class, LVEDP, and plasma BNP in patients with HCM. Patients with more severe clinical manifestations of heart failure present higher circulating levels of CgA correlating with NYHA class (Spearman coefficient 0.76, $P < 0.001$) (A), LVEDP (Pearson coefficient 0.83; $P < 0.001$) (B), and BNP (Pearson coefficient 0.85; $P < 0.001$) (C).

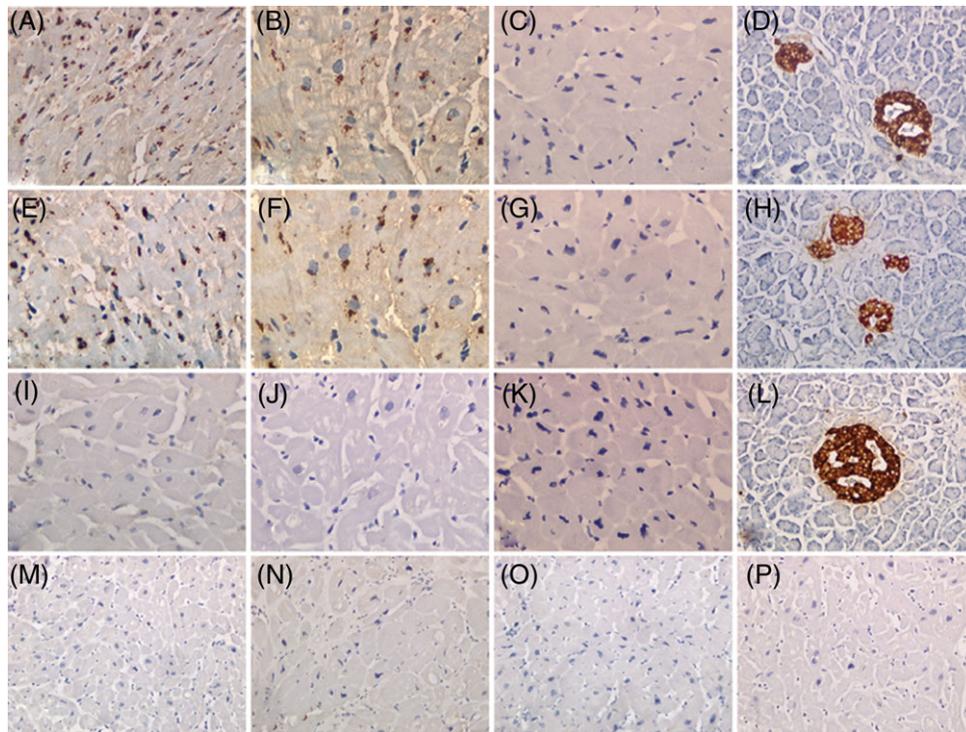


Figure 3 Immunohistochemistry with monoclonal IgG1 mouse anti-human CgA antibodies on myocardial tissue sections. (A–D) MAb B4E11 directed against vasostatin-1 domain of CgA, shows a diffuse granular staining of myocyte cytoplasm in DCM (A) and HCM (B) but not in normal myocardium (C); positive control represented by human pancreas sections presents a diffuse staining of endocrine cells (D). (E–H) MAb 5A8 recognizing a different region of vasostatin-1, produces a positive staining of myocardiocytes cytoplasm in both DCM (E) and HCM (F) but not in normal myocardium (G); endocrine pancreatic tissue is positively stained (H). (I–L) MAb DAK-3A recognizing the pancreastatin domain of CgA peptide shows no staining in both pathologic (I, J) and normal (K) myocardial tissue, but positive staining of pancreas (L). (M–P) Omission of primary antibody and unrelated monoclonal IgG1 antibody (mouse anti-human CD45RO) provide no staining of myocytes in both DCM (M, O) and HCM (N, P), respectively. (A–L: original magnification 200 \times) (M–P: original magnification 100 \times).

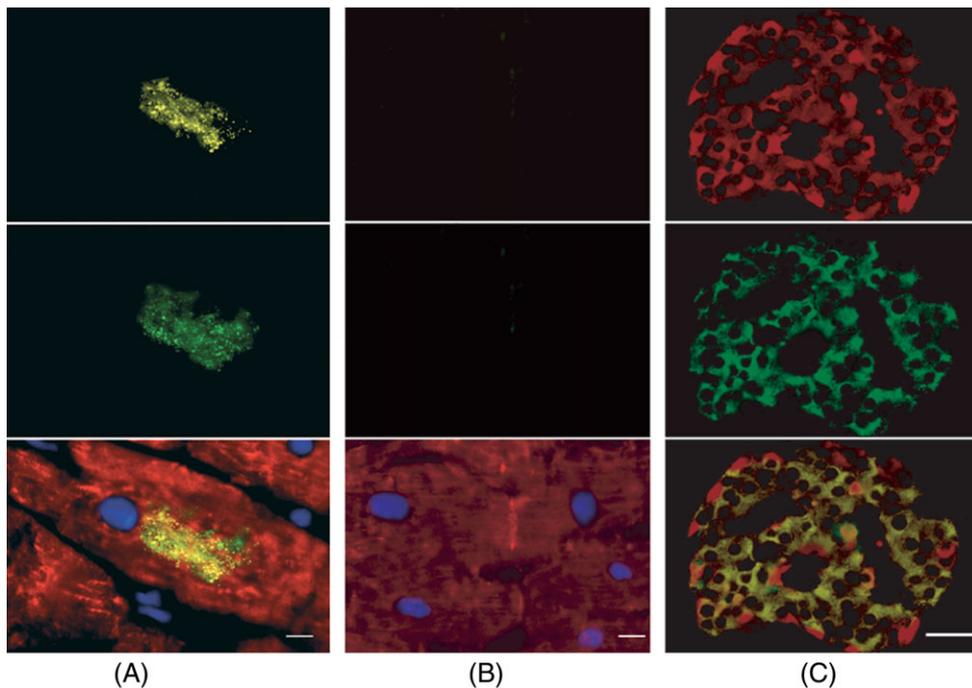


Figure 4 Confocal microscopy studies showing co-localization of BNP and CgA granules in the cytoplasm of a myocyte. (A) Upper panel illustrates BNP staining by yellow fluorescence; green fluorescence in middle panel corresponds to CgA labelling; lower panel depicts combination of BNP and CgA in myocyte cytoplasm, recognizable by red fluorescence of alpha-sarcomeric actin antibody staining. Nuclei are indicated by blue fluorescence of DAPI. (B) Omission of primary antibodies for BNP (upper panel) and CgA (middle panel) produces no staining of cardiomyocyte cytoplasm (lower panel). (C) Positive control represented by sections of pancreas: pancreatic islet cells identified by red fluorescence of anti-insulin antibody (upper panel) present diffuse cytoplasmic green staining (middle panel) with anti-CgA B4E11 antibody; lower panel shows the co-localization of the stainings. Scale bar = 10 μ m (A, B); scale bar = 50 μ m (C).

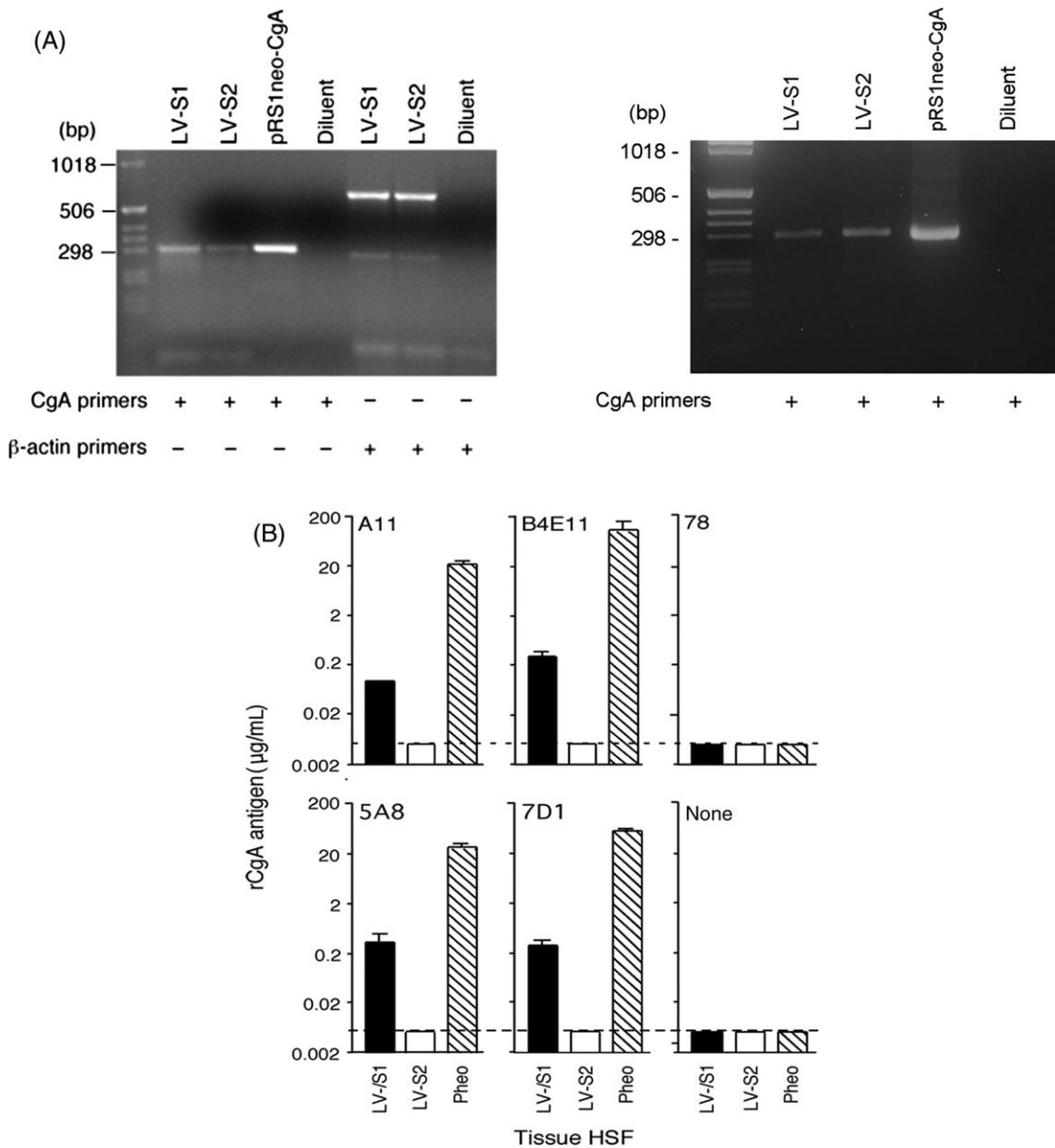


Figure 5 RT-PCR and ELISA on myocardial tissue extracts. (A) An intense band corresponding to CgA mRNA is present in both HCM patient (LV-S1) and normal control (LV-S2) tissue extracts (left panel) and in normal (LV-S1) and DCM (LV-S2) myocardial tissue (right panel). pRS1-neoCgA represents positive control. (B) A significant amount of CgA is detectable in myocardial tissue extract from HCM patient (LV-S1), while no signal is detectable in normal myocardium (LV-S2), with all ELISAs based on four anti-human CgA mAbs (B4E11, 7D1, 5A8, A11) recognizing different epitopes of CgA N-terminal region. Pheochromocytoma tissue extracts (Pheo) represent positive control. Unrelated antibody (mAb78, anti-human TNF) and primary antibody omission (upper and lower right panels, respectively) represent negative controls. Dotted lines represents detection limit.

(Figure 5B). From our data we estimated that the amount of CgA present in the myocardium from patients was $>0.5 \mu\text{g/g}$ (Table 2).

Gel-filtration HPLC of plasma samples

Gel-filtration HPLC showed that most of the circulating CgA antigen had a hydrodynamic size of 100 kDa, likely corresponding to intact CgA. Noteworthy, in most patients a small proportion of fragments with lower size was observed and some patients clearly showed immunoreactive peaks (Figure 6), likely corresponding to vasostatin-1 and -2.¹³

Physiology studies on rat-perfused heart

Basal conditions

After 20 min of equilibration, LVP was 89 ± 3 mmHg, heart rate was 280 ± 7 b.p.m., RPP was $2.5 \pm 0.1 \times 10^4$ mmHg b.p.m., coronary pressure was 63 ± 3 mmHg. $+(LV \text{ dP/dt})_{\text{max}}$ was 2492 ± 129 mmHg/s, Ttp was 0.08 ± 0.01 s, $-(LV \text{ dP/dt})_{\text{max}}$ was 1663 ± 70 mmHg/s, HTR was 0.05 ± 0.01 s, T/t or $+(LV \text{ dP/dt})_{\text{max}}/-(LV \text{ dP/dt})_{\text{max}}$ was -1.49 ± 1.84 . To assess the endurance and the stability of the preparation, the performance variables were measured every 10 min showing that the heart is stable up to 180 min.

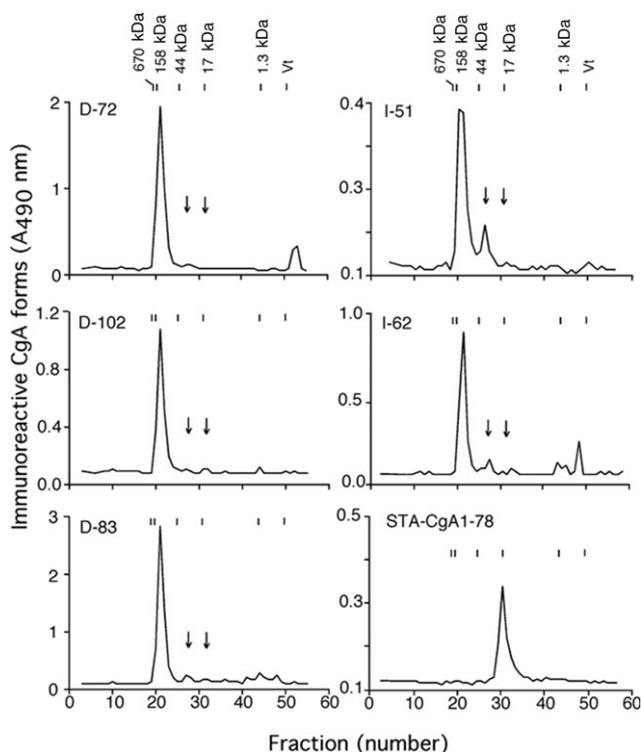


Figure 6 Gel-filtration HPLC of plasma samples from DCM (D-72, D-102, and D-83) and HCM (I-51 and I-62) patients. Fractions were analysed by sandwich ELISA based on mAb B4E11 and 5A8. The main peak correspond to a CgA form with a hydrodynamic size of 100 kDa. In addition, minor peaks corresponding to fragments immunoreactive with mAbs against the N-terminal domain were observed. Arrows indicate the expected elution volume for vasostatin-2 (left) and vasostatin-1 (right). Gel-filtration HPLC of recombinant N-terminal fragment STA-CgA 1–78 (vasostatin-1) represents positive control.

Vasostatin-1-stimulated preparation

The cardiac preparations were exposed to increasing peptide (hrSTA-CGA 1–78, vasostatin-1, and hrSTA-CGA 1–115, vasostatin-2) concentrations to generate the concentration–response curves. Exposure to single repeated doses of vasostatin-1 showed absence of desensitization (data not shown). Peptide effect reached its maximum after 5 min, being stable until 15 min and then gradually decreasing with time. Thus, cardiac parameters were measured at 10 min. Vasostatin-1 (11/165 nM) did neither affect heart rate nor coronary pressure. In contrast, it caused a significant concentration-dependent reduction of LVP and RPP (data not shown). The peptide-dependent negative inotropism was demonstrated by reduction of both $+(LV\ dP/dt)_{max}$ and time to peak of the isometric twitch (*Figure 7*). Analysis of myocardial effects of the peptide revealed also a negative lusitropic action, represented by a significant reduction of $-(LV\ dP/dt)_{max}$ in parallel with an increase of T/t , HTR being unchanged (*Figure 7*). Vasostatin-2 did not induce any inotropic or lusitropic effects on basal cardiac performance.

ISO-stimulated preparations

The positive chronotropic and inotropic effects induced by the β -adrenergic agonist ISO (5 nM) were significant until 5 min after drug application and gradually decreased with time. Preliminary experiments performed by consecutive

exposures of ISO (5 nM) on each heart showed absence of desensitization (data not shown).

To verify vasostatin ability to counteract the ISO-mediated lusitropic and inotropic effects, hearts were perfused with KHs containing 5 nM ISO plus a single concentration of vasostatin-1 (from 11 to 165 nM). The parameters were measured 5 min after drug application. Vasostatin-1 significantly blocked the ISO-mediated increase of LV dP/dt_{max} (positive inotropism) at all concentrations tested and without affecting Ttp. In addition, vasostatin-1 abolished the ISO-mediated positive lusitropism (i.e. $-LV\ dP/dt_{max}$), reduced the ISO-induced negative HTR and blocked the reduction of T/t at 33, 65, 110, and 165 nM of peptide (*Figure 8A and B*).

Discussion

Neurohormones play a central role in the pathophysiology of heart failure and cardiomyopathies and represent the main target of currently available therapeutic repertoire. The diagnostic and prognostic value of neurohumoural factors has been recently recognized and stimulated the development of new drugs. High levels of CgA have been recently reported in patients with heart failure and myocardial infarction, but no correlation between CgA and atrial natriuretic peptide nor other hormones such as norepinephrine, aldosterone, and renin activity was observed.¹¹ Thus, the source of CgA in cardiovascular disorders remained obscure.

In the present study, we provide the first demonstration that human ventricular myocardium produces and releases CgA. The myocardial production of CgA is supported by the immunohistochemical evidence of CgA-positive intracellular staining of cardiomyocytes by RT–PCR showing the presence of CgA–mRNA in myocardium, and by ELISA assays with four different monoclonal antibodies, measuring more than 0.5 μ g of CgA per gram of LV myocardial tissue. According to these results, assuming that CgA is constantly released by myocardial cells and considering that the plasma half-life of CgA is 18.4 min,²⁰ it is reasonable to speculate that the heart significantly contributes to the increased circulating CgA levels observed in our patients.

The hypothesis that myocardial CgA is released in the blood stream is supported by the results of confocal microscopy showing that CgA is co-localized with BNP in ventricular cardiomyocytes and by the strong correlation between CgA and BNP circulating levels. These findings suggest that both hormones are co-stored and co-released in circulation. Moreover, the significant correlation observed between CgA levels and LVEDP indicates that the stretch-induced release and transcriptional up-regulation mechanisms described for BNP²¹ could also be operative for CgA. Indeed, despite we could not detect CgA in normal myocardial tissue by immunohistochemistry and ELISA, the presence of CgA–mRNA in normal myocardium supports the existence of a physiological production of this protein in normal heart.

Although we could not clearly demonstrate the myocardial production of vasostatin, the results of immunohistochemistry with antibodies against the N-terminal and C-terminal domains, showing differential immunoreactivity patterns in cardiomyocytes and pancreas, suggest tissue-specific post-translational modifications. Accordingly, gel-

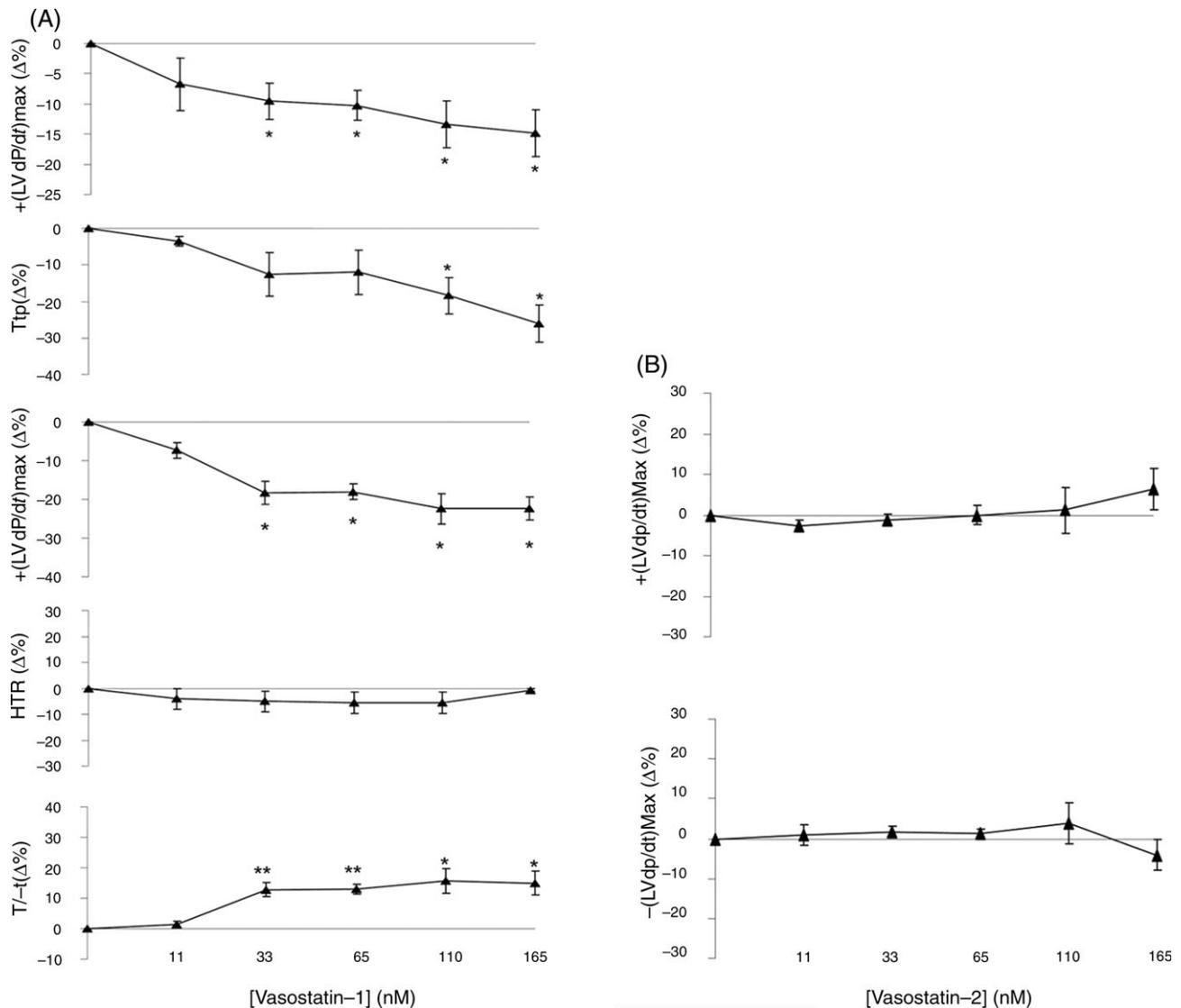


Figure 7 Effects of vasostatin-1 on inotropic and lusitropic properties of the isolated rat heart. Concentration-dependent effects of increasing concentrations (11/165 nM) of vasostatin-1 on $+(LV\ dP/dt)_{max}$, T_{tp} , $-(LV\ dP/dt)_{max}$, half-time relaxation, T/τ , or $+(LV\ dP/dt)_{max}/-(LV\ dP/dt)_{max}$ (A) and vasostatin-2 on $+(LV\ dP/dt)_{max}$ and $-(LV\ dP/dt)_{max}$ (B), in the rat isolated and Langendorff-perfused heart. Percentage changes were evaluated as mean \pm SD of seven experiments. * $P < 0.05$; ** $P < 0.01$ vs. control values.

filtration HPLC analysis of plasma samples showed that the main circulating form of CgA corresponds to a protein behaving as a globular protein of about 100 kDa, plus a significant proportion of lower molecular weight fragments immunoreactive with antibodies against the vasostatin-1 domain. Considering our previous report that 600, 100, and 55 kDa forms are present in different proportions in the blood of patients with neuroendocrine tumours,²⁰ it would appear that CgA is post-translationally modified in a different manner in patients with heart failure. Although it is difficult to speculate whether CgA is proteolytically cleaved in human myocardium, our recent finding that N-terminal fragments containing the vasostatin-1 domain are detectable in the rat heart suggests that proteolytic processing could indeed occur in myocardial tissue.²²

Our findings could have important pathophysiological implications related to the multiple cardiovascular functions potentially exerted by CgA and its N-terminal fragments. For

instance, it is well established that vasostatin-1 can inhibit adrenergic-mediated arterial vasoconstriction⁸ and may exert intrinsic negative myocardial inotropic effect counteracting β -adrenergic stimuli on isolated and perfused frog and eel heart.^{9,10} Here, we confirmed on mammalian heart the negative inotropic effects and described for the first time a vasostatin-induced negative lusitropic effect. Administration of vasostatin-1 in the rat-perfused heart produced a significant impairment of LV contraction and relaxation and significantly counteracted both the inotropic and lusitropic effects of β -adrenergic stimulation. With regard to the mechanisms underlying negative inotropic effects, we have recently shown in non-mammalian heart, that cytoskeleton reorganization appear to be a crucial event,²³ but it is still unclear to what extent these findings are transposable to mammalian tissue.

In addition to the effects on cardiac function, it has been previously shown that CgA and CgA N-terminal fragments

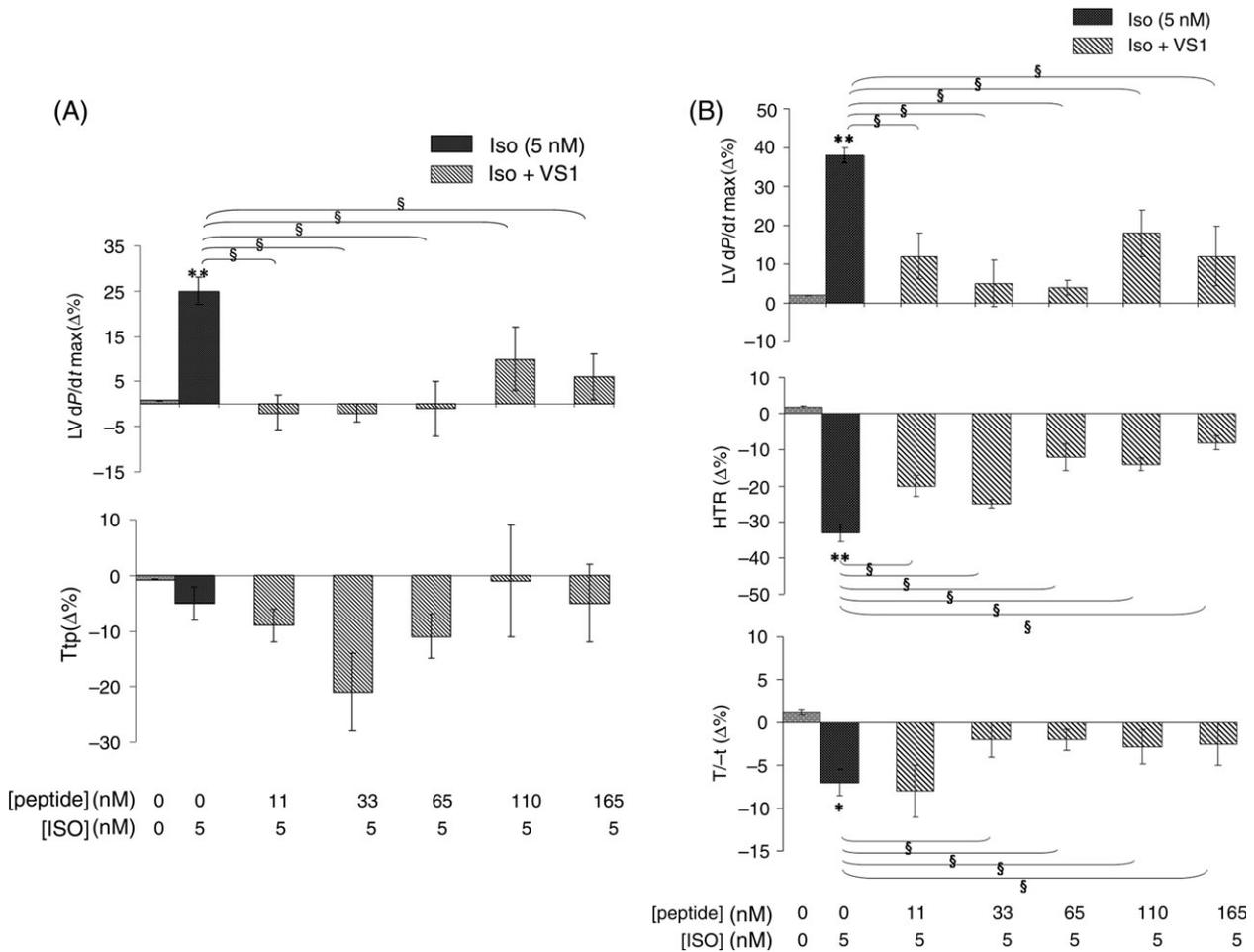


Figure 8 Effects of vasostatin-1 on the inotropic and lusitropic effects evoked by isoproterenol (ISO). Effects of vasostatin-1 (from 11 to 165 nM) on inotropic (A) and lusitropic (B) responses evoked by ISO on +(LV dP/dt)max, Ttp, –(LV dP/dt)max, half-time relaxation, and T/–t, or +(LV dP/dt)max/–(LV dP/dt)max, on the rat-isolated and Langendorff-perfused heart. Percentage changes were evaluated as mean ± SD of five experiments for each groups. *P < 0.05; **P < 0.01 vs. control values; §P < 0.05 for comparison between groups.

can modulate in a differential manner fibroblast and smooth muscle cell adhesion and spreading¹⁶ as well as endothelial cell to cell adhesion²⁴ and permeability.²⁵ Given the importance of these cells in determining tissue organization and architecture, myocardial overproduction of CgA could also contribute to regulate heart remodelling in patients with myocardial infarction, cardiomyopathies, and heart failure.

These findings suggest that CgA may play a detrimental role in cardiac failure contributing in an autocrine and paracrine fashion to systolic and diastolic dysfunction and to unfavourable remodelling. However, the potential effect of CgA on cardiac function must be considered in the wider context of neurohumoral activation occurring in heart failure, and in particular in relation to the natriuretic, vasodilating, and anti-fibrotic properties of BNP, co-stored in myocytes and probably co-released with CgA. In these settings, as observed with other molecules involved in neurohumoral compensatory response, an initial beneficial and homeostatic effect of CgA may become deleterious with the progression of the disease, probably in a dose-dependent manner. The high levels observed in our population and the significant correlation with the severity of clinical manifestations, support the notion that sustained overexpression of CgA may contribute to cardiac dysfunction in patients with diastolic and systolic heart failure.

Further studies are needed to better clarify the myocardial-specific post-translational processing of CgA, the effective content of vasostatin in human myocardial tissue and whether and to what extent CgA can indeed affect myocardial function in patients. In this view, abnormal production of CgA in the heart of patients could represent a new target for developing alternative pharmacological strategies aimed at modulating myocardial function in the treatment of heart failure.

Conflict of interest: none declared.

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