Catestatin, a chromogranin A derived peptide, is sympathoinhibitory and attenuates sympathetic barosensitivity and the chemoreflex in rat CVLM.

A. H. Gaede and P. M. Pilowsky

Australian School of Advanced Medicine, Macquarie University, Australia

Short Title: CARDIORESPIRATORY EFFECTS OF CATESTATIN IN CVLM

Word Count: 5,600

Figures: 6

Corresponding Author:
Paul M. Pilowsky
Australian School of Advanced Medicine
F10A, L1
Macquarie University, NSW 2109, AUSTRALIA
T: +61 2 9812 3560
F: +61 2 9812 3650
paul.pilowsky@mq.edu.au
www.pilowsky.org
ABSTRACT:

Hypertension is a major cause of morbidity. The neuropeptide catestatin (human chromogranin A-(352–372)) is a peptide product of the vesicular protein chromogranin A. Studies in the periphery and in vitro studies show that catestatin blocks nicotine-stimulated catecholamine release, and interacts with β-adrenoceptors and histamine receptors. Catestatin immunoreactivity is present in the rostral ventrolateral medulla (RVLM), a key site for blood pressure control in the brainstem. Recently we reported that microinjection of catestatin into the RVLM is sympathoexcitatory and increases barosensitivity. Here we report the effects of microinjection of catestatin (1mM, 50 nL) into the caudal ventrolateral medulla (CVLM) in urethane-anaesthetized, bilaterally vagotomized, artificially ventilated Sprague-Dawley rats (n = 8). We recorded resting arterial pressure, splanchnic sympathetic nerve activity, phrenic nerve activity, heart rate, and measured cardiovascular homeostatic reflexes. Homeostatic reflexes were evaluated by measuring cardiovascular responses to carotid baroreceptor and peripheral chemoreceptor activation. Catestatin decreased basal levels of arterial pressure (-23 ± 4 mmHg), sympathetic nerve activity (-26.6 ± 5.7%), heart rate (-19 ± 5 bpm) and phrenic nerve amplitude (-16.8 ± 3.3 %). Catestatin caused a 15% decrease in phrenic inspiratory period (T_i) and a 16% increase in phrenic expiratory period (T_e), but had no net effect on the phrenic interburst interval (T_{tot}). Catestatin decreased sympathetic barosensitivity by 63.6% and attenuated the peripheral chemoreflex (sympathetic nerve response to brief hypoxia; range decreased 39.9%; slope decreased 30.1%). The results suggest that catestatin plays an important role in central cardiorespiratory control.
KEYWORDS: hypertension, hypoxic chemoreflex, neuropeptides, neuropharmacology
INTRODUCTION:

Sympathetic tone, and the integration of adaptive reflexes, is dependent on bulbospinal pre-sympathetic excitatory neurons in the rostral ventrolateral medulla oblongata (RVLM). Pre-sympathetic sympathoexcitatory neurons integrate information from the center and periphery (7).

Chromogranin A (CgA) is packaged into large dense core secretory vesicles in nerve terminals throughout the peripheral and central nervous systems (45), but the function of this protein remains unclear. CgA is the precursor of several biologically active peptides that may play an important autocrine regulatory role in the neuroendocrine system (1, 12). One such cleavage product of CgA, catestatin, was first isolated from bovine adrenal medullary chromaffin cells, and initially shown to act as a potent non-competitive nicotinic acetylcholine receptor (nAChR) antagonist (24), and a β-adrenoceptor agonist in peripheral tissue (26).

Recently, we reported that catestatin plays an important role in regulating the responses of cardiorespiratory neurons in the central nervous system. Catestatin acts as a nicotinic receptor antagonist in the spinal cord, and microinjection of catestatin into the RVLM elicited pressor and sympathoexcitatory responses. At the same time, it was found that catestatin injected into the RVLM impaired the somatosympathetic reflex (SSR) and the hypoxic chemoreflex, but enhanced the sympathetic baroreflex (9, 10). In earlier studies, we addressed the role of catestatin at the level of the output motoneurons (9), specifically sympathetic preganglionic neurons (34), and the premotoneurons in the brainstem at the level of the RVLM (10).

Here, we focus on the cardiorespiratory effects of catestatin microinjected into the caudal ventrolateral medulla (CVLM). The CVLM is a crucial relay nucleus in the
brainstem circuitry that maintains sympathetic tone and integrates responses from the periphery, and the central nervous system. The output from the CVLM is a GABAergic inhibitory pathway that acts to restrain the activity of excitatory bulbospinal pre-sympathetic neurons in the RVLM. Activation of neurons in the CVLM is crucial for normal operation of the sympathetic baroreceptor reflex (36, 40).

We hypothesized that in the CVLM we would observe opposing effects on basal blood pressure and nerve activity levels to those seen in the RVLM. Specifically, we expected catestatin in the CVLM to significantly decrease mean arterial pressure (MAP), splanchnic sympathetic nerve activity (sSNA), and phrenic nerve activity amplitude (PNamp). We also hypothesized that catestatin would decrease barosensitivity, because the CVLM is thought to gate RVLM activity following baroreceptor loading. The objectives of the present study were four-fold: To determine the cardiovascular effects of catestatin in the CVLM in vivo, to determine the effects of catestatin on respiratory function, to investigate the role of catestatin in control of the sympathetic baroreflex, and finally to determine the effect of catestatin microinjected into the CVLM on the response to brief hypoxia.

METHODS:

All procedures and protocols were approved by the Macquarie University Animal Ethics Committee according to the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Experiments were conducted on adult male Sprague-Dawley rats (n = 8; 400–500 g body wt; Animal Resource Centre, Western Australia).
Surgical Procedures. Anesthesia was induced with a bolus dose of urethane (ethyl carbamate, 10% in 0.9% NaCl w/v; 1.3 g/kg, i.p.; Sigma-Aldrich). An arterial pressure change exceeding 10 mmHg, or the presence of a withdrawal reflex in response to hind-paw pinch was used to assess lightening of anesthesia. Small intravenous doses of sodium pentobarbital (10 mg in 20% solution) were given as needed, during surgery to maintain an appropriate level of surgical anesthesia. Sodium pentobarbital was occasionally necessary during surgery, but not during data acquisition, because of its fast-acting nature, and because each dose is metabolized relatively quickly (60-90 minutes). Following surgery, additional doses of urethane (20 mg in 10% solution w/v, i.v.) were given as required. The right jugular vein and common carotid artery were cannulated for drug administration and arterial pressure measurement, respectively. Rats were bilaterally vagotomized to remove parasympathetic outflow, so that splanchnic nerve activity measurements could be strictly attributed to presympathetic drive. The trachea was cannulated, and animals were subjected to neuromuscular blockade (pancuronium bromide; 0.8 mg i.v. initially, followed by 0.4 mg/h i.v.; Astra Pharmaceuticals). Rats were artificially ventilated to maintain expired CO₂ levels between 4 and 5% (pump frequency, 65–90 min⁻¹; tidal volume, 3–4 mL). The left phrenic nerve was exposed dorsolaterally, isolated, cut, and recorded. Similarly, the left greater splanchnic sympathetic nerve was dissected using a retro-peritoneal approach. Both nerves were recorded using bipolar recording electrodes. Data was acquired using an ADC system (model 1401, CED, Cambridge, UK). Nerve signals were amplified (×2000 for PNA; ×5000 for sSNA, CWE Inc., Ardmore, PA, USA), band pass filtered (0.03–3 kHz), sampled at 5 kHz, and recorded using Spike 2 analysis software (v. 7.07). The dorsal medullary surface was exposed and microinjections of glutamate (50 nL, 100 mM; Sigma-
Aldrich), rat catestatin (50 nL, 1 mM, Phoenix Pharmaceuticals, dissolved in phosphate buffered saline), and vehicle (phosphate buffered saline - PBS, 50 nL, 10 mM, pH7.4) were microinjected bilaterally into the CVLM using a single-barrel glass micropipette. The micropipettes were prepared using borosilicate glass capillary tubes (OD 1.0 mm, ID 0.25 mm; SDR Clinical Technology, Australia) and a laser pipette puller (P2000, Sutter Instrument Company, USA). Micropipettes were filled using suction applied by a syringe and tubing attached to the pipette. Pressure microinjections were performed using an air-filled syringe and attached tubing. The injection volume was determined by direct observation of the fluid meniscus against an attached calibrated grid (each square = 50 nL) using the operating microscope. The CVLM was located stereotaxically (Figure 1), and identified functionally, on the basis of a fall in MAP of at least 30 mmHg following unilateral pressure microinjections of L-glutamate (100 mM, 50 nL) as described previously (13, 29). The preliminary coordinates used to find the CVLM were 1.0 mm rostro-caudal, 1.8 mm medio-lateral, and 2.5 mm dorso-ventral to calamus scriptorius. The pipette was not removed from the electrode holder during the course of the experiment. The pipette was rinsed with vehicle after microinjection of glutamate. Vehicle control injections (e.g. Figure 2A, B) confirmed that no glutamate remained in the pipette after rinsing. Bilateral microinjections of catestatin were made after vehicle microinjections and homeostatic reflex challenges. On completion of the experiments, Pontamine Sky Blue (1% in saline w/v) was microinjected to label the injection site, and the brainstem was removed and fixed in 4% paraformaldehyde solution. Histology was performed to verify injection sites (Figure 1).

Intravenous drugs were dissolved in saline (0.9% w/v). Drugs for microinjection were dissolved in 10 mM PBS: L-glutamic acid (100 mM [5 nmol/50 nL]); catestatin (1
mM; e.g. Figure 2A, B). The concentration of catestatin used was selected based on previous studies in the brainstem (10).

**Experimental Design.** Homeostatic reflexes were evoked as previously described using sequential injections of phenylephrine (PE; 10 μg/kg i.v.) to load the baroreceptors, or by brief hypoxia (12s 100% N₂) to activate peripheral chemoreceptors (28). Reflexes were tested twice after bilateral microinjections of both vehicle and catestatin.

**Data Analysis.** Nerve recordings were calibrated using a 50-μV stimulus and integrated and smoothed for analysis (sSNA time constant = 1 s; phrenic nerve activity time constant = 50 ms). Baseline values were obtained by averaging 120 s of data prior to injection. Changes are expressed as percentage or actual change from baseline activity. The peak of each integrated and smoothed phrenic burst was used to create a channel for measuring PNamp. Phrenic interburst interval (Ttot) was calculated by measuring the duration of the phrenic cycle, triggered by the peak of each phrenic burst. Phrenic inspiratory period (Ti) is defined as the duration of each phrenic burst. Phrenic expiratory period (Te) is defined as the duration of silence between phrenic bursts. PNamp and Ttot were used to characterize inspiratory drive. Changes in MAP (mmHg), heart rate (HR; beats/min), sSNA (%Δbaseline), PNamp (%Δbaseline), Ttot (%Δbaseline), Ti (%Δbaseline), and Te (%Δbaseline) are expressed as means ± SEM. Sympathetic barosensitivity was acquired using the slope of a first-order polynomial fitted to the steepest portion of the curve obtained by plotting sSNA vs. MAP responses to PE. In addition, a Boltzmann sigmoid curve was fit to the MAP and sSNA responses to PE, individually. The range of the response was calculated and compared pre- and post-catestatin. For analysis of the peripheral chemoreflex, a
Boltzmann sigmoid curve was fit to the MAP and sSNA responses to hypoxia. Slope and range of the fitted curve were used to measure chemosensitivity. The range of the chemoreflex is expressed as a %Δ from a 60 s average of baseline activity prior to hypoxia.

Data are expressed as means and SEM. Statistical analysis was carried out using Prism 5 (v. 5.03, GraphPad Software, La Jolla, CA). Results are displayed as treatment vs. control. Student’s t-test was used to calculate peak effects. P<0.05 was considered significant.

RESULTS:

Catestatin in the CVLM is sympathoinhibitory, and decreases HR. In seven animals, with baseline levels of 103 ± 6 mmHg (MAP), 2.3 ± 0.6 µV (sSNA), and 441 ± 13 bpm (HR), bilateral microinjections of catestatin (1 mM, 50 nL) were made into the CVLM. Catestatin significantly decreased MAP, sSNA, and HR (Figure 2A). Grouped data shows the peak depressor response caused by catestatin in MAP (-23 ± 4 mmHg, P=0.0009; Figure 2B), and the decrease from baseline observed in sSNA (-26.6 ± 5.7%, P=0.004; Figure 2C) and HR (-19 ± 5 bpm P=0.007; Figure 2D). Peak effect on MAP, sSNA, and HR was observed 10-60 seconds after catestatin microinjection. The magnitude of the effects on MAP, sSNA, and HR due to catestatin was significantly greater than any seen after bilateral microinjection of vehicle (Figure 2B-D).

Catestatin decreases phrenic nerve amplitude, but not phrenic interburst interval. In five animals, having baseline levels of 11.6 ± 2.3 µV (PNamp), and 1.4 ± 0.06 s (Ttot),
Catestatin microinjection into the CVLM caused a significant decrease in PNamp (Figure 3A). Figure 3B shows grouped data demonstrating a maximum decrease of -16.8 ± 3.3 % in PNA (P=0.03) after catestatin. No net effect on T_{tot} was observed after treatment with catestatin (Figure 4A-B). However, catestatin microinjection resulted in a 14.9 ± 2.3% decrease in phrenic inspiratory period (T_{i}; P<0.05; Figure 4C) and a 15.6 ± 3.5% increase in phrenic expiratory period (T_{e}; P<0.05; Figure 4D).

Catestatin attenuates the sympathetic baroreflex. In six animals, PE was administered intravenously after both vehicle and catestatin microinjections into the CVLM to test the baroreceptor reflex (Figure 5A). The sSNA response to PE was plotted against the MAP response to the same stimulus in order to measure the sensitivity of the baroreflex. The slope of the line generated by this analysis was decreased by 62.6% after catestatin treatment (post-vehicle: slope = -0.090 ± 0.002; post-catestatin: slope = -0.034 ± 0.01; P = 0.0099; Figure 5B). Notably, the amplitude of the sSNA response to PE was significantly decreased post-catestatin by 34.8% (Figure 5C), while the MAP response was virtually unchanged.

Catestatin attenuates the peripheral chemoreflex. In five animals, brief hypoxia (12 s 100% N\textsubscript{2}) increased MAP and sSNA through activation of the carotid chemoreceptors. The range and slope of a sigmoid curve fitted to the sSNA response were both attenuated after catestatin injection (range decreased 39.9%; slope decreased 30.1%; Figure 6A-C). The blood pressure response to hypoxia was not significantly affected by catestatin microinjection into the CVLM when compared to vehicle injections.
DISCUSSION:

The major novel findings of this study are: 1) Catestatin microinjections into the CVLM elicit a sympathoinhibitory response associated with a decrease in MAP; 2) Catestatin attenuates PNamp, decreases the inspiratory period and increases the expiratory period, but does not affect phrenic frequency; 3) The sympathetic baroreflex is attenuated by catestatin; 4) The sSNA response to peripheral chemoreceptor stimulation (12 s hypoxia) is attenuated, while the blood pressure response is unaffected.

Catestatin and the brainstem circuitry that regulates blood pressure.

Catestatin is known to act as a non-competitive antagonist at nAChR, blocking catecholamine release from bovine chromaffin and rat PC-12 cells (21-24). In vivo studies show that catestatin causes vasodilation, due in part to the release of histamine (15). In addition to the effects of catestatin that occur outside the central nervous system, we have demonstrated that catestatin also acts on sympathetic preganglionic neurons (SPN) to attenuate responses to nicotine and isoproterenol (β-adrenoceptor agonist). This action on SPN suggests that catestatin may act as a modulatory agent to maintain basal blood pressure and sympathetic nerve activity levels (9).

Typically, peptides are associated with their own receptors. The ability of catestatin to impair actions at multiple receptors is unusual and is the subject of continuing investigation. Studies in human suggest that catestatin is an anti-hypertensive compound, and low levels may be implicated in the genesis of essential hypertension (8, 19, 32, 39). Alternatively, it may be that there is a defect in the physiology, or pharmacology, of catestatin that reduces its ability to act effectively in hypertensive, or pre-hypertensive, individuals. Despite the widespread distribution of CgA in the
central nervous system, the role played by catestatin in the regulation of central cardiorespiratory control mechanisms remains unclear.

*Actions of catestatin in the CVLM complement those seen in the RVLM:* In this study, we focused on the inhibitory neurons in the CVLM because of the key role that they play in setting the mean level of sympathetic tone and MAP by restraining sympathoexcitatory neurons in the RVLM. The crucial brainstem nuclei for central circulatory regulation include the CVLM, RVLM, and the nucleus tractus soltarius (NTS). Previously, we observed widespread catestatin-immunoreactivity in the brainstem, notably in the cell bodies of catecholaminergic, and non-catecholaminergic, RVLM neurons (10). We also demonstrated that catestatin microinjection into the RVLM is sympathoexcitatory and increases MAP. One aim of this study was to determine whether catestatin would have an opposing effect in the CVLM.

We show here that exogenous catestatin excites neurons in the CVLM, resulting in decreased sympathetic drive and a subsequent fall in MAP and HR. A bilateral vagotomy was performed in this study in order to obtain a preparation in which all activity was entirely due to sympathetic nerve outputs. Another benefit of vagotomizing the animals was the removal of influences from afferent pathways that could unnecessarily complicate interpretation of the data.

CVLM neurons are small GABAergic interneurons that project rostrally to inhibit bulbospinal pre-sympathetic neurons in the RVLM. Pre-sympathetic neurons in the RVLM form synapses with SPN (27, 33). Increases in arterial blood pressure activate baroreceptors in the periphery that in turn activate neurons in the NTS. Excitatory baroreceptor-activated interneurons in the NTS project to the CVLM. Subsequently,
activation of the CVLM causes a sympathoinhibitory and depressor response through decreased excitation of SPN, and thus decreased sympathetic outflow.

The effects seen in this study are consistent with the sympathoexcitation observed when catestatin is microinjected into the RVLM (10), suggesting that catestatin acts as an excitatory agent in the brainstem. Brainstem nuclei maintain blood pressure through two broad mechanisms. Glutamate and GABA primarily regulate short-term control of RVLM and CVLM neurons. Conversely, long-term control is mediated, at least in part, by metabotropic neurotransmitters (37), including peptides, such as neuropeptide Y (14), orexin (18), and others (46). Studies using this method – microinjection of neurotransmitters or neuropeptides into the CVLM or other brainstem nuclei – have revealed the differential effects on blood pressure and sympathetic outflow that result from these agents (2, 5), and suggest that the responses demonstrated here are unique to catestatin.

*Nicotine and catestatin in the CVLM:* Catestatin is a potent nAChR antagonist that inhibits catecholamine release in chromaffin cells (20, 24) and rat PC12 cells (23). Catestatin attenuates the sympathoexcitatory effects of nicotine on SPN, but itself has no obvious effect on resting MAP or sympathetic nerve activity (9). If catestatin were to act solely as a nAChR antagonist, then one would expect it to act differently than nicotine, a nAChR agonist, in the brainstem. In the *in vivo* rat model, microinjections of nicotine into the CVLM resulted in depressor and bradycardic responses. These responses were completely blocked by pretreatment with mecamylamine and α-bungarotoxin, proving that the responses were due to activation of nAChR (3). Similarly, catestatin microinjection into the CVLM causes a decrease in MAP and HR. In a similar manner, catestatin mirrored the response elicited by nicotine in the RVLM. We reported that catestatin microinjection into the RVLM significantly
increases MAP and sSNA. Microinjection of nicotine into the same region also elicited a pressor response that was blocked by hexamethonium (49). This suggests that catestatin is acting through an alternate mechanism in these nuclei, either in lieu of, or in addition to its action as an antagonist at nAChR.

**Catestatin regulation of respiratory function.**

PNamp is significantly decreased following microinjection of catestatin into the CVLM. Interestingly, $T_i$ was decreased after catestatin and $T_e$ was increased, so that $T_{tot}$ remained unchanged. Recently, we showed that catestatin injected into the RVLM causes an increase in PNamp, but has no effect on phrenic nerve frequency (PNf). Catestatin may exert its respiratory effects by activating interneurons in the CVLM that have downstream effects on respiratory sites. CVLM neurons affect central respiratory drive-related modulation of presympathetic RVLM neurons (25). This could explain the pattern change in phrenic firing that occurs after catestatin microinjection.

Another possible explanation for these respiratory responses is that the CVLM is intermingled with neurons in the pre-Bötzinger complex (preBötC), a site regarded as the rhythm generator for breathing (29, 44, 47). Respiratory neurons in the ventral respiratory column are likely to provide inputs to nearby cardiovascular neurons (35, 48). Neurons in the CVLM are governed by respiratory inputs, as well as excitatory inputs from the NTS, the caudal pressor area (CPA), and possibly other unknown inputs (25, 31, 40). However, the precise circuitry of the CVLM and its role in tonic blood pressure control is still in need of clarification.
Catestatin affects cardiovascular reflexes and autonomic function.

The CVLM restrains short-term changes in arterial pressure in response to changes in baroreceptor input. Neurons in the CVLM are also capable of tonically inhibiting neuronal activity in the RVLM by mechanism/s that are independent of arterial baroreceptors (6, 41, 42).

In the present study, we show that catestatin microinjected into the CVLM attenuates sympathetic barosensitivity in normotensive rats. This finding is consistent with our previous study where catestatin administered into the RVLM increases sympathetic barosensitivity. By exciting inhibitory neurons in the CVLM with catestatin, we expected to see effects that were opposite to those observed in the RVLM (17). CgA-null mice exhibit decreased HR barosensitivity (11) that could be rescued by intravenous delivery of catestatin. This suggests that catestatin plays an important role in the reflex control of arterial pressure, possibly by acting centrally to maintain a baseline level of barosensitivity.

Arterial pressure and sympathetic nerve activity responses to hypoxia were used to characterize the peripheral chemoreflex. Both the range and gain of the sSNA response to hypoxia were attenuated after catestatin microinjection, while the MAP response was not affected. A similar reduction in the range of the sSNA response was seen after catestatin was microinjected into the RVLM. A dissonance between the blood pressure response and the sympathetic response is not unusual, and is observed in many previous studies using many stimuli (28). Blood pressure changes in response to a summation of many variables, including: neural, hormonal and cardiovascular. Sympathetic nerve activity is affected by many fewer regulatory inputs. The CVLM and caudal portion of the RVLM anatomically overlap with inspiratory neurons of the
preBötC, which may explain the similarities observed in the chemoreflex responses (30). Catestatin may act to reduce the excitability of RVLM or preBötC neurons when injected into the RVLM.

While the CVLM does not receive direct inputs from chemosensitive afferent neurons, it may play a role in altering the effectiveness of the peripheral chemoreflex (16). Peripheral chemoreceptor afferent fibers project to the NTS. The NTS, in turn, provides excitatory projections to the RVLM, RTN, and the respiratory central pattern generator (CPG). The CPG projects to the RVLM, CVLM, and other respiratory sites. The CVLM, in turn, provides GABAergic inputs to bulbospinal presympathetic RVLM neurons. Therefore, a change in CVLM neuron activity may result in disinhibition, or further inhibition, of RVLM presympathetic neuron activity. The potential downstream effects include alterations in peripheral chemoreflex sensitivity.

**Potential mechanisms underlying the effects of catestatin.**

As noted above, catestatin appears to act in a manner different to other neuropeptides that exert their effects via direct action on specific receptors. To this extent, catestatin seems to belong to an entirely different class of neuropeptide, in that it has the capacity to modulate the effects of various types of neurotransmitters. The mechanisms by which this modulation occurs remain to be determined. Catestatin is known to exert its effects through activation of, or inhibition of, several receptor subtypes and by the activation of multiple intracellular signaling pathways including: nAChR (24), β-adrenoceptors (26), H1 histamine receptors (15), and the β2-adrenoceptor – Gi/o protein signaling pathway (4). The NO-cGMP-cCMP-dependent protein kinase (PKG) cascade was also shown to be involved in catestatin-dependent cardiosuppressive effects (4). We speculate that catestatin affects the activity, or
intracellular milieu, of neurons either by acting at classical receptors, or by translocating across the cell membrane to interact with intracellular signaling pathways directly (50). Catestatin shares many characteristics with other cell penetrating peptides (CPP), including being cationic and having an arginine-rich loop (38). In vitro studies have established that catestatin rapidly penetrates the cell membrane to activate calcium-independent phospholipase A₂, which leads to the production of lysophospholipids and the subsequent opening of store-operated calcium channels (43, 50). Increased calcium entry into the cell may explain the excitatory effects of catestatin in both the CVLM and RVLM.

PERSPECTIVES & SIGNIFICANCE:

Catestatin levels are decreased in hypertensive individuals (32). In this, and earlier studies, we show that, in normotensive rats, catestatin is excitatory in the RVLM (10) and CVLM. Our studies also suggest that in certain circumstances, catestatin may play a role in regulating adaptive reflexes including the sympathetic baroreflex and chemoreflex. Studies in human and CgA-knockout mice demonstrate that catestatin mechanisms are altered in hypertension (11, 19, 32). Catestatin also acts as a mediator between the neuroendocrine and immune systems. Understanding the mechanisms underlying the actions of catestatin, and how this peptide works in concert with other neurotransmitters, may point the way towards the development of therapeutic agents that affect this intriguing new class of peptide.

GRANTS:

Work in the Authors' laboratory is supported by grants from the National Health and Medical Research Council of Australia (457080, 457069, and 604002), the Australian
Research Council (DP110102110), and Macquarie University. AHG is supported by a Macquarie Research Excellence Scholarship.

**DISCLOSURES:**

No conflicts of interest are reported by the Authors.
REFERENCES:

5. **Burke PGR, Li Q, Costin ML, McMullan S, Pilowsky PM, and Goodchild AK.** Somatostatin 2A receptor-expressing presympathetic neurons in the rostral ventrolateral medulla maintain blood pressure. *Hypertension* 52: 1127-1133, 2008.


30. **Moraes DJA, Bonagamba LGH, Zoccal DB, and Machado BH.**


FIGURE LEGENDS:

Figure 1. Photomicrograph and illustration of brainstem coronal section showing microinjection site at the level of the CVLM. Sections were counterstained with neutral red. Sp5 = spinal trigeminal tract; NA = nucleus ambiguus; LRN = lateral reticular nucleus; IO = inferior olive; Pyr = pyramid.

Figure 2. Bilateral microinjections of catestatin (1 mM, 50 nL) into the CVLM significantly decrease mean arterial pressure (MAP), splanchnic sympathetic nerve activity (sSNA), and heart rate (HR) when compared with vehicle microinjections (n=7). [A] A sample trace illustrating MAP, sSNA, and HR responses to both vehicle and catestatin microinjections into the CVLM. Arrows indicate timing of injections. [B] Average peak MAP decrease = -22.7 ± 3.7 mmHg post-catestatin (P=0.0009). [C] Average peak decrease in sSNA = -26.6 ± 5.7% post-catestatin (P=0.004). [D] HR average decrease = -18.6 ± 4.5 bpm post-catestatin (P= 0.007). *** P<0.001, ** P<0.01.

Figure 3. Bilateral microinjections of catestatin (1 mM, 50 nL, n = 5) into the CVLM decreases the amplitude of phrenic nerve activity (PNamp). [A] Here we provide a sample trace showing the effects of catestatin microinjection on PNamp. Arrows indicate when catestatin was injected. PNA = phrenic nerve activity; top trace is smoothed and integrated, bottom trace is raw PNA. Black line on top PNA trace indicates PNamp. Expanded portions of the trace are shown in inserts (5 second duration). [B] Grouped data showing that PNamp is significantly decreased (-16.8 ± 3.3 %; (P=0.03)) after catestatin microinjection into the CVLM. * P<0.05.
Figure 4. Bilateral microinjections of catestatin (1 mM, 50 nL, n = 4) into the CVLM affect respiratory pattern. [A] Sample trace showing the phrenic interburst interval (T_{tot}); arrows indicate when catestatin was injected. Grouped data showing that T_{tot} does not change after microinjection [B], while phrenic inspiratory period (T_i) is significantly decreased ([C]; $P<0.05$) and expiratory period (T_e) is increased ([D]; $P<0.05$). * $P<0.05$

Figure 5. Catestatin microinjection into the CVLM attenuates the sympathetic baroreflex *in vivo* (n=6). [A] Sample trace showing responses to phenylephrine (PE) after pretreatment with vehicle or catestatin (1 mM, 50 nL microinjection to CVLM). [B] Slope indicating effectiveness of the baroreflex is decreased by 63.6% ($\mu$V/mmHg, $P<0.01$). [C] The range of the sSNA response to PE was significantly decreased after catestatin ($P<0.05$). * $P<0.05$, ** $P<0.01$.

Figure 6. Catestatin attenuates the peripheral chemoreflex response (n = 5). [A] Representative splanchnic nerve activity trace demonstrating the 12s N₂ exposures used to test the peripheral chemoreflex. The trace shows two nitrogen exposures given before and after bilateral microinjections of catestatin (Cts) into the CVLM. Expanded portions of the trace illustrate the sigmoid curve fit from which the slope and amplitude data were derived. The slope [B] and amplitude [C] of the sSNA response to hypoxia is significantly decreased after pretreatment with catestatin when compared with the vehicle response. * $P<0.05$, ** $P<0.01$. 