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The chromogranin A- derived N-terminal peptide vasostatin-I: *in vivo* effects on cardiovascular variables in the rabbit

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Abstract

This study is the first to report on vascular effect of the chromogranin A derived Vasostatin-I (CgA_{1-76}) *in vivo.* Cardiovascular parameters were recorded in 29 rabbits with sympathetically decentralized right carotid vascular bed. The recombinant human STA CgA_{1-78} (VS-1) was infused at 480 μg/kg over 25 min. Group I was kept awake while groups II-V were anesthetized with Ketamine-xylazine. VS-1 was given alone in groups I-II while in presence of either phentolamine, phentolamine plus propranolol or hexamethonium in groups III-V.

Serum VS-1 peaked at 2 μg/ml (200 nM) before onset of vascular effects and declined rapidly to ~200 ng/ml within 30 min. In all groups but III and IV VS-1 induced a brief vasoconstriction, being larger in intact than in sympathetically decentralized beds. The VS-1 induced vasoconstriction was not altered by hexamethonium but was abolished by phentolamine. In presence of the α-adrenergic blocker a long lasting vasodilatation, unaffected by propranolol, was apparent on both innervated and decentralized sides.

In conclusion, VS-1 induced an α-adrenoceptor-mediated vasoconstriction presumably brought about by noradrenaline release from sympathetic nerves when infused at a dose giving an initial serum concentration of ~200 nM. This initial vasoconstriction masked a persistent adrenoceptor-independent vasodilatation, consistent with previous reports from *in vitro* models.

Key words: sympathetic nervous system – vasoconstriction – vasodilatation – adrenergic blockades – ganglion blockade – conscious animal

1. Introduction

The exocytotic co-release of chromogranin A (CgA) with catecholamines (CA) from the adrenal medulla and sympathetic nerves suggests a significant role also for the circulating CgA by itself and/or as a prohormone for regulatory peptides involved in modulation of several homeostatic mechanisms [1]. At present it is accepted that CgA generates a number of structurally distinct peptides with defined biological activities concerning the regulation not only of plasma glucose and Ca^{2+} but also of vascular and heart contractility, release of adrenomedullary catecholamines (CA), alleviation of inflammatory responses and modulation of angiogenesis [2-6].

The main product of proteolytic processing of CgA in the bovine adrenal medulla is the N-terminal domain (CgA_{1-76}) termed vasostatin-I (VS-I) [7,8]. Evidence for cardiovascular responses to this domain has been accumulated since 1992 when it was first demonstrated that natural and synthetic VS-I exert a suppressive effect on precontracted human blood vessels *in vitro* [9]. In addition, VS-I appears to exert a wide spectrum of cardiac effects *in vitro* and *ex vivo*, which consists of a reduction in contractility as observed in the avascular hearts of eel and frog [10-13] and in the isolated, perfused rat heart [14-16]. In isolated rat hearts also a limitation of infarct size by pre-ischemic exposure to VS-1 has been reported [16]. Taken together, these *in vitro* and *ex vivo* findings point to an indirect, counter-adrenergic function of VS-I on the heart which could be due to an endocardial/endothelial release of nitric oxide (NO) [17], resulting in protection against excessive sympathetic stimulations such as those elicited by stress.

While the cardiovascular activity of VS-I is well documented *ex vivo* in isolated working hearts, data on *in vivo* animal models or in humans are not yet available. This study aims at investigating acute systemic and local cardiovascular effect of i.v. infusion of recombinant human VS-I (rhCgA1-78, VS-1). Based on a previously developed rabbit model [18], local vascular effects are investigated in intact and sympathetically decentralized vascular beds in order to understand the interaction between the putative dilatory action of VS-1 and the vasoconstrictor sympathetic tone in conscious and anesthetized animals. The time course of the concentration of the infused peptide in plasma has also been investigated and correlated with the observed hemodynamic effects and the plasma concentrations of noradrenaline (NA) and adrenaline (ADR) have been assessed before and at intervals during 150 min of peptide exposure.

2. Methods

2.1. Animals

Twenty-nine adult male European rabbits (*Oryctolagus cuniculus*) weighing 2.8 - 3.4 kg were kept in accordance with the principles of laboratory animal care. The experimental protocols conform with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No 5-23, revised 1996) and have been approved by the Ethical Committee for Animal Experiments at the University of Turin (Italy). At the end of all the experimental procedures the rabbits were killed with i.v. injection of a lethal dose of Urethane and autopsy was performed.

2.2. Anesthetics

Ketamine-xylazine (KX) was employed at doses of 20 or 5.5 mg/kg i.m., supplemented by continuous infusion of the same drugs (15-17 or 4-5 mg/kg/h i.v.). In the case of long lasting experiments and when the animals were killed immediately after the experiments, a single dose of Urethane (300 mg/kg i.v.) was added [19].

2.3. Animal groups

The effects of VS-1 were investigated in 29 rabbits divided in 7 groups:

Group 1 (n=5) rabbits in the awake state.

Group IIa (n=5): anesthetized, no other drugs.

Group IIb (n=4): anesthetized, no other drugs; blood samples collected at intervals before and after onset of VS-1 infusion to determine the serum concentration of VS-1.

Group IIc (n=4) anesthetized, no other drugs; blood samples collected at intervals before and after onset of VS-1 infusion to determine the serum concentration of noradrenaline (NA) and adrenaline (ADR).

Group III (n=5): anesthetized and in presence of α-adrenergic blockade.

Group IV (n=3) anesthetized and in presence of α- plus ß-adrenergic blockade.

Group V (n=3) anesthetized and in presence of ganglion blockade.

Figure 1 schematically shows the different surgical and pharmacological interventions targeting to different levels of the autonomic system.

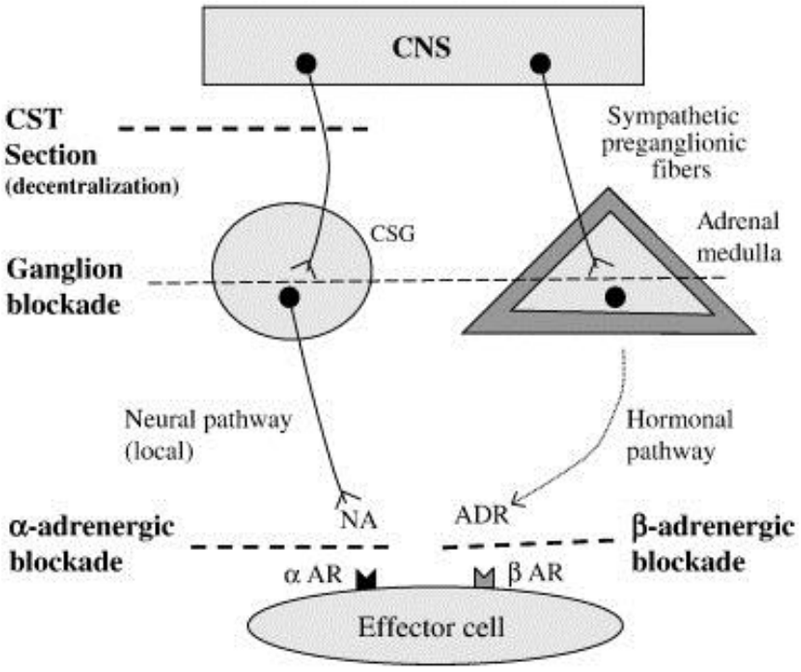

Figure 1. Schematic illustration of the experimental design. Simplified scheme of sympathetic pathways and of interruptions of sympathetic outflow (dashed lines) by the different surgical and pharmacological interventions (in bold). CNS: Central Nervous System, CSG: cervical sympathetic ganglion, CST: cervical sympathetic trunk, NA: noradrenaline, ADR: adrenaline, AR: adrenergic receptors.

2.4. Surgical procedures

2.4.1. Group 1: implant of chronic probes

Under KX-anesthesia a needle-cannula was inserted in the marginal ear vein for continuous administration of anesthetics (infusion pump Terumo STC-521, Tokyo, JP). Perivascular flow probes (model 1PRB, Transonic Systems Inc, Itaha, NY, USA) were implanted on both common carotid arteries (CCA). The probe wire was tunnelled below the platysma to the angle of the mandible and then subcutaneously around the neck. The small probe connector was left protruding from the dorsal cervical region, for subsequent connection to the flowmeter (2-channels TS420, Transonic) and thereafter sutured to the skin by means of a Delrin skin bouton (Transonic).

Arterial blood pressure (ABP) was measured through a telemetric system (PhysioTel PA-D70, DSI Instruments St Paul, MN USA) via a catheter connected with a pressure transducer pushed in the abdominal aorta via the right femoral artery. The ABP signal was radio-transmitted from the implanted transducer to a nearby located antenna. The radio transmitter was placed in a subcutaneous pouch in the abdomen.

Local sympathetic decentralization of the right CCA vascular bed was performed by cutting the corresponding cervical sympathetic trunk (CST) in the neck (preganglionic fibres). The efficacy of decentralization was tested by observing the changes in pupil diameter while supramaximally stimulating the CST (10 V, 0.5 ms, 20 imp/s). The manoeuvre was considered correct when the large pupil dilatation characteristic of an intact CST was abolished by the cut. Sterile procedures were used throughout surgery.

After anesthesia was discontinued, the group I animals underwent analgesic (Finadyne, 2 mg/kg i.m.) and antibiotic (Rubrocillin retard, 0.1 ml/kg i.m.) treatments and were allowed to recover from surgery for 7-10 days before peptide infusion. Then VS-1 injection was performed on the awake animal.

2.4.2. Group II-V: implant of probes for experiments on the same day

For studies of effects of VS-1 infusion under KX-anesthesia on the same day of surgery the skull was fixed in a stereotaxic frame through screws implanted in nasal and frontal bones. Trachea was intubated and a small well was built in the neck with skin flaps filled with saline solution to protect the artery-probe assembly, perivascular flow probes being located bilaterally around CCAs. The solution was kept at 37 °C with a heating lamp. Two venous catheters were inserted for separate infusion of anesthetics and other drugs, either bilaterally in the femoral veins or in a femoral vein and a marginal ear vein. In Group IIb rabbits a catheter was inserted in the left femoral artery to collect blood samples. ABP was measured via a catheter in the femoral artery as described above. The right CST was cut and efficacy of decentralization was tested as in Group 1 experiments. In two group IIa, rabbits an additional perivascular flow probe (model 1PRS or 1PRB, Transonic Systems Inc, Itaha, NY, USA) was implanted on the facial artery, on the CST-intact side. The technique of implantation, as well as the anatomical scheme of the facial artery supply in rabbits, were described in detail elsewhere [18].

In all experiments the animal condition and the level of anaesthesia were evaluated by continuously monitoring ABP, HR, respiratory movements through an inductive proximity sensor [20 and by estimating withdrawal reflexes. Body temperature was kept at 38 °C through a heating blanket regulated by feedback through a rectal thermistor probe (Harvard, UK).

2.5. Peptide infusions and analysis of serum VS-IR

In all groups VS-1 was given at a standard dose of 480 μg/kg dissolved in 5 ml of saline and given over 25 minutes via an infusion pump (Terumo STC-521, Tokyo, JP) through an ear vein in awake rabbits (Group I) and through a femoral or ear vein in the anesthetized rabbits (Groups II-V).

Infusion of 5 ml saline alone was made over 25 min in Group IIa rabbits (n=2) and found not to produce detectable changes in any of the cardiovascular parameters.

In preliminary experiments on rabbits prepared as Groups I and II, a wide range of peptide doses (4.8 - 550 μg/kg dissolved in 5 ml) were infused over 25 min for assessment of cardiovascular effects. Stable and reproducible parameters were recorded at and above VS-1 doses of 450 μg/kg. In the following experiments a standard dose of 480 μg/kg was therefore employed.

Serum concentration of immunoreactive VS-1 (VS-IR) was determined in Group IIb. Arterial blood was sampled every 5 to 10 min before and from onset of VS-1 infusion. Aliquots of 0.3 ml were centrifuged at 10.000 g for 5 min to extract serum and the supernatant was frozen. VS-IR was

detected by sandwich ELISA based on the anti-CgA monoclonal antibody 5A8 (capture step) and the biotinylated anti-CgA mAb B4E11 (detection step), essentially as previously described [21].

2.6. Plasma CA

Plasma concentration of CA was assessed in blood collected via a cannula inserted through the femoral artery into the abdominal aorta and sampled immediately prior to (basal) and at 30, 50, 80 and 150 min after onset of peptide infusion in KX-anesthetized animals (Group IIc, n=4). The blood samples were centrifuged (2000 g, 10 min, 4 °C) within 30 min of collection and the supernatants were stored at -30 °C until analysis for NA and ADR by HPLC technology (Baldi e Riberi Analysis Laboratory, S.Giovanni Battista University Hospital, Torino, Italy).

2.7. Peptide and pharmaca

Recombinant human CgA_{1-78} ($STACgA_{1-78}$,VS-1) was expressed in E. coli [22] and purified by reverse-phase HPLC and gel filtration chromatography as previously described [23]. Phentolamine methanesulphonate was obtained from Sigma (3 mg/kg i.v.), propranolol hydrochloride from AstraZeneca (2.5 mg/kg i.v.), hexamethonium choride from Sigma (20 mg/kg), Finadyne from Schering-Plough (2 mg/kg i.m.), Rubrocillin retard from Intervet (12500 UI/kg), Xylazin from Bayer, Ketalar from Parke-Davis and Urethane from Sigma.

2.8. Experimental protocols

ABP and the common carotid blood flow (CCABF) were continuously recorded, in two rabbits also the facial artery blood flow (FaBF). From these signals heart rate (HR), the common carotid artery conductance (CCAC) and facial artery conductance (FaC) were subsequently computed.

2.8.1. Group I, Awake animals

Three standardized tests known to produce different vasomotor responses were carried out on each rabbit in randomized sequences during a 2 h session on the day preceding peptide infusion 7-10 days after surgery. The air jet test consisted of a fine stream of compressed air (~50 l/min) directed toward rabbit's muzzle for ~60 s [24]. The noxious stimulus consisted of hypertonic saline (0.5 ml of 6% NaCl) [25] injected into a thigh muscle by means of a needle cannula inserted in the muscle a few minutes before the injection and connected to a syringe kept out of animal's sight. The feeding stimulus consisted of chewing pieces of tomato and carrot.

The next day these animals were tested for VS-1 responses in the awake condition. During experiments the animals were kept in a box (45 x 15 x 20 cm) closed in its anterior portion by a sliding cover to minimize disturbing visual stimuli [18]. After 30 min of stabilization VS-1 was infused by means of a remote syringe (Terumo infusion pump, Tokyo, JP) and the responses were recorded as long as the animal remained quiet, generally for 2.5 – 3 h.

2.8.2. Groups II-V, KX-anesthetized animals

VS-1 was infused after 60 min of stabilization of the hemodynamic condition and the effects were recorded for 3 to 7 h. VS-1 was tested alone (Groups IIa and IIb) and in the presence of phentolamine (Group III), phentolamine plus propranolol (Group IV) or hexamethonium hydrochloride (Group V).

2.9. Data acquisition and processing

CCABF, FaBF and ABP were recorded as analog voltage signals, then digitally sampled (1401micro, CED, UK) (sampling rate: 200 Hz), continuously acquired and stored in a personal computer. Acquisition and off-line processing were performed with Spike2 (CED, UK). Simple algorithms were implemented in the Spike2 script language aimed at identifying single cardiac cycles (based on systolic peak detection on the ABP signal), from which HR and time averages of the different signals (one value per cardiac cycle) were computed. CCAC and FaC were computed as CCABF/ABP and FaBF/ABP ratios, respectively.

2.10. Data analysis

All values in the text are presented as means ± SD while in the graphs as means ± SEM. Changes in the conductance responses are expressed as percent of the stabilized values prior to peptide infusion. The response of each variable was assessed at two time intervals, i.e. at 35'-50' and 120'-135' from onset of VS-1 infusion defined as "early phase" and "late phase" of the response, respectively, and compared to its own control value averaged in the 30-min interval preceding VS-1 infusion. Significance of the effects of VS-1 infusion on CCAC was first assessed in anesthetized and awake animals in the absence of other drugs by means of a 3-way ANOVA with the following factors: with *vs* without anesthesia, i.e. groups IIa/I), intact *vs* decentralized sympathetic innervation and early *vs* late phase of time. Then the effect of the different blocking agents on the VS-1 response was assessed with a 3-way ANOVA with the following factors: group (IIa *vs* III, IV and V), sympathetic innervation and time. Where applicable, the significance of specific comparisons were assessed by Tukey HSD post-hoc test.

3. Results

Experiments were initially performed on awake animals to avoid interference from anesthetics on the responses to VS-1. Despite previous training of the rabbits to stay calm in the cage for a few hours [18], it proved difficult to obtain stable recordings in the awake animals over a sufficient length of time. Thus, in some animals brisk movements caused large instability in the recordings and these experiments were therefore discarded. For this reason no more than 5 experiments were

successfully carried out on awake animals and all further studies were carried out on rabbits under general anesthesia.

3.1. Physiological changes in CCA conductance in the awake condition

Awake rabbits (Group I) were first tested for changes in CCA vascular conductance in response to three well defined and short-lasting physiological stimuli. As illustrated in Fig. 2 A, the air-jet stimulus (a) produced a marked vasoconstriction associated with an increase in ABP. A similar decrease in CCAC was observed in response to the nociceptive stimulus (not shown), ranging between 50 and 70% of control values. In contrast, brisk mastication (b) produced a clear vasodilatation and the increase in CCAC ranged between 70 and 100% of the pre-stimulus controls. These values served as reference for the effect of VS-1 described below.

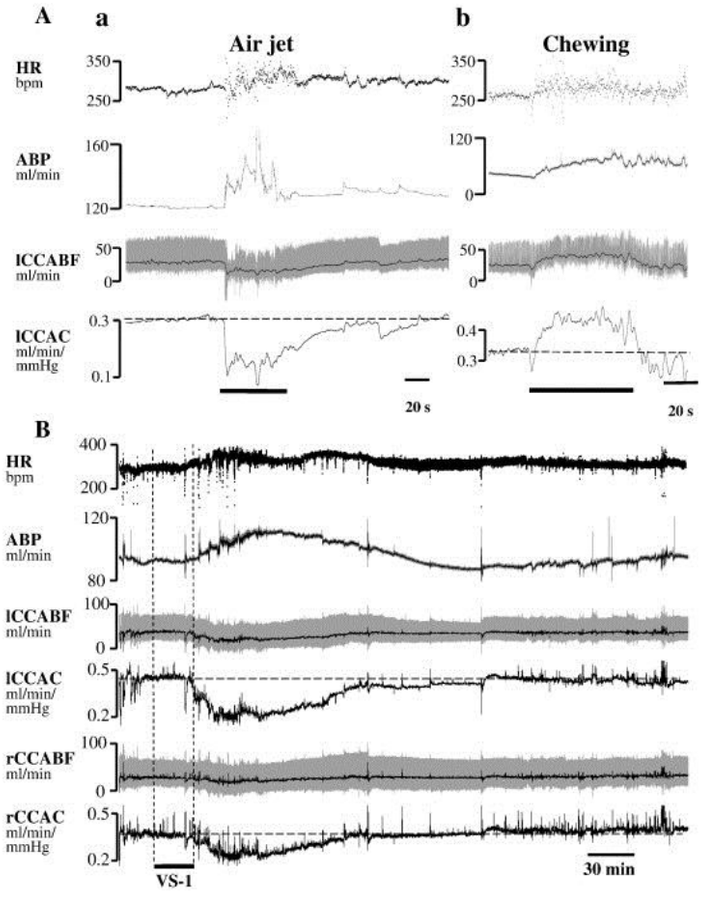

Figure 2. Cardiovascular changes occurring in the same awake rabbit (group I) (A) under different physiological conditions and (B) in response to VS-1. In (A) the responses are shown to (a) an air jet stressor producing a generalized vasoconstriction and (b) to mastication of carrot resulting in a local vasodilatation. The following variables were recorded: heart rate (HR), arterial blood pressure (ABP), left and right common carotid artery (CCA) blood flow (lCCABF and rCCABF) with average curves superimposed, left and right CCA conductance (lCCAC and rCCAC). Left cervical sympathetic trunk (CST) was intact and right CST cut. In (A) only left CCA records are shown. Times of air jet, mastication and VS-1 infusion are marked by thick bars. Note that the reduction in conductance induced by VS-1 in (B) (vasoconstriction) was more marked on the intact than on the sympathetically decentralized side (not shown).

3.2. Hemodynamic effects of VS-1 (Groups I and IIa)

A representative response to VS-1 in the same awake animal as in 2 A is given in fig. 2 B. The rise in HR was slight while ABP increased by ~18% concomitant with a decline in CCABF and CCAC on both the innervated and sympathetically decentralized sides. These responses began before the end of the infusion, peaked within the next 20-30 min and lasted about 100 min. The reduction in conductance was more marked in the innervated (-47%) than in the decentralized vascular bed (-33%).

The effects of VS-1 infusion on all recorded parameters is summarized in Fig. 3 showing average values ± SEM of ABP, HR and conductance on the sympathetically innervated (lCCAC) and sympathetically decentralized (rCCAC) sides in awake (Group I, filled symbols) and anesthetized animals (Group IIa, open symbols). With regard to systemic effect VS-1 induced small but significant increases in ABP in both awake and anaesthetised rabbits relative to controls (Fig. 3). Thereafter ABP returned to the control 3 to 4 hours after the peptide infusion started. Only in awake condition VS-1 induced some variability in HR, consisting of an initial increase (Figs 2 and 3) often followed by a decrease. However, neither of these changes reached statistical significance.

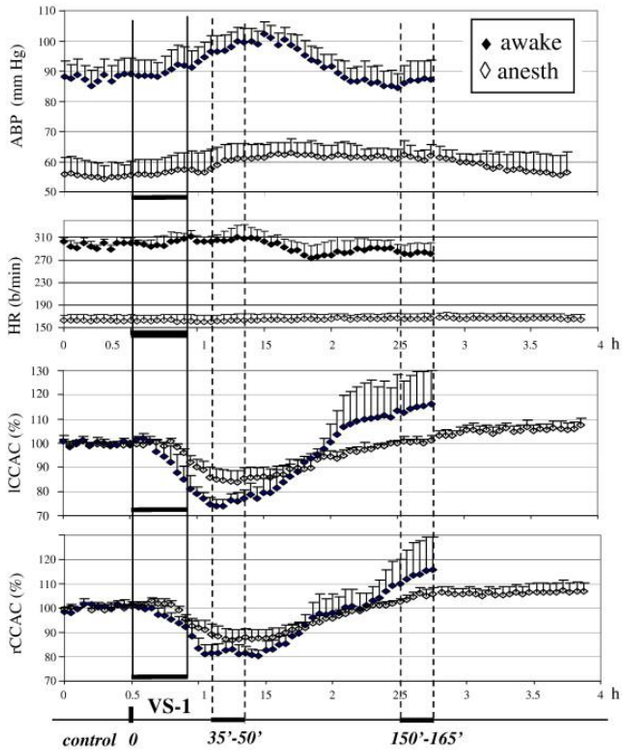

Figure 3. Effect of VS-1 infusion on arterial blood pressure (ABP), heart rate (HR) and conductance in the common carotid artery on the intact (lCCAC) and sympathetically decentralized (rCCAC) sides, in awake (filled symbols: group I, n=5) or anesthetized (open symbols: group IIa, n=5) rabbits. Duration of the experiments varied, being shortest in awake animals (see Methods). Time of VS-1 infusion is marked by horizontal bars and vertical thin lines. Two time windows of 15 min duration, during which the data for analysis were collected, are indicated by dotted lines and on the second x-axis, i.e. at 35'-50' and 150'-165' relative to zero time at onset of VS-1 infusion. The 30 min control period is also indicated. Traces of lCCAC and rCCAC are normalized and expressed in % relative to pre-infusion levels. Values are means of data ± SEM collected over 3 min in each group.

The most marked change in response to VS-1 concerned CCAC (Fig. 3 and Table 1). In general, the vasoconstrictor effect exhibited the same time course in awake and anesthetised rabbits; it was slightly bigger in the former group although not reaching statistical significance. Effect of VS-1 depended both on time ($P<0.01$) and on sympathetic innervation ($P<0.01$). Thus, by pooling together groups I and IIa, post-hoc test indicated a significant difference between innervated and decentralized sides in the early phase of response ($P<0.05$) and not in the late phase (Fig. 3 and Table 1).

Table 1. Effects of VS-I on conductance in common carotid arteries (CCAC) separately assessed for sympathetically innervated and decentralized sides, in the different groups.

Effect of infusion of VS-1 at zero time in the following animal groups:	Innervated side (ICCAC) (A) Change in % of pre-infusion level ± SD	Cut ide (rCCAC) (B) Change in % of pre-infusion level ± SD	Sides [c]	Groups Side A [d]
I. Awake (n=5)			A vs B,	I vs IIa
early phase [a]	- 25 ± 13 (p<0.01)[b]	- 18 ± 10 (p<0.05)	p<0.05	ns
late phase	+ 13 ± 26 (ns)	+ 13± 25 (ns)	ns	ns
IIa – V. Anesthetized (n=16)				
IIa. No blockers (n=5)			A vs B	IIa vs V
early	- 15 ± 10 (p<0.05)	- 12 ± 10 (p<0.05)	ns	ns
late	+ 1 ± 4 (ns)	+ 5± 10 (ns)	ns	ns
III. + phentholamine (n=5)			A vs B	III vs IIa
early	+ 28 ± 12 (p<0.01)	+ 33 ± 13 (p<0.01)	ns	p<0.01
late	+ 41 ± 23 (p<0.01)	+ 47 ± 20 (p<0.01)	ns	p<0.01
IV. + phentholamine + propranolol (n=3)			A vs B	IV vs III
early	+ 33 ± 16 (p<0.05)	+ 34 ± 18 (p<0.05)	ns	ns
late	+ 45 ± 24 (p<0.05)	+ 47 ± 16 (p<0.05)	ns	ns
V. + hexamethonium (n = 3)			A vs B	
early	- 14 ± 4 (p<0.05)	- 12 ± 3 (p<0.01)	ns	
late	- 3 ± 4 (ns)	- 4 ± 4 (ns)	ns	

[a] Time from onset of the 25'-lasting VS-1 infusion; early phase of the effect computed at the 35'-50' interval, late phase at the 120'-150' interval.

[b] Significance for difference from pre-infusion level.

[c] Significance for difference between A) innervated and B) CSN-cut (sympathetically decentralized) sides.

[d] Significance for difference between innervated side A in the respective groups.

Of note, in both conditions the conductance response to VS-1 was remarkably similar in latency and time course for the first 2 h regardless of differences in surgical history and presence or absence of anaesthesia. In some of the trials this initial phase of decrease in conductance was followed by an

increase which was most marked in the awake rabbits, however without reaching statistical significance.

In two of the rabbits in group IIa the effect of VS-1 infusion on blood flow through the facial artery and CCA on the CST-intact side were compared (Fig. 4). The response in the facial artery was a decrease in both flow and conductance (rFaC), consistent with vasoconstriction and was comparable in magnitude and time course of the conductance (rCCAC) apparent in CCA in the same experiments.

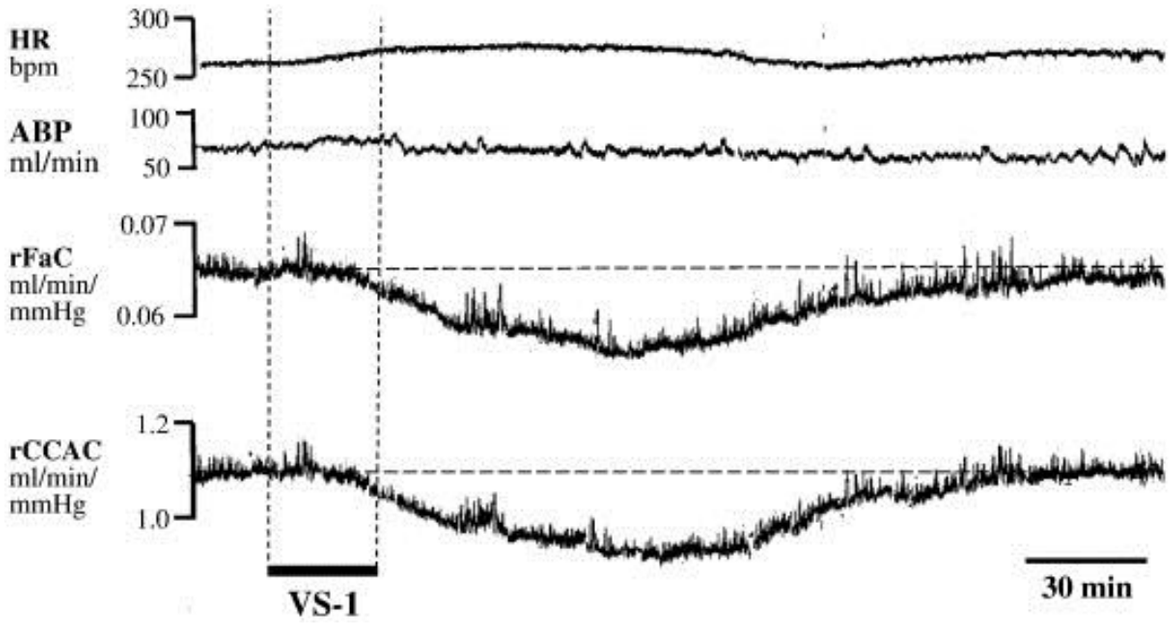

Figure 4. Comparison of the effect of VS-1 infusion on CCA and on the facial artery simultaneously recorded in the same anesthetized rabbit (group IIa). From top to bottom: heart rate (HR), arterial blood pressure (ABP), conductance in the facial artery (FaC), conductance in the common carotid artery (CCAC) on the same CST-intact side. Note that the percent reduction of conductance is very similar in the two arteries. VS-1 infusion is marked by a thick bar.

3.3. Effect of VS-I on plasma CA (Group IIc)

The mean plasma NA prior to peptide exposure was 116 ± 61 ng/L and no significant increase was observed during the 30 - 150 min period following onset of peptide infusion. Plasma ADR was of the same magnitude as NA, with a baseline value of 107 ± 94 ng/L. In response to VS-1 injection plasma ADR fluctuated more than plasma NA. However, the mean ADR values at 30, 50, 80 and 150 min were not significantly different from the mean baseline value.

3.4. Effect of VS-1 on CCA conductance in rabbits in presence of α-adrenergic blockade (Group III)

Figure 5 compares the effect of VS-I in the same anesthetized rabbit in the absence (A) and presence (B) of phentolamine. Infusion of the peptide under control conditions (Fig. 5 A) induced a decrease in CCAC in both innervated and sympathetically decentralized sides, with similar time

course as in the awake animals (Fig. 2). Vasoconstriction was no longer apparent when the peptide was infused in the presence of α-adrenoceptor blocker (Fig. 5 B). Instead, the response consisted of a gradual and long lasting rise in conductance, starting at about the end of the peptide infusion and reaching a plateau 35-45% above the baseline about 40 min later.

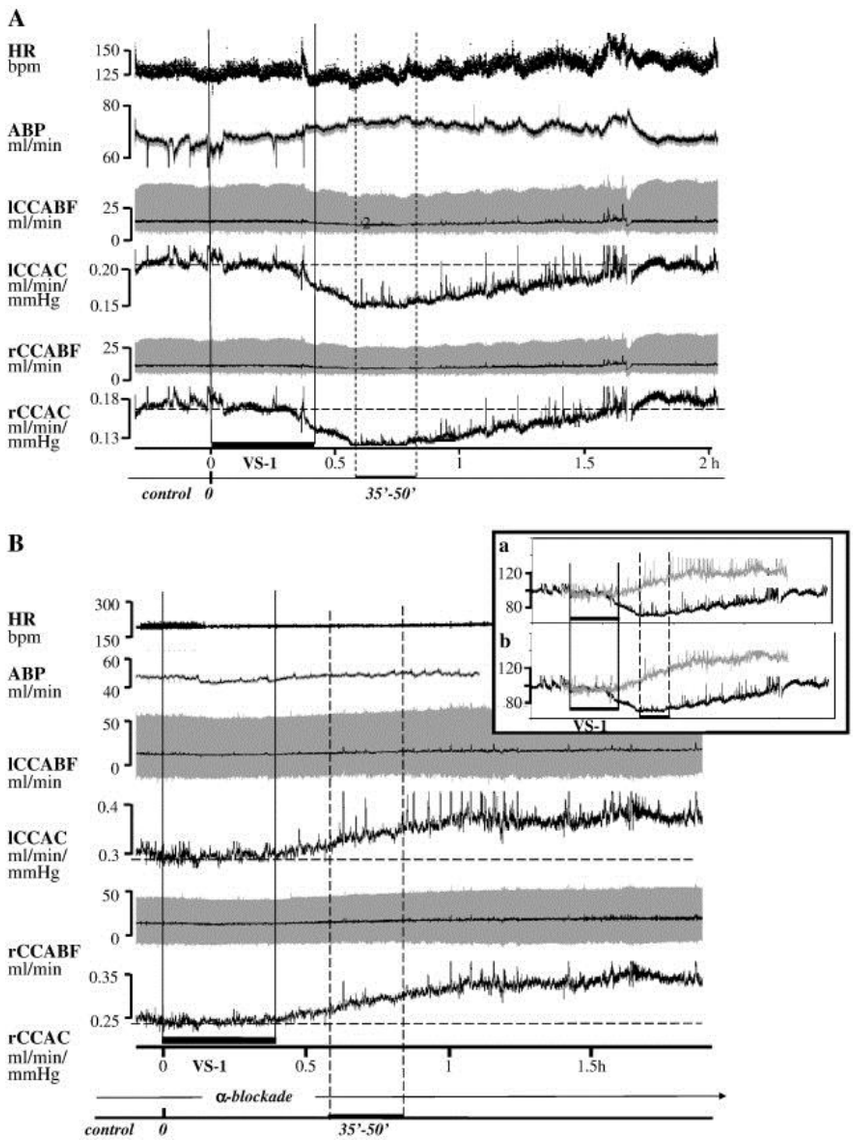

Figure 5. Effect of VS-1 infusion on cardiovascular parameters in an anesthetized rabbit (Group IIa) with right cervical sympathetic trunk (CST) cut, (A) in absence and (B) in presence of α-adrenergic blockade. Heart rate (HR), arterial blood pressure (ABP), left and right common carotid artery (CCA) blood flow (lCCABF and rCCABF) with average curves superimposed, left and right CCA conductance (lCCAC and rCCAC). The VS-1 -induced vasoconstriction in absence of phentholamine (A) was reverted to vasodilatation in (B) in the presence of α-adrenergic blockade. Two time windows of 15 min duration are indicated on the second x-axis, i.e. at 35'-50' and 150'-165' relative to zero time at onset of VS-1 infusion corresponding to the early and late phase of the effect, respectively. *Insert*: Superimposed CCAC curves normalized to pre-infusion levels and expressed in percent values in absence (black) and in presence (gray) of α-adrenergic blockade on (a) the intact and (b) the sympathetically decentralized sides.

In Fig. 5 B insert the recorded CCAC curves in the absence (black) and presence of phentolamine (grey) have been superimposed for comparison of time course of the respective vasoconstrictor and

dilator responses on the intact (a) and decentralized (b) side. Importantly, the latencies of the two oppositely directed responses were similar while differing with respect to the time to the peak.

Exposure to VS-I in the presence of α-blockade produced similar effects in all 5 animals to those in Fig. 5 B. The analysis of variance performed over groups IIa-V revealed a dependence on the group ($P< 0.01$) and on time ($P<0.01$) but not on sympathetic innervation. As reported in Table 1, in group III the mean increases in CCAC in the early phase of the response were highly significant for both innervated and sympathetically decentralized sides. In the late phase the CCAC values were further significantly increased (Table 1). In 3 of these animals the elevated levels of CCAC were maintained for 4 to 7 h.

Figure 6 A and B summarize the marked differences in conductance in the common carotid artery on the sympathetically intact side in response to infusion of VS-1 in the absence (A) and in the presence (B) of α-adrenergic blockade.

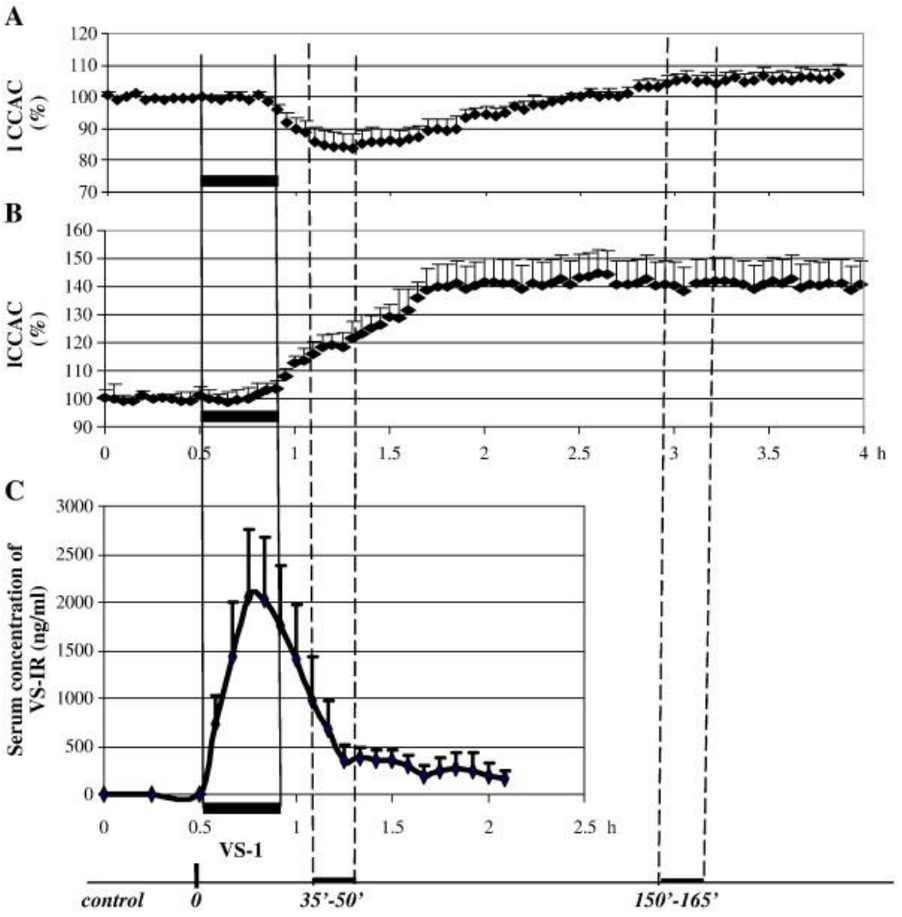

Figure 6. Comparison of the time course of effects of VS-1 infusion (480 µg/kg over 25 min, horizontal bars) on (A, B) the sympathetically innervated left CCAC, (A) in absence (group IIa, n=5) and (B) in presence of α-adrenergic blockade (group III, n=5). CCAC is expressed in % of pre-infusion level. Curves are mean values ± SEM of data averaged over 3 min. Note differences in time course of these two oppositely-directed effects with respect to development and duration. In (C): time course of the immunoreactive peptide (VS-IR) concentrations in serum. Values are means ± SEM from samples taken every 5 min (group IIb, n=4 rabbits). Note the near symmetrical peak, indicating a half life of 16-18 min of VS-IR in plasma. Two time windows of 35'-50' and 150'-165' duration relative to zero time are indicated on the second x-axis by bars and vertical dotted lines.

3.5. Effect of VS-1 infusion on the time course of immunoreactive VS-1 in serum (Group IIb)

As illustrated in Fig. 6 C the background of immunoreactive peptide (VS-IR) was close to zero, rising rapidly in response to infusion of VS-1. A mean peak value of 2 μg/ml was obtained between 16 and 18 min after the onset of the peptide infusion, declining rapidly to 1/10 of the peak value within the following 30 min. Two hours after the end of infusion plasma VS-IR was approximately down to pre-infusion background level.

3.6. Effect of VS-1 on CCA conductance in rabbits in the presence of α- and ß-adrenergic blockade (Group IV)

Infusion of VS-1 in the presence of phentolamine plus propranolol resulted in a marked vasodilatation with significant increases in CCAC on both sides (Table 1), increasing further at the late phase of the response. These results were not significantly different in size and time course from those observed in the presence of α-adrenergic blockade alone (Table 1 and Fig. 5 B). Thus, the VS-1-induced vasodilatation in presence of α-adrenergic blockade appeared insensitive to the β-adrenergic blocker and was therefore assumed to be distinct from a sympathetic ß-adrenergic action of the peptide. As already observed in the Group III rabbits this vasodilatory effect persisted for several hours.

3.7. Effect of VS-1 on CCA conductance in rabbits in presence of ganglion blockade (Group V)

VS-1 infusion in presence of the ganglion blocking agent hexamethonium (Table 1) induced a clear vasoconstriction with similar degrees of decline in CCAC on both sides (Table 1). The effect of VS-1 on these animals was not significantly different from the effect observed in absence of adrenergic blockers (Group IIa, Figs 2 B and 4 A) in terms of magnitude and time course of the vasoconstriction.

4. Discussion

4.1. The initial vasoconstrictor response to VS-1 *in vivo*

The present data provide novel information on cardiovascular effects of the CGA derived VS-1 in an animal model. Observations from awake and anaesthetized rabbits have demonstrated that intravenous infusion of a high dose of VS-1 markedly affects cardiovascular variables, notably vascular conductance in the common carotid vascular bed through an integration with, or modulation of, the catecholamine release. The most marked effect was a powerful vasoconstriction starting 18-25 min after onset of infusion and fading within the following 1.5 h. This response was

significantly smaller on the decentralized side and was completely suppressed in the presence of α-blockade (Table I), implicating release of CA.

Thus, the vasoconstrictor effect appears to be mediated by an additional peptide-induced increase in CA outflow, presumably involving both neural and extra-adrenal elements. The small hypertensive response that mirrors vasoconstriction in both common carotid and facial arteries indicates that the vasoconstrictor response to exogenous VS-1 is shared by other vascular beds, further supporting the concept of a systemic relevance for VS-1.

The activation of sympathetic pathways could be evoked at central or peripheral levels. However, the former possibility is excluded by the observation that the vasoconstrictor effect was unaffected by ganglionic blockade. This suggests that VS-1 action on the sympathetic system is exerted at a level distal to the sympathetic ganglion, as illustrated in Fig. 1. For example, at the high dose VS-1 might have potentiated the release of NA from postganglionic nerve terminals or varicosities (neural pathway) and/or medullary and extra-adrenomedullary chromaffin cells [26, 27] hormonal pathway).

The VS-1 -induced release of NA from nerve terminals acting directly and locally on blood vessels may also be the reason why the mean basal plasma level of NA was not significantly elevated in response to peptide infusion. This finding suggests that the initial vasoconstrictor response was a more sensitive marker of an additional release of NA than the chemical analysis.

The present data on the assessment of plasma CA in 4 animals failed to detect a consistent change in response to VS-1 infusion and call for some comments. The rabbit appears unique among mammals in that only ADR is released from the adrenal medulla [27, 28-30]. Thus, ADR concentration in arterial plasma is a particularly good indicator of the activity of the sympatho-hormonal pathway [31]. On the other hand, NA in samples collected at the level of the abdominal aorta as an average result of leakage from all body tissues is an approximate, not very sensitive index of the neurally released NA [32]. Moreover, in rabbits ADR has a prominent vasoconstrictor and pressor effect analogous to NA [31]. On this basis, the unchanged concentration of plasma ADR throughout the response suggests that ADR does not play a prominent role in the vasoconstrictor response and that the local NA release is the most likely factor although too weak or too localized to significantly increase its concentration in plasma at the level of the abdominal aorta.

Further studies are in progress to obtain insight into the underlying mechanisms of the VS-1 induced vasoconstrictor response.

4.2. The vasodilator response to VS-1

In the presence of α-adrenergic blockade the initial vasoconstriction was replaced by a vasodilatation with similar latency, yet larger in size and of much longer duration than the vasoconstriction occurring in the absence of α-adrenergic blockade. Intriguingly, the vasodilator effect was resistant to both phentolamine and propranolol. Hence, the non-adrenergic vasodilatation now demonstrated *in vivo* bears striking resemblance to the vasodilator effect reported for VS-1 *in*

vitro on pre-constricted human and bovine blood vessels attributed to an endothelium-independent mechanism [9, 33]. More recently, VS-I has been shown to protect the integrity of the bovine pulmonary and coronary endothelium [34] and to induce endotelium-dependent enhancement of coronary flow in response to VS-I attributed to NO-production in the rat heart [14-17]. The target and mechanism of dilatation in this *in vivo* experimental model remains to be identified.

4.3. Interaction between the VS-1 –induced vasoconstrictor and vasodilator effects

The results of this study are consistent with two overlapping, oppositely directed vascular responses to high doses of VS-1 (Figs 4B and 5A and B). These responses converge to shape the final, net response observed when the peptide is administered in the absence of adrenergic blockers. The results obtained prompted the following considerations. First of all, the response to VS-1 infusion in the absence of blockers was obviously the net sum of the oppositely directed vasoconstrictor and dilator effects, depending finally on their relative contributions. Secondly, the size of the vasoconstrictor response is certainly underestimated due to the concomitant vasodilatation, while instead the size of the vasodilatation could be correctly evaluated after α-adrenoceptor blockade. Last but not least, the much longer duration of the vasodilatation compared to the vasoconstriction may explain the occasional appearance of a late vasodilatation 90-120 min after onset of perfusion.

The present study was not designed to investigate the threshold doses and the dose-response relationships of VS-1 for these two oppositely directed effects. On the other hand, it may be argued that the oppositely directed effects might account for the unstable vasoconstrictor responses observed in our pilot experiments with doses of VS-1 ranging from 4.5 to 450 μ/kg.

Hence, the present findings provide strong support for a non-adrenergic vasodilator response to VS-I *in vivo,* consistent with that previously reported *in vitro,* although this vasodilatation may be masked by a concomitant contribution to the NA-evoked vasoconstriction *in vivo*.

Although the underlying mechanisms and the physiological implications of these oppositely directed effects of VS-1 are yet to be elucidated, it seems highly plausible that both vasoconstrictor and vasodilator effects in the CCA vascular bed are within the physiological range of CCAC changes, as assessed during the standard stress responses and masticatory activity (Figs. 2A).

4.4. The time course of rise and fall in plasma concentrations of immunoreactive VS-1

The circulating concentration of VS-1 is vital for an evaluation of the functional importance of our findings. The dose of VS-1 of 480 μg/kg in 5 ml saline was infused over 25 min and assumed to be evenly distributed in the plasma volume (40 ml /kg animal) [35] to reach a serum concentration of about to 12 μg/ml (1.3 μM). Contrary to this assumption the mean VS-IR peak in serum was markedly lower (2.0±1.2 μg/ml) and equivalent to ~200 nM. Moreover, the peak was reached 7 – 9 minutes before end of the infusion and the concentration declined rapidly to 0.25 μg/ml (25 nM)

within the following 30 min, i.e. to a fraction of the expected value. No decline in VS-1 concentration was detected in the injected solution at the end of the infusion, thus excluding loss of peptide by degradation or adhesion to the inner wall of the syringe during the infusion period. Accordingly, a rapidly activated counter-regulatory mechanism might account for the observed fast elimination of the peptide from the plasma phase.

Of note, the time course of the VS-IR disappearance from the blood resembles the two-pool distribution of plasma CgA during surgical removal of a CgA-producing pheochromocytoma as previously reported by Hsiao et al. [36]. These authors postulated a fast (t½ 16 min) and a slow (t½ 520 min) pool for the circulating human CgA. The predicted ratio of 1:24 between the plasma and extravascular pools led them to suggest a substantial tissue sequestration or binding of CgA. The time course and distribution pattern presently observed for the infused VS-1 points to a similar compartmentalization and short half life for plasma VS-1 in the rabbit.

4.5. Comparison of time course of vascular responses and plasma levels of VS-IR

When comparing the time course for the hemodynamic responses to that of the concentration of VS-IR in plasma (Fig. 6 A-C), it was evident that the onset of vasoconstriction corresponded to the peak of immunoreactive peptide. However, maximal vasoconstriction (Fig. 6 A) was obtained when the plasma pool of the peptide was down to a fraction of the expected value. When vasoconstriction was suppressed by α-adrenergic blockade, the unmasked vasodilatation (Fig. 6 B) showed the same latency in onset but reached a plateau that was maintained for several hours, long after the plasma concentration of the peptide had returned to background levels. The similar time lag in appearance of vasoconstriction and vasodilatation relative to onset of peptide infusion suggests that a rather high plasma concentration e.g., 200 nM, is required to trigger a rapid onset of both responses, but that vasodilatation peaks and is maintained at 1/10 of this plasma concentration.

4.6. Pathophysiological relevance for vascular responses to high plasma concentrations of VS-I

In our study the human recombinant VS-1 was added to the already circulating pool of VS-I. There are only two reports on VS-I in plasma [22, 37]. In a patient with metastatic pheochromocytoma a value of 2.8 nM VS-I was recorded pre-operatively versus <0.1 nM post-operatively. The latter value is consistent with the level in healthy individuals but is much below plasma VS-I in other mammals, ranging between 1.3 and 2.3 nM [37]. It therefore seems unlikely that the circulating levels of VS-I in normal subjects are sufficient to exert effects similar to those observed in our rabbit model. On the other hand, it should be kept in mind that plasma CgA in patients with neuroendocrine tumors may increase up to 10-1000 fold above normal [1, 36], i.e. to levels that could potentially generate VS-I levels similar to those presently observed. Moreover, it cannot be excluded that also the intact CgA may be biologically active via its VS-I domain, even without proteolytic processing. These issues call for future investigations.

4.7. Methodological considerations

It may be argued that the area supplied by the CCAs also includes autoregulated brain areas supplied by the internal carotid arteries and, for this reason, CCAs may not be representative of other vascular beds. However, blood flow in the internal carotid in the rabbit accounts for but a small fraction of the blood flow through the CCA [38]. In addition, stress-induced changes observed in the CCA were of the same order of magnitude as those observed in the facial artery, a branch of the external CCA [18, 31], suggesting that the CCAC changes mostly reflect regulation of extracranial territories. Moreover, VS-1 infusion was shown to produce parallel vasoconstrictions in the simultaneously recorded CCA and the facial artery.

4.8. Concluding remarks

CgA serves as a prohormone not only for VS-I but also for catestatin (bCgA362-372) in cardiovascular disease states such as essential hypertension [39]. While both peptides, distinctly different in sequence, may act synergistically to suppress basal and adrenergic myocardial contractility via PTX sensitive activation of endothelial production of nitric oxide (NO) in rats [14-17, 40], these two peptides also exert PTX-sensitive, antagonistic effects on angiogenesis in human venous umbilical endothelial cells [5, 6]. In addition, VS-1 protects against a PTX-sensitive impairment of endothelial integrity [41]. Hence, as hypothesized elsewhere [3], VS-I and catestatin most likely target to different PTX-sensitive Gαi/o subunits in endothelial cell membranes in a receptor-independent manner to account for their complex and diverging effects on significant endothelial mechanisms. Another mechanism by which VS-I may activate endothelial NO production has been reported for bovine aortic endothelial cells, involving membrane penetration, binding to heparin proteoglycans and eNOS phosphorylation through a P13K-dependent endocytosis [42]. In contrast, in fibroblasts, VS- I induces a proadhesive effect involving binding to membrane phospholipids and subsequent actin-cytoskeleton reorganization dependent on penetration of the C-terminal domain (CgA67-78) [43], Thus, VS-I appears to act via receptor-independent mechanisms that may be tissue and species-specific. Mechanisms behind the vascular responses to VS-I in rabbit tissues have yet to be elucidated.

The marked vascular effects of VS-1 reported in this *in vivo* study in the rabbit have been observed at high plasma concentrations of VS-IR. In the short term, an adrenergic constrictor effect, probably reflecting an increased NA release from sympathetic terminals, appears to prevail over a longer lasting and more pronounced non-adrenergic vasodilatation, at plasma concentrations at and above 25 nM. Further investigations are needed to evaluate the effects of lower concentrations, in particular to identify the dose - response relationship and time course of vasoconstriction and vasodilatation as described in the present study. Although concentration-dependent thresholds in various tissues and species may account for diverging results in different models, more long term observations are in addition needed to fully understand the complex role of this peptide in regulation of the cardiovascular system *in vivo*.

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