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Endogenous angiotensinergic system in neurons of rat and human trigeminal ganglia

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Abstract

To clarify the role of Angiotensin II (Ang II) in the sensory system and especially in the trigeminal ganglia, we studied the expression of angiotensinogen (Ang-N)-, renin-, angiotensin converting enzyme (ACE)- and cathepsin D-mRNA, and the presence of Ang II and substance P in the rat and human trigeminal ganglia. The rat trigeminal ganglia expressed substantial amounts of Ang-N- and ACE mRNA as determined by quantitative real time PCR. Renin mRNA was untraceable in rat samples. Cathepsin D was detected in the rat trigeminal ganglia indicating the possibility of existence of pathways alternative to renin for Ang I formation. *In situ* hybridization in rat trigeminal ganglia revealed expression of Ang-N mRNA in the cytoplasm of numerous neurons. By using immunocytochemistry, a number of neurons and their processes in both the rat and human trigeminal ganglia were stained for Ang II. Post *in situ* hybridization immunocytochemistry reveals that in the rat trigeminal ganglia some, but not all Ang-N mRNA-positive neurons marked for Ang II. In some neurons Substance P was found colocalized with Ang II. Angiotensins from rat trigeminal ganglia were quantitated by radioimmunoassay with and without prior separation by high performance liquid chromatography. Immunoreactive angiotensin II (ir-Ang II) was consistently present and the sum of true Ang II (1-8) octapeptide and its specifically measured metabolites were found to account for it. Radioimmunological and immunocytochemical evidence of ir-Ang II in neuronal tissue is compatible with Ang II as a neurotransmitter. In conclusion, these results suggest that Ang II could be produced locally in the neurons of rat trigeminal ganglia. The localization and colocalization of neuronal Ang II with Substance P in the trigeminal ganglia neurons may be the basis for a participation and function of Ang II in the regulation of nociception and migraine pathology.

Keywords

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1. Introduction

The trigeminal ganglion, a cranial analog of the dorsal root ganglia in the peripheral nervous system, is the site of primary sensory neurons involved in the regulation of nociception, thermoreception, proprioception and mechanoreception in the facial skin, eye, nasal and oral cavities, teeth and periodontal tissue and vibrissae [30] and provides sensory afferents to the cerebral blood vessels [15]. The trigeminovascular system plays an essential role in the pathophysiology of migraine [6,18], in the cerebrovascular vasospasm occurring after subarachnoid hemorrhage [23] and in chronic pain and inflammatory syndromes [13].

A multitude of transmitters and neuromodulators have been described in the trigeminal ganglion and its primary afferent neurons exhibit pathway-specific patterns of neurochemical expression and transmitter colocalization [30]. However, the presence and specific localization of renin-angiotensin system (RAS) components and role of angiotensin II (Ang II) has not been studied, in spite of the association of Ang II with central and peripheral sensory systems [4,5, 52,54] and its proposed role in the regulation of pain [22,43], cerebrovascular inflammation [2,57] and migraine [49,51].

Formation of circulating and local Ang II is mediated through activation of the RAS. The RAS includes a precursor, angiotensinogen (Ang-N) cleaved by the enzyme renin to produce the decapeptide angiotensin I (Ang I). In turn, Ang I is cleaved by angiotensin converting enzyme (ACE) to generate the active RAS principle, the octapeptide Ang II [11].

Existence of an endogenous angiotensinergic system in the neurons of rat and human sympathetic coeliac ganglia innervating mesenteric resistance arteries has been reported recently [37]. Here we address the issue of the possible formation and localization of Ang II in the trigeminal ganglion by determination of Ang-N-, Renin-, ACE- and Cathepsin D-mRNA by qRT-PCR in rat trigeminal ganglia, by using *in situ* hybridization of the Ang II precursor Ang-N mRNA in rat trigeminal ganglia and the expression of Ang II by immunocytochemistry in rat as well as in human trigeminal ganglia. Several studies substantiated the role of substance P in the regulation of sensory transmission in the trigeminal ganglion [17,24,25,30] and the involvement of Ang II in the regulation of Substance P release [14,28]. Hence we attempted to determine the possible formation and localization of Ang II in the neurons of trigeminal ganglion and colocalization with Substance P in the same neurons.

2. Methods

2.1. Rat and human trigeminal ganglia

We purchased 8-week-old, male Wistar Kyoto (WKY) rats (approximately 200 g body weight) from the Central Animal Facilities of the University of Bern. Adequate measures were taken to minimize pain or discomfort, in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with Animal Protocols approved by the Animal Care and Use Committee, NIMH, NIH, USA. Rats were anaesthetized intraperitoneally with 100 mg/kg thiopentane sodium and were perfused transcardially with 150 ml Ringer solution containing 1000 U heparin at 37 °C followed by 300 ml 2% freshly prepared formaldehyde at 4 °C. Trigeminal ganglia were carefully dissected and incubated by immersion fixation in 2% formaldehyde for 28 h at 4 °C. Subsequently, ganglia were immersed for 14 h in phosphate-buffered saline (PBS-Dulbecco) containing 18% sucrose at 4 °C. Fixed ganglia were frozen in isopentane at −50 °C and 30 μm thick sections were cut on a cryostat and subsequently used as free-floating sections for immunocytochemistry. For some experiments after perfusion and immersion fixation rat ganglia were embedded in paraffin. Paraffin sections, 7 μm thick, were used for immunocytochemical as well as for *in situ* hybridization experiments.

For extraction of total RNA and angiotensin components rats were shortly anesthetized with ether and subsequently sacrificed by decapitation. Fresh rat trigeminal ganglia were dissected and instantly transferred into RNA later (Ambion), frozen in liquid nitrogen, and then processed for total RNA extraction (Ambion). For angiotensin component extraction, trigeminal ganglia were rapidly removed, rinsed with cold Ringer solution, blotted by filter paper and wet weight was measured. The ganglia were frozen in liquid nitrogen and stored at −70 °C.

Human trigeminal (semilunar) ganglia were procured from three adult individuals for whom a permit for clinical autopsy (informed written consent by next of kin) had been obtained according to state law, in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). After removal of the brain with transection of the cranial nerve roots along the brainstem and harvest of the pituitary gland from the sella, the superficial dura of the middle cranial fossa was removed by traction with forceps. The semilunar ganglion was then easily detached from its bed (Meckle's cave) and the three trigeminal branches transected at their passage through the foramina. Human trigeminal ganglia were fixed by immersion in freshly prepared 2% formaldehyde for 3 days and then used for cryosectioning or embedded in paraffin.

2.2. RNA isolation and quantitative realtime RT-PCR (qRT-PCR)

Total 6 trigeminal ganglia from different WKY rats were taken for total RNA extraction. RNA integrity was confirmed for each sample on the Agilent Bioanalyzer using the RNA 6000 Nano kit (Agilent Technologies). 1 μg of total RNA was reverse transcribed using Superscript II (Invitrogen) and random hexamers according to the manufacturer's protocol. For realtime PCR, reverse transcribed material corresponding to 40 ng RNA was amplified with the TaqMan assays described below in 25 μl Universal PCR Master Mix, No AmpErase UNG on the SDS 7000 (Applied Biosystems) using the standard thermal protocol. Average values and standard deviations of relative mRNA levels of each sample, normalized to relative 18S rRNA levels, are from four measurements and were calculated using the relative differences. The following TaqMan assays were used for qRT-PCR at a final concentration of 250 nM TaqMan probe and 900 nM of each primer:

Angiotensinogen

Forward primer 5′-CACGACTTCCTGACTTGGATAAAGA-3′;

Reverse primer 5′-CTGCGGCAGGGTCAGA-3′;

TaqMan probe 5′-FAM CCTCGGGCCATCC GMGB- 3′;

manufactured as Assays-by-Design (RATG-EJ3) by Applied Biosystems.

Renin Assay-on-demand Rn00561847_m1 from Applied Biosystems.

ACE Assay-on-demand Rn00561094_m1 from Applied Biosystems.

Cathepsin D Assay-on-demand Rn00592528_m1 from Applied Biosystems.

18S rRNA Predeveloped Assay Reagent 431-9413E from Applied Biosystems.

2.3. In situ hybridization

2.3.1. DIG-labelled RNA probe preparation—By using an appropriate cDNA template for Ang-N [31], a 403 bp long fragment corresponding to nucleotides 221–623 was generated by digestion with restriction enzymes *EcoRV* and *Bam HI*. The obtained fragment was cloned between *Stu I* and *Bam HI* into pBluescript I KS+ (Stratagene). Digoxigenin-labelled probes were prepared using the DIG-RNA-labelling Mix (Roche) according to the manufacturer's protocol. T7 RNA polymerase was used to generate antisense riboprobe using the *Hind III*

linearized template, and the sense strand (used as a control) was generated by T3 RNA polymerase using the same template linearized with *Bam HI* (for gel images please see online supplementary method). The RNA concentration was estimated comparing dot-blot tests and NanoDrop measurements. Filter hybridization with RNA extracts from trigeminal ganglia (RNaqueous-4PCR kit from Ambion) was used for binding tests.

2.3.2. Procedure for in situ hybridization—For *in situ* hybridization, 6 μm thick paraffin sections were rehydrated in a graded alcohol series (2 times Xylol for 10 min, EtOH 100% (2 times), 96%, 70%, 50%, DEPC H_2O each step 5 min) using DEPC-treated H_2O for the dilution of all reagents and solutions. The sections were equilibrated in proteinase K buffer (100 mM Tris, 50 mM EDTA, pH 7.5) for 5 min and then treated with proteinase K (19 μg/ml) at 37 °C for 2 min, after a wash with DEPC-treated H_2O. Following a wash in DEPC-water, sections were post fixed with freshly prepared 4% formaldehyde for 5 min, followed by two subsequent washes in DEPC-water for 5 min each. Sections were then incubated in prehybridization solution (SIGMA) at 45 °C for 2 h, followed by incubation with heat denatured sense and antisense riboprobes (5–10 ng/μl) in 30 μl hybridization mix (SIGMA) for 48 h at 45 °C in a humid chamber (saturated with 2× SSC). Subsequently, the sections were incubated with 2× SSC for 30 min at room temperature, followed by 1 h in 2× SSC at 45 °C and 1 h in 0.1× SSC at 45 °C. Sections were equilibrated for 5 min with buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5), then incubated with alkaline phosphatase-coupled anti-digoxigenin antibody (Roche) 1:500 diluted in buffer 2 (10× blocking solution diluted with buffer 1) for 2 h at room temperature, followed by two 5 min washes with buffer 1. Finally, after 5 min equilibration with buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), the color reaction containing NBT and BCIP in buffer 3 was completed according to manufacturer's protocol (Sigma).

2.4. Immunocytochemistry

For immunocytochemical incubations, a protein G purified murine monoclonal antibody against Ang II (Mab-Trap G II column, Amersham Sciences) was used at a concentration of 0.3 μg/ml in buffer solution. This is a self generated monoclonal antibody against the synthetic peptide to human Ang II. The specificity for the monoclonal antibody against Ang II (4B3) has been previously documented and it produced the same staining as a polyclonal antibody against Ang II in rat adrenal glands [20]. For immunization of the mice, Ang II peptide was cross-linked with glutaraldehyde to keyhole limpet hemocyanin. In dot blot assay, the monoclonal antibody against Ang II (4B3) showed total cross reactivity with Ang III (2–8), Ang 3–8, Ang 4–8 and Ang 5–8. It showed no cross reaction with human plasma angiotensinogen, Ang I (1–10) and angiotensin 1–7. Pre-absorption was performed by using a batch procedure with the synthetic Ang II-peptide that was covalently linked through its N-terminus to CH-Sepharose 4 B. The gel was loaded into a glass column and the monoclonal anti Ang II-antibody solution effluent was used for immunocytochemical preabsorption controls. Further controls were done with mouse non-immune serum and for the secondary goat anti-mouse Cy3 antibody without the primary antibody. All these additional controls showed absolute no staining within the sections. The pre-absorbed monoclonal antibody after low pH-elution revealed the same staining as the Mab-Trap G II purified antibody.

Substance P was detected with a mouse monoclonal antibody to the synthetic peptide Substance P from Novus Biologicals (SP-DE4-21). Goat anti-mouse immunoglobulins (GAM Cy3; Jackson ImmunoResearch) pre-incubated with rat serum prior to immunocytochemistry were used as secondary antibodies. Sections were incubated free floating for 36 h at 4 °C with the primary antibody, washed and incubated with the secondary antibody for 90 min at room temperature and counterstained with DAPI (4′,6-diamidino-2-phenylindole). After washing, cryosections were mounted on gelatin-coated slides and air-dried. The stained sections were

embedded with Glycergel (DAKO) and cover-slipped. The same procedure as for free floating cryosections has been used for paraffin sections mounted on glass slides. Pictures were taken by a normal fluorescent light microscope (LEICA DM 6000B) using a digital camera or by a confocal laser scanning microscope (LEICA SP2 with AOBS).

2.5. Colocalization studies

Directly adjacent free-floating cryosections of rat trigeminal ganglia were immunostained for Ang II and Substance P, as mentioned under methods (2.4 Immunocytochemistry). Pictures were taken by a normal fluorescent light microscope (LEICA DM 6000B) using a digital camera LEICA-DFC350FX. The total number of neurons was counted for each adjacent section, and nuclei which were counterstained with DAPI in blue. Ang II and Substance P immunopositive neurons were counted in respective adjacent sections. For colocalization studies Substance P staining was digitally changed to yellow from red, taking the advantage of directly adjacent sections we superimposed the Ang II and Substance P sections by using Corel Draw and colocalized neurons where counted.

2.6. Measurement of angiotensin peptides

Immunoreactive Ang II (ir-Ang II) as well as Ang-(1–8) octapeptide, Ang-(2–8) heptapeptide, Ang-(3–8) hexapeptide, Ang-(4–8) pentapeptide, Ang-(1–10) decapeptide and Ang-(2–10) nonapeptide were measured in rat trigeminal ganglia by radioimmunoassay without and with prior separation by high performance liquid chromatography (HPLC) [34]. Measurement of true angiotensin was applied also for Ang I, and peptide extraction from tissue was adapted for additional freeze-thaw procedures to ensure the lysis of cells [35,36]. Briefly, trigeminal ganglia weighing 14.8–21.9 mg were homogenized in 1.5 ml water, shock frozen in ethanol/ dry ice, thawed in a 37 °C water bath. The freeze-thaw procedure was repeated twice. Samples were sonicated for 10 min at 37 °C, centrifuged at 3500 g and the supernatant in an albumin-coated polypropylene tube was dried at 37 °C under nitrogen. The residue was dissolved in 2.2 ml Tris buffer (0.1 M, pH 7.5) containing bovine serum albumin (5 g/l). Solid-phase extraction on phenylsilylsilica, HPLC and radioimmunoassay were performed according to the routine procedures using very sensitive antisera for Ang II and Ang I with cross-reactivities of 53% for Ang-(2–8) heptapeptide as well as for Ang-(3–8) hexapeptide, 52% for Ang-(4–8) pentapeptide, and 33% for Ang-(2–10) nonapeptide, respectively [35,36]. Recoveries for the different Ang peptides and for ir-Ang II were consistently above 67% except for the nonapeptide (34±2%, mean±SD). Therefore, no corrections were made for recovery losses. Results are presented as fmol Ang per gram wet weight. Detection limits were variable according to the various weights of individual ganglia. Results below detection limits were taken as zero. All measurable Ang concentrations were at least at 150% of the detection limit except for one hexapeptide result at the detection limit. Instead of homogenates, controls of 1.5 ml water were extracted and quantitated with every analysis and any traces of Ang peptides found in these "blanks" were subtracted to obtain final ganglial concentrations. In 3 rats, ir-Ang II of one trigeminal ganglion was compared with the sum of the specifically measured Ang peptides of the contra lateral trigeminal ganglion. Immunoreactivity of the metabolites was calculated using the cross-reactivity as a factor.

3. Results

3.1. Quantitative real time RT-PCR

In order to demonstrate the existence of Ang II in the trigeminal ganglion we investigated the presence of different RAS components. We detected substantial amounts of Ang-N- and ACE-mRNA in the rat (Fig. 1a and c) trigeminal ganglion by qRT-PCR. No renin mRNA was detected (Fig. 1b). We have discovered cathepsin D mRNA, a possible alternative pathway for Ang I formation (Fig. 1d). RNA from tissues with previously reported high levels of the

respective mRNAs were used as positive controls, and all mRNA values were normalized to 18S rRNA.

3.2. In situ hybridization and post in situ hybridization immunocytochemistry

With paraffin sections, Ang-N mRNA was identified in most, if not all the neurons studied in the rat trigeminal ganglion (Fig. 2a and b). Ang II immunoreactivity was present in both the neuronal cytoplasm and neuronal processes in the rat trigeminal ganglion (Fig. 3). However some neurons were not stained for Ang II (Fig. 3). Additionally we demonstrate Ang II staining in the rat central nervous system (CNS) in the spinal trigeminal tract (Fig. 4a and b).

In the human trigeminal ganglion Ang II immunoreactivity was observed in the cytoplasm (see movie 2, as online supplement) of many neurons and in their processes with both paraffin and free-floating cryosections (Fig. 5a and b).

We used rat paraffin sections for post *in situ* hybridization immunocytochemistry to determine the colocalization of Ang-N mRNA and Ang II in trigeminal ganglia neurons. In the rat, we found that some, but not all the Ang-N mRNA positive neurons (Fig. 6a) were stained for Ang II (Fig. 6b) as revealed in Fig. 6c.

3.3. Immunocytochemistry and colocalization studies

In directly adjacent free-floating cryosections of rat trigeminal ganglia, we detected a number of Ang II (Fig. 7a) and of Substance P-positive neurons (Fig. 7b) and their respective processes. In consecutive paraffin sections we found that out of total neurons, approximately 25% neurons were immunopositive for Ang II (Fig. 8a) and 7 to 8% for Substance P (Fig. 8b). In sum of Ang II and Substance P positive neurons approximately 7% neurons indicated Ang II colocalized with Substance P. The number of Ang II neurons was higher than that of Substance P neurons, and some Ang II positive cells did not contain Substance P (Fig. 8c).

3.4. Concentration of angiotensin peptides in trigeminal ganglia

Immunoreactive Ang II was found in 9 of 10 extracted trigeminal ganglia (76±53 fmol/g, mean ±SD, median 53 fmol/g). Ang I or Ang-(1–10) decapeptide was never (n=10) found above any detection limit (≥4 fmol/g) and no Ang-(2–10) nonapeptide was found in a single experiment. Ang-(1–8) octapeptide was measurable only in one of eight individual ganglia, but in 2 out of 3 pooled ganglia extracts. Only 3 of 6 ganglia contained Ang-(2–8) heptapeptide (33±28 fmol/g, median 27 fmol/g), but 5 of 6 ganglia contained Ang-(3–8) hexapeptide (32±22 fmol/g, median 22 fmol/g). Significant amounts of Ang-(4–8) pentapeptide were present in 4 of 6 ganglia (55± 49 fmol/g, median 87 fmol/g). Table 1 presents levels of Ang I, Ang II, Ang III, Ang IV and Ang V and corresponding detection limits measured in trigeminal ganglia extracts pooled from 2 rats: All Ang peptides except Ang I were found well above detection limits. For individually examined rats, the sum of true Ang II (Ang-1–8) and specifically measured metabolites Ang III, Ang IV and Ang V of the left sided trigeminal ganglion corresponded well with ir-Ang II extracted from the right sided trigeminal ganglion (Fig. 9).

4. Discussion

The proposal of important roles of Ang II in peripheral sympathetic and sensory ganglia [37, 38] and our clear demonstration of the intraneuronal expression of Ang II in the trigeminal ganglia, raises the issue of the possibility of local Ang II formation in these neuronal tissues.

The existence of an endogenous angiotensinergic system in the neurons of rat and human sympathetic coeliac ganglia has been reported recently [37]. In our current work we provide evidence for local formation of Ang II in sensory neurons of trigeminal ganglia. First, we report

with the use of qRT-PCR, the presence of Ang-N mRNA and ACE mRNA in the tissue extracts of trigeminal ganglia from rats. Second, *in situ* hybridization confirmed the existence of Ang-N mRNA at cellular level in the cytoplasm of rat trigeminal ganglia neurons. Ang-N mRNA detection by *in situ* hybridization demonstrates that Ang-N could be locally formed in this tissue, indicating that Ang-N uptake from the circulation is not necessary for the local formation of Ang II. With the exception of one earlier communication on the presence of Ang-N mRNA in the rat trigeminal ganglion extracts [7] ours is the first report on the presence of angiotensin system components at cellular resolution in this structure.

We did not detect the expression of mRNA for renin, the enzyme classically considered necessary for cleaving Ang-N into the Ang I precursor of angiotensin II [38], in the rat trigeminal ganglion. Instead, we detected expression of cathepsin D mRNA in the trigeminal ganglia, at levels higher in comparison with those found in rat liver and kidney, but lower in comparison to those recently reported in rat coeliac ganglia [37]. Cathepsin D is a protease capable of cleaving Ang-N into angiotensin I [48], a non-renin alternative pathway for Ang I formation. Our results are in agreement with an earlier report of the existence of cathepsin D enzymatic activity in rat trigeminal ganglion neurons [1]. These results indicate the existence of an alternate pathway for Ang II synthesis in trigeminal ganglion sensory neurons. Alternatively, neurons of trigeminal ganglion may take up circulating renin to support local synthesis of endogenous Ang II [21]. These mechanisms have been proposed for other tissues such as the heart, where strong evidence exists for uptake of extracellular renin from the circulation [38,10,53,44], while the presence of renin in cardiac tissue is still a matter of controversial debate [16,38–40,45,50,53,56].

In the brain, Ang-N is localized to multiple cell types, predominantly in astrocytes but also in neurons [26]. The expression of the Ang-N gene in selective groups of neurons has been conclusively demonstrated in the murine central nervous system [55]. Our *in situ* hybridization studies demonstrate the presence of Ang-N mRNA specifically in most, but not all, the neuronal cell bodies of the trigeminal ganglia, and not in surrounding cells (Fig. 6a). Not all Ang-N mRNA containing neurons stained for the same extent for Ang II (Fig. 6c). There are several possibilities for this result, including lack of Ang II internalization or storage in these neurons, Ang II production in amounts too low to be detected with our immunocytochemical methods, or to different roles for Ang-N, not related to Ang II formation [9,29].

We have detected Ang II-like immunoreactivity in the cytoplasm of neurons and their processes (Figs. 3, 5 and 7a) within the trigeminal ganglia. Ang II immunoreactivity was found in higher concentration in small and medium sized neurons as compared to large neurons of the rat and human trigeminal ganglia. The use of polyclonal, affinity-purified, monospecific antibodies to Ang II (BODE 1) [5] have revealed similar distribution of Ang II immunoreactivity within the neurons of the rat trigeminal ganglia as with the applied monoclonal anti Ang II (4B3) antibody. We could also detect angiotensinergic processes in the rat CNS in the spinal trigeminal tract (sp5), indicating that Ang II may act as a neurotransmitter in the CNS (Fig. 4a and b). Hence, we can hypothesize that locally formed Ang II may be transported to the terminal fields innervated by the trigeminal ganglion in order to act as a neurotransmitter for sensory transmission, a function similar to that demonstrated in the sympathetic nervous system [37].

Our results demonstrate the presence of Ang II and its metabolites in extracts of trigeminal ganglia of Wistar–Kyoto rats (Table 1) at concentrations well comparable with other tissue concentrations of Ang II established in Wistar rats with similar methodology [36]: These tissue levels were depending on the organ about tenfold higher than plasma concentrations (4.4±0.4 fmol/ml), in heart (10.1±1.2 fmol/g), muscle (30.3.±3.5 fmol/g), liver (69.7±6.9 fmol/g), lung (79.5± 9.7 fmol/g), kidney tissue (192±23 fmol/g), but low in whole brain (3.2±0.5 fmol/g) and very high in adrenal gland (3061±159 fmol/g). The Ang II concentration in WKY rat

trigeminal ganglia is thus six fold higher than whole brain Ang II in Wister rats. Interestingly, brain Ang I (decapeptide) levels (3.0±0.7 fmol/g) in Wister rats were comparable to Ang II levels, but similar concentrations in trigeminal ganglia would have been below detection limit (<3.95 fmol/g) of the present work since maximally 121 mg pooled ganglial tissue was available. It cannot be concluded that Ang I is absent in trigeminal ganglia, but if it would be present it had to be fivefold below Ang II levels and less than half of all other measured Ang peptides.

The reliability of our Ang measurements is well documented by the close relation between ir-Ang II of one-sided ganglia extract and the sum of immunoreactivities of all specifically measured Ang peptides of the contra lateral trigeminal ganglion. There can be little doubt that the Ang II visualized by immunocytochemistry in trigeminal ganglia is indeed Ang II or its metabolites. In the context of perivascular Ang II immunocytochemical stainings and visibility of corresponding varicosities as synapses en passant, a role for angiotensin as a neurotransmitter is very likely [37].

Additionally, we attempted to determine whether Ang II was associated with other neuropeptides in the trigeminal ganglion. From the multiple neurotransmitters and neuromodulators identified in this ganglion, we chose Substance P, a neuropeptide established as a key regulator of sensory transmission in the trigeminal ganglion [17,25,30]. We found that Ang II was colocalized with Substance P in trigeminal ganglion neurons (Fig. 8), supporting the hypothesis of a close integration between these two systems, and the previous reports of regulation of Substance P release by Ang II [14,28,22]. We found that in the total of 826 neurons of trigeminal ganglia 214 were stained for Ang II, 60 were stained for Substance P, whereas 20 neurons were showing colocalization of Ang II and Substance P (Fig. 8c). Neuronal colocalization suggests the possible interaction of Ang II with Substance P and further involvement of Ang II in trigeminal neuralgia and nociception [12,42,43,46]. Angiotensin receptor type 1 have been reported in the nucleus tractus solitarri (NTS) of rat brain [19,47], moreover Substance P receptors are also localized in NTS of rat brain [32].

Our results are not without clinical interest. The trigeminal ganglion provides sensory afferents to the cerebral blood vessels [15] and the trigeminovascular system is involved in the pathophysiology of migraine, cerebrovascular vasospasm, chronic pain and inflammatory syndromes [6,13,23]. RAS inhibition through ACE inhibitors or Ang II type 1 receptor blockade appears to be effective for the prevention of migraine, independently of their blood pressure lowering effects [8,49,51], to increase resistance to brain ischemia [27,33] and to reduce cerebrovascular inflammation [2,57]. It is reasonable to propose that Ang II, formed in the neurons of trigeminal ganglia, plays an important role in cerebrovascular pathophysiology either directly and/or in association with other transmitter systems such as Substance P or calcitonin gene-related peptide [30]. Ang II has an antinociceptive effect during high pain sensitivity [42]. Interactions between the Substance P and RAS are also proposed in the pineal gland, which receives Substance P innervation from the trigeminal ganglia [46] and expresses a local RAS system [3].

In conclusion, our findings strongly indicate the presence of a local angiotensinergic system in neurons of the trigeminal ganglia, where Ang II may act as a neurotransmitter. The roles of Ang II in the trigeminal ganglia possibly include the regulation of sensory pathways including pain and the functions of other local neurotransmitters.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.
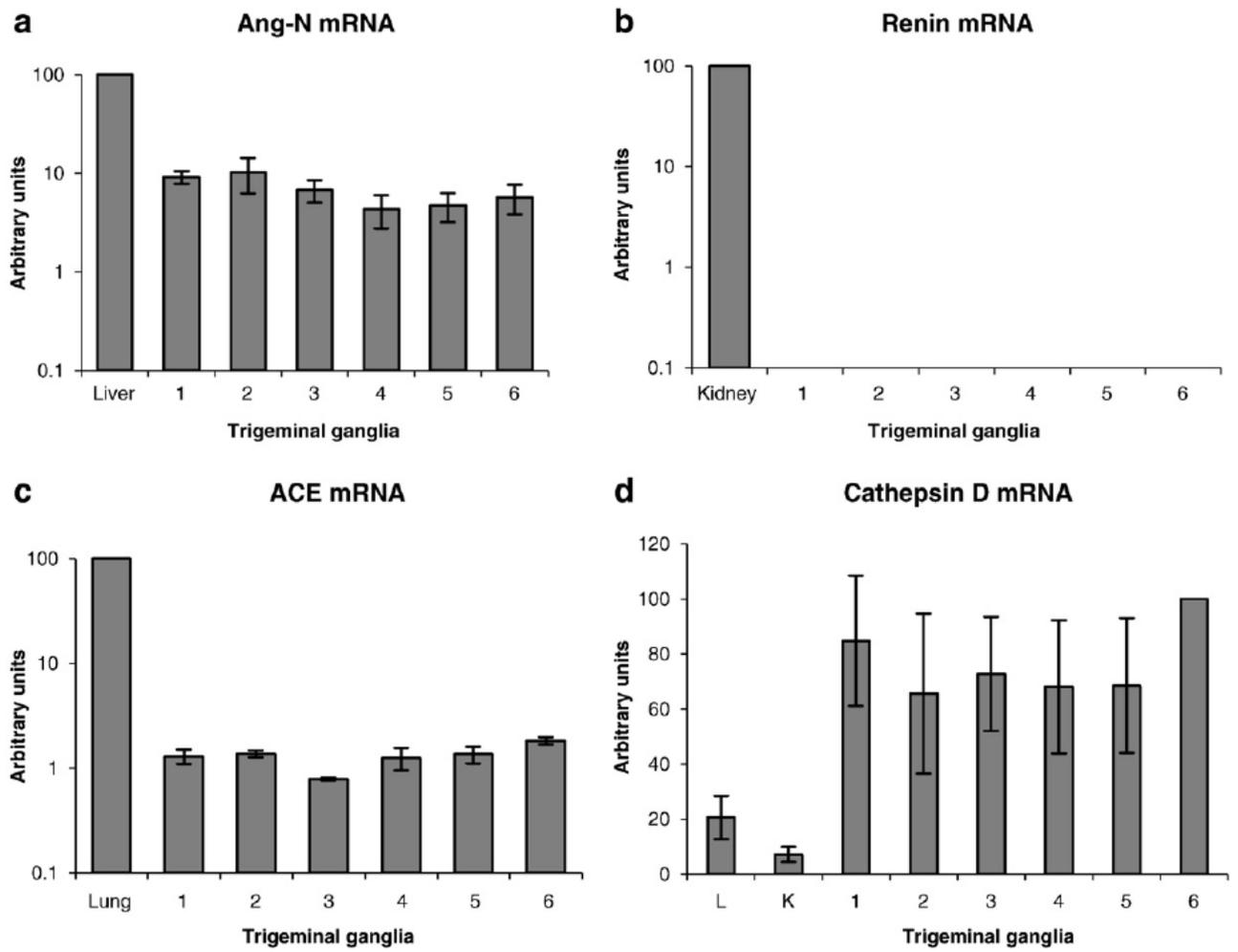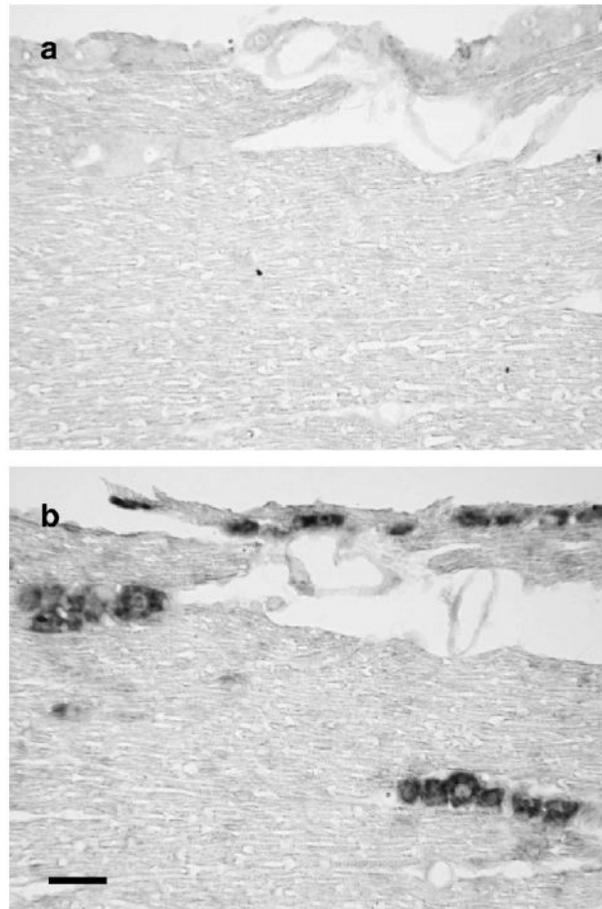
Acknowledgments

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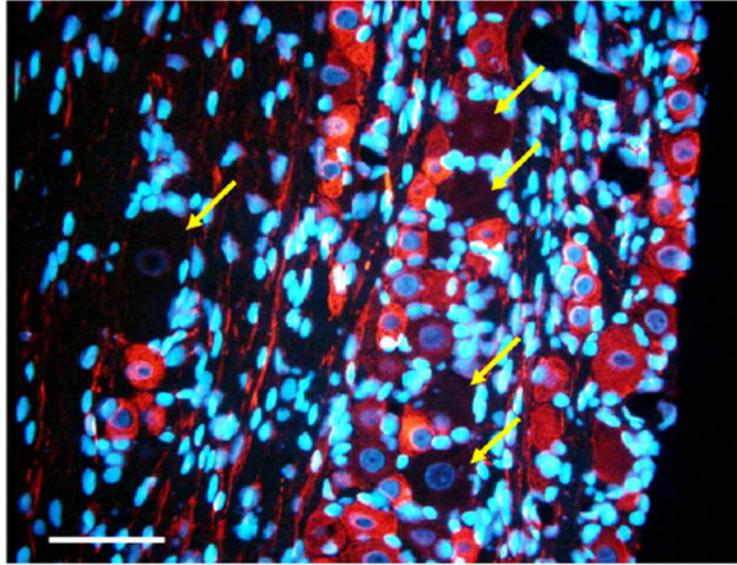
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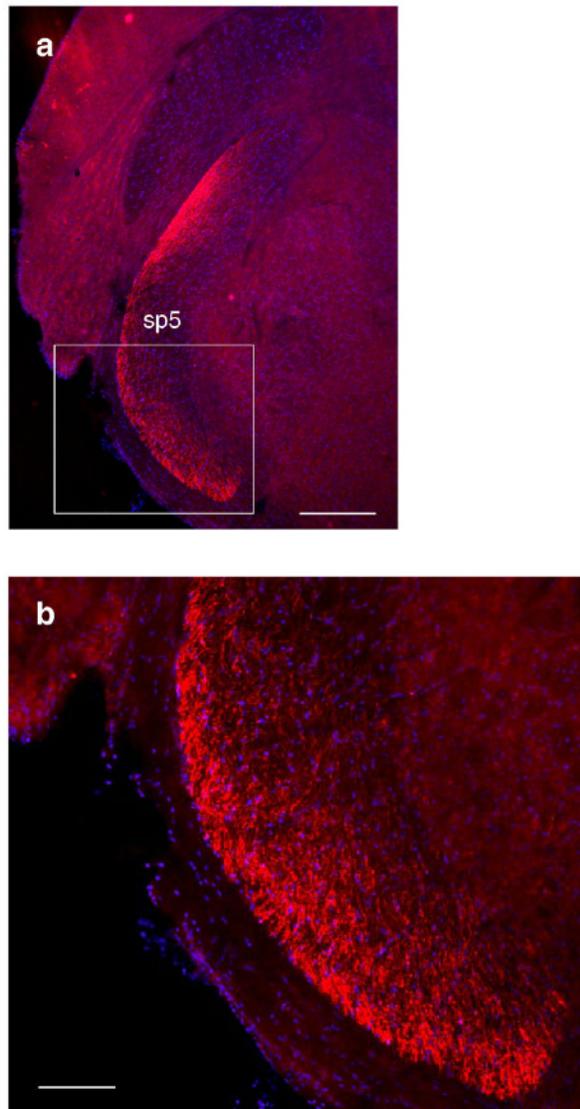
Fig. 1.
Determination of relative mRNA levels in rat trigeminal ganglia. Reverse transcribed total RNA extracts of rat trigeminal ganglia from different rats were tested by qRT-PCR for the presence of Ang-N mRNA (a), for renin mRNA (b), for ACE mRNA (c) and cathepsin D mRNA (d). Total RNA extracts from liver (a), kidney (b), lung (c) and liver–kidney (d) were used as a reference samples expression. All relative mRNA values are normalized to 18S rRNA levels. Average values and standard deviations of 4 qRT-PCR measurements are shown.

Fig. 2.
In situ hybridization in rat trigeminal ganglion for detection of Ang-N mRNA, 6 μm thick consecutive paraffin sections were processed as described in methods. a) sense probe. b) with antisense probe, revealing positive staining in the cytoplasm of numerous neurons within the ganglion. Bar: 100 μm.
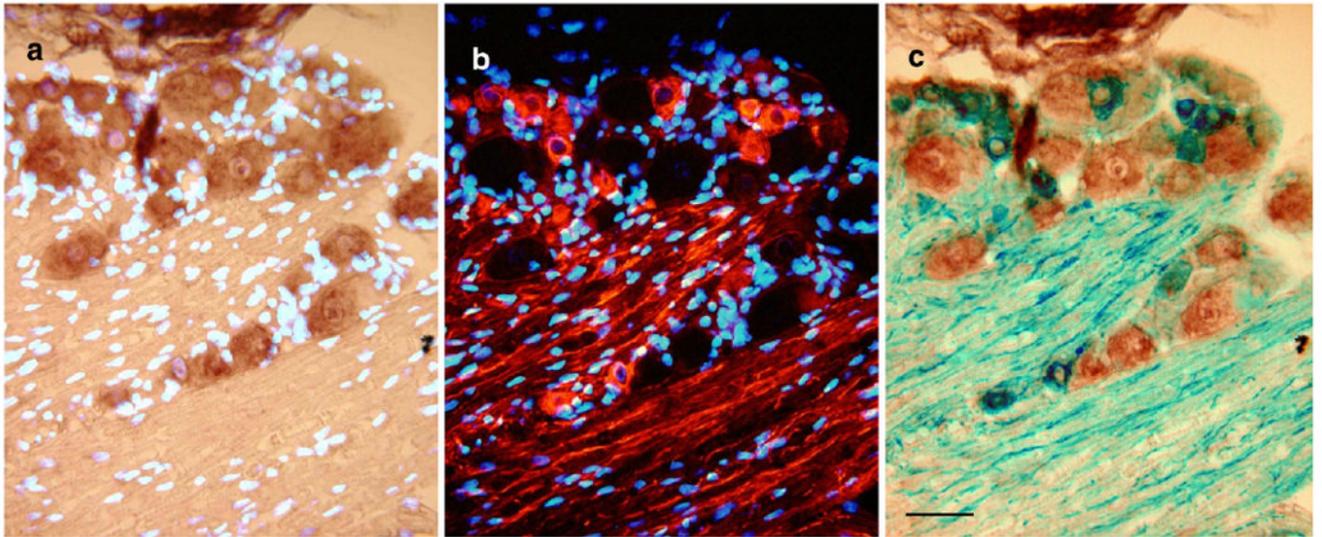
Fig. 3.
Ang II immunocytochemistry in rat trigeminal ganglion. 30 µm thick free-floating cryosections were prepared and incubated with our murine monoclonal antibody against Ang II [20], stained with goat anti-mouse (GAM^Cy3) in red and DAPI in blue for nuclei, as described under methods. Note, the intensity of the staining for Ang II varies among the neurons and their projections, some neurons are not stained for Ang II (see arrows). Bar: 50 µm. See movie 1 in supplementary material.
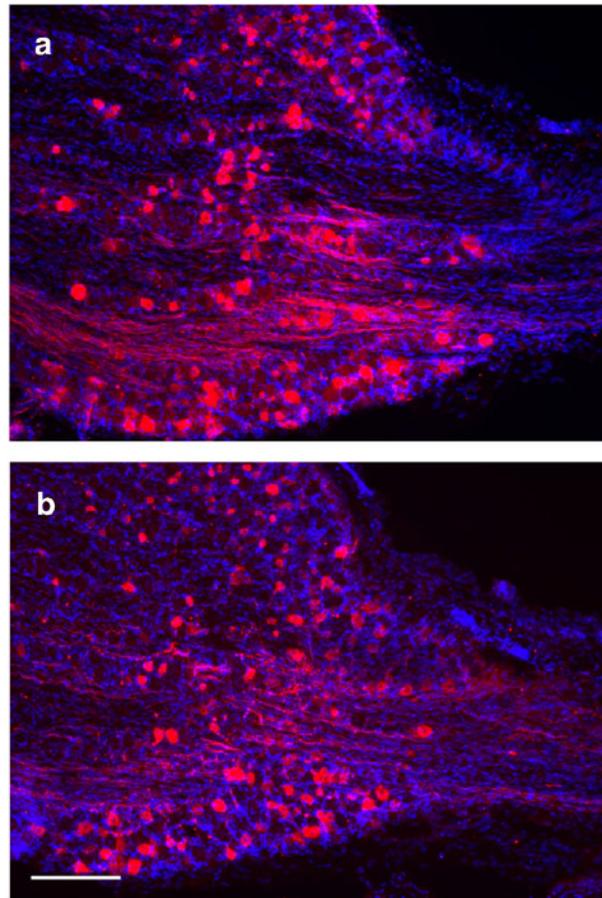
Fig. 4.
Angiotensinergic neuronal processes within the central nervous system in the rat spinal trigeminal tract (sp5) [41]. a. Bar: 200 μm. b. Bar: 100 μm.
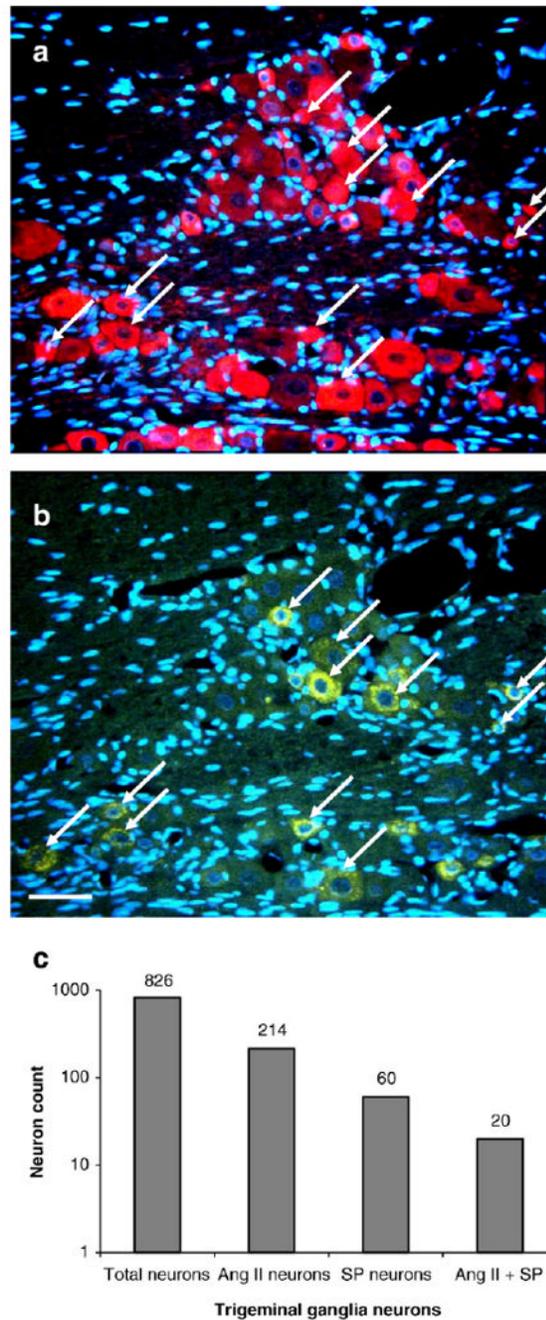
Fig. 5.
Ang II immunocytochemistry in human trigeminal ganglion. a. A 6 μm thick paraffin section, incubated with murine monoclonal anti Ang II antibody [20], stained with goat anti-mouse (GAM^Cy3) in red and DAPI in blue for nuclei. Note, Ang II staining in neuronal cytoplasm and projections and the intensity of the staining varies among the neurons. Autofluorescence of lipofuscin granules is shown in yellow, Bar: 50 μm. b. A free floating 30 μm thick cryosection, incubated with murine monoclonal anti Ang II antibody stained with goat anti-mouse (GAM^Cy3) in red and DAPI in blue, as described under methods. Ang II staining in trigeminal neurons is cytoplasmic and in processes (see arrows). The picture was taken with a confocal laser scanning microscope. Bar: 50 μm. See movie 2 in supplemented material.
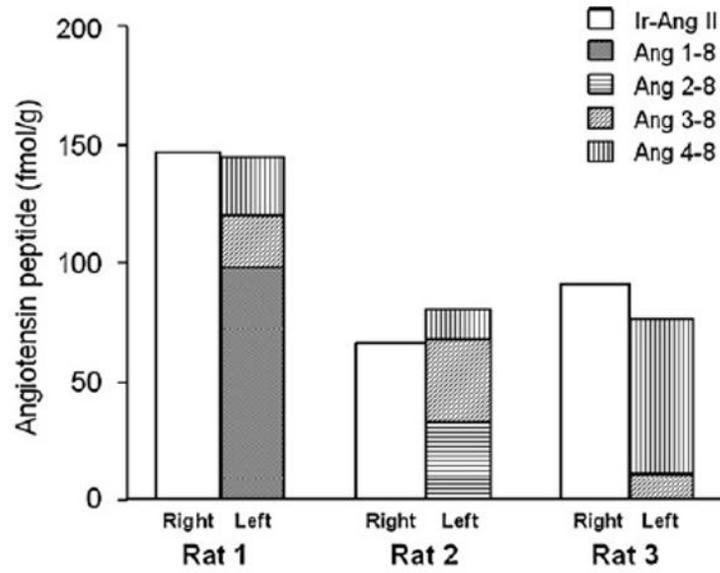
Fig. 6.
Colocalization of Ang-N mRNA and Ang II immunoreactivity in the rat trigeminal ganglion. A 7 μm thick paraffin section was processed for *in situ* hybridization with the antisense probe to detect Ang-N mRNA (a) followed by immunocytochemistry for Ang II as described in methods (b) post *in situ* hybridization immunocytochemistry (c) Here angiotensinergic staining in neurons and projections is in greenish-blue color. Note that not all Ang-N mRNA-expressing neurons stain for Ang II to the same extent. Bar: 100 μm.

Fig. 7.
Ang II and Substance P immunocytochemistry in rat trigeminal ganglion. Directly adjacent, 30 μm free-floating cryosections were incubated with Ang II (a) or Substance P (b) antibodies and stained with goat anti-mouse (GAM^{Cy3}) as secondary antibodies in red and DAPI in blue as nuclear stain, as described under methods. Bar: 200 μm.

Fig. 8.
Colocalization of Ang II and Substance P (SP) immunoreactivity in rat trigeminal ganglion neurons. Directly adjacent, 6 μm thick paraffin sections were incubated with Ang II (a) or Substance P (b) antibodies and then stained with goat anti-mouse (GAM^{Cy3}) as secondary antibodies in red, DAPI for nuclei in blue. In order to perform the colocalization studies Substance P originally stained in red, was digitally changed to yellow. Arrows indicate colocalized, positive neuronal staining Bar: 50 μm. Note that more neurons were stained for Ang II than for Substance P, while some neurons show colocalization of Ang II with Substance P. Statistical analysis showing total neurons of trigeminal ganglia sections, Ang II-, Substance P-positive neurons and their colocalization (c).

Fig. 9.
Concentration of ir-Ang II (RIA) and specifically measured Ang peptides (HPLC-RIA) in trigeminal ganglia of 3 rats: The sum of individually measured Ang peptides of one trigeminal ganglion parallels levels of ir-Ang II as measured in the contralateral trigeminal ganglion.

Table 1

Angiotensin concentration of rat trigeminal ganglia using HPLC-RIA (fmol/g wet weight; 4 ganglia of 2 rats were pooled).

Peptide name	Ang-(1–10) decapeptide	Ang-(1–8) octapeptide	Ang-(2–8) heptapeptide	Ang-(3–8) hexapeptide	Ang-(4–8) pentapeptide
Trivial name	Ang I	Ang II	Ang III	Ang IV	Ang V
Peptide content (fmol/g)	<4.0	19.7	8.2	13.4	17.9
Detection limit (fmol/g)	4.0	2.0	3.8	3.8	3.8