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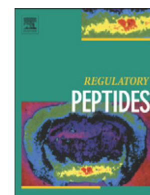

Endogenous angiotensinergic system in neurons of rat and human trigeminal ganglia

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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.

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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

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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 2x SSC). Subsequently, the sections were incubated with 2x SSC for 30 min at room temperature, followed by 1 h in 2x SSC at 45 °C and 1 h in 0.1x 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 (10x 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

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