Delayed Maturation of Catecholamine Phenotype in Nucleus Tractus Solitarius of Rats With Glial Angiotensinogen Depletion

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Abstract—Cerebral catecholamines and angiotensins are both involved in the regulation of cardiovascular function. Recent in vitro studies have suggested that angiotensin II modulates noradrenergic neurotransmission by controlling both the expression and neuritic trafficking of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis. To assess the potential existence of such mechanisms in vivo, we compared TH phenotype ontogeny in the nucleus tractus solitarius (NTS), which is the first central relay of the baroreflex, between control Sprague-Dawley rats and TGR(ASrAOGEN) rats (TG) with glial specific angiotensinogen (AOGEN) depletion. TG displayed a delayed increase in both TH-mRNA and TH protein levels, which sharply rises in the NTS of control rats within the fourth week. The delayed maturation of TH phenotype also affected the presence of TH protein in the neuropil, not only within the NTS region but also within the ventrolateral medulla. This was evidenced by a large decrease in the density of TH-containing neuronal processes in TG at 4 weeks only, without noticeable modification of the labeling of the neuritic marker MAP2, suggesting that neuritic trafficking of TH protein was transiently altered. These results indicate that glial AOGEN is crucial to coordinate within the fourth week the mechanisms driving the maturation of NTS catecholaminergic neurons and suggest that impairment of the central angiotensinergic system early in development can lead to cardiovascular dysfunction related to altered maturation of catecholaminergic neurons located in both the dorsal and the ventrolateral medulla. (Hypertension. 2003;42:978-984.)

Key Words: rats, transgenic ■ angiotensin ■ angiotensinogen ■ catecholamines ■ gene expression

Catecholamine neurons of Nucleus Tractus Solitarius (NTS), which is the first central relay of the baroreflex, and cerebral angiotensins, in particular angiotensin II (Ang II), play an important role in the regulation of blood pressure.2–6 This effect of Ang II involves its interaction with NTS neurons through angiotensin type 1 (AT1) receptor.7–10 Within this structure, catecholamine (noradrenergic and adrenergic) neurons might be those targeted by Ang II, since (1) they express AT1 receptors at their cell body surface,11,12 and (2) recent in vitro studies have revealed that Ang II operates chronotropic actions on noradrenergic hypothalamus and brain stem neuronal cocultures from newborn rats.13 In addition, Ang II has been shown to modulate at longer term the noradrenergic metabolism of these cultured neurons through unusual molecular mechanisms. Indeed, in these neurons, Ang II, through AT1 receptors, not only stimulates the expression of tyrosine hydroxylase (TH),14,15 the rate-limiting enzyme in catecholamine biosynthesis,16 but also controls the neuritic trafficking of TH protein.17 However, such potential modulations of TH phenotype by Ang II have only been characterized in vitro and deserve to be verified in vivo within the NTS.

Cerebral angiotensins can be produced by both glial and neuronal cells in vivo, which have been shown to express angiotensinogen (AOGEN), the only known precursor of all angiotensins.18 However, within the NTS region, only glial cells have been immunostained for AOGEN throughout postnatal development and at the adult stage.19 Therefore, to determine whether angiotensins derived from glial AOGEN may participate in regulating TH phenotype in the NTS, we investigated the developmental profile of TH phenotype in the NTS region of transgenic TGR(ASrAOGEN)680 rats (TG), characterized by a specific downregulation of glial AOGEN synthesis. These rats display at adulthood a decreased blood pressure, an increased cardiac baroreflex sensitivity, and a reduced drinking response to intracerebroventricular renin administration.20,21 They express an antisense (AS) RNA targeted against AOGEN-mRNA, under the control of glial fibrillary acidic protein (GFAP) promoter. Since GFAP promoter is activated early during embryogenesis,22 TG is a helpful model to investigate the in vivo regulatory effects of the depletion of glial AOGEN–derived peptides on TH phenotype maturation in the NTS during development.

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Methods

Animals and Tissue Preparation
All studies were performed according to the Guiding Principles in the Care and Use of Animals, corresponding to the American physiological society guidelines. All TG were obtained from our breeding colony, and control Sprague-Dawley rats (mother strain) were purchased from Iffa-Credo.

In a first set of experiments, 10 OFA Sprague-Dawley (control SD) rats and TG, ages 2, 3, 4, 7, and 12 weeks, were anesthetized with intraperitoneal injection of sodium pentobarbital (0.4 mg/kg; Sanofi); the brains were removed frozen in isopentane and stored at −80 °C. Tissue homogenization was performed in distilled water, and the resulting homogenates were divided to perform protein and total mRNA extractions.

Proteins, after quantification of total protein by the Bradford technique, were extracted with the use of Trizol (Invitrogen), following the manufacturer’s instructions. After treatment of the homogenate fractions with a 0.1% perchloric acid–0.1% EDTA solution and centrifugation at 4 °C, catecholamines were measured in the supernatant.

A second set of experiments, 7 SD rats and 5 TG, 3 and 12 weeks of age, were injected with intraperitoneal NSD-1015 (100 mg/kg; RBI) and were decapitated 20 minutes later to determine in vivo activity of TH, by the measurement of L-dihydroxy phenylalanine (L-DOPA) accumulation. Brains were frozen in isopentane. Five hundred micrometers coronal cryostat-cut sections were made throughout the brain medulla as described above. L-DOPA was extracted as described above for catecholamines.

In a third set of experiments, 3 SD rats and 3 TG (4 weeks old) and 4 SD rats and 4 TG (12 weeks old) were given transcardial perfusion through the brain medulla as described above. L-DOPA was measured by the dot blot technique, with the use of Trizol (Invitrogen), the proteins and total mRNA extractions.

TH-Immunohistochemistry

Tissue TH level was measured by the dot blot technique, with the use of a mouse monoclonal anti-TH antibody (Roche Diagnostics; final concentration, 13 ng/mL) and 100 μCi/mL [3H] protein (Amer- sham; SA, 30 μCi/mg), as described previously. Membranes were then exposed onto special crystal ray–sensitive screens (Molecular Dynamics). Quantification of spots was performed with the use of ImageQuant software (Molecular Dynamics). The immunohistauro-diographic labeling was calibrated with the use of a scale of standards of TH protein, obtained by diluting adult rat adrenal gland extracts (rich in TH) in adult rat cerebellum extracts (poor in TH), spotted on the nitrocellulose membrane. One unit of TH (U.TH) was defined as the mean TH protein content of 10 μg (wt weight) of adult adrenal gland. Data were expressed in U.TH per mg of proteins, after quantification of total protein by the Bradford technique.

Reverse Transcription and PCR Amplification

We used Superscript II (Invitrogen) to reverse-transcribe total mRNAs, using a mix of random primers and oligo dT, (Invitrogen), and to reverse-transcribe the angiotensinogen antisense transcript RNA (AS-RNA), using a mix containing AS sequence specific primer 5′-ATA GCT GTG CTT GTC TGG GC 3′. Sequences of the primers used for PCR amplification were 5′-GGG AGG TGC TCT TGC TGT AG 3′ (AS primer sense) and 5′-ATA GCT GTG CTT GTC TGG GC 3′ (AS primer antisense), 5′-ACT GTC CGC CCG TGA TTT TC 3′ (TH primer sense; primer position: 262 to 281; Genbank accession number NG_003700) and 5′-AAG AAC TGG ATC TTC TCC TC 3′ (GFAP primer sense; primer position: 599–618; Genbank accession number NG_003700), 5′-CCC AAA AAC CAA AAA GAA GAT TGA 3′ (MAP2 primer sense; primer position: 1931–1941; Genbank accession number NG_003700) and 5′-AAT CAA GCC AGC ACA TAG CGA 3′ (MAP2 primer antisense; primer position: 2230 to 2250; Genbank accession number NG_003700) and 5′-GCT GGT GCT GAT TGT GGT GA 3′ (GAPDH primer sense; primer position: 336–355; Genbank accession number NG_003700). Amplification of AS DNA and TH, GFAP, GAPDH cDNAs was performed by PCR, with the use of Platinum Taq DNA polymerase (Invitrogen). PCR products were loaded on agarose gels containing ethidium bromide, and bands were revealed under UV rays. Quantification of the bands (AS: 275 bp; TH: 273 bp; GFAP: 357 bp; GAPDH: 425 bp) was performed with the use of the Kodak Electrophoresis documentation and analysis System 120 and Kodak Digital Science 1 software (Kodak). PCR amplification of MAP2 cDNA was performed with the LightCycler (Roche Molecular Dynamics) with the Quantitect SYBR Green PCR kit (Qiagen). Results for all genes are expressed in arbitrary units (A.U.) corresponding to the ratio target cDNA/GAPDH cDNA, with GAPDH considered as a house keeping gene.

Immunohistochemistry Assays

Paraformaldehyde-fixed, 25-μm-thick coronal sections were incubated in a mouse monoclonal anti-TH antibody solution diluted at 1:1000 (Roche Diagnostics), in a mouse monoclonal anti-microtubule associated protein 2 (MAP2) antibody diluted 1:6000 (Sigma) or in a rabbit polyclonal anti–glial fibrillary acidic protein (GFAP) antibody diluted at 1:2000 (Chemicon). Sections were then incubated in a biotinylated horse anti-mouse IgG antibody solution diluted at 1:1000 (Vector) for TH and MAP2 detection and in a biotinylated donkey anti-rabbit IgG antibody diluted at 1:1000 (Jackson Immunoresearch) for GFAP detection. TH, MAP2, and GFAP signals were revealed through the use of a peroxidase-conjugated avidin-biotin complex (Vector) and diaminobenzidine (Sigma). Sections were then dehydrated, defatted, and coverslipped. Immunolabeled sections were digitized, and the density of TH-immunolabeled elements was quantified through the use of Leica QWin software (Leica). For this purpose, sections referred to the same anatomic plane (corresponding in adults to IA –3.80 mm²) were superimposed by using different anatomic landmarks (the medial longitudinal fasciculus and the ventral border of the 4th ventricle). Once the best overlap was attained, a system of reference measurement boxes. For each section, a color code was used to highlight the TH-specific immunohistochemical labeling. The software provided the percentage of the surface occupied by the TH-specific labeling within each of the two boxes delineated as an index of the density of TH-immunopositive elements.

Catecholamines and L-DOPA Measurement

Catecholamines and L-DOPA levels were directly measured with the use of a high-pressure liquid chromatography technique coupled to an electrochemical detection, as previously described. Results are expressed in picograms or nanograms of noradrenaline or L-DOPA, respectively, per milligram of wet tissue.

Statistical Analysis

The influence of age on levels of TH protein and mRNA in control SD rats was tested with the use of ANOVA I. The influence of the strain (control SD rats versus TG) on the postnatal development of

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Results

Developmental Profile of TH Phenotype in NTS of Control SD Rats

In the first part of our study, the postnatal ontogeny of TH protein and mRNA levels was analyzed within the NTS of control SD rats for the first time. TH protein level showed a sharp rise between 3 and 4 weeks, followed by a plateau (Figure 1A). This increase in TH protein synthesis between 3 and 4 weeks may be supported by the rise in TH-mRNA level observed during the same period (Figure 1B). TH protein contained in cell bodies was previously estimated to represent only 2% of the total TH measured in the NTS region of adult rats, with the majority of TH protein being present in the catecholaminergic neuronal processes of the NTS. Thus, it is likely that the increase in TH protein level that we saw between 3 and 4 weeks in the NTS occurred in neuronal processes of NTS neurons, probably as the result of both an elevated TH protein synthesis at the cell body level and an increased outflow of the protein toward neuronal processes, as previously reported in the locus coeruleus during the same postnatal period.

AOGEn Depletion Alters Developmental Profile of TH Phenotype in NTS of TG

AOGEn protein depletion in TG is dependent on the expression of an AS-RNA directed against AOGEn-mRNA. AS-RNA directed against AOGEn-mRNA. AS-RNA expression is controlled by GFAP promoter activation; this was demonstrated by the highly significant linear correlation established in this study between GFAP-mRNA (x) and AS-RNA (y) levels determined over the entire postnatal period studied (y=0.7033x+0.1034; R=0.778, P<0.01).

Our data show that the rise in TH protein and TH-mRNA levels normally occurring between 3 and 4 weeks in the NTS of control SD rats was delayed in TG, reaching control values only at 7 weeks. Thus, chronic astrocytic AOGEn depletion, as a consequence of the expression of the AS-RNA, triggers a sharp decrease in TH expression at 4 weeks (Figures 2 and 3) at both protein and mRNA levels (~70% of control SD rats) (Figures 2B and 3B). These results argue in favor of a crucial role of central AOGEn-derived peptides in the maturation of the noradrenergic phenotype not only in vitro, as suggested previously, but also in vivo during the postnatal development of NTS. Whether this role of AOGEn depletion on TH phenotype maturation is direct or not in vivo remains to be elucidated.

We verified whether this difference in TH content between control rats and TG was due to a modification of the ultrastructural distribution of the protein within catecholaminergic neurons. By using immunohistochemistry, we observed that the presence of TH protein was dramatically decreased in fibers at 4 weeks in TG as compared with control rats. At this age, control SD rats had TH-immunolabeling within the dorsal medulla that was mainly localized in dendrites and axon terminals (Figure 4A), whereas it was almost restricted to neuronal cell bodies in TG (Figure 4B). Quantification of the density of TH-immunolabeled fibers coursing between the NTS and the ventrolateral medulla,
within a region corresponding to the intermediate reticular nucleus region (Figures 4C and 4D), showed a decrease reaching up to 65% in TG versus control SD rats (Figure 4E). Similar observations were made at the ventrolateral medulla level (Figures 5A and 5B). However, both TH protein level within the NTS region (Figure 2) and density of TH-immunolabeled neuronal processes displayed control SD rat features at 12 weeks (Figures 5C and 5D and Figure 6).

It is likely that the alterations seen at 4 weeks were specific to catecholaminergic neurons or affected a limited number of medulla cell populations only. Indeed, the analysis of both the general neuronal marker MAP2 and the general astrocytic marker GFAP did not reveal discernible impairments in TG, both in terms of tissue mRNA levels (GFAP in TG: −14±8%, P = 0.24, Student t test; MAP2 in TG: −4±6%, P = 0.82, Student t test; percentage of variation compared with control SD) and pattern of immunohistostaining of the related proteins (not shown). Thus, these results suggest (1) that the regulatory role of glial AOGEn–derived peptides on TH phenotype maturation might be dominant at critical periods of postnatal development only (after weaning, between 3 and 4 weeks) and (2) that mechanisms independent of glial AOGEn–derived peptides are involved in vivo in the regulation of TH phenotype in the (nor)adrenergic neurons of the medulla beyond 4 weeks.

**AOGEn Depletion Alters Tissue NA Concentration in NTS of 12-Week-Old TG**

Even if TH phenotype was restored in the NTS of adult TG, the sustained glial AOGEn depletion throughout postnatal development may have caused robust changes in the catecholaminergic metabolism within this structure. We found that tissue NA concentration was increased (561±37 pg/mg protein versus 457±26 pg/mg protein, P < 0.05, Student t test) in the NTS of TG at 12 weeks, whereas TH protein and TH-mRNA levels displayed values found in control SD rats (Figures 1 and 2). Furthermore, this greater concentration of NA in TG could not be explained by an alteration of TH-specific activity, since in vivo TH activity that we measured in the NTS region did not differ from control SD rats (0.54±0.02 ng DOPA/mg of tissue versus 0.52±0.06 ng DOPA/mg of tissue, P = 0.74, Student t test). The increased tissue NA concentration of TG at 12 weeks may correspond to a prolonged presence of the neurotransmitter in the extracellular compartment, due to either a decreased degradation of the monoamine or a decreased activity of the NA transporter. Indeed, Ang II was demonstrated to stimulate (1) in vitro and in vivo, the expression of macrophage inhibitory
Discussion

The significance of this study is that it supports, for the first time in vivo with the use of transgenic rats with specific depletion of glial AOGEN, the hypothesis that angiotensins derived from glial AOGEN participate in the regulation of TH phenotype setting-up within the NTS region, which is the first central relay of the baroreflex. The observations made in this work suggest that specific depletion of glial AOGEN-derived peptides early in development profoundly modified different mechanisms involved in NA neurotransmission within the NTS, which may explain part of the cardiovascular dysfunctions observed in these rats at the adult stage.

Transgenic rats of the TG strain have been proposed as a model of specifically downregulated glial AOGEN-derived peptides and at adulthood show cardiovascular dysfunction. Nevertheless, cerebral angiotensin depletion has not yet been reported in TG. However, the level of vasopressin, which is mainly synthesized in the paraventricular nucleus of the hypothalamus under the straight control of cerebral angiotensins, was reduced in the plasma of TG. It is possible that the cellular mechanisms that drive TH phenotype maturation within the fourth week are those usually activated by AT1 receptors, involving the Ras-Raf-MAP kinase signaling pathway. These receptors are present at the cell surface of (nor)adrenergic neurons both in the NTS and VLM. The inhibition of TH trafficking within (nor)adrenergic neuronal processes that may occur in TG during the fourth postnatal week to drive correct maturation of TH phenotype within the NTS and the ventrolateral medulla (VLM). The reduced presence of TH protein within neurons of the NTS and the VLM was associated with a decreased density of TH-immunolabeled neuronal processes, without any modification in the immunolabeling of the neuritic marker MAP2. These results thus support the hypothesis that glial AOGEN–derived angiotensins can regulate the targeting of TH toward neuronal processes, as previously suggested for Ang II from in vitro studies, without affecting the morphological maturation of catecholaminergic neuronal processes. Other studies aimed at precisely analyzing the morphology of TH-containing processes are obviously needed to further strengthen this hypothesis.

It is possible that the cellular mechanisms that drive TH phenotype maturation within the fourth week are those usually activated by AT1 receptors, involving the Ras-Raf-MAP kinase signaling pathway. These receptors are present at the cell surface of (nor)adrenergic neurons both in the NTS and VLM. The inhibition of TH trafficking within (nor)adrenergic neuronal processes that may occur in TG during the fourth week in this study could involve the disruption of the AT1-βPKC-MARCKS signaling pathway, which was strongly associated with neuritic distribution of TH protein in cultured noradrenergic neurons.

Factors other than glial AOGEN–derived peptides appear to be involved in the maturation of TH phenotype in vivo. Indeed, the downregulation of TH gene expression and TH protein distribution described at 4 weeks in TG was transient. However, even if these unknown factors helped to recover a correct TH phenotype in the NTS of TG later in development, other mechanisms involved in NA metabolism (NA uptake and/or degradation) might have been profoundly altered as the result of the early depletion of glial AOGEN–derived peptides.
peptides. Indeed, NA tissue concentration was significantly higher in the NTS of adult TG, in appearance independent of TH enzyme, since in vivo TH activity was not modified at this stage as compared with control rats. If the increased tissue concentration of NA was associated with prolonged presence of NA in the extracellular compartment within the NTS, caused by a reduced clearance of the neurotransmitter, then a greater stimulation of postsynaptic adrenoceptors might have occurred. Such a possibility may partially explain the increased sensitivity of the baroreflex control of heart rate noticed in adult TG.6,20,21 This hypothesis is corroborated by the increased sensitivity of the baroreflex control of heart rate might have occurred. Such a possibility may partially explain the early inhibition of the central angiotensinergic system caused in adult rats sustained alterations of the mechanisms involved in noradrenaline turnover within the medulla. Future studies will help to elucidate whether the increased noradrenaline tissue concentration observed in transgenic rats with glial angiotensinogen depletion is associated with increased presence of the neurotransmitter within the extracellular compartment and with altered binding capacities onto adrenoceptors within the medulla. Finally, it will be interesting to know whether local infusions of Aogen or derived peptides within the medulla of transgenic rats with glial angiotensinogen depletion before the fourth postnatal week can help to restore normal maturation of TH phenotype. If this is the case, it will be worth knowing whether TH phenotype restoration can counteract the development of cardiovascular dysfunctions.

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References


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