Increased Neural Atrial Natriuretic Factor Generation in Spontaneously Hypertensive Rats

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The increased neural atrial natriuretic factor (ANF) concentrations in spontaneously hypertensive rats compared with Wistar-Kyoto control rats prompted us to examine the basis of this phenomenon. Central and peripheral neural tissues from both strains were processed under rigorous antiproteolytic conditions, which were monitored by degradation of purified and iodo-labeled proANF or synthetic ANF[Ser⁹, Tyr¹²]. The extracted material was separated on a reverse-phase high-pressure liquid chromatography system, and eluates were determined for immunoreactive ANF with radioimmunoassays, employing antibodies directed against two different fragments of the ANF prohormone. The chromatographic pattern of immunoreactive-ANF elution showed the clear presence of proANF in all neural tissues studied. This pattern also included an intermediate ANF and COOH-terminal of proANF whose relative varying content was tissue dependent. Superior cervical ganglia contained most of the immunoreactive ANF corresponding to proANF and intermediate ANF. Total immunoreactive ANF in the hypothalamus, brainstem, and upper and lower parts of the spinal cord was always higher in spontaneously hypertensive than in Wistar-Kyoto rats. However, there was no difference in the content of the main molecular forms of ANF in any of their neural tissues. We suggest that the increase in neural ANF in spontaneously hypertensive rats does not arise from posttranslational alterations and may indicate an enhanced ANF synthesis rate or accumulation, or both.

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Earlier studies on atrial natriuretic factor (ANF) have shown that the hypothalamus of the spontaneously hypertensive rat (SHR) contains more immunoreactive ANF than that of the Wistar-Kyoto (WKY) control rat, a finding which has been confirmed by several groups, including our own laboratory. In addition, we have demonstrated that ANF levels are higher also in the brainstem, spinal cord, and in some sympathetic ganglia. These observations suggest that a genetic drive enhances the expression of this peptide in the SHR nervous system to a higher level than in control rats; alternatively, it may reflect a phenomenon secondary to high blood pressure. Independent of either possibility, such differences might be due to an impairment of posttranslational peptide maturation. In previous reports, ANF was detected with one anti-COOH terminal antibody, which has a different cross-reactivity towards low- and high-molecular weight ANF. This problem could, therefore, not be addressed because tissue-extract immunoreactivity corresponded to unidentified ANF molecular forms. We recently isolated proANF chromatographically in neural tissues and noted its appreciable contribution to total immunoreactivity, provided that proteolytic degradation was minimized under conditions that were quite different from those used previously. Even if gel filtration analyses showed the same pattern of hypothalamic ANF in the SHR and WKY rat, almost exclusively low–molecular weight COOH-terminal ANF was detected. Therefore, in this study, we evaluated the molecular forms of neural ANF in the hypertensive SHR with two different antibodies directed against two extreme ends of the proANF molecule, and we carefully monitored proteolytic ANF degradation in both rat strains.

Materials and Methods

Male 12-week-old SHRs and WKY rats (Taconic Farm Inc., Germantown, New York) were main-
Debinski et al ANF in Genetic Hypertension 805

FIGURE 1. High-pressure liquid chromatography profile of $^{125}$I-ANF[Ser$^{99}$, Tyr$^{126}$] processed (lower panel) or not (upper panel) with rat lumbosacral spinal cord. The eluted radioactivity is expressed as percentage of the main peak found besides void volume.

FIGURE 2. High-pressure liquid chromatography profile of $^{125}$I-proANF processed with the hypothalamus of spontaneously hypertensive or Wistar-Kyoto rats. The eluted radioactivity is expressed as percentage of the main peak found besides void volume.

tained under controlled conditions on normal rat chow and tap water ad libitum. Blood pressure was measured by the tail-cuff method under light ether anesthesia.

Neural tissues were removed after decapitation and stored as described previously. They were boiled for 10 minutes before homogenization in a Polytron homogenizer (Teflon, Gaframco Wierton, Ontario, Canada) in 1.0 M CH$_3$COOH containing endogenous protease inhibitors: 5 mM EDTA, 10 µM phenylmethylsulfonyl fluoride, 5 µM pepstatin, 1 µM phosphoramidon (all reagents from Sigma Chemical Co., St. Louis, Missouri), 1 µM aprotinin (Bayer (Miles) AG, Leverkusen, FRG), and 10 µM captopril (Squibb Canada, Montreal, Quebec, Canada). The homogenates were centrifuged at 10,000×g at 4°C for 30 minutes. The supernatants were then dry-evaporated in a Speed-Vac (Savant, Hicksville, New York) concentrator. The residues were dissolved in the equilibration buffer of a high-performance liquid chromatography (HPLC) column shortly before injection. Immunoreactivity was measured with two assays: in the first, antibodies were directed against immunoreactive COOH-terminal ANF[Ser$^{99}$, Tyr$^{126}$] (irANF-C), and in the second, against immunoreactive ANF[Asp$^{11}$, Ala$^{37}$] (irANF-N), as described previously. Cross-reactivity to proANF is 66% in the first assay and 100% in the second. Tissue extracts were processed in an HPLC system, as outlined elsewhere. ProANF and ANF[Ser$^{99}$, Tyr$^{126}$] (Bio-Mega, Laval, Quebec, Canada) were labeled and purified according to established techniques.

Results

SHRs had body weight significantly lower (250±3 g) than WKY rats (290±7 g), p<0.05, and blood pressure elevated (128±8 vs. 87±3 mm Hg; p<0.05) at age 12 weeks. $^{125}$I-ANF[Ser$^{99}$, Tyr$^{126}$] and proANF standards were eluted at 22 and 52 minutes, respectively. When processed with neural tissues, the low-molecular weight peptide had a tendency to shift to one fraction earlier, but radioactivity was confined only to this region (Figure 1), strongly suggesting that it did not interact with neural proteins that could change its chromatographic pattern and eventually be mistaken for higher molecular weight peptides. Figure 2 presents a typical chromatographic pattern of $^{125}$I-proANF processed with the hypothalamus of SHRs or WKY rats. It is also representative of other organs. The proANF peak was broadened with added tissue when compared with standard elution, and some radioactivity appeared in a void volume. Interestingly, radioactive peptides were eluted at and after 36 minutes, obviously the degradation products of proANF. No significant difference was noted between chromatograms obtained from hypertensive and normotensive rats.
A typical chromatogram of the brainstem appears in Figure 3. The main irANF-C peak was eluted at 16 minutes, where the ANF standard[Ser<sup>99</sup>, Tyr<sup>126</sup>] was found. An appreciable amount of immunoreactivity appeared between 34 and 40 minutes. It is noteworthy that the position of this peptide was similar to that of [<sup>125</sup>l]proANF degradation compounds. The smallest but clear irANF-C peak coeluted with nonlabeled proANF at 54 minutes. A significant quantity of irANF-N was demonstrated only in this region with the highest peak corresponding to the position of proANF. Again, there was no difference in the chromatographic patterns of irANF between SHRs and WKY rats; only the SHR brainstem values quantitatively exceeded those of WKY rats. The content of irANF-C in three main regions of HPLC profile in SHRs was 128±17% in lumbosacral spinal cord, 174±27% in brainstem, and 142±8% in hypothalamus when compared with WKY rats (100%) (n=three HPLC runs). The ANF HPLC pattern of the cervicothoracic and lumbosacral spinal cord was similar to that of the hypothalamus, but with a somewhat larger relative amount of proANF and the other higher molecular weight ANF compared with low-molecular weight COOH-terminal ANF. These patterns contrasted with those in the sympathetic ganglia (Figure 4), where most of the irANF-C was found to be coeluted with both high-molecular weight ANF and a minute quantity with standard ANF[Ser<sup>99</sup>, Tyr<sup>126</sup>]. The difference in superior cervical ganglia ANF between SHRs and WKY rats was not so evident, which is in accordance with our previous results.

**Discussion**

The availability of proANF enabled us to perform controlled studies on the extraction of different ANF molecular forms present in the nervous sys-
Our results identified proANF in neural tissues with its varying relative contribution to total immunoreactivity being dependent on the tissue type. ProANF content was lowest in the hypothalamus and highest in the superior cervical ganglia with the spinal cord values being between both. ProANF was detected by its coelution with the prohormone standard and recognition by the two different antibodies according to their affinity toward the propeptide. We also demonstrated that proANF may undergo partial degradation during the extraction procedure, even if processed under rigorous antiproteolytic conditions. That is probably why, in our initial report, we predominantly obtained low-molecular weight ANF in superior cervical ganglia extracted with weaker acid and without boiling. The follow-up of such degradation leads us to propose that the intermediate peak detected in all studied tissues represents a degradation product of proANF, probably as a consequence of nonspecific proteolysis in tissue homogenates. It is also possible that we still did not extract all the proANF, which is degraded faster than our experimental protocol can cope with, implying that ANF is stored mainly in its precursor form and possibly cleaved shortly before or during release. The ANF results obtained in the adrenal medulla favor such a possibility. ANF would thus represent a rare example of a neuropeptide stored in the form of a prohormone. The ANF HPLC profile in superior cervical ganglia was evidently closer to that seen in the atria, where only proANF is stored in granules.

Most of the COOH-terminal ANF was found at the elution position of ANF\[Ser\(^{29}\), Tyr\(^{126}\)]. Our experimental conditions did not allow precise evaluation of the homogeneity of irANF-C eluted in this region since, for example, atriopeptin III (ANF \[Ser\(^{30}\), Tyr\(^{126}\]) was eluted in the same region. Therefore, it is still possible that this immunoreactivity corresponds to peptides shorter than ANF\[Ser\(^{29}\), Tyr\(^{126}\)], as suggested previously.

Under these conditions, we could again detect a great increase in neuronal ANF content in 12-week-old SHR when compared with age-matched WKY rats. There was no indication of a different fate of ANF extracted from the hypothalamus, brainstem, and spinal cord; thus, the tissues from both strains express similar, specific, or nonspecific proteolytic activity. In addition, the HPLC profiles of irANF-C and irANF-N were, in practice, identical in SHRs and WKY rats.

We therefore conclude that the observed higher ANF content in the SHR is not related to any alteration in posttranslational processing of the peptide. This increase may be caused either by enhanced neural ANF generation in the SHR or altered release of the peptide. The former possibility seems more probable since ANF receptors are down-regulated (or their occupancy is much higher) in the SHRs compared with the WKY rats, which suggests a greater availability of releasable ANF pools in the nervous system. More detailed studies on subelements of ANF in the central and peripheral nervous system are required to explore the link between neural ANF and blood pressure regulation and its possible involvement in the development of hypertension in the SHR. The levels of ANF messenger RNA in these rats would also be of interest. It is noteworthy that blood pressure reduction diminishes the ANF content of hypothalami in the SHR with established hypertension. We are presently studying the effect of hypertension prevention on neural ANF in the SHR.

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