Atrial Natriuretic-Like Peptide and its Prohormone Within *Metasequoia* (44403)

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Abstract. **Metasequoia glyptostroboides was one of the dominant conifers in North America, Asia, and Europe for more than 100 million years since the late Cretaceous Albian Age, but Quaternary glaciations drove the *Metasequoia* population to apparent extinction. A small pocket of *Metasequoia*, however, was found in central China in the 1940s representing the only surviving population of this "living fossil" species. Atrial natriuretic peptide, a 28-amino-acid peptide hormone that causes sodium and water excretion in animals, has been found to be part of the first peptide hormonal system in lower plants. The existence of this hormonal system has never been examined within trees of any genus. High-performance gel permeation chromatography of the leaves and stems (i.e., branches) of *Metasequoia* followed by atrial natriuretic peptide radioimmunoassay revealed an ANP-like peptide and its prohormone (i.e., ≈ 13,000 mol wt) were present in both leaves and stems of this conifer. The elution profile of ANP-like peptide in stems of *Metasequoia* had a shoulder to the left of where pure synthetic ANP elutes suggesting the possibility of a slightly larger peptide eluting within this shoulder secondary to alternate processing of the ANP-like prohormone and similar to what occurs with the kidney of animals. The elution profile of ANP-like peptide in the leaves of *Metasequoia* revealed two peaks; one where ANP elutes and a second peak suggesting a smaller peptide that has been metabolically processed. The presence of the ANP-like prohormone strongly suggests that ANP-like gene expression is occurring in both leaves and stems of *Metasequoia* since this prohormone is the gene product of this hormonal system. The presence of the ANP-like hormonal system in trees implies that this hormonal system may have been present early in land plant evolution to allow trees to reach heights of greater than 30 feet where a water flow-enhancing substance is absolutely necessary for water flow to occur to these heights.** [P.S.E.B.M. 1999, Vol 221]

A small number of *Metasequoia glyptostroboides* were discovered in central China in the 1940s by Hu and Cheng (1). This has been considered one of the most fascinating botanical findings in this century and has attracted worldwide attention (2); before then, the genus *Metasequoia* was only known in fossils (3). The natural population of *Metasequoia glyptostroboides* is presently restricted to the border area of Hubei, Sichuan, and Hunan Provinces in central China. In geologic history, however, *Metasequoia* was one of the dominant conifers in the temperate floras of the northern hemisphere during Late Cretaceous and early Tertiary epochs, widely flourishing in North America, Asia, and Europe, where abundant fossils occur in sedimentary strata (4–7). By Pliocene time (5.3–1.8 million years ago), the distribution of *Metasequoia* retreated to isolated areas (Japan, northern China, and eastern Khazakstan), and the Quaternary glaciations drove the *Metasequoia* populations to near extinction, with only one small population left in central China (1, 2, 6, 7). In North America, the dominance of this deciduous tree was replaced by the giant, evergreen redwoods *Sequoia sempervirens* and *Sequoiadendron giganteum* (5).

Atrial natriuretic peptide (ANP) a 28-amino-acid peptide hormone that causes sodium and water excretion in

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humans (8, 9) and animals (10) has recently been found to be part of the first peptide hormonal system in plants (11, 12). This hormonal system in plants causes the movement of water and solute up through the stems to the leaves and flowers (12). Part of the mechanism of movement of solute in plants is *via* transpiration of water through leaves (12–14). ANP-like peptide immunoaffinity purified from English ivy (*Hedra helix*) and rose (*Rosa damascena*) leaf extracts induce the opening of stomata of *Tradescantia sp* in a concentration-dependent manner (13) similar to that observed utilizing rat ANP to open the stomata of *Tradescantia sp*, allowing transpiration to take place (14). It has been speculated that the opening of stomata by ANP-like peptide involves stimulating K^+ influx much like the nonpeptide phytohormones do (13). Whether atrial natriuretic peptide is present in conifers such as *Metasequoia*, or for that matter trees of any genus, has not been examined. We hypothesize that the atrial natriuretic peptide-like hormonal system must have been present early in the evolutionary process to have allowed plants to survive on earth. Since *Metasequoia* has been present on earth for over 100 million years (2, 15) with a unique evolutionary history (1, 2, 6, 7), *Metasequoia* was chosen as the first tree to examine for the presence of the atrial natriuretic peptide-like hormonal system. The present investigation was designed to determine if atrial natriuretic like-peptide and its prohormone are present within the leaves and branches of *Metasequoia glypotostroboides* utilizing high-performance gel permeation chromatography (HP-GPC) followed by a specific radioimmunoassay to atrial natriuretic peptide.

Materials and Methods

Metasequoia. *Metasequoia glyptostroboides* leaves and branches were collected in the natural population near the border area of Hebei, Sichuan, and Hunan Province in central China and immediately frozen with liquid nitrogen. Four grams of the leaves and branches, respectively, of *Metasequoia* were cut into 0.5-cm squares with scissors and then pulverized with a mortar and pestle. The crushed stems and leaves were then separately placed in saline containing 15% ethylenediaminetetraacetic acid (1.5 g tetrasodium salt of EDTA/10 ml of saline) to prevent the proteolytic breakdown of any peptides that might be present. This resulted in a dark green mixture for the *Metasequoia* leaves and medium brown mixture for the *Metasequoia* branches. After centrifugation at 3,000*g* for 15 min, extraction of the samples was performed with 100% ethanol (1:1 dilution) that was added to the supernatant fractions, which were then allowed to stand for 30 min at 4°C. When handled in this manner, we found in a series of experiments, comparing samples in EDTA alone with samples collected in EDTA plus the protease inhibitors aprotinin and/or leupeptins, that additional protease inhibitors are not necessary (16). EDTA alone gave identical results for ANP to that with additional protease(s) inhibitors present (16). After 30 min at 4°C, the samples were centrifuged at 3,000*g* for 15 min, and the supernatants were taken to dryness *via* controlled airflow. This drying process took several days and resulted in a very gelatinous brown mass for both leaves and stems.

Atrial Natriuretic Peptide Radioimmunoassay. ^{125}I-labeled and unlabeled atrial natriuretic peptide (ANP) and antisera used for radioimmunoassays were from Peninsula Laboratories (Belmont, CA). The specific activity of ^{125}I-ANP was 2,000 Ci/mmol. The respective high-performance gel permeation chromatography (described below) eluted samples of *Metasequoia* leaves and branches were examined by our ANP assay devised to amino acids 99–126 of the ANP prohormone as described in detail previously (16–18). Each sample was reconstituted in 100 μl of 0.1 *M* phosphate buffer (pH 7.4) containing 0.05 *M* NaCl, 0.01% bovine serum albumin, 0.1% Triton X-100, and 0.01% NaN_3. To the redissolved sample, 100 μl (0.03 mg) of rabbit immunoglobulin G plus 100 μl of ^{125}I-labeled ANP (10,000 cpm) were added, mixed, and incubated for 18 hr at 4°C. The precipitation of the antibody-bound tracer was accomplished by adding 100 μl goat antirabbit globulin after the above 18-hr period and incubating this mixture for 2 hr at room temperature. Each tube was then centrifuged at 3,000*g* for 20 min. The supernatant was aspirated, and the pellet was counted in a gamma counter. All determinations were performed in triplicate. The intraassay and interassay coefficients of variation for the ANP radioimmunoassay were 5.7% and 6.9%, respectively. The lowest detectable concentration of ANP with this assay is 1.4 fmol/tube (4.3 pg/tube). Nonspecific binding was 2.8% for the ANP radioimmunoassay.

High-Performance Gel Permeation Chromatography. The molecular forms of immunoreactive ANP-like peptide(s) in extracts of *Metasequoia* leaves and stems were determined by high-performance gel permeation chromatography (HP-GPC) as described in detail previously (12, 19, 20). The dried ethanol-extracted samples were resuspended in 400 μl of HP-GPC column mobile phase (10 m*M* trifluoroacetic acid containing 0.3 *M* NaCl and 30% acetonitrile) for HP-GPC assay. The resuspended extracts were centrifuged for 10 min in an Eppendorf microcentrifuge (Beckman Instruments Inc., Westbury, NY), which resulted in large brown pellets that were solid for the stem and friable for the leaves. Neither was gelatinous, unlike after the ethanol extraction. Both the stems and leaves had brown supernatants that were decanted into Eppendorf tubes and recentrifuged for 10 min in an Eppendorf microcentrifuge. This step was repeated one more time with the resultant supernatant for the leaves in mobile phase being a transparent brown-tinged solution whereas the stem supernatant was a dark greenish-brown color. After the three microcentrifugations there was no longer any pellet present. Samples (100 μl) of these supernatants were applied to a TSK-GEL G2000SW column (7.5 × 600 mm equipped with a guard column, 7.5 × 75 mm; Toyo Soda, Tokyo, Japan) and eluted isocratically with HP-GPC column mobile phase at a flow rate of 0.3 ml/min. Fractions (0.3 ml) were collected, dried,

then assayed for ANP with the radioimmunoassay described above. Blue dextran (2,000,000 mol wt) and p-aminohippuric acid were used to determine the void and total volumes of the column. To calibrate the column, carbonic anhydrase (29,000 mol wt), myoglobulin (16,900 mol wt), cytochrome *c* (12,384 mol wt), vasoactive intestinal peptide (3300 mol wt), and Tyr-somatostatin (1730 mol wt) were used. The elution positions of ANP were determined using ^{125}I-labeled and unlabeled synthetic human forms of ANP. Recovery of ANP-like peptide was 77%.

Statistical Analysis. Statistical significance between the fractions containing ANP-like immunoreactivity versus the fractions not containing immunoreactivity was determined using analysis of variance for *Metasequoia* leaves and stems group comparisons. In all cases $P < 0.05$ was found to be present and was used as the criterion for statistical significance.

Results

High-performance gel permeation chromatography (HP-GPC) followed by atrial natriuretic peptide (ANP) radioimmunoassay of branches (i.e., stems) of *Metasequoia* revealed that an ANP-like peptide and its prohormone (i.e., ≈ 13 kDa) were both present (Fig. 1). Examining the area under the elution profiles in Figure 1 reveals that more of ANP-like peptide than its prohormone was present in the branches. The elution profile for ANP-like peptide was fairly broad as one observes in Figure 1. The ANP assay did not immunologically recognize proANP 1–98 in plants (i.e., the N-terminus of the ANP prohormone) that would elute at 9800–10,000 mol wt (i.e., 9.8–10 kDa) (Fig. 1). Evaluation of the leaves of *Metasequoia* by HP-GPC followed by ANP radioimmunoassay also revealed two peaks, one that eluted where the pure synthetic form of ANP elutes and a second peak consistent with the ANP-like prohormone (Fig. 2). The area under the ANP-like peak was slightly larger than the ANP-like prohormone peak and eluted as a biphasic peak that had not been observed previously in plants (12), or animals (20). It is important to note in Figure 2 that proANP 1–98 is not immunologically recognized in *Metasequoia* leaves by this evaluation.

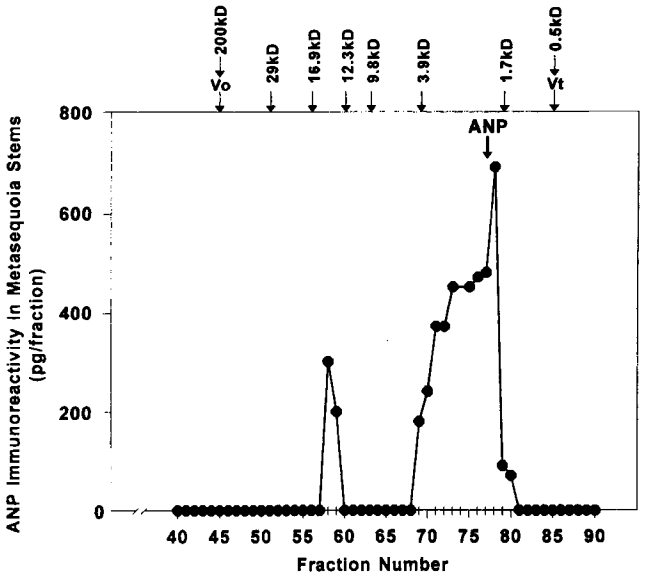

Figure 1. High-performance gel permeation chromatography evidence that atrial natriuretic-like peptide (ANP) and the atrial natriuretic peptide-like prohormone exist in *Metasequoia glyptostroboides* stems (i.e., branches). In evaluation of *Metasequoia* stems, a sharp peak consistent with the ANP prohormone was observed at 13,000 mol wt (13 kDa). A second, broader peak that eluted where the pure synthetic form of ANP (arrow) elutes with a shoulder of 0.3 ml fractions to the left of the ANP-like peak suggesting a slightly larger peptide than ANP was also present. Elution profiles of molecular weight markers are denoted by arrows on the top of the graph. Vt = total column volume; para-aminohippuric acid used to characterize this peak elutes in fractions 84–90 with peak in fraction 87 (arrow). To insure that we detected all of the immunoreactive peptide present we assayed through fraction 90. Vo = void volume. Each fraction equals 0.3 ml with fractions 40–90 being evaluated.

Discussion

The present investigation revealed that the leaves and stems of *Metasequoia* contain an atrial natriuretic-like peptide and the atrial natriuretic peptide-like prohormone. Thus, the stems and leaves of this conifer are similar to other plants in this regard as all plants smaller than trees appear to contain this peptide hormonal system (12). Although the present investigation is the first demonstration of a peptide hormonal system in a tree of any genus, one would suspect that all trees in addition to *Metasequoia* may contain this hormonal system for the transpiration-osmosis mechanism of water flow cannot cause water to flow mechanically to a height of more than 30 feet (21). Since the majority of conifers, *Sequoia sempervirens* (North American Redwoods), oak (Fagaceae), pine and even Date palm (Palmaceae) trees grow to heights of 100 feet and above, they each need a hormonal system such as the atrial natriuretic peptide hormonal system to actively move water above 30 feet. Plants could not have grown to heights greater than 30 feet without an active process mediated by a substance with water flow-enhancing properties.

HP-GPC evaluation of leaves of the Florida Beauty (*Dracaena godseffiana*) did not demonstrate that the ANP-like prohormone as well as ANP-like peptide was present (12). Thus, the results of the HP-GPC evaluation of *Metasequoia* in the present investigation were distinctly different from the evaluation on Dracaena since immunologic evidence of the ANP-like peptide prohormone was found in *Metasequoia*. The presence of the ANP-like prohormone strongly suggests that ANP-like gene expression is occurring in *Metasequoia* leaves since the ANP prohormone is the gene product of ANP gene expression of this hormonal system (22, 23). In animals wherever the ANP prohormone was found, ANP gene expression was found to occur (22, 23). This also appears true for plants for when examining this question in English ivy (*Hedra helix*) with Northern blot analysis, we found that ANP-like prohormone steady-state mRNA was present in the stem of English ivy indicating ANP-like prohormone synthesis was occurring there

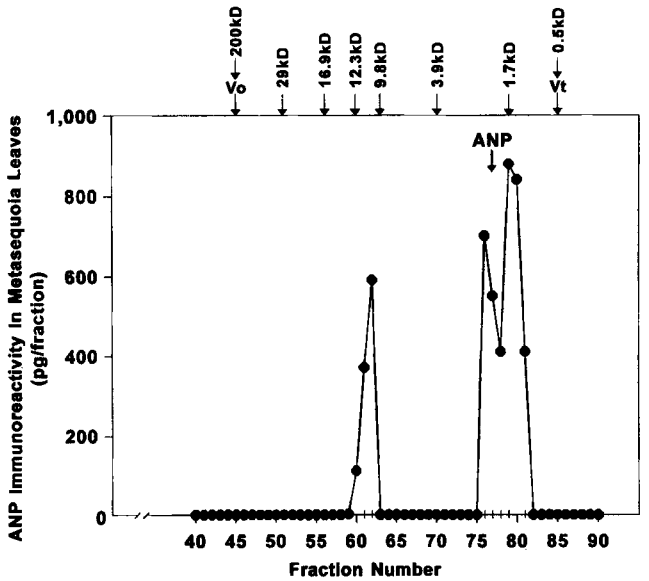

Figure 2. Evaluation of *Metasequoia* leaves by high-performance gel permeation chromatography followed by atrial natriuretic peptide radioimmunoassay of 0.3-ml fractions 40 through 90 obtained from this evaluation revealed two elution peaks. One peak was observed at 12,000 mol wt (12 kDa), which is consistent with this peak being the ANP-like prohormone. The second peak was a biphasic peak with the left-hand portion of this peak eluting where the pure synthetic form of ANP (arrow) elutes and the right-hand portion of this peak suggesting a peptide-shortened form of ANP-like peptide that recognizes the epitope of ANP. Thus, there is the possibility that plant ANP-like peptide has been partially metabolically degraded as atrial natriuretic peptides are known to do when incubated *in vitro* with human plasma (25, 26). Molecular weight markers are denoted by arrows on the top of the graph. Vt = total column volume; para-aminohippuric acid used to characterize this peak elutes in fractions 84–90 with peak in fraction 87 (arrow). To insure that we detected all of the immunoreactive peptide present, we assayed through fraction 90. Vo = void volume. Each fraction equals 0.3 ml.

(Vesely DL, Overton RM, Gower WR Jr., unpublished observations). The probe for this Northern blot analysis was a full-length cDNA rat probe. Hybridization was performed under low stringency conditions that allowed some mismatching between the sequences of the probe and the target mRNA. The finding of the ANP-like prohormone within the leaves of *Metasequoia* was different from the leaves of the English ivy where the ANP-like prohormone could not be demonstrated to be present (Vesely DL, Overton RM, and Gower WR Jr., unpublished observation). This local production of the ANP-like prohormone may well be an adaptive measure by taller plants (i.e., trees) to help with water and solute flow that occurs over greater distances in trees than in shrubs or single-stem flowering plants.

There was immunologic evidence that the ANP-like prohormone was also present in the stem (i.e., branches) of *Metasequoia*. This was consistent with what was found previously (i.e., that ANP-like prohormone gene synthesis of steady-state mRNA occurs within the stems of plants such as the English ivy (Vesely DL, Overton RM, Gower WR Jr., unpublished observation)). The present investigation demonstrates that atrial natriuretic-like peptide prohormone within the stem provides a mechanism for the flow of water in taller plants since ANP-like peptide would be immediately available from its storage form (i.e., the ANP-like prohormone) no matter how high the stem or trunk of a tree, to actively increase the flow of water and nutrients. Recent studies have demonstrated that plants have the endogenous machinery to transport large molecules the size of atrial natriuretic-like peptide between cells through plasmodesmata to their target cells or vessels (24). Atrial natriuretic-like peptides, however, have not as yet been shown to be transported between cells in plants.

The elution profiles of ANP-like peptide isolated from *Metasequoia* leaves and stems were different from its elution profile found previously in other plants (12) and its elution profile within the circulation of healthy humans (19). In *Metasequoia* stems the elution profile was broader than seen previously with elution being present where synthetic ANP elutes. There was also a shoulder to the left of where pure synthetic ANP elutes suggesting the possibility of a slightly larger peptide also eluting within this shoulder. This would suggest that within the stem of *Metasequoia* there may be an alternate processing of the ANP-like prohormone to create a slightly larger ANP-like peptide. There is precedent for this in animals. Within the kidney of animals the ANP prohormone is processed to form a larger ANP-like peptide consisting of amino acids 95–126 of the ANP prohormone whereas in all other tissues in animals ANP is formed by proteolytic processing between amino acids 98–99 to form a 28-amino-acid peptide consisting of amino acids 99–126 of this 126-a.a. prohormone (22, 23).

The elution profile of ANP-like peptide within leaves of *Metasequoia* was also different from that found previously in that its elution revealed two peaks, one where synthetic ANP elutes and a second peak suggesting a somewhat smaller peptide. This would suggest that within leaves of *Metasequoia* ANP-like peptide is metabolically processed to a smaller peptide that may or may not have biologic activity. In humans there is metabolic processing of atrial natriuretic peptides within plasma when atrial natriuretic peptides are incubated *in vitro* with plasma at 37°C (25, 26), so this finding within leaves has some precedent also. The processing of atrial natriuretic peptides occurs at different rates. Thus, ANP (amino acids 99–126 of ANP prohormone) and kaliuretic peptide (amino acids 79–98 of the same prohormone) when incubated with plasma are completely degraded into smaller peptides within 2 hr whereas long acting natriuretic peptide (amino acids 1–30 of this prohormone) and vessel dilator (amino acids 31–67 of ANP prohormone) are ≈ 25% metabolically processed at 2 hr. It would appear from these studies that the animal and plant kingdoms are much more similar than previously thought. It should be pointed out that although very similar, preliminary studies (12) suggest that plants and animals are not identical with respect to atrial natriuretic-like peptides, and water or solute movement as ANP-like peptide in plants has fewer solute movement properties than other atrial natriuretic-like peptides from the same prohormone. Since the

"living fossil" *Metasequoia* has been in existence on earth for over 100 million years (2), this suggests that the atrial natriuretic-like peptide hormonal system may have been present early in evolution to allow plants to adapt to earth. Further evidence that the atrial natriuretic-like peptide hormonal system was important to allow plants to evolve is that this hormonal system is present (12) even in single-celled plants (i.e., Euglena) where its function in single cells appears to be the regulation of cell volume by causing the extrusion of water out of the cell, thereby decreasing and maintaining cell volume (27).

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