Abstract—Aminopeptidase N (APN) is an abundant metallohydrolase in the brush border of kidney proximal tubule cells that degrades angiotensin III (Ang III) to angiotensin IV (Ang IV) and, along with dipeptidylaminopeptidase, degrades Ang IV. We examined the impact of a high-salt diet on renal APN activity and transcript abundance in the Sprague-Dawley and Dahl salt-sensitive (SS/Jr) rat strains. APN transcript abundance and protein abundance were 2-fold greater ($P<0.05$; $n=6$) in the kidneys of Sprague-Dawley and Lewis rats ingesting 8% versus 0.3% salt diets, suggesting that increased aminopeptidase activity may contribute to decreased renal sodium uptake during adaptation to a high-salt diet. In contrast, renal APN transcript abundance and activity were the same in Dahl SS/Jr rats ingesting 8.0% versus 0.3% salt diets. The APN gene was mapped, using a radiation-hybrid panel, to known quantitative loci on chromosome 1 for blood pressure in the Dahl SS/Jr rat. The results suggest that the APN gene is a good candidate for salt-sensitivity in the Dahl SS/Jr rat. (Hypertension. 2004;43:282-285.)

Key Words: sodium ▪ kidney ▪ Dahl rat ▪ angiotensin ▪ hypertension

Angiotensin II (Ang II) is metabolized to angiotensin III (Ang III) (Arg-Val-Tyr-Ile-His-Pro-Phe) by aminopeptidase A. In turn, APN metabolizes Ang III to Ang IV (Val-Tyr-Ile-His-Pro-Phe) and, along with dipeptidylaminopeptidase, degrades Ang IV. Ang III shares many of the physiological functions of Ang II in the cardiovascular and nervous systems, including stimulation of vasopressin, aldosterone, and catecholamine secretion. Similar to Ang II, it acts as a pro-inflammatory agent by stimulating MCP-1 cytokine production and activating NF-kB and AP-1. Ang III has been postulated to contribute to the inflammatory response and cell growth with progression of renal disease. However, Ang IV is thought to have minor biological activity because Ang II receptors AT1 and AT2 have poor affinity for Ang IV, and Ang IV infusion does not elicit Ang II-dependent effects. Recently, receptors for Ang IV have been detected in cortical collecting ducts and mesangial cells and Ang IV, in contrast to Ang III, has been shown to increase renal blood flow and cerebral vasodilation. Thus, APN may be in a critical position to modulate salt uptake in nephrons and renal vascular resistance by controlling the metabolism of Ang III to Ang IV. We hypothesized that APN may play a mechanistic role in salt adaptation and hypertension.

APN is a major constituent of the brush-border membranes of kidney proximal tubule cells and enterocytes. APN (IUBMB enzyme nomenclature ec 3.4.11.2, ap-n), known also as microsomal aminopeptidase or aminopeptidase M, is a homodimeric, membrane-bound, zinc-dependent peptidase particle-bound aminopeptidase that preferentially releases neutral amino acids from the N-terminal end of oligopeptides and has specificity similar to that of cytosolic leucine aminopeptidase. APN belongs to the M1 family of the MA clan of peptidases, which includes membrane-bound type II glycoproteins and has been cloned from 6 different mammalian species. In the present study, we examined the impact of dietary salt content on renal APN in normotensive and Dahl salt-sensitive (SS) rats.

Methods

Animals

The animal-use protocols were approved by the Chicago-West Side Veterans Administration Institutional Animal Care and Use Committee. Male Sprague-Dawley, Lewis, and Dahl SS/Jr rats weighing 250 to 300 grams and obtained from Harlan Sprague-Dawley (Indianapolis, Ind) were administered basal (0.3%) and high-salt (8%) rat-chow diets (Purina series 5500) for 10 days. Urinary sodium was measured to confirm diets. Intra-aortic measurements of blood pressure were performed as previously described. Telemetric blood pressure measurements of these arrivals have previously been reported.

RNA Preparation

Kidneys were removed and fast-frozen in liquid nitrogen. Total RNA was isolated with TRIzol (Invitrogen), with subsequent removal of residual contaminants by using the RNeasy Total RNA Isolation Kit (Qiagen). For high-quality poly(A)+ mRNA, total RNA prepared by TRIzol was followed by use of the Oligotex mRNA kit (Qiagen).
Real-Time Polymerase Chain Reaction
SYBR green polymerase chain reaction (PCR) amplifications were performed as previously described. APN primers were designed on the basis of GenBank database accession number AFO39890 (Rattus norvegicus APN gene, exon 2, and partial cds) using Primer Express Software version 1.0 (PE Applied Biosystems) and checked by running virtual PCR (forward: 5’ TCCTTGAGCCTGGTAGAATCC 3’; reverse: 5’ GGAAGGGGCTGACTAATT 3’). Primers for GAPDH were as follows: forward = 5’ GAAAGGCTATGCACCA-CAGT 3’; reverse = 5’ GGATGCAGGATGTGTTCT 3’. PCR yielded unique bands of the predicted sizes. Sequencing of these cloned products verified gene specificity. Near-log–linear amplification was observed, with amplification occurring between cycles 26 and 32. Each sample and reaction was replicated 3 times. Expression was normalized to GAPDH as an endogenous reference and relative to basal salt gene expression as a calibrator.

Western Analysis
Immunoblot analysis of kidney homogenates was performed as previously described. Membranes were immunoprobed with mouse polyclonal antihuman APN antibody (CD13 Ab-3 Laboratory Vision). Secondary antibody was a goat antimouse IgG HRP-conjugated antibody (Santa Cruz Biotechnology). Blots were controlled for differences in sample loading by normalization to actin.

APN Activity
APN activity was measured in total kidney extract (prepared as described for Western blots) by the methods of Ryan et al. except we used fluorogenic substrate Arg-Phe-AFC (Enzyme System Products, Livermore, Calif) rather than radioactive substrate. The rate of fluorescence increase was linear for 20 minutes. Substrate without kidney extract was used as blank. The enzyme activity was expressed as micromoles of AFC produced per minute per milligram of protein using authentic AFC as a calibrator.

Radiation Hybrid Mapping
A rat–hamster hybrid panel consisting of 106 clones with an average locus retention rate of 28% created by Peter Goodfellow (Cambridge, UK) (Research Genetics, Huntsville, AL) was used. Each marker was tested separately (none was multiplexed) and screened at least twice. The presence or absence of the aminopeptidase gene was determined by PCR using gene-specific primers (forward: 5’ ATT-TGCCCCCCTTTTACGAG 3’; reverse: 5’ ACTGCATGGGAAT-GTCACAA 3’; predicted product size: 448 bp). PCR products were resolved on a 3% agarose gel and analyzed using the Bio-Rad gel documentation system. The data were submitted to the Otsuka Gene Institute (http://e4000.otsuka.gr.jp:8012/cgi-bin/SH/ RhNgv.pl) for testing against framework maps.

Gene locus markers were integrated into the virtual comparative map (http://rgd.mcw.edu/tools/vcmapper?ver=5.0) and related to known quantitative trait locus (QTL) and rat hormone markers using Medical College of Ohio (http://home.mco.edu/depts/physiology/research/rat/markers.html), Massachusetts Institute of Technology (http://www-genome.wi.mit.edu/rat/public/Rat/oxford/ (http://www.eubiotic.org/Rat/mapping_resources), and Arb (http://www.niams.nih.gov/rtbc/rtbdatabase/index.htm) linkage and rat hormone markers.

Statistics
Transcript/protein abundance and activities within strains were compared using ANOVA and change between strains by 2-sided t test. Significance was set at a level of P<0.05.

Results
Blood Pressure and Weights
In Sprague-Dawley rats, arterial blood pressure was the same on 0.3% and 8% salt diets (116±4 mm Hg versus 116±7 mm Hg). In Dahl SS rats, blood pressure was greater on 8% versus 0.3% salt diet (147±8 mm Hg versus 116±6 mm Hg, respectively; P<0.01).

Renal APN Transcript and Protein Abundance
Renal APN transcript abundance (Figure 1A) in kidneys was the same in Sprague-Dawley, Lewis, and Dahl SS/Jr rats on 0.3% salt diets. On 8% salt diets, transcript abundance was 2.5-fold greater than in 0.3% salt (P<0.01) in the Sprague-Dawley rats and the same in the Dahl SS/Jr rats on 0.3% salt. Renal APN protein abundance (Figure 1B) in the three rat strains on 0.3% salt diets was the same. On 8% salt, abundance was 3-fold and 2-fold greater in Sprague-Dawley and Lewis rats, respectively, than in Dahl SS/Jr rats (P<0.05). Thus, protein and transcript abundance parallel each other in both strains.

Renal APN Activity
APN activity was measured in membrane extracts from kidneys after 10 days on either 8% or 0.3% salt diets (Figure 2). On a 0.3% salt diet, activity was 3-fold and 2-fold greater in Dahl SS/Jr and Lewis rats versus Sprague-Dawley rats. APN activity was increased by 10-fold and 2-fold in Sprague-Dawley and Lewis rats, respectively, on 8% versus 0.3% salt diets. However, there was no change in activity in the Dahl SS/Jr rats on the same diets.
Chromosomal Mapping of APN Gene

The marker was detected in 26% of the hybrids. LOD score from 2-point analysis showed close correlation of the locus to D1Rat42, D1Rat38, D1Got122, and D1Got115. The closest linkage was with D1Rat42 (H9258/H11005 0.086, LOD H11005 17.749). D1Rat42 and D1Rat38 were within blood pressure QTL 1b, which was congenically defined in Dahl SS/Jr X Lewis.15 The relationship to other markers and crosses are indicated in Figure 3.

Discussion

The results suggest that APN may mediate salt-adaptation and play a role in the pathogenesis of SS hypertension. Adaptation to a high-salt diet in the Sprague-Dawley and Lewis rats is accompanied by increased APN abundance and activity, suggesting that increased metabolism of Ang III to Ang IV may be a mechanism for reducing Na\(^+\) uptake and renal vascular resistance in adaptation to a high-salt diet. From the present studies, it is not possible to determine whether a change in APN activity contributes to the development versus maintenance of pressure in salt-adaptation, because the temporal relationship of the increase in blood pressure to the increase in APN transcript in the rats was not evaluated. In contrast, a high-salt diet does not increase renal APN abundance or activity in Dahl SS/Jr rats. This is consistent with the hypothesis that reduced APN activity contributes to salt-sensitivity. Because Ang III acts on AT\(_2\) receptors, which are present in the thick ascending loop of Henle, less metabolism of Ang III in Dahl SS rats on an 8% salt diet may be a mechanism for increased sodium uptake in the thick ascending loop of Henle in the Dahl SS rat.16–18

Because reduced plasma Ang I and Ang II, renin, and aldosterone levels have been found in the Dahl SS rats,19–25 the RAS system has generally been thought to undergo normal compensatory changes in response to hypertension and not play a role in the pathogenesis of hypertension in this strain. However, the present findings raise the possibility that Ang III and IV play a mechanistic role in salt adaptation and in the pathogenesis of hypertension. These measurements may also help explain the finding of greater renal APN activity in the Dahl SS/Jr than in the Sprague-Dawley on a 0.3% salt diet, even though there is greater renal uptake of Na\(^+\) in the Dahl SS/Jr rat. To further address this requires measurements of angiotensin metabolites, including angiotensins II, III, and IV, in these rat strains. The possibility that other APN substrates, which include a variety of aliphatic branched N-terminal amino acids,26 play a role in blood pressure cannot be ruled out.

Further support for APN as a candidate gene for hypertension in the Dahl SS/Jr rat is provided by the finding that it maps to a reported blood pressure QTL on chromosome 1.27,28 This is consistent with, but does not demonstrate, APN as the culprit gene within the QTL that affects blood pressure. Because this QTL was determined from a series of congenic rat strains with segments of chromosome 1 from Lewis rats introgressed into Dahl SS/Jr,27,28 this provides additional support for APN as a candidate gene in the Dahl SS/Jr rat, because we have identified it by comparing the Lewis strain and the Sprague-Dawley rat strain, which is the progenitor strain, to the Dahl SS/Jr rat.

Because selection in the present study was based on differences in gene transcript abundance, we speculate that functional polymorphisms/alleles of the APN gene associated with SS hypertension are in noncoding flanking and/or intronic regions of the APN gene, because there is altered regulation of transcript abundance. The APN gene consists of 20 exons that encode segments differing in size from 18 to 205 amino acids.11 However, the possibility that there are functional differences in the coding region cannot be excluded, especially because APN activity is different in Sprague-Dawley versus Lewis and Dahl SS/Jr rats on 0.3% salt diet.
salt diets, even though APN protein abundance is the same. If post-translational modifications of APN, rather than the APN gene, are important in the pathogenesis of hypertension, genes for the culprit modifiers (eg, kinases, phosphatases, and the like) should cosegregate with hypertension rather than the APN gene. Sequencing and further cosegregation analysis should help to resolve this in the future.

Perspectives

The role that aminopeptidases and APN play in human blood pressure remains to be determined. Thirty-three polymorphisms in the adipocyte-derived leucine aminopeptidase gene have been identified, one of which (a Lys528Arg polymorphism) recently has been associated with essential human hypertension.29 The APN gene has been assigned to the long-arm region (q11-qter) of human chromosome 15, a region in which there is evidence for a QTL for blood pressure.30

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