Development of Hypertension and Kidney Hypertrophy in Transgenic Mice Overexpressing ARAP1 Gene in the Kidney

Deng-Fu Guo, Isabelle Chenier, Julie L. Lavoie, John S.D. Chan, Pavel Hamet, Johanne Tremblay, Xiang Mei Chen, Donna H. Wang, Tadashi Inagami

Abstract—Angiotensin II regulates blood pressure via activation of the type 1 receptor. We previously identified a novel angiotensin II type 1 receptor–associated protein and demonstrated that it promotes receptor recycling to the plasma membrane. To delineate the pathophysiological function of the ARAP1 in the kidneys, we generated transgenic mice that overexpress rat ARAP1 cDNA specifically in proximal tubules and tested the hypothesis that proximal tubule-specific overexpression of ARAP1 causes hypertension. Two lines of male transgenic mice, 650 and 670, displayed kidney-specific transgene expression. Systolic blood pressure was significantly elevated by 20 to 25 mm Hg in these lines of mice at 20 weeks of age compared with their nontransgenic litter mates. Urine volume, but not water intake, was significantly decreased in both lines compared with nontransgenic controls. The kidney/body weight ratio was significantly increased in both lines compared with their nontransgenic litter mates at 12 and 20 weeks of age. In contrast, no difference was observed in the ratio of brain, spleen, heart, and testis to body weight between male transgenic and nontransgenic animals. Inhibitions of the renin–angiotensin system completely normalized the systolic blood pressure of transgenic mice. Moreover, low salt intake prevented the development of hypertension, whereas high salt intake exacerbated the increase in blood pressure in transgenic mice. Therefore, our data show that proximal tubule-specific overexpression of ARAP1 leads to hypertension, suggesting that renal ARAP1 plays an important role in the regulation of blood pressure and renal function via activation of the intrarenal renin–angiotensin system.

Hypertension is a major risk factor for cardiovascular and renal diseases. Although numerous studies have implicated a role for the renin–angiotensin system (RAS) in the development of hypertension, its mechanisms remain incompletely understood. Traditionally, the hypertensive effects of the RAS were considered to be the actions of circulating components of this system. According to this view, angiotensin II (Ang II) in the systemic circulation is generated from angiotensinogen (AOGEN), which is acted on by renin from the kidneys to proteolytically produce angiotensin I, a substrate being further processed by angiotensin-converting enzyme (ACE) to form biologically active Ang II. However, recent convincing evidence accumulated from physiological, biochemical, and molecular studies has demonstrated other pathways of Ang II production. Among these, the intrarenal RAS is of special interest. Renal proximal tubules contain all of the components of the RAS.1–3

It has been demonstrated that activation of the Ang II type 1 receptor (AT1) stimulates the apical sodium–hydrogen exchanger in proximal tubules4 and augments epithelial sodium channel (ENaC) activity in the collecting ducts,5,6 leading to modulation of blood pressure (BP) and fluid homeostasis. Furthermore, dysregulation of the intrarenal RAS has been implicated in certain models of hypertension, nephrogenesis, and renal repairment.7–9 For example, AOGEN-null mice display lesions in the renal cortex, interstitial inflammation, tubular atrophy, reduced renal papillary, and hypotension.10,11 Knockout mice, which lack AT1 expression, show a phenotype similar to that of mice lacking AOGEN and ACE gene expression.12–16 These animals present hypotension, shorter survival, and marked abnormalities in renal development. Conversely, transgenic mice carrying both human AOGEN and renin genes incur glomerulosclerosis and phenotypic alterations in mesangial cells.17,18

Biochemical and pharmacological studies have revealed that AT1 undergoes rapid internalization on agonist stimula-
tion and then recycles back to the plasma membrane.\textsuperscript{19–22} Although the recycling of receptors to the plasma membrane is a notable event, the molecular mechanisms of this process are not well understood. AT\textsubscript{1}, receptor-associated protein (ARAP1) has been identified to directly interact with AT\textsubscript{1}, and promote receptor recycling to the plasma membrane in vitro.\textsuperscript{23}

The objective of the present study was to investigate the pathophysiological function of ARAP1 gene in the kidneys of transgenic mice. For this purpose, we generated transgenic mice overexpressing rat ARAP1 driven by the renal androgen-regulated protein (KAP) promoter in the renal proximal tubules. This promoter targets the gene of interest to the proximal tubules, where the transgene will then respond to androgen. We report here that transgenic mice overexpressing ARAP1 gene in the proximal tubules developed hypertension and kidney hypertrophy.

Methods

Generation of KAP2-ARAP1 Transgenic Mice

KAP2-ARAP1 transgenic mice were generated to produce proximal tubule-specific rat ARAP1 expression by inserting rat ARAP1 cDNA into a construct containing the KAP promoter and noncoding region of DNA including exons 3 to 5 of the human AOGEN gene (Figure 1A). Further details of generation of the transgenic mice are given in the online supplement available at http://hyper.ahajournals.org.

Analysis of Transgene Expression

Total RNA of various tissues were purified and used to test tissue specificity of transgene expression. Detailed methods of analysis of transgene expression are given in the online supplement.

Figure 1. Schematic map of the KAP2-ARAP1 construct. A, The ARAP1 gene with Myc epitope tag on its 5'-terminus was inserted into the NotI site of the KAP2 vector. The transgene was excised as a SpeI and Ndel fragment for microinjection. The probe for Southern blot hybridization, restriction enzymes, KAP promoter, and human AOGEN are indicated. B, Southern blot analysis of tail biopsies for the F2 generation. Twenty milligrams of genomic DNA were digested with BamHI and electrophoresed in 0.8% agarose gel. F1 DNA served as a positive control. Homozygous transgenic animals express markedly increased copy numbers (5- and 8-fold) for lines 650 and 670, respectively.

Physiological Parameters

Systolic BP (SBP) was measured by tail-cuff plethysmography (BP-2000 system; VisiTech System). Animals were trained for 30 minutes per day for 5 days before the measurement of baseline BP for a period of 1 week. We also used radiotelemetry (Data Sciences International) to confirm SBP in the male transgenic mice as described previously.\textsuperscript{24}

To confirm that the hypertension development of male transgenic mice was indeed driven by the KAP promoter linked to ARAP1 transgene, transgenic females at 16 weeks of age were anesthetized with isoflurane and implanted surgically with placebo pellets (n=4) or 5-mg testosterone pellets (n=4; 21-day release schedule). The pellet was implanted subcutaneously in the back and tunnelled to the nape of the neck with a 10-gauge trocar. The incision was closed with a stainless steel staple, and the mice were given 2 days to recover. After 2 days of testosterone administration to induce renal ARAP1 expression, BP was monitored by tail-cuff pressure machine 3 times a week for 24 days.

To investigate whether RAS inhibitors can reverse SBP, male nontransgenic litter mates and transgenic mice were divided into 3 groups (n=5): placebo, losartan treatment (30 mg/kg per day in drinking water), and perindopril (5 mg/kg per day in drinking water). SBP was measured every 2 to 3 days during treatment.

Urine volume and water intake were measured by housing the mice in metabolic cages for 2 days and recorded for an additional 2 days. At the end of the study, body and tissues, including the brain, heart, spleen, testes, and kidneys, were weighed.

Effects of Salt Diets and Amiloride on SBP in the Transgenic Mice

Five male transgenic and 6 male nontransgenic mice at 16 weeks of age were fed a high-salt diet (8% NaCl) for 4 weeks, or 5 male transgenic and 6 male nontransgenic mice at 17 weeks of age were fed a low-salt diet (0.2% NaCl) for 2 weeks, and their SBP was measured. To study whether ENaC inhibition affects SBP in transgenic mice, 5 male transgenic mice and 6 male nontransgenic mice at 13 weeks of age were treated with 2 mg/kg per day of amiloride in drinking water for 2 weeks, and SBP was measured.

Figure 2. Transgene expression of line 650. RT-PCR product showing differential tissue expression of transgene mRNA in (A) 12-week-old male mice and (B) 12-week-old females treated with or without testosterone for 2 weeks before RNA isolation. C, Transgene product expression in different tissues of 20-week-old male mice by immunoprecipitation and Western blot detection. Br indicates brain; Li, liver; Lu, lungs; Ki, kidneys; Sp, spleen; Mu, skeletal muscle; Te, testes.
Gene Expression of the RAS Components, Aquaporins, ENaC, and Sodium-Hydrogen Exchanger in KAP2-ARAP1 Transgenic Mice

To explore possible mechanisms of hypertension development, we examined mRNA expression of the RAS components: aquaporins 1, 2, and 7 (AQP1, AQP2, and AQP7); the 3 subunits of ENaC; and sodium-hydrogen exchanger (NHE3) in the whole kidneys of nontransgenic and KAP2-ARAP1 male mice at 20 weeks of age, with details in the online supplement.

Statistical Analysis

The data are expressed as means±SE. The statistical significance of differences between the experimental groups was evaluated by 1-way ANOVA and the Bonferroni test. P<0.05 values were considered to be statistically significant.

Results

Tissue-Specific Expression of the ARAP1 Transgene in the Transgenic Mice

KAP2-ARAP1 transgenic mice were generated to produce proximal tubule-specific rat ARAP1 expression by inserting rat ARAP1 cDNA into a construct containing the KAP promoter and noncoding region of DNA including exons 3 to 5 of the human AOGEN gene (Figure 1A). Southern blot analysis revealed the presence of the transgene in heterozygous and homozygous animals but not in wild-type mice in the F2 stage (Figure 1B). Transgene mRNA expression was highly expressed in the kidneys and testes and detectable in the brain but not in the liver, lungs, spleen, and skeletal muscles of male transgenic line 650 (Figure 2A). The second transgenic line, 670, displayed similar tissue distribution of the transgene. Because the KAP promoter is regulated by androgen, we examined whether transgene mRNA was expressed in the same tissues of transgenic females with or without testosterone implantation for 2 weeks. As seen in Figure 2B, transgene mRNA expression was significantly higher in the kidneys of testosterone-treated female animals compared with testosterone-untreated controls. Transgene protein expression was detected in the kidneys and testes but not in other tissues (Figure 2C), confirming its mRNA expression pattern.

Development of Hypertension in the Transgenic Mice

As shown in Figure 3A, SBP was significantly elevated in both transgenic mice lines 650 (P<0.001) and 670 (P<0.001) by 20 and 25 mm Hg, respectively, compared with their nontransgenic litter mates, indicating that transgenic males developed hypertension. To test whether transgenic females developed a similar phenotype, SBP was monitored in female mice implanted with placebo or 5-mg testosterone pellet. As seen in Figure 3B, SBP was significantly higher in females given testosterone compared with mice given placebo pellet after 4 days (P<0.01) and showed the highest level after 10 to 15 days (P<0.001), remained hypertensive after 19 days (P<0.01), and returned to baseline after 23 days of testosterone implantation. In contrast, no SBP increase was observed in transgenic females implanted with placebo pellet and in nontransgenic females implanted with 5-mg testosterone pellet. To further confirm this phenotype, radiotelemetry was used to record direct SBP measurements in transgenic males at 20 weeks of age. SBP was significantly elevated at both day and night times by 20 and 23 mm Hg, respectively (n=4; P<0.01).

To define the underlying mechanism(s) of hypertension in the transgenic mice, water intake and urine volume in transgenic mice and their nontransgenic litter mates were examined and are shown in Table 1. Although water intake did not differ between transgenic mice and nontransgenic litter mates, urine volume was significantly reduced in transgenic mice at ages 12 and 20 weeks compared with their nontransgenic litter mates (P<0.01 for line 650 and P<0.001 for line 670), indicating water retention in the transgenic mice. Serum hematocrit was significantly lower in transgenic mice (41.4%) compared with their nontransgenic litter mates (45.9%, n=6; P<0.01), supporting

TABLE 1. Water Intake and Urine Volume (mL/24 h per 20 g) of Transgenic and Nontransgenic Mice at Different Ages

<table>
<thead>
<tr>
<th>Volume</th>
<th>8 Weeks</th>
<th>12 Weeks</th>
<th>20 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NT</td>
<td>650</td>
<td>670</td>
</tr>
<tr>
<td>Water intake (mL/24 hours per 20 g)</td>
<td>3.9±0.5</td>
<td>3.7±0.4</td>
<td>4.1±0.6</td>
</tr>
<tr>
<td>Urine volume (mL/24 hours per 20 g)</td>
<td>1.2±0.2</td>
<td>1.1±0.2</td>
<td>1.1±0.2</td>
</tr>
</tbody>
</table>

NT indicates nontransgenic.

*P<0.05 and †P<0.01.
the notion of increased extracellular fluid volume in transgenic mice affecting SBP. As depicted in Figure 4, the AT1 antagonist losartan or the ACE inhibitor perindopril completely normalized the SBP of transgenic mice compared with that of nontransgenic mice.

We observed that the α-ENaC subunit mRNA expression, but not β- and γ-ENaC, was significantly increased in the kidney of transgenic mice compared with their nontransgenic control (Figure I in the online supplement). To examine whether inhibition of ENaC activity affects BP, transgenic mice at 13 weeks of age were treated with amiloride (2 mg/kg in drinking water), an inhibitor of ENaC, for 2 weeks, and their SBPs were measured. As shown in Figure 5, SBP was significantly lowered by 12 mm Hg reaching to 120 mm Hg (n=5; P<0.05) in transgenic mice after 4 days of amiloride treatment but not completely normalized as seen in mice treated with RAS inhibitors and remained at the same level for the rest of the experiments. In contrast, SBP was not changed by amiloride in nontransgenic mice throughout the experiment. Interestingly, SBP was significantly increased to 145 mm Hg in transgenic mice (n=5; P<0.01) after 4 weeks of high-salt diet (Figure 6A), whereas no change in SBP was observed in nontransgenic animals (n=6) fed a high-salt diet. In contrast, SBP was significantly lowered to 112 mm Hg in transgenic mice after 7 days of low-salt intake (Figure 6B, n=5; P<0.001), whereas no change in SBP was observed in nontransgenic mice fed a low-salt diet (n=6).

Development of Kidney Hypertrophy in the Transgenic Mice

As shown in Table 2, there was no significant difference in body weight between transgenic and nontransgenic mice in all of the ages examined. However, a significant increase in the kidney:body weight ratio was observed in both male transgenic lines at 12 and 20 weeks but not 8 weeks of age compared with their nontransgenic mice (P<0.01 for 650 and P<0.05 for 670), whereas no significant change was observed in the ratio of the brain, heart, spleen, and testes to body weight.

Histological Parameters

Transgenic mice at 8 weeks of age displayed normal kidneys compared with nontransgenic litter mates. However, ~5% to 10% of renal tubules in transgenic mice at 12 weeks of age and 20% to 30% of renal tubules in transgenic mice at 20 weeks of age presented renal morphological changes compared with nontransgenic litter mates that had normal kidney (Figure 7A). Kidneys of male transgenic mice exhibited cellular edema, reabsorption of droplets, and enlargement of epithelia cells in proximal tubules (Figure 7B). To determine whether droplets were water or fat in content, oil red-O staining was undertaken using frozen kidneys. No positive
Figure 7C. was not observed in the kidneys of nontransgenic litter mates the transgenic mice at 20 weeks of age (Figure 7D), which hydropic degeneration of renal proximal tubules occurred in nontransgenic mice at 20 weeks of age, suggesting that staining was observed in the kidneys of the transgenic and transgenic mice. Five male transgenic and 6 nontransgenic mice 16 weeks of age were fed either (A) high-salt diet (8% NaCl) for 4 weeks or (B) low-salt diet (0.2% NaCl) for 2 weeks. *P<0.01 and **P<0.001.

staining was observed in the kidneys of the transgenic and nontransgenic mice at 20 weeks of age, suggesting that droplets were formed by water reabsorption. Moreover, hydropic degeneration of renal proximal tubules occurred in the transgenic mice at 20 weeks of age (Figure 7D), which was not observed in the kidneys of nontransgenic litter mates (Figure 7C).

Figure 6. Effect of low- and high-salt diet on SBP in the transgenic mice. Five male transgenic and 6 nontransgenic mice 16 weeks BW indicates body weight (g); Br, brain; He, heart; Ki, kidney; Sp, spleen; Te, testes (mg/g); NT, nontransgenic. *P<0.01 and † P<0.05.

Discussion

ARAP1 was cloned as a binding protein to the AT1 cytosolic C-terminal domain. This protein has been shown to accelerate AT1 recycling back to the plasma membrane. Because AT1 plays versatile physiological roles, the present study focused on defining the pathophysiological role of ARAP1 in the renal proximal tubules on its tissue-specific transgenic expression driven by a proximal tubule-specific promoter KAP. We found that proximal tubular ARAP1 transgenic mice have a significant elevation of BP by 20 to 25 mm Hg, which is salt sensitive and can be normalized by inhibition of the RAS by an ACE inhibitor or AT1 receptor blocker. These results, obtained by limiting the expression ARAP1 to the proximal tubules, indicate potential pathophysiological important roles of ARAP1 in regulating BP.

Abbreviated ARAP1 in genomic databases is used for Centaurin-α2 (Arf-GAP, p-GAP, ankyrin repeat, and pleckstrin homology domain—containing proteins 1), which is a totally different gene identified by our group. Further searching of databases revealed that ARAP1 identified by us and used in the present study is identical to angiopoietin-related protein 2 precursor (ANGPTL2).

To assess the pathophysiological significance of the ARAP1 gene in proximal tubules, we generated transgenic mice that overexpress rat ARAP1 gene in the kidneys under control of the KAP promoter. The reliability of the KAP promoter in transgenic models in which the kidneys are specifically targeted to express the transgene has proven repeatedly to be valuable in many other studies. ARAP1 transgene expression in male transgenic mice was at ≈8 weeks of age, higher during maturation, and decreased on aging. In addition, the transgene expression of transgenic females was detected in the kidneys of mice treated only with testosterone but not placebo, confirming previous reports.

The male transgenic mice developed not only kidney hypertrophy but also hypertension. Interestingly, low-salt diet reversed higher SBP to a normal level in 1 week after dietary treatment, whereas high-salt diet further increased SBP 4 weeks after high-salt intake, strongly indicating that the hypertension development of transgenic mice is salt sensitive. Dahl’s salt-sensitive rats have been widely used for the investigation of the underly-
ing mechanisms for increased salt sensitivity. Sixteen genomic regions containing quantitative trait loci (QTLs) for BP regulation have been reported in this strain.\textsuperscript{28–30} However, the genes responsible for salt-sensitive hypertension have not been unequivocally identified regardless of intensive genetic studies. Garrett et al\textsuperscript{28} reported the existence of 9 BP QTLs (chromosomes 1, 2, 3, 5, 8, 10, 16, 17, and 18) using F2 derived from Dahl’s salt-sensitive×Lewis rats. Interestingly, the rat and human ARAP1 gene is located in chromosome 3 and 9, respectively.

Long-term BP control is closely tied to sodium balance and extracellular fluid volume regulation, both of which are controlled in part by the RAS.\textsuperscript{31} Ang II has important nonrenal effects that are instrumental in BP control by being a vasoconstrictor and a regulator of aldosterone secretion. In addition, Ang II has direct effects on renal tubules, and on regulating NaCl reabsorption via sodium channels, presumably ENaC in connecting tubules through the collecting ducts and NHE3 in the proximal tubule.\textsuperscript{32} The present study demonstrates that elevated a-ENaC mRNA expression, but not NHE3 (details in the online supplement), accompanies hypertension in the transgenic mice. Moreover, blockade of ENaC by amiloride lowers SBP, indicating that ENaC contributes at least in part to the hypertension development of the transgenic mice. An explanation for the partial normalization of BP may be that ENaC inhibition leads to activation of other sodium channels (eg, NHE3), sodium chloride cotransporter, or sodium potassium chloride cotransporter 2 in the kidneys to compensate for the function of ENaC. Further studies are underway to verify these possibilities.

Both transgenic lines had significantly increased BP, indicating that hypertension is likely the result of specific overexpression of the ARAP1 gene rather than the ARAP1 transgene randomly inserted into chromosome that caused adverse effects. At the present time, we cannot rule out the possibility that activation of the RAS in the central nervous system may contribute to elevated BP in transgenic mice given the fact that ARAP1 transgene was slightly expressed in the brain. Future studies are required to determine whether activation of RAS in the central nervous system by ARAP1 affects BP. Administration of RAS blockers significantly reduced BP in these mice, indicating that activation of the intrarenal RAS plays an important role in this process. Perindopril seemed to be more effective than losartan in lowering BP in these mice. A similar perindopril efficiency has been reported in humans.\textsuperscript{33,34} Taken together, the present study suggests that a link exists among ARAP1 gene expression, hypertension, and kidney hypertrophy.

**Perspectives**

The present study shows that the transgenic mice manifest a significant increase in BP and develop kidney hypertrophy, which is influenced by salt intake. Although the precise mechanism(s) by which BP increases and causes kidney hypertrophy in this transgenic mouse model remains unclear, several possible hypotheses arise from these observations. One is that ARAP1 produced in renal proximal tubules evokes AOGEN expression and that AOGEN is converted to active Ang II, which is transported to the collecting ducts where it activates ENaC and leads to hypertension. This hypothesis remains to be proven in future studies. Long-term studies will reveal whether the transgenic mice incur protein urine because KAP2-AOGEN transgenic mice develop kidney injury.\textsuperscript{35} It is of interest to analyze whether QTL containing ARAP1 correlates with BP in salt-sensitive hypertension in future studies. Gene expression profiling in the kidneys of the transgenic mice may be helpful to identify genes associated with hypertension and kidney hypertrophy. These animal models will provide useful tools for the investigation of a novel paradigm for defining the underlying mechanism(s) of hypertension and for the development of new therapeutic approaches for the treatment of hypertensive patients.

**Acknowledgments**

We thank Dr Curt D. Sigmund for providing the KAP2 transgenic mouse vector. The editorial assistance of Ovid M. Da Silva, editor, Research Support Office, Research Centre, Centre hospitalier de l’Université de Montréal, is acknowledged.
Sources of Funding
This work was supported in part by grants from the Canadian Institutes of Health Research (MT-14726 to D.F.G.), the National Institutes of Health (grant HL58205 to T.I. and grants HL-57853, HL-73287, and DK67620 to D.H.W.), and the Main State Basic Research Development Program of the People’s Republic of China and the Creative Research Group Fund of the National Foundation Committee of Natural Science of the People’s Republic of China (30121005 to X.M.C.).

Disclosures
None.

References
Development of Hypertension and Kidney Hypertrophy in Transgenic Mice
Overexpressing ARAP1 Gene in the Kidney
Deng-Fu Guo, Isabelle Chenier, Julie L. Lavoie, John S.D. Chan, Pavel Hamet, Johanne Tremblay, Xiang Mei Chen, Donna H. Wang and Tadashi Inagami

Hypertension. 2006;48:453-459; originally published online June 26, 2006; doi: 10.1161/01.HYP.0000230664.32874.52
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/48/3/453

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2006/06/26/01.HYP.0000230664.32874.52.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org/subscriptions/