Validation of Uromodulin as a Candidate Gene for Human Essential Hypertension

Lesley A. Graham, Sandosh Padmanabhan, Niall J. Fraser, Satish Kumar, James M. Bates, Hajamohideen S. Raffi, Paul Welsh, Wendy Beattie, Shoujin Hao, Sabine Leh, Michael Hultstrom, Nicholas R. Ferreri, Anna F. Dominiczak, Delyth Graham,* Martin W. McBride*

Abstract—A recent genome-wide association study identified a locus on chromosome 16 in the promoter region of the uromodulin (UMOD) gene that is associated with hypertension. Here, we examined the hypertension signal with functional studies in Umod knockout (KO) mice. Systolic blood pressure was significantly lower in KO versus wild-type (WT) mice under basal conditions (KO: 116.6±0.3 mmHg versus WT: 136.2±0.4 mmHg; P<0.0001). Administration of 2% NaCl did not alter systolic blood pressure in KO mice, whereas it increased in WT mice by ≈33%, P<0.001. The average 24-hour urinary sodium excretion in the KO was greater than that of WT mice (P<0.001). Chronic renal function curves demonstrate a leftward shift in KO mice, suggesting that the relationship between UMOD and blood pressure is affected by sodium. Creatinine clearance was increased during salt loading with 2% NaCl in the KO mice, leading to augmented filtered Na+ excretion and further Na+ loss. The difference in sodium uptake that exists between WT and KO strains was explored at the molecular level. Urinary tumor necrosis factor-α levels were significantly higher in KO mice compared with WT mice (P<0.0001). Stimulation of primary thick ascending limb of the loop of Henle cells with exogenous tumor necrosis factor-α caused a reduction in NKCC2A expression (P<0.001) with a concurrent rise in the levels of UMOD mRNA (P<0.001). Collectively, we demonstrate that UMOD regulates sodium uptake in the thick ascending limb of the loop of Henle by modulating the effect of tumor necrosis factor-α on NKCC2A expression, making UMOD an important determinant of blood pressure control. (Hypertension. 2014;63:551-558.) • Online Data Supplement

Key Words: hypertension ■ mice ■ mice, knockout ■ Umod protein, mouse ■ uromodulin

Hypertension (HTN) is an independent, heritable, and modifiable risk factor for cardiovascular and renal disease.1 Blood pressure (BP) is controlled by physiological pathways involved in extracellular volume homeostasis, cardiac contractility and vascular tone with multiple lines of evidence;2,3 Mendelian forms of syndromic hypotension/HTN, and renal transplant studies4 that point to the crucial role of the kidney in HTN causation. However, the genetic dissection of polygenic HTN has been challenging.5 The largest genome-wide association study (GWAS) meta-analysis of >200,000 subjects identified 29 validated single-nucleotide polymorphisms associated with systolic BP (SBP) and diastolic BP but explained <1% of the population variance of the trait.6,7 Recently, we performed a GWAS on a population cohort consisting of BP extremes in which a single-nucleotide polymorphism (rs13333226 at position 16:20365654) in the 5′ region of the uromodulin (UMOD) gene associated with HTN was identified and validated.7 The minor G allele of rs13333226 was associated with lower risk of HTN, decreased urinary UMOD excretion, and higher glomerular filtration rate (GFR).3 Furthermore, the association with HTN was shown to be independent of renal function2 indicating a possible pleiotropic effect because single-nucleotide polymorphisms highly correlated with rs13333226 were shown to be associated with kidney function in independent GWAS. The genotype association of rs13333226 and urinary UMOD excretion was more pronounced on low salt intake and blunted with high salt intake, indicating a possible gene–environment interaction.7 Expression of UMOD, also known as Tamm-Horsfall glycoprotein, is restricted to the medullary region of the thick ascending limb of the loop of Henle (TAL). This region of
the kidney reabsorbs ~25% of filtered NaCl. The Na⁺-K⁺-2Cl⁻ cotransporter (NKCC2), which is selectively expressed in the TAL also, transports the majority of NaCl reabsorbed by the TAL. Inactivating mutations of the NKCC2 gene (SLC12A1) cause a significant reduction in BP and have a severe salt-wasting phenotype (Bartter syndrome).9,10 It has recently been shown that UMOD can modulate NKCC2 activity and NaCl reabsorption in TAL cells.11 Furthermore, it is known that UMOD can bind several cytokines, including tumor necrosis factor-α (TNF-α), via its epidermal growth factor (EGF) domains; TNF-α is produced by the TAL and acts in an autocrine manner to downregulate NKCC2A expression, lowering NaCl reabsorption at this site.11–14

To understand the role that UMOD plays in BP regulation, we have studied BP, sodium excretion, and renal function under conditions of normal sodium and high sodium intake in knockout (KO; Umod−/−) and wild-type (WT; Umod+/+) mice.

Methods

Experimental Animals
The KO and WT mice were generated by Bates et al15 (see Methods in the online-only Data Supplement for details) and have been maintained as breeding colonies at Glasgow University since 2010. The mice were housed under controlled environmental conditions, fed standard rat chow (rat and mouse No. 1 maintenance diet, Special Diet Services), and water provided ad libitum. All animal procedures performed were approved by the Home Office according to regulations regarding experiments with animals in the United Kingdom.

Hemodynamic Parameters
Cardiovascular monitoring was conducted in male mice from 12 weeks of age. Hemodynamic parameters were measured by tail cuff plethysmography (Harvard Instruments) and radiotelemetry (Data Sciences International; see Methods in the online-only Data Supplement). Baseline SBP was measured in 12-week-old KO and WT mice for a 21-day period. The impact of salt loading was investigated for a 6-week period by administration of ±2% NaCl in the drinking water.

Physiological Measurements and Ex Vivo Analysis
During week 6 of the salt-loading period, urine samples from KO and WT mice (±2% NaCl) were obtained by 24-hour metabolic cage collection. Electrolyte analysis in urine was assessed. On euthanasia, blood samples were taken for biochemical analysis, and body weight, kidney weight, and tibia length were measured. All tissue weights were normalized to tibia length. Estimated GFR was measured by quantitative determination of creatinine, glucose, and urea from WT and KO mice ±2% NaCl in the plasma and urine using Roche/Hitachi cobas c systems kits (Roche Diagnostics, Burgess Hill, United Kingdom). Values were then normalized to kidney weight and urine flow per minute.

Umod⁺/+ (WT) and Umod⁻/- (KO) Histology
Three-micrometer-thick sections were stained with periodic acid–Schiff and visualized using the EnVision detection system for mouse primary antibodies (K5007, Dako, Glostrup, Denmark). All slides were imaged using an Aperio scanner XT at ×20 magnification producing a resolution of 0.495 μm/pixel and investigated using the ImageScope software (Aperio v12.0.0.5039).

Mouse TAL Cell Isolation and UMOD Knockdown Studies
Male WT mice of 5 to 7 weeks old were used for TAL cell isolation. Isolation of mouse TAL (mTAL) cells was performed as previously described by Eng et al16 (see Methods in the online-only Data Supplement). On confluence, mTAL cells were transfected with lipofectamine 2000 (Invitrogen) for a 4-hour period with either small interfering RNA (10 nmol/L) to knockdown UMOD expression or scrambled control (Ambion, Manchester, United Kingdom) and then quiesced overnight with RPMI (Gibco, Manchester, United Kingdom) before a 6-hour period of TNF-α treatment (5 nmol/L; PeproTech, NJ).

Gene Expression
Quantitative real-time polymerase chain reaction of total RNA extracted from mTAL cells from WT mice and small interfering RNA treated mTAL using TRIzol (Life Technologies, Grand Island, NY). cDNA was prepared using GoScript Reverse Transcription System (Promega, Madison, WI). Determination of mRNA abundance of specific genes was assessed by quantitative real-time polymerase chain reaction using 1-step 48-well Real Time PCR System and Fast SYBER Green Master Mix (Applied Biosystems, CA). Results were normalized to the housekeeper GAPDH (primers sequences are included in Methods in the online-only Data Supplement).

Figure 1. Knockout (KO; umod⁻/-) mice have lower systolic blood pressure (SBP) compared with wild-type (WT; umod⁺/+ ) mice. SBP was measured by radiotelemetry for a 21-day period under normal salt conditions. KO mice have significantly lower SBP than WT, n=6 per group, ***P<0.0001. Results are displayed as daily averaged daytime and night-time means±SEM. Analyzed with repeated-measures ANOVA. Week 1 was a recovery period after surgery and was not included in the analysis. Umod indicates uromodulin.
ELISA for TNF-α
The measurement of urinary TNF-α by ELISA was performed using commercially available mouse TNF-α ELISA kits (BD Sciences, CA). The procedure can be found in the Methods in the online-only Data Supplement.

Statistical Analysis
The results are expressed as mean±SEM. GraphPad Prism 4 was used to analyze all data sets. One-way ANOVA (followed by Tukey or Dunnett post hoc test) was used for comparisons among groups, and an unpaired Student t test was used to compare data between groups. Radiotelemetry data were analyzed by repeated-measures ANOVA. A P value <0.05 was considered statistically significant. Gene expression data were made using 2(−ΔΔCT) method and submitted to ANOVA statistical analysis. A generalized estimating equation model, which accounts for correlation between measurements in longitudinal studies, was used to study the effect of normal salt intake and high salt intake on BP and water intake using repeated weekly measurements of these variables for 6 weeks in KO and WT mice. Urine sodium excretion over 24 hours was measured in week 6. Strain and salt intake effects were tested using generalized linear models.

Results

Hemodynamic Parameters in WT (Umod+/+) and KO (Umod−/−) Mice Were Investigated Under Basal and Salt-Loaded Conditions
Baseline SBP measured by radiotelemetry for a 3-week period was lower in KO mice compared with WT (KO: 116.6±0.3 mmHg versus WT: 136.2±0.4 mmHg; ***P<0.0001; n=6 per group; Figure 1). During the 6-week 2% NaCl-loading period, SBP, measured by tail cuff plethysmography, was elevated in WT mice from the third week of treatment onward (WT control versus WT +2% NaCl; ***P<0.0001; n=10 per group; Figure 2A). During the 6-week salt-loading period, the Umod KO did not affect SBP (Figure 2B). The difference in hemodynamic response to salt between WT and KO mice was confirmed by radiotelemetry where the weekly averaged daytime and night-time SBP in salt-loaded WT mice was elevated compared with KO animals (KO: 122.2±7.0 mmHg versus WT: 144.4±6.3 mmHg; **P<0.001; n=8 per group; Figure 3A). Figure 3B illustrates elevated weekly averaged daytime and night-time pulse pressure in salt-loaded WT compared with KO mice (18.3±5.7 mmHg versus 5.5±1.0 mmHg; WT versus KO; ***P<0.0001; n=8 per group). In the general linear model (GLM), after adjusting for salt intake, the KO mice had a 27.2 mmHg lower SBP compared with WT animals, with no difference in volume intake during the 6 weeks (P<0.0001; Table). There were no significant differences between 2% salt-loaded WT and KO mice for diastolic pressure, mean arterial pressure, heart rate, or motor activity (Table S1 in the online-only Data Supplement). The chronic renal function curves (Figure 3C and 3D) showed a leftward shift in KO mice compared with the WT strain, suggesting that UMOD affects BP through altering sodium excretion. Figures 1 and 3C are 2 independently generated data sets. Figure 1 data represent continuous (real-time) daily averaged data points in animals measured from 12 to 15 weeks of age. Figure 3A represents weekly averaged data points in animals measured from 12 to 18 weeks of age. First week data that were collected during essential periods of recovery (for radio telemetry) and training (for tail cuff) were not included in any statistical analyses.

Urinary Electrolyte Analysis in WT (Umod+/+) and KO (Umod−/−) Mice Under Baseline and Salt-Loaded Conditions
Detailed analysis of fluid intake, urine output, and urine electrolytes were performed at week 6 of the study. Fluid intake was increased in WT mice at week 1 and 6 of the NaCl-loading period (P=0.02; n=10 per group) compared with normal salt intake (Figure 4A). The KO mice showed increased fluid intake at weeks 1, 3, and 5 when treated with 2% NaCl (*P=0.03, **P=0.005, and ***P=0.009 at weeks 1, 3, and 5, respectively; n=10 per group; Figure 4A). Body weight was unchanged in WT mice during salt loading (Figure 4B), whereas KO mice had a lower body weight at weeks 1, 2, 4, 5, and 6 (**P<0.05; ***P<0.01). This suggested that, on a high-salt diet, the KO animals retained less Na+ and had reduced extracellular fluid and blood volumes compared with the WT strain. Analysis of weekly fluid intake and SBP during 6 weeks of normal salt and 6 weeks of salt loading showed that the WT mice had increased fluid intake and SBP (P<0.0001; n=10 per...
group), whereas the KO more than doubled their fluid intake accompanied by negligible change in SBP (*P <0.0001; n=10; Table S2). In the generalized estimating equation model, after adjusting for the salt effect, KO mice had an increased fluid intake of 1.1 mL compared with WT animals (Table). Urinary Na+ was increased in KO mice after 6 weeks of salt loading with 2% NaCl (*P =0.02; n=5 per group; Figure 4C). The generalized estimating equation analyses for urine volume and electrolytes at 6 weeks were adjusted for both salt and fluid volume intake to dissect strain-specific effects. KO mice had significantly greater urine volume, as well as urine sodium, potassium, and chloride excretion compared with WT (Table). The urinary Na+/K+ ratio was similar in both strains. At week 6, after adjusting for salt intake, the average 24-hour urinary sodium excretion in KO mice was appreciably greater than observed for the WT strain (**P <0.001). The lower arterial pressure observed in KO mice may be explained in terms of increased Na+ loss because this is accompanied by reduced extracellular and blood volume which, in turn, lowers cardiac output and BP.

Renal Function in WT (Umod+/+) and KO (Umod−/−) Mice After Salt Loading With 2% NaCl
At baseline, KO mice had significantly decreased GFR, estimated by measurement of creatinine clearance (CrCl), compared with WT (**P<0.001; Figure 5A). Salt loading resulted in increased GFR in KO mice compared with all other groups (**P<0.001; n=7 per group). There was no difference in CrCl after treatment with 2% NaCl in the WT mice (P=0.07). Renal mass index was similar between strains ±2% NaCl (P>0.5; n=10 per group; Figure 5B). Histological evaluation showed normal cortical and outer medullary structure without arterial lesions, glomerulosclerosis, adhesions, tubular atrophy, or interstitial fibrosis, with no signs of necrosis or apoptosis. However, diffuse edema with cellular and interstitial swelling was observed in the papillary tissue of KO animals, both before and after salt loading (Figure 5C).

Interactions Between UMOD, TNF-α, and NKCC2 in the TAL
mTAL cells secrete the cytokine TNF-α, which acts in an autocrine manner to inhibit Na+ uptake. Furthermore, TNF-α

Table. Cardiovascular Parameters Measured by Radiotelemetry in WT (umod+/+) and KO (umod−/−) Mice After 2% NaCl Loading

<table>
<thead>
<tr>
<th>Period</th>
<th>Variables</th>
<th>umod+/+</th>
<th>umod−/−</th>
<th>GEE/GLM</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SBP, mmHg</td>
<td>Normal Salt</td>
<td>High Salt</td>
<td>Normal Salt</td>
<td>High Salt</td>
</tr>
<tr>
<td>Week 6</td>
<td>Water intake, μL/24 h</td>
<td>6412.7 (1516)</td>
<td>9174.6 (2149.)</td>
<td>5904.8 (1191)</td>
<td>7476.2 (449.4)</td>
</tr>
<tr>
<td></td>
<td>Urine volume, μL/24 h</td>
<td>555.6 (316.7)</td>
<td>777.8 (370.1)</td>
<td>1044.4 (342.3)</td>
<td>1388.9 (422.6)</td>
</tr>
<tr>
<td></td>
<td>Urine Na+, μmol/24 h</td>
<td>54.9 (35.7)</td>
<td>457.4 (314.2)</td>
<td>217 (85.1)</td>
<td>1258 (372.1)</td>
</tr>
<tr>
<td></td>
<td>Urine K+, μmol/24 h</td>
<td>64.4 (37.4)</td>
<td>177.5 (106.2)</td>
<td>310.7 (192.4)</td>
<td>425.1 (87.4)</td>
</tr>
<tr>
<td></td>
<td>Urine Cl−, μmol/24 h</td>
<td>88.2 (50.7)</td>
<td>532.4 (358.7)</td>
<td>408.3 (217.2)</td>
<td>1407.4 (364.1)</td>
</tr>
<tr>
<td></td>
<td>Urine Na+/K+ ratio</td>
<td>1.2 (0.91)</td>
<td>2.51 (0.27)</td>
<td>1.05 (0.76)</td>
<td>2.94 (0.55)</td>
</tr>
</tbody>
</table>

GEE/GLM β coefficients are presented as KO effect with respect to WT after adjustment for covariates. All data were normalized to urine volume collected over a 24-h period and kidney weight on animal euthanasia. GEE indicates generalized estimating equation; KO, knockout; SBP, systolic blood pressure; Umod, uromodulin; and WT, wild type. Covariates in GEE/GLM: *strain, salt intake; †strain, salt intake, water intake (n=8 per group).
production by TAL cells is enhanced in response to stimuli such as angiotensin II.19,20 UMOD was originally identified as a ligand for renal cytokines including interleukin-1 and TNF-α.21 We thought it was possible that in the KO mouse, the absence of UMOD may result in an increased accumulation of TNF-α in urine. To test this hypothesis, we measured the TNF-α content of urine samples collected from both KO and WT mice at 6 weeks with or without salt loading. Under baseline conditions, urinary TNF-α levels were significantly greater in KO mice compared with WT (*P<0.01; n=3 per group; Figure 6A). This increase was ≈5-fold in the KO mice on salt loading (**P<0.001). Quantitative real-time polymerase chain reaction on RNA isolated from kidney outer medulla showed an increase in the abundance of TNF-α gene transcripts in both WT and KO animals on salt loading, reaching statistical significance in KO mice (**P<0.01; n=3; Figure 6B). To test whether the sodium loss observed in KO mice was because of increased levels of extracellular TNF-α, the mRNA levels of the major functional splice variant of NKCC2 (NKCC2A) was measured in mTAL cells±TNF-α, ±UMOD small interfering RNA (Figure 6C). In the presence of UMOD, stimulation with TNF-α (6-hour incubation with 5 nmol/L TNF-α) decreased relative NKCC2A mRNA levels (***P<0.001; n=3), consistent with previous findings.22 UMOD knockdown caused an even greater reduction in NKCC2A transcript levels (**P<0.001; n=3) that could not be further lowered by addition of exogenous TNF-α (Figure 6C). This suggested to us that, in the absence of UMOD, TNF-α secreted by the TAL cells, acting in an autocrine manner, was sufficient to cause maximal downregulation of NKCC2A gene expression. In mTAL cells, stimulation with TNF-α was found to increase the relative levels of UMOD mRNA by ≈3.5-fold (***P<0.0001; n=3; Figure 6D). This raises the intriguing possibility of a negative feedback loop in which the TNF-α–induced reduction in NKCC2A gene expression is switched off by TNF-α through increasing the production of cell-surface UMOD (which binds TNF-α at high affinity).

Discussion

In this study, we have followed up our HTN GWAS and provide functional evidence that UMOD is involved in BP regulation through altering sodium excretion. To our knowledge, we are the first to show clear BP differences between UMOD KO mice and WT animals in that they display lower baseline BP and are insensitive to salt-induced changes in BP. The lower BP in KO mice can be explained by a leftward shift in the chronic renal function curves. Furthermore, we provide evidence that this may be because of an inverse association between the expression levels of UMOD and NKCC2A.

Steady-state electrolyte handling has been investigated previously in Umod KO mice that demonstrated no differences compared with the WT strain. These findings are consistent with our study.12 A defect in renal function related to renal damage does not seem to contribute to this effect because no differences in renal mass index or renal structures were observed between the strains. However, diffuse edema with cellular and interstitial swelling was observed in the KO mice.

Figure 4. Electrolyte analysis reveals altered Na+ handling in knockout (KO; umod−/−) mice. A. Fluid intake was significantly increased at week 6 in wild-type (WT) mice when salt loaded compared with the control WT animals (*P=0.02; n=10 per group). The salt-loaded KO mice displayed increased fluid intake at weeks 1, 3, and 5 compared with the non–salt-loaded control animals (P=0.03, **P=0.005, and ***P=0.009; n=10 per group). B. Body weights were significantly reduced in KO mice at weeks 1, 2, 4, 5, and 6 of the salt-loading period compared with the non–salt-loaded control (*P<0.05; **P<0.01). Body weights were unchanged in WT after salt loading. C. Urinary Na+ excretion was significantly increased in the KO mice after 6 weeks of salt loading (2% NaCl; *P<0.01; n=5 per group). Results displayed as mean±SEM. 

Analyzed with unpaired Student t test. UMOD indicates uromodulin.
before and during salt loading. The KO mice show lower CrCl under normal salt conditions, consistent with previous reports,\textsuperscript{12,15} and higher CrCl when salt loaded compared with WT mice. This increase in CrCl suggests that KO mice have a greater capacity to excrete Na\textsuperscript{+}, thereby maintaining body homeostasis despite increased Na\textsuperscript{+} intake. The lower baseline CrCl in KO mice is in contrast to previous human GW AS that showed alleles associated with low UMOD excretion are associated with high estimated GFR.\textsuperscript{5,8} Because KO mice displayed high CrCl only in relation to high salt intake, it is possible that the associations found in these GWAS were confounded by high dietary salt intake in the population cohorts used. Our group previously reported that the minor G allele of rs13333226 (allele frequency, 0.18 in Europeans) was associated with a lower risk of HTN, lower urinary UMOD excretion, and increased proximal tubular sodium reabsorption.\textsuperscript{7} We also showed that the lower urinary UMOD excretion associated with the G allele was present only on low-salt diets and that this association was lost when the sodium intake was high. In the human study, the observation of increased proximal tubular sodium reabsorption was observed in studies in which salt intake was not strictly controlled, so it is difficult to ascertain whether this is a consequence of increased GFR or a compensatory reaction to a primary decrease in distal reabsorption.\textsuperscript{7} Together, the previous human population and animal studies described here indicate that UMOD plays an important role in regulating BP, and further detailed human studies are merited to disentangle the relationship between salt intake, renal function, UMOD excretion, and BP. Given the salt effects noted with the KO mice, however, future human studies investigating UMOD genotype–phenotype association should be performed with strict dietary salt control.

The Umod KO mice in our study display increased levels of urinary TNF-\(\alpha\), which is further increased on salt loading. These data are consistent with a recent study showing that salt loading increased TNF-\(\alpha\) production by the kidney,\textsuperscript{23} and more specifically the TAL,\textsuperscript{22} an effect that is greatly accentuated in Umod KO mice. The elevated levels of urinary TNF-\(\alpha\) observed in KO mice suggests that cell-surface UMOD in the WT animals sequesters TNF-\(\alpha\), limiting the amount of cytokine that is excreted. In TAL cells, Na\textsuperscript{+} uptake, across the apical cell membrane, is primarily mediated by the electroneutral cotransporter NKCC2 (which is essential for salt conservation and water regulation).\textsuperscript{24} Loss-of-function point mutations in NKCC2 can lead to Bartter syndrome that is characterized by low BP, an inability to concentrate urine, and reduced NaCl reabsorption.\textsuperscript{25}
Previously, it has been suggested that UMOD facilitates the cotransport of NKCC2 to the apical membrane of TAL cells.11

Here, we show that TNF-α can reduce the levels of NKCC2A mRNA in primary mTAL cells and that this effect is enhanced in the absence of UMOD. NKCC2A is part of a signaling pathway26 that activates TNF production by the mTAL, functioning as part of a negative feedback loop to inhibit total NKCC2 activity. This suggests that UMOD plays a critical role in BP regulation by mediating the effect of TNF-α on NKCC2A, thus maintaining sodium homeostasis. Umod KO mice on high salt have a mild Bartter phenotype, with reduced BP and sodium reabsorption compared with WT mice, consistent with UMOD exerting its effect on BP via regulation of NKCC2 expression. In mTAL cells, stimulation with TNF-α was found to increase the relative levels of UMOD mRNA, creating a negative feedback loop in which the TNF-α–induced reduction in NKCC2A gene expression is switched off through increasing the production of cell-surface UMOD (which is known to bind with high affinity to TNF-α).21

Our cardiovascular characterization has shown BP differences in Umod KO mice in parallel with a leftward shift in the chronic renal function curve. This work has also shown that UMOD plays a direct role in BP regulation by modulating the effect of TNF-α on NKCC2 expression. We have provided evidence of a novel functional pathway of BP regulation by UMOD that impacts renal sodium/volume homeostasis. Our future work will focus on elucidating the fine details of the molecular mechanism by which UMOD affects sodium transport in the TAL.

**Perspectives**

Although GWAS have been successful in identifying replicated and valid signals for complex traits, the major weakness of GWAS has been that these replicated signals are not causative and in many cases not clearly associated with genes. The true value of GWAS to date has been in the identification of novel pathways, and our study exemplifies this. We have extended this GWAS discovery in humans, using a umod KO mouse model, identifying a novel pathway linking UMOD, sodium homeostasis, and HTN. Further work needs to be prioritized to elucidate the underpinning molecular mechanisms, so that this novel pathway can be translated into new drugs for HTN.

**Sources of Funding**

This work was supported by the British Heart Foundation through Chair and Programme Grant funding to A.F. Dominiczak (CH/98001 and RG/07/005), EU community’s Seventh Framework Programme (FP7/2007-2013) under grant agreement (Health-F4-2010-241504 EURATRANS) awarded to A.F. Dominiczak, a 4-year PhD studentship to M.W. McBride (FS/09/052/28032), and a special project grant to A.F. Dominiczak and S. Padmanabhan (SP/08/005/25115).

**Disclosures**

None.

**References**

What Is New?

- We show that there are differences in systolic blood pressure (BP) between an uromodulin (umod) knockout (KO) mouse and a wild-type strain.
- We identify, for the first time, the important role that UMOD plays in BP regulation by contributing to sodium homeostasis.

What Is Relevant?

- Our data show that in the absence of UMOD, there is increased Na+ loss which impacts pressure natriuresis.
- We also show that UMOD can regulate sodium uptake in the thick ascending limb of the loop of Henle by modulating the effect of tumor necrosis factor-α on NKCC2A expression.

Novelty and Significance

We have confirmed a significant genome-wide association study signal for hypertension in the region of the human UMOD promoter by performing a series of functional studies in Umod KO mice. Systolic BP was significantly lower in the KO versus wild-type mice under basal conditions. Furthermore, 2% NaCl salt loading had no effect on systolic BP in KO animals, whereas it increased in wild-type mice by ≈33%. The chronic renal function curves show a leftward shift in KO mice compared with wild-type mice, suggesting that the relationship between UMOD and BP is affected by sodium.
Validation of Uromodulin as a Candidate Gene for Human Essential Hypertension
Lesley A. Graham, Sandosh Padmanabhan, Niall J. Fraser, Satish Kumar, James M. Bates, Hajamohideen S. Raffi, Paul Welsh, Wendy Beattie, Shoujin Hao, Sabine Leh, Michael Hultstrom, Nicholas R. Ferreri, Anna F. Dominiczak, Delyth Graham and Martin W. McBride

Hypertension. 2014;63:551-558; originally published online December 9, 2013;
doi: 10.1161/HYPERTENSIONAHA.113.01423

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/63/3/551

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2013/12/09/HYPERTENSIONAHA.113.01423.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/
VALIDATION OF UROMODULIN AS A CANDIDATE GENE FOR HUMAN ESSENTIAL HYPERTENSION

Lesley A. GRAHAM 1, Sandosh PADMANABHAN1, Niall J. FRASER1, Satish KUMAR2, James M. BATES2, Hajamohideen S. RAFFI2, Paul WELSH1, Wendy BEATTIE1, Nicholas R. FERRERI3, Shoujin HAO3, Sabine LEH4, Michael HULTSTROM5, Anna F. DOMINICZAK1, Delyth GRAHAM1, and Martin W. McBRIDE1.

1 Institute of Cardiovascular & Medical Sciences, BHF Glasgow Cardiovascular Research Centre, University of Glasgow, 126 University Place, Glasgow G12 8TA, United Kingdom.
2 Department of Medicine, University Oklahoma Health Sciences Centre and Veterans Affairs Medical Center, Oklahoma City, Oklahoma 73104, USA.
3 Department of Pharmacology, New York Medical College, Valhalla, NY 10595, USA.
4 Department of Pathology, Haukeland University Hospital, Bergen, N-5021 Norway.
5 Department of Medical Cell Biology, Uppsala University, Uppsala, SE-751 05, Sweden.

Short Title: UMOD variation on blood pressure control

Word count: 5823

Corresponding author:
Dr Martin W. McBride

Institute of Cardiovascular & Medical Sciences, BHF Glasgow Cardiovascular Research Centre, University of Glasgow, 126 University Place, Glasgow G12 8TA, United Kingdom.

Tel: +44 (0) 141 330 2911

Fax: +44 (0) 141 330 1763

Email: Martin.McBride@glasgow.ac.uk
Supplementary Methods

Experimental Animals

The THP-/- (KO) and THP+/+ (WT) mice were generated by Bates et al [1]. Mouse THP cDNA isolated from 129/sv mice was transfected into developing blastocysts of C57Bl/6 mice. Chimeric mice were bred with Black Swiss mice and screened by polymerase chain reaction (PCR) to obtain mice heterozygous for THP deficiency. The heterozygous mice were bred with each other to obtain THP+/+ (WT) and THP-/- (KO) offspring. The mice were backcrossed onto the 129/sv strain for seven generations to produce THP+/+ and THP-/- offspring with a similar genetic background. The KO mice lack a 2kb segment 5' of the cap site of the UMOD gene and the first four exons plus the intervening intron. The genotypes of KO and WT mice were verified using end point PCR of tail genomic DNA with specific primers (Umod KO; forward: 5' gaagggactgtgttgtg 3' and reverse: 5' aatatcagggtagccacgg 3' and WT; forward: 5' aggctttacagggaggttg 3' and reverse: 5' gattgcactcagggaggt 3').

Hemodynamic parameters

Tail cuff plethysmography was performed on adult male mice using a Panlab Non-Invasive Blood Pressure System for rodents (Harvard Apparatus, UK). Mice were heated to 30°C, wrapped in a cloth and an inflatable cuff plus pulse transducer placed on the tail (Model LE5160M) for measurement of systolic and diastolic blood pressure. An average of 6 readings were taken for each mouse per sitting and a mean value calculated.

The Dataquest IV telemetry system (Data Sciences International, USA) was used for direct measurement of systolic pressure, diastolic pressure, pulse pressure, heart rate and locomotor activity using a PA-C10 pressure transmitter. Adult male mice were anesthetized using 2% isoflurane. The common carotid artery was isolated by a midline skin incision from chin to manubrium. A blunt trocar was passed under the skin from the neck incision to the flank region for tunnelling of the transmitter catheter. The catheter was placed into the common carotid artery and the transmitter body was placed under the skin in the flank region. The skin was sutured and carprofen analgesia administered. The mice were then allowed to recover for 7 days prior to haemodynamic assessment. Baseline blood pressure measurements were recorded continuously over a three week period using mice of 12 to 15 weeks of age. This data set represents continuous (real time) daily averaged data i.e 24 hour cycles for blood pressure measurements. Weekly averaged data points were performed in an independent group of animals at 12 to 18 weeks of age. Due to the short battery life of telemetry probes it is not possible to carry out a longitudinal continuous (real time) study for six weeks. In this study weekly averaged data points were generated from three consecutive day time and night time cycles. Data collected in the recovery week post surgical procedure was not used in any analysis. Equally, the training week for tail cuff plethysmography was not included in any analysis.

mTAL cell isolation and UMOD knockdown studies

Isolation of mTAL cells was performed as previously described by Ferreri et al [2]. Mice were anesthetised and kidneys perfused with sterile 0.9% saline solution via retrograde perfusion of the aorta. Excised kidneys were cut along the corticopapillary axis, to expose the
medulla. The inner stripe of the outer medulla was then excised and digested with 0.1% collagenase solution that was gassed with 95% oxygen. The cell suspension was sedimented on ice and mixed with Hanks Balanced Saline (HBSS) containing 2% BSA, and the crude suspension of tubules was collected. The remaining undigested tissue was collagenase treated a further three times. The supernatants were spun and resuspended in HBSS. The resuspension was passed over a 52µm nylon mesh membrane. The filtered solution was discarded and the tubules collected on the mesh were washed with HBSS and centrifuged. The supernatant was aspirated and the cells were resuspended with REGM media (Lonza, Preston, UK). The tubule suspension was grown on cell culture inserts to insure polarisation. The cells were grown to approximately 70-80% confluence prior to any experiment, with fresh media changes every 48 hours.

**Gene expression**

Primer sequences are as follows: Forward primer: 5’cacggeaatctaggggc3’ and Reverse primer: 5’ggtggtagagccagctgt3’). Primer sequences are as follows; UMOD forward 5’ggaaagcagaaaacctggtg3’ and reverse 5’gagacagggcttcatacat3’, NKCC2A forward primer 5’ggtacctctgctcggtgt3’ and reverse 5’gtttctggtgggtccacca3’.

**Enzyme Linked Immunosorbent Assay for TNF-α**

TNF-α levels were measured in urine from Umod KO and WT mice. Briefly, 96 well flat bottom plates were coated with either anti TNF-α monoclonal antibodies overnight to capture TNF present in the urine samples. Any unbound material was washed off and a horseradish peroxidise conjugated secondary antibody was added to bind to the captured TNF-α. A substrate solution was added to initiate peroxidase catalyzed colour change, which was then stopped by acidification. Absorbance readings were measured at 450 nm, from which TNF-α concentrations were determined.

**References**


Supplementary Table S1. The remaining hemodynamic parameters did not change (n=8 per group). Displayed as weekly averaged daytime and night time Mean ± SEM. Analysed with repeated measure ANOVA.

<table>
<thead>
<tr>
<th>Week</th>
<th>(B) DBP (mmHg) p=0.88 n=8 per group WT vs. KO</th>
<th>(C) MAP (mmHg) p=0.10 n=8 per group WT vs. KO</th>
<th>(E) HR (bpm) p=0.06 n=8 per group WT vs. KO</th>
<th>(F) Act p=0.25 n=8 per group WT vs. KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>114.0 ± 4.1 vs. 121.2 ± 3.0</td>
<td>121.4 ± 3.8 vs. 121.4 ± 3.6</td>
<td>474.2 ± 19.8 vs. 438.0 ± 16.3</td>
<td>2.7 ± 0.4 vs. 2.4 ± 0.1</td>
</tr>
<tr>
<td>1</td>
<td>105.4 ± 4.8 vs. 116.5 ± 2.7</td>
<td>112.3 ± 5.6 vs. 119.2 ± 3.2</td>
<td>441.1 ± 16.1 vs. 442.5 ± 9.2</td>
<td>1.7 ± 0.4 vs. 1.6 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>105.8 ± 4.0 vs. 107.6 ± 3.9</td>
<td>112.3 ± 5.0 vs. 110.6 ± 3.8</td>
<td>449.2 ± 24.1 vs. 461.9 ± 15.7</td>
<td>1.8 ± 0.4 vs. 2.1 ± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>104.0 ± 3.8 vs. 104.2 ± 6.2</td>
<td>111.2 ± 4.6 vs. 107.1 ± 6.0</td>
<td>444.5 ± 14.8 vs. 446.2 ± 15.9</td>
<td>2.1 ± 0.3 vs. 1.9 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>110.0 ± 4.4 vs. 104.8 ± 2.9</td>
<td>117.9 ± 5.5 vs. 107.5 ± 2.8</td>
<td>449.8 ± 29.0 vs. 446.2 ± 17.0</td>
<td>2.0 ± 0.3 vs. 1.5 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>111.4 ± 3.3 vs. 104.0 ± 2.7</td>
<td>120.1 ± 4.3 vs. 106.7 ± 2.5</td>
<td>9 457.3 ± 29.8 vs. 424.1 ± 15.4</td>
<td>2.8 ± 0.5 vs. 2.0 ± 0.3</td>
</tr>
<tr>
<td>6</td>
<td>104.4 ± 3.9 vs. 101.3 ± 4.2</td>
<td>113.5 ± 5.3 vs. 103.6 ± 4.0</td>
<td>428.3 ± 26.1 vs. 423.3 ± 11.2</td>
<td>2.7 ± 0.6 vs. 1.5 ± 0.3</td>
</tr>
</tbody>
</table>
Supplementary Table S2. GEE analysis of weekly fluid intake and SBP during 6 weeks of normal salt and 6 weeks of salt loading

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Umod+/+</th>
<th>Umod/-/</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal salt</td>
<td>SALT</td>
</tr>
<tr>
<td>SBP mmHg</td>
<td>133.5 (3.1)</td>
<td>153.5 (17.3)</td>
</tr>
<tr>
<td>Intake ml</td>
<td>6.3 (0.5)</td>
<td>7.3 (1.2)</td>
</tr>
</tbody>
</table>