Human Vascular Smooth Muscle Cells Express a Urate Transporter

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An elevated serum uric acid is associated with the development of hypertension and renal disease. Renal regulation of urate excretion is largely controlled by URAT1 (SLC22A12), a member of the organic anion transporter superfamily. This study reports the specific expression of URAT1 on human aortic vascular smooth muscle cells, as assessed by reverse transcription–PCR and Western blot analysis. Expression of URAT1 was localized to the cell membrane. Evidence that the URAT1 transporter was functional was provided by the finding that uptake of 14C-urate was significantly inhibited in the presence of probenecid, an organic anion transport inhibitor. It is proposed that URAT1 may provide a mechanism by which uric acid enters the human vascular smooth muscle cell, a finding that may be relevant to the role of uric acid in cardiovascular disease.


Urate is generated as a result of purine metabolism. In most species, this is an intermediate product that is degraded further by the hepatic enzyme uricase to allantoin, which then is excreted freely in the urine (1). In humans, however, urate is the final breakdown product as a result of a mutation that renders the uricase gene nonfunctional (2); as a consequence, humans have higher serum urate levels (2 mg/dl) compared with most mammals (<2 mg/dl) (1).

Hyperuricemia, usually defined as >7 mg/dl in men and >6 mg/dl in women (1), has been identified as a risk factor in the development of hypertension and renal disease (3–6). We showed previously that raising uric acid in rats via administration of an uricase inhibitor leads to a thickening of the afferent arteriole, endothelial dysfunction, activation of the renin-angiotensin system, and hypertension (7–11). Similarly, uric acid stimulates rat vascular smooth muscle cell (VSMC) proliferation in vitro with increased expression of platelet-derived growth factor (PDGF), cyclooxygenase-2, and monocyte chemoattractant protein-1 (12,13). Uric acid also stimulates human VSMC proliferation and synthesis of C-reactive protein (CRP) (14). These observations raise the question of how uric acid enters the VSMC and the transporters involved. In the kidney, uric acid is reabsorbed and secreted primarily by the organic anion transporter (OAT) superfamily, which consists of OAT1 (SLC22A6) (15), OAT2 (SLC22A7) (16), OAT3 (SLC22A8) (17), OAT4 (SLC22A9) (18), and the recently cloned URAT1 (SLC22A12) (19). The expression of these transporters has been investigated in proximal tubular epithelial cells (20) and rat VSMC (21). With regard to human VSMC, we previously reported that probenecid (an organic anion transport inhibitor) can significantly inhibit uric acid–induced proliferation and CRP expression (14). Therefore, we hypothesized that the human VSMC may express an OAT similar to that expressed in the proximal tubular cell. We demonstrate that URAT1 may be the transporter by which uric acid enters human VSMC.

Methods and Materials

Cell Culture

Human aortic VSMC were obtained from Prof. Elaine Raines (University of Washington, Seattle, WA) and cultured as described previously (22). Briefly, cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 20% FBS (Invitrogen), 25 mM HEPES (Invitrogen), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen). Cells were subcultured 1:3 on confluence. All experiments were performed on at least three independent occasions with cells between passages 4 and 8.

Uric Acid Stimulation

Cells were grown to 70% confluence, serum-starved 24 h before experimentation and challenged with varying concentrations of uric acid (3 to 12 mg/dl) for 6 h to collect RNA and 24 h to collect protein. In addition, RNA and protein were collected from nonstimulated cells at the same time points for comparison.

Reverse Transcription–PCR Amplification

RNA was isolated using Tri-Reagent (Sigma, St. Louis, MO) and extracted with isopropanol (Sigma) followed by ethanol precipitation. One microgram of RNA was used to create cDNA, according to providers’ instructions (Bio-Rad Laboratories, Hercules, CA). Two micro-

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liters of cDNA product was used in a 50-µl final volume reaction that contained 1.5 mM MgCl₂, 200 µl dNTP, iTaq buffer (200 mM Tris-HCl [pH 8.4] and 500 mM KCl), 100 nM of both sense and antisense primers, and 1.25 U of iTaq DNA polymerase (Bio-Rad Laboratories). cDNA preparations from human kidney, human liver, and human placenta poly A⁺ RNA (Clontech, San Jose, CA) were used as positive controls for the appropriate gene: human kidney for OAT1, OAT3, and URAT1 (15,17,19); human liver for OAT2 (16); and human placenta for OAT4 (18). A negative control that consisted of the PCR mixture excluding template cDNA was included. The PCR primers and conditions used are shown in Table 1. Results shown are representative agarose gels of at least three independent experiments. In addition, the identity of the PCR products produced was confirmed by forward and reverse sequence analysis (Sigma Genosys, The Woodlands, TX).

Western Blot Analysis for URAT1

Cells were lysed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris buffer [pH 8.0]), and Western blotting was performed using 15 µg of protein as described previously (23). Briefly, after electrophoresis and transfer by electroblotting, membranes were blocked in 5% nonfat milk for 1 h before incubation with rabbit anti-human URAT1 (1:500; Alpha Diagnostic Inc., San Antonio, TX) overnight at 4°C. Appropriate horseradish peroxidase antibodies (Dako, Carpinteria, CA) were then used, and bands were detected by chemiluminescence (Amersham Biosciences, Piscataway, NJ). Blots were stripped and reprobed with human glyceraldehyde-3-phosphate dehydrogenase (1:300; Chemicon International, Temecula, CA), to assess equal loading. The result of the Western blot shown is representative of at least three independent experiments.

Total Membrane Isolation

Human VSMC that were grown to 70% confluence were washed three times with ice-cold Krebs Ringer Buffer (128 mM NaCl, 4.7 mM KCl, 1.25 mM MgSO₄·7H₂O, 1.25 mM CaCl₂·2H₂O, and 5 mM phosphate salts), collected in Buffer A (20 mM Tris-HCl, 1 mM EDTA, and 255 mM sucrose [pH 7.4]) that contained protease inhibitors, homogenized on ice, and centrifuged at 55,000 rpm for 70 min at 4°C. The cell pellet was resuspended in 200 µl of Buffer A, and the protein concentration was determined with a Bio-Rad protein assay (Bio-Rad Laboratories). To confirm membrane localization of URAT1, we performed indirect immunofluorescence staining. VSMC were fixed in 3% paraformaldehyde, quenched in 50 mM ammonium chloride and treated with 0.1% Triton X-100 for 10 min. Cells then were incubated overnight at 4°C using an anti-human URAT1 N-terminal polyclonal antibody followed by a donkey anti-rabbit antibody conjugated to Texas Red (Jackson ImmunoResearch, West Grove, PA) for 45 min at room temperature. Nuclei were counterstained by 4′,6-diamidino-2-phenylindole. As a negative control, staining was performed with omission of the primary antibody.

Urate Uptake by VSMC

VSMC (1 × 10⁵) were incubated with 50 µM ¹⁴C-urate (American Radiolabeled Chemicals, St. Louis, MO) in Hanks medium (Invitrogen) supplemented with 1 mM L-glutamine (Invitrogen) and 100 µM sodium pyruvate (Invitrogen) for 0, 5, 15, 30, and 60 min at 37°C in a 5% CO² incubator. To stop the reaction, we removed the incubation medium and washed the cells three times with ice-cold Hanks medium. The cells were lysed with 0.1 N sodium hydroxide (Sigma) for 20 min, collected into 4-ml scintillation fluid (Fisher Scientific, Pittsburgh, PA) and measured in a β counter (Beckman Coulter Inc., Fullerton, CA). For determination of the specificity of urate uptake, the OAT inhibitor probenecid (1 mM; Sigma) was added to the reaction for the same time course, and samples were collected and measured as described above. All uptake experiments were performed on three separate occasions, and an average value was taken. Data were assessed using a one-way ANOVA with Bonferroni analysis.

Results

First, we examined the mRNA expression of OAT1, OAT2, OAT3, OAT4, and URAT1 in nonstimulated or uric acid–stimulated human aortic VSMC (Figure 1). No detectable expression of OAT1 (573 bp), OAT2 (530 bp), OAT3 (902 bp), or OAT4 (434 bp) mRNA was demonstrated in nonstimulated or uric acid–stimulated VSMC. Nevertheless, expression of OAT1 and OAT3 was present in human kidney, whereas OAT2 and OAT4 were expressed in human liver and placenta, respectively, consistent with their known sites of expression (15–19) (Figure 1).

A band consistent with URAT1 mRNA was observed in both nonstimulated and uric acid–stimulated human VSMC and also in human kidney (365 bp; Figure 1). Forward and reverse

Table 1. Primer sequences for the human organic anion transporters

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Corresponding Nucleotides</th>
<th>Annealing Temperature</th>
<th>Number of Cycles</th>
<th>Amplicon Size</th>
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<tbody>
<tr>
<td>hOAT1</td>
<td>Forward: CCA CCT CTG CCT CTC</td>
<td>1266 to 1289</td>
<td>60°C</td>
<td>25</td>
<td>573 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTC TGT TTC CCT CTC</td>
<td>1838 to 1815</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hOAT2</td>
<td>Forward: CTA TCC CCA GGC TCT</td>
<td>252 to 275</td>
<td>62°C</td>
<td>25</td>
<td>530 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAA GCC ATC GCC AGT</td>
<td>781 to 758</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hOAT3</td>
<td>Forward: GCT CTT CCT ATC TCT</td>
<td>740 to 761</td>
<td>60°C</td>
<td>20</td>
<td>902 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTG GCT CTT TCT TGT</td>
<td>1642 to 1619</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hOAT4</td>
<td>Forward: TGC CTT CCT GAT TTT</td>
<td>1550 to 1571</td>
<td>60°C</td>
<td>30</td>
<td>434 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTC TGG GTC TGG ATT</td>
<td>1983 to 1962</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hURAT1</td>
<td>Forward: TTG ATT GCC AGG AGG</td>
<td>2355 to 2374</td>
<td>60°C</td>
<td>35</td>
<td>365 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGT TAA GTG GAG TCG</td>
<td>2719 to 2700</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
sequencing showed that the PCR products had >99.0% homology with the expected human URAT1 gene sequence (data not shown).

URAT1 protein also was detected in the human VSMC by Western blotting (Figure 2A, top). A band of 40 kD was detected in the nonstimulated and uric acid–stimulated cells. Equality of loading was confirmed by comparative glyceraldehyde-3-phosphate dehydrogenase expression (Figure 2A, bottom).

URAT1 is expressed on the apical membrane of epithelial cells of the human proximal tubule (19). We therefore examined whether URAT1 was expressed on the membrane of human VSMC by Western blot analysis and immunocytochemistry. As can be seen from the representative blot (Figure 2B), URAT1 was expressed on the nonstimulated and uric acid–stimulated (6 h) human aortic VSMC, as assessed by RT-PCR. The results shown are representative of at least three independent experiments. The specificity of the URAT1 observations was also confirmed by both forward and reverse sequencing.

Figure 1. Reverse transcription–PCR (RT-PCR) analysis of URAT1 in nonstimulated and uric acid–stimulated human aortic vascular smooth muscle cells (VSMC). M, 100-bp DNA ladder; +ive, positive control; −ive, without cDNA; 0, VSMC cDNA; 3, VSMC cDNA + 3 mg/dl UA; 6, VSMC cDNA + 6 mg/dl UA; 9, VSMC cDNA + 9 mg/dl UA; 12, VSMC cDNA + 12 mg/dl UA. The organic anion transporter (OAT) URAT1 was expressed in the nonstimulated and uric acid–stimulated (6 h) human aortic VSMC, as assessed by RT-PCR. The results shown are representative of at least three independent experiments. The specificity of the URAT1 observations was also confirmed by both forward and reverse sequencing.

Figure 2. Western blot analysis of URAT1 in human VSMC. (A) Total protein in nonstimulated and uric acid–stimulated VSMC. 0, VSMC cDNA; 3, VSMC cDNA + 3 mg/dl UA; 6, VSMC cDNA + 6 mg/dl UA; 9, VSMC cDNA + 9 mg/dl UA; 12, VSMC cDNA + 12 mg/dl UA. The OAT URAT1 (40 kD) was expressed at similar levels in the nonstimulated and uric acid–stimulated (24 h) human aortic VSMC. Equality of loading was confirmed by comparative glyceraldehyde-3-phosphate dehydrogenase expression. (B) Membrane preparations of human VSMC. The OAT URAT1 was expressed on the membrane of the human aortic VSMC. In both panels, the results shown are representative of at least three independent experiments.

Figure 3. Immunolocalization of URAT1 on human VSMC. Human VSMC were stained overnight with anti-human URAT1 N-terminus affinity-purified antibody followed by donkey anti-rabbit antibody conjugated to Texas Red. (A) URAT1 expression was localized to the cell membrane. (B) Negative control consisted of omission of the primary antibody. For visualization of cell nuclei, cells were stained with 4′,6-diamidino-2-phenylindole.

Discussion

We examined the expression of various OAT in human VSMC. mRNA expression of OAT1, OAT2, OAT3, and OAT4 was not detected in nonstimulated or uric acid–stimulated human aortic VSMC. In contrast, URAT1 mRNA and protein were expressed by both nonstimulated and uric acid–stimulated human VSMC. Consistent with URAT1 being a transporter, we demonstrated the presence of URAT1 on the membrane of these cells. We further showed the presence of a functional OAT in human aortic VSMC, because the cells actively took up urate over a 60-min time course and the uptake was reduced by probenecid. These studies are consistent with previous studies in Xenopus oocytes that express URAT1 (19), in which uptake of urate also was significantly inhibited by probenecid.
counts per minute (on three separate occasions, and the data are displayed as mean and 60 min with probenecid). The experiments were performed that drugs such as probenecid also may have direct effects on tion that URAT1 also is expressed on human VSMC suggests inhibit urate reabsorption in the proximal tubule. The observa-

largely has been assumed that these agents are acting solely to
commonly used to treat hyperuricemia in patients with gout. It
relating hyperuricemia to mutations in URAT1.

leading to necrosis. Currently, there are no published studies
urate and accumulation of urate crystals in kidney tubules,
a functional URAT1 transporter results in lower levels of blood
species that occurs in muscle during exercise (25,26). The lack of
generated by the increased production of urate and reactive oxygen
is characterized by exercise-induced acute renal failure, trig-

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zbromarone may be more specific for URAT1 (29). Indeed, to
uptake completely. In addition, other inhibitors such as ben-
probenecid are suggestive of a functional uric acid transporter
in human VSMC. However, it should be noted that the concen-
tration of probenecid used may be too low to block urate
uptake completely. In addition, other inhibitors such as benz-
romarone may be more specific for URAT1 (29). Indeed, to
prove definitively that URAT1 is a functional transporter, ex-
periments with antisense constructs or small interfering RNA
need to be performed.

Figure 4. Urate uptake by human VSMC. The function of the
URAT1 transporter was assessed by incubating the cells (1 × 10^6) with 14Curate over a 60 min time course. At timed inter-
vals, the cells were lysed and the level of urate uptake was
measured. Probenecid (1 mM), an inhibitor of OAT, was used
to determine specificity of uptake by URAT1 (P < 0.05 for 30
and 60 min with probenecid). The experiments were performed
on three separate occasions, and the data are displayed as mean
counts per minute (± SD).

URAT1 is a recently cloned member of the OAT superfamily
and consists of 555 amino acid residues with 12 predicted
putative transmembrane domains with both intracellular
amino and carboxyl termini (19). Previous studies have dem-
strated that URAT1 is expressed prominently on the apical
membrane of the proximal tubules but not that of distal tubules
in the renal cortex (19). URAT1 transports urate across the
apical membrane of proximal tubular cells, with various or-
ganic anions being transported in exchange into the tubular
lumen to maintain electrical balance (19). Urate then moves
across the basolateral membrane into the capillaries via another
OAT, most likely OAT1 and OAT3 (24). Here we demonstrate
the first evidence of a more generalized expression of URAT1 in
human VSMC. This novel finding may provide insights into the
mechanisms by which uric acid may influence vascular re-
sponses in normal and disease states.

The clinical importance of URAT1 is demonstrated by recent
studies showing that mutations in the human gene cause idio-
pathic renal hypouricemia (25,26). This rare disorder occurs
with a prevalence of 0.12% in most populations, with a higher
frequency in Japanese (27,28) and Iraqi-Jews (24). The disorder
is characterized by exercise-induced acute renal failure, trig-
gered by the increased production of urate and reactive oxygen
species that occurs in muscle during exercise (25,26). The lack of
a functional URAT1 transporter results in lower levels of blood
urate and accumulation of urate crystals in kidney tubules,
leading to necrosis. Currently, there are no published studies
relating hyperuricemia to mutations in URAT1.

Uricosuric agents such as probenecid and benzbioradone are
commonly used to treat hyperuricemia in patients with gout. It
largely has been assumed that these agents are acting solely to
inhibit urate reabsorption in the proximal tubule. The observa-
tion that URAT1 also is expressed on human VSMC suggests
that drugs such as probenecid also may have direct effects on
vascular cells. Further studies are planned to determine the role
of VSMC expression of URAT1 in normal individuals and
patients with cardiovascular disease.

There are several caveats that need to be considered when
interpreting the results of this study. First, although we per-
formed forward and reverse sequencing on the PCR products
that were obtained from nonstimulated and uric acid–stimu-
lated cells, it would have been optimal to clone and sequence
the entire cDNA of URAT1. Second, it would be interesting to
explore whether uric acid stimulation alters the expression of
URAT1. Although our data suggest that uric acid does not
change URAT1 expression, the methods used are nonquantita-
tive. Therefore, further studies need to be performed using

techniques such as real-time PCR or Northern analysis. Finally,
the data obtained with radiolabeled urate and the addition of
probenecid are suggestive of a functional uric acid transporter
in human VSMC. However, it should be noted that the concen-
tration of probenecid used may be too low to block urate
uptake completely. In addition, other inhibitors such as ben-
romarone may be more specific for URAT1 (29). Indeed, to
prove definitively that URAT1 is a functional transporter, ex-
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