Molecular Mechanisms Underlying Urate-Induced Enhancement of Kv1.5 Channel Expression in HL-1 Atrial Myocytes

Nani Maharani, MD; Ya Kuang Ting, MD, PhD; Jidong Cheng, MD, PhD; Akira Hasegawa, PhD; Yasutaka Kurata, MD, PhD; Peili Li, MD, PhD; Yuji Nakayama, PhD; Haruaki Ninomiya, MD, PhD; Nobuhiro Ikeda, PhD; Kumi Morikawa, PhD; Kazuhiro Yamamoto, MD, PhD; Naomasa Makita, MD, PhD; Takeshi Yamashita, MD, PhD; Yasuaki Shirayoshi, PhD; Ichiro Hisatome, MD, PhD

Background: Hyperuricemia induces endothelial dysfunction, oxidative stress and inflammation, increasing cardiovascular morbidities. It also raises the incidence of atrial fibrillation; however, underlying mechanisms are unknown.

Methods and Results: The effects of urate on expression of Kv1.5 in cultured mouse atrial myocytes (HL-1 cells) using reverse transcriptase-PCR, immunoblots, flow cytometry and patch-clamp experiments were studied. Treatment with urate at 7 mg/dl for 24 h increased the Kv1.5 protein level, enhanced ultra-rapid delayed-rectifier K+ channel currents and shortened action potential duration in HL-1 cells. HL-1 cells expressed the influx uric acid transporter (URAT), URATv1, and the efflux UATs, ABCG2 and MRP4. An inhibitor against URATv1, benzbromarone, abolished the urate effects, whereas an inhibitor against ABCG2, KO143, augmented them. Flow cytometry showed that urate induced an increase in reactive oxygen species, which was abolished by the antioxidant, N-acetylcysteine (NAC), and the NADPH-oxidase inhibitor, apocynin. Both NAC and apocynin abolished the enhancing effects of urate on Kv1.5 expression. A urate-induced increase in the Kv1.5 proteins was accompanied by phosphorylation of extracellular signal-regulated kinase (ERK), and was abolished by an ERK inhibitor, PD98059. NAC abolished phosphorylation of ERK by urate.

Conclusions: Intracellular urate taken up by UATs enhanced Kv1.5 protein expression and function in HL-1 atrial myocytes, which could be attributable to ERK phosphorylation and oxidative stress derived from nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase. (Circ J 2015; 79: 2659–2668)

Key Words: Atrial fibrillation; ERK; Kv1.5; Oxidative stress; Urate

Compared to other mammals whose serum urate levels are 0.5–1 mg/dl or less, humans have a higher serum urate level due to loss of uricase activity.1 Hyperuricemia, defined as a condition with the serum urate level exceeding 6.8 mg/dl, is a risk factor for gout, kidney stone and renal failure.2,3 Recently, the guideline in Japan has indicated that hyperuricemia is defined as the serum urate level of more than 7 mg/dl.4 It is caused by an imbalance between urate synthesis and its renal excretion; the predominant cause is reduced excretion due to altered expression of uric acid transporters (UATs) in the kidney. UATs in renal proximal tubular cells play a pivotal role in the regulation of serum urate levels.5,6

Hyperuricemia has been reported to be associated with various kinds of diseases such as hypertension, metabolic syn-
dromes, diabetes mellitus, as well as chronic kidney disease. It increases oxidative stress, endothelial dysfunction and inflammation, thereby increasing cardiovascular morbidity and mortality. Two possible mechanisms have been proposed for hyperuricemia-related cardiovascular dysfunction: (1) xanthine oxidase causes oxidative stress; and (2) urate induces vascular smooth muscle cell proliferation, decreases nitric oxide production, and activates the renin-angiotensin system.

Nitric oxide synthase and NADPH oxidase cause oxidative stress; and (2) urate induces vascular hyperuricemia-related cardiovascular dysfunction: (1) xanthine oxidase causes oxidative stress; and (2) urate induces vascular smooth muscle cell proliferation, decreases nitric oxide production, and activates the renin-angiotensin system.

AF is triggered by ectopic activity in the atrium or pulmonary vein and is sustained by reentry that is characterized by shortening of the effective refractory period (ERP), unidirectional conduction block and slow conduction. Electrical properties of atrial myocytes in AF depend on electrical remodeling, namely, altered expressions of atrial ion channels. Shortening of the atrial action potential duration (APD) shortens ERP, which plays an important role in the initiation and sustainment of AF. Both rate and rhythm control are essential in AF management, however, their application must be considered wisely due to serious adverse effects and limited long-term efficacy.

Kv1.5 channels (voltage-gated potassium channel, shaker-related subfamily, member 5), encoded by the KCNA5 gene, confer the ultra-rapid delayed-rectifier potassium channel currents (I_Kr) that strongly influence APD. It is more abundantly expressed in the human atrium than in the ventricle. The increases in mRNA and protein levels of Kv1.5 channels contribute to the shortening of atrial APD, causing electrical remodeling with atrial APD shortening in AF; therefore, inhibition of Kv1.5 channel expression may be one of the therapeutic approaches for AF.

Kv1.5 channels, as reported in several publications.

Taken together, we hypothesized that urate could enhance the expression of Kv1.5 in atrial myocytes. To verify this hypothesis, we studied the effects of urate on the expression and function of Kv1.5 channels using HL-1 cells, and explored subcellular mechanisms underlying the hyperuricemia-induced increase in AF incidence and alteration of Kv1.5 channels to facilitate AF occurrence.

**Methods**

**Cell Culture**

HL-1 cells were cultured in Claycomb medium supplemented with 10% Fetal bovine serum, 1% nonpeneprin, 1% penicillin-streptomycin, and 1% L-glutamin, on dishes coated with 0.02% gelatin-fibronectin in an incubator at 37°C with 5% CO₂, before being subjected to the assays.

**Reagents**

Urate (uric acid sodium salt) (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 10mM/L NaOH. It was administered to cells at 60–70% confluence at the final concentration of 7 mg/dL. We also used the influx UAT (URAT1) inhibitor, benzembrarone, and efflux UAT (ABCG2) inhibitor, KO143 (Sigma Aldrich), antioxidant N-acetylcysteine (NAC) (Sigma Aldrich), NADPH-oxidase inhibitor, apocynin (Abcam Biochemicals), and an extracellular signal-regulated kinase (ERK) inhibitor, PD98059 (Sigma Aldrich).

**mRNA Extraction and Reverse Transcriptase-PCR**

Total RNA was extracted from HL-1 cells and mouse kidney using a RNeasy kit (Qiagen). cDNA was synthesized using PrimeScript with gDNA eraser (Takara Bio Inc, Kusatsu, Japan). PCR was performed using primers designed from the mouse uric acid transporter sequences listed in Table 1. PCR products were sequenced to validate the primer design.

**Table 1. Sequences of Mouse Uric Acid Transporters’ cDNA Primers**

<table>
<thead>
<tr>
<th>No.</th>
<th>Transcript</th>
<th>Reference sequence</th>
<th>Product (bp)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>URAT1</td>
<td>NM_009203.3</td>
<td>431</td>
<td>CAGTGTATGTCCTGCTGG</td>
<td>AGGTGGGCCCAGCTGATGAG</td>
</tr>
<tr>
<td>2</td>
<td>URATv1</td>
<td>NM_001102414.1</td>
<td>576</td>
<td>GAGGGAGGACAAAGAATGTTCC</td>
<td>ATCACTCGAAGCAGCTGATGAG</td>
</tr>
<tr>
<td>3</td>
<td>ABCG2</td>
<td>NM_011920.3</td>
<td>496</td>
<td>CCTCAACACCTGCTGGAT</td>
<td>ATGACGACGCTCCACAGC</td>
</tr>
<tr>
<td>4</td>
<td>NPT1</td>
<td>NM_009198.3</td>
<td>665</td>
<td>GATGTCTTTGCTCTCCACAC</td>
<td>TGGTGAAGAGGTTCCAGGACG</td>
</tr>
<tr>
<td>5</td>
<td>NPT4</td>
<td>NM_001164743.1</td>
<td>527</td>
<td>TCACACTGTAGGCGAGAAT</td>
<td>ACTAATGATGGCGGGCAGAAT</td>
</tr>
<tr>
<td>6</td>
<td>OAT1</td>
<td>NM_008766.3</td>
<td>331</td>
<td>ACCTTGTGCTCTCTCGAG</td>
<td>AACTGCGCCAAAGCTGAGAC</td>
</tr>
<tr>
<td>7</td>
<td>OAT3</td>
<td>NM_031194.5</td>
<td>308</td>
<td>CTTCGGATTCTGTTGGCT</td>
<td>TAGGCAAGCAGGGAGGAAG</td>
</tr>
<tr>
<td>8</td>
<td>MRP4</td>
<td>NM_001163676.1</td>
<td>426</td>
<td>CGTTAATGACGCTCGGTT</td>
<td>GGTTAGAGGTGCGCCAGAATC</td>
</tr>
<tr>
<td>9</td>
<td>MCT9</td>
<td>NM_025807.3</td>
<td>330</td>
<td>CTTCCTCAAAGGCTCGCCA</td>
<td>CCCCAAGAAAGTCTGGCCAC</td>
</tr>
</tbody>
</table>

**ABCG2, ATP-binding cassettes subfamily G second member 2 (also known as the human breast cancer resistance protein [BCRP]); bp, base pairs; MCT, monocarboxylate transporter; MRP, multidrug resistance protein; NPT, Na+/phosphate cotransporter; OAT, organic anion transporter; URAT, urate transporter.**
After treatment with urate, Kv1.5 channel currents corresponding to IKur were measured. Outward membrane currents were elicited every 6 s by 300-ms test pulses ranging from –60 to +80 mV (in 10 mV increments) with a holding potential (HP) of –60 mV. A specific inhibitor of Kv1.5 channel currents, 4-aminopyridine (4-AP), at a dose of 100 µmol/L was used to isolate IKur. To eliminate the contamination by another outward current, rapidly activating delayed rectifier potassium channel current (IKr), the outward currents were recorded in the presence of 5 µmol/L E4031, which blocked IKr almost completely. Thus, the currents measured here were 4-AP-sensitive, E4031-insensitive currents. The extracellular solution contained (in mmol/L) 140 NaCl, 4 KCl, 1.8 CaCl2, 0.53 MgCl2, 0.33 NaH2PO4, 5.5 glucose, and 5 HEPES, with the pH adjusted to 7.4 by NaOH. The internal pipette solution contained (in mmol/L) 100 K-aspartate, 20 KCl, 1 CaCl2, 5 Mg-ATP, 5 EGTA, 5 HEPES, and 5 creatine phosphate dipotassium (pH 7.2 with KOH). Patch pipettes had a resistance of 5–10 MΩ when filled with the pipette solution. Series resistance (Rs) was determined by fitting a single exponential function to the capacitive current decay to estimate its time constant and membrane capacitance. After the Rs compensation of 50–60%, the voltage errors arising from Rs were estimated to be less than 5 mV. The membrane potential was not corrected for the liquid junction potential, which was estimated to be <10 mV.

**Western Blot Analysis**

Cells were collected in lysis buffer containing 0.01 M phosphate buffered saline (PBS), 1% nonidet P-40 (w/w), 0.5% sodium deoxycholate (w/v), 0.1% sodium dodecyl sulphate (w/v), 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 1 mmol/L phenylmethylsulfonyl fluoride, and were lysed by repeat pipetting. Insoluble materials were removed by centrifugation, and the protein concentration of the supernatant was determined by using the Bradford Protein Assay method. An aliquot of 10–15 µg protein was subjected to sodium dodecylsulfate poly-acrylamide gel electrophoresis and electrotransferred to a polyvinylidene fluoride membrane. After being blocked with 5% skim milk, membranes were probed with primary antibodies against Kv1.5 (1:400; Alomone Labs, Jerusalem, Israel), α-tubulin (1:5,000; Abcam, Tokyo, Japan), phosphorylated ERK (1:500; Cell-signaling Technology) or total ERK (1:1,000; Cell-signaling Technology). The secondary antibodies were either anti-rabbit IgG or anti-mouse IgG (1:3,000); both are horseradish-peroxidase-linked (GE-Healthcare Limited, Buckinghamshire, UK). The blots were developed using the enhanced chemiluminescence (ECL) system (Amersham Bioscience, Piscataway, NJ, USA). The band intensities were quantified using Image J version 1.42q software (NIH, Bethesda, MD, USA).

**Electrophysiological Recordings**

After treatment with urate, Kv1.5 channel currents corresponding to IKur were measured. Outward membrane currents were elicited every 6 s by 300-ms test pulses ranging from –60 to +80 mV (in 10 mV increments) with a holding potential (HP) of –60 mV. A specific inhibitor of Kv1.5 channel currents, 4-aminopyridine (4-AP), at a dose of 100 µmol/L was used to isolate IKur. To eliminate the contamination by another outward current, rapidly activating delayed rectifier potassium channel current (IKr), the outward currents were recorded in the presence of 5 µmol/L E4031, which blocked IKr almost completely. Thus, the currents measured here were 4-AP-sensitive, E4031-insensitive currents. The extracellular solution contained (in mmol/L) 140 NaCl, 4 KCl, 1.8 CaCl2, 0.53 MgCl2, 0.33 NaH2PO4, 5.5 glucose, and 5 HEPES, with the pH adjusted to 7.4 by NaOH. The internal pipette solution contained (in mmol/L) 100 K-aspartate, 20 KCl, 1 CaCl2, 5 Mg-ATP, 5 EGTA, 5 HEPES, and 5 creatine phosphate dipotassium (pH 7.2 with KOH). Patch pipettes had a resistance of 5–10 MΩ when filled with the pipette solution. Series resistance (Rs) was determined by fitting a single exponential function to the capacitive current decay to estimate its time constant and membrane capacitance. After the Rs compensation of 50–60%, the voltage errors arising from Rs were estimated to be less than 5 mV. The membrane potential was not corrected for the liquid junction potential, which was estimated to be <10 mV.

**Assay of Reactive Oxygen Species (ROS) Level**

HL-1 cells were treated with urate (7 mg/dl) alone, urate (7 mg/dl) with either NAC (10 mmol/L) or apocynin (1 mmol/L), or the solvent NaOH (10 mmol/L) as a control, washed with PBS, and incubated in the presence of 20 µmol/L 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) (Sigma Aldrich) for 30 min. Cells were then washed with PBS, trypsinized, resuspended in fluorescence-activated cell sorting (FACS) buffer, and analyzed by BD FACSARia™ flowcytometry (BD Bioscience, San Jose, CA, USA).
control, 36.7±3.0 pF with urate). Summary data of the voltage-dependent activation of $I_{Kur}$ in HL-1 cells in the absence and presence of urate indicate that urate significantly augmented $I_{Kur}$ in a potential range from –30 to +60 mV without affecting the threshold potential (Figure 2B). Urate did not affect the L-type calcium channel currents or protein level, as shown in Figures S1 and S2.

Figure 3 shows the representative action potentials recorded from HL-1 cells treated with (black and blue) or without urate (red and green), and averaged APDs at 50% repolarization (APD$_{50}$) and 90% repolarization (APD$_{90}$). Urate significantly shortened APD$_{50}$ (32.83±6.51 ms in control, 17.74±5.85 ms with urate) and APD$_{90}$ (113.18±18.51 ms in control, 53.28±23.29 ms with urate) without changes in resting membrane potentials (–69.38±2.85 mV in control; –69.60±1.77 mV with urate). The details on action potential parameters are presented in Table 2. To ensure that the changes in APDs were specifically caused by the increase of $I_{Kur}$, we also compared APDs in the control and urate groups in the absence and presence of 4-AP. The results showed that after addition of 4-AP, the APDs in urate-treated cells were significantly prolonged, but shorter than those in the control group.

Inhibition of UATs Influenced a Urate-Induced Increase in Kv1.5 Channel Proteins in HL-1 Cells

We evaluated the mRNA expression of 9 UATs in HL-1 cells used. Analysis of variance (ANOVA) followed by Fisher’s Least Significant Difference (LSD) post-hoc test was used to assess the difference between multiple groups. A P value of <0.05 was considered significant. All of the statistical analyses were performed by using OriginPro 9.1.0 (OriginLab Corporation, Northampton, MA, USA).

Results

Urate Increased Kv1.5 Protein Levels and Enhanced $I_{Kur}$

Figure 1 shows the representative Western blot of Kv1.5 proteins expressed by HL-1 cells treated for 24 h with or without urate (7 mg/dl). The summarized data on the density of Kv1.5 proteins normalized to α-tubulin density confirmed that urate significantly increased the protein level of Kv1.5. To examine whether urate increases the activity of Kv1.5 channels on the cell surface, we measured $I_{Kur}$ through Kv1.5 channels.

Figure 2A shows original current traces of 4-AP-sensitive $I_{Kur}$ in HL-1 cells treated with or without urate (7 mg/dl). Depolarizing test pulses elicited outward currents, which were almost completely blocked by 4-AP at 100 µmol/L. As HL-1 cells were known to express another component of outward currents, $I_{Kr}$, we eliminated $I_{Kr}$ contamination using the $I_{Kr}$ inhibitor, E4031. Treatment with urate for 24 h increased the amplitude of $I_{Kur}$ elicited by the depolarizing pulses from a HP of –60 mV. Urate did not influence the capacitive currents (35±3.2 pF in control, 36.7±3.0 pF with urate). Summary data of the voltage-dependent activation of $I_{Kur}$ in HL-1 cells in the absence and presence of urate indicate that urate significantly augmented $I_{Kur}$ in a potential range from –30 to +60 mV without affecting the threshold potential (Figure 2B). Urate did not affect the L-type calcium channel currents or protein level, as shown in Figures S1 and S2.

Figure 3 shows the representative action potentials recorded from HL-1 cells treated with (black and blue) or without urate (red and green), and averaged APDs at 50% repolarization (APD$_{50}$) and 90% repolarization (APD$_{90}$). Urate significantly shortened APD$_{50}$ (32.83±6.51 ms in control, 17.74±5.85 ms with urate) and APD$_{90}$ (113.18±18.51 ms in control, 53.28±23.29 ms with urate) without changes in resting membrane potentials (–69.38±2.85 mV in control; –69.60±1.77 mV with urate). The details on action potential parameters are presented in Table 2. To ensure that the changes in APDs were specifically caused by the increase of $I_{Kur}$, we also compared APDs in the control and urate groups in the absence and presence of 4-AP. The results showed that after addition of 4-AP, the APDs in urate-treated cells were significantly prolonged, but shorter than those in the control group.

Inhibition of UATs Influenced a Urate-Induced Increase in Kv1.5 Channel Proteins in HL-1 Cells

We evaluated the mRNA expression of 9 UATs in HL-1 cells
Urate-Induced Enhancement of the Kv1.5 Channel

Figure 3. Effects of urate on action potentials of HL-1 cells. (A) Action potentials recorded from HL-1 cells treated with (blue and pink) or without (black and red) urate at 7 mg/dl before and during application of 4-aminopyridine (4-AP). (B) Action potential durations at 50% repolarization (APD50) and 90% repolarization (APD90) measured for the control and urate-treated cells. Both APD50 and APD90 values were significantly shorter in the urate-treated cells than in control cells (n=10; *P<0.05).

Table 2. Action Potential Parameters

<table>
<thead>
<tr>
<th>No.</th>
<th>Treatment group</th>
<th>Resting membrane potential (mV)</th>
<th>AP amplitude (mV)</th>
<th>Overshoot (mV)</th>
<th>APD50 (ms)</th>
<th>APD90 (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>−69.38±2.85</td>
<td>90.73±6.69</td>
<td>21.35±7.03</td>
<td>32.83±6.51</td>
<td>113.18±18.51</td>
</tr>
<tr>
<td>2</td>
<td>Urate</td>
<td>−69.60±1.77</td>
<td>87.45±5.21</td>
<td>17.85±6.50</td>
<td>17.74±5.85</td>
<td>53.28±23.39</td>
</tr>
</tbody>
</table>

APD50, action potential durations at 50% repolarization; APD90, action potential durations at 90% repolarization.

using RT-PCR (Figure 4A), which detected the expressions of an influx transporter (URATv1) and two efflux transporters (ABCG2 and MRP4). All of these expressed UATs were also expressed in human embryonic stem cell-derived cardiomyocytes (Figure S3).

To test how the expressed UATs could be involved in the urate-induced enhancement of Kv1.5 protein expression, we examined the effects of UATs inhibitors on the Kv1.5 protein expression in HL-1 cells. Figure 4B shows the representative Western blots of expressed Kv1.5 proteins after a 24-h treatment with or without urate. Pretreatment with benz bromarone (20µmol/L), an inhibitor of the influx transporter, URATv1, abolished the urate-induced increases in Kv1.5 protein expression. Figure 4C shows the representative Western blots of Kv1.5 proteins after a 24-h treatment with or without urate in the absence and presence of KO143 (100nmol/L), an inhibitor of the efflux urate transporter, ABCG2. KO143 (100nmol/L) enhanced the urate-induced increase in Kv1.5 protein expression. Either benz bromarone or KO143 alone did not affect the Kv1.5 expression (data not shown).
Oxidative Stress Is Involved in Urate-Induced Upregulation of Kv1.5 Channel Proteins

We also examined whether oxidative stress was involved in the urate-induced enhancement of Kv1.5 expression. Treatment with urate increased ROS by approximately 30%, and this effect was cancelled by simultaneous treatment with an antioxidant, NAC (Figure 5A Left). Figure 5B (Left) shows the representative Western blots of Kv1.5 proteins after 24-h treatment with or without urate in the absence and presence of NAC. Treatment with NAC (10 mmol/L) for 1 h prior to urate exposure abolished the enhancing effect of urate on Kv1.5 protein expression, while NAC alone did not affect the Kv1.5 expression (data not shown).

As ROS generation induced by urate has been reported to involve nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, we tested the effects of a NADPH-oxidase
shown). Each of the flow cytometry experiments was confirmed by another experiment.

**ERK Pathway Is Involved in Urate-Induced Kv1.5 Upregulation**

We further examined an involvement of the ERK pathway, the downstream signaling pathway activated by oxidative stress, in the urate-induced enhancement of Kv1.5 expression. Figure 6A shows the representative Western blots of Kv1.5 proteins after 24-h treatment with or without urate. Treatment with apocynin on urate-treated cells abolished the urate-induced enhancement of Kv1.5 expression. Apocynin alone did not affect Kv1.5 expression (data not shown).
Figure 6. Involvement of the extracellular signal-regulated kinase (ERK) pathway in the urate-induced upregulation of Kv1.5. (A) Effect of the ERK-inhibitor, PD98059, on Kv1.5 expression. HL-1 cells were treated with NaOH at 10 mmol/L (Control), urate (7 mg/dl) alone, or urate (7 mg/dl) + PD98059 (20 µmol/L) for 24 h. Protein lysates were taken, and subjected to Western blot analyses (Right). The averaged density of Kv1.5 proteins normalized to that of α-tubulin (Left) showed a significant reduction of Kv1.5 expression in the PD98059-treated group (n=3; *P<0.05). (B) Effect of PD98059 on phosphorylated-ERK. HL-1 cells were treated as per the Kv1.5 expression analysis described above. Western blots using an antibody against phosphorylated ERK (pERK) revealed an increase of pERK level in the urate-treated cells (right). The averaged density of pERK as a percentage to the total ERK showed significant inhibition of the urate-induced pERK increase by PD98059 (Left; n=3; *P<0.05). (C) Effect of N-acetylcysteine (NAC) on phosphorylated-ERK. HL-1 cells were treated with NaOH at 10 mmol/L (Control), urate (7 mg/dl) alone, or urate (7 mg/dl) + NAC (20 mmol/L) for 24 h. Western blots using anti-pERK antibody revealed an increase of pERK level in the urate-treated cells (Right). The averaged density of pERK as a percentage to the total ERK showed significant inhibition of the urate-induced pERK-increase by NAC (Left; n=3; *P<0.05).
24-h treatment with or without urate in combination with an ERK inhibitor, PD98059. PD98059 (20 µmol/L) abolished the urate effect. Urate increased phosphorylated ERK (pERK) in HL-1 cells (Figure 6B), and PD98059 (20 µmol/L) abolished this enhancement. We finally examined effects of NAC on the level of pERK in urate-treated cells (Figure 6C). NAC abolished the increase of pERK caused by urate, indicating that the urate-induced enhancement of ERK phosphorylation is due to oxidative stress.

**Discussion**

In the present study, we investigated whether urate, a soluble form of uric acid, could alter the expression and function of Kv1.5 channels in HL-1 cells. Treatment with urate increased the protein level of Kv1.5, which was accompanied by an increase in I_{Kur} and shortening of APDs.

Kv1.5 channels, encoded by the KCNA5 gene, confer I_{Kur}. In humans, the Kv1.5 channel is selectively expressed in atrial myocytes where it is an important contributor for action potential repolarization and controls APD. Overexpression of Kv1.5 in rat cardiomyocytes shortens APD, leading to the shortening of ERP, which plays an important role in the generation of reentry circuits to cause tachyarrhythmia. Yamashita et al reported that the enhancement of Kv1.5 expression by rapid pacing resulted in APD shortening and AF. In the present study, the urate-induced increase in Kv1.5 expression was associated with APD shortening. This is the first study to report that intracellular elevation of urate could shorten atrial APD and ERP via enhancement of Kv1.5 channel currents. Elevation of the serum urate level has been reported to increase the incidence of AF, and a recent study has indicated that hyperuricemia is an independent risk factor of AF. Our findings are consistent with these reports.

The intracellular urate level is regulated by UATs. Intraacellular accumulation of urate via activation of influx UATs could cause cellular damage through several signaling pathways. In a previous report by Kang et al, urate induced vascular smooth cell proliferation through activation of an influx UAT, voltage-driven urate transporter 1 (URAT1v1, also known as SLC22A12). There is no report regarding the expression of UATs in human atrium, while there is a report of UATs expressed in human umbilical endothelial cells (HUVECs). This report indicated that URAT1v1, as well as ATP-binding cassettes subfamily G member 2 (also known as the human breast cancer resistance protein, BCRP; ABCG2), multidrug resistance protein 4 (MRP4) and monocarboxylate transporter 9 (MCT9) were expressed in HUVECs; thus, the types of UATs in HUVECs were similar to those in HL-1 cells. We confirmed that the expressed UATs were also expressed in human embryonic stem cell-derived cardiomyocytes (Figure S3). Based on these data, there might be significant expressions of URAT1v1 and other UATs in human atrium as well. However, further experiments would be necessary to clarify whether hyperuricemia really shortens the refractory period in human atrium via the enhancement of Kv1.5 channel expression.

In the present study, HL-1 cells expressed mRNA of the influx transporter, URAT1v1, as well as the efflux transporters, ABCG2 and MRP4. The authentic URAT1v1 inhibitor benzbro-maronol abolished, but the ABCG2 inhibitor, KOI43, enhanced the urate-induced increases of Kv1.5 proteins. These findings support our hypothesis that the intracellular accumulation of urate via UATs increases Kv1.5 proteins in HL-1 cells.

Urate-induced oxidative stress is implicated in various physiological states. Urate stimulates the production of oxidant in both adipocytes and vascular smooth muscle cells. We observed an increase of ROS in urate-treated HL-1 cells. This increase was proportional to the increase of Kv1.5 proteins. Reversal of the increase by the antioxidant, NAC, confirms an involvement of oxidative stress. ROS is well-known to be generated by NADPH oxidase and urate-induced ROS production via activation of NADPH oxidase. The NADPH oxidase inhibitor, apocynin, reduced the ROS level and inhibited the urate-induced enhancement of Kv1.5 expression. Chao et al reported that urate stimulated the expression of endothelin-1 and NADPH oxidase in human aortic smooth muscle cells. Taken together, we conclude that urate-induced oxidative stress via activation of NADPH oxidase increased the protein level of Kv1.5.

Several studies reported involvement of the ERK 1/2 pathway as part of the downstream signaling of urate-derived ROS. In the present study, treatment with urate increased the level of pERK, which was suppressed by pretreatment with the antioxidant, NAC. This finding is consistent with previous reports for vascular smooth muscle cells, adipocytes and pancreatic β-cells. The present study demonstrates that urate enhances Kv1.5 expression through xanthine oxidase and NADPH oxidase-dependent oxidative stress, and activation of the ERK1/2 pathway.

Some limitations in this study should be considered. The Kv1.5 channel is known to have a complex regulatory mechanism, starting from its transcription, translation, and post-translational modifications affecting its glycosylation, trafficking, surface expression and degradation. Our study measured only the total expressed Kv1.5; further studies are needed to clarify which part of the regulations is actually affected. The mean current increased 3-fold, while the Kv1.5 protein expression increased by only approximately 30%. This discrepancy might be caused by the involvement of other factors that we did not observe. In this study, we used the HL-1 mouse atrial myocytes cell line, of which electrophysiological and biological properties have been well characterized. However, experiments using primary cultured cardiomyocytes or in vivo studies are necessary in the future. Another limitation is that we selected only some of the commonly known UATs to be analyzed for their presence in HL-1 cells. Thus, the presence of other UATs in HL-1 cells is unknown.

In summary, urate enters the cell through UATs, and enhances Kv1.5 protein expression. This effect is exerted by NADPH oxidase-dependent oxidative stress and the ERK pathway. Because the atrium with increased Kv1.5 currents is predisposed to AF, the use of influx UAT inhibitors and antioxidants may be beneficial for the prevention of AF in patients with hyperuricemia. Confirming this finding can lead to a more comprehensive treatment of hyperuricemia, particularly for the prevention of an overexpression of K+ channels, which may increase the risk of tachyarrhythmias, including AF.

**Acknowledgments**

We sincerely thank Professor William C Claycomb for the original HL-1 cells.

**Source of Funding**

None.

**Disclosures**

None.


