Soluble Uric Acid Promotes Atherosclerosis via AMPK (AMP-Activated Protein Kinase)-Mediated Inflammation

Yoshitaka Kimura, Tamiko Yanagida, Akiko Onda, Daisuke Tsukui, Makoto Hosoyamada, Hajime Kono

OBJECTIVE: Uric acid is supposed but not yet determined to be associated with atherosclerosis. Uric acid is released from damaged cells to form urate crystal, which is recognized by the immune system to produce IL (interleukin)-1. Danger signals and IL-1 have been shown to play an important role in atherosclerosis. We determined whether the physiological level of soluble uric acid promotes inflammation and develops atherosclerosis.

APPROACH AND RESULTS: The secretion of IL-1β from human peripheral blood mononuclear cells mediated by NLRP3 (NACHT, LRR, and PYD domain-containing protein 3) inflammasome was promoted by physiological levels in serum uric acid. This augmentation of inflammation was mediated by the regulation of the AMPK (AMP-activated protein kinase)-mTOR (mammalian target of rapamycin) mitochondrial reactive oxygen species and HIF-1α (hypoxia-inducible factor-1α) pathway. In both of uricase transgenic and xanthine oxidase inhibitor–treated mice, decreased levels of uric acid resulted in the activation of AMPK and attenuation of the development of atherosclerotic plaques. Further, acute uric acid reduction by the administration of benzbromarone in healthy humans for 2 weeks significantly decreased plasma IL-18—an inflammasome-dependent cytokine.

CONCLUSIONS: The data indicate that the development of atherosclerosis and inflammation is promoted by uric acid in vivo. Moreover, the lowering of uric acid levels attenuated inflammation via the activation of the AMPK pathway. This study provides mechanistic evidence of uric acid–lowering therapies for atherosclerosis.

Key Words: humans • inflammation • interleukin-1 • mice • uric acid

Hyperuricemia has been thought to be one of the possible risk factors for atherosclerosis. Although many epidemiological studies have shown that high serum uric acid (SUA) level was associated with the incidence or mortality of cardiovascular diseases, some studies provide conflicting results. Furthermore, most of the patients with hyperuricemia have metabolic comorbidities such as hypertension, diabetes mellitus, or metabolic syndrome. These confounding factors hinder the understanding of the relation of hyperuricemia with atherosclerosis. To the best of current knowledge, whether hyperuricemia should be treated to prevent the development of atherosclerosis is not yet known.

Several speculated mechanisms of hyperuricemia involvement in atherosclerosis have been reported. Uric acid is produced by the oxidation of xanthine and hypoxanthine by xanthine oxidase—one of the primary sources of reactive oxygen species (ROS). The hyperuricemia accompanies excessive production of oxidative stress, which presumably promotes the development of atherosclerotic lesions. The other mechanisms of the effect of hyperuricemia on atherosclerosis are vascular endothelial dysfunction caused by decreased NO levels, smooth muscle cell proliferation, or angiotensin II production. Inflammation plays essential roles in various stages of atherosclerotic development. Damaged cells or
tissues release molecules to activate the innate and acquired immune systems. These molecules are called danger signals or damage-associated molecular patterns. They play essential roles in not only acute inflammation but also in chronic inflammatory diseases, including atherosclerosis.6

Uric acid is the first molecule identified as an intracellular danger signal. In hominoids, uric acid is an end product of purine metabolism. Prolonged, high uric acid levels lead to the crystallization of uric acid as monosodium urate crystals in articular cavities. Monosodium urate crystals induce an acute inflammation mediated by NLRP3 (NACHT, LRR, and PYD domain-containing protein 3) inflammasomes and IL (interleukin)-1.7 By uric acid–lowering therapies for atherosclerosis.

In this study, we identified that NLRP3 inflammasome-mediated inflammation was promoted by the physiological concentration of soluble uric acid. Furthermore, we found that inflammation was regulated via the AMPK (AMP-activated protein kinase) pathway and attenuated the development of atherosclerosis. By using uricase transgenic mice, activated AMPK (AMP-activated protein kinase) and mTOR (mammalian target of rapamycin) pathway.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<tr>
<td>FBS</td>
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<td>HFD</td>
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<td>HIF-1α</td>
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<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<td>NLRP3</td>
<td>NACHT, LRR, and PYD domain-containing protein 3</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>ssUOXTg</td>
<td>secretable uricase transgenic</td>
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<td>SUA</td>
<td>serum uric acid</td>
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<td>TLR4</td>
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MATERIALS AND METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

Highlights

- Soluble uric acid of physiological levels augmented inflammasome-dependent inflammation by regulation of the AMPK (AMP-activated protein kinase)–mTOR (mammalian target of rapamycin) pathway.
- Lowering uric acid levels in 2 model mice by the administration of xanthine oxidase inhibitor, and by using uricase transgenic mice, activated AMPK pathway and attenuated the development of atherosclerosis.
- Uric acid reduction by the administration of benzbromarone suppressed inflammasome-dependent inflammation in human.
- This study provides mechanistic evidences of uric acid–lowering therapies for atherosclerosis.

Ethics Approval

All animal experiments were performed in accordance with the institutional regulations, complying with the Act on Welfare and Management of Animals and the related guidelines in Japan; the experiments were approved by the animal experimentation ethics and the genetically modified organism experimental safety committees of Teikyo University. All experiments on humans were approved by the Ethics Committee of the Teikyo University and performed under monitoring by an outsider. All participants provided informed consent before the study.

Mice

Mice were housed in approved specific pathogen-free conditions in a temperature-controlled environment with a 12-hour light/dark cycle. Mouse strains are summarized in the Major Resources Tables in the online-only Data Supplement. C57BL/6 N mice were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). ApoE<sup>−/−</sup> (B6.129P2-Apoetm1Unc/J, No. 002052) and Ldlr<sup>−/−</sup> (B6.129S7-Ldlrtm1Her/J, No. 002207) mice were purchased from the Jackson Laboratory. intUOXTg (intracellular uricase transgenic) and ssUOXTg (secretable uricase transgenic) mice were kindly donated by Prof K.L. Rock of the University of Massachusetts Medical School and backcrossed to C57BL/6 N >10×. Uricase transgenic mice were backcrossed to C57BL/6 JapoE<sup>−/−</sup> Ldlr<sup>−/−</sup> mice several times to produce apoE<sup>−/−</sup> Ldlr<sup>−/−</sup> uricase transgenic. These were then backcrossed to C57BL/6 JapoE<sup>−/−</sup> or C57BL/6 J Ldlr<sup>−/−</sup> mice again and the progeny genotyped and used for experiments. Uricase activity in serum and leukocytes was determined using the methods described below; mice with no uricase activity were used as control. In all experiments, the littermates were used for ensuring the genetic homogeneity. In all experiments, mice were euthanized using isoflurane (Pfizer).

Atherosclerotic Mouse Models

ApoE<sup>−/−</sup> and Ldlr<sup>−/−</sup> mice had free access to drinking water or allopurinol (Sigma) solution (45 μg/mL) and were fed a high-fat diet (HFD; D12079B; Research Diets) for 16 weeks from 8 weeks of age. The uricase transgenic mice were fed an HFD for
16 weeks from 6 weeks of age. Male mice were used because estrogen is known to affect the development of atherosclerosis and the expression of urate transporters. After euthanasia, the blood was collected from the inferior vena cava and the heart was removed. The hearts were fixed with formalin, and serial frozen sections (8 μm) were taken along the aortic sinus. The sections were stained with hematoxylin–eosin and captured by using a BX53 microscope (Olympus). The size of atherosclerotic plaques from the initial appearance to the complete disappearance of the aortic valve was measured for every 10 slices using ImageJ software. The volumes of atherosclerotic plaques were calculated by totaling the area of atherosclerotic plaques in each slice and multiplying by the thickness of 10 slices (80 μm). The area of plaques stained Mac-2 and the number of Ly-6G/Ly-6C–positive cells were evaluated in the slice where the area of plaque was the largest among the serial sections.

The blood was hemolyzed; the leukocytes were used for the measurement of mitochondrial ROS and AMPK activity. The leukocytes were incubated with APC-Cy7 rat anti-mouse Ly-6G antibody (Biolegend) in the presence of mAb 2.4G2 (Bioxcell). Next, they were incubated with 5 μM Mitosox (Invitrogen) for 10 minutes, and mean fluorescence levels were measured using flow cytometry. The other cells were lysed in 300 μL lysis buffer.

**Measurement of Uricase Activity**

Uric acid (Sigma) mixed with 0.1 M boric acid solution was used as a standard. The optical density of the solution at 292 nm was adjusted to around 0.7. Blood was collected from tail veins of 4- to 5-week-old mice. The blood was hemolyzed, and residual leukocytes were incubated with standard solution at 37°C. For the secratable-type uricase transgenics, serum was separated, added to standard solution, and incubated at 37°C. After 0, 4, and 12 hours, the optical density at 292 nm was measured. The samples for which the optical density value decreased after incubation were considered to be positive for uricase activity.

**Analysis of Plasma Uric Acid Levels by Using High-Performance Liquid Chromatography**

Plasma uric acid levels in mice were measured using high-performance liquid chromatography as described previously. Blood was collected from the tail vein and placed on ice with heparin. Plasma was separated by centrifugation at 4°C. Next, 2 μL of plasma was added to 40 μL of 80% acetonitrile, and proteins were removed by filtering through a 0.22-μm PVDF filter (Millipore) by centrifugation. The flowthrough was evaporated. The deproteinized sample was resuspended with 10 μL of high-performance liquid chromatography mobile phase (20 mmol/L ammonium formate). Separation was achieved at a flow rate of 0.200 mL/min on a 250×2-mm (5 μm particle size) ODS column, Unison US-C18 (Imtakt) at 25°C. Uric acid was detected at 234 nm by using a UV detector (Chromaster 5410; Hitachi).

**Infiltration of Inflammatory Cells Into the Peritoneal Cavity by Injection of Cholesterol Crystals**

The infiltration of inflammatory cells into the peritoneal cavity was measured as described previously. Cholesterol (Sigma) was crystallized as described previously. Cholesterol crystals were suspended in 200 μL of PBS and injected into the peritoneal cavities of mice. At 6 hours after injection, mice were euthanized, and their peritoneal cavities were washed with 6 mL DMEM with 2% fetal bovine serum (FBS) containing 3 mmol/L EDTA and 10 U/mL heparin. The cells were stained with PE rat anti-mouse Ly-6C antibodies (BD Biosciences) and APC-Cy7 rat anti-mouse Ly-6G antibodies (BD Biosciences) for 15 minutes at 4°C in the presence of mAb 2.4G2 (Bioxcell). Total numbers of neutrophils or monocytes in the peritoneal exudate cells were determined by counting Ly-6G<sup>lo</sup>Ly-6C<sup>hi</sup> or Ly-6G<sup>lo</sup>Ly-6C<sup>lo</sup> cells, respectively, by flow cytometry (Cell Analyzer EC800; Sony).

**Separation of Human Peripheral Blood Mononuclear Cells**

Blood samples were collected in heparin-coated tubes from healthy adults. Human peripheral blood mononuclear cells (hPBMCs) were separated using density gradient centrifugation with Histopaque-1077 (Sigma). The hPBMCs were resuspended at 2×10<sup>7</sup> cells/mL in PBS.

**Stimulation of hPBMCs With Lipopolysaccharide and Cholesterol**

Separated hPBMCs were seeded at 5×10<sup>5</sup> cells/well in a 96-well plate and then incubated in RPMI-1640 with 10% FBS with or without uric acid for 24 hours. In experiments involving uricase, the uric acid solution was added to 0.5 mUnit/mL uricase (Sigma) or 0.5 mUnit/mL deactivated uricase (boiled at 100°C for 5 minutes). Cells were then stimulated with 300 pg/mL lipopolysaccharide (LPS-EK Ultrapure; InvivoGen) or after 3 hours stimulated with 100 μg/mL cholesterol crystals.

The following inhibitors and an activator were added to the hPBMCs: N-acetyl-L-cysteine (Sigma), mitoquinone (Focus Biomolecules), KC7F2 (Sigma), rapamycin (Calbiochem), and AICAR (Adipogen).

**ELISA**

IL-1β in supernatants and pro–IL-1β in cell lysates were measured using the Human IL-1β/IL-1F2 DuoSet ELISA Development System (R&D). TNF (tumor necrosis factor)-α was measured using the Human TNFα DuoSet ELISA Development System (R&D). Mouse serum IL-1 and TNFα were measured using the Mouse IL-1β/IL-2 QuantiKine HS ELISA Kit (R&D) and the Mouse TNFα Quantikine HS ELISA Kit (R&D). Human plasma IL-1β was detected using the Human IL-1β DuoSet ELISA Development System (R&D).

**RT-PCR**

RNA was extracted using the ReliaPrep RNA cell Miniprep System (Promega) and reverse transcribed into cDNA using the ReverTra Ace qPCR Master Mix (Toyobo). The mRNA was analyzed using the Thunderbird Sybe qPCR Mix (Toyobo) and a 7500 Real-Time PCR System (Applied Biosystems). The following primers were used: human IL-1β forward: 5′-GCCCTAACCAGATGAAGTG CTC-5′ reverse: 3′-GAACCAGCATCTTCCTCAG-5′.
human β-actin forward: 3'-CATTGCGACAGGATGCA-5' and reverse: 3'-CATCTGCAGGAAAGTGGACAG-5'.

Relative mRNA expression levels were analyzed using the comparative computed tomography method with β-actin mRNA as the reference.

**Generation of NLRP3 Inflammasome Activation Reporter Cells**

We generated NLRP3 inflammasome activation reporter cells as described previously. C-terminal caspase recruitment domain (ASC) cerulean expression vector (pRP_ASC-LmCerule) was kindly provided by Prof Eicke Latz (University of Bonn). The plasmid was transfected into HEK293T (Riken, Tokyo, Japan) cells using Fugene 6 transfection reagent (Promega). The cells were selected using 0.5 μg/mL puromycin (InvivoGen). Next, full-length human NLRP3 cDNA was excised from the pEF-PYPAF-1 plasmid (Riken), and the NLRP3 gene fragments were ligated into pMSCVhygro vectors. The plasmids were transfected into the ASC-cerulean overexpressing cell line. The cells were selected using 120 μg/mL hygromycin B (Wako), and surviving cells were stimulated with nigericin sodium salt (Sigma) as the reference.

**Formation of NLRP3 Inflammasomes**

Inflammasome activation reporter cells were plated at 1.0×104/well in a 96-well plate in DMEM +10% FBS+0.5 μg/mL puromycin+120 μg/mL hygromycin B (Wako), and surviving cells were selected using nigericin sodium salt (Sigma)–an NLRP3 inflammasome activator. The cells in which speck formation was observed were selected as NLRP3 and ASC-cerulean overexpressing cell lines.

**Measurement of Intracellular ROS**

To measure intracellular ROS levels, we used the DCFDA Cellular ROS Detection Kit (Abcam). The hPBMCs were cultured in RPMI-1640 without FBS, with or without uric acid, at 2.0×105 cells/well in a 96-well plate for 18 hours. Lipopolysaccharide (300 pg/mL) was added; after 6 hours, the cells were collected and the fluorescence levels were measured; cells were incubated with 5 μM Mitosox (Invitrogen) to detect NLRP3 inflammasome activation. Sections were permeabilized with 1% BSA in TBS, they were incubated with the following primary antibodies against phosphorylated AMPKα (T172; Cell Signaling Technology), phosphorylated Akt (S473; Cell Signaling Technology), NLRP3 (Cell Signaling Technology), and phosphorylated LKB1 (S431; Santa Cruz Biotechnology). The secondary antibody was HRP-conjugated rabbit anti-rabbit antibody (Cell Signaling Technology), anti-Akt rabbit antibody (Cell Signaling Technology), anti-LKB1 (Santa Cruz Biotechnology), anti-α-tubulin rabbit antibody (Proteintech), or anti-β-actin rabbit antibody (Proteintech) and detected as described above.

**Measurement of Mitochondrial ROS in Peritoneal Macrophages**

Murine peritoneal macrophages were isolated as described previously. The cells were plated at 5×104 cells/well in a 96-well plate with DMEM/F-12+10% FBS. After 12 hours, they were washed with PBS and the medium was changed to DMEM without FBS, with or without uric acid. Cells were incubated overnight, and then, mitochondrial ROS levels were measured; cells were incubated with 5 μM Mitosox (Invitrogen) for 10 minutes, and mean fluorescence levels were measured using flow cytometry.

**Western Blotting**

Proteins in the cell lysate were separated by SDS-PAGE on a 10% polyacrylamide gel and transferred to polyvinylidene fluoride membranes (GE or ATTO). After the membranes were blocked with 1% BSA in TBST, they were incubated with the following primary antibodies against phosphorylated AMPKα (T172; Cell Signaling Technology), Akt (S473; Cell Signaling Technology), NLRP3 (Cell Signaling Technology), and phosphorylated LKB1 (S431; Santa Cruz Biotechnology). The secondary antibody was HRP-conjugated goat anti-rabbit IgG (Cappel) and HRP-conjugated rabbit anti-mouse IgG (Invitrogen). Antibodies were detected using Chemi-Lumi One Ultra (Nakarai).

**Immunohistochemistry and Immunofluorescence**

Sections were immersed in HistoVT One (Nacalai) for antigen activation. Sections were permeabilized with 0.1% Triton X-100 in TBS for staining of intracellular proteins. Sections were blocked with 0.3% H2O2 in methanol and Blocking One Histo (Nacalai). Sections were incubated with anti–p-AMPKα (T172) rabbit antibody (Cell Signaling Technology) and anti–Mac-2 antibody (Biolegend) at 4°C overnight. The secondary antibody was HRP-conjugated goat anti-rabbit IgG (Cappel) and HRP-conjugated rabbit anti-rat IgG (Abcam). The Peroxidase Stain DAB kit (Nacalai) was used as a chromogenic agent.

For immunofluorescence of Gr-1, sections were performed antigen activation, subsequently blocked with Blocking One Histo (Nacalai). Sections were incubated with anti–Gr-1 rat IgG (Biolegend) at 4°C overnight. The secondary antibody was FITC-conjugated goat anti-rat IgG (Proteintech). Sections were incubated with Vector TrueVIEW Autofluorescence Quenching Kit (Vector) for reducing the autofluorescence emission.
Administration of Benzbromarone to Healthy Adults

We enrolled participants who were over 20 years of age and had no history of any disease, including hyperuricemia and gout. They were orally administrated a 150-mg daily dose of benzbromarone (Nichiko, Toyama, Japan) for 2 to 3 weeks and Uralyt-U (Sawai, Osaka, Japan) to prevent the formation of uric acid stones. Before the treatment and 14 days after, blood samples were collected in heparinized tubes in triplicate. PBMCs and plasma were separated. PBMCs were cultured in RPMI-1640 containing 10% FBS in a 96-well plate. After seeding, cells were stimulated with 100 pg/mL lipopolysaccharide, followed by the addition of 100 pg/mL cholesterol crystals after 3 hours. After 24 hours, supernatants were collected. TNFα levels of supernatants stimulated with lipopolysaccharide and IL-1β levels in supernatants stimulated with lipopolysaccharide and cholesterol crystals were measured using ELISA. Plasma uric acid levels were measured using the Fuji Drychem System (Fujifilm). Plasma IL-18 levels were measured using ELISA.

Statistical Analysis

All data are expressed as mean±SEM. An assumption of normality of data was tested using a normality test. F test or the Brown-Forsythe test was used for testing the equality of variances. Differences between groups were analyzed using Student t test and 1-way ANOVA followed by Dunnett test or 2-way ANOVA followed by Bonferroni post hoc test. If normality was rejected, results were further analyzed using the Mann-Whitney U or Kruskal-Wallis tests, followed by Dunn post hoc test. Values with P<0.05 were considered statistically significant. All analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA).

For further information, please see the Major Resources Table in the online-only Data Supplement.

RESULTS

Acute Inflammation by Cholesterol Crystals Is Attenuated in Uricase Transgenic Mice

In atherosclerotic lesions, cholesterol crystals are deposited from the early stage of plaque progression and play a significant role in pathogenesis via IL-1β and NLRP3 inflammasome-mediated inflammation.19 To determine the role of uric acid in cholesterol crystal–induced inflammation, we injected cholesterol crystals into the peritoneal cavities of uricase transgenic mice in which the metabolism of uric acid was increased and the amount of uric acid in the body was decreased.

Two types of uricase transgenic mice (secretable and intracellular) were used. The former (ssUOXTg) mice express uricase with a signal peptide for extracellular secretion and the transgens’ SUA level is lower than that of controls. The latter (intUOXTg) express uricase without a signal peptide, so the uricase remains inside cells; therefore, in these mice, the pool of intracellular uric acid is decreased. We utilized that using the 2 types of uricase transgenic mice to clarify where uric acid affects inflammation pathways in extracellular or intracellular milieu. The animals were without any obvious phenotypic abnormalities.13

In a previous study, the cell death–induced inflammatory response such as acute drug-induced liver injury and infiltration of inflammatory cells was reduced in uricase transgenic mice.15 In this study, infiltration of neutrophils in response to cholesterol crystals was significantly decreased in both uricase transgenic mouse lines (Figure 1A). For monocytes, cell infiltration was significantly decreased in ssUOXTg mice, whereas the number of infiltrated cells was lower in intUOXTg mice but not significantly different from that of WT (wild type; Figure 1B).

Soluble Uric Acid Promotes the Secretion of IL-1β Dependent on NLRP3 Inflammasomes via Activation of HIF-1α and Mitochondrial ROS

The infiltration of inflammatory cells induced by sterile crystals has been shown to be dependent on IL-1β.19 Secretion of IL-1β from inflammatory cells occurs via 2 pathways: signal 1 and signal 2. The signal 1 pathway leads to the production of pro–IL-1β or NLRP3. The signal 2 pathway leads to the conversion of pro–IL-1β to mature IL-1β via the activation of NLRP3 inflammasomes. We incubated human hPBMCs in medium containing physiological levels of uric acid and then stimulated them with lipopolysaccharide and cholesterol crystals, which were activators of signal 1 and signal 2, respectively. IL-1β was not secreted by hPBMCs stimulated with either lipopolysaccharide or cholesterol crystals alone. Stimulation with both lipopolysaccharide and cholesterol crystals induced the secretion of IL-1β, and the enhancement of IL-1β was observed with the increase in uric acid levels (Figure 2A). Decreasing the
Figure 2. Soluble uric acid promotes the secretion of IL (interleukin)-1β via activation of HIF-1α (hypoxia-inducible factor-1α) and mitochondrial reactive oxygen species (ROS).

A, Secretion of IL-1β from human peripheral blood mononuclear cells (hPBMCs), which were incubated with uric acid and stimulated with lipopolysaccharide (LPS; 300 pg/mL) or cholesterol crystals (100 µg/mL). Representative data from 3 independent experiments are shown (n=2–6). Kruskal-Wallis test. B, hPBMCs cultured with uric acid oxidase, uricase, or boiled uricase, and then stimulated with LPS and cholesterol crystals with multiple concentrations of uric acid. Representative data from 3 independent experiments are shown (n=4). Two-way ANOVA, compared with UA (0 mg/dL). ****P<0.0001, ####P<0.0001. C, NLRP3 (NACHT, LRR, and PYD domain-containing protein 3) inflammasome activation reporter cells cultured with or without uric acid and stimulated with 2 mM nigericin. A light speck represented an NLRP3 inflammasome complex. Images are fluorescence images of specks at 0 and 4 h after stimulation. Scale bar=50 µm. The number of specks per cell area was shown (n=4). D, The mitochondrial ROS levels of peritoneal macrophages derived from WT (wild type; C57BL/6) and intUOXTg (intracellular uricase transgenic) cultured with or without uric acid for 24 h (n=6–7). It was detected by MitoSOX Red. Images are representative images of MitoSOX red (mitochondrial ROS) and Hoechst33342 (nuclear) staining. Scale bar=50 µm. E, hPBMCs stimulated with LPS and cholesterol crystals. The mitochondrial inhibitor (100 nmol/L mitoquinone, MitoQ) or HIF-1α inhibitor (10 µmol/L KC7F2) were added. Data are combined from the results of 3 independent experiments. C–E, Two-way ANOVA. *P<0.05, **P<0.01. A–E, Data are shown as mean±SEM. NS indicates not significant.
Uric acid levels by addition of uricase significantly attenuated the secretion of IL-1β (Figure 2B).

Next, we investigated the association of soluble uric acid with the signal 1 pathway. Expression of pro–IL-1β mRNA and protein and NLRP3 protein was not affected by uric acid levels by stimulating with lipopolysaccharide alone (Figure IA through IC in the online-only Data Supplement). However, after stimulation with both lipopolysaccharide and cholesterol crystals, the expression of pro–IL-1β was significantly increased by uric acid (Figure ID in the online-only Data Supplement). Uric acid has been shown to augment the expression of HIF-1α (hypoxia-inducible factor-1α),20 which upregulates pro–IL-1β expression by binding its enhancer element.21 The increase in pro–IL-1β by uric acid was attenuated by the addition of KC7F2—an HIF-1α inhibitor—while secretion of IL-1β was not affected by KC7F2 (Figure ID in the online-only Data Supplement).

Next, we investigated the effect of soluble uric acid on the signal 2 pathway. To perturb the signal 2 pathway without being affected by signal 1, we developed inflammasome activation reporter cells, which are HEK293T cells stably overexpressing NLRP3 and apoptosis-associated speck-like protein containing the ASC coupled to cerulean (ASC-cerulean).

First, reporter cells were incubated in medium with or without uric acid. Incubation with uric acid alone did not affect the formation of the inflammasome complex. Second, we cultured the reporter cells with medium containing 0 or 4 mg/dL of uric acid and then stimulated them with nigericin—an NLRP3 inflammasome activator—by damaging the cell membrane. After stimulation with nigericin, inflammasome formation was significantly increased in the reporter cells incubated with uric acid (Figure 2C).

ROSs have been reported to be involved in the activation of NLRP3 inflammasomes.22 We found that ROS in the reporter cells incubated with uric acid (Figure 2C).

Next, we investigated the association of soluble uric acid with AMPK in vivo. Uricase transgenic and control WT mice were provided high fructose solution. The AMPK activity of the leukocytes of both intUOXTg and ssUOXTg mice was higher than those of WT controls (Figure 3D).

As described above, lowering uric acid level resulted in attenuation of inflammation and decreasing of infiltration of inflammatory cells. In these mice with HFD,
the serum inflammatory cytokines were measured. The serum IL-1β level was significantly lower in ssUOXTg mice than in the controls, while the serum TNFα level was significantly lower in intUOXTg mice (Figure IIIB in the online-only Data Supplement). Furthermore, mitochondrial ROS levels in peripheral neutrophils was decreased in intUOXTg mice (Figure IIIC in the online-only Data Supplement). As for infiltration of inflammatory cells into atherosclerotic plaques, the number of Gr-1-positive cells (neutrophils and monocytes) tend to be smaller in uricase transgenic mice (Figure IIID in the online-only Data Supplement). The area of a macrophage marker, Mac-2, in plaques was also significantly decreased in uricase transgenic mice (Figure 4B).
To better understand the role of uric acid, we assessed the development of atherosclerotic plaques in mice treated with the xanthine oxidase inhibitor allopurinol. In ApoE\(^{-/-}\) mice administered allopurinol, leukocyte AMPK activity was increased (Figure IVA in the online-only Data Supplement). The size of atherosclerotic plaques...
in the aortic roots was significantly reduced by allopurinol treatment in 
ApoE<sup>−/−</sup> and Ldlr<sup>−/−</sup> mice (Figure IVB in the online-only Data Supplement). By immunostaining for phospho-AMPK (Thr172), the activity of AMPK was higher in mice treated with allopurinol (Figure IVB in the online-only Data Supplement).

**Benzbromarone Decreased Inflammasome Activation in Humans**

We next measured the effect of decreased amount of uric acid in the body on inflammation induction in vivo in humans. Eight healthy adults (4 men; 4 women; age, 21–50 years; mean, 29.5±4.1) with no underlying disease, including hyperuricemia or gout, were identified. They were treated with benzbromarone (150 mg/d, p.o.)—a uricosuric agent—for 2 weeks. Blood samples were collected before and after benzbromarone treatment.

The average plasma uric acid level dropped from 6.0±0.6 to 1.9±0.2 mg/dL after benzbromarone treatment (Figure 5A). Mean plasma IL-18 levels were significantly decreased from 273±59 to 248±53 pg/mL (Figure 5B). IL-18 is a cytokine related to the activation of NLRP3 inflammasomes. We also found that plasma IL-18 levels were positively, but not significantly, correlated with plasma uric acid levels (Figure V in the online-only Data Supplement).

**DISCUSSION**

Here, we showed that soluble uric acid enhances NLRL3 inflammasome activation via AMPK-mTOR-mitochondrial ROS pathway. Uric acid promotes the ROS-mediated activation of NLRP3 inflammasomes. Soluble uric acid suppresses the activity of AMPK followed by the activation of mTOR. The alterations of these pathways by uric acid result in the generation of mitochondrial ROS and activation of HIF-1α. Thus, soluble uric acid promotes the secretion of IL-1β and enhances innate inflammation.

![Figure 5. Benzbromarone decreases inflammasome activation in humans.](https://example.com/figure5)

Eight healthy adults were treated with benzbromarone (150 mg/d) for 2 wk. **A,** The changes of plasma uric acid levels. **B,** The changes of plasma IL (interleukin)-18 levels. **C** and **D,** PBMCs stimulated with lipopolysaccharide (LPS; **C**) or LPS and cholesterol crystals (**D**). **C,** TNF (tumor necrosis factor)-α and (**D**) IL-1β levels in supernatants after 24 h of stimulation. Symbols connected by a line represent mean data before and after treatment for 1 subject. Two-way ANOVA.
Soluble uric acid has been shown to increase the production of mitochondrial ROS. In addition, our results showed that uric acid was involved in the production of pro–IL-1β via HIF-1α; however, this pathway was independent of the TLR4 (toll-like receptor 2) pathway. Since the effect of uric acid on the production of pro–IL-1β was noted after stimulation with lipopolysaccharide and cholesterol crystals, the effect was hypothesized to be caused by molecule(s) released after pyroptosis. Further studies are required for the molecular identification of this effector produced by treatment with cholesterol crystals.

In the cell culture experiments, uric acid was used in concentrations normally found in plasma. However, the concentration of proteins in the cell culture medium was lower than that in extracellular fluid. Some proteins among them may bind to uric acid. Then, there is a possibility that the actual working concentration of uric acid in the cell culture medium is artificially high. However, we showed that decreasing of uric acid levels by benzbro- 

marone in vivo resulted in suppression of inflammatory response. It indicated that uric acid affected inflammation even in the condition of higher concentration of proteins.

AMPK is a serine/threonine kinase that monitors intracellular energetic conditions and regulates energy metabolism. Recently AMPK has received attention as a key player connecting metabolism with inflammation. Activation of AMPK activity attenuates inflammation responses. Suppression of AMPK activity has been also reported to activate NLRP3 inflammasomes in several pathways, including inhibition of autophagy, the disorder of homeostasis of damaged mitochondria, and the attenuation of ER stress. Those data are consistent with our data from partial inhibition of the uric acid–mediated augmentation of IL-1β by inhibiting both HIF-1α and mitochondrial ROS.

In this study, we showed that the peritoneal infiltration of inflammatory cells induced by cholesterol crystals was suppressed in uricase transgenic mice. The AMPK pathway of inflammatory cells was activated in uricase transgenic mice (Figure VI in the online-only Data Supplement). AMPK has been reported to suppress neutrophil infiltration and release of IL-1β and CXCL1 by monosodium urate crystals. Taken together, these results indicated that uric acid promoted the sterile crystal-induced inflammation via regulation of AMPK.

We measured the effect of uric acid on the development of atherosclerosis in mice. Several reports have shown an association of SUA levels with atherosclerosis in ApoE−/− mice given allopurinol or benzbro- 
mazone. These results could potentially be explained by off-target effects of the drugs used. It has been reported that inhibition of xanthine oxidase by allopurinol resulted in the reduction of oxidative stress and prevention of the differentiation of macrophages to foam cells in plaques. In this study, we utilized a uricase transgenic atherosclerotic mouse model. To our knowledge, this is the first study to show an effect of uric acid on the development of atherosclerosis in mice without uric acid–lowering drugs. Lowering uric acid levels by both alteration of production or degradation resulted in attenuation of development of atherosclerosis. These results indicate that uric acid itself is involved in the pathogenesis of atherosclerosis. Serum inflammatory cytokines of uricase transgenic mice were decreased. IL-1β was decreased in intUOXTg, while TNFα was in ssUOXTg mice. The result suggested that the effect of uric acid on inflammation might be different between intracellular and extracellular milieu. Furthermore, the infiltration of inflammatory cells and the accumulation of macrophages were decreased in uricase transgenic mice. Monocytes infiltrated into plaques differentiated to macrophages. It supported that uric acid affected the development of atherosclerosis via regulation of inflammatory response.

The effect of uric acid–lowering treatment on cardiovascular risks has been investigated in several clinical studies. In a cohort study of hyperuricemic patients, allopurinol treatment was associated with a decreased cardiovascular risk. In a meta-analysis by Bredemeier et al, xanthine oxidase inhibitors did not significantly reduce the risk of major adverse cardiovascular events and death but reduced total adverse cardiovascular events and hypertension. Especially, allopurinol protected for myocardial infarction. As for comparison among xanthine oxidase inhibitors, all-cause mortality and cardiovascular mortality were higher in patients treated with febuxostat—a nonpurine xanthine oxidase inhibitor—than in those treated with allopurinol—a purine base analogue of xanthine oxidase inhibitor.

Recently, the importance of AMPK in atherosclerosis has been confirmed. The AMPK activators suppressed sizes of plaques in ApoE−/− mice. AMPK activation caused inhibition of chemotaxis of monocytes, antinflammatory effects via suppression of STAT3, and inhibition of differentiation of monocytes to macrophages. In uricase transgenic mice consuming fructose or on an HFD, AMPK phosphorylation was elevated. Also, administration of allopurinol to mice on an HFD activated AMPK in ApoE−/− mice. The activation of AMPK was detected in endothelial side of plaques. These data indicate that soluble uric acid affects endothelial cells of plaques or infiltrating cells into plaques from vessels. Finally, atherosclerotic lesions were increased in endothelial cell–specific Ampk−/− mice. We showed that the administration of benzbro- 

mazone significantly decreased plasma IL-18 levels and plasma uric acid levels in healthy human subjects. Pro- 

IL-18 is constantly expressed and matured to IL-18 by NLRP3 inflammasomes. In a cohort study of patients with hyperuricemia, SUA levels were found to be positively correlated with serum TNFα, high-sensitivity CRP, and IL-18. Atherosclerosis has been shown to be
prevented in IL-18 and ApoE double-knockout mice. Further, high plasma IL-18 levels were reported to be an independent predictor of cardiovascular risk. Therefore, decreasing IL-18 levels by the administration of benzbromarone might provide a treatment for atherosclerosis via the regulation of inflammasome activity.

In this study, we showed that the development of atherosclerosis was prevented in 2 uric acid–lowering model mice. Uric acid worked as a danger signal by induction of inflammasome-dependent inflammation via the suppression of AMPK activity. Furthermore, benzbromarone reduced plasma uric acid levels and suppressed inflammasome-dependent inflammation in vivo in humans. This study indicated that uric acid–lowering therapy could be able to reduce the residual risks of cardiovascular diseases.

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Affiliations
From the Department of Internal Medicine, Faculty of Medicine (Y.K., T.Y., A.O., D.T., H.K.) and Department of Human Physiology and Pathology, Faculty of Pharma-Sciences (M.H.), Teikyo University, Tokyo, Japan.

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Disclosures
None.

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