Communication to the Editor

The Mechanism of False in Vitro Elevation of Uric Acid Level in Mouse Blood

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Thirty minutes incubation at room temperature elevates the uric acid (UA) level of mouse blood in a test tube, and has previously been reported as “false in vitro elevation of the uric acid level.” However the UA level of human blood does not elevate using the same incubation. We clarified the mechanism of the false in vitro UA elevation using mice with highly active hypoxanthine phosphoribosyl transferase (Hprt) of B6-ChrXCMSM, a consomic mouse strain with the chromosome portion of Mus musculus morocinus in the Hprt gene site, or mice with a targeted deletion of the urate oxidase gene (Uox) (Uox-knockout (KO)). The plasma levels of UA, hypoxanthine, and xanthine, determined by HPLC, were compared with those of C57BL/6J laboratory mice used as controls. The uric acid level of Uox-KO mice was approximately 10 times higher than that of control, but was not elevated after incubation in the test tube. With allopurinol, the hypoxanthine levels of B6-ChrXCMSM and Uox-KO were not significantly lower than that of controls. Without allopurinol, the UA and xanthine levels of B6-ChrXCMSM were significantly lower than those of C57BL/6J mice. Even with allopurinol, the UA and xanthine levels were still significantly lower than that of controls. In conclusion, “false in vitro elevation of uric acid level” seems to be caused by low levels of erythrocyte HPRT activity and the low plasma uric acid level of laboratory mice.

Key words uric acid; hypoxanthine; hypoxanthine phosphoribosyl transferase; urate oxidase

In our previous study, false elevation of the uric acid (UA) level was demonstrated in mouse blood that had been incubated in the test tubes for 30 min after blood sampling. As part of the purine degradation pathway, xanthine oxidase oxidizes hypoxanthine to xanthine, and xanthine to UA. Although UA is decomposed into allantoin by urate oxidase in the liver hypoxanthine to xanthine, and xanthine to UA. Therefore, it is necessary to use urate oxidase gene (Uox)-knockout (KO) mice, to study purine metabolism in humans. Instead of elevation of the UA level, the hypoxanthine level in mouse plasma was elevated during the incubation with allopurinol, a xanthine oxidase inhibitor. Therefore, UA was produced from hypoxanthine in the test tubes by xanthine oxidase in mouse plasma. Hypoxanthine was supposed to be released from the blood cells during incubation. However, false elevation of the plasma UA level appears to rarely occur in human blood after the same incubation. In the purine salvage pathway, hypoxanthine guanine phosphoribosyl transferase (HPRT) recycles hypoxanthine using 5-phosphoribosyl-1-pyrophosphate (PRPP) for re-synthesizing inosine monophosphate (IMP). As the activity of HPRT in human erythrocytes was reported to be as much as ten times higher than that in mice, it is hypothesized that hypoxanthine should not be released from blood cells, but should be phosphoribosylized to IMP in human erythrocytes via HPRT. Loss of urate oxidase activity is a well-known species difference in purine metabolism between humans and mice, as well as low activity of HPRT in mouse erythrocytes.

The activity of HPRT in erythrocytes of wild-derived strains of mice carrying the hypoxanthine phosphoribosyl transferase (Hprt) a allele (Mus musculus castaneus) is higher than that of inbred mouse strains carrying the Hprt b allele (C57BL/6J). In the above hypothesis, hypoxanthine will not be released from erythrocytes carrying the Hprt a allele. Therefore, it is expected that false elevation of the plasma UA level will be suppressed in wild-derived mouse blood.

We used Uox-KO mice, which lack urate oxidase and so more closely mimic purine metabolism in humans, compared to the C57BL/6J mouse and B6-ChrXCMSM mice, to examine the causes of varied UA levels in plasma due to species differences.

The purpose of this study is to elucidate whether false elevation of the UA level will be suppressed in the plasma of consomic B6-ChrXCMSM mice carrying the Hprt a allele, or in the plasma of urate oxidase gene targeted knockout mice (Uox-KO), whose UA level is similar to that in human plasma.

MATERIALS AND METHODS

Animals Male C57BL/6J mice weighing 27–30 g were purchased from Sankyo Lab. Service (Tokyo, Japan). B6-ChrXCMSM mice weighing 27–32 g were purchased from the National Institute of Genetics (Shizuoka, Japan). Uox-KO mice weighing 31–46 g were purchased from the Jackson Laboratory (U.S.A.). This study was approved by the animal ethics committee of Teikyo University.

Genotyping of Hprt Gene Allelic discrimination of the Hprt gene was conducted using TaqMan® GTXpress™ Master Mix (Applied Biosystems™) with the following oligonucleotide primers and minor groove binder (MGB) probes: F-CCCTCTCAGACCCTTTTG; R-TCTGTGAGTC CCCCCTT; VIC-CCCCGTCATCGAC-MGB; fluorescein amide (FAM)-CCCGTC ATGCGAC-MGB. The PCR amplifications were carried out in final volume of 10 µL consisting of a master mix, 0.9 µM of each primer, and 0.2 µM of 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein (VIC)-labeled and FAM-labeled probes, respectively. The holding stage before PCR was performed at 25°C for 1 min. PCR cycling was conducted first at 95°C for 20 s, followed by 40 cycles at 95°C for 3 s each, and finally at 60°C for 30 s. The holding stage after PCR was performed at 25°C for 1 min.

Sampling of Blood and Drug Treatment in Vitro Mice were anesthetized with diethyl ether followed by intra-peritoneal injection of 50 µg/g body weight pentobarbital. After
abdominal incision, blood was collected from the inferior vena cava and dispensed into a MiniCollect® tube (Greiner bio-one, Kremsmunster, Austria) containing heparin lithium for plasma preparation. For 1 µM allopurinol treatment in vitro, 1 µL of 100-times concentrated drug in saline was added to the blood in the MiniCollect® tube to make a total volume of 100 µL. The blood in the MiniCollect® tube was incubated for 0 and 30 min. After incubation, plasma was separated by centrifugation at 3000 x g for 15 min. For sampling of human blood, 1 mL volume of peripheral blood was drawn from healthy donors. The study was approved by the Ethics Committee of the University of Teikyo (Nos. 15–185).

Uric Acid and Hypoxanthine Analysis Using HPLC  As described previously,21 20 µL of the separated plasma or serum was deproteinized with 80% acetonitrile by centrifugation at 12000 x g for 4 min. The deproteinized sample was resuspended with 20 µL of HPLC mobile phase (20 mM ammonium formate) after evaporation. Separation was achieved at a flow rate of 0.200 mL/min on a 250 mm x 2 mm, 5 µm particle size octadecyl silica (ODS) column, Unison US-C18 (Imtakt, Japan) at 28°C (Shimadzu Prominance). Plasma creatinine levels, detected at 234 nm by UV detector (SPD-20A, Shimadzu), in each mouse at 0 min were used as internal controls to standardize the levels of UA, hypoxanthine, and xanthine levels after the 30 min incubation with or without allopurinol.

Statistical Analyses  All data were expressed as the mean ± standard error of the mean (S.E.M.). Differences between groups were analyzed statistically using Student’s t-tests. p Values of <0.05 were considered significant.

RESULTS

Genomic sequencing revealed the 4th base of the Hprt gene was to be cytidine in C57BL/6J mice, but guanine in B6-ChrXc<sup>MSM</sup> mice (Fig. 1A), confirming that B6-ChrXc<sup>MSM</sup> mice carry the Hprt<sup>a</sup> allele, similarly to Mus musculus cast-erreus.

The inclusion of the Hprt<sup>a</sup> allele in B6-ChrXc<sup>MSM</sup> mice was also confirmed in Fig. 1B. Genotyping of the Hprt<sup>a</sup> and b alleles was performed by allelic discrimination plots using TaqMan® GTXpress™ Master Mix (Applied Biosystems™). Two allelic genes could be discriminated and classified into the wild type, heterozygous, or homozygous types.

In the plasma of C57BL/6J mice, the UA level significantly elevated from 0 to 30 min after incubation, but no elevation was noted at 30 min after the addition of allopurinol. In contrast, no elevation in the UA level in the blood of humans was noted after 30 min incubation (Table 1).

Plasma UA (Fig. 2A), hypoxanthine (Fig. 2B), and xanthine (Fig. 2C) levels were observed at 0 and 30 min after incubation, as well as 30 min after the addition of allopurinol in the blood of C57BL/6J, B6-ChrXc<sup>MSM</sup>, and Uox-KO mice. After 30 min incubation, the UA level was elevated to 20.5 and 14.3 µM in the plasma of C57BL/6J and B6-ChrXc<sup>MSM</sup>, respectively. The plasma UA level in Uox-KO was 194.8 ± 31.7 µM at 0 min, approximately 10 times higher than that in C57BL/6J.

Table 1. The Uric Acid Levels of Blood Incubated in Collection Tubes

| Incubation at room temperature | n   | Uric acid (µM)          |
|-------------------------------|-----|-------------------------|
|                               |     | 0 min | 30 min | 30 min with 1 µM allopurinol |
| Human                         | 3   | 194.8 ± 31.7 | 215.1 ± 32.2 | 202.3 ± 36.4 |
| C57BL/6J mice                 | 5   | 19.5 ± 1.9 | 39.2 ± 3.8 | 14.8 ± 2.1 |

Table 1 Mean ± S.E.M.

Fig. 1. Genotyping of the Hprt Gene in Xc<sup>MSM</sup> Mice

A: Sequences of mouse Hprt<sup>a</sup> and b allele. Hprt<sup>a</sup> is a c.C4G (p.P2A) mutation of Hprt<sup>b</sup>. B: Allelic Discrimination Plot of the Hprt Gene. Red, green, and blue circles represent Hprt<sup>b/b</sup>, Hprt<sup>a/b</sup>, and Hprt<sup>a/a</sup>, respectively.
controls. No elevation was noted in the blood of \textit{Uox}\textsuperscript{-KO} mice after incubation for 30 min or 30 min after the addition of allopurinol, unlike samples taken from the other 2 mouse strains. Hypoxanthine levels in the plasma of C57BL/6J and B6-ChrXC\textsuperscript{MSSM} mice at 0 min were 6.4\pm0.2 and 0 \mu M, respectively, 18.1\pm7.1 and 13.2\pm8.1 \mu M at 30 min, respectively, and 76.4\pm5.4 and 39.2\pm10.1 \mu M after the addition of allopurinol, respectively. In the blood of C57BL/6J and B6-ChrXC\textsuperscript{MSSM} mice, the addition of allopurinol elevated plasma hypoxanthine to approximately 4 and 3 times higher levels than those at 30 min after incubation, respectively. The plasma hypoxanthine level after the addition of allopurinol in the blood of B6-ChrXC\textsuperscript{MSSM} mice was 50.3\% of that of C57BL/6J controls, demonstrating a significant inhibition \((p<0.01)\). The plasma hypoxanthine level in \textit{Uox}-KO mice was 1.3\pm1.3 \mu M at 30 min and 28.8\pm4.1 \mu M at 30 min after the addition of allopurinol, which is significantly lower than the plasma of C57BL/6J controls; however, no hypoxanthine was detected in human
plasma (Table 1). Plasma xanthine levels were unchanged in all mice.

The UA and xanthine levels in the plasma of B6-ChrXC<sup>MSM</sup> mice at 30 min after incubation were 41.1±3.8 µM, significantly lower than those of C57BL/6J controls, 54.6±2.5 µM (Fig. 2D). The sum of the plasma levels of UA, hypoxanthine, and xanthine in B6-ChrXC<sup>MSM</sup> mice were 27.7±1.8 µM, significantly lower than those of C57BL/6J controls, 42.2±1.6 µM at 0 min. Moreover, 30 min after the addition of allopurinol, the sum of the plasma levels of UA, hypoxanthine, and xanthine in B6-ChrXC<sup>MSM</sup> mice were 63.2±9.9 µM, significantly lower than those of C57BL/6J controls, 105.9±3.3 µM (Fig. 2E).

DISCUSSION

Unlike C57BL/6J mice carrying the Hprt<sup>b</sup> allele, expressing the low-active HPRT B protein, B6-ChrXC<sup>MSM</sup> mice possess the Hprt<sup>a</sup> allele, expressing the highly active HPRT A protein. It has been shown that the erythrocyte HPRT activity levels in Mus spretus and Mus m. castaneus possessing the Hprt<sup>a</sup> allele were approximately 70 and 25 times higher, respectively, than those in C57BL/6J controls possessing Hprt<sup>b</sup>.<sup>5</sup> It is speculated that HPRT activity is enhanced because alanine is present at the 2nd amino acid position of HPRT A protein, and this region is not readily degraded by the ubiquitin-proteasome system, compared with HPRT B protein in which proline is present at the same position.<sup>9</sup> In addition, a high HPRT activity level in erythrocytes of Mus m. morocinus, similar to those in M. spretus and M. m. castaneus, has been reported.<sup>8</sup> Suggesting that the erythrocyte HPRT activity level is also high in B6-ChrXC<sup>MSM</sup> mice, a consomic mouse strain with the chromosome portion of Mus m. morocinus in the Hprt gene site.

In B6-ChrXC<sup>MSM</sup> mice, in which the HPRT activity level is higher than that in C57BL/6J controls, conversion of hypoxanthine to IMP is enhanced in erythrocytes, reducing the hypoxanthine level in these cells, which may reduce their release of hypoxanthine. In fact, the plasma UA and xanthine levels in B6-ChrXC<sup>MSM</sup> mice after 30 min incubation was significantly smaller than that in C57BL/6J controls (<i>p</i>=0.019). Moreover, the hypoxanthine level of B6-ChrXC<sup>MSM</sup> mice at 30 min after the addition of allopurinol was significantly smaller than that in C57BL/6J controls (<i>p</i>=0.012). As allopurinol inhibited the generation of UA and xanthine from hypoxanthine as a xanthine oxidase inhibitor, a difference in the hypoxanthine levels was observed between C57BL/6J controls and B6-ChrXC<sup>MSM</sup> mice.

When the plasma UA level was adjusted to similar levels as in human, as in Uox-KO mice by deleting Uox, elevations in the plasma hypoxanthine level were inhibited after 30 min incubation and at 30 min after the addition of allopurinol. As hypoxanthine and UA are transported through a common transporter in the human erythrocyte membrane,<sup>9</sup> it is possible that the high plasma UA levels inhibited hypoxanthine release from erythrocytes. Hiroshige et al. reported that the hypoxanthine level in human plasma doubled from 0.82 µM after 30 min incubation.<sup>10</sup> As this hypoxanthine level was below the detection limit of our system, we could not detect the elevation of hypoxanthine levels in human plasma in this study. Moreover, the activity of xanthine oxidase in human plasma is too small to oxidize hypoxanthine to UA. Thus, the elevation of UA levels in human plasma was not related to the elevation of hypoxanthine levels.

In addition, despite the plasma UA level being high in Uox-KO mice, allopurinol elevated the plasma hypoxanthine level. Since the HPRT activity level in laboratory mouse erythrocyte is lower than that in humans, PRPP may not have been consumed, and may have been metabolized to hypoxanthine through the de novo synthesis system. In fact, it has been reported that the PRPP levels in mouse erythrocytes is approximately 10 times higher than that in human erythrocytes.<sup>23</sup>

In conclusion, we revealed that the false <i>in vitro</i> elevation in the UA level in mouse plasma after blood sampling was due to a low HPRT activity level in mouse erythrocytes and low a plasma UA level, which leads to hypoxanthine release and its conversion to UA as a result of high xanthine oxidase activity in mouse plasma.

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Conflict of Interest The authors declare no conflict of interest.

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