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Establishment of the Process in Blood Sampling and Sample Handling as a Biomarker of Hypoxia-Inducible Diseases; Plasma Hypoxanthine and Xanthine Measurement

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Abstract

Aim: Plasma oxypurines (hypoxanthine and xanthine) levels elevate as soon as blood is collected, there is no method to accurately measure them in clinical settings. We aimed to establish a standard operation in which the plasma oxypurines level for diagnosis or research is measured in clinical site, and verified the efflux mechanism.

Method: Seventeen healthy volunteers were consented. Blood samples were divided into micro-tubes and stored at 4°C in each study. Blood collecting-tubes and operating time-course after blood sampling were evaluated. The inhibitors (nitrobenzylthioinosine (NBMPR), dipirydamole, decynium-22) and the protein expression (equilibrative nucleoside transporter (ENT) 1 and 2.) for nucleobase transporter were also evaluated. Plasma oxypurines and glucose as well as intraerythrocytic oxypurines, ATP and lactic acid levels were measured.

Results: Plasma and intraerythrocytic oxypurine levels time-dependently increased after blood collection. Dipirydamole and Decynium-22 inhibited the plasma hypoxanthine level, but NBMPR did not. And plasma xanthine did not alter it. The *PAX* gene in the collection-tube did not increase plasma hypoxanine level until 9 hrs without adenosine-5'-triphosphate(ATP) expenditure despite ENT1 and 2 protein expressions were observed. Thus, erythrocytic hypoxanthine efflux to plasma was from partial ENT2 and equilibrative nucleobase transporter (ENBT)-1, but ENT1.

Conclusion: The process of blood-sampling and sample handling for plasma hypoxanthine and xanthine measurement as a biomarker of hypoxia-inducible diseases was established.

Keywords: Erythorocyte; Equilibrative Nucleoside Transporters (ENTs); Salvage pathway; Hypoxanthine; Xanthine; Biomarkers

Introduction

Hypoxanthine and xanthine (oxypurines) are a kind of purine nucleobase that is metabolized to xanthine and uric acid by xanthine oxidoreductase (XOR; EC1.1.3.22). Hypoxanthine simultaneously synthesizes purine nucleotide, inosine 5-monophosphate (IMP) by hypoxanthine phosphoribosyltransferase (HPRT; EC2.4.2.8) in human. The latter enzymatic reaction is called as salvage pathway, which recycles the basic materials for reconstitution of DNA, RNA and purine nucleotides, ATP, AMP, GTP, GMP without ATP expenditure, and cooperates with *de novo* pathway [1].

Plasma hypoxanthine is well known as an indicator of hypoxia [2-4], and is also used as a tool for the diagnosis of ischemic heart disease [5,6]. Patients receiving hemodialysis treatment show elevated plasma hypoxanthine level compared to healthy subjects [7,8], and patients in acute respiratory distress syndrome have higher level of plasma hypoxanthine and have high mortality [9]. Patients with cardiovascular disease, respiratory disease and haemolytic disorders are most likely in hypoxia condition, which might lead to disease progression, organ injury and failure [10]. Thus, it is important to accurately measure plasma hypoxanthine level.

It has been reported that there are three of sodium-dependent nucleobase transporter 1 (SNBT1/SLC23A4), equilibrative nucleoside transporters (ENT's), and equilibrative nucleobase transporter 1(ENBT1/SLC43A3) in the purine nucleobase transport system.

Although SNBT1 has been found in rat, but not in human [11], ENTs exist in a variety of mammalian cells and tissue [12], they can be classified into ENT1 (SLC29A1) and ENT2 (SLC29A2) according to the sensitivity to their transporter inhibitor, nitrobenzylthioinosine (NBMPR) [12,13]. Although, dipyridamole can inhibit the activity of widely ENTs, NBMPR can inhibit the activity of ENT1, but not ENT2. Furthermore, decynium-22 can inhibit the activity of ENBT1. In recently, Fujikawa et al. [14] reported that equilibrative nucleobase transporter 1(ENBT1/SLC43A3) is abundantly expressed in the liver and lung, and can convey the purine nucleobases and be inhibited the transport by decynium-22.

To date, there are some reports that determine the plasma oxypurines level. Simmonds et al. [15] reported that plasma hypoxanthine levels increased at 37°C and 4°C rather than room temperature, and Murase et al. [16] reported that the plasma hypoxanthine and xanthine levels after blood collection increased time-dependently at 4°C. On the contrary, Roselyne et al. [17] and Hiroshige et al. [18] reported that

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plasma hypoxanthine levels elevated more at room temperature when compared to on ice or 4°C, respectively. Thus, at present, it seems that blood sample should be centrifuged immediately to accurately measure the level of plasma hypoxanthine [17,19]. However, it is difficult to process the blood samples fast enough to measure it with accuracy in clinical settings. Furthermore, it has been suggested that oxypurines leak into plasma through the membrane by means of ATP degradation in erythrocyte [15,20]. To overcome these issues, we aimed to establish the way in which blood should be collected and handled to determine accurate level of plasma hypoxanthine and xanthine concentrations in clinical site and research.

Material and Methods

Reagents

Dipyridamole was purchased from Wako Pure Chemical Industries (Osaka, Japan), and nitrobenzylmercaptopurine riboside (NBMPR) and Decynium-22 were purchased from Sigma (St. Louis, MO, USA). Blood-collecting tube; EDTA-2K (#367846), 3.8% Buffered Sodium Citrate (#369741) and *PAX* gene DNA (#761165) were purchased from Becton, Dickinson and Company (NJ, USA). [$^{13}C_2$,$^{15}N_2$] uric acid, [$^{13}C_2$,$^{15}N_2$] xanthine, and [$^{13}C_3$,^{15}N] hypoxanthine were synthesized in our laboratory. Human ENT1 and ENT2 antibodies were purchased from Santa Cruz Biotechnology Inc., Cat# sc-377283-HRP (Dallas, TX, USA) and Bioss Antibodies, Cat# bs-13078R-HRP (Woburn, MA, USA), respectively. Monoclonal Anti-β-Actin Clone AC-15, Cat# A5441 and anti-mouse IgG antibody, Cat#NA931V were purchased from Sigma (St. Louis, MO, USA) and GE Healthcare UK Ltd (Amersham Place, Little Chalfont, Buckinghamshire, UK), respectively. Complete protease inhibitor cocktail and phosphatase inhibitors were purchased from Roche (Mannheim, Germany). All other chemicals used were of molecular biology grade.

Blood collection and preparation of plasma and erythrocyte

This study was approved by the Committee of Hirosaki Univercity and Sanwa Kagaku Kenkyusho Co., Ltd., and was performed with the blood sample from 17 healthy volunteers (male 9, female 8) with informed consent. 10 mL blood was collected from brachial veni under the over 10-hrs fasting. Blood samples was divided into an aliquot (0.5 mL), and were kept at 4°C until centrifugation (2000 × g, 4°C, 10 min) to separate plasma and erythrocyte after collection. The erythrocyte was removed with buffy coat.

The efflux effect of hypoxanthine and xanthine by transporter inhibitors

Blood samples mixed with each inhibitor (Dipyridamole, Decynium and NBMPR) were kept 3 hrs at 4°C. Dipyridamole was prepared with the concentration of 1, 10, 100, 500 and 1000 μM, decynium-22 was prepared with the concentration of 0.1, 1, 10, 50 and 100 μM, and NBMPR was prepared with the concentration of 0.005, 0.05, 0.5, 5 and 50 μM. Each inhibitor was reconstituted with dimethylsulfoxide (DMSO). The mixture was centrifuged (2000 × g, 4°C, 10 min), then the supernatant was measured the concentration of hypoxanthine and xanthine using liquid chromatography/triple quadrupole mass spectrometry (LC/TQMS) (Thermo Fisher Scientific Inc, Waltham, MA, USA). Additionally, to confirm the degree of hemolysis according to these drugs or DMSO, the plasma was measured hemoglobin concentration using automatic analyzer (7870 Hitachi), and were expressed as 4 grade by the degree of absorbance, (−); 0–25 mg/mL, (±); 25–50 mg/dL, (+); 50–100 mg/dL, (++); 100–200 mg/dL.

The efflux effect of hypoxanthine on each blood-collection tube

Blood samples withdrawn by blood-collecting tube; EDTA-2K, dipyridamole (100 μM), decynium-22 (10 μM), NBMPR (0.5 μM), *PAX* gene DNA and 3.8% Buffered Sodium Citrate were kept 9 hrs at 4°C. The sample was centrifuged (2000 × g, 4°C, 10 min), then the supernatant was measured the concentration of hypoxanthine using LC/TQMS.

The time-dependent efflux effect of hypoxanthine on each blood-collection tube

Blood samples withdrawn by blood-collecting tube; EDTA-2K, dipyridamole (100 μM), *PAX* gene DNA and 3.8% Buffered Sodium Citrate were kept at 4°C, and then were centrifuged (2000 × g, 4°C, 10 min) at 0, 3, 6, 9 and 24 hrs after blood sampling. The supernatant was measured hypoxanthine, xanthine and glucose level and the pellets were measured the concentration of hypoxanthine, xanthine, lactic acid and ATP as described below.

Purine bodies and glucose determination in plasma or erythrocyte

Packed erythrocyte were hemolyzed with purified water containing [$^{13}C_2$,$^{15}N_2$] xanthine, [$^{13}C_3$,^{15}N] hypoxanthine and [$^{13}C2$,$^{15}N_2$] uric acid as internal standard (IS mixture). Plasma samples were added into MeOH containing the IS mixture, and then they were centrifuged at 2,000 × g, 4°C for 10 min. The supernatants were dried using a centrifugal evaporator and reconstituted with 150 μL distilled water. These samples were filtered through an ultrafiltration membrane (Amicon® Ultra-0.5, 3K, Millipore), and hypoxanthine and xanthine measured using LC/TQMS. Plasma glucose was determined by glucose CII test WAKO Pure Chemical Industries (Osaka, Japan).

Determination of intra-erythrocytic ATP and lactic acid concentration

The samples of packed erythrocyte were vigorously mixed with water using a vortex mixer for several seconds, and were filtered through ultrafiltration membrane (Amicon® Ultra-0.5, 3K, Millipore). The filtrate were measured by luminescent measurement (Spectra MAX GEMINI EM, Molecular Devices, LLC., San Jose, CA, USA) using ATP determination kit, TA100 (TOYO B-Net Co., Ltd., Tokyo, Japan), lactic acid concentration was determined using Determiner-LA (KYOWA medics Co., Ltd, Tokyo, Japan).

Electrophoresis and western blot

Red blood cell membrane protein preparation was performed as described previously [21]. In brief, 490 μL of water including in proteinase and phosphatase inhibitors was added to 50 μL of packed erythrocyte, and was aggressively mixing by vortex for several seconds. 60 μL of approximately 100 mM phosphate-buffer saline (PBS) was added, and was centrifuged at 20,000 × g for 20 min 4°C. The pellet was pooled when it was washed 2 times in ice cold PBS of approximately 10 mM, and then was dissolved in 100 μL of RIPA buffer including in protease inhibitor (Complete; Cat.No. 04693126001, Roche, Mannheim Germany) and phosphatase inhibitor (PhosSTOP; Cat.No. 4906837001, Roche, Mannheim Germany), and stored −80°C until analysis. Protein concentration was measured by the BCA protein assay kit (Thermo Fisher Scientific K.K. Tokyo, Japan). Protein samples were boiled in

Laemmli sample buffer (Bio-Rad Laboratories, Inc, Hercules, CA, USA) for 5 min at 95°C, and were electrophoresed using 7.5% SDS-PAGE gel (Bio-Rad Laboratories, Inc, Hercules, CA, USA). Then, western blotting for ENT1 and ENT2 protein was performed, the protein was transferred to PVDF membrane (Cat#170-4156, Bio-Rad Laboratories, Inc, Hercules, CA, USA) by Trans-BLOT TURBO(Bio-Rad Laboratories, Inc, Hercules, CA, USA), and was captured for 10 to 30 seconds using ECL Prime Western Blotting Detection Reagent, RPN2232 (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK) by ChemiDock XRS Plus (Bio-Rad Laboratories, Inc, Hercules, CA, USA). Both primary antibodies were used at 1:500 with 2% skim milk. And then, re-blotting for the β-actin was performed using the PVDF membrane bathing application of Re-blot Plus Mild Antibody Stripping Solution (2502 EMO Millipore Corp. USA), anti-β-actin antibody and the mouse IgG secondary antibody were used at 1:2000 with Can Get Signal Immunoreaction solution 1 (Toyobo Co., Ltd. Osaka, Japan) and Can Get Signal Immunoreaction solution 2 (Toyobo Co., Ltd. Osaka, Japan), respectively.

Statistical analysis

Data were expressed as means ± standard deviation and were statistically analyzed using the statistical analysis software (SAS) program version 8.0 (SAS Institute, Cary, NC, USA). Statistical analyses were performed by Williams test on the time course study and by Steel's-test following Bartlett analysis for plasma hypoxanthine level at 9 hrs after blood-sampling, respectively. Less than 0.025 or 0.05 of P-value was regarded as statistically significant in the Williams test or Steel test, respectively.

Results

The efflux effect of hypoxanthine and xanthine by mean of nucleobase transporter inhibitor, NBMPR, dipyridamole and decynium-22

To investigate which transporter was in charge with the efflux to the plasma, purine nucleobase transporter inhibitors, NBMPR, dipyridamole and decynium-22 were evaluated. NBMPR did not alter the plasma hypoxanthine level in the all concentration at all, on the contrary, dipyridamole potently decreased at over 500 μM and decynium-22 declined weakly at over 10 μM (Figures 1A-1C). On the other hand, xanthine did not influence the plasma level by these inhibitors (Figures 1D-1F). According to the plasma hemoglobin level, dipyridamole and decynium-22 showed severely (++) the degree of hemolysis condition at over 500 μM and at over 50 μM, respectively. O'Connor et al. reported [22] that hemolysis let you complicate a diagnosis. Thus, in the following studies, diypridamole and decynium-22 were applied to the concentration at 100 μM and 10 μM, respectively.

The concentration of plasma hypoxanthine at 9 hrs after blood-sampling on each blood collection tube

The plasma hypoxanthine levels at 9 hrs after blood-sampling using blood collection tube, EDTA-2K, increased significantly relative to that of the initial, and dipyridamole, decynium-22, NBMPR and sodium citrate did not deceased it. Contrary, the collection-tube, *PAX* gene significantly suppressed the level compared with EDTA-2K (Figure 2).

ENT1 and 2 protein expression in erythrocyte

To confirm the protein of the nucleobase transporter, western analysis following SDS-PAGE was performed, and evaluated qualitatively. The ENT1 and ENT2 protein expressions in erythrocyte plasma membrane were confirmed at 9 hrs after blood sampling in all 7 tubes (Figure 3).

The time-dependent plasma and intraerythrocytic hypoxanthne concentration on each blood-collection tube

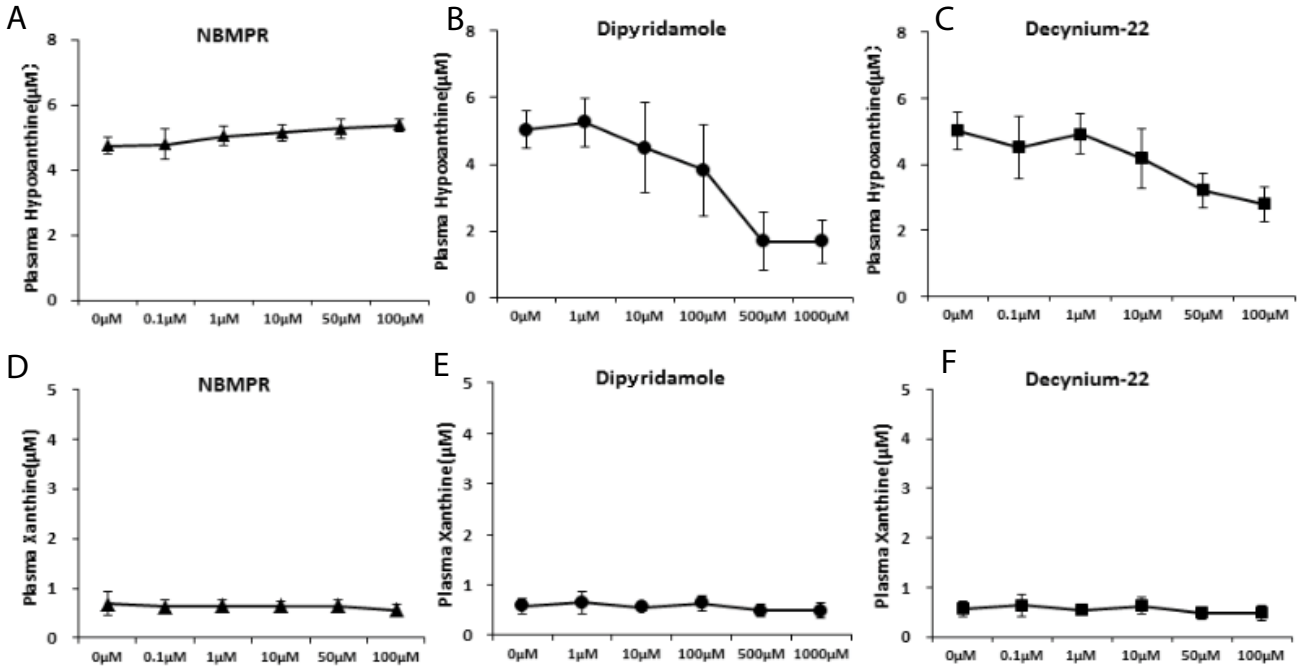

Figure 1: The efflux effect of hypoxanthine **(A-C)** and xanthine **(D-F)** by nucleobase transporter inhibitor, NBMPR, dipyridamole and decynium-22 at 3hr from blood collection using EDTA-2K. Data are expressed as mean ± SD (n=4).

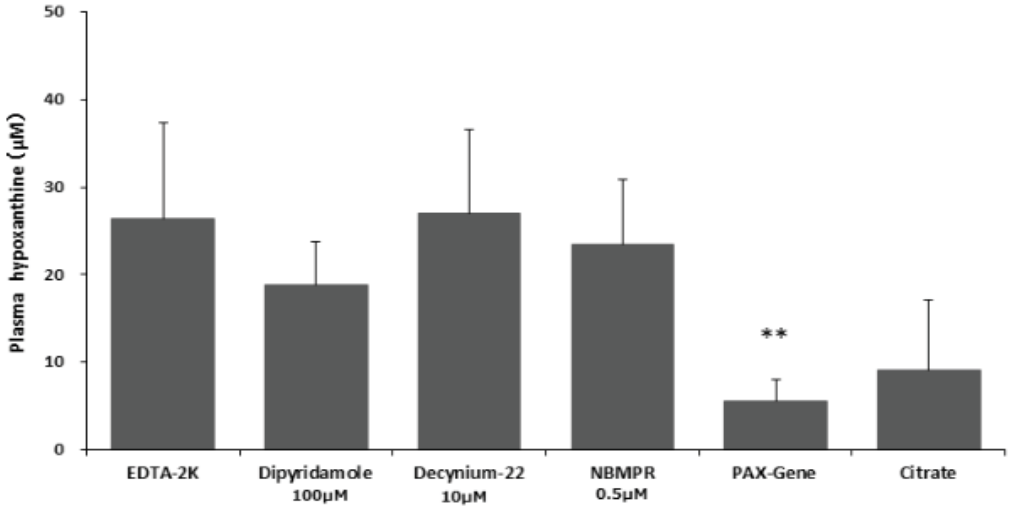

Figure 2: The concentration of human plasma hypoxanthine at 9hr after blood-sampling in each blood collection tube. Data are expressed as mean ± SD (n=7). ¨P<0.01 vs. EDTA-2K group (Steel's-test). Less than 0.05 of P-value was regarded as statistically significant.

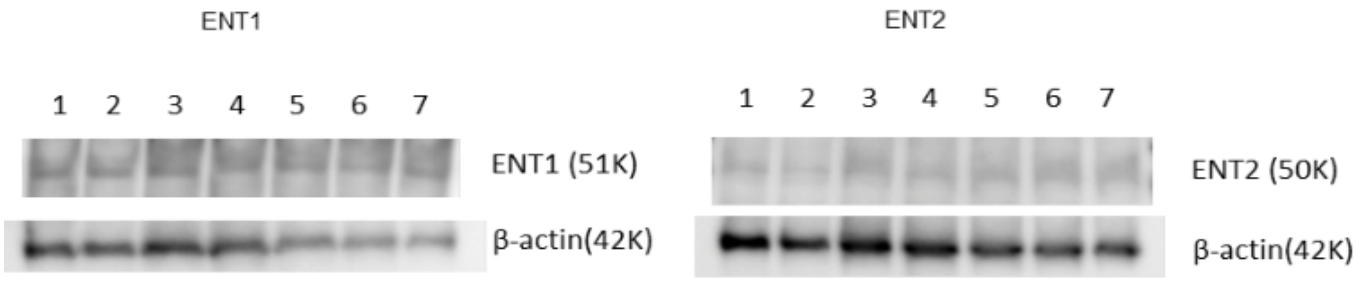

Figure 3: The protein expression of the nucleobase transporter, ENT1 and 2 in pool erythrocyte gathered from three persons after blood sampling in each blood-collecting tube; Lane 1: EDTA-2K at 0 hr, Lane 2: EDTA-2K, 9 hr, 4°C, Lane 3: Dypiridamole, 9 hr, 4°C, Lane 4: Decynium-22, 9 hr, 4°C, Lane 5: NBMPR, 9 hr, 4°C, Lane 6: PAX gene, 9 hr, 4°C, Lane 7: Sodium citrate, 9 hr, 4°C.

At 0 hrs after blood sampling, plasma hypoxanthine concentration in collection-tube, sodium citrate was 1.44 ± 0.5 μM, which was the lowest than that of others (EDTA-2K; 2.09 ± 0.6 μM, dypiridamole; 1.82 ± 0.5 μM, *PAX*gene; 1.93 ± 0.4 μM, n=4). Plasma hypoxanthine concentration after blood-sampling using blood-collection tube, EDTA-2K and dypiridamole increased time-dependently and the collection tube, *PAX* gene and sodium citrate significantly suppressed the level until 9 hrs (Figures 4A-4D). Additonally, intraerythrocytic hypoxanthine excursion was almost the same as that of plasma (Figures 4E-4H).

The time-dependent plasma glucose and intraerythrocitec ATP and lactic acid levels on each blood-collection tube

The plasma glucose levels after blood sampling in all blood collection-tube exception for sodium citrate were time-dependently decreased (Figures 4I-4L). Intraerythrocytic ATP levels in each collection-tube were almost plateau until 9 hrs after blood sampling, and EDTA-2K was dramatically decreased the level at 24 hrs after blood sampling, but dipirydamole, *PAX* gene and sodium citrate were kept until 24 hrs (Figures 4M-4P). Intraerythrocytic lactic acid concentrations were time-dependently increased (Figures 4Q-4T).

Discussion

Murase et al. [16] reported that the plasma hypoxanthine and xanthine levels after blood-sampling increased time-dependently. It was suggested that the risen hypoxanthine and xanthine in blood specimen were released via nucleobase transporter on the erythrocytic membrane [20]. Thus, to elucidate which transporter was in charge with the efflux to the plasma, each transporter inhibitor (NBMPR for ENT1, Dipyridamole for ENT1 and 2, and Decynium-22 for ENBT1) was evaluated the plasma oxypurines levels after blood-sampling. NBMPR in the micromolar levels did not alter the plasma hypoxanthine concentrations at all (Figure 1A), suggesting that ENT1 might be poor contribution of the hypoxanthine efflux in the red blood cells. However, Yao et al. reported [23] that NBMPR in the concentration of 1 and 10 μM inhibited the uptake of the hypoxanthine by recombinant human ENT1 produced in Xenopus oocytes. This contradicting result might be attributed to the difference between erythrocyte and oocyte, or influx and efflux study. In the present study, dipyridamole and decynium-22 suppressed the plasma hypoxanthine levels at over 100 μM and 10 μM, respectively (Figures 1B and 1C). Thus, it was assumed that both inhibitors inhibited the hypoxanthine efflux in the micromolar range. Therefore, it was suggested that plasma hypoxanthine was from erythrocyte via ENT2 and ENBT1, but not ENT1 on the erythrocytic membrane (Figure 5).

On the other hand, there was no change in the plasma xanthine

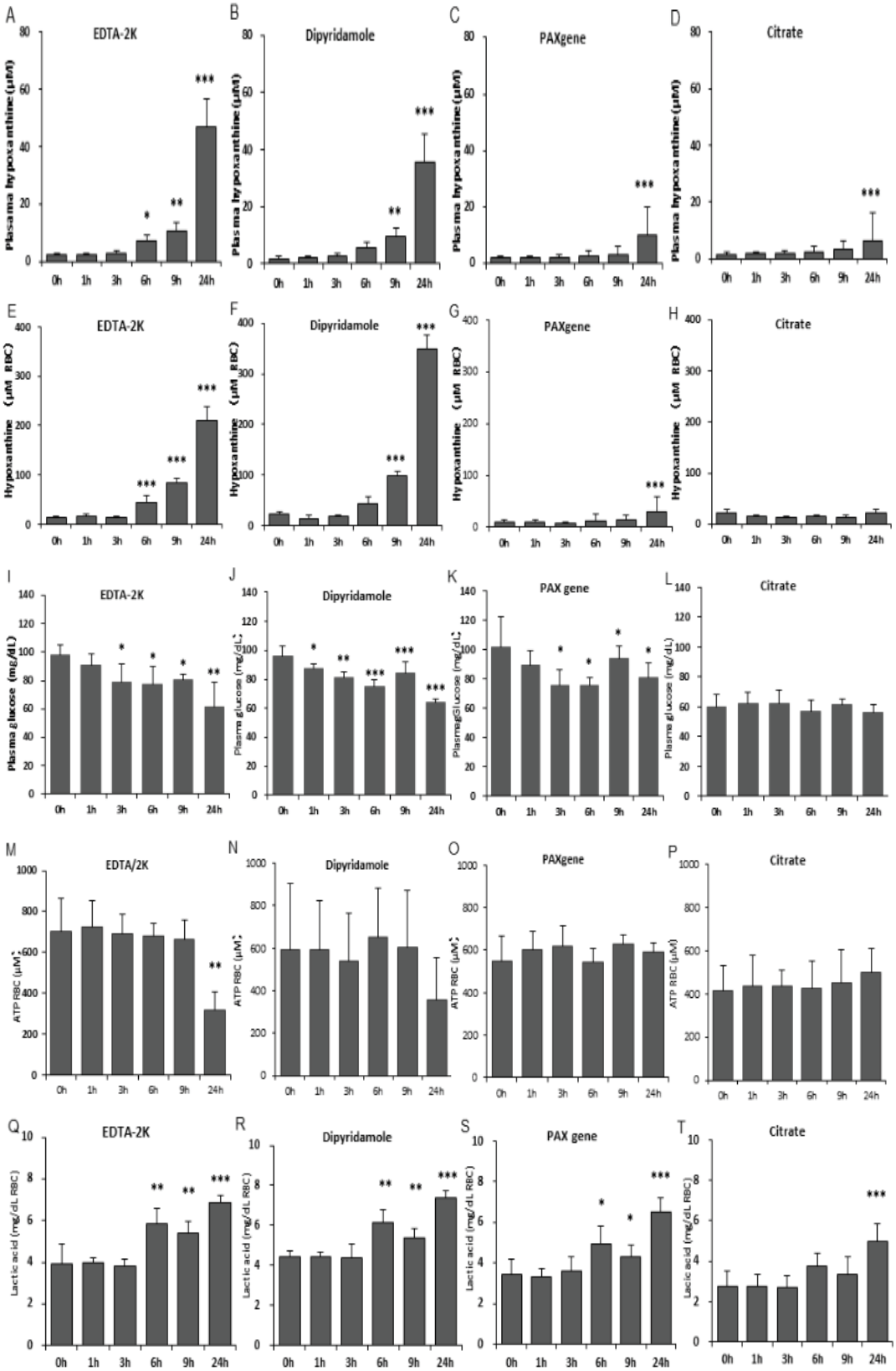

Figure 4: The time course of plasma hypoxanthine (A-D), Erythrocyte hypoxanthine (E-H), plasma glucose (I-L), erythrocyte ATP (M-P), and lactic acid (Q-T) from blood collection on each blood collection tube. Data are expressed as mean ± SD (n=4). *p<0.025, **p<0.005, ***p<0.0005 vs 0h (Williams tests).

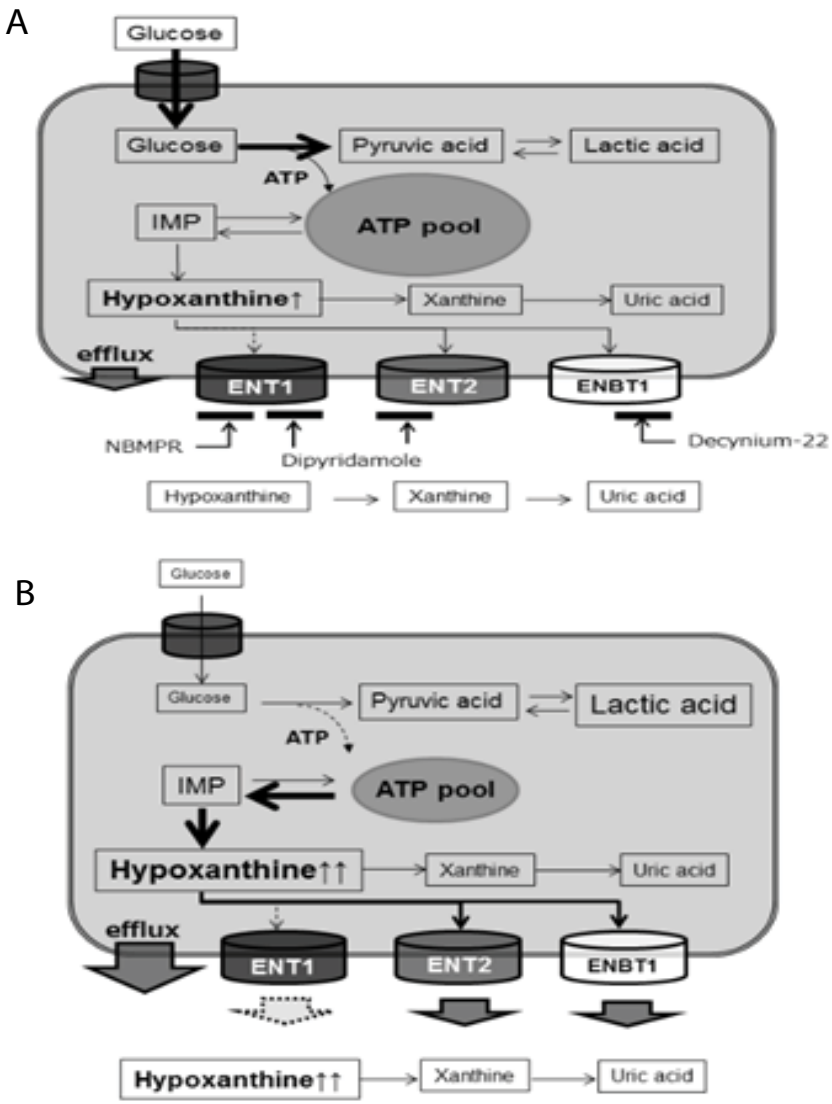

Figure 5: Intraerythrocytic enargy flow until at 9 hour **(A)** and at 24 hour **(B)** after blood-sampling. A shows glucose uptake and the utilization in the early stage after blood-sampling. B shows ATP catabolism owing to reduction of glucose uptake and incerase efflux of intraerythrocytic hypoxanthine to the plasma via ENT2 and ENBT1.

level by transporter inhibitors (Figures 1D-1F). In generally, purine nucleobases are hydrophilic, which can permeate plasma membrane by mean of facilitative diffusion depending on the concentration of the substrate [13]. The plasma and erythrocytic hypoxanthine concentration at 0 hrs after blood-sampling using blood collection-tube, EDTA-2K were 2.09 ± 0.62 μM (n=4) and 13.4 ± 3.1 μM (n=4), respectively (Figures 4A and 4E), and the ratio (intraerythrocytic / plasma) was 6.58 ± 1.1 (n=4). Thus, it was suggested that the time-dependently elevated hypoxanthine in the red blood cell diffused into the plasma. However, the ratio of xanthine level was 1.01 ± 1.1 (n=4), the concentration gradient of xanthine in intra-and extra-erythrocyte was almost equal at each blood-sampling time (Supplementary Figure 1). Thus, the xanthine efflux to plasma was weaker than that of hypoxanthine. Therefore, no transporter inhibitors observed the reduction of the plasma xanthine level (Figures 1D-1F).

Additionally, in the present study, the expression of the ENT2 protein on human erythrocytic membrane was observed for the first time. Despite the fact that the protein expression of ENT1 and 2 in red blood cells at 9 hrs after blood-sampling was ubiquitously confirmed in the all blood collection-tube (Figure 3), the plasma hypoxanthine in *PAX* gene and sodium citrate was decreased relatively to that of EDTA-2K (Figures 4C and 4D). Thus, it suggested that the *PAX* gene and sodium citrate might be inactivation of the transport activity, but not the degradation.

The plasma hypoxanthine concentration at 0 hrs after blood-sampling in collection-tube, sodium citrate was the lowest value as 1.44 ± 0.5 μM relative to that of others (Figure 4D). This was because that the collection-tube was filled with 0.6 mL aliquot of the citrate buffer. Thus, the usage of the sodium citrate in blood collection tube was estimated to be lower than that of others.

In the view point of plasma oxypurines (hypoxanthine and

xanthine), Harmanjit et al. reported [24] that the ratio of hypoxanthine/xanthine was significantly elevated in patients of obstructive sleep apnea syndrome, Lorenzi et al. [25] that plasma oxypurines are significantly elevated in patients with gastric and colorectal cancer, and are related to the stage of the tumors. In the present study, the ratio at 9 hrs after blood-sampling using blood collection-tube, EDTA-2K increased increase to 18.1 ± 5.99 from 3.5 ± 0.76 (n=4) (Supplementary Figure 2), suggesting that the increase in the ratio might be also an indicator of tissue hypoxia. Therefore, plasma hypoxanthine as well as xanthine might become a biomarker to diagnosis or treat for these diseases, it is important to be able sequentially and accurately to measure it.

The natural cause of intraerythrocytic ATP level after blood-sampling using collection-tube, EDTA-2K was almost plateau until 9 hrs and decreased at 24 hrs, but plasma glucose level decreased time-dependently (Figures 4I and 4M) and intraerythrocytic lactic acid level increased time-dependently (Figure 4Q). These results were suggested that intraerythrocytic glucose might be utilized faster than ATP. Matured red blood cells can not synthesize purine via the *de novo* pathway [26], so that need to be supplied energy through glycolysis for the survival (Figure 5). Actually, in the preliminary study, in blood collecting tube, sodium fluoride which is enolase inhibitor in glycolysis, the plasma hypoxanthine level increased time-dependently up to 59.4 ± 6.3 μM (n=3) at 9 hrs after blood-sampling (Supplementary Figure 3). Thus, the intraerythrocitic ATP concentration at 24 hrs after blood-sampling was dramatically declined to be accelerated purinenucleoside catabolism. Nevertheless, the intraerythrocytic ATP concentration at 24 hrs after blood sampling in the *PAX* gene and sodium citrate blood collecting tube did not alter (Figures 4O and 4P). It was considered that the biological activity including in the enzyme and transporters in the erythrocyte might be inactivated. The evaluation time that concerns erythrocyte hypoxanthine efflux on each blood collection tube was set up to 9 hrs after blood-sampling. This is because health professionals work 8-9 hrs a day. Thus, this setting is considered reasonable.

Conclusion

This is the first report which overcame some issues that encounters throughout the process of blood-sampling and handling the samples in clinical setting to accurately determine the plasma hypoxanthine and xanthine levels. Plasma hypoxanthine was from intraerythrocytic hypoxanthine via ENT2 and ENBT1, but not ENT1, but plasma xanthine was not clear. Thus, it is necessary to need some kind of strategy to measure plasma oxypurines, which it should be the usage of blood collecting tube such as dypiridamole, *PAX* gene and citrate with EDTA-2K. Especially, *PAX* gene enabled medical-workers to determine the plasma oxypurines level, which can help as a biomarker for clinical diagnosis or research of hypoxia-inducible diseases such as cardiovascular or respiratory disease, sleep apnea syndrome and cancer, etc.

Author contributions

T.N. and T.M. wrote the manuscript. C.H., H.K., and H.T reviewed manuscript. T.N., T.M., C.H., N.K., and Y.N. designed the research. T.M. and E.S. performed the research. T.N., H.K., and H.T. analyzed the data.

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Conflicts of Interest

The authors have no conflicts of interest to disclose.

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