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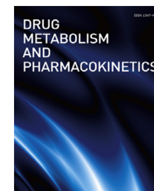

Regular Article

Investigation of the transport of xanthine dehydrogenase inhibitors by the urate transporter ABCG2

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ABSTRACT

Hyperuricemia induces gout and kidney stones and accelerates the progression of renal and cardiovascular diseases. Adenosine 5′-triphosphate-binding cassette subfamily G member 2 (ABCG2) is a urate transporter, and common dysfunctional variants of ABCG2, non-functional Q126X (rs72552713) and semi-functional Q141K (rs2231142), are risk factors for hyperuricemia and gout. A recent genome wide association study suggested that allopurinol, a serum uric acid-lowering drug that inhibits xanthine dehydrogenase, is a potent substrate of ABCG2. In this study, we aimed to examine the transport of xanthine dehydrogenase inhibitors via ABCG2. Our results show that ABCG2 transports oxypurinol, an active metabolite of allopurinol, whereas allopurinol and febuxostat, a new xanthine dehydrogenase inhibitor, are not substrates of ABCG2. The amount of oxypurinol transported by ABCG2 vesicles significantly increased in the presence of ATP, compared to that observed with mock vesicles. This indicates that the serum level of oxypurinol would increase in patients with ABCG2 dysfunction. Since the half-life of oxypurinol is longer than that of allopurinol, the xanthine dehydrogenase-inhibiting effect of allopurinol mainly depends on its metabolite, oxypurinol.
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1. Introduction

Uric acid (UA) is the end product of purine metabolism in humans. Elevated serum uric acid (SUA) levels cause gout and accelerate the progression of renal and cardiovascular diseases [1–4]. Genetic risk factors for gout and hyperuricemia have been investigated through large genome-wide association studies (GWAS) [5–8]. Recently the adenosine 5′-triphosphate (ATP)-binding cassette (ABC) subfamily G member 2 (ABCG2) gene was shown to be associated with gout [9–11]. ABCG2, also known as breast cancer resistance protein (BCRP), encodes a multi-specific efflux transporter [12–14]. It mediates drug resistance and affects the pharmacological behavior of many compounds [15,16]. ABCG2 is expressed on the apical membrane in several tissues, including intestine, liver, and kidney [17,18]. Previously, we identified ABCG2 as a high-capacity urate exporter that mediates renal and/or extra-renal urate excretion, and characterized the ABCG2 mutants Q141K and Q126X [9,19]. The Q141K mutation results in a Gln to Lys substitution at position 141 and causes about 50% loss of the urate transport function of ABCG2 [9]. The nonsense mutation Q126X leads to complete dysfunction of the urate transport. Q126X and Q141K are assigned to different risk haplotypes and we showed that dysfunctional genotype combinations of ABCG2 genes (Q126X and Q141K) were major causes of hyperuricemia and gout [9,20,21].

Allopurinol is a SUA-lowering drug for hyperuricemia and gout [22] and is metabolized to oxypurinol. Both compounds act as inhibitors of xanthine dehydrogenase (XDH), the enzyme that converts hypoxanthine to xanthine and xanthine into UA, thereby lowering SUA levels [23,24]. In addition, febuxostat (FBX) is an alternative treatment for hyperuricemia and gout [25,26]. FBX is a non-purine selective inhibitor of XDH and has no effect on other enzymes involved in purine and pyrimidine metabolism [27]. ABCG2 regulates the effect of many cytosolic and cytotoxic drugs by ATP-dependent efflux. If ABCG2 is involved in the elimination of XDH inhibitors, their pharmacokinetics will differ in patients with ABCG2 dysfunctional mutations. Recently, Wen et al. conducted a GWAS that examined the genetic factors affecting allopurinol-related SUA reduction [28]. They identified ABCG2 as a

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determinant of response to allopurinol. ABCG2 has been reported to transport purine analogs and affect their cytotoxicity and tissue distribution [29]. Cell accumulation studies in ABCG2-overexpressed cells have shown accumulation of [^{3}H]-labeled allopurinol and oxypurinol in the presence of an ABCG2 inhibitor [28]. From these results, it was suggested that ABCG2 is involved in the efflux of allopurinol and oxypurinol. In other words, ABCG2 and its dysfunction are thought to affect allopurinol-based therapy for hyperuricemia and gout.

In this study, to examine the transport of XDH inhibitors via ABCG2, we performed ABCG2 transport assay using the plasma membrane vesicles. Allopurinol, oxypurinol, and FBX were incubated with the ABCG2-expressed vesicles, and the transport amounts were analyzed using high-performance liquid chromatography (HPLC).

2. Materials and methods

2.1. Materials

Monosodium urate, allopurinol, oxypurinol, and horseradish peroxidase (HRP) substrate ImmunoStar Zeta kits were obtained from Wako Pure Chemical Industries (Osaka, Japan). FBX was donated by Teijin Pharma (Tokyo, Japan). The structure of compounds in this study was shown in Fig. S1 (Supplementary file). Ko143 hydrate was obtained from Sigma-Aldrich (St. Louis, MO, USA). ATP, adenosine 5′-monophosphate (AMP), creatine kinase (CK), and creatine phosphate (CP) were purchased from Oriental Yeast (Tokyo, Japan). Mouse anti-ABCG2 antibody BXP-21 and the HRP-conjugated anti-mouse IgG antibody were obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

2.2. Preparation of plasmids

Human ABCG2 cDNA was cloned from mRNA of the human hepatocellular carcinoma cell line HepG2 (Riken Bioresource Center, Tsukuba, Japan). Reverse transcription-PCR was carried out using the PrimeScript first strand cDNA synthesis kit (Takara Bio Inc., Shiga, Japan) and KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan). The following specific primers were used for PCR: sense 5′-CCGGATC-CATGTCTTCCAGTAATGTCGA-3′ and antisense 5′-GGCTCGAGTTAA-GAATATTTTTTAAGAA-3′. The PCR reaction consisted of 40 cycles of 98 °C for 10 s, 44 °C for 30 s, and 68 °C for 1 min. The resulting PCR product was inserted into the pBluescript II SK (+) vector (Agilent Technologies, Santa Clara, CA, USA). The ABCG2 cDNA was removed from the vector by *Bam*HI/*Xho*I digestion. The digested ABCG2 cDNA was inserted into the *Bam*HI and *Xho*I sites of the pcDNA 3.1 expression vector (Thermo Fisher Scientific, Waltham, MA, USA) using the Takara DNA ligation kit v. 2.1 (Takara Bio, Shiga, Japan). Finally, *Escherichia coli* JM109 competent cells were transformed with the ligated product.

2.3. Cell culture

Human embryonic kidney (HEK) 293 cells were obtained from Riken Bioresource Center (Tsukuba, Japan). They were maintained in high-glucose Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.4. Expression of ABCG2 in HEK 293 cells

HEK 293 cells were seeded at 4×10^6 cells/dish in 10-cm dishes and incubated for 24 h at 37 °C. The transfection protocol was modified from Reed et al. [30]. The pcDNA-ABCG2 plasmid was mixed with 150 mM NaCl at a concentration of 40 μg/500 μL. Linear polyethylenimine (PEI, Polysciences, Warrington, PA, USA) was also added to the 150 mM NaCl and the final concentration was 0.10 μg/500 μL. The PEI mixture and the plasmid mixtures were mixed together and incubated for 10 min at room temperature. One milliliter of this mixture was added to the culture medium in each dish. Mock cells were prepared by transfecting HEK 293 cells with the empty pcDNA 3.1 vector. Forty-eight hours after transfection, cells were harvested by centrifugation (200×*g*, 3 min at room temperature). Cells were subsequently washed with phosphate-buffered saline (PBS) and stored at −80 °C until use.

2.5. Preparation of membrane vesicles

Plasma membrane vesicles were prepared by the method of Kondo et al. with minor modifications [31]. In brief, the frozen cell pellet was diluted with a hypotonic buffer (1 mM Tris-HCl/0.5 mM EDTA, pH 7.5) containing Complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), and rotated for 1 h at 4 °C. The cell suspension was centrifuged at 100,000×*g* for 1 h at 4 °C and the pellet was suspended in Tris-sucrose (TS) buffer (0.25 M sucrose and 10 mM Tris-HCl, pH 7.4), then homogenized with a Potter-Elvehjem homogenizer. The crude homogenate was layered over 38% (w/v) sucrose-containing 5 mM Hepes-KOH, pH 7.4, and centrifuged at 200,000×*g* for 2 h at 4 °C. The turbid layer at the interface was collected and suspended in TS buffer. The suspension was centrifuged at 100,000×*g* for 2 h at 4 °C and the pellet was suspended in TS buffer. Vesicles were formed by passing the suspension 30 times through a 25-gauge needle. The protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Aliquots of the vesicles were stored at −80 °C until use.

2.6. Western blotting

One microgram of the prepared vesicles was subjected to electrophoresis on a 10% SDS-polyacrylamide gel and were electro-blotted onto Protran BA85 nitrocellulose membranes (GE Healthcare UK, Little Chalfont, Buckinghamshire, UK). The blots were blocked for 30 min at room temperature with PBS containing 5% skim milk. After three washing steps with PBS containing 0.1% tween 20 (PBS-T), the blots were incubated with mouse anti-ABCG2 antibody (1:1000 dilution in 0.1% bovine serum albumin/PBS) for 1 h at room temperature. After washing, the secondary HRP-conjugated anti-mouse IgG antibody (1:2000 dilution in 0.1% bovine serum albumin/PBS) was added to the blots and incubated for 1 h at room temperature. Then, the blots were washed three times with PBS-T. HRP-dependent luminescence was developed using an Immunostar Zeta kit and was detected using an Image Analyzer LAS-1000 (GE Healthcare UK, Little Chalfont, Buckinghamshire, UK).

2.7. Transport studies

Membrane vesicles (10 μg of protein) were incubated with the compound (UA, allopurinol, oxypurinol, or FBX) at the indicated concentration in 20 μL of incubation buffer. To clarify whether ABCG2 transports allopurinol, oxypurinol, and FBX, their experimental concentrations were relatively high (0–3 mM), comparing to the ordinary peak blood concentrations (10, 30 and 50 μM, respectively). The incubation buffer comprised 10 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose, 10 mM $MgCl_2$, 100 μg/mL CK, 100 mM CP and 5 mM of ATP. For the ATP(−) sample, ATP was replaced with AMP. UA was dissolved by ultra-sonication. After

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