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Hyperuricemia influences tryptophan metabolism via inhibition of multidrug resistance protein 4 (MRP4) and breast cancer resistance protein (BCRP)

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ABSTRACT

Hyperuricemia is related to a variety of pathologies, including chronic kidney disease (CKD). However, the pathophysiological mechanisms underlying disease development are not yet fully elucidated. Here, we studied the effect of hyperuricemia on tryptophan metabolism and the potential role herein of two important uric acid efflux transporters, multidrug resistance protein 4 (MRP4) and breast cancer resistance protein (BCRP). Hyperuricemia was induced in mice by treatment with the uricase inhibitor oxonic acid, confirmed by the presence of urate crystals in the urine of treated animals. A transport assay, using membrane vesicles of cells overexpressing the transporters, revealed that uric acid inhibited substrate-specific transport by BCRP at clinically relevant concentrations (calculated IC_{50} value: 365 ± 13 μM), as was previously reported for MRP4. Moreover, we identified kynurenic acid as a novel substrate for MRP4 and BCRP. This finding was corroborated by increased plasma levels of kynurenic acid observed in $Mrp4^{-/-}$ (107 ± 19 nM; $P = 0.145$) and $Bcrp^{-/-}$ mice (133 ± 10 nM; $P = 0.0007$) compared to wild type animals (71 ± 11 nM). Hyperuricemia was associated with >1.5 fold increase in plasma kynurenine levels in all strains. Moreover, hyperuricemia led to elevated plasma kynurenic acid levels (128 ± 13 nM, $P = 0.005$) in wild type mice but did not further increase kynurenic acid levels in knockout mice. Based on our results, we postulate that elevated uric acid levels hamper MRP4 and BCRP functioning, thereby promoting the retention of other potentially toxic substrates, including kynurenic acid, which could contribute to the development of CKD.

1. Introduction

Uric acid is a weak organic acid and the end-product of purine nucleotides degradation in humans. One of the enzymes involved in this process is xanthine oxidoreductase, which enables the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid. During this reaction, reactive oxygen species are generated as by-product [1,2]. Therefore, uric acid is recognized as a marker for oxidative stress. However, the molecule itself has antioxidant properties and can act as a free radical scavenger and chelator of transitional metal ions which are converted into poorly reactive forms [3]. Hyperuricemia, i.e. elevated plasma uric acid levels (≥360 μM) [4], is related to a variety of pathologies, including gout, cardiovascular disease and chronic kidney disease (CKD). Gout is the most common form of inflammatory arthritis caused by sodium uric acid crystal precipitation, which is followed by phagocytosis of the crystals by neutrophils and macrophages and activation of acute

Abbreviations: AHR, aryl hydrocarbon receptor; ANOVA, analysis of variance; BCRP, breast cancer resistance protein; CKD, chronic kidney disease; Ct, cycle threshold; E_1S, estrone sulphate; EDX, energy-dispersive X-ray; EM, electron microscopy; eYFP, enhanced yellow fluorescent protein; FVB, friend leukemia virus B; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; HE stain, hematoxylin and eosin stain; HEK293 cells, human embryonic kidney cells; IC_{50}, half maximal inhibitory concentration; IDO, indoleamine 2,3-dioxygenase; Kim-1, kidney injury molecule-1; LC–MS/MS, liquid chromatography-tandem mass spectrometry; MRP4, multidrug resistance protein 4; MTX, methotrexate; Ngal, neutrophil gelatinase-associated lipocalin; OAT, organic anion transporter; SEM, standard error of mean; SLC, solute carrier family; SNP, single nucleotide polymorphism; URAT1, urate transporter 1

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inflammation and tissue injury [5]. Epidemiological studies show that prevalence and incidence are still increasing [6,7]. Formation of uric acid crystals is also the cause of nephrolithiasis, i.e. kidney stones, which is significantly more common among patients diagnosed with metabolic syndrome, obesity and type 2 diabetes [8]. Hyperuricemia also correlates with the development and progression of cardiovascular diseases [9–11], potentially via interfering with nitric oxide function. In animal models, it has been shown that mild hyperuricemia contributes to the development of hypertension as a result of endothelial dysfunction and reduction of nitric oxide levels [10,12,13]. Recently, hyperuricemia has received attention as a possible risk factor for CKD [4,14], which affects approximately 10% of the adult population in developed countries [15]. Hyperuricemia has been associated with a hazard ratio of 2.1 and 1.3 for men and women for developing CKD [4], respectively. Several mechanisms were proposed via which uric acid could contribute to the development of CKD, including uric acid-induced glomerular hypertrophy and endothelial dysfunction [4,16]. However, the pathophysiological mechanism has as of yet not been fully elucidated.

In healthy individuals, two-thirds of uric acid is excreted by the kidney and one-third by the intestine due to breakdown of urate by gut bacteria. Purine ingestion, endogenous synthesis of purines from nonpurine precursors and reutilization of preformed purine compounds are the sources of uric acid production, a process that, under steady-state conditions, is in balance with the uric acid disposal [17,18]. Hyperuricemia can develop due to overproduction or a diminished excretion of uric acid. Maintaining uric acid homeostasis is highly dependent on kidney function and regulated by a number of transporters, including the urate transporter 1 (URAT1; *SLC22A12*) – responsible for up to 99% of uric acid reabsorption after glomerular filtration – the facilitated glucose transporter (solute carrier family 2 member 9 (*SLC2A9*)) [19], several organic anion transporters including OAT1 (*SLC22A6*) and OAT3 (*SLC22A8*) [5], and the ATP-dependent urate efflux transporters multidrug resistance protein 4 (MRP4; *ABCC4*) [20] and breast cancer resistance protein (BCRP; *ABCG2*) [21–23].

As uric acid is one of the important factors in a variety of pathologies, tight regulation of this metabolite is of key importance. The vital role of transporters in uric acid homeostasis can clearly be observed in patients suffering from hyperuricemia due to single nucleotide polymorphisms (SNPs) that render the transporters inactive, such as the common SNP *C421A* encoding the Q141K mutation of BCRP [21,22,24] and several genetic variants for *SLC2A9* [19]. Next to genetic factors, high plasma levels of uric acid might also result in a reduced transporter activity [20]. Since these transporters are also involved in the excretion of a wide variety of other compounds, changes in transport efficacy could result in metabolic disturbances. This hypothesis is corroborated by two recent studies showing that high uric acid levels in patients with acute gout were associated with altered tryptophan concentrations in plasma and urine [25,26]. Therefore, the aim of our study was to investigate the effect of hyperuricemia on tryptophan metabolism and the potential role herein of two important uric acid efflux transporters, MRP4 and BCRP. Both transporters are expressed in the apical membrane of renal proximal tubule cells, amongst other tissues, and are involved in the urinary excretion of a multitude of endogenous compounds and drugs [27]. Using Mrp4$^{-/-}$ and Bcrp$^{-/-}$ mice, we show that hyperuricemia is associated with the accumulation of tryptophan and associated metabolites, most likely due to transporter dysfunction.

2. Material and methods

2.1. Transduction of human embryonic kidney cells and preparation of membrane vesicles

Overexpression of MRP4 and BCRP in human embryonic kidney cells (HEK293; American Type Culture Collection, Manassas, VA) was established using baculoviruses, which were produced using the Bac-to-Bac and the Gateway system (Invitrogen, The Netherlands), as described previously [28,29]. As a control, the enhanced yellow fluorescent protein (eYFP) was introduced as mock protein into the baculovirus expression system. Crude membranes of HEK293-MRP4, -BCRP and -mock cells were isolated, resuspended in TS buffer (10 mM Tris–HEPES and 250 mM sucrose, pH 7.4) and membrane vesicles were prepared according to a previously described method [28] by means of ultracentrifugation. Crude membrane vesicles were dispensed in aliquots, snap frozen in liquid nitrogen, and stored at −80 °C until further use.

2.2. Membrane vesicle inhibition and uptake assays

The effects of uric acid and oxonic acid on MRP4 and/or BCRP activity were assessed by a well-established assay in our laboratory [28–31]. In brief, a reaction mix consisting of TS buffer supplemented with 4 mM ATP/AMP, 10 mM $MgCl_2$ and 250 nM [^{3}H]-methotrexate (MTX; for MRP4) or [^{3}H]-estrone sulphate (E_1S; for BCRP) at pH 7.4 was added to 7.5 μg of membrane vesicles (based on total protein content). After incubation at 37 °C to enable ATP-dependent uptake, the reaction was stopped by placing the samples on ice and by addition of ice-cold TS buffer. Reaction mix was removed and the vesicles were washed by means of a rapid filtration technique using filter plates (Millipore, Etten-Leur, The Netherlands). Scintillation fluid was added to the filters and the amount of radioactivity was determined using a scintillation counter (Tri-Carb® 2900TR; Perkin Elmer, Waltham, MA, USA). Reference samples were measured to calculate the amount of transported MTX and E_1S. ATP-dependent transport was calculated by subtracting values measured in the presence of AMP from those measured in the presence of ATP. Net transporter-mediated substrate uptake was calculated by subtracting ATP-dependent uptake in HEK293-mock vesicles from that of HEK293-transporter vesicles.

Uptake of kynurenic acid into MRP4-overexpressing membrane vesicles was established using the same assay. Vesicles were incubated with 0.1 mM kynurenic acid in the presence of AMP or ATP. After the described washing step, kynurenic acid was determined by LC–MS/MS.

2.3. Oxonic acid-mediated induction of hyperuricemia in mice

All experiments were approved by the local Animal Welfare Committee of the Radboud University Nijmegen Medical Centre (RU-DEC 2012-018), in accordance with the directive for animal experiments (2010/63/EU) of the European Parliament. The effects of hyperuricemia in vivo were examined in wild type (WT) Friend leukemia virus B (FVB) mice as well as Mrp4$^{-/-}$ and Bcrp$^{-/-}$ mice (both FVB background). The WT FVB and Bcrp$^{-/-}$ mice were kindly provided by Dr. A. Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands) and the Mrp4$^{-/-}$ mice by Dr. J. Schuetz (St. Jude Children's Research Hospital, Memphis, TN, USA) and Dr. P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands), all animals were bred and housed at the Central Animal Laboratory of the RUNMC. The animals (N = 9) received the uricase inhibitor oxonic acid (2% w/v; pH 7) via their drinking water, ad libitum, to induce hyperuricemia [32]. The animals were individually caged and housed under controlled conditions. Parallel control groups were also individually caged and received normal tap water at equal pH. After 14 days, mice were placed individually in metabolic cages (Techniplast, Germany GmbH) to collect 24 h urine samples, with access to water (with or without oxonic acid 2% w/v) and pulverized standard chow ad libitum. Next, blood was collected from the orbital sinus in lithium–heparin tubes via a terminal procedure performed under isoflurane anesthesia and centrifuged for 15 min at 3000 ×g to obtain plasma. Animals were sacrificed by cervical dislocation. Isolated kidneys, plasma and urine were immediately snap frozen in