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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

liquid nitrogen and stored at −80 °C until further analysis. Biochemical parameters were determined by routine clinical chemistry.

2.4. Energy-dispersive X-ray (EDX) microanalysis

Transmission electron microscopy and EDX were performed for identification of the ultrastructure and composition of the insoluble crystals found in the urine samples of oxonic acid-treated mice. Urine samples were spotted onto copper grids (100 mesh) coated with a support film, air dried, negative stained with uranyl acetate and examined using a Jeol 1200 EX II. For EDX measurements, the grids were examined using a Jeol 1200/STEM in combination with a Thermo Noran microanalysis six system. Accelerated voltage of 60 keV was used for X-ray microanalysis. X-ray spectra for element distribution were acquired. In each sample, 3–5 measuring points were selected.

2.5. RNA isolation and quantitative PCR

Effects of hyperuricemia on kidney injury was evaluated by determining mRNA expression levels of early renal injury markers kidney injury molecule-1 (Kim-1) and neutrophil gelatinase-associated lipocalin (Ngal) in kidneys of treated and control mice. Frozen kidneys were homogenized using a Mikro-dismembrator U (Sartorius B. Braun Biotech Int., Melsungen, Germany). Subsequently, total RNA was isolated using a NucleoSpin® RNA II kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Immediately, a reverse transcriptase reaction was performed with 250 ng RNA using random primers (Invitrogen, Breda, The Netherlands) and an Omniscript® RT kit (Qiagen, Hilden, Germany), following manufacturer's recommendations. Synthesized cDNA was used for quantitative PCR, performed in a StepOnePlus™ Real-Time PCR system by means of the TaqMan® protocol (Applied Biosystems, Warrington, UK). *Kim-1* and *Ngal* mRNA concentrations were normalized to the mRNA concentration of the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*). The primer-probe sets were obtained from Applied Biosystems (*Gapdh*; Mm999999915_g1, *Kim-1*; havcr1 Mm00506686_m1, *Ngal*; lcn2 Mm01324470_m1).

2.6. Tryptophan, kynurenine and kynurenic acid measurements by LC–MS/MS

After the vesicle uptake experiments, kynurenic acid was extracted from the filters in 3.3% perchloric acid. Plasma samples were diluted in H_2O (1:1) prior to LC–MS/MS measurements and deproteinized with perchloric acid (final concentration 3.3%). Samples were centrifuged at 12,000 ×*g* for 3 min. Clear supernatant was injected into the LC–MS/MS system that consisted of an Accela HPLC system (Thermo scientific, Breda, The Netherlands) equipped with a C18 HPLC column (VisionHT C18 B 10,062 mm, 1.5 mm; Grace). Tryptophan, kynurenine and kynurenic acid were measured in the same run. Deuterated kynurenic acid was added as an internal standard for quantification. Measurements were performed as published earlier [30].

2.7. Kinetic analysis and statistics

Statistics were performed using GraphPad Prism 5.02 via one-way analysis of variance (ANOVA) followed by the Dunnett's Multiple Comparison Test to study differences between all groups as well as an unpaired Student's *t*-test to study the impact of oxonic acid treatment within each strain. Differences between groups were considered to be statistically significant when $P < 0.05$. GraphPad Prism was also used to perform non-linear regression analysis.

3. Results

3.1. Oxonic acid induces hyperuricemia

To study the impact of hyperuricemia on murine physiology, wild type (WT), Mrp4$^{-/-}$ and Bcrp$^{-/-}$ mice were treated with the uricase inhibitor oxonic acid as described before [32]. Two weeks of oxonic acid treatment did neither affect overall weight of the mice, nor plasma levels of urea, sodium and calcium (Table 1). Interestingly, water intake was strongly increased after oxonic acid treatment in all strains investigated, with an increase up to four times that of controls in Bcrp-deficient mice. This was accompanied by a two-fold increase in urine flow in treated WT animals; whereas no significant changes were observed in knockout animals. Yet baseline urine flow of Bcrp$^{-/-}$ mice tended to be increased as compared to WT animals.

Following oxonic acid treatment, the urine collected from all animals was turbid due to presence of crystals (Fig. 1A–D). Energy-dispersive X-ray microanalysis (EDX) revealed that the major constituents of the urinary crystals were sodium, potassium, phosphorus and calcium; the copper signals arose from the sample grid used in the analysis (Fig. 1E). Two types of crystals could be distinguished: first, crystals that showed an archetypical peak pattern corresponding to uric acid crystals, which consisted primarily of sodium, calcium and phosphorus (Fig. 1F). Second, crystals that mainly contained calcium and potassium (Fig. 1G), which was likely due to the treatment with oxonic acid potassium salt. These data are in accordance with the development of hyperuricemia in oxonic acid-treated mice.

3.2. Uric acid inhibits BCRP activity

Plasma urate levels are controlled by an interplay of transporters expressed in the kidney proximal tubules. MRP4 and BCRP have been associated with active urinary urate efflux, and MRP4 was also sensitive to inhibition by urate [20]. We determined the inhibitory properties of uric acid on BCRP activity using membrane vesicles prepared from BCRP-overexpressing HEK293 cells. Uric acid dose-dependently inhibited BCRP-mediated uptake of the substrate E_1S (Fig. 2A) with a calculated half maximal inhibitory concentration (IC_{50}) value of 365 ± 13 μM. Complete inhibition of BCRP activity was found at the highest uric acid concentration used (i.e. 1 mM). These findings indicate that uric acid can reduce transport activity of two important efflux pumps at concentrations demonstrated in patients with hyperuricemia (≥360 μM).

3.3. MRP4-mediated transport of kynurenic acid

There is some evidence linking hyperuricemia to changes in tryptophan levels [25]. In addition, we recently reported that kynurenic acid, a tryptophan derivative, can interact with MRP4 and BCRP [30], and we identified kynurenic acid as a BCRP substrate (Dankers et al., submitted for publication). Here, we investigated whether kynurenic acid is also a substrate for MRP4. Fig. 2B shows that the ATP-dependent uptake of kynurenic acid in MRP4-overexpressing vesicles is 7-fold higher as compared to controls with an average rate of 21.6 pmol/mg ∗ min^{-1}. Furthermore, Fig. 2B demonstrates that non-specific, AMP-dependent, uptake is very low. These results indicate that kynurenic acid is indeed transported by MRP4.

3.4. Retention of tryptophan and its metabolites during hyperuricemia

As uric acid can inhibit MRP4 and BCRP-mediated transport and kynurenic acid is a substrate for both pumps, we determined the effects of hyperuricemia on tryptophan metabolism and the role of the efflux pumps herein. Plasma tryptophan levels were similar in all untreated groups, but oxonic acid treatment led to increased tryptophan levels in Mrp4$^{-/-}$ mice (125 μM) as compared to untreated

Table 1
General characteristics and serum and urine biochemistry of experimental groups.

	WT	WT + oxonic acid	Mrp4$^{-/-}$	Mrp4$^{-/-}$ + oxonic acid	Bcrp$^{-/-}$	Brcp$^{-/-}$ + oxonic acid
General characteristics						
Weight d.0 (g)	22.4 ± 0.8	25.3 ± 1.1	23.4 ± 1.4	25.0 ± 1.5	23.8 ± 1.1	25.7 ± 1.0
Weight d.14 (g)	24.6 ± 1.0	24.9 ± 1.2	23.5 ± 0.9	24.6 ± 0.9	24.5 ± 0.7	24.6 ± 0.6
Water intake (ml/24 h)	1.6 ± 0.4	5.5 ± 0.8***	2.9 ± 0.6	5.5 ± 1.0*	2.1 ± 0.7	8.5 ± 0.5***
Urine flow (ml/24 h)	0.3 ± 0.1	0.6 ± 0.1*	0.3 ± 0.1	0.7 ± 0.3	0.7 ± 0.2	0.7 ± 0.1
Plasma						
Urea (mM)	12.5 ± 0.8	11.8 ± 0.7	11.4 ± 0.4	10.5 ± 0.5	12.5 ± 0.5	11.7 ± 0.6
Sodium (mM)	149.7 ± 1.1	151.1 ± 1.4	147.6 ± 1.8	151.7 ± 0.8	149.4 ± 0.9	148.0 ± 1.0
Calcium (mM)	2.3 ± 0.02	2.4 ± 0.03	2.2 ± 0.02	2.3 ± 0.02	2.3 ± 0.01	2.3 ± 0.02
Urine						
Creatinine (μmol/24 h)	1.4 ± 0.6	1.6 ± 0.2	1.1 ± 0.2	1.5 ± 0.3	2.4 ± 0.8	1.3 ± 0.3
Sodium (μmol/24 h)	139.7 ± 63.1	126.3 ± 14.1	93.4 ± 17.4	150.8 ± 42.7	196.0 ± 49.2	138.8 ± 21.4

Individually caged mice were treated with oxonic acid via their drinking water for 14 days. Mice were weighed before and after treatment period. Water intake, urine flow and urine content were determined after a 24 h-period in metabolic cages at day 14. Plasma was collected at day 14. Data represent mean ± SEM of 9 mice per group. *$P < 0.05$ and ***$P < 0.001$ compared to untreated mice from the same strain using a Student's *t*-test. Differences between the strains were not significant (one-way ANOVA).

animals (93 μM; Fig. 3). Baseline plasma levels of the intermediate tryptophan metabolite, kynurenine, were similar in knockout animals as compared to WT animals. And oxonic acid treatment led to a >1.5-fold increase in kynurenine plasma levels in all three strains, without differences between strains. In contrast, baseline plasma kynurenic acid levels of knockout mice were elevated compared to WT mice, which was significant for Bcrp$^{-/-}$ mice. Hyperuricemia did not further increase plasma kynurenic acid levels in knockout mice, but led to significantly elevated levels in WT animals to the levels of untreated knockout mice. Furthermore, IDO activity, represented by the ratio between tryptophan and kynurenine, was similar in untreated strains, but significantly increased after oxonic acid treatment in Bcrp$^{-/-}$ mice (0.5 vs 0.7). Thus, hyperuricemia clearly affects tryptophan metabolism.

3.5. Oxonic acid does not influence MRP4 and BCRP activity

To exclude the possibility that oxonic acid itself inhibited the efflux pumps resulting in metabolite retention, we investigated the effect of oxonic acid on MRP4 and BCRP transport activity. Membrane vesicle uptake studies revealed that oxonic acid itself did not affect MRP4-mediated MTX uptake and BCRP-mediated E_1S uptake with more than 15% in a concentration range of 1 μM to 1000 μM (Fig. 4).

3.6. Hyperuricemia induces expression of the early kidney injury marker Ngal

Finally, we studied whether hyperuricemia and changes in tryptophan metabolism coincided with an amelioration of kidney function. Renal damage was evaluated by assessing mRNA expression of the early renal injury markers Kim-1 and Ngal in kidneys of treated and control mice. As shown in Fig. 5A, Kim-1 was not differentially expressed in oxonic acid-treated mice compared to untreated mice. In contrast, renal Ngal expression (Fig. 5B) was increased after oxonic acid-induced hyperuricemia. These increases were significant for both knockout strains, and in Mrp4$^{-/-}$ mice expression levels rose up to 2.3 times that of untreated mice. With regard to kidney function, no significant effect was observed on creatinine and sodium

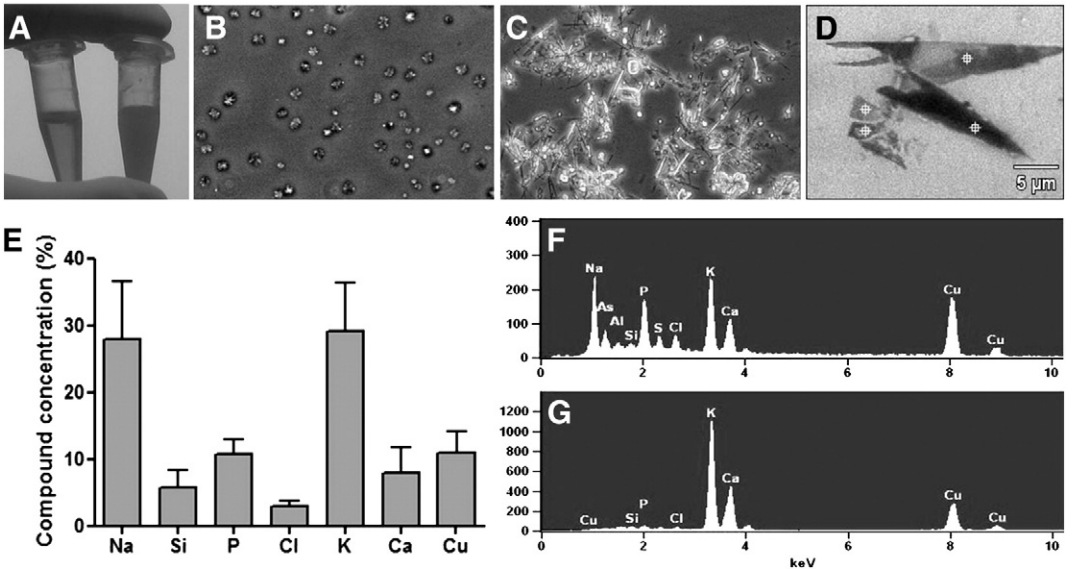

Fig. 1. Analysis of urine samples of WT, Mrp4$^{-/-}$ and Bcrp$^{-/-}$ mice after 14 days of treatment with oxonic acid via their drinking water. A representative sample of clear urine of an untreated animal (A, left and B) and turbid, crystal-filled urine of an oxonic acid-treated animal (A, right and C) is shown by a photograph and micrographs (magnification 10×). The contents of the insoluble crystals were determined by energy dispersive X-ray microanalysis (EDX). In each sample, 3–5 measuring points were selected (D). The components of all urinary crystals of three oxonic acid-treated WT mice were depicted as mean + SEM of twelve measurements (E). Mainly two types of crystals were found. A typical EDX spectrum of urate crystals, containing sodium, calcium and phosphorus, is depicted in panel F, while panel G represents an EDX spectrum of oxonic acid crystals consisting of calcium and potassium.

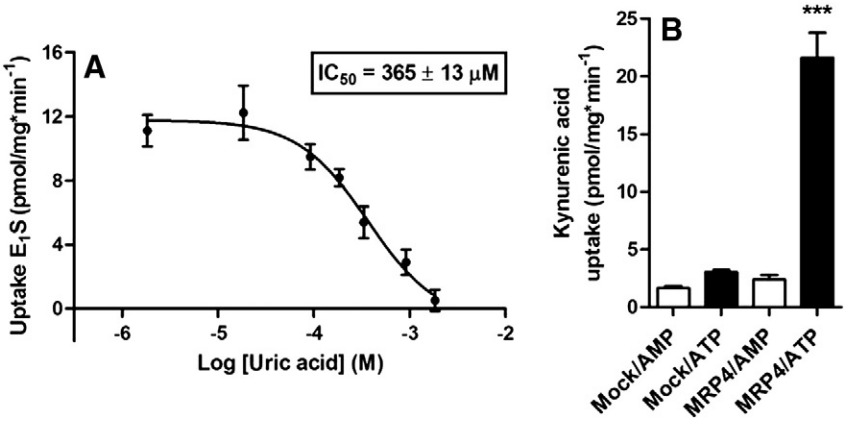

Fig. 2. Concentration-dependent inhibition of net BCRP-mediated [^{3}H]-estrone sulphate (E_1S) uptake by uric acid and MRP4-mediated kynurenic acid uptake into membrane vesicles. (A) Membrane vesicles were incubated with 250 nM E_1S and increasing concentrations of uric acid in the presence of AMP or ATP, for 60 s at 37 °C. Net BCRP-mediated E_1S uptake was calculated by subtraction of corresponding mock values. Curve fitting was performed by non-linear regression analysis using GraphPad Prism software (version 5.02, GraphPad Software Inc., San Diego, CA, USA). Graph represents means ± SEM of three independent experiments. (B) Kynurenic acid uptake was assessed by LC–MS/MS analysis after incubating membrane vesicles with 0.1 mM kynurenic acid in the presence of AMP or ATP, for 5 min at 37 °C. Bars represent mean + SEM of three independent experiments. ***$P < 0.001$ compared to other bars by one-way ANOVA followed by Dunnett's post-hoc test.

excretion (Table 1), suggesting the absence of overt kidney damage which was confirmed by histology. Light microscopic evaluation of HE-stained kidney slices (Fig. 5C) revealed intact brush borders and absence of casts or destroyed tubules in exposed animals. Taken together, these results indicate that two weeks of oxonic acid-induced hyperuricemia reveals, at most, early signs of kidney damage.

4. Discussion

This study reports for the first time that hyperuricemia is associated with disturbances in tryptophan metabolism, most likely due to uric acid-induced dysfunction of the renal efflux pumps MRP4 and BCRP. High levels of uric acid are associated with an increased risk for the development of various diseases and the common mode of action is the formation of crystals (e.g. gout and nephrolithiasis) or by negatively influencing the endothelium (e.g. hypertension). Here, we hypothesized that hyperuricemia could also contribute to disease development in an indirect manner by promoting the retention of other potentially toxic metabolites. Our results revealed that oxonic acid-induced hyperuricemia resulted in elevated plasma levels of tryptophan, kynurenine and kynurenic acid in vivo. The findings are in agreement with two recent studies by Liu et al. [25,26], who reported that plasma levels of tryptophan were increased in patients with acute gout while their urinary concentrations were decreased. Hence, there appears to be a link between hyperuricemia and disturbances in tryptophan metabolism.

The efflux transporters MRP4 and BCRP are important in regulating uric acid levels, but also essential for the clearance of many exogenous and endogenous waste products. Recently, we demonstrated that kynurenic acid could interact with MRP4 and BCRP activity in

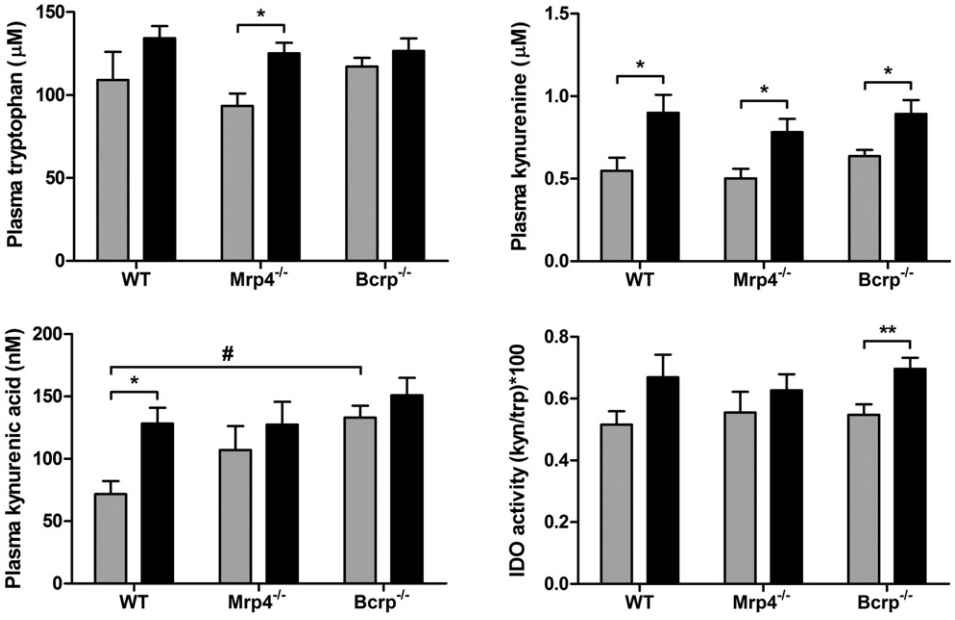

Fig. 3. Plasma tryptophan metabolism in WT, Mrp4$^{-/-}$ en Bcrp$^{-/-}$ mice. Plasma tryptophan, kynurenine and kynurenic acid levels and IDO activity of untreated (gray) and oxonic acid-treated (black) WT, Mrp4$^{-/-}$ en Bcrp$^{-/-}$ mice after 14 days of treatment via drinking water, determined by LC–MS/MS analysis. IDO activity is expressed as the ratio between kynurenine and tryptophan * 100. Bars represent mean + SEM of 9 mice per group. Statistical analysis was performed using both one-way ANOVA followed by the Dunnett's Multiple Comparison Test and an unpaired Student's *t*-test. *$P < 0.05$ and **$P < 0.01$ by Student's *t*-test and #$P < 0.001$ by one-way ANOVA.

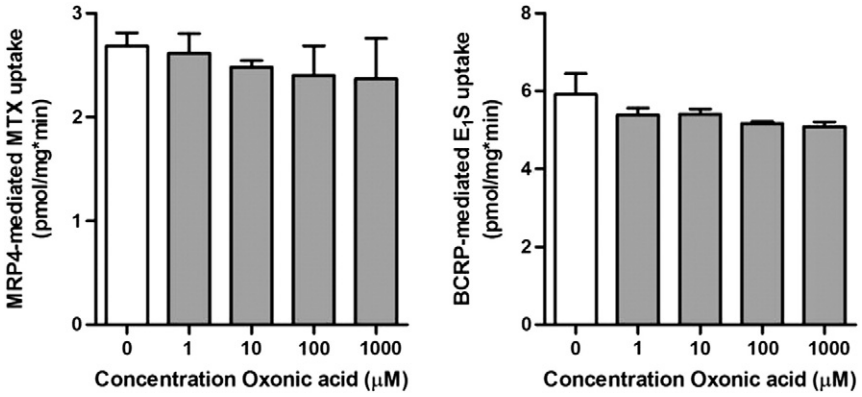

Fig. 4. Oxonic acid does not interfere with MRP4-mediated MTX uptake and BCRP-mediated E_1S uptake. Membrane vesicles were incubated with 250 nM [^{3}H]-MTX or [^{3}H]-E_1S and indicated concentrations of oxonic acid for 5 min at 37 °C in the presence of AMP or ATP. AMP values were subtracted from ATP values. Net transporter-mediated uptake was expressed as means + SEM of triplicate measurements in a representative experiment. Results were analyzed by one-way ANOVA followed by Dunnett's post-hoc test.

the membrane vesicle transport assay, suggesting that the metabolite is a possible substrate for both transporters [30]. Using the same assay, we have shown that kynurenic acid is indeed transported by BCRP (Dankers et al., submitted for publication) and here we report that kynurenic acid is also a substrate for MRP4. These findings are in agreement with the observed increase in plasma kynurenic acid levels in Mrp4$^{-/-}$ and Bcrp$^{-/-}$ mice. Therefore, MRP4 and BCRP could be potential novel therapeutic targets for the regulation of kynurenic acid levels in a variety of diseases.

Kynurenic acid is a widely studied antagonist of the *N*-methyl-D-aspartate-receptor and the α7-nicotinic acetylcholine receptor, and elevated levels of kynurenic acid are related to several neurological disorders [33]. Another target of kynurenic acid is the orphan G-protein-coupled receptor GPR35, of which kynurenic acid is one of the most potent endogenous agonists currently known. The receptor is highly expressed in the intestine and in several immune cells, including monocytes and T cells. Kynurenic acid also alters the release of multiple growth factors such as nerve growth factor and fibroblast growth factor-1 [34]. Thus, perturbations in kynurenic acid levels can result in marked effects on receptor activation and changes in growth factors. With regard to pathophysiological effects, classic experiments have demonstrated that kynurenic acid inhibited pro-insulin synthesis in

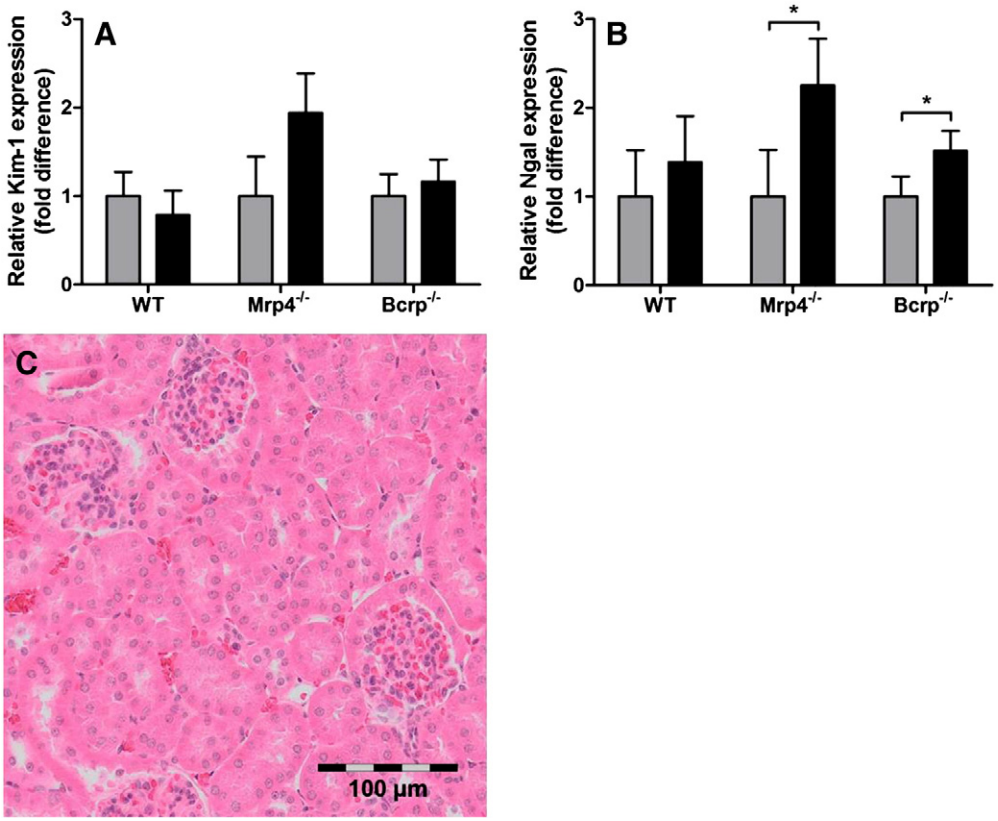

Fig. 5. Expression of kidney injury markers and histology after oxonic acid treatment. Relative mRNA expression levels of kidney injury markers Kim-1 (A) and Ngal (B) in kidney tissue of untreated (gray) and oxonic acid-treated (black) mice obtained by QPCR. Animals were exposed to oxonic acid via their drinking water for 14 days. Cycle threshold (Ct) values were normalized for the endogenous reference gene *Gapdh* and expressed as mean fold difference from untreated animals + SEM (N = 9). *$P < 0.05$ by Student's *t*-test. Both genes were not differentially expressed in untreated animals of the different strains. Panel C depicts a representative micrograph of an oxonic acid-treated WT mice and shows that no kidney damage was observed in oxonic acid-treated mice (HE staining), which was comparable in treated knockout mice.

isolated rat pancreatic islets and increased the release of insulin in rats, suggesting a role in diabetes [35,36]. Furthermore, in CKD patients, kynurenic acid accumulates [30] and increased levels correlate positively to multiple markers of endothelial dysfunction, namely von Willebrand factor, thrombomodulin and soluble adhesion molecules (sICAM-1, sVCAM-1) [37,38]. In addition, kynurenic acid is suggested to be an important early mediator of leukocyte recruitment [39]. Moreover, kynurenic acid reduces glucuronidation activity of UDP-glucuronosyltransferases, as shown in proximal tubule cells, thereby affecting the metabolic capacity of the kidney [40]. Hence, elevated levels of kynurenic acid induced by hyperuricemia, as observed in our study, might play a pivotal role in the pathophysiological effects currently attributed to uric acid.

Our results further demonstrated that kynurenine levels were similar in untreated WT and knockout animals, suggesting that the levels of this metabolite are not influenced by MRP4 and BCRP activity. Following induction of hyperuricemia, kynurenine levels were markedly increased in all groups. The observed increase might be due to a reduced activity of a uric acid transporter other than MRP4 and BCRP, elevated tryptophan levels, as seen in Mrp4$^{-/-}$ mice, and/or increased activity of indoleamine 2,3-dioxygenase (IDO), as observed in Bcrp$^{-/-}$ mice. Interestingly, IDO is involved in immune regulation and enzyme activity is higher during chronic inflammation and in CKD patients [41]. The exact interaction between hyperuricemia and IDO requires further investigation. For a long time, kynurenine was regarded as an intermediate of tryptophan metabolism with little biological activity. Yet Opitz et al. [42] recently demonstrated that this metabolite is a ligand for the aryl hydrocarbon receptor (AHR) and can promote tumor cell survival and suppress antitumor immune responses. The AHR signaling pathway is involved in a myriad of cellular processes, including embryogenesis, inflammation and phase I and phase II metabolism. Fascinatingly, another tryptophan metabolite, indoxyl sulphate, is also reported to activate the AHR in primary human hepatocytes and human umbilical vein endothelial cells [43,44]. Thus, by disturbing tryptophan metabolism, hyperuricemia could indirectly be involved in AHR activation and subsequent pathologies.

Several polymorphisms in transporter genes are associated with elevated serum uric acid levels. For instance, Woodward et al. [22], described that the common single nucleotide polymorphism (SNP) rs2231142 encoding the Q141K mutation of BCRP caused hyperuricemia-based gout. Also in a Japanese population, Q141K was shown to be a common dysfunctional form of BCRP causing gout [21]. Another transporter recently implicated in uric acid metabolism is SLC2A9. Using *Xenopus* oocytes, Vitart et al. [19] demonstrated that SLC2A9-mediated uptake of uric acid was sevenfold higher in SLC2A9-expressing oocytes compared to URAT1-expressing oocytes. These findings have been confirmed by others [10,45]. Moreover, multiple genome-wide association studies reported a relationship between uric acid levels and SNPs in several transporters including SLC17A3, SLC22A11 and SLC22A12 [23,46,47]. Taken together, when studying the link between uric acid and disease progression it is important to take into consideration the presence of possible polymorphisms in transporter genes as well as changes in metabolite levels of transporter-specific substrates.

Two weeks of hyperuricemia induced early signs of kidney damage, as observed by an increased mRNA expression of Ngal in knockout animals. In contrast, no signs of renal damage were seen in WT animals. These findings suggest that knockout animals were more prone to the development of renal failure. Since, kynurenic acid levels were already elevated in Mrp4$^{-/-}$ and Bcrp$^{-/-}$ animals before the induction of hyperuricemia, one could argue that other tryptophan metabolites are responsible for induction of Ngal observed in knockout mice. To better understand the effect of hyperuricemia on the development of CKD, future studies should include longer treatment periods (>2 weeks) and also determine the levels of other intermediates and end-products of tryptophan metabolism.

In conclusion, our results showed that uric acid dose-dependently inhibited BCRP activity and inhibition occurred at physiologically relevant levels as was reported previously for MRP4 [20]. Moreover, we demonstrated that Mrp4- and Bcrp-deficiency as well as hyperuricemia are associated with alterations in tryptophan metabolism and the retention of kynurenine and kynurenic acid, two metabolites with a broad array of biological activities. Therefore, 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. These findings underline the complex relation between hyperuricemia and linked pathologies, which should be taken into account when interpreting results obtained using in vivo hyperuricemia models.

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