

Original Paper

Different Pharmacological Properties of GLUT9a and GLUT9b: Potential Implications in Preeclampsia

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Key Words

Glucose transporter type 9 (GLUT9) • Uric acid • Uric acid transport • Iodine • Electrophysiology

Abstract

Background/Aims: Glucose transporter 9 (GLUT9/SLC2A9) is the major regulator of uric acid homeostasis in humans. Hyperuricemia due to impaired regulation by GLUT9 in pregnancy is closely associated with preeclampsia. While GLUT9 is expressed in two alternative splice variants, GLUT9a and GLUT9b, with different subcellular localizations, no functional differences of the two splice variants are known to date. The aim of this study was to investigate the function of both GLUT9 isoforms. **Methods:** To characterize the different pharmacological properties of GLUT9a and GLUT9b electrophysiological studies of these isoforms and their modified variants, i.e. NmodGLUT9a and NmodGLUT9b, were performed using a *Xenopus laevis* oocytes model. Currents were measured by an electrode voltage clamp system. **Results:** Functional experiments unveiled that uric acid transport mediated by GLUT9a but not GLUT9b is chloride-dependent: Replacing chloride by different anions resulted in a 3.43 ± 0.63 -fold increase of GLUT9a- but not GLUT9b-mediated currents. However, replacement by iodide resulted in a loss of current for GLUT9a but not GLUT9b. Iodide inhibits GLUT9a with an IC_{50} of $35.1 \pm 6.7 \mu M$. Modification of the N-terminal domain leads to a shift of the iodide IC_{50} to $1200 \pm 228 \mu M$. Using molecular docking studies, we identified two positively charged residues H23 and R31 in the N-terminal domain of hGLUT9a which can explain the observed functional differences. **Conclusion:** To the best of our knowledge, this is the first study showing that the N-terminal domain of hGLUT9a has a unique regulatory function and the potential to

interact with small negatively charged ions like iodide. These findings may have significant implications in our understanding of hyperuricemia-associated diseases, specifically during pregnancy.

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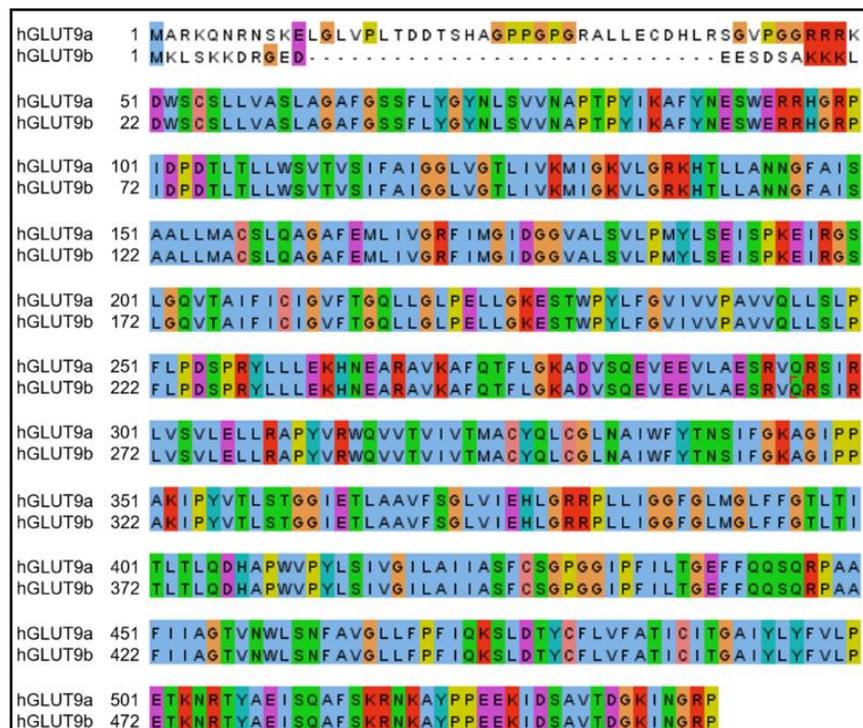
Introduction

Transport of monosaccharides, polyols and other small carbon compounds across the plasma membranes is mediated by proteins of the glucose transporter family that are encoded by the SLC2 gene family. The human genome currently comprises 14 isoforms that differ in their tissue distribution, kinetic properties and substrate specificity. A common structural feature shared among all members of the glucose transporter family (SLC2 family) is the presence of 12 transmembrane helices with N- and C-termini facing the cytoplasm and an N-linked glycosylation site. GLUT9a and GLUT9b are splice variants of the gene SLC2A9 which only differ in their N-terminus (Fig. 1) [1, 2]. Human GLUT9a is 540 amino acids in length and is encoded by 12 exons whereas GLUT9b is comprised of 512 amino acids and is encoded by 13 exons. In both humans and mice GLUT9b is expressed only in the placenta, liver and kidney whereas GLUT9a is present in many more tissues, including placenta liver, kidney, intestine, leukocytes, and chondrocytes [3]. GLUT9a is expressed in the basolateral side of the proximal tubular cell, whereas GLUT9b is expressed in the apical side of the collecting duct cell in the kidney [4].

GLUT9 is an exception in the glucose transporter family: although it was initially considered to transport glucose and fructose [5, 6], its main substrate is uric acid [7, 8]. GLUT9a and GLUT9b transport uric acid with the same kinetics (K_m for uric acid ~ 0.6 mM), and transport is not inhibited by the presence of an excess glucose or fructose. Uric acid transport by GLUT9 is electrogenic and depends on the membrane potential [8]. GLUT9 is believed to be one of the major uric acid transporter, along with URAT1, OAT4, ABCG2 and OAT10 [9].

Humans and higher primates have high plasma uric acid levels, as the final oxidation step is missing due to mutational silencing of the liver enzyme uricase. In most other

Fig. 1. Alignment of hGLUT9a and hGLUT9b. GLUT9a and GLUT9b are equal from amino acid 51 (GLUT9a). Alignment has been performed using T-COFFEE alignment tool applying a CLUSTALX color code [36, 37].



mammalian species (including rodents), uric acid is further oxidized in the liver to allantoin by uricase. Therefore, humans have relatively high levels of plasma uric acid levels (180–420 $\mu\text{mol/L}$) compared with the majority of mammals (30–120 $\mu\text{mol/L}$) [10]. Uric acid is a potent antioxidant, but due to its limited solubility, its tissue accumulation can lead to significant clinical pathologies. Human plasma uric acid levels are regulated within relatively tight limits and already small increases above normal levels show significant correlation with the incidence of gout, kidney stone disease, metabolic disease (including diabetes), cardiovascular morbidity and hypertension [11–16]. Plasma uric acid levels reflect a balance between production and excretion. The kidney plays an important role in uric acid homeostasis, since it reabsorbs around 90% of the secreted uric acid. In the proximal tubule of the kidney, GLUT9 transports uric acid across the basolateral membrane into the blood, as part of the reabsorption process. Although other uric acid transporters in the renal tubular system contribute to uric acid homeostasis, GLUT9 remains functionally the most important uric acid transporter while, at the renal proximal tubule apical membrane URAT1 plays an important role in uric acid reabsorption.

In pregnancy, serum uric acid concentrations decrease during the first trimester by at least 25%, and return to normal levels between the second and third trimester of pregnancy [17]. It has been shown that maternal serum uric acid concentrations rise in multiple pregnancies [18–21], suggesting that the presence of fetus and/or placenta contributes to the increased production of uric acid. Preeclampsia is a pregnancy-specific disease, characterized by hypertension and proteinuria, which contributes substantially to the incidence of iatrogenic preterm deliveries with its sequelae. Hyperuricemia is a key biochemical feature in preeclampsia and is associated with severity of preeclampsia and adverse perinatal outcome [22]. Moreover, recent findings indicate that iodine deficiency is associated with preeclampsia [23, 24]. Vidal et al. showed that in trophoblastic cells deprived of iodine reactive oxygen species were increased and migration processes were decreased while the differentiation marker human chorionic gonadotropin is upregulated indicating that iodine plays an important role in placentation [25]. We speculate that besides the above-mentioned mechanisms iodide has also an impact on the regulation of uric acid transport capacity.

In this study we analyzed the pharmacological properties of both hGLUT9 isoforms and investigated a possible functional link between iodine deficiency and hyperuricemia, both being common features in preeclampsia.

Materials and Methods

*Functional GLUT9 expression in *Xenopus laevis* oocytes*

Electrophysiological studies (n=5–8 for each experiment) of hGLUT9a, hGLUT9b, NmodGLUT9a and NmodGLUT9b were performed using the *Xenopus laevis* oocytes model as described before [26]. The two electrode voltage clamp system Roboinject (Multi Channel Systems Reutlingen, Germany) was used to measure currents. The use of animals was permitted by permission BE26/12 canton of Bern, Switzerland. All electrophysiological recordings were done using an automated two electrode voltage clamp system (Multi Channel Systems, Reutlingen, Germany).

Chloride replacement experiments

X. laevis oocytes (n=8) expressing hGLUT9a were clamped at -30 mV. The oocyte was exposed twice to buffer M1 containing 500 μM uric acid for 20 s, followed by a washing period with buffer (M2–M6). After the washing period the oocytes were exposed to buffer (M2–M6) containing 500 μM uric acid for 20 s. The same procedure has been done with *X. laevis* oocytes injected with either NmodGLUT9a or hGLUT9b.

Chloride was replaced by the use of media given in Table 1.

Dose-response experiments

Oocytes were exposed 2-3 times to 500 μ M uric acid for 20 s, followed by a 1 min washing period with buffer M1 containing iodine (1–10000 μ M). After the washing period the oocytes were exposed to buffer M1 containing 500 μ M uric acid and different iodine concentrations (1–10000 μ M) for 20 s. Current was normalized to the iodine-free current (n=5). The curves were fitted with the equation: $y=100/[1+10^{((\text{Log}(\text{EC50-X})\cdot\text{hill slope})]$.

Computational Biology - Molecular Docking

Molecular docking of uric acid was performed on the published predictive hGLUT9 structure [26] using the Vina AutoDock software. 778 million random combinations were tested resulting in 9 thermodynamically favorable positions of uric acid. The best result was obtained with a predicted binding affinity of -6.2 kcal/mol. A surface modeling at 3Å was realized to determine which amino acids are potentially interacting with uric acid.

Statistics

All data are shown as mean \pm standard deviation (SD). Statistical analysis was performed with SigmaStat (version 5.0 for Windows, USA). Data were compared using an unpaired Student's t-test. A p-value of <0.05 was considered significant.

Results

Physiological properties of GLUT9a and GLUT9b

To investigate the physiological properties of both GLUT9 isoforms, experiments with the *X. laevis* oocytes model were performed. Therefore, GLUT9a and GLUT9b cDNA were cloned into a vector containing a T7-promotor for *in vitro* transcription. cRNA of hGLUT9a and hGUT9b were microinjected into *X. laevis* oocytes. 48 hours following injection the *X. laevis* oocytes were used for functional experiments: Two electrodes voltage clamp techniques with exposure to 500 μ M uric acid for 20 s were performed. Oocytes expressing either one or the other GLUT9 isoform showed equal outward currents, due to uric acid influx, indicating a similar functional expression level. To analyze the impact of chloride on the GLUT9a-mediated uric acid transport, the extracellular chloride concentration was reduced from 106 mM to 6 mM by replacing sodium chloride with sodium pyruvate. Interestingly, at 6 mM chloride concentration application of 500 μ M uric acid led to an inward current for less than 1 s returning into a 3.43 ± 0.63 -fold increased outward current (1 ± 0.05 versus 3.43 ± 0.63 , $p<0.001$, Student's T-test) (Fig. 2A). To rule out the possibility that this observation might be due to a negative charge competition effect, chloride was replaced by sodium fluoride, sodium nitrate, sodium pyruvate and 2-morpholinoethanesulfonic acid (MES). Under these conditions the 3.43 ± 0.63 -fold increase was not attenuated indicating that no negative charge competition is involved. Each condition led to a quick initial inward current following the uric acid application before returning to the usually observed outward current. Interestingly GLUT9a modified by adding a N-terminal tag (decahistidine-FLAG-HRV3C-HA) (NmodGLUT9a) showed a 2.10 ± 0.84 -fold increase in current under the same condition, but lacking this fast initial inward current (Fig. 2B). However, GLUT9b-mediated current was not affected by this reduction of chloride (Fig. 2C). To our surprise further experiments in an analogous setting replacing sodium chloride by sodium iodide resulted in a complete loss of current for GLUT9a (n=8; Fig. 3A) while no effect of currents mediated by GLUT9b (n=8; Fig. 3B) was found. The inward current observed was almost inexistent (15nA peak). Replacement

Table 1. Media composition used to replace chloride (pH was adjusted to 7.4 using NaOH)

Salt (mM)	M1	M2	M3	M4	M5	M6	M7
NaCl	100						
KCl	2	2	2	2	2	2	2
NaMES		100					
NaPyruvate			100				
NaNO ₃				100			
NaF					100		
NaI						100	
NaBr							100
MgCl ₂	1	1	1	1	1	1	1
CaCl ₂	1	1	1	1	1	1	1
HEPES	10	10	10	10	10	10	10

of chloride by bromide resulted in a reduction of the GLUT9a-mediated current from $100 \pm 3 \%$ to $90 \pm 6 \%$ ($n=5$, $p=0.01$, Student's T-test; Supplementary Fig. S1A – for all supplemental material see www.cellphysiolbiochem.com).

To characterize the impact of iodide on the uric acid transport in chloride-containing medium dose-response experiments were performed: GLUT9a showed an inhibition of transport with an IC_{50} of $35.1 \pm 6.7 \mu M$ ($n=6$) for iodide (Fig. 4). In contrast GLUT9b-mediated uric acid transport was not affected by iodide (Fig. 4) even when the iodide concentration was increased to highly supraphysiological levels, i.e. 20 mM ($n=8$) (Supplementary Fig. S1B).

Localization of the iodide interaction

The observation that iodide inhibited uric acid transport in hGLUT9a but not in hGLUT9b strongly indicates that hGLUT9a includes an iodide binding domain. Moreover, the fact that hGLUT9b-mediated current is unaffected by reduced chloride concentration suggests that the N-terminal domain of hGLUT9a has a regulatory function. The only difference between hGLUT9a and hGLUT9b is the peptide sequence in their N-terminal domains. Therefore, this domain of hGLUT9a was modified by adding an N-terminal tag (decahistidine-FLAG-HRV3C-HA). NmodGLUT9a was characterized regarding expression and function: NmodGLUT9a showed no difference in both expression and functional levels and exhibited a similar electrophysiological behavior when compared to native hGLUT9a in *X. laevis* oocytes (Supplementary Fig. S1C). However, iodide inhibition IC_{50} of NmodGLUT9a showed a 34-fold increase when compared to the IC_{50} of hGLUT9a ($1200 \pm 228 \mu M$ versus $35.1 \pm 6.7 \mu M$, $p < 0.001$, Student's T-test, Fig. 4). The same modification of GLUT9b (NmodGLUT9b) did not result in a change of current in the presence of iodide or chloride (Fig. 4).

In order to elucidate the functional difference between hGLUT9a and -b, we performed molecular docking analysis on hGLUT9a structure using uric acid as substrate. The best result was obtained

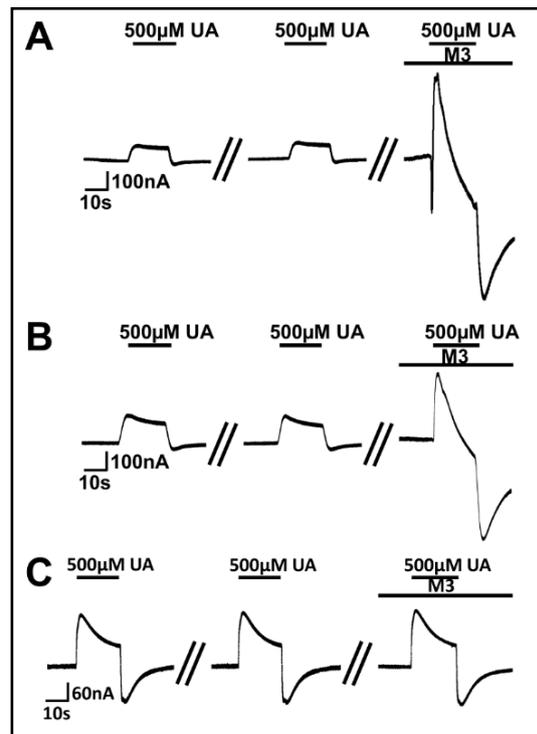


Fig. 2. Chloride dependence of hGLUT9a, NmodGLUT9a hGLUT9b. Replacement of chloride with pyruvate: A: hGLUT9a-mediated current was 3.43 ± 0.63 -fold increased. B: NmodGLUT9a-mediated current was 2.10 ± 0.84 -fold increased. C: GLUT9b-mediated current was not affected.

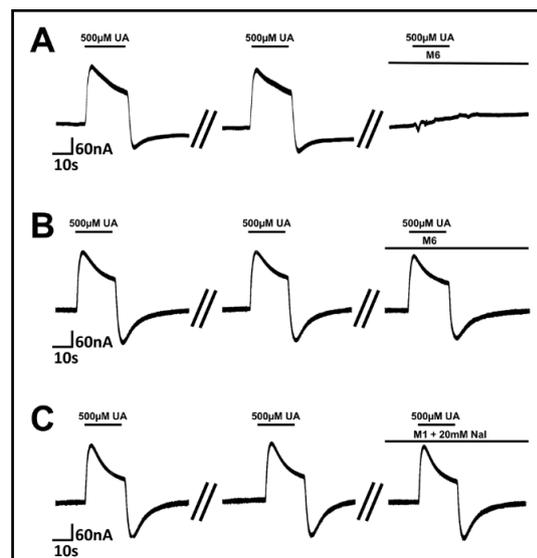


Fig. 3. Effect of replacing chloride by iodine on hGLUT9a and hGLUT9b. Replacement of chloride with iodine: A: hGLUT9a-mediated current was abolished. B: GLUT9b-mediated current was not affected. C: Addition of 20mM Nal had no effect on GLUT9b-mediated current.

with a predicted binding affinity of -6.2 kcal/mol. A surface modeling at 3\AA was realized to determine which amino acids are probably interacting with the substrate (Fig. 5). The resulting cavity (green mesh) is bounded by the amino acids H23/R31 (TM1), L182 (TM4), Q203/A206 (TM5), Q328 (TM7) and W459 (TM11) which are potentially important in the binding of the uric acid (Fig. 5A). In addition, some amino acids are observed to be close to the substrate: L332/N333 (TM7), F426 (TM10) and N462 (TM11). Finally, the predictive

Fig. 4. Iodine dose response curve for hGLUT9a, NmodGLUT9a, hGLUT9b, NmodGLUT9b. *X. laevis* oocytes expressing hGLUT9a (filled circles), NmodGLUT9a (open circles), GLUT9b (filled triangles) or NmodGLUT9b (open triangles). Iodine inhibits hGLUT9a-mediated current in a dose-dependent manner. Iodine inhibition of NmodGLUT9a is 34-fold decreased when compared to hGLUT9a (IC_{50} : $35.1 \pm 6.7 \mu\text{M}$ versus $1200 \pm 228 \mu\text{M}$, respectively, $p < 0.001$, Student's T-test). hGLUT9b- and NmodGLUT9b-mediated current is not affected by iodine.

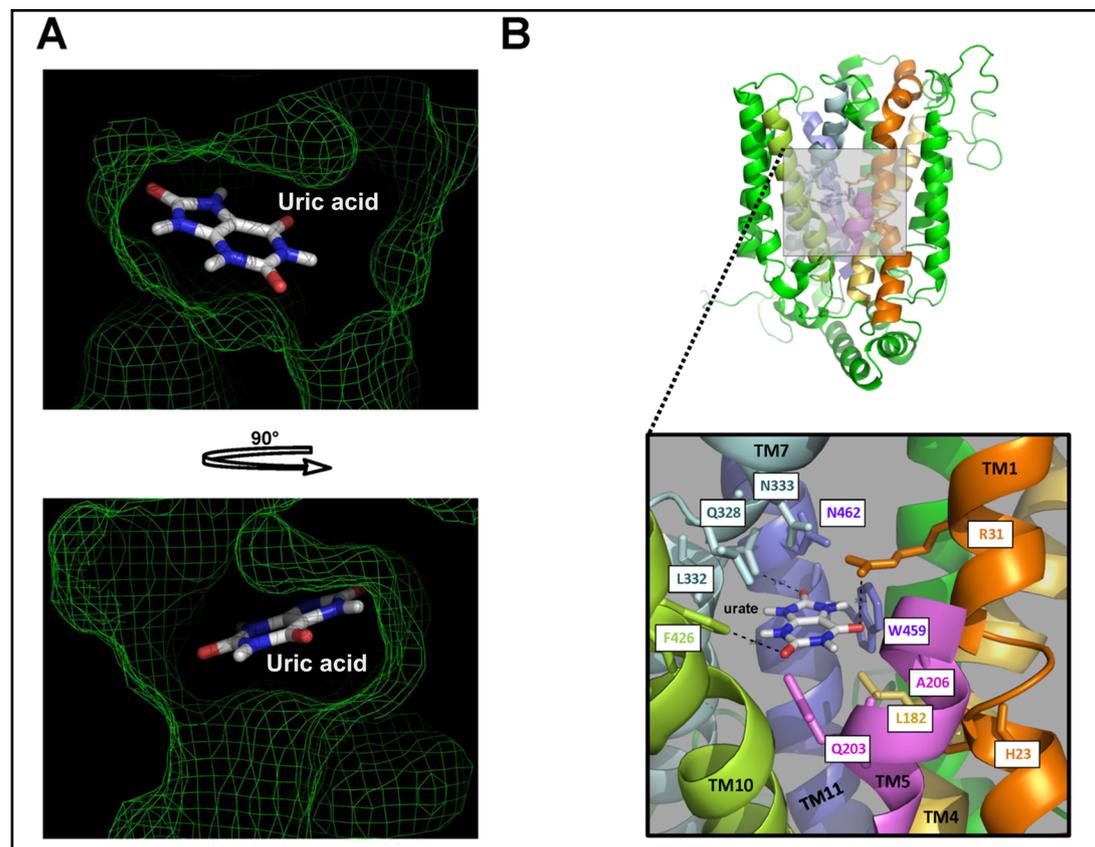
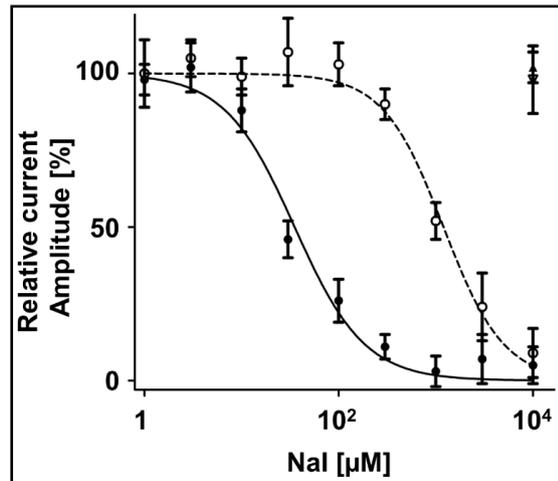


Fig. 5. The uric acid binding site of hGLUT9a. A: The surface was modeled at 3\AA around the uric acid substrate forming a binding site pocket (green mesh). B: The putative binding site is formed by amino acids: H23, R31, L182, Q203, A206, Q328, L332, N333, F426, W459 and N462 represented with sticks. Potential interactions of uric acid with R31, Q328 and F426 are shown (dotted lines).

uric acid binding site domain is indicated in orange (TM1), yellow (TM4), purple (TM5), green (TM10) and blue (TM11) in the Fig. 5B. In summary the putative binding site is formed by the following amino acids: H23, R31, L182, Q203, A206, Q328, L332, N333, F426, W459 and N462 (Fig. 5B). L332, Q328 and N333 are very close to the ketone group of the five atoms ring of uric acid allowing hydrogen bonding; these interactions may also play a role between the side chains of R31 and F426 and the other ketone groups of uric acid as depicted by dotted lines (Fig. 5B).

Discussion

Uric acid is a weak organic acid which generally acts as an antioxidant [27]. However, under hypoxic conditions or in the absence of ascorbic acid, uric acid acts as a prooxidant. Uric acid is generated by the enzyme xanthine dehydrogenase/oxidase (XDH/XO). Hyperuricemia is involved in the pathogenesis and/or aggravation of various human diseases such as gout, metabolic syndrome and kidney stones. Recent data suggest that hyperuricemia is not only associated with preeclampsia, but also plays a direct role in the pathogenesis of preeclampsia. The kidney takes on a major role in uric acid homeostasis [28, 29]. In the present study, we have focused on the investigation of the physiological properties of hGLUT9, one of the key regulators of uric acid homeostasis in humans: Both hGLUT9a and hGLUT9b isoforms exhibited similar electrophysiological characteristics in experimental settings when using the two electrodes voltage clamp technique in the *X. laevis* oocyte expression system. Interestingly only hGLUT9a- but not hGLUT9b-mediated uric acid transport turned out to be chloride-dependent. A direct negative charge competition was ruled out by the partial replacement of chloride by four differently sized anions (fluoride < nitrate < pyruvate < MES). Both, hGLUT9a- and NmodGLUT9a-mediated currents were increased in all these replacement conditions, whereas hGLUT9b-mediated currents were not affected. hGLUT9a-mediated current resulted in a reversed current for less than 1s, when chloride concentration was reduced. This could be due to a chloride-leak pathway under non-physiological low-chloride concentrations. In contrast NmodGLUT9a did not exhibit this initial inward current, indicating that this chloride-leak pathway under the aforementioned non-physiological low-chloride condition is a specific feature of the N-terminal domain of hGLUT9a. However, under physiological conditions, this brief initial inward current was non-existent in hGLUT9a-mediated uric acid transport.

Decreased serum iodine concentrations were identified as risk factor for preeclampsia and other hypertensive pregnancy complications [23, 30]. In preeclampsia, which is strongly associated with hyperuricemia, low iodine concentrations were found in maternal blood as well as in the placenta, whereas increased iodine levels were observed in the fetal circulation [31].

Therefore, we speculated that iodine has an impact on the regulation of the uric acid transport system in preeclampsia and possibly other hyperuricemia-associated diseases. This is in line with our findings that hGLUT9a-mediated uric acid transport is completely abolished by the reduction of chloride from 106 mM to 6 mM and replacing it by 100 mM iodide. Replacement of chloride by bromide resulted only in a small reduction of the current. The size of bromide is 198 pm which is between the size of chloride (181 pm) and iodide (220 pm) [32]. Further dose-response experiments revealed that in this cell model iodide inhibits hGLUT9a-mediated uric acid transport with an IC_{50} of $35.1 \pm 6.7 \mu\text{M}$. These findings demonstrate that iodide is able to regulate hGLUT9a-mediated uric acid transport in the *Xenopus laevis* oocytes model. However, the measured apparent affinity might be altered in different models [33] or *in vivo*.

Interestingly, analogous experimental settings using hGLUT9b uric acid transport were not affected by iodide, even when the iodide concentration was drastically increased to 20 mM in the presence of 106 mM chloride. We therefore speculated that hGLUT9a-mediated uric acid transport capacity can be regulated. The only difference between the two

hGLUT9 isoforms is the peptide sequence located at the N-terminal domain. Therefore, we hypothesized that the N-terminal domain may directly interact with iodide. We modified this domain without affecting either NmodGLUT9a expression or uric acid transport activity, compared to the properties of native hGLUT9a. The iodide-mediated inhibition substantially decreased to an IC_{50} of $1200 \pm 228 \mu M$. These data validate our hypothesis that the N-terminal domain of hGLUT9a has a regulatory function in uric acid transport activity.

In order to investigate the mechanism of the functional differences in GLUT9a and -b mediated uric acid transport regulated by iodide, we performed molecular docking analysis. Both the N- and C-terminal domains are expressed in the cytoplasm, however our molecular docking studies unveiled that the N-terminal domain of hGLUT9a faces (at least partially) the uric acid binding site. To our surprise we identified two positive charged residue in the N-terminal domain of hGLUT9a, i.e. H23 and R31, which were absent in hGLUT9b. The close proximity of these two residues to uric acid bound to its binding site (less than 3 \AA) indicates that they could play a major role during the uric acid transport cycle. Interestingly both residues, i.e. histidine and arginine are positively charged allowing interaction with anions such as chloride and iodide. Since there are no functional differences in uric acid transport capacity and uric acid binding affinity between hGLUT9a and hGLUT9b [34], it is unlikely that these two residues interact directly with uric acid. However due to their positive charges and their localization in the binding site, an interaction between these residues and iodine with subsequent alteration of hGLUT9a-mediated uric acid transport capacity is plausible. The exact mechanisms how uric acid interacts with GLUT9a and -b remain to be elucidated. Interestingly murine GLUT9a (mGLUT9a)-mediated uric acid transport capacity was not affected by low extracellular chloride concentration [8]. Alignment studies comparing hGLUT9a with mGLUT9a revealed that the mGLUT9a lacks the positive charged residues H23 and R31 (Supplementary Fig. S1D). These findings further support our concept that hGLUT9a-mediated uric acid transport is modulated by iodide, bromide and chloride. However only iodide has the potential to effectively regulate GLUT9a-mediated uric acid transport *in vivo* since its inhibitory effect is substantially higher than those of chloride and bromide.

As already noted, uric acid homeostasis is dependent on uric acid production and renal excretion. Since the fetus and the placenta produce uric acid, the fetoplacental unit contributes (at least partially) to the maternal uric acid homeostasis. GLUT9 was shown to be expressed in placental tissue [35]. Further studies investigating the placental uric acid transport system in normal pregnancy and in pregnancy diseases such as preeclampsia are needed to assess the contribution of total uric acid production. Such insights in the placental uric acid transport system may facilitate the development of novel preventive and therapeutic strategies. However, even in these pregnancy complications characterized by high uric acid serum levels, the maternal kidney seems to play a predominant role in uric acid homeostasis compared to the fetoplacental unit.

We propose that, *in vivo*, the renal uric acid transport system is functionally regulated at least partly by iodide. Indeed several studies showed an association between iodine deficiency and preeclampsia [23, 30]. Therefore, iodine intake might be beneficial in human hyperurecemic diseases such as preeclampsia as already suggested by other authors [31].

Conclusion

In conclusion, to the best of our knowledge, this is the first study showing that the N-terminal domain of hGLUT9a has a unique regulatory function. Our data provide clear evidence that hGLUT9a-mediated uric acid transport is modulated by iodine, in contrast to hGLUT9b, a finding which may have significant implications in our understanding of the development of pregnancy complications such as preeclampsia and GDM. Further studies investigating the role of the uric acid transport system and its regulation in human diseases are needed. The gained insights into the uric acid transport system may facilitate the

development of therapeutic and preventative strategies for the treatment of hyperuricemia-related diseases.

Abbreviations

GLUT9 (glucose transporter 9); SLC (solute carrier); *X. laevis* (*Xenopus laevis*); TM (transmembrane); XDH/XO (xanthine oxidase); N-mod (N-terminally modified).

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Disclosure Statement

The authors have no conflicts of interest to declare.

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