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Discovery of pyridoxal reductase activity as part of human vitamin B6 metabolism

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ABSTRACT

Background: Pyridoxal 5′-phosphate (PLP) is the active form of vitamin B6. Mammals cannot synthesize vitamin B6, so they rely on dietary uptake of the different B6 forms, and *via* the B6 *salvage* pathway they interconvert them into PLP. Humans possess three enzymes in this pathway: pyridoxal kinase, pyridox(am)ine phosphate oxidase and pyridoxal phosphatase. Besides these, a fourth enzyme has been described in plants and yeast but not in humans: pyridoxal reductase.

Methods: We analysed B6 vitamers in remnant CSF samples of PLP-treated patients and four mammalian cell lines (HepG2, Caco2, HEK293 and Neuro-2a) supplemented with PL as the sole source of vitamin B6.

Results: Strong accumulation of pyridoxine (PN) in CSF of PLP-treated patients was observed, suggesting the existence of a PN-forming enzyme. Our *in vitro* studies show that all cell lines reduce PL to PN in a time- and dose-dependent manner. We compared the amino acid sequences of known PL reductases to human sequences and found high homology for members of the voltage-gated potassium channel beta subunits and the human aldose reductases. Pharmacological inhibition and knockout of these proteins show that none of the candidates is solely responsible for PL reduction to PN.

Conclusions: We show evidence for the presence of PL reductase activity in humans. Further studies are needed to identify the responsible protein.

General significance: This study expands the number of enzymes with a role in B6 *salvage* pathway. We hypothesize a protective role of PL reductase(s) by limiting the intracellular amount of free PL and PLP.

1. Introduction

Vitamin B6 is present in the human body as six interconvertible vitamers: pyridoxal (PL; aldehyde group at C4′), pyridoxine (PN; alcohol group at C4′), pyridoxamine (PM; amine group at C4′), and their 5′-phosphate esters pyridoxal 5′-phosphate (PLP), pyridoxine 5′-phosphate (PNP) and pyridoxamine 5′-phosphate (PMP) [20] (Fig. 1). Vitamin B6 is catabolized to 4-pyridoxic acid (PA) which is excreted in urine (Hufft and Perlzeig, [11]). PLP, the metabolically active form of vitamin B6, is an essential cofactor in human metabolism. A total of 56 PLP-dependent enzymes are currently known to exist in humans according to the B6 database (Percudani and Peracchi, 2009). Most PLP-dependent reactions involve the metabolism of amino acids, neurotransmitters (such as γ-aminobutyric acid, dopamine, serotonin, epinephrine and norepinephrine), nucleic acids and carbohydrates [24].

Although all organisms depend on vitamin B6 to survive, only microorganisms and plants can synthesize it *de novo* [5]. Mammals acquire B6 vitamers from the diet and convert them to PLP, using the vitamin B6 *salvage* pathway [4] (Fig. 1). Vitamin B6 enters the cells after hydrolysis of the phosphorylated forms by the membrane-bound tissue non-specific alkaline phosphatase (TNSALP; EC 3.1.3.1) (Waymire et al., 1995). Once inside the cells, PL kinase (PDXK; EC 2.7.1.35) phosphorylates the hydroxymethyl group of PL, PN and PM to their respective 5′-phosphate forms [9]. Pyridox(am)ine phosphate oxidase

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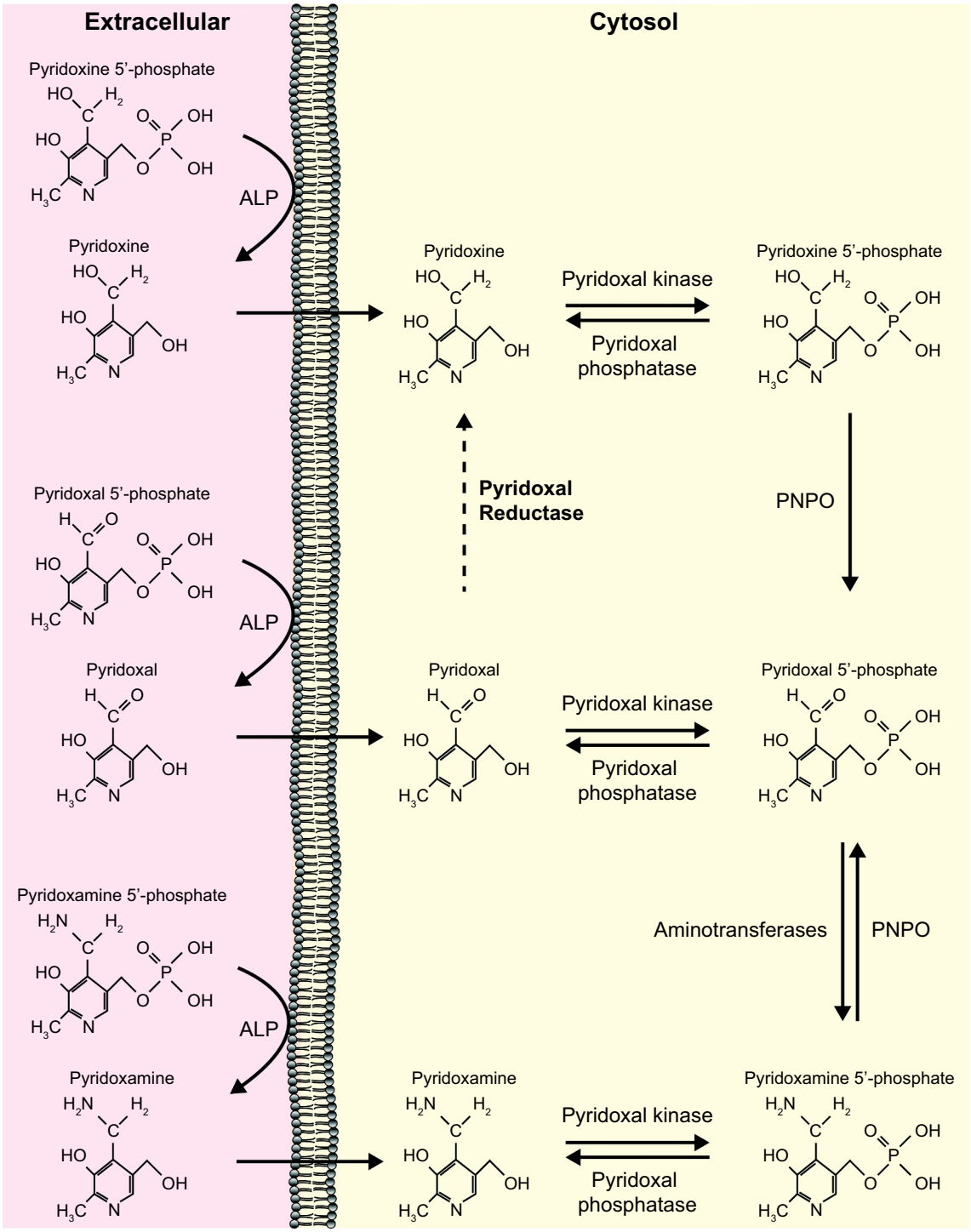

Fig. 1. The human vitamin B6 metabolic pathway. Membrane-bound alkaline phosphatase (ALP) dephosphorylates circulating PLP, PMP and PNP to their corresponding unphosphorylated forms (PL, PM and PN), in order to cross the cellular membrane. Inside the cells, PL kinase phosphorylates the hydroxymethyl group of PL, PN and PM into their respective 5′-phosphate forms. Pyridox(am)ine phosphate oxidase (PNPO) catalyses the oxidation of PNP and PMP to PLP. Dephosphorylation of PLP, PNP and PMP is catalysed by PL phosphatase. Aminotransferases use PLP during the interchange of the amino group between one amino acid and an α-keto acid, producing PMP as an intermediary in the first part of the reaction. The dashed arrow represents the place that PL reductase may occupy in vitamin B6 metabolism.

(PNPO; EC 1.4.3.5) catalyses the oxidation of PNP and PMP to PLP [16]. Dephosphorylation of PLP, PNP and PMP, as catalysed by PL phosphatase (PDXP; EC 3.1.3.74), is one of the mechanisms that cells have to control the amount of intracellular PLP concentrations [12]. An additional enzyme with a role in the B6 *salvage* pathway has been reported in yeast and plants, but never in humans. This enzyme, PL reductase (EC 1.1.1.65), is a member of the aldo-keto reductase (AKR) family [18] and catalyses the reduction of PL to PN, simultaneously oxidizing NADPH to $NADP^+$ [7]. PL reductase activity was first reported in the budding yeast *Saccharomyces cerevisiae*. Guirard and Snell purified the enzyme and showed that between a pH of 6.3 and 7.1 (the intracellular pH of *S. cerevisiae*) its equilibrium lies towards PN

formation. In addition, the authors proposed that formation of PLP followed the route: PL → PN → PNP → PLP, since PN was the preferred substrate for PL kinase in this yeast [7].

In 1998, Yagi et al. reported that the fission yeast *Schizosaccharomyces pombe* accumulated PN intracellularly not only when exposed to PN, but also during incubation with PL [27]. The same group subsequently purified the PL reductase of *S. pombe* and characterized its catalytic activity [18]. Several years later, *plr1*$^{+}$ was identified as the PL reductase-encoding gene in *S. pombe* [17], but the existence of other enzyme(s) with PL reductase activity was also proposed since strains of *S. pombe* with deleted *plr1*$^{+}$ still produced low amounts of PN when incubated with PL [17].

The presence of proteins with PL reductase activity has also been reported in plants. AtPLR1 was shown to catalyse the reduction of PL to PN in *Arabidopsis thaliana* [10]. At*PLR1* knockdown lines were shown to increase the expression of two other salvage pathway genes (*PDX3*, encoding the PL (PN, PM) kinase and *SOS4*, encoding PNP(PMP) oxidase), while little to no changes were observed in the expression of the *de novo* pathway genes (*PDX1.1*, *PDAX1.2*, *PDX1.3* and *PDX2*). In addition, At*PLR1* was significantly up-regulated when *PDX3* and *SOS4* were knocked down, leading to the conclusion that the AtPLR1-protein plays a role in the vitamin B6 salvage pathway [10].

In this study, we provide evidence for the presence of protein(s) with PL reductase activity in humans. Analysis of B6 vitamers in cerebrospinal fluid (CSF) samples of PLP-treated patients revealed surprisingly high concentrations of PN, thus suggesting the existence of a PN-forming enzyme. In addition, using *in vitro* studies, we discovered a cell-type-, time- and dose-dependent behaviour of PL reductase activity in various mammalian cell lines. Further studies are needed to identify the protein responsible for PL reductase activity in humans. Knockout models for several genes encoding the potential human PL reductase and pharmacological inhibition studies of these candidates unfortunately did not allow for the discovery of the responsible enzyme.

2. Material and methods

2.1. Cell culture

2.1.1. General steps

HepG2, Neuro-2a, Caco2 and HEK293 cells were purchased from the ATCC Cell Biology Collection. Dulbecco's modified eagle medium (DMEM) GlutaMAX™ (31966), vitamin B6-free DMEM GlutaMAX™ (custom made 31966-like), fetal bovine serum (FBS; 10270), penicillin-streptomycin (P/S; 15140) and trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, 0.5%) were purchased from Gibco (Invitrogen Life Technologies). Pyridoxal hydrochloride (PL-HCl) was purchased from Sigma-Aldrich (Steinheim, Germany). Cells were grown in 75 cm^2 flasks and maintained in DMEM GlutaMAX™ (supplemented with 10% heat-inactivated FBS (FBS-HI) and 1% P/S), in a humidified atmosphere of 5% CO_2 at 37 °C. When cells reached optimal confluence (> 80%) they were washed twice with room temperature (RT) PBS and plated in 96-well plates by trypsinization with 0.05% trypsin-EDTA.

2.1.2. Evaluation of PL reduction to PN

Cells were grown in 96-well plates and kept in DMEM GlutaMAX™ (supplemented with 10% FBS-HI and 1% P/S), in a humidified atmosphere of 5% CO_2 at 37 °C. *Media* were refreshed with DMEM GlutaMAX™ (suppl. with 10% FBS-HI and 1% P/S) 24 h before exposure to different PL concentrations. On the day of exposure, confluent wells were pre-incubated for 1 h with vitamin B6-free DMEM GlutaMAX™ (with 10% FBS-HI and 1% P/S). Vitamin B6-free DMEM GlutaMAX™ medium with FBS-HI was analysed and the contribution to the B6 vitamers concentrations proved to be minimal: PL 10 nM; PN 5 nM; PM 2 nM; while no phosphorylated vitamers (PLP, PNP and PMP) were detectable. The pre-incubation step was introduced to remove intracellular PN from the cells. After pre-incubation, cells were exposed to different experimental conditions: cells were either exposed to vitamin B6-free DMEM GlutaMAX™ (with 10% FBS-HI and 1% P/S) without PL (or any other source of vitamin B6) or to medium supplemented with PL (0.1, 100 and 1000 μmol/L), in a humidified atmosphere of 5% CO_2 at 37 °C. Triplicates of each condition were collected at t = 4, 24 and 48 h after exposure. Media of the four PL conditions (0, 0.1, 100 and 1000 μmol/L), kept at 37 °C in the absence of cells, were collected at the same time points. In the absence of cells, no spontaneous PN formation was observed under the experimental conditions. The residual content of PN (derived from the 10% FBS-HI supplement and PL-supplement impurity) was subtracted to PN secreted by cells.

2.2. Generation of knockout clones by CRISPR-Cas9 in HepG2 cells

HepG2 cells were transiently transfected with pSpCas9(BB)-2A-GFP (PX458) [19] encoding sgRNAs targeting KCNAB2, AKR1B1 or AKR1B10. sgRNAs were designed with http://crispor.tefor.net/ [8] and target coding sequences just upstream of a series of conserved residues, which in KCNAB2 have been defined as catalytic residues by Weng et al. [26] (see also Fig. 3). For KCNAB2 the following sgRNAs were used: sgRNA2: ATCTTACCTCATCGGTGATC, sgRNA3: ATCGGTGATCTGGCC TCCGA or sgRNA4: AGACTTCTGCTGTATCGAAG; for AKR1B1 sgRNA2: GTGAAGGTGGCCATTGACGT and for AKR1B10 sgRNA1: GTGGCCAT TGATGCAGGATAT. In all cases GFP-positive cells were sorted using a FACSAria II flow cytometer (BD) and plated in 10 cm dishes. Colonies were picked from these plates after 1 week and expanded. To confirm KCNAB2 absence, genomic DNA from candidate clones was isolated using the QIAamp DNA Micro kit (QIAGEN). The targeted genomic region was amplified by PCR using the forward primer 5′-CTGAGCAC CGACGGGATAAT-3′ and the reverse primer 5′-GCTGCATTCCCAATGA CCAA-3′. PCR fragments were sequenced and analysed by TIDE (https://tide.nki.nl/). If TIDE predicted PCR products to only contain indels resulting in reading frame shifts, PCR fragments were cloned into pJET (Thermo Scientific). Multiple pJET clones were sequenced to identify the exact nucleotide sequence of targeted alleles. In most cases, sequencing results were consistent with TIDE predictions. HepG2 clones with out of frame alleles only were considered to be mutant. For AKR1B10 the same approach was used but the genomic locus was amplified with the forward primer: 5′-TCCCTTGGGGTTATTTAGAG-3′, and reverse primer: 5′-AGAGTTCTTGCTGCCAACC-3′. Absence of AKR1B1 was demonstrated by Western blot analysis following standard molecular biology procedures, using a rabbit anti-AKR1B1 polyclonal antibody (Abcam cat. Ab62795; diluted 1:5000); mouse monoclonal anti-GAPDH (EMD Millipore cat. MAB374; diluted 1:5000) and anti-RaIA (BD transduction cat. 610,222; diluted 1:5000) antibodies were used as loading controls. Coding sequences of KCNAB2, AKR1B1 and AKR1B10 and protein sequences are provided in Supplementary Fig. 1.

2.3. Pharmacological inhibition of PL reductase activity

3,4-Dihydroxyphenylacetic acid (DOPAC), rutin, resveratrol, zopolrestat, tolrestat and oleanolic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Stock solutions (100 mmol/L) of each inhibitor were prepared in 1% DMSO. HepG2 cells were grown in 96-well plates. When optimal confluence was reached, each well was washed twice with RT PBS and pre-incubated for 1 h with vitamin B6-free DMEM GlutaMAX™ (with 10% FBS-HI and 1% P/S). After pre-incubation, cells were incubated for 20 min with vitamin B6-free medium and 100 μmol/L of each inhibitor (DOPAC, rutin, resveratrol, zopolrestat, tolrestat and oleanolic acid). Subsequently, cells were incubated with medium containing equimolar concentrations (100 μmol/L) of each inhibitor and of PL. Medium, in triplicate, was collected at t = 15 and 30 min after exposure to the experimental conditions. The amount of PN present in these media was subtracted from the residual content of PN (derived from the 10% FBS-HI supplement and PL-supplement impurity).

2.4. Vitamin B6 vitamer analysis in CSF samples of PLP-treated patients and medium samples of the different mammalian cell lines

Vitamin B6 vitamers were quantified in remnant CSF samples of two PLP-treated patients according to the UPLC-MS/MS method described by van der Ham et al. [25]. The medium samples from our *in vitro* studies were analysed using the same method. Apart from adapting the range of the calibrators to the samples' concentrations, and dilution of the medium samples (1:10 and 1:100) with TCA, no further adaptations were needed for sample preparation or vitamer analysis.

2.5. Statistical analysis

Unpaired two-tailed *t*-tests were performed using GraphPad Prism 6 (version 6.0.2, GraphPad Software Inc.) software.

3. Results

3.1. Pyridoxine accumulates in cerebrospinal fluid of children on PLP treatment

B6 vitamers were analysed in CSF samples of two children treated with PLP (Table 1). In addition to PL, PLP, PM and PA, metabolites commonly elevated in vitamin B6-treated patients, PN was strongly elevated (368 and 168 nmol/L; ref. range: < 0.03 nmol/L [1]) (Table 1). Contamination of the PLP supplement with PN, as a potential cause for the high PN level, was excluded: a single tablet of PLP (50 mg) was found to contain only 15 ng of PN (0.00003%, *i.e.* < 0.1 nmol). In addition to the patients described above, Table 1 displays the plasma B6 vitamer profile of reported PLP-treated patients [6,14].

3.2. Mammalian cells reduce pyridoxal to pyridoxine

Four mammalian cell lines (mouse neuroblastoma (Neuro-2a) cells; human embryonic kidney (HEK293) cells, human hepatocellular carcinoma (HepG2) cells, and human colorectal adenocarcinoma (Caco2) cells) were incubated with increasing concentrations of PL to study *in vitro* reduction of PL to PN. Secretion of PN was observed in all the tested cell lines in a dose- and time-dependent fashion, when PL was added to the culture medium (Fig. 2). In contrast, no PN secretion was observed in any of the tested cell lines when PL was absent from the culture medium (data not shown). The highest secretion of PN was observed in HepG2 cells, followed by Caco2, Neuro-2a and HEK293 cells (Fig. 2). Therefore, subsequent studies on reduction of PL to PN were performed in HepG2 cells.

3.3. Human homologs of PL reductase

To identify the human pyridoxal reductase enzyme, the *Basic Local Alignment Search Tool* (BLAST, NCBI) was used to identify possible homologs of PL reductase from yeast ($plr1^+$ in *S. pombe*; accession number: CAB16409.1; GI number: 2414666) and plant (AtPLR1 in *A. thaliana*; accession number: NP_200170.2; GI number: 30696358). The highest homology scores for both $plr1^+$ and AtPLR1 were for members of the auxiliary beta subunits of the voltage-gated potassium channel family (KCNAB; EC 1.1.1.-) and the human aldose reductase family (AR; EC 1.1.1.21). Based on sequence homology (Fig. 3), we tested three candidate proteins as human pyridoxal reductases: the auxiliary β2 subunit of the voltage-gated potassium channel (KCNAB2), and the aldose reductase (AKR1B1) and aldose reductase-like (AKR1B10) proteins.

Table 1
B6 vitamer concentrations in CSF and plasma samples of children treated with pyridoxal 5′-phosphate.

	Ref.	Body fluid	Diagnosis and clinical information	Age	Therapy, dosage	Vitamin B6 vitamers (nmol/L)						
						PLP	PL	PMP	PM	PNP	PN	PA
Ref. range[a]		**CSF**		**< 2 weeks**		**19–221**	**16–199**	**< 5.4**[d]	**0.3–3.3**	**nd**	**< 0.03**[d]	**1.9–52**
				> 2 weeks		**8–76**	**14–103**	**< 5.4**[d]	**0.3–1.4**		**< 0.03**[d]	**0.9–11**
1		CSF	No ATQ nor PNPO deficiencies	1 d	PLP, 90 mg/day	54	**3940**	nd	**63**	nd	**368**	**548**
2		CSF	ATQ deficiency	17 d	PLP, 100 mg/day	**129**	**2078**	nd	**19**	nd	**168**	**136**
Ref. range[b]		**Plasma**		**4.3 y – 16 y**		**46–321**	**4.6–18.1**	**nd-9.3**	**nd**	**nd**	**nd-0.62**	**16.4–139**
Known IEM affecting vitamin B6 metabolism												
3	b	Plasma	PNPO deficiency	2 y 2 m	PLP, 30 mg/kg/day	**580**	**426.8**	**18**	**192.7**	**43**	**575**	**792.8**
4	b	Plasma	PNPO deficiency	10 y 2 m	PLP, 30 mg/kg/day	**632.6**	**5798**	**101**	**2731**	**77.2**	**598.8**	**7926.3**
Seizures fully responsive to vitamin B6 (No ATQ nor PNPO deficiencies)												
5	b	Plasma	PLP responsive	6 y 5 m	Lamotrigine and PLP, 30 mg/kg/day	**709.4**	**7893**	nd	**28**	nd	**32**	**7331**
Seizures partially responsive to vitamin B6 (No ATQ nor PNPO deficiencies)												
6	b	Plasma	Asperger Syndrome, seizures, PLP responsive	13 y 3 m	PLP, 30 mg/kg/day	306.4	**8452.5**	4.1	**5.1**	nd	**2.7**	**5031.9**
7	b	Plasma	Partially PLP responsive	2 y 7 m	PLP, 30 mg/kg/day	**478.4**	**102.4**	6.8	nd	nd	0.31	**144.6**
Ref. range[c]		**Plasma**		**< 18 y**		**10–289**	**4–85**	**na**	**< 1**	**na**	**< 1**	**5–564**
8	c	Plasma	PNPO deficiency	3 y	PLP, 50 mg/day	**1080**	**4180**	na	**1180**	na	**1210**	**3340**
				4 y	PLP, 690 mg/day	**919**	**6800**	na	**1180**	na	**1570**	**9610**
9	c	Plasma	PNPO deficiency	na	PN + PLP, 100 + 90 mg/day	**412**	**7640**	na	**2050**	na	**11,100**	**4940**

[a] [1]
[b] [6].
[c] [14].
[d] Determined limit of quantification (LOQ) of this B6 vitamer. nd, not detected; na, not available; PLP, pyridoxal 5′-phosphate; PL, pyridoxal; PMP, pyridoxamine 5′-phosphate; PM, pyridoxamine; PNP, pyridoxine 5′-phosphate; PN, pyridoxine; PA, 4-pyridoxic acid; PNPO, pyridox(am)ine 5′-phosphate oxidase; ATQ, antiquitine; CSF, cerebrospinal fluid; y, years; m, months; d, days. Reference ranges were established using samples from patients not affected by disorders of vitamin B6 metabolism and not treated with vitamin B6. Values outside the reference range are shown in bold.

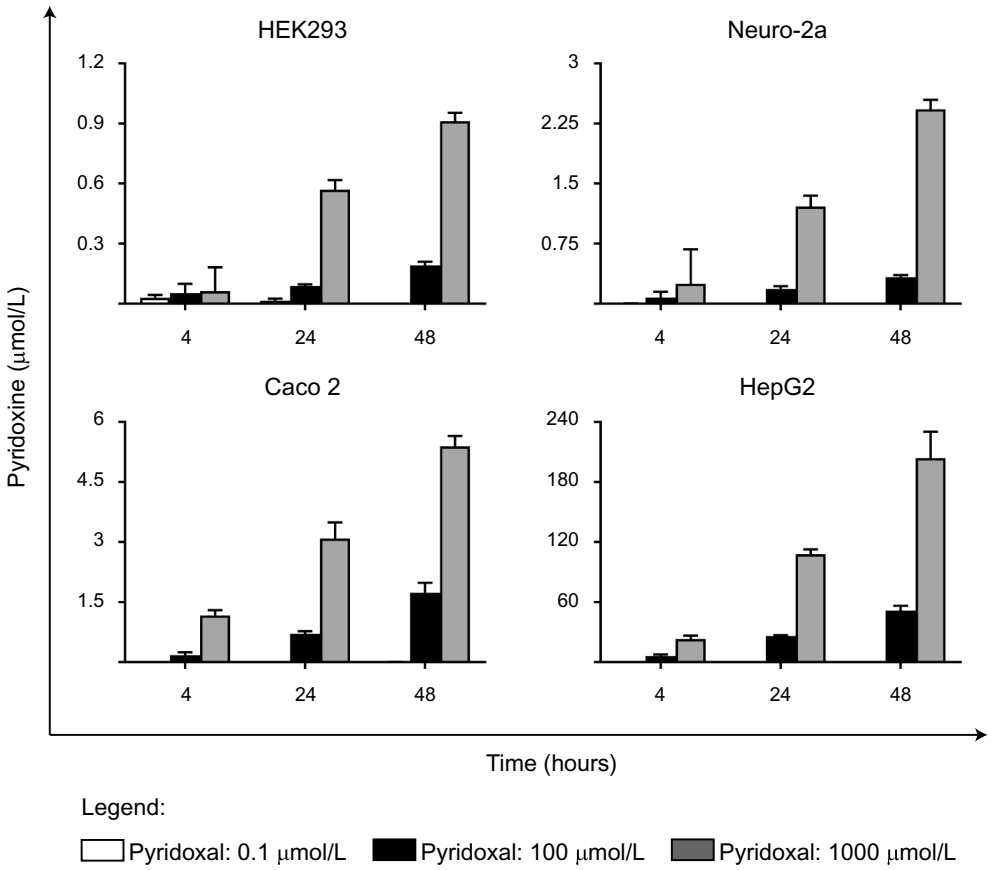

Fig. 2. Pyridoxine accumulates in the medium of four mammalian (Hek293, Neuro2a, Caco2 and HepG2) cell lines. Pyridoxine secretion in the culture medium is dose- and cell type-dependent, and observed in all of the four studied cell lines, upon PL supplementation.

3.4. The β2 subunit of the voltage-gated potassium (Kv) channel

To verify whether KCNAB2 represents the human PL reductase, KCNAB2$^{-/-}$ HepG2 cells were generated by CRISPR/Cas9 technology. Despite complete absence of KCNAB2 protein (Fig. 4A), the amount of PN secreted to the culture medium, after 4 h of incubation with 100 μmol/L of PL, was not reduced when compared to control cells, thus disproving the hypothesis of KCNAB2 representing the sole human PL reductase (Fig. 4B). Pharmacological inhibition studies with KCNAB2-specific inhibitors (3,4-dihydroxyphenylacetic acid (DOPAC), rutin and resveratrol) [2] showed that none of these inhibitors lead to significant decreases of PN secretion (Fig. 4C), thus corroborating the results obtained with the KCNAB2-knockout clones.

3.5. The human aldose reductase (AR)

The human proteins with the second highest sequence homology to plr1^{+} and AtPLR1 were the aldose reductase and aldose reductase-like proteins (AKR1B1 and AKR1B10, respectively). To study the role of these proteins in reduction of PL to PN, we generated AKR1B1$^{-/-}$, AKR1B10$^{-/-}$ and AKR1B1/10$^{-/-}$ knockout HepG2 cells. Absence of AKR1B1$^{-/-}$ and AKR1B1/10$^{-/-}$ was confirmed by Western Blot analysis (Fig. 5A and B), while absence of AKR1B10 was confirmed by sequencing (Fig. 5C). No differences in PN secretion, after 4 h of incubation with 100 μmol/L PL, were observed between HepG2 control cells and AKR1B1$^{-/-}$, AKR1B10$^{-/-}$ and AKR1B1/10$^{-/-}$ knockout clones (Fig. 5D).Pharmacological inhibition studies with AKR1B1-specific inhibitors (tolrestat and zopolrestat) and the AKR1B10-specific inhibitor (oleanolic acid) [28] were also performed. Zopolrestat strongly inhibited PN secretion (47% ($P < .05$) inhibition at $t = 15$ min and 61% inhibition ($P < .05$) at $t = 30$ min) (Fig. 5E), while the other two inhibitors did not significantly affect PN secretion.

4. Discussion

Three decades have passed since PL reductase activity was discovered in yeast [7], followed more recently by its description in plants [10]. No mammalian PL reductase has been described to date. However, the presence of a reductase acting on PL is suggested by reports on increased PN concentrations in plasma of PLP-treated patients (with PNPO deficiency or with other seizure disorders responsive to vitamin B6) [6,14].

Presence of PN in CSF and plasma has mainly been observed in PN-treated patients, being absent in untreated subjects [6,14]. Here, we confirm the *in vivo* reduction of PL to PN by demonstrating the presence of PN in CSF of two PLP-treated patients. Furthermore, experiments in cultured cells reveal that PN is rapidly formed from PL in a time- and dose-dependent and cell-type specific fashion. Liver (HepG2) cells secrete the highest amount of PN, followed by intestinal (Caco2), neuronal (Neuro-2a) and kidney (HEK293) cells. These data suggest that PL reductase activity shows tissue dependence. Likewise, there is tissue dependency of other enzymes acting on PL. PL can be phosphorylated by pyridoxal kinase, converted into pyridoxic acid by aldehyde oxidase and/or aldehyde dehydrogenase and, as presented by our work, reduced to PN. The interplay between uptake of PL and these enzymes determines the amount of PN formed.

Based on homology to the known PL reductase amino acid sequences of *S. pombe* and *A. thaliana*, we tested three candidate proteins as human pyridoxal reductases: the auxiliary β2 subunit of the voltage-gated potassium (Kv) channel (KCNAB2) and the aldose reductase and

A

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Sp_PLR1         ------------------------------------------------------------
At_PLR1         ------------------------------------------------------------
Hs_KCNAB1       --------MLAARTGAAGSQISEENTKLRRQSGFSVAGKDKSPKKASENAKDSSLSPSGE
Hs_KCNAB2       --------MYPESTTGSPARLSLRQTGSPGMIYSTRYGSPKRQ-----------------
Hs_KCNAB3       MQVSIACTEQNLRSRSSEDRLCGPRPGPGGGNGGPAGGGHGNPPGGGGSGPKARAALVPR
Hs_AKR1B1       ------------------------------------------------------------
Hs_AKR1B10      ------------------------------------------------------------

Sp_PLR1         -------------------------------------MPIVSGFKVGPIGFGLMGLTW---
At_PLR1         --MALTLSTTKTFTNINCSNNTSNITTFKPLKLPLFWPWQKVKMGPLSVSPMGFGTWAWG
Hs_KCNAB1       SQLRARQLALLREVEMNWYLKLCDLSSEHTTVCTTGMPHRNLGKSGLRVSCLGLGTWVT-
Hs_KCNAB2       -------------------------------------LQFYRNLGKSGLRVSCLGLGTWVT-
Hs_KCNAB3       PPAPAG--------------------ALRESTGRGTGMKYRNLGKSGLRVSCLGLGTWVT-
Hs_AKR1B1       ----------------------------------------MASRLLLNNGAKMPILGLGTWKS-
Hs_AKR1B10      ------------------------------------MATFVELSTKAKMPIVGLGTWKS-
                                                                  **

Sp_PLR1         -----KPKQTPDEEAFEVMNYALSQGSNYWDAGEFYGVDPPTSNLDLLARYFEKYP---
At_PLR1         NQLLWGYQTSMDDQLQQAFELALENGINLFDTADSYGTGRLNGQSERLLGKFIKESQGLK
Hs_KCNAB1       -----FGGQISDEVAERLMTIAYESGVNLFDTAEVYAAG----KAEVILGSIIKKK---G
Hs_KCNAB2       -----FGGQITDEMAEQLMTLAYDNGINLFDTAEVYAAG----KAEVVLGNIIKKK---G
Hs_KCNAB3       -----FGSQISDETAEDVLTVAYEHGVNLFDTAEVYAAG----KAERTLGNILKSK---G
Hs_AKR1B1       -----PPGQVT-----EAVKVAIDVGYRHIDCAHVYQNE-----NEVGVAIQEKLREQ-V
Hs_AKR1B10      -----PLGKVK-----EAVKVAIDAGYRHIDCAYVYQNE-----HEVGEAIQEKIQEK-A
                                *   *    *    *              *

Sp_PLR1         ENANKVFLSVKGGLDFKTLVPDGNPDFVSKSVENVIAHLRGTKKLDLFQCAR-VDPNVPI
At_PLR1         GKQNEVVVATKFAAYP-----WRLTSGQFVNACRASLDRLQIDQLGIGQLHWSTASYAPL
Hs_KCNAB1       WRRSSLVITTKLYWGGKAETERGLSRKHIIEGLKGSLQRLQLEYVDVVFANR-PDSNTPM
Hs_KCNAB2       WRRSSLVITTKIFWGGKAETERGLSRKHIIEGLKASLERLQLEYVDVVFANR-PDPNTPM
Hs_KCNAB3       WRRSSYVITTKIFWGGQAETERGLSRKHIIEGLRGSLERLQLGYVDIVFANR-SDPNCPM
Hs_AKR1B1       VKREELFIVSKLWCT-------YHEKGLVKGACQKTLSDLKLDYLDLYLIHW-PTGFKPG
Hs_AKR1B10      VKREDLFIVSKLWPT-------FFERPLVRKAFEKTLKDLKLSYLDVYLIHW-PQGFKSG
                        *

Sp_PLR1         ETTMKTL--KGFVDSGKISCVGLSEVSAETIKRAHA------VVPIAAVEVEYSLFSRDI
At_PLR1         QELVLWDGLVQMYEKGLVRAVGVSNYGPQQLVKIHDYLKTR-GVPLCSAQVQFSLLSMGK
Hs_KCNAB1       EEIVRAM--THVINQGMAMYWGTSRWSAMEIMEAYSVARQFNMIPPVCEQAEYHLFQREK
Hs_KCNAB2       EETVRAM--THVINQGMAMYWGTSRWSSMEIMEAYSVARQFNLTPPICEQAEYHMFQREK
Hs_KCNAB3       EEIVRAM--TYVINQGLALYWGTSRWGAAEIMEAYSMARQFNLIPPVCEQAEHHLFQREK
Hs_AKR1B1       KEFFPLD--DESGNVVPSDTN---------ILDTWAAMEELVDEGLVKAIGISNF-NHLQ
Hs_AKR1B10      DDLFPKD--DDKGNAIGGKAT---------FLDAWEAMEELVDEGLVKALGVSNFSHFQI

Sp_PLR1         ETNGIMDICRKLSIPIIAYSPFCRGLLTGRIKTVEDLKEFAKSFPFLEYLDRFSPDVFAK
At_PLR1         EQLEIKSICDELGIRLISYSPLGLGMLTG--------KYSSSKLPTGPRSLLFRQILPGL
Hs_KCNAB1       VEVQLPELYHKIGVGAMTWSPLACGIISG--------KYGNGVPESSRASLKCYQWLKEE
Hs_KCNAB2       VEVQLPELFHKIGVGAMTWSPLACGIVSG--------KYDSGIPPYSRASLKGYQWLKDD
Hs_KCNAB3       VEMQLPELYHKIGVGSVTWYPLACGLITS--------KYDGRVPDTCRASIKGYQWLKDD
Hs_AKR1B1       VEMILNKPGLKYKPAVNQIECHPYLTQEKLIQYCQSKGIVVTAYSPLGSPDRPWAKPEDP
Hs_AKR1B10      -EKLLNKPGLKYKPVTNQVECHPYLTQEKLIQYCHSKGITVTAYSPLGSPDRPWAKPEDP

Sp_PLR1         NLPFLQAVEQLAKKFG---------MTMPEFSLLFIMASGNGLVIPIPGSTSVSRTKSN
At_PLR1         EPLLLALSEIAKKRGK----------TMPQVAINWCICK---GTVPIPGIKSVRHVEDN
Hs_KCNAB1       RIVSEEGRKQQNKLKDLSPIAERLGCTLPQLAVAWCLRNEGVSSVLLGSSTPEQLIENL
Hs_KCNAB2       KILSEEGRRQQAKLKELQAIAERLGCTLPQLAIAWCLRNEGVSSVLLGASNADQLMENI
Hs_KCNAB3       KVQSEDGKKQQAKVMDLLPVAHQLGCTVAQLAIAWCLRSEGVSSVLLGVSSAEQLIEHL
Hs_AKR1B1       SLLEDPRIKAIAAKHNK-----------TTAQVLIRFPMQRNLVVIPKSVTPERIAENF
Hs_AKR1B10      SLLEDPKIKEIAAKHKK-----------TAAQVLIRFHIQRNVIVIPKSVTPARIVENI

Sp_PLR1         LNALNKSLSPEQFKEAKEVLSKYPIYGLRYNEQLAGTLSV--
At_PLR1         LGALGWKLTNDEQLQLEYAAKESPKSMIQNIFQTR-------
Hs_KCNAB1       GAIQVLPKMTSHVVNEIDNILRNKPYSKKDYRS---------
Hs_KCNAB2       GAIQVLPKLSSSIIHEIDSILGNKPYSKKDYRS---------
Hs_KCNAB3       GALQVLSQLTPQTVMEIDGLLGNKPHSKK--------------
Hs_AKR1B1       KVFDFELSSQDMTTLLSYNRNWRVCALLSCTSHKDYPFHEEF
Hs_AKR1B10      QVFDFKLSDEEMATILSFNRNWRACNVLQSSHLEDYPFNAEY
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(caption on next page)

Fig. 3. A. Amino acid sequence alignment of two known PL reductases from yeast (plr1$^+$ in *S. pombe*) and plant (AtPLR1 in *A. thaliana*) with the human KCNAB1, KCNAB2, KCNAB3, AKR1B1 and AKR1B10 sequences. The alignment was done with ClustalX and manually improved on the basis of secondary structure prediction using Jpred (http://www.compbio.dundee.ac.uk/jpred). Predicted alpha-helices are marked yellow, beta-sheets are marked turquoise. Catalytic residues that function in aldo-keto reductase activity in KCNAB2 [26] and are conserved in AKR proteins are marked by red asterisks below the sequence alignment. Cas9 nuclease sites (−3 relative to the PAM sequence) are located in (KCNAB2, AKR1B1) or immediately downstream of (AKR1B10) codons that have been marked green. B. Pairwise alignment of the proteins shown in A using the NCBI Basic Local Alignment Tool (BLAST). For each combination the percentage of identity is shown in bold with the number of residues used to calculate this percentage below. In between brackets the percentage of similarity is shown. Note that the regions of yeast PLR1 aligned to AKR-members are short. However, PLR1 from Arabidopsis, which has also been functionally characterized, does show significant similarity over extended regions to both KCNAB and AKR members. This is also reflected in the *E*-values (shown in grey cells).

aldose reductase-like (AKR1B1 and AKR1B10, respectively) proteins.

The voltage-gated potassium channels are the most complex class of the voltage-gated ion channels both from a functional and a structural point of view [21]. Their diverse functions include regulation of neurotransmitter release, heart rate, insulin secretion, neuronal excitability, epithelial electrolyte transport, smooth muscle contraction, and cell volume [21]. The β-subunits of the potassium channels are cytoplasmic and associate in a 4:4 stoichiometry with the N-terminus of the membrane-spanning α-subunits [15,22,23]. Furthermore, the β-subunits are members of the AKR superfamily, containing an active site composed of conserved catalytic residues, a coenzyme and a substrate binding site [3,13]. The Kvβ2-subunit is the most strongly expressed Kv1-associated β-subunit protein [15]. Although the catalytic and kinetic mechanisms of Kvβ2 are poorly understood and its substrate specificity is unknown, valuable information on its substrate preference is available in the literature. The β2-subunit has a strong affinity for the reduced pyridine coenzyme NADPH, suggesting that the protein functions mainly as a reductase rather than as an oxidase [13]. Besides oxidation of NADPH to NADP$^+$, the β2-subunit catalyses the reduction of both aldehydes and ketones. Interestingly, the β2-subunit reduces preferably aldehydes and aromatic substrates [21], all chemical characteristics of PL, which has an aromatic pyridine ring and an aldehyde functional group at C4' (Fig. 1). Deletion of KCNAB2 and the use of KCNAB2-specific inhibitors however, failed to decrease PL to PN reduction, suggesting that KCNAB2 does not function as the sole human PL reductase. However, redundancy in enzyme activity between the three β-subunits (β1, β2 and β3) may occur, minimizing the deleterious effect of a β2-subunit deletion.

The second family of proteins with the highest amino acid homology to the known PL reductases were the human aldose reductases. Even though the primary structure of the yeast PL reductase shows low identity with the known human aldo-keto reductases (AKRs), the secondary structure was reported to be similar to that of the human aldose reductase [18].

The use of zopolrestat, an AKR1B1-specific inhibitor, strongly inhibited PN-secretion in our HepG2-WT cell model. Nevertheless, we were unable to confirm PL reductase function of AKR1B1 in a genetic AKR1B1-knockout cell line, showing that zopolrestat may be inhibiting other proteins, rather than AKR1B1, with a role in reducing PL to PN. The use of oleanolic acid, the AKR1B10-specific inhibitor, failed to inhibit PL reduction. Redundancy between AKR1B1 and AKR1B10 but also with other cellular reductases likely occurs. To study the redundancy between both aldose reductases we generated AKR1B1/AKR1B10$^{-/-}$ knockouts. These double knockouts secreted the same amounts of PN as the control cell lines. Interestingly, when PL reductase was identified in *S. pombe* [17], the authors had already proposed the presence of other enzymes with PL reductase activity in those yeast strains besides the one encoded by the *plr1*$^+$ gene.

Although our *in vitro* studies were unable to identify the enzyme responsible for reducing PL to PN, we present strong evidence for its existence. We clearly show that CSF samples of patients treated with PLP have increased PN levels, corroborating the existence of enzymes with PL reductase activity in humans. Similar results have been reported in plasma samples of PLP-treated patients, even though up to date no explanation was given for this puzzling finding. In our study, all mammalian cell lines reduced PL to PN and secreted PN to the culture medium in a time- and dose-dependent way. Although these findings are not entirely understood, PL reductase(s) may play an important role

B

GENE	Sp_PLR1	At_PLR1	Hs_KCNAB1	Hs_KCNAB2	Hs_KCNAB3	Hs_AKR1B1	Hs_AKR1B10
Sp_PLR1	100	2 E^{-22}	4 E^{-16}	8 E^{-16}	2 E^{-14}	7 E^{-2}	3 E^{-2}
At_PLR1	**25** (41) 79/312	100	1 E^{-25}	7 E^{-28}	2 E^{-24}	3 E^{-12}	1 E^{-14}
Hs_KCNAB1	**22** (42) 68/310	**26** (49) 82/319	100	0.0	0.0	3 E^{-07}	1 E^{-10}
Hs_KCNAB2	**21** (42) 68/331	**27** (49) 83/310	**84** (95) 277/329	100	0.0	2 E^{-07}	1 E^{-09}
Hs_KCNAB3	**21** (41) 68/331	**26** (48) 82/308	**75** (88) 248/329	**74** (89) 241/325	100	9 E^{-05}	6 E^{-05}
Hs_AKR1B1	**22** (42) 17/76	**23** (40) 74/316	**29** (50) 25/85	**24** (43) 41/174	**27** (45) 23/85	100	2 E^{-178}
Hs_AKR1B10	**20** (42) 15/76	**23** (41) 72/313	**24** (43) 54/222	**24** (43) 52/222	**24** (45) 26/108	**71** (83) 223/316	100

Fig. 3. (*continued*)

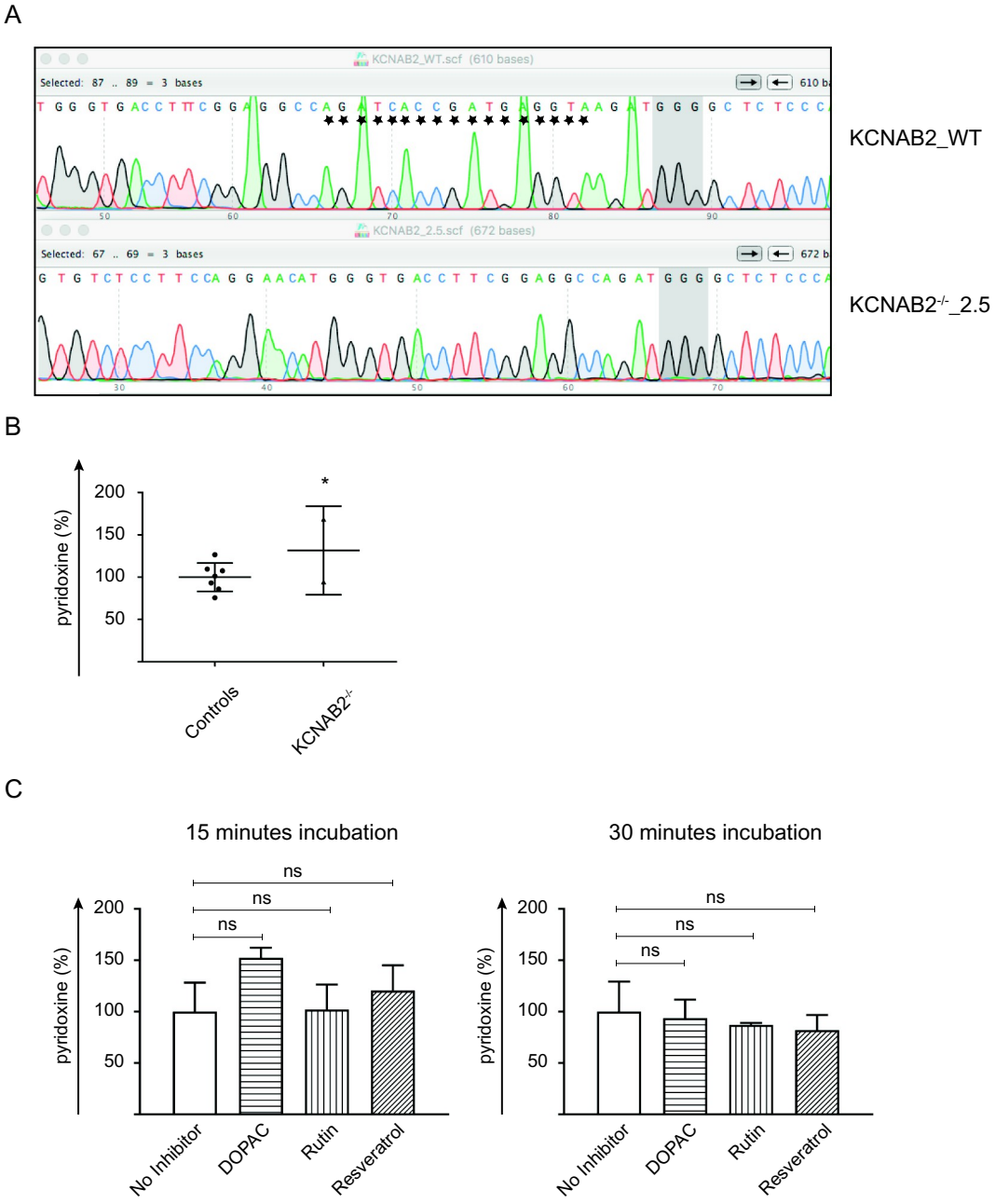

Fig. 4. KCNAB2 protein as a potential human pyridoxal reductase. A. Confirmation of KCNAB2-knockout in HepG2 cells, after CRISPR/Cas9, was achieved by sequencing. Representative clone: comparison of the sequence of KCNAB2 wild type (WT) cells and KCNAB2$^{-/-}$_2.5 clone, which contains a homozygous deletion of 17 nucleotides (marked with an asterisk in the wild type sequence). The proto-spacer adjacent motif (PAM) sequence is marked in grey. B. PN secretion in KCNAB2-deficient HepG2 clones. All results are represented as the mean of triplicates ± SD; * $P < .05$. C. The effect of specific KCNAB2 inhibitors on PN secretion. All results are represented as the mean of triplicates ± SD; ns, not significant.

in cellular detoxification and protection, since it is a well-known fact that PLP, and to a lesser extent PL, react non-enzymatically with primary amino groups of amines and amino acids through their adehyde group at C4' [4]. The electrophilic characteristics of the aldehyde group at C4' derive from the existence of a protonated pyridinium hydrogen (N1) and a phenoxide anion at C3'. These stabilize the protonated state of the imine nitrogen during the formation of the Shiff base between PL (P) and substrates [4]. PN, on the other hand, has a hydroxymethyl group (-CH_2OH) at C4', leading to a lower reactivity towards amino groups and therefore less cellular toxicity.

5. Conclusions

Here, we show that mammalian cells, like plants and yeast, secrete

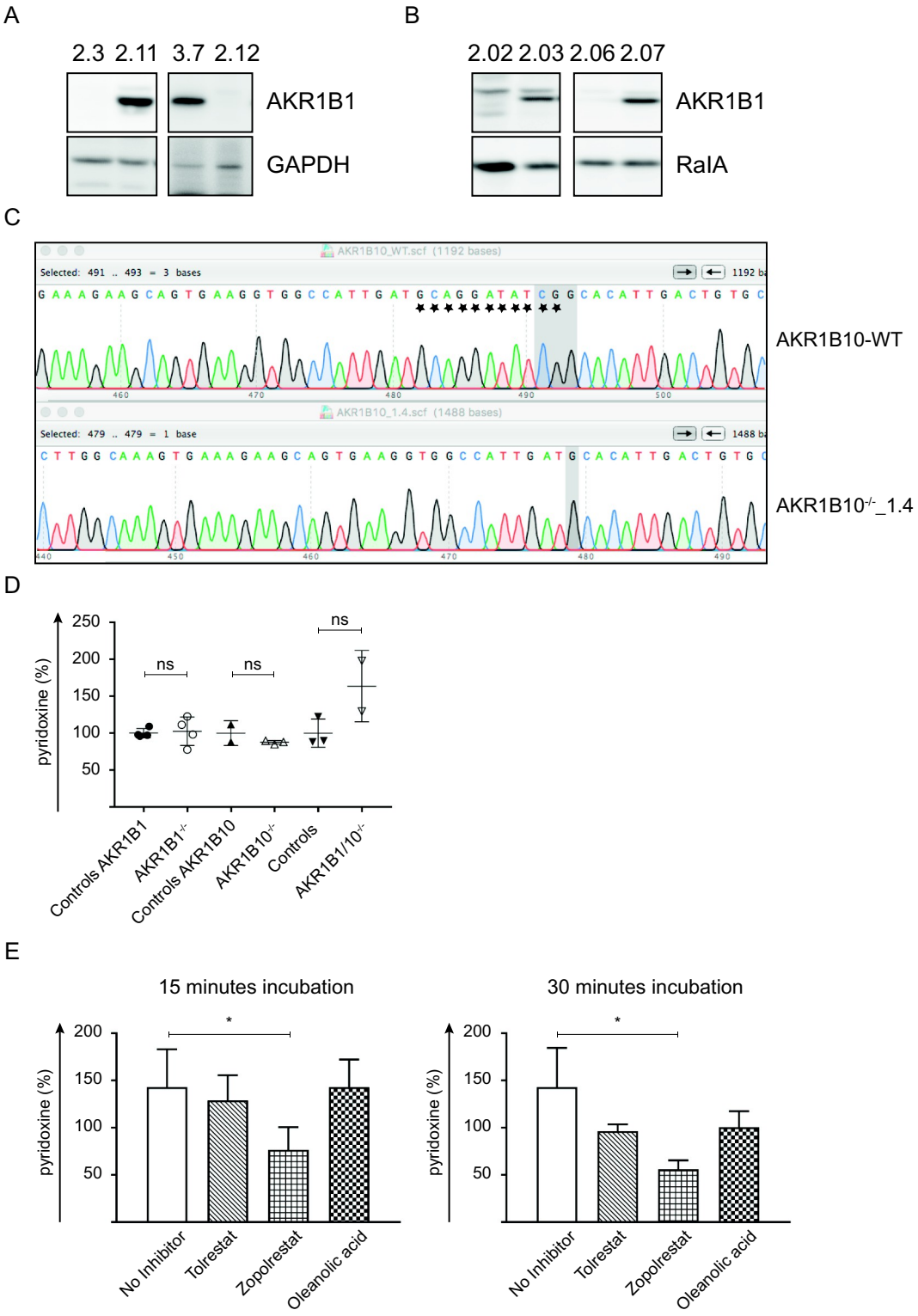

Fig. 5. AKR1B1 and AKR1B10 proteins as potential human pyridoxal reductases. A. Confirmation of AKR1B1-knockout in the HepG2 cells, after CRISPR/Cas9, was achieved by Western Blot analysis. Complete absence of AKR1B1 in clones 2.3 and 2.12 at the protein level is observed, while clones 2.11 and 3.7 contain an intact AKR1B1 gene. GAPDH is shown as a loading control. B. Confirmation of AKR1B1/10-double knockouts, after CRISPR/Cas9: Western blot showing absence of AKR1B1 in AKR1B10$^{-/-}$ clone 1.4 (clones 2.02 and 2.06) at the protein level. Clones 2.03 and 2.07 contain an intact AKR1B1 gene. RalA is shown as a loading control. C. AKR1B10-knockout confirmation, after CRISPR/Cas9, was achieved by sequencing. Representative clone: comparison of the sequence of AKR1B10 wild type cells and AKR1B10$^{-/-}$_1.4 clone, which contains a homozygous deletion of 11 nucleotides (marked with an asterisk in the wild type sequence). The PAM sequence is marked in grey. D. PN secretion in AKR1B1$^{-/-}$ AKR1B10$^{-/-}$ and double knockout HepG2 clones. All results are represented as the mean of triplicates ± SD; ns, not significant. E. The effect of AKR1B1- and AKR1B10-specific inhibitors on PN secretion. All results are represented as the mean of triplicates ± SD; * $P < .05$.

PN in a PL (time and dose) dependent fashion, suggesting the existence of PL reductase activity. Physiologically, PL reductase(s) may serve to limit intracellular accumulation of the reactive aldehydes, PL and PLP, protecting the cell from unwanted reactions that could lead to inactivation of important metabolites.

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Clause from BBA (Authorship)

Rúben Ramos, Judith Jans and Nanda Verhoeven-Duif designed the study. Rúben Ramos, Fried Zwartkruis, Susan Zwakenberg, Esmee Vringer and Marjolein Bosma performed the study and collected the data. Rúben Ramos, Fried Zwartkruis, Monique Albersen, Judith Jans and Nanda Verhoeven-Duif interpreted the data. Rúben Ramos, Judith Jans and Nanda Verhoeven-Duif wrote the article. All authors revised it critically and approved the final version to be submitted.

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