

NAD metabolism in HPRT-deficient mice

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Abstract The activity of hypoxanthine-guanine phosphoribosyltransferase (HPRT) is virtually absent in Lesch-Nyhan disease (LND), an X-linked genetic disorder characterized by uric acid accumulation and neurodevelopmental dysfunction. The biochemical basis for the neurological and behavioral abnormalities have not yet been completely explained. Prior studies of cells from affected patients have shown abnormalities of NAD metabolism. In the current studies, NAD metabolism was evaluated in HPRT gene knock-out mice. NAD content and the activities of the enzymes required for synthesis and breakdown of this coenzyme were investigated in blood, brain and liver of HPRT and control mice. NAD concentration and enzyme activities were found to be significantly increased in liver, but not in brain or blood of the HPRT mice. These results demonstrate that changes in NAD metabolism occur in response to HPRT deficiency depending on both species and tissue type.

Keywords Hypoxanthine-guanine phosphoribosyltransferase deficiency · Lesch-Nyhan disease · Knockout mice · NAD · Pyridine nucleotides · Purine nucleotides

Introduction

Lesch-Nyhan disease (LND) is an inborn X-linked disease caused by complete deficiency of the enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT);

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E.C. 2.4.2.8), due to mutation in the encoding gene, a constitutively expressed housekeeping gene located in X-chromosome (Jinnah et al. 2000; Lesch and Nyhan 1964).

HPRT is a purine salvage enzyme, with notably higher expression in mammalian brain and testes. LND patients exhibit uric acid urinary stones and a devastating neurological syndrome with dystonia and self-injurious behavior (Jinnah et al. 2006). The mechanism of uric acid overproduction has been clarified, while the connection between HPRT deficiency and the neurobehavioral manifestations remains unclear, though evidence has accumulated for dysfunction of basal ganglia dopamine systems (Nyhan and Wong 1996).

The biochemical aspects of LND have been explored extensively in patient cells (red blood cells, fibroblasts, lymphoblasts) in autopsied brain specimens, and in different HPRT cultured cell lines. The results are often inconsistent, suggesting significant differences depending upon both species and tissue source (Shirley et al. 2007).

Animal models, such as the HPRT knock-outs and 6-hydroxydopamine-treated rats, have been developed to reproduce the disease (Jinnah et al. 1992; Allen and Davis 1999). Such animals do not develop the neurological symptoms associated with the complete deficiency of HPRT in man, but their brains are not entirely normal (Jinnah et al. 1994). The HPRT knock-out mice have metabolic aberrations, such as increased “de novo” purine synthesis and dopamine depletion in the basal ganglia (Jinnah et al. 1993; Jinnah et al. 1999). They were also found to have an increased response to drugs interacting with dopamine system. On the basis of the above observations they are considered useful tools for studying the metabolic abnormalities following HPRT deficiency, but not for the expression of neuro-behavioral defects.

Several metabolic abnormalities are known to occur in LND, including grossly increased *de novo* purine synthesis, with raised uric acid production and excretion. Peculiar metabolic alterations have also been reported to accompany HPRT deficiency in erythrocytes, such as GTP depletion, increased phosphoribosylpyrophosphate and UDP-glucose, and grossly increased NAD. A number of related enzymes have also been reported to display abnormally higher activities in HPRT deficient erythrocytes, such as adenine phosphoribosyltransferase (APRT, E. C.2.4.2.7) (Simmonds et al. 1988), 5′nucleotidase (5′NT) (Pesi et al. 2000), and two enzymes committed to NAD synthesis, nicotinate phosphoribosyltransferase (NAPRT; E.C. 2.4.2.11) and NAD synthetase (NADs, E.C. 6.3.5.1) (Micheli et al. 1999). In contrast, LND fibroblasts exhibit NAD, ATP and GTP depletion (Fairbanks et al. 2002). Together these findings suggest disturbed pyridine metabolism accompanying purine perturbation in different cell types.

Cellular NAD concentration results from a balance between synthesis and utilization, the latter occurring in different processes utilising its adenylyl moiety, such as DNA repair (polyADPR synthesis), calcium mobilization (cADPR synthesis) or protein post-translational modifications (protein ADP-ribosylation) (Ziegler 2000). NAD depletion in HPRT deficient nucleated cells, such as LND fibroblasts, might be related to NAD over-utilisation by poly (ADP-ribose) polymerase in DNA repair, unlike erythrocytes, known to lack this pathway.

The synthesis of NAD can be accomplished through a “salvage” pathway from preformed pyridine rings (nicotinic acid or nicotinamide), or through an aerobic de

novo pathway, starting from tryptophan, leading to quinolinate. Tryptophan is also used in the bipterin-requiring pathway leading to 5-hydroxy-tryptamine (serotonin). Connections between the two tryptophan-derived pathways have been demonstrated in human and rat neurodegenerative events (Foster et al. 1985) and in rats undergoing treatment with drugs affecting one or both pathways. Such observations suggested possible involvement in LND pathogenesis.

This study was devised to determine whether alterations of NAD concentration and metabolism similar to those found in HPRT-deficient human erythrocytes or fibroblasts could also be seen in the HPRT⁻ mouse model of LND. The present study focused on NAD synthesis through the salvage pathway and on its breakdown in blood, brain and liver. The results show significant changes in NAD metabolism in the liver, but no changes in brain or blood. They provide further support for important differences in the metabolic consequences of HPRT deficiency among different tissues and species.

Materials and methods

Materials

Reagents of analytical grade were purchased from SIGMA (St. Louis MO, USA). Other Chemicals for HPLC separation were of the highest quality available.

Animal tissues

Frozen tissue samples from 6 control and 6 HPRT⁻ mice were derived from breeders carrying a deletion mutation spanning the first two exons of the HPRT gene. Control and HPRT⁻ male mice, 8–12 weeks old, belonging to the same strain congenic with C57BL/6J (Jackson Laboratories in Bar Harbor, Maine, USA) were used. Blood, brain and liver were obtained through decapitation of six HPRT⁻ mice and six normal littermate controls; specimens were divided into aliquots, immediately frozen in liquid nitrogen, shipped on dry ice, and stored at -80°C until examination.

Blood samples

Perchloric acid extracts were prepared from frozen whole blood samples obtained from HPRT⁻ and control mice, to measure nucleotide concentration. Two volumes of 0.6 M ice-cold perchloric acid were added to 100 µl aliquots of frozen blood in microfuge tubes and allowed to thaw slowly while homogenising on ice with a plunger. The precipitated proteins were discarded by centrifugation and the clear supernatant was brought to neutrality by adding 3.5 M K₂CO₃. The perchlorate precipitate was discarded following centrifugation and the clear supernatant was used for HPLC analysis.

Lysates were obtained by adding 4 volumes of water to 100 µl aliquots of frozen blood in microfuge tubes and allowed to thaw slowly while vortex mixing. Suspensions were freeze-thawed twice, mixed with activated charcoal to eliminate

endogenous nucleotides and centrifuged 10 min at $14,000 \times g$. Supernatants were used for enzyme activity and protein assays.

Brain and liver samples

Aliquots of frozen whole brain and liver were used for biochemical assays. Samples were quickly weighed and acid and alkaline protein-free extracts were prepared for nucleotide studies, according to modifications of previously described methods (Micheli et al. 1993; Cappiello et al. 2000).

Acid extracts were prepared to detect purine nucleotides and oxidized pyridine compounds: frozen tissue specimens were quickly added to ice-cold 0.6 M perchloric acid (10 volumes) and homogenised on ice in a Potter-Elvehjem homogeniser. Precipitated proteins were discarded following centrifugation ($14,000 \times g$, 3 min) and clear supernatant brought to neutrality by adding 3.5 M K_2CO_3 . The precipitated perchlorate was then discarded by centrifugation ($14,000 \times g$, 2 min) and the clear supernatant used for HPLC analysis.

Alkaline extracts were performed to detect reduced pyridine coenzymes, NADH and NADPH. Frozen tissue specimens were quickly added to ice-cold 0.5 M KOH (4 volumes) and homogenised on ice in a Potter-Elvehjem homogeniser; 3 volumes of cold water were added to the homogenate, and proteins were eliminated by ultrafiltration (centrifugation in Amicon Centrifree, $2,000 \times g$, 10 min). The ultrafiltrate was brought to pH 7.0 with 0.1 M KH_2PO_4 and immediately used for HPLC analysis.

Crude homogenates were prepared from brain and liver for enzyme activity assays.

Frozen specimens were added to ice-cold 50 mM sodium phosphate buffer (pH 7.5) containing 5 mM dithiothreitol, and homogenised on ice in a Potter-Elvehjem homogeniser. Homogenates were used directly (only for NADase activity determination), or mixed with activated charcoal to eliminate endogenous nucleotides, shaken for 20" and centrifuged (10 min, $14,000 \times g$). Pelleted charcoal and cell debris were discarded and the clear supernatant used to measure enzyme activities and protein concentration.

Enzyme assays

Enzyme activity assays of HPRT, APRT, NAPRT, NAMPRT (nicotinamide phosphoribosyltransferase; E.C. 2.4.2.12.) NMN-/NAMN-AT (nicotinate mononucleotide /nicotinamide mononucleotide adenylyltransferase, E.C. 2.7.7.1 and 2.7.7.18, respectively), NADs and NADase were performed in blood, brain and liver homogenates according to slight modifications of HPLC-linked methods previously described for human cells (Micheli et al. 1999; Sestini et al. 2000; Micheli and Sestini 1997). The term NADase was used to indicate any enzyme splitting NAM from NAD, which may be accomplished by different enzyme activities leading to different adenylyl products: either free ADPR (proper NAD-glycohydrolase), cADPR (ADP-ribosyl cyclase, E.C.3.2.2.5), poly-ADPR (PARPs, E.C. 2.4.2.30) or else an ADP-ribosylated protein (ADP-ribosyl transferase, E.C. 2.4.2.30). Such total activity was detected by measuring free NAM production (Ceroni et al. 2007).

Non-radioactive methods were used for all assays, except NAPRT and NAmPRT activities in tissue homogenates, which required the use of [^{14}C]-labeled nicotinate and nicotinamide, respectively, and long incubation times (up to 6 hours).

At the end of all the enzyme reactions protein-free perchloric extracts of assay mixtures were obtained as described above and analysed by HPLC to quantify substrates and products.

HPLC analysis

Analysis of tissue nucleotide content or enzyme activities was conducted on protein-free extracts by modifications of previously described elution methods (Micheli et al. 1999; Micheli et al. 1993; Micheli and Sestini 1997). The HPLC apparatus consisted of a Beckman System Gold Module 126, with a model 168 Nouveau diode array detector, equipped with an in-line model 171 radioisotope detector (Beckman San Ramon, CA, U.S.A.). Phenomenex Luna C18 columns (3 μm particle size, 75 \times 4.6 mm) equipped with guard columns (Phenomenex Security guard 4 mm L \times 3 mm ID) were used. Suitable gradients of 0.1 M potassium phosphate buffer containing 6 mM tetrabutylammonium phosphate, pH 5.5 (eluant A) and methanol (eluant B) were used for the different analyses, elution time varying from 12 and 22 min. The absorbance was monitored at 260 and 280 nm. Retention time, coelution with added internal standards, absorption spectra and/or 260/280 nm absorbance ratios confirmed peak identities.

Concentration/peak area linear plots were developed with known standard solutions for quantification of each compound of interest. Radioactive compounds (nicotinic acid, nicotinamide and their mononucleotides in NAPRT and NAmPRT assays, respectively) were quantified on the basis of cpm/peak area linear plots with known standard solutions.

Protein determination

Protein content was measured according to Bradford (Bradford 1976) for quantification of both nucleotide concentration and enzyme activities; Bio-Rad Dye Reagent (Bio-Rad Laboratories, Munich, Germany) was used. Biochemical parameters were expressed both per gram of wet tissue (when applicable) and per milligram of proteins.

Statistical analysis was performed by ANOVA.

Results

Nucleotide concentrations

Significantly higher amounts of purines and pyridines (AMP, ADP, ATP, GTP, GDP, NAD, NADP and NADPH) were found in liver extracts from HPRT⁻ mice compared with controls (Table 1). The difference was evident despite considerable sample variance, either when expressed per milligram of protein or per gram of wet tissue (latter data not shown).

Table 1 Nucleotides in blood, brain and liver from control and HPRT-deficient mice

	AMP	ADP	ATP	GDP	GTP	NAD	NADP	NADH	NADPH	EC
Blood										
WT	0.07±0.02	0.40±0.08	2.2±0.4	0.10±0.01	0.7±0.1	0.16±0.02	0.06±0.004	nd	nd	0.90±0.01
KO	0.05±0.02	0.40±0.01	2.1±0.3	0.10±0.03	0.7±0.1	0.15±0.02	0.06±0.03	nd	nd	0.90±0.02
Brain										
WT	10.1±3.4	2.4±1.1	0.51±0.25	1.4±0.8	0.75±0.35	1.9±1.0	0.08±0.03	0.12±0.03	nd	0.13±0.02
KO	9.8±2.8	2.2±0.6	0.48±0.15	1.2±0.4	0.72±0.26	1.7±0.4	0.08±0.03	0.14±0.004	nd	0.13±0.03
Liver										
WT	8.9±0.8	2.1±1.7	0.8±0.6	0.3±0.1	0.8±0.4	1.1±0.7	0.36±0.16	0.32±0.22	0.14±0.06	0.13±0.07
KO	14.7±5.0*	5.6±2.7*	2.3±1.1*	0.8±0.3**	1.6±0.5*	3.7±0.9**	1.00±0.30**	0.74±0.41	0.42±0.21*	0.22±0.06

Data are expressed as nmoles/mg protein: * $P < 0.05$, ** $P < 0.01$

EC energy charge, *nd* not determined

Energy charge was also increased in the liver from the HPRT⁻ mice, though not significantly. Adenine and guanine nucleotides displayed a considerable degradation in brain and liver, due to rapid dephosphorylation, as previously described (Jinnah et al. 1993; Clark and Dar 1988).

Purine nucleotide concentrations measured in blood and brain extracts did not show any significant difference, in agreement with a prior report for brain (Jinnah et al. 1993), neither did the amount of pyridine dinucleotides NAD(H) and NADP show any difference between HPRT⁻ mice and controls in these tissues (Table 1).

Enzyme activities

Analysis of purine phosphoribosyltransferases in control mice showed that APRT activity exceeded HPRT in blood (APRT/HPRT ratio: 1.58); the two activities were almost equal in liver (APRT/HPRT ratio: 0.84), while APRT was much lower than HPRT activity in brain (APRT/HPRT ratio: 0.08).

Comparison of HPRT⁻ mice with controls showed significantly higher NADase, NAPRT and NAMPT activities in liver (Table 2). No significant difference was found between HPRT⁻ mice and controls for any of the measured enzymes in blood (APRT, NAPRT, NMN/NAMN-AT, NADs) or brain (APRT, NAPRT, NMN/NAMN-AT, NADase) (Table 2).

Discussion

The present study demonstrates that increased NAD turnover occurs in the liver of HPRT⁻ mice. The increased amount of nucleotides in liver, possibly a result of the increased de novo purine synthesis rate (Jinnah et al. 1993), might stimulate NAD

Table 2 Enzyme activities in blood, brain and liver from control and HPRT-deficient mice

	HPRT	APRT	NAPRT	NAmPRT	NMN-AT	NAMN-AT	NADs	NADase
Blood								
WT	2.8±0.9	4.5±2.3	0.10±0.05	nd	0.07±0.02	0.07±0.02	0.36±0.10	nd
KO	0	6.6±0.9	0.10±0.03	nd	0.08±0.02	0.08±0.02	0.36±0.07	nd
Brain								
WT	520±132	46.9±20.4	0.6±0.2	nd	13.0±4.7	19.4±5.2	nd	819±56
KO	0	40.7±12.6	0.6±0.1	nd	13.0±6.5	26.5±13.2	nd	1069±350
Liver								
WT	76.1±13.6	64.2±15.2	2.8±0.9	0.25±0.06	20.5±7.0	37.8±17.5	nd	2116±641
KO	0	77.0±13.9	6.2±1.1*	0.56±0.33*	29.6±18.3	48.5±35.9	nd	3158±732*

Data are expressed as nmoles/h/mg protein: * $P < 0.05$

nd not determined

synthesis. Moreover NAPRT and NamPRT activities were also raised in HPRT⁻ mouse liver, as well as NAD breakdown rate, demonstrated by increased NADase activity (such term including all different enzymatic activities leading to nicotinamide removal from NAD, namely proper NAD glycohydrolase, ADPR cyclase, PARP, ADPRibose transferase). Thus the NAD abnormality found in LND patients can be replicated in HPRT⁻ mouse, but it is limited to liver, pointing to important tissue differences in the metabolic consequences of HPRT deficiency. HPRT-mediated purine recycling may be less important in mouse than in human, particularly in some tissues, such as erythrocytes, displaying a higher APRT/HPRT ratio compared to human and no change in NAD, or even brain (Wu and Melton 1993; Engle et al. 1996; Adams and Harkness 1976). NAD metabolism has never been examined in human LND liver or brain, but the implication from the mouse results is that it may be altered as well.

Conclusions

These studies leave open the question of NAD metabolism in the pathogenesis of LND. Altered NAD metabolism is observed in various HPRT⁻ human cells, but nothing is known for HPRT⁻ human brain. Altered NAD metabolism is observed in HPRT⁻ mouse liver, but not brain. The lack of NAD abnormality in mouse brain might account for the lack of behavioral phenotype. On the other hand we cannot exclude that NAD metabolism might not be altered in human as in mice brain.

Present results provide further information on metabolic aberrations following HPRT deficiency in mouse compared to man, possibly related to dissimilar role and importance of this enzyme between rodents and humans. The consequences of HPRT deficiency are known to be determined not only by species, but by tissue too (Shirley et al. 2007). Further studies are needed to clarify if present results represent a species difference or a tissue difference, which would be relevant for the understanding of LND pathogenesis.

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