Z-nucleotide accumulation in erythrocytes from Lesch-Nyhan patients.

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Research Article

5-Amino-4-imidazolecarboxamide riboside 5'-monophosphate (ZMP) is an intermediate in the purine de novo synthetic pathway that may be further metabolized to inosine 5'-monophosphate, degraded to the corresponding nucleoside (5-amino-4-imidazole-carboxamide riboside; Z-riboside), or phosphorylated to the corresponding 5'-triphosphate (ZTP). Accumulation of ZTP in microorganisms has been associated with depletion of folate intermediates that are necessary for the conversion of ZMP to inosine 5'-monophosphate and has been postulated to play a regulatory role in cellular metabolism. We have shown the presence of Z-nucleotides in erythrocytes derived from five individuals with the Lesch-Nyhan syndrome. Erythrocyte folate levels were within the normal range, although guanosine triphosphate levels were significantly reduced below those in normal controls (P less than 0.01). A small amount of Z-nucleotide accumulation was also found in one individual with partial deficiency of the enzyme hypoxanthine guanine phosphoribosyltransferase and in two individuals with other disorders of purine overproduction. In contrast, no Z-nucleotides were detected in 13 normal controls or in three individuals with hyperuricemia on allopurinol therapy. We conclude that Z-nucleotide formation may result from markedly increased rates of de novo purine biosynthesis. It is possible that metabolites of these purine intermediates may play a role in the pathogenesis of the Lesch-Nyhan syndrome.

Z-Nucleotide Accumulation in Erythrocytes from Lesch-Nyhan Patients

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Abstract

5-Amino-4-imidazolecarboxamide riboside 5'-monophosphate (ZMP) is an intermediate in the purine *de novo* synthetic pathway that may be further metabolized to inosine 5'-monophosphate, degraded to the corresponding nucleoside (5-amino-4-imidazolecarboxamide riboside; Z-riboside), or phosphorylated to the corresponding 5'-triphosphate (ZTP). Accumulation of ZTP in microorganisms has been associated with depletion of folate intermediates that are necessary for the conversion of ZMP to inosine 5'-monophosphate and has been postulated to play a regulatory role in cellular metabolism. We have shown the presence of Z-nucleotides in erythrocytes derived from five individuals with the Lesch-Nyhan syndrome. Erythrocyte folate levels were within the normal range, although guanosine triphosphate levels were significantly reduced below those in normal controls ($P < 0.01$). A small amount of Z-nucleotide accumulation was also found in one individual with partial deficiency of the enzyme hypoxanthine guanine phosphoribosyltransferase and in two individuals with other disorders of purine overproduction. In contrast, no Z-nucleotides were detected in 13 normal controls or in three individuals with hyperuricemia on allopurinol therapy. We conclude that Z-nucleotide formation may result from markedly increased rates of *de novo* purine biosynthesis. It is possible that metabolites of these purine intermediates may play a role in the pathogenesis of the Lesch-Nyhan syndrome.

Introduction

5-Amino-4-imidazolecarboxamide riboside 5'-monophosphate (ZMP)[1] is a metabolic intermediate in the *de novo* synthetic pathway of purines that subsequently enters the purine nucleotide pool at the level of inosine 5'-monophosphate (IMP) (Fig. 1). The conversion of ZMP to IMP is a two-step reaction that requires the presence of the folate intermediate, 10-formyl-tetrahydrofolate. Alternatively, ZMP may be directly converted to the corresponding triphosphate (5-amino-4-imidazolecarboxamide; riboside 5'-triphosphate; ZTP) in a reaction catalyzed by 5-phosphoribosyl-1-pyrophosphate (PP-ribose-P) synthetase (E.C. 2.7.6.1.) (1). ZTP was identified as a metabolite in microorganisms depleted of guanine nucleotides and it was postulated that a depletion of GTP led to a secondary deficiency of folate intermediates derived from guanine nucleotides (2). Consequently, ZMP could not be metabolized to IMP and was available as a substrate for PP-ribose-P synthetase and conversion to ZTP. Support for this hypothesis was obtained from other experiments in which direct depletion of folate intermediates by inhibitors of dihydrofolate reductase led to marked increases in ZTP. ZTP was thus postulated to act as an "alarmone" for C-1-folate deficiency in *Salmonella* (2), but has never been described as a metabolite in human cells.

While examining the erythrocytes from patients with the Lesch-Nyhan syndrome for alterations in intracellular guanine nucleotide pools, we discovered the presence of 5-amino-4-imidazolecarboxamide (Z)-nucleotides in these cells. The Lesch-Nyhan syndrome is an X-linked disease characterized by the marked overproduction of purines leading to hyperuricemia and by a severe neurologic disorder characterized by mental retardation, spasticity, choreoathetosis, and self-mutilation (3). The disorder results from a complete deficiency of the enzyme hypoxanthine guanine phosphoribosyltransferase (HPRT; see ref. 4). HPRT is responsible for the salvage of guanine to guanosine monophosphate (GMP) in a reaction utilizing PP-ribose-P, and it has been postulated, although never conclusively demonstrated, that guanine nucleotide depletion and/or folic acid deficiency may contribute to the neurologic manifestations of the syndrome (3). We have therefore measured the nucleotide pools in erythrocytes from these patients and compared them with those in individuals with other disorders causing purine overproduction.

Methods

Materials. Z-riboside, Z-base, Z-nucleotides, adenine and guanine nucleotides, yeast hexokinase, and calf intestinal alkaline phosphatase were purchased from Sigma Chemical, Co., St. Louis, MO. Venom phosphodiesterase was obtained from Worthington Diagnostics Div., Millipore Corp., Freehold, NJ.

Nucleotide pool determinations. Erythrocytes were separated from heparinized peripheral blood by the Dextran sedimentation technique and an aliquot of the packed, washed erythrocyte pellet was diluted 1:10 (vol/vol) in Hanks balanced salt solution. The erythrocytes were counted and the suspension was heated for 2 min in a boiling water bath. The supernatant was then filtered through a 0.45-μm filter and analyzed directly by high pressure liquid chromatographic techniques.

Nucleotides were separated on a Partisil-10 SAX anion exchange column using a gradient of 100% Buffer A (0.002 M $NH_4H_2PO_4$, pH 2.8) to 60% Buffer B (0.75 M $NH_4H_2PO_4$, pH 3.9) over 80 min at a flow

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1. *Abbreviations used in this paper:* GDP, guanosine diphosphate; GMP, guanosine monophosphate; GTP, guanosine triphosphate; HPRT, hypoxanthine guanine phosphoribosyl transferase; IMP, inosine 5'-monophosphate; PNP, purine nucleoside phosphorylase; PP-ribose-P, 5-phosphoribosyl-1-pyrophosphate; s-AMP, succinyl adenosine 5'-monophosphate; Z, 5-amino-4-imidazolecarboxamide; ZMP, 5-amino-4-imidazolecarboxamide riboside 5'-monophosphate; ZTP, 5-amino-4-imidazolecarboxamide riboside 5'-triphosphate.

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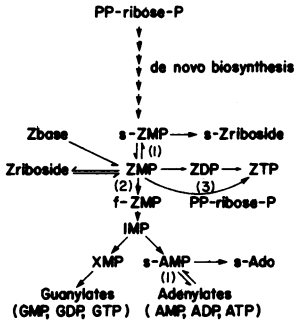

Figure 1. Metabolic pathways involving Z-nucleotides (1) adenylosuccinate:AMP lyase; (2) 10-formyl-tetrahydrofolate:ZMP formyltransferase; (3) PP-ribose-P synthetase. f-ZMP-10-formylZMP. The succinyl derivatives of AMP, ZMP, and adenosine are designated as s-AMP, s-ZMP, and s-Ado, respectively.

rate of 1.5 ml/min. Nucleotides were quantitated using a high pressure liquid chromatograph (Waters Associates, Millipore Corp., Milford, MA) equipped with a Model 440 absorbance detector and a Model 730 data module. Nucleoside diphosphates and triphosphates were detected and quantitated by comparison with nanomol amounts of authentic standard. Nucleosides were separated and quantitated on a C_8 reverse-phase column (Waters Associates) using a linear gradient of 20 mM KH_2PO_4, pH 5.6, to 60% methanol over 35 min at a flow rate of 1 ml/min.

Absorbance data were obtained on peaks as they were eluted from the anion exchange column using an absorbance detector (model 1040A; Hewlett-Packard Co., Palo Alto, CA) and data processor.

Results

Fig. 2 shows a representative high pressure liquid chromatogram of an erythrocyte extract prepared from a Lesch-Nyhan patient (*A*) compared with a control extract from a normal individual (*C*). Although these chromatograms are not corrected for erythrocyte number, there is a relative decrease in the size of both the guanosine triphosphate (GTP) peak (retention time, 72 min) and the guanosine diphosphate (GDP) peak (retention time, 48 min) in the patient's extract. In addition, there are new peaks

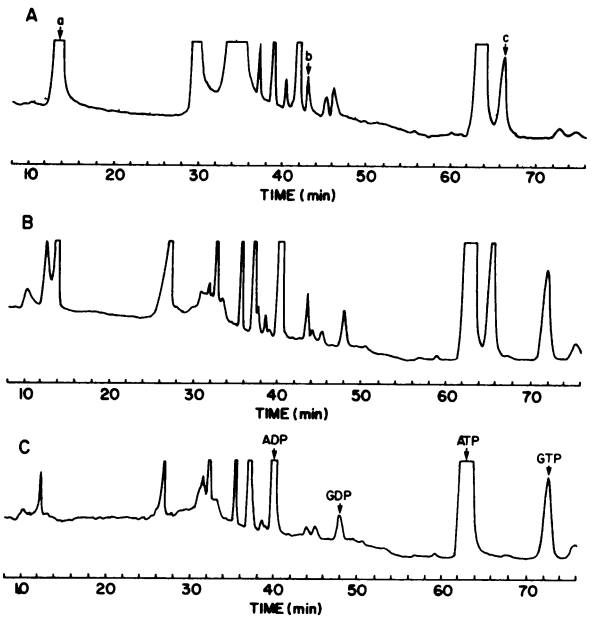

Figure 2. High pressure liquid chromatography fractionation of erythrocyte nucleotides. (*A*) Lesch-Nyhan syndrome patient; (*B*) normal erythrocytes incubated with 100 μM 5-amino-4-imidazolecarboxamide for 2 h; (*C*) normal erythrocytes. a, b, and c designate putative ZMP, ZDP, and ZTP peaks, respectively.

eluting with retention times of 12, 42, and 65 min, which we have designated as a, b, and c, respectively. The comigration of peak c with pure ZTP standard led us to suspect the presence of Z-nucleotides in these erythrocytes. Consequently, the normal erythrocytes were washed and resuspended in RPMI 1640 medium containing 100 μM 5-amino-4-imidazolecarboxamide (Z), the base of Z-riboside. Z can be converted to the corresponding Z-nucleotides in a phosphoribosylation reaction catalyzed by adenine phosphoribosyltransferase. After a 4-h incubation, peaks corresponding in retention times to a, b, and c were also found in these cells (Fig. 2, *B*). Confirmation of the identities of peaks a, b, and c as ZMP, ZDP, and ZTP, respectively, was obtained as follows: (*a*) erythrocyte extracts were incubated for 30 min in the presence of 2 U hexokinase (400 U/ml), 8 mM $MgCl_2$, and 84 mM D-glucose (5). The peaks corresponding to ATP and GTP, as well as peak c, disappeared and there was an associated increase in the peaks corresponding to ADP, GDP, and peak b. Hence, peak c represents a nucleoside triphosphate; (*b*) treatment of patient erythrocyte extracts with phosphodiesterase shifted peak c and peak b, as well as the known nucleoside diphosphates and triphosphates, to the mononucleotide region of the chromatogram; (*c*) digestion of Lesch-Nyhan extracts with phosphodiesterase and alkaline phosphatase (6) resulted in a new peak that comigrated with Z-riboside when run on a C_8 reverse-phase column; and (*d*) a U.V. spectrum of peak c obtained by direct analysis from the ammonium phosphate gradient demonstrated a λ_{max} of 267 nm and an absorbance spectrum identical to that obtained with a ZTP standard run on the same gradient.

To determine the specificity of ZTP accumulation in the Lesch-Nyhan syndrome, we examined erythrocyte lysates from 13 healthy controls, 5 Lesch-Nyhan patients, 1 patient with partial HPRT deficiency, 3 patients with a history of gout with normal HPRT levels on allopurinol therapy, 1 child with purine nucleoside phosphorylase deficiency, and 1 patient with PP-ribose-P synthetase overactivity. The latter two disorders have both been associated with purine overproduction (7, 8). Z-nucleotides were not detected in extracts from normal individuals or from individuals on allopurinol therapy (Table I). ZTP levels of >390 pmol/10^8 cells were observed in four of the Lesch-Nyhan patients, with substantially lower levels in a fifth patient. In contrast, very small but easily identifiable peaks comigrated with ZTP in samples from patients with inherited PP-ribose-P synthetase overactivity, purine nucleoside phosphorylase (PNP) deficiency, and partial deficiency of HPRT. GTP levels were significantly lower ($P < 0.01$) in Lesch-Nyhan erythrocytes than in normal controls and were also markedly diminished in the PNP-deficient erythrocytes, consistent with previously reported data (9).

Discussion

We have considered several possible explanations for the natural occurrence of Z-nucleotides in the Lesch-Nyhan syndrome. First, the inability to salvage guanine by HPRT-deficient cells could lead to guanine nucleotide depletion, secondary folic acid deficiency, and inability to utilize ZMP. Although we have documented low GTP levels in these erythrocytes, this sequence of events is unlikely for three major reasons: (*a*) erythrocyte folate levels in the four Lesch-Nyhan patients in whom they were measured were within the normal range. In addition, intracellular folate intermediates should be easily maintainable on a diet containing adequate amounts of folic acid; (*b*) erythrocytes do not

Table I. Nucleotide Pools in Erythrocytes of Lesch-Nyhan Patients, Normal Controls, and Patients with Other States of Purine Overproduction

Nucleotide	Controls (n = 13)	Lesch-Nyhan syndrome (n = 5)	Partial HPRT deficiency (n = 1)	Hyperuricemia on allopurinol (n = 3)	PNP deficiency (n = 1)	PP-ribose-P synthetase overactivity (n = 1)
	pmol/10⁸ erythrocytes	*pmol/10⁸ erythrocytes*	*pmol/10⁸ erythrocytes*	*pmol/10⁸ erythrocytes*	*pmol/10⁸ erythrocytes*	*pmol/10⁸ erythrocytes*
ADP	2,514±817*	1,825±248	2,074	2,045±399	2,483	3,961
GDP	83±27	37±15	57	77±16	18	142
ATP	12,633±3,988	13,319±3,634	13,077	11,242±2,713	8,213	14,733
GTP	398±120	248±93	183	414±82	51	555
ZTP	UD‡	394, 669, 1,305 566, 78	83	UD‡	38	27

* Values represent mean±SD. Determinations were performed in duplicate on each patient. n = number of patients. ‡ UD, undetectable (<15 pmol/10⁸ cells).

have the ability to synthesize purines *de novo,* which makes endogenous ZMP generation unlikely (10); and (*c*) although GTP levels were lower in the PNP-deficient patient's cells than in any of the Lesch-Nyhan erythrocytes, Z-nucleotides were barely detectable in these erythrocytes.

A second more likely possibility is that exogenous Z or Z-riboside generated by the accelerated rate of *de novo* purine biosynthesis in the Lesch-Nyhan syndrome in tissues other than erythrocytes is metabolized to the corresponding Z-nucleotides by erythrocytes. Previous studies have shown that normal erythrocytes incubated with either Z or Z-riboside accumulate the corresponding 5′-monophosphates and triphosphates, as well as small amounts of ZDP (11), a finding that we have confirmed (Fig. 1 *B*). In addition, the urinary excretion of Z has been determined to be 10-fold greater than that in normal subjects in five Lesch-Nyhan patients, all of whom had normal serum folate levels (12). Although we have been unable to detect Z in the plasma of these patients by our high performance liquid chromatography methods, its elevated urinary excretion most probably reflects the fact that it is present in increased concentrations in the plasma. Furthermore, since erythrocytes are capable of metabolizing Z and Z-riboside to IMP (13), the accumulation of nucleotides implies both an excess of either or both of these substrates and a rate-limiting step in the enzymatic conversion of ZMP to IMP. The lower but detectable levels of Z-nucleotides in PNP-deficiency and PRPP synthetase overactivity states are consistent with the lesser degree of overproduction of purines in these disorders.

Whether Z-nucleotide accumulation plays any role in the pathogenesis of the Lesch-Nyhan syndrome remains uncertain. Z-riboside has been shown to be toxic to cultured fibroblasts at a concentration of 200 μM while lower and higher concentrations had far less effect (14). Growth inhibition at this concentration appeared to be mediated by inhibition of pyrimidine biosynthesis. However, we were unable to detect comparable Z-riboside concentrations in the plasma of Lesch-Nyhan patients. In addition, pyrimidine nucleotide pools were not diminished in the mononuclear cells from these patients. A second potential mechanism of toxicity resulting from metabolites in this pathway is the inhibition of the enzyme adenylosuccinate lyase (E.C. 4.3.2.2) by ZMP. Infusion of Z-riboside into dogs over a 40-min period resulted in accumulation of Z-riboside, ZMP, and ZTP in cardiac and skeletal muscle (15). In these and other studies (16), accumulation of ZMP was shown to inhibit adenylosuccinate lyase, an enzyme integral to the formation of AMP from IMP, and resulted both in increased levels of IMP and an increased degree of purine catabolism. Inhibition of this enzyme by Z-riboside infusion also produces skeletal muscle dysfunction in mice and results in increased levels of its substrate, adenylosuccinate (s-AMP), in this tissue (17). The inhibition constant (K_i) of the lyase for ZMP is relatively low in erythrocytes (10 μM) (18), and ZMP is clearly present in Lesch-Nyhan erythrocytes (Fig. 2). Unfortunately, human erythrocytes lack the synthetase activity that catalyzes the formation of adenylosuccinate from IMP (10), so that the detection of lyase inhibition by measuring s-AMP levels is not possible in these cells.

It is of considerable interest in this regard, however, that an apparent inherited deficiency of adenylosuccinate lyase activity has recently been associated with the occurrence of psychomotor retardation and autism in three individuals, all of whom had detectable levels of succinylpurines derived from the substrates of this enzyme in cerebrospinal fluid (19). In contrast to erythrocytes, cells of the central nervous system have the capacity to synthesize purines *de novo* and some evidence exists that the rate of purine synthesis in the brains of Lesch-Nyhan patients is accelerated (3). In addition, the purine nucleotide cycle appears to be intact and transient increases in s-AMP levels have been demonstrated after electrical shock treatment (20). These observations raise the additional possibility that Z-nucleotides may be generated within the central nervous system of these individuals and that they may interfere with neuronal function on the basis of altering local levels of succinylpurines.

The only available data on neurotransmitter abnormalities within the brains of Lesch-Nyhan patients demonstrate a loss of dopaminergic nerve terminals with a specific decrease in dopamine and homovanillic acid levels, as well as dopa decarboxylase and tyrosine hydroxylase activities (21). However, dopamine levels were normal in the cell bodies. Thus, it is difficult to implicate an overall defect in dopamine synthesis; also, we cannot specifically relate an accumulation of Z-nucleotides to interference with any steps in this metabolic pathway. It has also been postulated that excessive utilization of folates by the high rate of *de novo* purine biosynthesis could contribute to both the central nervous system manifestations and the megaloblastic anemia of the Lesch-Nyhan syndrome (3). Since ZMP reacts directly with 10-formyl-tetrahydrofolate, accumulation of ZMP

in the central nervous system might also result from this mechanism. Validation of any hypotheses relating the documented increase in erythrocyte Z-nucleotides to the central nervous system will have to depend on direct biochemical analysis of postmortem brain tissue from Lesch-Nyhan patients or, preferably, on reproduction of these metabolic abnormalities in an animal model.

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