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Pathways for α-D-ribose utilization for nucleobase salvage and 5-fluorouracil activation in rat brain

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Abstract

Recently, interest has increased in the use of α-D-ribose (Rib) as a naturally occurring nutriceutical for enhancement of cardiac and muscular performance. Most likely the elevation of available PRPP, following Rib administration, plays a key role in the salvage of purine nucleobases, thus, accelerating the restitution of ATP pool. In addition, administration of Rib improves some of the neurological symptoms in patients with adenylosuccinase deficiency. In this paper, we show that rat brain extract can catalyze the Rib-mediated salvage of both adenine and uracil, as well as the activation of the pyrimidine pro-drug, 5-fluorouracil (5-FU). The results strongly support that the pentose may be converted to both PRPP and Rib1-P for the salvage of the adenine and uracil, respectively. Most likely two-reaction pathway, composed of ribokinase and PRPP synthetase, is responsible of the PRPP formation, needed to salvage adenine to adenine nucleotides. A two-reaction pathway, composed of ribokinase and phosphopentomutase, appears to be responsible of the Rib1-P formation, needed to salvage uracil to uracil-nucleotides and to activate 5-FU to cytotoxic 5-FU-ribonucleotides. α-D-2′-Deoxyribose (deoxyRib) has a negligible effect on both the salvage of natural nucleobases to deoxyribonucleotides and on the activation of 5-FU to cytotoxic 5-FU-deoxynucleotides.

Keywords: α-D-Ribose; α-D-Deoxyribose; Purine salvage; Pyrimidine salvage; 5-Fluorouracil activation; Rat brain

1. Introduction

Rib, the sugar moiety of ATP, has recently received interest as a metabolic supplement for the heart [1–5]. Administration of Rib accelerates the repletion of ATP in rat and human myocardium during recovery from reversible ischemia [6–8] as well as in human cardiomyocytes and endothelial cells [9,10]. Moreover, Rib is capable of maintaining ATP at higher level in preserved rat heart [11] and dog kidney [12]. Recently, Salerno *et al.* have shown that the administration of Rib improves some of the neurological symptoms in a patient with adenylosuccinase deficiency [13], a human inherited disorder discovered by Jaeken and Van den Berghe [14], thus, suggesting that the pentose might exert its effects not only in heart, but also in central nervous system.

Rib must be transformed into one of its phosphorylated derivatives in order to be incorporated into purine and pyrimidine nucleotides without cleavage of its ribofuranosidic ring. The elevation of available PRPP is thought to be responsible of the Rib-enhanced synthesis of ATP. However, we recall that in normal rat tissues pyrimidine salvage, as well as 5-FU activation, is a Rib1-P rather than PRPP-dependent process [15–17]. Therefore, other mechanisms must be operative in the Rib-enhanced synthesis of pyrimidine nucleotides, as reported by Geisbuhler and Schwager [18].

This paper gives an "*in vitro*" insight of the biochemical mechanisms leading to Rib-enhanced synthesis of purine and pyrimidine nucleotides and to 5-FU activation. The results show that rat brain can readily anabolyse Rib into both PRPP and Rib1-P, for the salvage of purine and pyrimidine bases, respectively.

2. Materials and methods

2.1. Materials

[8-^{14}C]Adenine (55 mCi/mmol), [2-^{14}C]Uracil (54 mCi/mmol), [2-^{14}C]5-FU (55 mCi/mmol), Rib, deoxyRib,

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Abbreviations: Rib, α-D-ribose; deoxyRib, α-D-2′-deoxyribose; PRPP, 5-phosphoribosyl 1-pyrophosphate; Rib1-P, ribose 1-P; G-1,6-P_2, glucose-1,6-bisphosphate; deoxyATP, 2′-deoxyadenosine 5′-triphosphate; 5-FU, 5-fluorouracil; 5-FUrd, 5-fluorouridine; 5-FUMP, 5-FUDP; 5-FUTP, 5-fluorouridine 5′-mono-, -di- and -triphosphate.

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G-1,6-P_2, PRPP, dithiothreitol, bases, nucleosides, and nucleotides were from Sigma. Hi Safe II Scintillation liquid was purchased from Wallac. Polyethyleneimine (PEI)-cellulose precoated thin-layer plastic sheets (0.1 mm thick) were purchased from Merck and prewashed once with 10% NaCl and three times with deionized water before use. All other chemicals were of reagent grade. Three-month-old male Sprague-Dawley rats were killed according to the Guilding Principles in the Care and Use of Animals (DHWEW publication, NIH 86-23). The brain was removed and kept frozen at −80° until needed. Storage times did not exceeded 3 months.

2.2. Preparation of rat brain extracts

Rat brain was cut in small pieces, washed with cold saline and homogenized with a hand-driver Potter homogenizer in three volumes of 100 mM Tris–HCl buffer, pH 7.4, with 20 mM KCl and 1 mM dithiothreitol. The homogenate was centrifuged at 4° at 39,000 *g* for 1 hr. The supernatant fluid obtained was dialyzed overnight at 4° in dialysis bags against 10 mM Tris–HCl buffer, pH 7.4, supplemented with 1 mM dithiothreitol, and is referred to as crude extract.

2.3. Incubation procedures

Crude extracts, containing 50–150 μg of protein, were incubated in a total reaction volume of 70 μL containing 3 mM of either Rib or deoxyRib, 5 mM Tris–HCl buffer, pH 7.4, 1 mM [8-^{14}C]Adenine (8000 dpm/nmol) or [2-^{14}C]Uracil (12,000 dpm/nmol) or [2-^{14}C]5-FU (12,000 dpm/nmol), 3.6 mM of either ATP or deoxyATP, and 8 mM $MgCl_2$. The reaction was started by the addition of crude extract. At the different time intervals, the reaction was stopped by rapidly drying portions of 10 μL of the incubation mixture on PEI-cellulose precoated thin-layer plastic sheets and a chromatogram was developed in *n*-butanol/glacial acetic acid/H_2O (4:2:1, v/v) to separate adenosine and adenine-ribonucleotides or with *n*-propanol/NH_3/trichloroacetic acid (100%)/H_2O (75:0.7:5:20, v/v) to separate Urd and uracil-ribonucleotides, deoxyUrd and uracil-deoxyribonucleotides, 5-FUrd and 5-FU-ribonucleotides, 5-FdeoxyUrd and 5-FU-deoxyribonucleotides. In all separation, appropriate standards were used and detected as ultraviolet absorbing areas which were excised and counted for radioactivity with 8 mL of scintillation liquid. Appropriate controls were always run in parallel, in which ATP, or Rib or deoxyRib were omitted. In the absence of added ATP, no nucleobase salvage or 5-FU activation was observed. In the absence of added Rib some adenine-, uracil-, and 5-FU-nucleotides formation was observed after prolonged incubation. These data were subtracted from those obtained with ATP plus Rib.

2.4. Protein concentration

Protein concentration was determined by the Coomassie blue binding assay, using bovine serum albumin as standard [19].

3. Results

In order to assess the pathways of Rib anabolism in rat brain, we have investigated the effect of Rib on adenine and uracil salvage, respectively. The rational of our experimental approach was the following. Since in rat brain adenine salvage is a PRPP-dependent process [20], we reasoned that a Rib-mediated adenosine-nucleotide biosynthesis would necessarily imply that the pentose is anabolized to PRPP. Since uracil salvage is a Rib1-P-dependent process [15–17], a Rib-mediated uridine-nucleotides biosynthesis would necessarily imply that

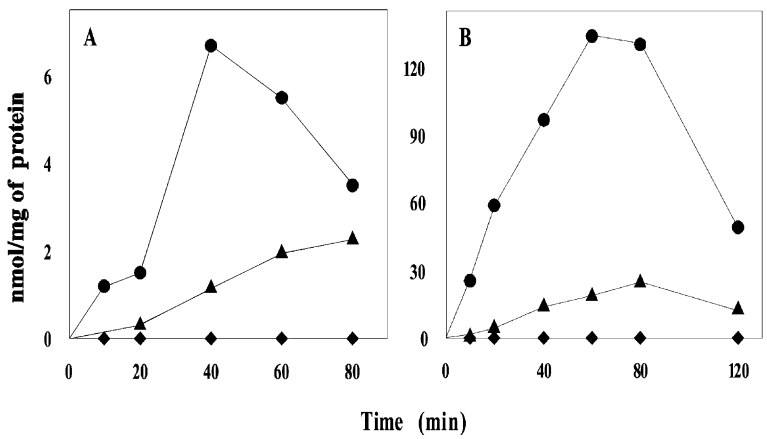

Fig. 1. Time course of Rib-mediated (A) and PRPP-mediated (B) adenine salvage catalyzed by rat brain extract. (A) The incubation mixture contained, in a final volume of 70 μL, 210 nmol of Rib, 70 nmol of [8-^{14}C]Adenine (8000 dpm/nmol), 252 nmol of ATP, 560 nmol of $MgCl_2$, and 100–150 μg of protein in 50 mM Tris–HCl buffer, pH 7.4. (B) Rib was substituted with 210 nmol of PRPP. ADP + ATP (●); AMP (▲); adenosine (◆).

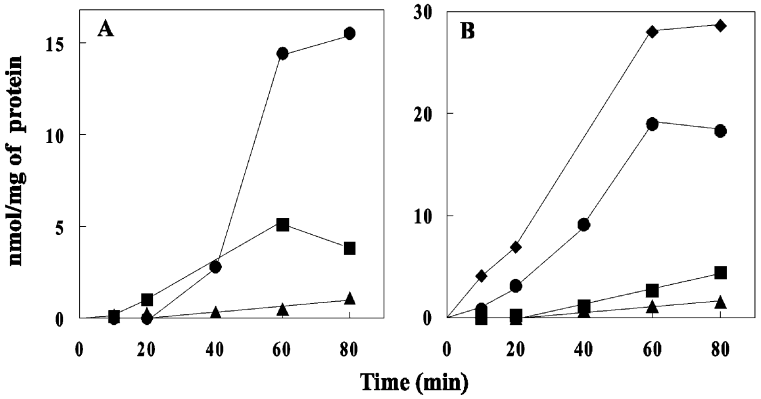

Fig. 2. Time course of the Rib-mediated uracil salvage catalysed by rat brain extract. (A) The incubation mixture contained, in a final volume of 70 μL, 210 nmol of Rib, 70 nmol of [2-^{14}C]Uracil (12,000 dpm/nmol), 252 nmol of ATP, 560 nmol of $MgCl_2$, and 100–150 μg of protein in 50 mM Tris–HCl. (B) ATP was substituted with 252 nmol of deoxyATP. Urd1 (●); UMP (▲); UDP + UTP (■); Urd (◆) formed when 3.5 nmol of G-1,6-P_2 and 7 nmol of $MnCl_2$ were added and to the incubation mixture.

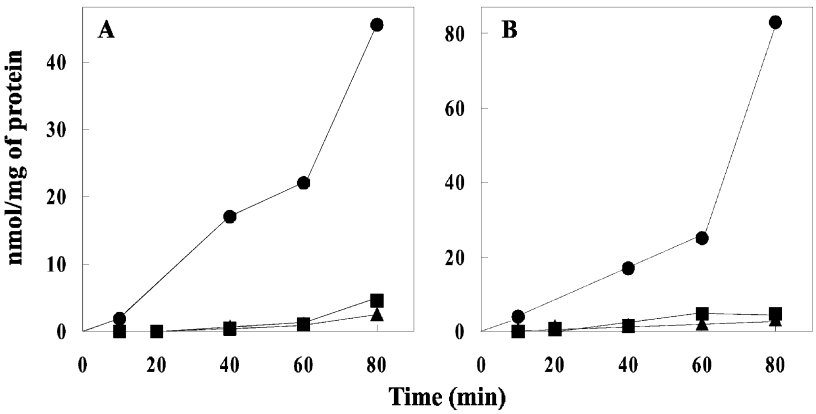

Fig. 3. Time course of the Rib-mediated 5-FU activation catalysed by rat brain extract. (A) The incubation mixture contained, in a final volume of 70 μL, 210 nmol of Rib, 70 nmol of [2-^{14}C]5-FU (12,000 dpm/nmol), 252 nmol of ATP, 560 nmol of $MgCl_2$, and 100–150 μg of protein in 50 mM Tris–HCl. (B) ATP was substituted with 252 nmol of deoxyATP. 5-FUrd (●); 5-FUMP (■); 5-FUDP + 5-FUTP (▲).

the pentose is anabolized to Rib1-P. Fig. 1A shows the time courses of adenine salvage catalyzed by crude extracts of rat brain incubated with Rib, ATP and [8-^{14}C]Adenine. The lack of any adenosine formation as an intermediate shows that ribosylation of adenine did not occur, and that AMP formed was not dephosphorylated during the entire incubation period. Rather, it was further phosphorylated to ADP and ATP. However, it cannot be excluded that adenosine could be formed and recycled into AMP by adenosine kinase, and/or degraded to inosine and hypoxanthine. When deoxyATP was substituted for ATP as phosphate donor, neither adenosine, nor adenosine-nucleotide formation could be detected. Taken together, these data strongly suggest that in our experimental conditions Rib was converted to PRPP (see Section 4). Therefore, we have repeated the experiment of Fig. 1A, by substituting PRPP for Rib. With the exception of the higher amount of adenosine-nucleotides formed, the time course was similar to that observed with Rib (Fig. 1B).

Contrary to the Rib-mediated adenine salvage, in the Rib-mediated uracil salvage considerable ribosylation of the nucleobase to the corresponding nucleoside occurred (Fig. 2A). Moreover, ATP could be substituted by deoxyATP as phosphate donor, and G-1,6-P_2, a coenzyme of phosphopentomutase reaction, increased the amount of Urd formation of about 36% (Fig. 2B).

The time courses of Rib-mediated 5-FU activation catalyzed by crude extracts of rat brain incubated with [2-^{14}C]5-FU, and either ATP or deoxyATP appear in Fig. 3A and B, respectively. In analogy to the Rib-mediated uracil salvage, the nucleoside 5-FUrd was found as an intermediate, and deoxyATP could substitute ATP as phosphate donor.

4. Discussion

The mean plasma Rib concentration in normal subjects treated with 26 mmol/kg per day of Rib reached values

high as 1–3 mM [13]. Understanding the precise pathways by which the pentose is anabolyzed to its phosphorylated derivatives is of paramount importance to rationalize its nutriceutical and therapeutic effects. The Rib effect in accelerating the ATP repletion during recovery from reversible ischemia is enhanced if nucleobases are co-administered [2,4], suggesting that the pentose stimulates the "salvage" pathway, rather than the "*de novo*" nucleotide synthesis. In rat brain, which does not possess the "*de novo*" nucleotide synthesis [21], the salvage pathway is particularly active [15,20].

4.1. Rib pathways for adenine salvage

The results presented in Fig. 1A show that rat brain extract catalyze the Rib-mediated conversion of [8-^{14}C] Adenine into the corresponding [8-^{14}C]Adenosine 5′-mono-, -di-, and -triphosphates. The lack of intermediate adenosine formation may be explained by the absence of any adenosine phosphorylase activity in mammals [22], and also excludes that AMP might be formed by the nucleoside-phosphotransferase activity of brain cytosolic 5′-nucleotidase [23]. Moreover, adenosine is a poor substrate of this enzyme [24,25]. AMP was further phosphorylated to ADP and ATP during the entire incubation period, rather than being dephosphorylated to adenosine. The higher incorporation of adenine into ADP + ATP as compared with AMP might reflect the adenylate kinase equilibrium. Moreover, the decrease in ATP and ADP after 40–60 min incubation might reflect their degradation by nonspecific phosphatases. Since the salvage of adenine is a PRPP-dependent process, it is evident that in our experimental conditions Rib was converted into PRPP via a two-step pathway catalyzed by ribokinase and PRPP synthetase (Fig. 4). This idea is strengthened by the striking observation that, when deoxyRib was substituted for Rib, or when both deoxyATP and deoxyRib were substituted for ATP and Rib, respectively (Table 1), two conditions in which PRPP formation can be excluded "*a priori*", no adenine-ribo- or adenine-deoxyribonucleotide biosynthesis was observed. On the contrary, when PRPP was substituted for Rib, consistent formation of adenine-ribonucleotides was observed, and again no adenosine could be detected (Fig. 1B). PRPP synthetase has an absolute requirement of Pi. It is conceivable that some endogenous Pi, and also the release of Pi from added ATP and Rib1-P might have been responsible of the PRPP synthetase activation.

4.2. Rib pathways for uracil salvage and 5-FU activation

The lack of uracil phosphoribosyltransferase, together with the high levels of Urd phosphorylase and Urd kinase activities in rat brain shown by us in previous papers [15–17], renders the salvage of uracil a Rib1-P-dependent process. And in fact the results presented in Fig. 2A and B show that rat brain extracts catalyze the Rib-mediated

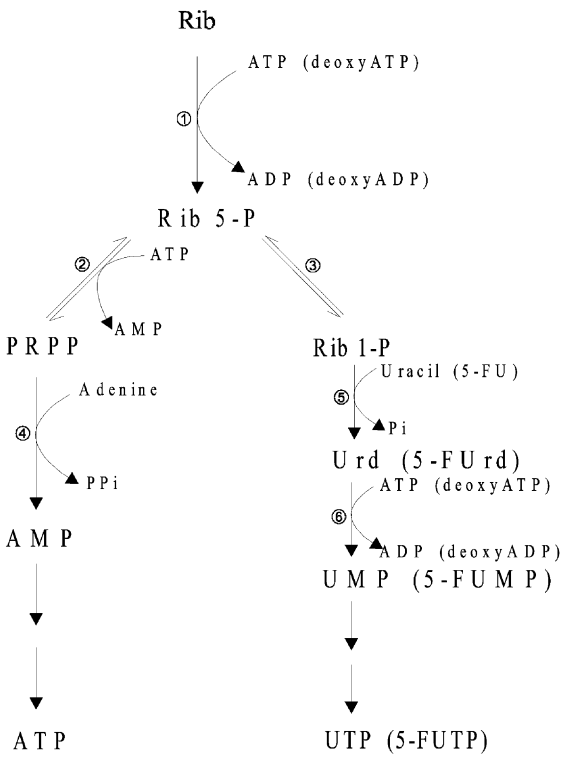

Fig. 4. Pathways of Rib anabolism for adenine and uracil salvage and for 5-FU activation in rat brain. Ribokinase (1); PRPP synthetase (2); phosphopentomutase (3); adenine-phosphoribosyltransferase (4); Urd-phosphorylase (5); Urd-kinase (6).

Table 1
DeoxyRib-mediated adenine and uracil salvage and 5-FU activation catalysed by rat brain extracts[a]

Base	Incubation time (min)	Phosphate donor					
		Deoxyribonucleoside (nmol/mg of protein)		Deoxyribonucleoside monophosphate (nmol/mg of protein)		Deoxyribonucleoside di- and -triphosphates (nmol/mg of protein)	
		dATP	ATP	dATP	ATP	dATP	ATP
Adenine	40	0.00	0.00	0.00	0.00	0.00	0.00
	80	0.00	0.00	0.00	0.00	0.00	0.00
Uracil	40	2.60	7.32	0.00	0.31	0.00	1.02
	80	6.80	26.99	0.00	0.53	0.00	3.28
5-FU	40	4.85	2.70	0.48	0.68	0.00	0.64
	80	8.81	6.97	0.73	0.76	0.00	5.38

[a] The incubation mixture contained, in final volume of 70 μL, 210 nmol of deoxyRib, 70 nmol of either [8-^{14}C]Adenine, or [2-^{14}C]Uracil, or [2-^{14}C]5-FU, 252 nmol of either ATP or deoxyATP, 560 nmol of $MgCl_2$, 100–150 μg of protein in 50 mM Tris–HCl, pH 7.4.

conversion of [2-^{14}C]Uracil into their corresponding [2-^{14}C]Urd-nucleotides with the intermediate [2-^{14}C]Urd formation. It is conceivable that in our experimental conditions Rib was anabolyzed via the two-step pathway, catalyzed by ribokinase and phosphopentomutase (Fig. 4). This idea is strengthened by the observation that G-1,6-P_2, a coenzyme of phosphopentomutase, increases the amount the nucleoside synthesized. The higher amount of Urd formed when deoxyATP was used as phosphate donor (Fig. 2B) might be explained by the higher affinity of ribokinase for deoxyATP with respect to ATP when Rib is the Pi acceptor [26]. The results presented in Fig. 3A and B show that Rib exerts an even more pronounced effect on 5-FU activation to 5-FUrd-nucleotides, with both ATP and deoxyATP as phosphate donors. 5-FUrd was an intermediate. Even through the intervention of orotidine phosphoribosyltransferase, and thus, of PRPP, when ATP was used as Pi donor, cannot be excluded "*a priori*", we recall that in rat brain 5-FU activation is a Rib1-P-dependent pathway [17]. As a consequence, it is conceivable that the pathway of Rib utilization for 5-FU activation in rat brain is the same as that of Rib utilization for uracil salvage (Fig. 4). Moreover, Table 1 shows that deoxyRib had a small effect on uracil salvage and 5-FU activation to their respective deoxyribonucleotides, with both ATP and deoxyATP as phosphate donors, even though deoxyuridine and 5-fluorodeoxyuridine accumulation was observed.

In conclusion, our results give a clear picture of the normal pathways for Rib utilization and should be taken into consideration when the pentose is administered as a nutriceutical or for therapeutic purposes.

Acknowledgments

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