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EFFECT OF DIPYRIDAMOLE ON INOSINE TRIPHOSPHATE PYROPHOSPHOHYDROLASE ACTIVITY AND INOSINE TRIPHOSPHATE CONTENT IN FRESH HUMAN ERYTHROCYTES INCUBATED WITH ADENOSINE

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The activity of inosine triphosphate pyrophosphohydrolase (ITPH) in human erythrocytes was found to be $1.50 \pm 0.39 \, \mu \text{mol} \, \text{min}^{-1} \, \text{per g Hb}$, and no measurable amount of ITP was detected. When dipyridamole was added to the medium composed of adenosine, pyruvate and inorganic phosphate, ITPH activity was $1.18 \pm 0.41$, and at the same time ITP accumulation was $0.61 \pm 0.31 \, \mu \text{mol/g Hb}$. The negative correlation between ITPH activity and accumulation of ITP was $r = -0.87$ at $P<0.001$.

Until early 1960s the presence of inosine triphosphate (ITP) in red blood cells was not mentioned in the literature. In 1976 Vanderheiden [1] showed that fresh red cells contained trace amounts of ITP.

The synthesis of inosine triphosphate in human erythrocytes incubated with inosine or hypoxanthine has been proved by several investigators [2-9]. ITP has also been found in the erythrocytes incubated in the medium containing adenosine, pyruvate and inorganic phosphate (APP medium) [10, 11]. The intracellular concentration of ITP in erythrocytes and various animal tissues is controlled by the activity of a hydrolytic enzyme, nucleoside triphosphate pyrophosphohydrolase (EC 3.6.1.19) specific for inosine triphosphate [12-16].

Dipyridamole (2,6-bis-[diethanolamino]-4,8-dipiperidinopyrimido(5,4-d) pyrimidine; persantin) a vasoactive substance, is a well known inhibitor of adenosine deaminase [17] and of the uptake of purine and pyrimidine nucleosides into a variety of cells [18-21]. This drug inhibits also the inward and outward movement of inorganic phosphate through the erythrocyte membranes [22].
We have found previously [23] that 10^{-4} M dipyridamole raised IMP content by about 3.5 fold (the content of IMP in red cells after incubation in APP medium was 1.52 \pm 0.78 \mu mol/g Hb but after incubation in this medium supplemented with dipyridamole it was 5.30 \pm 2.33 \mu mol/g Hb) and lowered at the same time ITP content by about 53\% (from 1.40 \pm 0.84 \mu mol/g Hb to 0.65 \pm 0.26 \mu mol/g Hb). This effect could be due to stimulation of inosine triphosphate pyrophosphohydrolase (ITPH) activity by dipyridamole. The aim of the present study was to verify this supposition.

MATERIALS AND METHODS

Fresh human blood was collected from healthy donors into heparinized glass bottles and centrifuged at 4°C. Plasma and buffy coat were removed, and red cells were washed three times with an excess of chilled 0.9\% NaCl solution.

Erythrocytes were incubated at 37°C in the medium containing adenosine (10 mM), pyruvate (10 mM), inorganic phosphate (50 mM), NaCl (75 mM) and dipyridamole (0.1 mM) (APPD medium). The pH of the medium was adjusted to 7.35. Washed erythrocytes were suspended in the medium (haematocrit of about 20\%) and agitated for 2 h in a water bath at 37°C. A control sample of erythrocytes was extracted with perchloric acid at 0 time [24]. The samples incubated with dipyridamole were rapidly chilled, the medium was centrifuged off and the cells were washed three times with an excess of cold 0.9\% NaCl. The acid-soluble phosphate compounds were extracted in the same manner as controls [24]. The phosphate compounds were separated by ion-exchange chromatography on Dowex 1 [25, 4]. Hypoxanthine nucleotides in eluates were determined spectrophotometrically [3].

ITP pyrophosphohydrolase activity was determined simultaneously in fresh and incubated erythrocytes according to Vanderheiden [26]. Washed red cells were haemolysed with a double volume of cold distilled water. Directly before incubation the haemolysate was mixed with an equal volume of 0.1 M dithiothreitol. The assay mixture contained in the final volume of 4 ml: 8 \mu mol ITP, 200 \mu mol MgCl_2, 40 \mu mol dithiothreitol in 0.1 M Tris/HCl buffer, pH 7.5, and 0.8 ml haemolysate (30-40 mg haemoglobin). In the blank sample, the haemolysate was replaced by 0.8 ml of distilled water. The samples were incubated at 37°C for 30 min. The reaction was terminated by the addition of 1 ml of 20\% perchloric acid. The mixture was cooled and neutralized with 40\% KOH solution. The KCIO_4 precipitate was removed by centrifugation, and the supernatant was applied onto the column (0.8 × 20 cm) filled with Dowex 1 × 4, 200-400 mesh, formate form. The formic acid-ammonium formate gradient was used to elute the products of ITP hydrolysis. Fractions of 3.5 ml were collected and the absorbance was monitored at 260 nm (spectrophotometer Pye-Unixam SP 500 series 2). Identification of the nucleotides eluted was based on separation of standard nucleotides (IMP, IDP, ITP) and the
hypoxanthine/phosphate molar ratios. For quantitative calculations the molar extinction coefficient of $8.1 \times 10^{-3}$ was used. The amount of ITP remaining after incubation was calculated as based on chromatographic separations of the incubated blank and the test sample [27].

ITPH activity was expressed in μmol of ITP hydrolysed per minute per gram Hb.

RESULTS AND DISCUSSION

Before incubation fresh red blood cells did not contain a measurable amount of ITP under our assay conditions. After 2 h incubation in APPD medium the mean ITP level in red cells reached the value of $0.69 \pm 0.31$ μmol/g Hb (20 expériments). The mean ITPH activity in fresh cells was $1.50 \pm 0.39$ μmol hydrolysed ITP/g Hb per minute and after 2 h incubation in the APPD medium this activity was $1.18 \pm 0.41$ μmol hydrolysed ITP/g Hb per minute (Fig. 1). ITPH activity was highly negatively correlated ($r = -0.87; P < 0.001$) with ITP concentration (Fig. 2).

A similar highly negative correlation was found previously [28] between ITPH activity and ITP accumulation ($r = -0.89; P < 0.001$) in erythrocytes incubated in the APP medium (without dipyridamole). Although the correlation coefficients in these experiments were closely similar, the regression equations were different due to different ITP values.

As we know, ITPH controls inosine triphosphate level in red cells [12] but both ITP content and ITPH activity were found to vary largely [5, 29]. Fraser et al. [5] showed that accumulation of ITP is characteristic for a given individual and is relatively constant. According to Vanderheiden & Zarate-Moyano [30] in all cases in which ITP was detected, the enzyme was shown to be deficient. In our experiments ITP has not been found in fresh red blood cells [23, 28], however, on incubation in either APP [28] or APPD [23] medium, ITP accumulated. This could be due either to the decreased ITPH activity or/and to enhanced formation of ITP. More recent data of Soder et al. [31] support those of Vanderheiden [26] as in their experiments low ITPH activity in erythrocytes was associated with a high ITP level. Our present results are consistent with our earlier data [28].

We have shown that, in erythrocytes incubated in APP medium, dipyridamole lowered ITP content and raised IMP content, whereas ITPH activity was diminished by about 21%. These results are not clear. The lower rate of nucleoside synthesis in erythrocytes incubated in APPD medium might be caused by P$_i$ deficiency inside red cells, because dipyridamole considerably reduced penetration of P$_i$ through erythrocyte membranes [32, 33, 34].
Fig. 1. ITPH activity of fresh red blood cells and after 2 hours of incubation in APPD medium (medium containing adenosine, pyruvate, inorganic phosphate and dipyridamole 10:10:50:0.1 mM respectively). Experimental conditions were as described in Material and Methods.

Fig. 2. Relationship between accumulation of ITP in red blood cells and the activity of ITPH after 2 hours of incubation in APPD; experimental conditions were as described in Material and Methods.
REFERENCES


