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Fluctuations in phosphoribosyl pyrophosphate levels in monolayer tumor cell lines

Effects of drugs

G.J. Peters, E. Laurensse, A. Leyva and H.M. Pinedo

Laboratory of Biochemical Pharmacology, Department of Oncology, Free University Hospital, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands

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The concentration of PRPP (phosphoribosyl pyrophosphate) measured in tumor cells grown in monolayer showed a large variation with the various harvesting methods examined, including trypsinization. This variation could be reduced by a 1-h incubation of trypsinized cells as a suspension in Dulbecco's medium. After this preincubation these cell suspensions were suitable for the study of modulation of PRPP. One μM methotrexate caused a 2–3-fold increase and 1 mM *N*-phosphonoacetyl-L-aspartate a slight increase, but inosine and deoxyinosine drastically reduced PRPP concentrations. 5-Fluorouracil had no effect. This study demonstrates that metabolic parameters such as PRPP concentrations can be studied conveniently in suspensions of cells which are commonly cultivated as monolayers.

Phosphoribosyl pyrophosphate *Monolayer tumor cell* *Modulation*
Pyrimidine antimetabolite *Purine*

1. INTRODUCTION

Phosphoribosyl pyrophosphate (PRPP) plays a crucial role in cellular metabolism [1]. It is a high-energy phosphate, that serves as a substrate for the phosphorylation of purine bases and for rate-limiting steps in the de novo synthesis of purine, pyrimidine and pyridine nucleotides. The enzymes involved in these reactions, phosphoribosyltransferases, also catalyze the conversion of various antimetabolites such as 5FU (5-fluorouracil) and 6-mercaptopurine to their active forms [2]. Other antimetabolites such as methotrexate (MTX) increase PRPP levels [3,4]. Biochemical studies on PRPP modulation by antimetabolites have been carried out predominantly in leukemic cells that grow in suspension. Unfortunately, in culture, solid tumors only grow in monolayers, which are available for biochemical studies only after detaching them from the plastic surface of culture flasks, usually performed by trypsinization. Studies with fibroblasts demonstrated that PRPP concentration in these cells is influenced by various factors like the harvesting technique, the passage number and the culture medium [5,6]. Such variables make it difficult to study modulation of PRPP in monolayer cells. To be able to perform such studies we compared several forms of harvesting monolayer cells prior to the measurement of PRPP concentrations. The study of PRPP modulation could be carried out most conveniently in monolayer cells harvested by trypsinization. The cells were preincubated as a suspension for 1 h prior to addition of drugs.

2. EXPERIMENTAL

All chemicals and drugs were obtained from sources described [7–9]. Cell culture was performed

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in 75-cm^2 Falcon flasks in Dulbecco's medium supplemented with 15% dialyzed, heat-inactivated fetal bovine serum. Origins of the murine B16 melanoma and the human 1GR3 melanoma were described in [7]. Cells were passaged every 2–3 days. Cells for metabolic studies were always harvested from cultures in logarithmic growth. The monolayers were washed with Hank's balanced salt solution (HBBS) without Ca^{2+} and Mg^{2+} and trypsinized at room temperature. Cells were suspended in an isotonic Tris–saline buffer (50 mM Tris–HCl, 100 mM NaCl, pH 7.4) or in Dulbecco's medium supplemented with 7.5% dialyzed, heat-inactivated fetal bovine serum. The cells were counted with a hemocytometer, centrifuged at 200 × *g* and suspended in an appropriate incubation medium or assay buffer (50 mM Tris–HCl containing 1 mM EDTA, pH 7.4) for PRPP determinations. PRPP was assayed either immediately after harvesting or after storage of the cell pellet at −70°C. PRPP concentrations were determined after suspension of the cell pellet in the Tris–EDTA buffer. Fresh cells were lyzed by sonication (2 cycles of 5 s at 50 W output, Branson sonifier). Frozen cells did not require further lysis after suspension of the cell pellet in the Tris–EDTA buffer. PRPP concentrations in fresh and frozen cells were similar. PRPP concentrations were determined as in [9,10] by the method based on the release of $^{14}CO_2$ from [*carboxy*-^{14}C]orotic acid. The amount of $^{14}CO_2$ was proportional to the amount of PRPP present in the cell extract (0.2–5 × 10^6 cells). To prevent interference of PRPP metabolizing enzymes, cell extracts were denatured before the assay by incubating them during 45 s in a boiling water bath. Recovery of PRPP during this procedure was measured by addition of known amounts of PRPP just before boiling to some additional flasks containing cell extract [9,10]. Recovery of PRPP was consistently higher than 90% with both cell lines. The presence of EDTA prevented consumption of PRPP by other enzymes during the heating procedure [10]. The compounds tested for their effects on PRPP concentration did not interfere with the assay, neither by inhibiting the yeast orotate phosphoribosyltransferase nor by affecting other enzymes since these enzymes were denatured before addition of the yeast enzyme. Furthermore, the concentration of drugs in the assay would be too low to cause interference since only cell pellets were used for the assay and not the medium.

3. RESULTS AND DISCUSSION

The most suitable buffer for isolation of cells prior to PRPP determination in erythrocytes and lymphoid cells appeared to be Tris–saline [9,10]. With monolayer cells a large variation in PRPP concentration was found with this buffer, whereas the variation was smaller with Dulbecco's medium (fig.1, *t* = 0). However, Tris–saline contains no nutritional compounds that stimulate PRPP synthesis or consume PRPP. Therefore PRPP concentrations measured in cells isolated in Tris–saline reflect the actual concentration of the cells in the monolayers. Further evidence was obtained by using harvesting techniques that avoid possible ef-

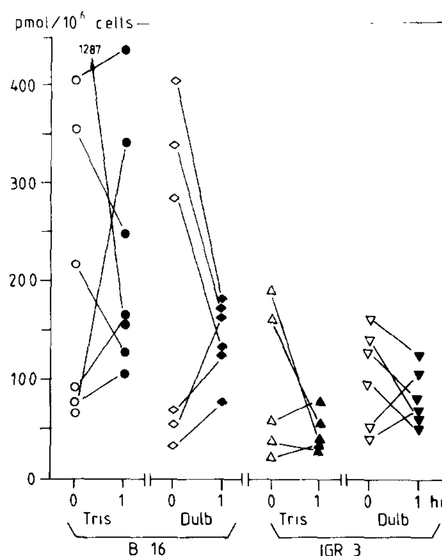

Fig.1. Effect of a 1-h incubation in Dulbecco's medium on the PRPP concentration of B16 and IGR3 melanoma cells. The buffers in which the cells were washed were Tris–saline and Dulbecco's medium and are indicated as Tris and Dulb, respectively. In one part of the cells PRPP concentration was measured immediately after harvesting by suspension of the cell pellet in Tris–EDTA buffer as described in section 2 (0 h). After centrifugation, the other part of the cells was suspended in fresh culture medium and incubated at 37°C in a shaking water bath. PRPP concentration was measured in these cells after a 1 h incubation by centrifugation of the cells and suspending them in the Tris–EDTA buffer. Lines connect the 0 and 1 h values for the same sample.

fects of trypsinization. Cells were harvested by incubating monolayers in 2 mM EDTA in HBSS without Ca^{2+} and Mg^{2+} at 37°C for 5–10 min. By binding bivalent ions EDTA not only promotes detachment of the cells from the plastic surface but also inhibits enzymic synthesis and degradation of PRPP [10]. The PRPP concentration in B16 melanoma cells harvested in this way was comparable to that measured in Tris–saline isolated cells from duplicate cultures. Rapid freezing with liquid nitrogen fixes cells in their metabolic state and appeared to be an adequate method to measure PRPP levels in hamster ovary cells [11]. Monolayer cells grown in 6-well cluster plates were rapidly frozen with liquid nitrogen after removal of the medium. Cells were subsequently lyophilized and PRPP concentration was measured after suspension in the Tris–EDTA buffer. Again the concentration of PRPP was comparable to that in cells isolated in Tris–saline.

PRPP concentrations differed in cultures started at different time points and harvested in Tris–saline prior to assay (fig.1, $t = 0$). However, PRPP concentrations measured in duplicate flasks or dishes grown and harvested simultaneously, were comparable.

The variation in PRPP concentration depending on growth conditions, was also reported for fibroblasts [5,6]. The fluctuations in PRPP concentrations in fibroblasts are partly due to the limited life-span of these non-tumor cells. Yet, the condition of the medium appears to be the major cause of variation with fibroblasts from a low passage number [5]. Substrates for phosphoribosyl-transferases such as hypoxanthine may be absent and this may lead to the increased PRPP concentrations. Isolation buffers that contain substrates for PRPP utilization or precursors for PRPP synthesis such as glucose, influence the PRPP concentration [9]. With melanoma tumor cells isolation methods that use buffers without nutritional compounds, gave comparable results. Therefore the fluctuating concentrations measured with Tris–saline represent actual concentrations in these cells.

Although PRPP concentrations could be measured reliably by these methods, none of these methods could be used to study drug effects in monolayer cells which was the main aim of this study. Variation in PRPP concentration due to culture effects was higher than the effects of drugs (not shown). To minimize culture effects we incubated cells as a suspension in fresh culture medium in a shaking water bath at 37°C. The range of PRPP concentration declined in cells isolated in either Tris–saline or Dulbecco's medium with serum and incubated in Dulbecco's medium. The concentration of PRPP measured in 11 and 7 separate samples of B16 and IGR3 cells, respectively, isolated in Dulbecco's medium and incubated for 1 h in Dulbecco's medium were 200 ± 20 and 87 ± 10 pmol/10^6 cells (means ± SE).

Since longer incubations (up to 5 h) did not significantly affect PRPP concentrations, these cell suspensions were used to study modulation of PRPP concentrations (table 1). MTX was included in these studies since an enhancement of PRPP concentrations has been reported for this drug for various leukemic cell lines [3,4]. The increase found with B16 cells demonstrates that this method can be used to study modulation of PRPP in monolayer cell lines. PALA (*N*-phosphonoacetyl-L-aspartate), an inhibitor of aspartate transcarbamylase, slightly increased PRPP concentrations, possibly by depletion of orotic acid (not shown). In order to affect cell growth 5FU has to be converted to nucleotides. This conversion takes place in a

Table 1

Modulation of the PRPP concentration in B16 melanoma cells

Drug	PRPP concentration
None	253 ± 40
PALA, 0.1 mM	364 ± 38
PALA, 1 mM	346 ± 38
5FU, 25 μM	252 ± 52
5FU, 50 μM	242 ± 52
MTX, 1 μM	607 ± 108
Inosine, 0.4 mM	41 ± 8
Deoyxinosine, 0.4 mM	26 ± 4

Concentrations (in pmol/10^6 cells) are means ± SE of 3–5 separate experiments. Cells were harvested by trypsinization and suspended in Dulbecco's medium supplemented with 7.5% dialyzed serum. Drugs were added after a 1 h preincubation in fresh culture medium and PRPP concentrations were measured 2 h later by centrifugation of the cells and suspension of the cell pellet in Tris–EDTA buffer

two-step reaction catalyzed by uridine phosphorylase and uridine kinase or directly in a reaction catalyzed by orotate phosphoribosyl-transferase [12] with PRPP as a co-substrate; 5FU did not significantly influence PRPP levels, indicating that under these conditions 5FU phosphorylation does not consume PRPP or that PRPP consumption by 5FU phosphorylation is compensated by an increased synthesis. The purine nucleosides, inosine and deoxyinosine, decreased PRPP concentration. Various mechanisms may account for this decrease [13]. Inhibition of PRPP synthetase does not seem probable since both nucleosides do not inhibit the enzyme [14]. Hypoxanthine, the phosphorolysis product of inosine and deoxyinosine, can reduce PRPP levels through its conversion to IMP [3], a step which consumes PRPP. IMP is also an inhibitor of PRPP synthetase [14,15]. However, inhibition of PRPP synthesis may predominantly be due to a decrease in orthophosphate levels caused by phosphorolysis of inosine [13]. The availability of orthophosphate which is an allosteric activator of PRPP synthetase, is an important control mechanism of PRPP synthesis [13,16] and may account for a number of biological effects of nucleosides.

In conclusion, we described a method for the measurement of PRPP concentration in monolayer tumor cell lines using Tris–saline as an isolation buffer. Short-term modulation of PRPP concentrations could be carried in cells isolated in Dulbecco's medium and preincubated for 1 h in this medium. These cell suspensions might also be used for the study of other metabolic parameters, when intact cells are required.

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