Biochimica et Biophysica Acta, 772 (1984) 227–230
Elsevier

BBA Report

BBA 70154

NUCLEOSIDE TRANSPORT IN HUMAN ERYTHROCYTES

NITROBENZYLTHIOINOSINE BINDING AND URIDINE TRANSPORT ACTIVITIES HAVE SIMILAR RADIATION TARGET SIZES

SIMON M. JARVIS [a], DARON A. FINCHAM [c], J. CLIVE ELLORY [d], ALAN R.P. PATERSON [b] and JAMES D. YOUNG [c]

[a] *Department of Physiology and* [b] *Cancer Research Group (McEachern Laboratory), University of Alberta, Edmonton, Alberta, T6G 2H7 (Canada),* [c] *Department of Biochemistry, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T. (Hong Kong), and* [d] *Physiological Laboratory, University of Cambridge, Cambridge, CB2 3EG (U.K.)*

(Received September 22nd, 1983)
(Revised manuscript received February 23rd, 1984)

Key words: Nucleoside transport; Nitrobenzylthioinosine; Radiation inactivation; (Human erythrocyte)

Intact human erythrocytes were irradiated in the frozen state with a high-energy electron beam. Nitrobenzylthioinosine-sensitive uridine influx, equilibrium exchange uridine influx and high-affinity nitrobenzylthioinosine binding were inactivated as a simple exponential function of the radiation dose, indicating an in situ target size of 122000. The results suggest that the nitrobenzylthioinosine-binding site(s) and the permeation site(s) of the transporter are present on the same transporter element.

Movement of nucleosides into human erythrocytes and a variety of other cell types occurs via a common broad-specificity facilitated diffusion process (see Refs. 1 and 2 for review). Transport by this route is inhibited by nitrobenzylthioinosine (6-(4-nitrobenzyl)thio-9-β-D-ribofuranosylpurine) (NBMPR) which binds specifically to functional nucleoside transport sites (apparent K_d 0.1–1 nM) [3,4]. We have previously reported that the NBMPR-binding entities in human erythrocyte 'ghosts' have an apparent M_r in situ of 122000 when estimated by radiation inactivation analysis [5]. Although much of the available evidence [3,6–9] favours the view that the erythrocyte NBMPR binding site is identical to the nucleoside translocation site of the carrier, the possibility of NBMPR binding at a distinct site (perhaps a regulatory site) cannot at this stage be ruled out. We report here experiments in which the apparent target size of the nucleoside transporter in human erythrocytes has been determined by radiation inactivation of uridine fluxes. Values obtained are compared with M_r estimates based on radiation inactivation of NBMPR binding in the same cells.

The principal features of the method were as follows: (i) human erythrocytes were frozen in liquid nitrogen in the presence of cryoprotective compounds [10], (ii) frozen cells were irradiated at liquid nitrogen temperature with high energy electrons, and (iii) assays for NBMPR-sensitive uridine fluxes and high-affinity NBMPR binding activity were compared in treated and untreated cells. It was necessary to irradiate the cells at low temperature in order to minimize the possibility of secondary radiation effects [11]. Control experiments established that the freezing and thawing of fresh human erythrocytes did not result in significant loss of either NBMPR-sensitive uridine flux or high-affinity NBMPR binding activity (Table I). Furthermore, the NBMPR-insensitive flux of uridine, which represents passive diffusion of

Abbreviations: NBMPR, 6-(4-nitrobenzyl)thio-9-β-D-ribofuranosylpurine or nitrobenzylthioinosine.

TABLE I

EFFECT OF FREEZING AND THAWING ON URIDINE TRANSPORT AND HIGH-AFFINITY NBMPR BINDING BY HUMAN ERYTHROCYTES

Fresh erythrocytes, collected from healthy volunteers into heparinized tubes, were washed three times in 150 mM NaCl, 10 mM tris-HCl (pH 7.4 at 22°C) and the buffy coat and plasma were discarded. Equal volumes of packed cells and the freezing solution (containing 280 g of glycerol, 28 g of sorbitol and 720 ml of 150 mM NaCl [10]) were mixed and the cells equilibrated for 30 min at 4°C. Aliquots (0.5 ml) were added to 15 ml centrifuge tubes and frozen by immersion in liquid nitrogen. The cells were thawed by the addition of 10 ml of prewarmed (37°C) freezing solution diluted 50% with 150 mM NaCl. The thawed cells were centrifuged at $1000 \times g$ for 5 min, washed once in 600 mM NaCl [21], followed by three washes in 145 mM NaCl, 5 mM KCl, 5 mM glucose and 15 mM Tris-HCl (pH 7.4 at 22°C). Initial rates of zero-*trans* uridine influx (extracellular uridine concentration 0.05–3 mM, 3 s assay interval) and uridine equilibrium exchange influx (5 mM uridine, 5 s assay interval) were determined at 22°C by a method which employed NBMPR (10 μM) as a transport 'stopper' and separated cells from extracellular medium by pelleting under oil [6,7]. Transport rates were measured in the presence and absence of 5 μM NBMPR and the difference between these two rates is referred to as 'NBMPR-sensitive transport'. High-affinity NBMPR binding to erythrocytes at 22°C was determined by the method of Jarvis and Young [3]. The mean values of two separate experiments are given.

		Fresh cells	Frozen and thawed cells
Uridine influx	V_{max} (10^{-18} mol/cell per s)	2.0	1.9
	K_m (mM)	0.15	0.15
Uridine equilibrium exchange influx	V_{max} (10^{-18} mol/cell per s)	3.9	3.6
NBMPR binding	B_{max} (10^{-21} mol/cell)	16.6	15.3
	K_d (nM)	0.35	0.36

uridine across the membrane [6], increased by less than 10% in cells frozen and then thawed as compared to fresh cells (data not shown).

Irradiation of frozen human erythrocytes was found to inactivate NBMPR-sensitive uridine influx, equilibrium exchange uridine influx, and high-affinity NBMPR binding in a dose-dependent manner (Fig. 1). When the logarithm of the uridine influx and NBMPR binding activity was plotted against the radiation dose, a linear relationship was observed. The extrapolated dose required to reduce the transport and binding activities to 37% of their original activity (D_{37}) was approx. 12.5 Mrad. Assuming single-hit target theory [11,12], and using the equation,

$$\text{target size} = \left(6.4 \cdot 10^5 / D_{37} \text{ in Mrad}\right)$$

which is derived both from theoretical and empirical work [11,12], we calculate a target size for both uridine flux and high-affinity NBMPR binding activities of $51\,000 \pm 9000$. The estimated target size is 42% of the previous figure of 122 000 determined for the apparent molecular weight of the NBMPR-binding complex of freeze-dried human erythrocyte membranes irradiated at 30°C [5]. Under our irradiation conditions with intact cells at liquid nitrogen temperature (−196°C), glucose-6-phosphate dehydrogenase activity, used as an internal standard, also gave an apparent molecular weight of 42% of that estimated by conventional methods (42 100, mean of three separate experiments; Fig. 2) [13]. A control experiment using freeze-dried haemolysates confirmed that glucose-6-phosphate dehydrogenase inactivated with its normal molecular weight when irradiation was performed at 30°C (see also Fig. 2). These results agree with previous studies which have demonstrated that an empirical correction factor needs to be applied to molecular weight estimates obtained at low temperature [11,14,15]. Using our glucose-6-phosphate dehydrogenase correction factor of 2.39, we therefore calculate that both uridine transport and high-affinity NBMPR binding in intact human erythrocytes inactivate with the same apparent molecular weight as that determined previously for the NBMPR binding complex in freeze-dried membranes [5].

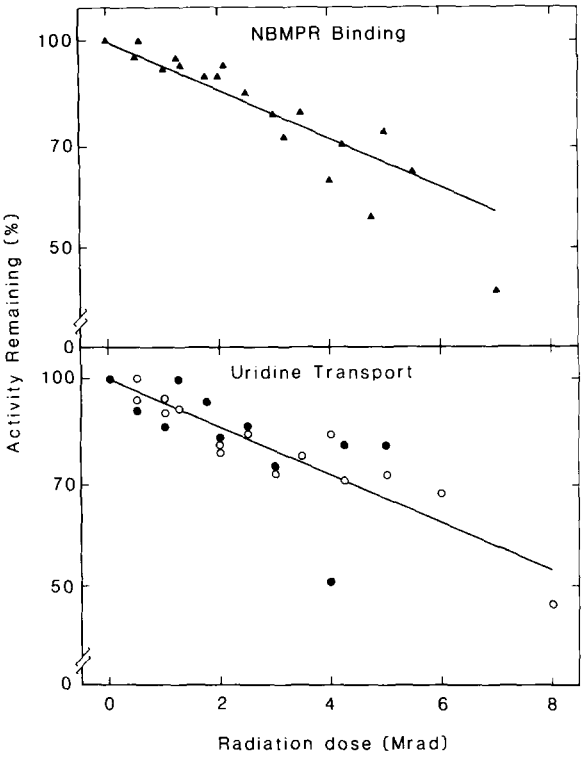

Fig. 1. Effect of increasing radiation dose on uridine transport and high-affinity NBMPR binding activity in intact human erythrocytes. Samples of erythrocytes equilibrated in the freezing solution as described in Table I, 0.5 ml in volume, were added to 6-mm tubes sealed at one end and frozen by immersion in liquid nitrogen. The tubes were immediately sealed under vacuum and stored in liquid nitrogen. The frozen samples were irradiated with high-energy electrons using a clinical Phillips-MEL SL 75/20 20 MeV linear accelerator, at the Department of Radiotherapeutics, Addenbrooke's Hospital, Cambridge, at a dose rate of 2.0 Mrad·min^{-1} as described by Ellory et al. [22]. During irradiation the frozen samples were contained in a NMR Dewar finger flask filled with liquid nitrogen. After irradiation, the frozen cells were thawed by the procedure described in the legend to Table I. Initial rates of NBMPR-sensitive zero-*trans* uridine influx (●) and uridine equilibrium exchange influx (○) were determined at saturating concentrations of uridine, 3 and 5 mM, respectively. The maximum number of high-affinity NBMPR binding sites (▲) was measured at a saturating concentration of [^{3}H]NBMPR (initial concentration 30 nM). The data are plotted as log % remaining activity (log scale) versus radiation dose (Mrad); linear least-squares analyses were used to fit the lines shown. The results were normalized for differences in cell number at each radiation dose by measuring the final haemoglobin content of cells in each transport or binding assay. The data points represent the pooled results of four separate experiments.

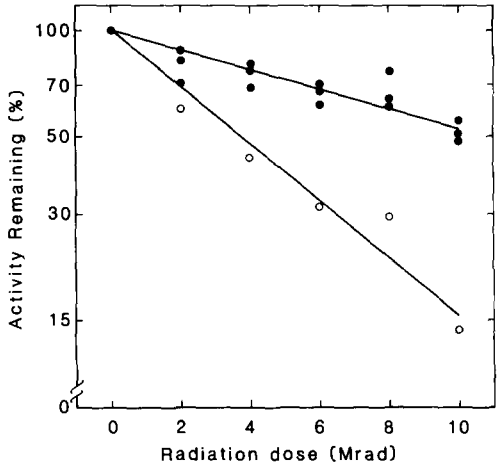

Fig. 2. Effect of increasing radiation dose on glucose-6-phosphate dehydrogenase activity in human erythrocytes. In the first series of experiments, intact human erythrocytes were frozen and irradiated at −196°C as described in the legends to Table I and Fig. 1. In the other experiment, human erythrocyte haemolysates (0.5 ml, 20% haematocrit equivalent) were freeze-dried in 6-mm tubes and irradiated in vacuo under identical conditions except that the irradiation temperature was maintained at 30°C. Enzyme activity was assayed as described by Beutler [23] and the results normalized for differences in cell number at each radiation dose by measuring the final haemoglobin content of each assay sample. The data are plotted as log % remaining activity (log scale) versus radiation dose (Mrad) and linear least-squares analyses used to draw the fitted lines. The data points for the intact cell study (●) represent the pooled results of three separate experiments. The haemolysate data points (○) are from a single experiment. The estimated molecular weight values are 42100 and 118000, respectively.

In conclusion, the present results demonstrate that the nucleoside transporter has an apparent molecular weight in situ of 122000, as estimated directly by uridine flux inactivation or indirectly by the binding of transport inhibitor, NBMPR. The results suggest that the NBMPR binding site and the permeation site(s) of the transporter are present on the same transporter element (or polypeptides). Recently, radioactively labeled NBMPR and azidobenzyladenosine have been used to covalently label the human erythrocyte nucleoside transporter [16]. Selective incorporation of radioactivity was associated with a broad band on sodium dodecyl sulphate polyacrylamide gels in the molecular weight range of 45000–65000 (band 4.5). Thus, it seems probable that the nucleoside

may exist in the membrane as a dimer. The glucose transporter, another band 4.5 polypeptide (for references see Refs. 17 and 18), also appears to be a multerimeric protein in the intact cell [19,20]. However, one noticeable difference between the erythrocyte glucose and nucleoside transport systems is that the target size for D-glucose-sensitive cytochalasin B binding and 3-*O*-methyl-D-glucose flux is approximately 185 000 [19,20]. Furthermore, the target size estimated for the glucose transporter is reported to be independent of temperature.

Acknowledgments

We thank R. Gouldstone, Department of Radiotherapeutics, Addenbrooke's Hospital, Cambridge for help with the linear accelerator. The work was supported by grants from the Alberta Heritage Foundation for Medical Research (AHFMR), the Wellcome Trust and the Cancer Research Campaign, U.K. Presently, S.M.J. is an AHFMR Scholar and during part of these studies was a Research Fellow, Cancer Research Grants Program (Alberta Cancer Board) of the Alberta Heritage Savings Trust Fund.

References

1 Paterson, A.R.P., Kolassa, N. and Cass, C.E. (1981) Pharmacol. Ther. 12, 515–536
2 Young, J.D. and Jarvis, S.M. (1983) Biosci. Rep. 3, 309–322
3 Jarvis, S.M. and Young, J.D. (1980) Biochem. J. 1980, 377–383
4 Cass, C.E., Kolassa, N., Uehara, Y., Dahlig-Harley, E., Harley, E.R. and Paterson, A.R.P. (1981) Biochim. Biophys. Acta 649, 769–777
5 Jarvis, S.M., Young, J.D. and Ellory, J.C. (1980) Biochem. J. 190, 373–376
6 Jarvis, S.M., McBride, D. and Young, J.D. (1982) J. Physiol. (Lond.) 324, 31–46
7 Jarvis, S.M. and Young, J.D. (1982) J. Physiol. (Lond.) 324, 47–66
8 Cass, C.E. and Paterson, A.R.P. (1976) Biochim. Biophys. Acta 419, 285–294
9 Jarvis, S.M., Janmohamed, S.N. and Young, J.D. (1983) Biochem. J. 216, 661–667
10 Mohn, J.F., Bowman, H.S. and Cunningham, R.K. (1970) Vox Sang. 19, 508–521
11 Kempner, E.S. and Schlegel, W. (1979) Anal. Biochem. 92, 2–10
12 Kepner, G.R. and Macey, R.I. (1968) Biochim. Biophys. Acta 163, 188–203
13 Bonsignore, A., Cancedda, R., Lorenzoni, I., Cosulich, M.E. and De Flora, A. (1971) Biochem. Biophys. Res. Commun. 43, 94–101
14 Schlegel, W., Kempner, E.S. and Rodbell, M. (1979) J. Biol. Chem. 254, 5168–5176
15 Kempner, E.S. and Haigler, H.T. (1982) J. Biol. Chem. 257, 13297–13299
16 Young, J.D., Jarvis, S.M., Robins, M.J. and Paterson, A.R.P. (1983) J. Biol. Chem. 258, 2202–2208
17 Carter-Su, C., Pessin, J.E., Mora, R., Gitomer, W. and Czech, M.P. (1982) J. Biol. Chem. 257, 5419–5425
18 Shanahan, M.F. (1982) J. Biol. Chem. 257, 7290–7293
19 Jung, C.Y., Hsu, T.L., Hah, J.S., Cha, C. and Haas, M.N. (1980) J. Biol. Chem. 255, 361–364
20 Cuppoletti, J., Jung, C.Y. and Green, F.A. (1981) J. Biol. Chem. 256, 1305–1306
21 Rowe, A.W., Lenny, L.L., Dayain, G. and Mayer, K. (1973) Transfusion 13, 355–356
22 Ellory, J.C., Green, J.R., Jarvis, S.M. and Young, J.D. (1979) J. Physiol. (Lond.) 295, 10P–11P
23 Beutler, E. (1975) Red Cell Metabolism, A Manual of Biochemical Methods (2nd Edn.), pp. 66–67, Grune and Stratton, New York