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TIAZOFURIN-INDUCED CHANGES IN INOSITOL LIPID CYCLE IN NUCLEI OF FRIEND ERYTHROLEUKEMIA CELLS

A.M. Billi, L. Cocco°, A.M. Martelli*, R.S. Gilmour§, and G. Weber#

Istituto di Anatomia Umana Normale, V. Irnerio, 48, 40126 Bologna, Italy

*Dipartimento di Morfologia Umana, V. Manzoni, 16, 34138 Trieste , Italy

§Department of Molecular and Cellular Physiology, AFRC, Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, England, U.K.

#Laboratory for Experimental Oncology, Indiana University School of Medicine, Indianapolis, IN 46202-5200

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When murine erythroleukemia cells are treated up to 96 hrs with the antitumor drug tiazofurin the induction of erythroid differentiation is accompanied by changes in both synthesis and breakdown of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5 bisphosphate in the nucleus. These changes, which parallel the effect of dimethylsulfoxide, a well known inducer of erythroid differentiation *in vitro*, are due to the inhibition of the nuclear phosphoinositidase C activity. Therefore the present data indicate the nuclear inositol lipid cycle as a target of tiazofurin in addition to its inhibition of IMP dehydrogenase (EC 1.1.1.205) activity.

The erythroid differentiation programme of cultured K562 human erythroleukemic cells can be activated by a number of chemical agents and also by the antitumor drug tiazofurin (1).
Although the exact mechanism of DMSO induction is not known, differentiation has been shown to involve dramatic changes in a recently discovered phosphoinositide lipid component of the nucleus in murine Friend erythroleukemia cells (2). Indeed during the course of differentiation there is a progressive accumulation of phosphatidylinositol 4,5 bisphosphate in the Friend cell nucleus (3). The inositol lipid cycle is characterised by the presence of both lipid kinases which synthesise PtdInsP and $PtdInsP_2$ and PIC which determines the cleavage of the inositol phosphates from the diacylglycerol moiety of the phosphoinositides (4).

° To whom correspondence should be addressed.

Abbreviations are: PtdInsP, phosphatidylinositol 4-phosphate; $PtdInsP_2$, phosphatidylinositol 4,5-bisphosphate DMSO, dimethyl sulfoxide; PIC, phosphoinositidase C; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; PtdOH, phosphatidic acid; $InsP_3$, inositol 1,4,5-trisphosphate.

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Little is known about the initial mechanisms which precede tiazofurin mediated differentiation. This drug is an anti-tumor nucleoside that inhibits IMP dehydrogenase and consequently lowers guanylate levels (5). Moreover, in cultured K562 cells the induction of erythroid differentiation is accompanied by down-regulation of c-Ki-*ras* protooncogene (1). These data suggest involvement of a signalling cascade in the antitumor effect exhibited by tiazofurin. Differentiation can be prevented by the addition of guanosine, suggesting that guanine nucleosides are pivotal to this process. Because of the previously reported changes in nuclear inositol lipids occurring during erythroid differentiation of murine erythroleukemia cells (3), in this paper we ask whether tiazofurin-induced differentiation is also accompanied by changes in phosphoinositides of Friend cell nuclei and we further illuminate on the mechanisms involved in tiazofurin action.

MATERIALS AND METHODS

Cell culture. Murine erythroleukemia cells (Friend cells, clone 707) were cultured as previously described, and haemoglobin synthesis was induced by addition of 1.5% (v/v) DMSO (2) or 5 μM tiazofurin (1). In some experiments guanosine (50 μM) was added to the medium for inhibition of tiazofurin differentiation (1).

Isolation of nuclei and preparation of cytoplasmic fraction. A hypotonic shock combined with non-ionic detergent was employed. Briefly, cells were collected by centrifugation and washed once in PBS, then resuspended to 1.5 x 10^7/ml in 10 mM Tris-Cl, pH 7.4, 2 mM $MgCl_2$, 0.5 mM PMSF, 10 μg/ml leupeptin, 0.3 μM aprotinin, 15 μg/ml calpain I inhibitor, 7.5 μg/ml calpain II inhibitor (temperature of the buffer was 10°C). After the addition of Triton X-100 to 0.3% (w/v), cells were sheared by one up and down passage through a 22 gauge needle fitted to a 30 ml plastic syringe. Mg^{2+} concentration was brought to 5 mM and nuclei were separated from the cytosol by low speed centrifugation (400 g for 8 min). The nuclear pellet was washed once in 10 mM Tris-Cl, pH 7.4, 5 mM $MgCl_2$, 0.5 mM PMSF, 10 μg/ml leupeptin, 0.3 μg/ml aprotinin, 15 μg/ml calpain I inhibitor, 7.5 μg/ml calpain II inhibitor.
The cytoplasmic fraction was obtained by homogenizing cells with 20 strokes in a Dounce homogenizer in 10 mM Tris-Cl, pH 7.8, 2 mM $MgCl_2$ plus protease inhibitors as above and then pelletting the nuclei at 400 g.

Phosphoinositidase C activity. The phosphoinositidase C activity was carried out in isolated nuclei from control and both tiazofurin and DMSO treated cells using as exogenous substrate [^{3}H]PtdInsP_2 at the optimal conditions previously described by Martelli et al. (6). [^{3}H]InsP_3 released after 30 min of incubation at 37°C was analyzed by HPLC exactly as in ref. 6.

RESULTS AND DISCUSSION

Exposure of Friend cells to DMSO or tiazofurin for 4 days gave rise to erythroid differentiation as detected by the benzidine test (1). With DMSO and tiazofurin the percentage of benzidine-positive cells was 87% and 34% respectively over a basal level of 3%. The *in vitro* phosphorylation of isolated nuclei (Fig. 1) shows a pattern quite similar to that seen with tiazofurin and DMSO, characterised by an accumulation of PtdInsP_2 above that of the control

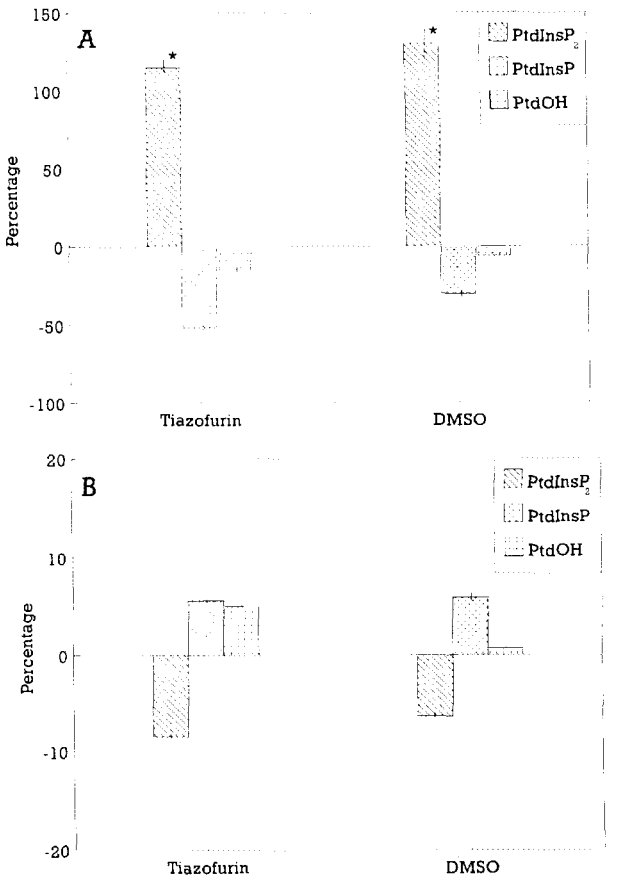

Fig. 1. Histogram showing the effect of tiazofurin and DMSO on the *in vitro* synthesis of both nuclear (A) and cytoplasmatic (B) polyphosphoinositides. Values are the means ± S.E. of 5 separate experiments and are expressed as percentage increase or decrease of phosphorylation over the control. The average of total radioactivity in control nuclei (300 μg nuclear protein) is 8,500 ± 61.0 cpm and percentages of radioactivity in PtdOH, PtdInsP and $PtdInsP_2$ are respectively 8.2, 78.1, 13.7. The average of total radioactivity in control cytoplasm (300 μg cytoplasmatic protein) is 127,500 ± 770 cpm and percentages of radioactivity in PtdOH, PtdInsP and $PtdInsP_2$ are respectively 8, 64.8, 27.2. *Significant differences ($p < 0.005$, Student's *t*-test) from controls.

only in the nuclear fraction whilst cytoplasm was not affected. This strengthens the contention that nuclear polyphosphoinositides are involved in erythroid differentiation (2).

Since the previous observations dealing with DMSO-induced differentiation of Friend cells suggested at changes of inositol lipid concentrations (2), we were prompted to analyse the nuclear PIC activity after both tiazofurin and DMSO treatment. Fig. 2 shows that PIC activity is markedly reduced by treatment with both agents. However, the addition in the tissue culture medium of guanosine, which inhibited the erythroid differentiation in tiazofurin but not in DMSO-treated cells, restored the PIC activity only in nuclei from cells treated with tiazofurin but not in nuclei from DMSO- induced cells.

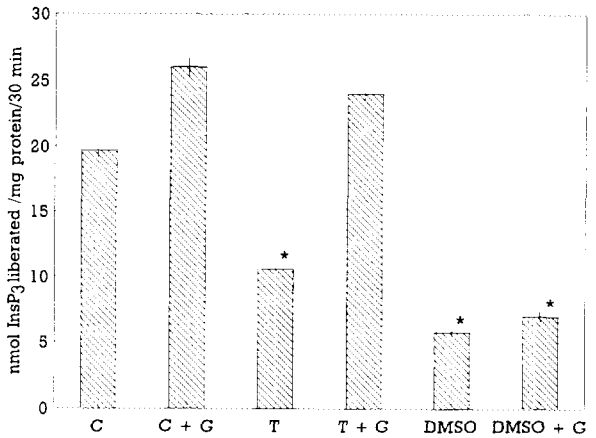

Fig. 2. PIC activity in isolated nuclei from Friend cells treated with tiazofurin or DMSO. ^{3}H-$PtdInsP_2$ was used as exogenous substrate. Values are expressed as nmoles of $InsP_3$ liberated/mg protein/30 min incubation at 37°C and are the means ± S.E. of 5 separate experiments. The percentage of benzidine-positive cells in the presence of guanosine are: Control + guanosine 3%; tiazofurin + guanosine 4%; DMSO + guanosine 85%. C, control; C+G, control cells plus guanosine; T, tiazofurin treated cells; T+G, tiazofurin treated cells in the presence of guanosine; DMSO, DMSO treated cells; DMSO+G, DMSO treated cells in the presence of guanosine. * Significant differences ($p < 0.005$, Student's *t*-test) from controls.

A common feature of erythroid differentiation by DMSO and tiazofurin in Friend cells is the inhibition of nuclear PIC activity and a concomitant accumulation of nuclear $PtdInsP_2$. This suggests that the resulting shut-down of nuclear polyphosphoinositide turnover is a component of the programmed pathway of differentiation although the mechanisms whereby the two compounds trigger the onset of the process may be quite different. This is emphasised by the observation that exogenous guanosine can prevent only tiazofurin-induced differentiation while it has no effect on induction by DMSO. A simple interpretation of this might be the restoration of GTP concentration by guanosine through activity of the salvage enzyme, guanine phosphoribosyltransferase (EC 2.4.2.8), thereby permitting the cells to continue dividing as undifferentiated cells. The fact that c-Ki-*ras* mRNA decreases within 12 hours of tiazofurin induction (1) may result from a reduction of GTP pools rather than a direct step in the differentiation pathway. Further experiments are required to determine the time-course of events over the first 24 hours of induction and the relationship between nuclear inositol lipid accumulation and Ki-*ras* expression. However, the results support a hypothesis that polyphosphoinositide cycle is linked with the *in vitro* erythroid differentiation of Friend cells. This agrees with the operation of an autonomous polyphosphoinositide signalling system in the nucleus, which responds to mitogenic stimuli (6). Moreover, while mitogenic stimulation with insulin-like growth factor I activates nuclear PIC, differentiating agents, which also inhibit cell growth (i.e. DMSO and tiazofurin), behave in the opposite way by inducing inhibition of nuclear PIC activity. The present results point out regulation of nuclear polyphosphoinositide cycle as a key step in the cascade of molecular events leading to cell differentiation.

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