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Journal of Chromatography B, 678 (1996) 344–348

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Facile analysis of ribonucleotides in D,L-α-difluoromethylornithine-treated mouse lymphoid cells by high-performance anion-exchange column chromatography

Su-Che Cho

Department of Biology, School of Health Sciences, Fujita Health University, Toyoake-shi, Aichi-ken 470-11, Japan

Received 31 August 1995; revised 16 November 1995; accepted 27 November 1995

Abstract

Using high-performance liquid chromatography with the strong anion-exchange resin column CDR-10 for analysis of ribonucleotides, the effects of D,L-α-difluoromethylornithine (DFMO) on ribonucleotides of mouse leukemic lymphoid cells SC-1 have been studied. More than 16 nucleoside mono-, di and triphosphates, and unknown peaks were clearly separated and measured without increase in baseline rise using the gradient systems of phosphate buffer. The ATP level in DFMO-treated SC-1 cells was reduced, but was reversed by exogenous putrescine. These facts may suggest that polyamine depletion by DFMO during a short time (6 h) caused mitochondrial damage in mammalian cells.

Keywords: Ribonucleotides; D,L-α-Difluoromethylornithine

1. Introduction

D,L-α-Difluoromethylornithine (DFMO) is an enzyme-activated, irreversible inhibitor of ornithine decarboxylase, the first enzyme in the polyamine biosynthetic pathway [1]. A number of studies have shown that the intracellular putrescine and spermidine content of cultured mammalian cells is depleted after treatment with DFMO [2–4]. Nevertheless, the total adenine nucleotide content was greater in DFMO-treated Ehrlich ascites tumor cells for 72 h than in untreated cells [5–7], while DFMO damaged mitochondrial structure and function in DFMO-treated rat brain tumor cells [8]. To clarify this discrepancy it is necessary to estimate the changes in ribonucleotides in DFMO-treated cells during the short time without cell division. There are only a few reports on the changes of ribonucleotide levels in DFMO-treated mammalian cells without cell division. It is conceivable that mitochondrial damage due to DFMO-treated interphase cells causes the changes in ATP and other ribonucleotide levels.

Anion-exchange and reversed-phase high-performance liquid chromatography (HPLC) are the principal methods used for nucleotide separation [9]. HPLC using a strong anion-exchange resin column for analysis of ribonucleotides has a excellent long column lifetime and a high reproducibility, but the disadvantage of anion-exchange resin column is the use of highly concentrated eluent buffers. In the present experiment, HPLC using the anion-exchange resin column CDR-10 and the buffer system of Hartwick and Brown [10] showed more facile and sensitive separation of ribonucleotides than when acetate buffer systems were used [11]. The present study reports that SC-1 mouse lymphoid cells treated

0378-4347/96/$15.00
SSDI 0378-4347(95)00546-3

with DFMO for 6 h particularly reduced ATP level, and that polyamine depletion by DFMO might affect the mitochondrial function.

2. Experimental

2.1. Cell culture

Mouse lymphoid leukemia SC-1 cells [12] were grown in suspension for 5 days in medium containing 8% horse serum. The cell cultures were incubated at 37°C in 5% CO_2. The growth medium used contained Dulbecco's modified Eagle's minimum essential medium (Nissui, Japan) supplemented with penicillin (30 UI/ml), streptomycin (75 μg/ml), glucose (1.5 g/l) and $NaHCO_3$ (1.3 g/l). DFMO was kindly donated by Merrell Dow Research Institute (Cincinnati, OH, USA).

2.2. Extraction of intracellular ribonucleotides

Cells were disrupted by sonication using cell disruptor (Model MS-50, Heat Systems-Ultrasonics, USA) in 10 volumes ice-cold 0.5 *M* $HClO_4$ (approx. 0.9 ml). The homogenates were kept 10 min at 4°C and centrifuged at 16 000 *g* for 3 min. To the supernatant was then added 0.9 ml 0.5 *M* KOH, and the pH of the solution was adjusted with KOH or $HClO_4$ between 5.5 and 7.0. The solution was maintained at 0°C for 5 min to maximize the precipitation of potassium perchlorate, followed by recentrifugation at 16 000 *g* at 4°C for 5 min. The supernatant was immediately frozen and lyophilized with the vacuum concentrator. The residue was dissolved in 250 μl 10 m*M* HCl and centrifuged to dispense with the rest of the perchlorate, followed by filtration through a 0.45-μm HA membrane (Nihon Millipore Kogyo, Japan).

2.3. High-performance liquid chromatography

The HPLC apparatus consisted of CCPM solvent pumps (Tosoh, Japan); a 7125 Rheodyne syringe loading sample injector (Rheodyne, Cotati, CA, USA); a PX8010 CCP controller (Tosoh); a DE-8000 gradient system (Tosoh); a S-533 UV spectrometer (Irikakiki, Japan); a DG1200 on-line degasser (Labo-Quatec, Japan); and a T-80 column heater (Irikakiki, Japan).

The strong anion-exchange resin column (MCI CDR-10, 7μm pore size, 250×4.6 mm I.D.) [13] was obtained from Mitsubishi Kasei (Japan). The ribonucleotide assay was performed with gradients of phosphate buffer [10,14]. Buffer A, 0.007 *M* KH_2PO_4 at pH 4.0, and buffer B, 0.25 *M* KH_2PO_4–0.5 *M* KCl pH 4.5, were used as eluants. The elution program was as follows: 0 min, A only; 0–50 min, linear increase in B from 0 to 100%; 50–55 min, B only; 55–65 min, A only. The flow-rate was 0.8 ml/min, and column temperature was 60°C. The chart speed was 25 cm/h. A 20-μl aliquot of $HClO_4$-denatured filtrate was injected and peaks were determined at 254 nm, 0.08 AUFS.

2.4. Measurement of peaks

The ribonucleotide levels were calculated from the chromatographic height obtained from known amounts of standard ribonucleotides and corrected according to the recovery of the internal standard (XMP). The recovery of XMP itself was approx. 80–90%.

3. Results and discussion

Fig. 1 shows the separation of standard purine and pyrimidine nucleotides by a MCI CDR-10 column using gradients of phosphate buffer. Nucleoside monophosphates, diphosphates and triphosphate were eluted in that order, except that XMP appeared before IDP. As XMP was not detected in SC-1 cells examined, it was used as the internal standard for recovery. Table 1 shows the retention times of the nucleotides in cell extracts and standards. In Table 1, besides a little disagreement of IMP and AMP, almost all retention times in the standard nucleotides were in accord with those of the cell extracts.

Comparison of the system described in this paper with the HPLC method established with previously published acetate buffer systems [11] highlighted the three main advantages of the strong anion-exchange resin column CDR-10. Firstly, the sensitivity range of 0.08 AUFS achieved by this method was more

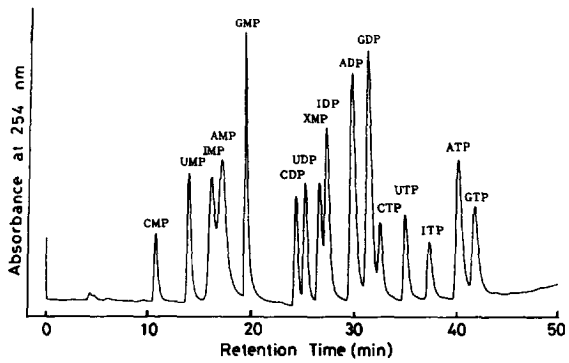

Fig. 1. Chromatograms obtained with UV detection of a standard mixture of various ribonucleotides. A mixture (40 μl) of ribonucleotides, containing nucleoside monophosphate (4 μg each: CMP 10.84 nmol, UMP 10.8 nmol, IMP 10.78 nmol, AMP 11.52 nmol, GMP 9.9 nmol), nucleoside diphosphate and triphosphate (8 μg each: CDP 15.9 nmol, UDP 18.73 nmol, XMP 18.3 nmol, IDP 15.6 nmol, ADP 16.07 nmol, GDP 15.23 nmol, CTP 14.2 nmol, UTP 15.77 nmol, ITP 13.93 nmol, ATP 13.22 nmol, GTP 13.16 nmol) was injected onto the column. As described in Section 2.3, the elution profile was composed of an initial isocratic phase (buffer A) and a linear gradient from buffer A to buffer B for 50 min, followed by an isocratic phase with buffer B (5 min).

sensitive than that of 0.16 AUFS in the previously published acetate buffer systems [11]. Secondly, the analysis time was shortened from approximately 65 min [11] to 45 min. Therefore, more samples can be measured in the same period of time. Thirdly, in the present study there is almost no increase in both the baseline rise and the column inlet pressure compared to previously published acetate buffer systems [11].

Table 1
Retention time of nucleotides in HPLC

Nucleotide	Retention time (min)		
	Standard	Control	10^{-5} *M* DFMO
CMP	10.66	10.87	10.87
UMP	13.76	13.91	13.70
IMP	15.89	17.17	16.52
AMP	16.86	18.04	17.61
GMP	19.19	19.13	20.00
CDP	24.22	24.13	22.83
UDP	25.19	25.00	24.78
XMP	26.36	26.09	26.09
IDP	27.13	–	–
ADP	29.46	29.13	29.35
GDP	31.20	31.09	31.09
CTP	32.56	32.39	31.96
UTP	34.88	35.00	34.57
ITP	37.40	–	–
ATP	39.92	39.78	39.78
GTP	41.67	41.74	41.52

McCormick et al. [14] reported that ATP and adenine nucleotides exhibited the greatest change in the cell cycle of a synchronized mouse fibroblast cell line. On the other hand, in the present study, the DFMO treatment time of 6 h has the advantage of allowing directly measurable effects of DFMO on ribonucleotides of SC-1 cells without fluctuations in the intracellular pool levels of ribonucleotides within the cell cycle. Fig. 2a shows chromatographic separation ribonucleotides extracted from 5-day-old SC-1 cells. Fig. 2b shows the effects of 10^{-5} *M* DFMO treatment (6 h) on the ribonucleotide of 5-day-old SC-1. In Fig. 2, the peaks of ATP in 10^{-5} *M* DFMO-treated cells were particularly lowered.

Fig. 3a shows that no clear decrease in the contents of ribonucleotide monophosphate was observed in any of the DFMO treatment. However, the result of GMP is not shown in Fig. 3, as the peak height of GMP is too high due to the too high sensitivity for GMP of this column system, while the result of the GMP content in the addition of putrescine alone is shown in Fig. 5. In contrast, Fig. 3b and Fig. 3c demonstrate that 10^{-5} *M* DFMO-treated SC-1 cells decreased ribonucleoside diphosphate and ribonucleotide triphosphate contents. The decrease of intracellular ribonucleotide contents resulting from the treatment with 10^{-5} *M* DFMO is reversed by exogeneous putrescine (10^{-4} *M*) (Fig. 4). The ATP content of putrescine alone control is similar to that of untreated control (Fig. 5). This result seems to be in accord with the fact that putrescine has no observable effect on mitochondria [8].

However, Oredsson et al. [5,6] showed that the total adenine nucleotide pool increased, rather than decreased, as a result of DFMO treatment in Ehrlich acites tumor cells, the major contributors to the increase being ATP and ADP. These results indicated the possibility that part of the antiproliferative effect of polymine depletion may be attributable to the accumulation of these ribonucleotides. The increase in ribonucleotide content occurring during DFMO-induced growth inhibition (72 h) may partly be due to a decreased utilization of ribonucleotides for nucleic acid synthesis [5,6]. Accordingly, this problem may suggest the correlation with the time scale

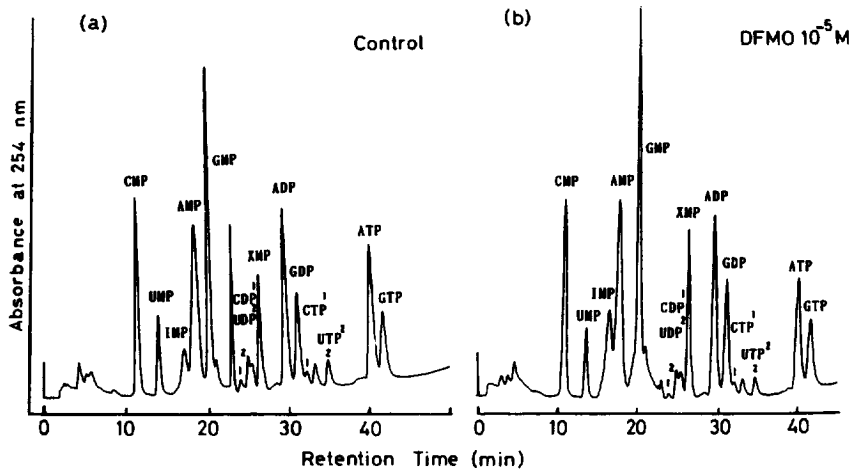

Fig. 2. Chromatographic separation of ribonucleotides from SC-1 cells. The buffers used and the gradient elution profile are described in Section 2.3. For an analysis of nucleotides, 20 μl of each extract was injected onto the chromatograph. (a) Nucleotide pattern of 5-day-old SC-1 cells. (b) Effects of 10^{-5} *M* DMFO on the nucleotide composition of 5-day-old SC-1 cells after 6 h of DFMO administration.

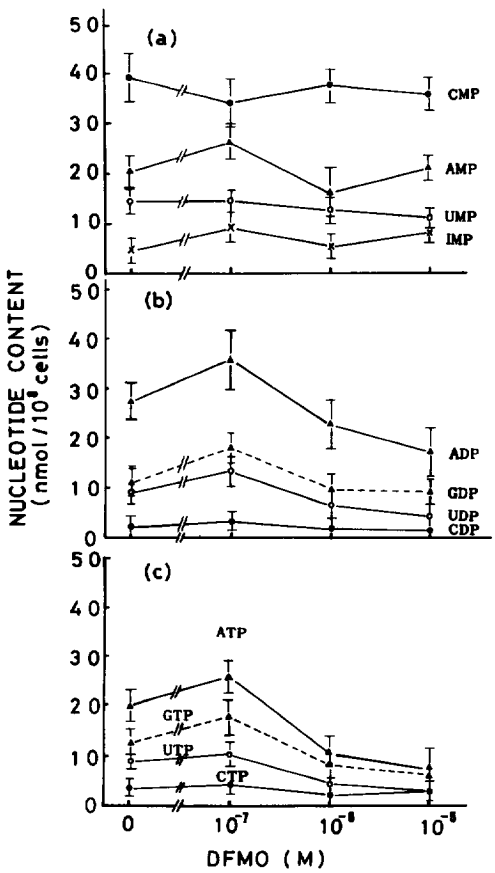

Fig. 3. Concentrations of ribonucleotides in SC-1 cells after 6 h incubation with and without DFMO added. Vertical lines show means±S.D. (n=4).

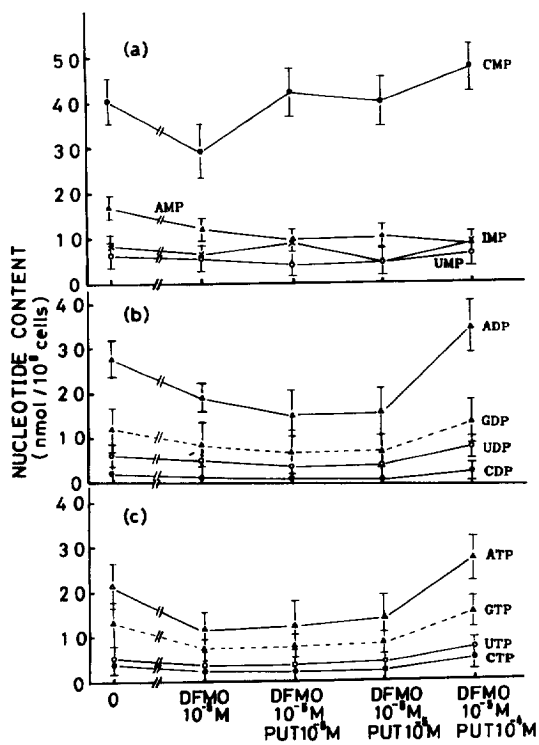

Fig. 4. Reversal of the effect of 10^{-5} *M* DFMO on the ATP level by 10^{-4} *M* exogenous putrescine. Vertical lines show means±S.D. (n=4).

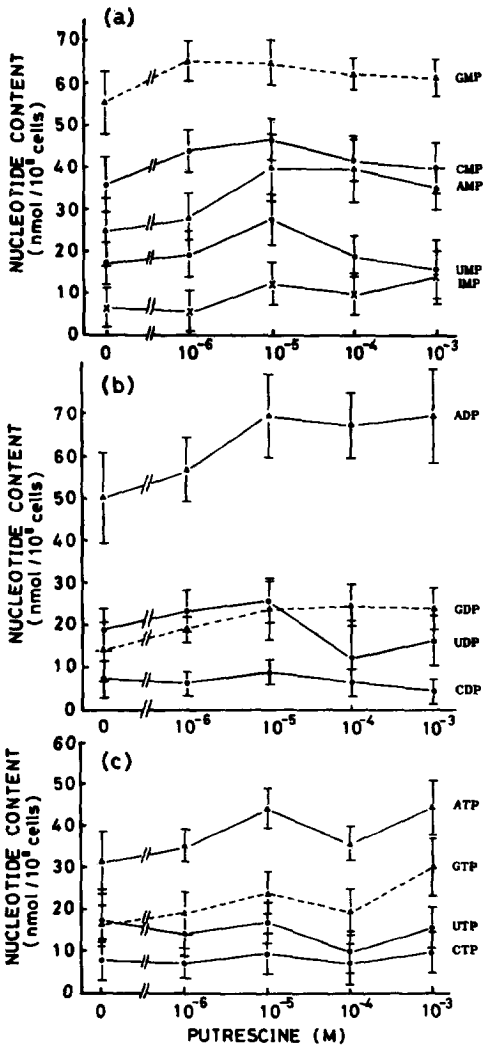

Fig. 5. The effect of putrescine alone on the ribonucleotide content of 5-day-cultured SC-1 cells. Vertical lines show means±S.D. (n=4).

of DFMO treatment of cells. In the present study, the results obtained may suggest that the decrease in the ATP level caused by the administration of 10^{-5} M DFMO to SC-1 cells results from the mitochondrial damage, since nucleic acid synthesis does not rise for new cell division during relatively short time (6 h) of administration of DFMO, and the decrease of the main ribonucleotides (ATP, GTP, etc.) in DFMO-treated SC-1 cells was reversed by exogenous putrescine (Fig. 4).

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