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Effects of peroxisomal β-oxidation antagonist on 2′,3′-cyclic-nucleotide 3′-phosphohydrolase, membrane lipid compositions, and membrane fluidity in C-6 glial cells

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Abstract

In order to understand the relationship between peroxisomal dysfunction and clinical manifestations of peroxisomal disorders, the effect of thioridazine, a peroxisomal β-oxidation antagonist, on the differentiation, membrane lipid composition and membrane fluidity of C-6 glial cells was examined. In our study, induction of 2′,3′-cyclic-nucleotide 3′-phosphohydrolase (CNP), which was considered to be a membrane-associated enzyme closely associated with myelination, was inhibited with supplementation of thioridazine, followed by an increase in the relative concentration of longer chain fatty acids in cell membrane lipids, indicating that thioridazine causes impaired differentiation in the glial stem cell system. Membrane fluidity of C-6 glial cells was examined using a fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH). The DPH anisotropy value was decreased in the glial cells treated with thioridazine. These results indicate that the alteration of the membrane lipid composition caused by thioridazine affects the differentiation of glial cells via the changes in membrane properties.

Keywords: C-6 glia; Thioridazine; 2′,3′-Cyclic-nucleotide 3′-phosphohydrolase; Lipid composition; Membrane fluidity

1. Introduction

In patients with peroxisomal disorders such as Zellweger syndrome, severe neurological abnormalities are present [1–3]. Such patients show a number of metabolic abnormalities including an accumulation of very long chain fatty acids and pipecolic acid in the tissue and the body fluid [4,5], and a decreased level of plasmalogen in tissues [6]. Pathological cytoarchitectonic abnormalities resulting from migration arrest have been described in developmental neuronal tissues [7–9]. However, the role of the metabolic abnormalities in the dysfunction of the nervous system in peroxisomal disease remains unknown.

One of the cell culture models of the developing brain, C-6 glial cells are considered to have properties of glial stem cells [10]. They have oligodendroglia-like properties when they are cultured in serum-free medium or at high density. Induction of 2′,3′-cyclic-nucleotide 3′-phosphohydrolase (CNP), a marker enzyme of myelin and oligodendroglia, occurs upon transfer of C-6 glial cells to a serum-free medium [11–14]. It was reported that thioridazine, one of the phenothiazine derivatives, inhibits peroxisomal β-oxidation in hepatocytes [15,16]. Since the inhibition of β-oxidation is the major abnormality in peroxisomal disorders, investigating the effect of thioridazine on developing neural cells could be an aid to understand the mechanism of neurological manifestations.

The effect of thioridazine on CNP activity was examined in this study using cultured C-6 glial cells. In addition, the changes in the nature of C-6 cell membrane such as fatty acid composition and membrane fluidity upon culture with thioridazine were studied.

Abbreviations: CNP, 2′,3′-cyclic-nucleotide 3′-phosphohydrolase; DMEM, Dulbecco's modified Eagle's medium; DPH, 1,6-diphenyl-1,3,5-hexatriene.

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2. Materials and methods

2.1. Cell culture

C-6 glial cells were obtained from the American Type Culture Collection (Rockville, MD) and routinely cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. The stock of C-6 glial cells were cultured for 3 days and washed with phosphate buffer. To induce the differentiation of C-6 glial cells, they were cultured for additional 2 days in serum-free medium with 0 to 0.04 mM thioridazine. They were harvested mechanically and the cell pellets were frozen in liquid N_2 until the enzyme assay was performed.

2.2. CNP activity

CNP activity was measured by the previously reported method [10]. Essentially, C-6 glial cells were homogenized and solubilized with sodium deoxycholate. The solution was added to 2′,3′-cyclic-monophosphate, and the amount of inorganic phosphorus released from adenosine 2′,3′-cyclic-monophosphate was determined by the methods of Ames [17]. Protein assay was done by the method of Lowry et al. [18].

2.3. Lipids extraction and fatty acid analysis

Fatty acid analysis was done by the method of MacGee and Allen [19]. Fatty acids were extracted with n-hexane after saponification of total lipids from the homogenized cells and were neutralized. Then trimethyl-α,α,α-trifluoro-*m*-tolyl ammonium hydroxide was added to take up free fatty acids from the hexane phase, followed by their methylation on the capillary column (Omegawax 320 0.32 mm, 30 m, Supeluco) of a gas chromatograph (GC-14A, Shimadzu). The initial temperature was 150° C and the temperature was raised 10° C per min to the final temperature of 270° C. The peak area was calculated with Chromatopack (C-R5A, Shimadzu).

2.4. Determination of total cellular cholesterol and phospholipid

Cells were suspended in 0.9% (w/v) NaCl solution and lipids were extracted with NaCl solution/methanol/chloroform (3:4:8 by volume). An aliquot of the chloroform layer was used for quantitation of total cellular cholesterol and lipid-bound phosphorus. Total cholesterol was measured by the colorimetric assay with cholesterol oxidase (Boehringer-Mannheim). Total lipid-bound phosphorus was measured by the method of Ames [17].

2.5. Membrane fluidity

Membrane fluidity was monitored according to the method of Kawato et al. [20] In brief, a lipophilic probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) was added to the homogenized C-6 glial cells and embedded in the membrane by incubation at 37° C for 20 min. Fluorescence intensity was measured with a fluorescence spectrophotometer (FP-2070, JASCO). The excitation wavelength was 360 nm, and the emission light (426 nm) was polarized vertically (I_v) and horizontally (I_h) by a polarizing filter. The steady-state emission anisotropy (r) was calculated as follows.

$$r = \frac{I_v - I_h}{I_v + 2I_h}$$

All chemicals and solvents were of reagent grade. Thioridazine was obtained from Sandoz Yakuhin (Tokyo).

3. Results

3.1. CNP activity

The effect of thioridazine on CNP activity is shown in Table 1. The activity of CNP decreased as a function of thioridazine concentration. The induction of CNP activity at the concentration of 0.04 mM thioridazine was sup-

Table 1
Effect of thioridazine on CNPase activity in C-6 glial cells

Medium	CNP (nmol/min per mg protein)	Protein (mg/flask)
DMEM with 10% FCS	149 ± 11	1.9 ± 0.1
Serum-free DMEM	408 ± 26	1.9 ± 0.1
Serum-free DMEM		
+0.01 mM thioridazine	375 ± 11	2.1 ± 0.2
+0.02 mM thioridazine	321 ± 30	1.9 ± 0.2
+0.04 mM thioridazine	214 ± 11	2.0 ± 0.3

C-6 glial cells were cultured in DMEM medium with 10% fetal calf serum for 3 days. Then glial cell differentiation was induced by culture for 2 days in serum-free medium. Results of CNPase activity and protein assay are the means for three determinations.

Table 2
Fatty acid composition in C-6 glial cells cultured in serum-free medium with or without thioridazine

	% of total fatty acids	
	serum-free medium	serum-free medium with thioridazine
C14:0	2.1 ±0.2	4.2 ±0.4
C16:0	18.6 ±1.5	22.1 ±1.4
C16:1	6.8 ±0.8	6.1 ±0.4
C18:0	13.0 ±1.0	14.4 ±1.1
C18:0	40.4 ±2.4	33.0 ±2.5
C20:0	1.2 ±0.1	1.0 ±0.1
C20:4	2.2 ±0.2	2.9 ±0.2
C22:0	0.4 ±0.1	0.8 ±0.1
C22:6	1.3 ±0.4	1.5 ±0.3
C24:0	1.0 ±0.2	1.4 ±0.1
Others	13.7 ±2.4	18.6 ±1.5
A, sum of C14–20	93.7 ±0.6	90.0 ±1.1
B, sum of C22–24	4.23 ± 0.3	6.16 ± 0.25
B/A	0.045 ± 0.004 *	0.068 ± 0.003 *

C-6 glial cells were cultured in DMEM medium with 10% fetal calf serum for 3 days. To induce glial cell differentiation, cells were transferred to serum-free medium. Thioridazine was added to the serum-free medium. Fatty acid compositions in the total lipid extracts were examined by GLC.
* P,0.01.

pressed to half of the level in cells cultured with serum-free medium. The amount of protein in each flask measured concomitantly with CNP activity was unchanged, indicating that thioridazine does not affect cell proliferation. Since CNP is a marker enzyme for oligodendroglia, this result indicates that thioridazine suppresses the differentiation of C-6 glial cells. CNP activity was induced in serum-free media after transfer of cells to medium containing no thioridazine (data not shown), indicating that the effect of thioridazine on C-6 glial cell differentiation is reversible.

3.2. Fatty acid composition in C-6 glial cell

The relative concentration of longer chain fatty acids was increased by culture of the cells in the presence of 0.04 mM thioridazine (Table 2). The ratio of C22-C24/C14-C20, which was 0.045 ± 0.004 in the C-6 glial cells cultured in serum-free medium containing no thioridazine, increased to 0.068 ± 0.003 in those subsequently cultured in serum-free medium supplemented with thioridazine.

3.3. Concentration of total cellular cholesterol and phospholipids

Concentrations of total phospholipids and cholesterol in C-6 glial cells are shown in Table 3. Contents of phospholipids and cholesterol per mg of protein decreased upon transfer to serum-free medium. The concentrations of both cholesterol and phospholipids were increased in the cells cultured in the presence of 0.04 mM thioridazine. How-

Table 3
Effect of thioridazine on the ratio of phospholipids to cholesterol in C-6 glial cells

C-6 glial cells	PL (nmol/mg protein)	Chol (nmol/mg protein)	Ratio PL/Chol
FCS medium	57.1 ± 3.8	191 ± 2.4	0.30
FCS-free medium	44.6 ± 2.4	142 ± 5.7	0.31
FCS-free medium with thioridazine	72.6 ± 4.1	234 ± 9.0	0.31

Phospholipids (PL) and cholesterol (Chol) were quantitated as described in the text. Values are the means of three separate experiments.

Table 4
Fluorescent anisotropy values in the C-6 glial cell membrane treated with DPH

	Fluorescent anisotropy value				
	FCS medium	FCS-free medium			
Thioridazine (mM):	0	0	0.01	0.02	0.04
	0.190 ± 0.002	0.189 ± 0.002	0.174 ± 0.001 *	0.169 ± 0.005 * *	0.166 ± 0.003 *

2 ml of cell suspension in PBS (0.5 mg protein/ml) was added to 2 μl of DPH solution (190 mM) in a cuvette and the fluorescent anisotropy was measured after incubation at 37° C for 20 min. The values denote mean ± S.D. from four separate determinations. Values marked with asterisk and double asterisk differ from control (FCS medium without thioridazine) with P values of 0.01 and 0.02, respectively.

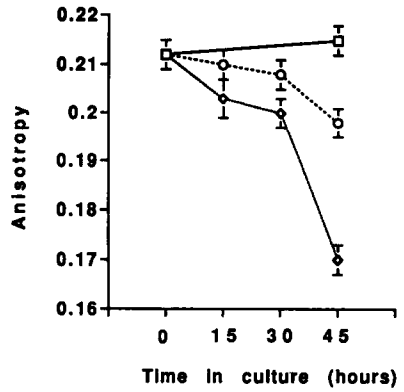

Fig. 1. The effect of 0.04 mM thioridazine added to serum-free medium (◇) on membrane fluidity of C-6 glial cells as a function of time. Membrane fluidity of cells cultured with serum-free medium (○) and medium containing 10% fetal calf serum (□) was also monitored by DPH fluorescence anisotropy. Results of anisotropy of each cell are the means for three determinations and the error bars represent S.D.

ever, no difference in the molar ratio of total phospholipid/cholesterol was observed among the cells cultured in serum-supplemented medium, and those cultured in serum-free medium with or without thioridazine.

3.4. Membrane fluidity

The effect of thioridazine on steady-state anisotropy values of DPH-labeled C-6 glial cells was determined after treatment of the cells with various concentrations of thioridazine as shown in Table 4. The anisotropy values for C-6 glial cells cultured with and without 10% fetal calf serum at 37° C were 0.190 ± 0.002 and 0.189 ± 0.002, respectively. When thioridazine was added to the serum-free medium, the anisotropy values decreased with increasing concentration of thioridazine. The steady-state anisotropy value for C-6 glial cells cultured in the presence of 0.04 mM thioridazine was 0.166 ± 0.003, which was a 12% reduction from that for the control cells. Thus, although the membrane fluidity was not changed upon transfer to serum-free medium, the addition of thioridazine to serum-free medium resulted in a significant increase of membrane fluidity compared with those cells cultured in fetal calf serum (FCS)-medium containing no thioridazine. The effect of thioridazine as a function of time is shown in Fig. 1. DPH anisotropy significantly decreased 2 days after the medium change with added 0.04 mM thioridazine.

4. Discussion

Several investigators have studied the possible relationship between the induction of CNP and lipid components of glial cells. Volpe and Obert demonstrated that one of the major cellular lipids, cholesterol was critical for manifestation of oligodendroglial differentiation in C-6 glial cells [21]. In a study on another major cellular lipid, phospholipid, CNP activity in C-6 glial cells was suppressed by incorporation of the choline analogue *N,N'*-dimethylethanolamine into cellular membrane [22]. Yavin et al., reported that membrane fluidity in cultured neuroblastoma cells increased by incubation of the cells with *N,N'*-dimethylethanolamine [23]. Based on these results, Volpe et al., suggested that an increase of membrane fluidity resulted in suppression of induction of CNP [22]. In the present study, we also demonstrated that the decreased CNP activity was closely correlated with the increased membrane fluidity and that the change in membrane fluidity of the cells cultured in the presence of thioridazine was due to the accumulation of relatively longer chain fatty acids in C-6 glial cells. Although an elevated levels of very long chain fatty acids should lower membrane fluidity, the increased membrane fluidity in our study might be related to the elevation of unsaturated fatty acids ($C > 20$). The effect of thioridazine on membrane fluidity was dependent on time of supplementation. In order to assess the direct effect of thioridazine on membrane fluidity measurement with DPH, homogenized C-6 glial cells grown in serum-free medium were mixed with thioridazine solution just before the anisotropy assay. The DPH-labelled anisotropy values (data not shown) increased indicating decreased membrane fluidity. Therefore, it was suggested that changes in membrane fluidity of C-6 glial cells cultured with thioridazine was not due to the direct perturbation of membrane but the alteration of membrane fatty acid composition via inhibition of peroxisomal function. In accordance with the results that incubation with thioridazine led to increased synthesis of phospholipids [24], the concentration of phospholipids in the cells cultured in serum-free medium with thioridazine increased to a level 1.6-times higher than that of cells cultured without thioridazine in the recent study. Simultaneously, the concentration of cholesterol increased in the cells upon culture with thioridazine, so that there was no difference in the ratios of cholesterol to phospholipids between the cells treated with or without thioridazine. Zubenko and Cohen examined the effect of thioridazine on the physical properties of platelet membrane and showed a significant increase in fluorescent polarization of DPH [25]. The effect of phenothiazines on the molecular dynamics of lipid bilayers is complex and might depend on the membrane system examined [26,27]. Therefore, the effect of phenothiazines on neural cells which are rich in lipid might differ from that on platelet membrane. In our study, thioridazine, a peroxisomal β-oxidation antagonist, affected C-6 glial cell differentiation, presumably through the changes in fatty acid composition and membrane fluidity. Although the changes which present in membrane properties are still unclear in peroxisomal disorders with the multiple metabolic abnormalities, it is implicated that cellular dysfunction in peroxisomal disorders might be relevant to the altered membrane fluidity in neural cells. Further study is necessary to understand the role of altered membrane fluidity in vivo.

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References

[1] Kelley, R. (1983) Am. J. Med. Genetics. 16, 503–517.
[2] Lazarow, P.B. and Moser, H.W. (1983) in The Metabolic Basis of Inherited Disease 5th edition (Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L. and Brown, M.S., eds.), pp. 1479–1509, McGraw-Hill, New York.
[3] Schutgens, R.B.H., Heymans, H.S.A., Wanders, R.J.A., Van den Bosch, H. and Tager, J.M. (1986) Eur. J. Pediatr. 144, 430–440.
[4] Brown III, F.R., McAdams, A.J., Cummins, J.W., Konkol, R., Singh, I., Moser, A.B. and Moser, H.W. (1982) Johns Hopkins Med. J. 151, 344–361.
[5] Danks, D.M., Tippett, B., Adams, C. and Campbell, P. (1975) J. Pediatr 86, 382–387.
[6] Heymans, H.S.A., Schutgens, R.B.H., Tan, R., Van den Bosch, H. and Borst, P. (1983) Nature 306, 69–70.
[7] Volpe, J.J. and Adams, R.D. (1972) Acta Neuropathol. 20, 175–198.
[8] Agamanolis, D.P., Robinson, H.B. and Timmons, G.D. (1976) J. Neuropathol. Exp. Neurol. 35, 226–246.
[9] Evrard, P., Caviness, V.S., Prats-Vinas, J. and Lyon, G. (1978) Acta Neuropathol. (Berl.) 41, 109–117.
[10] Maltese, W.A. and Volpe, J.J. (1979) J. Cell Physiol. 101, 459–470.
[11] Sprinkle, T.J., Zaruba, M.E. and Mckhann, G.M. (1978) J. Neurochem. 30, 309–314.
[12] Snyder, D.S., Zimmerman, T.R., Jr., Muhammad, Farooq, Norton, W.T. and Cammer, W. (1983) J. Neurochem. 40, 120–127
[13] Liao, C.L., Eng, L.F., Herman, M.M. and Bensch, K.G. (1978) J. Neurochem. 30, 1181–1186.
[14] Poduslo, S.E. and Norton, W. (1972) J. Neurochem. 19, 727–736.
[15] Leighton, F., Persico, R. and Necochea, C. (1984) Biochem. Biophys. Res. Commun. 120, 505–511.
[16] Branden, C.V.D. and Roels, F. (1985) FEBS Lett. 187, 331–333.
[17] Ames, B.N. (1966) Methods Enzymol. 7, 115–118.
[18] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[19] MacGee, J. and Allen, K.G. (1974) J. Chromatogr. 100, 35–42.
[20] Kawato, S., Kinosita, K. and Ikegami, A. (1977) Biochemistry 16, 2319–2324.
[21] Volpe, J.J. and Obert, K.A. (1983) J. Neurochem. 40, 530–537.
[22] Volpe, J.J., Iimori, Y., Haven, G.G. and Goldberg, R.I. (1986) J. Neurochem. 46, 475–482.
[23] Yavin, E. and Zutra, A. (1979) Biochim. Biophys. Acta 553, 427–437.
[24] Villarruel, M.C., Fernandez, G., De Ferreyra, E.C., De Fenos, O.M. and Castro, J.A. (1990) Toxicol. Lett. 51, 13–21.
[25] Zubenko, G.S. and Cohen, M. (1985) Psychopharmacolgy 86, 369–373.
[26] Seeman, P. (1972) Pharmacol Rev. 24, 583–655.
[27] Frenzel, J., Arnold, K. and Nuhn, P. (1978) Biochim. Biophys. Acta 507, 185–197.