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Peroxisomal Disease Cell Lines with Cellular Plasmalogen Deficiency Have Impaired Muscarinic Cholinergic Signal Transduction Activity and Amyloid Precursor Protein Secretion

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We tested whether alterations in membrane lipid composition associated with peroxisomal diseases affect muscarinic cholinergic signal transduction activity and amyloid precursor protein (APP) secretion in cultured human skin fibroblasts and Chinese hamster ovary (CHO) mutants. We found that in cell lines from patients with peroxisomal disorders where plasmalogen levels were low, the low-Km GTPase activity was not induced by carbachol, and APP secretion was reduced. This effect on signal transduction activity was not associated with decreased levels of the M1-muscarinic cholinergic receptor or its associated heterotrimeric G-protein. Specifically, this decrease was associated with a plasmalogen deficiency since a CHO cell line with only a deficit in plasmalogens was as severely affected as were generalized peroxisomal disorder cell lines. Thus, plasmalogens appear to be implicated in muscarinic cholinergic signal transduction and secretion of APP. These results provide new insights about the pathophysiology of peroxisomal diseases and may be relevant to Alzheimer's disease where reduced plasmalogen levels have been reported.

Key Words: **peroxisome; plasmalogen; amyloid precursor protein; signal transduction; muscarinic cholinergic receptor.**

Several lines of evidence suggest that peroxisomal dysfunction plays a role in the aging process (1, 2). Peroxisomal β-oxidation and catalase activity diminish in aging rodents (3, 4). The levels of docosahexaenoic acid (DHA or 22:6n-3), which requires the peroxisome for its synthesis (5), are diminished in the retina of aged rats (6). Moreover, the level of ethanolamine plasmalogen, which also requires the peroxisome for its synthesis (7), has been reported to be reduced in the brains of patients with Alzheimer's disease (AD) (8). Deficits in the cholinergic transmitter system in AD are well known and form the basis of current therapeutic regimens (9). Decrements in cholinergic signal transduction activity in aging and AD brain have been documented (10, 11) and include defective coupling of muscarinic cholinergic receptors to heterotrimeric G-proteins as assessed by carbachol-stimulated low-Km GTPase activity (a measure of G-protein-coupled muscarinic cholinergic transduction) (12) as well as accumulation of the β-amyloid peptide, a fragment of the amyloid precursor protein (APP) (11, 13). These changes in receptor signal transduction have been associated with changes in different membrane parameters: the sensitivity to free-radicals, the bilayer width (12), and lipid composition (14).

Genetically determined peroxisomal disorders are associated with marked alterations in the levels of DHA and plasmalogens, with patterns that vary with the gene defect (15). Cultured skin fibroblasts have proven of value in investigating the pathophysiology of both AD (16) and peroxisomal diseases (17). In this study, we report alterations in muscarinic cholinergic signal transduction activity and secretion of amyloid precursor protein in fibroblast cell lines of patients with four different types of peroxisomal disorders, as well as in CHO mutants, and suggest that plasmalogens are involved in both mechanisms.

MATERIAL AND METHODS

Chemicals. Carbachol was purchased from Sigma (St. Louis, MO) and [γ-^{32}P]GTP (30 Ci/mmol) from New England Nuclear (Boston,

0006-291X/98 $25.00

MA). An antibody against M1-AChR was purchased from Research and Diagnostic Antibodies (Berkeley, CA) and those against $G_{\alpha q/11}$ and $G_{\beta\gamma}$ from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against APP (clone 22C11) and a Complete protease inhibitor were obtained from Boehringer (Mannheim, Germany).

Cell culture. Human skin fibroblasts were grown in MEM (GIBCO, Gaithersburg, MD) supplemented with 10% FBS, 50 U penicillin/streptomycin, and 2 mM L-glutamine. We used normal (CTL), Zellweger syndrome (ZW), rhizomelic chondrodysplasia punctata (RCDP), bifunctional enzyme deficiency (BIF), and X-linked adrenoleukodystrophy (X-ALD) human skin fibroblast cell lines. RCDP phenotypes included cell lines deficient in PEX7, the receptor for Peroxisome Targeting Sequence 2 (18) and those deficient in dihydroxyacetone phosphate acyltransferase and dihydroxyacetone phosphate synthase.

Chinese hamster ovary (CHO) cells were grown in RPMI-1640 (GIBCO, Gaithersburg, MD) supplemented with 3% FBS, 50 U penicillin/streptomycin, and 2 mM L-glutamine. We used CHO-K1, a control cell line, CHO-ZR82, a peroxisome-deficient cell line, and CHO-NZel-1, a cell line with only a defect in plasmalogen synthesis (19).

To obtain optimal alterations in membrane lipid composition, human skin fibroblast cell lines were grown and harvested 2-3 days post-confluency, and CHO cell lines were harvested 24 hours post-confluency.

For analysis of APP secretion, the culture medium was changed after cells reached confluence. The cells were then incubated for 24 hours. Aliquots of the culture medium were collected and frozen until Western-blot analysis. After collection of the culture medium, the number of cells in each culture was determined. The amount of medium loaded for SDS-PAGE separation was normalized to represent the secretion activity per unit of cells (approx. 5300).

Preparation of crude membranes. Membranes were prepared at 0°C. Each cell line (fibroblasts or CHO) was washed three times with PBS. Cells were scraped off the dish with a rubber scraper into lysis buffer (10 mM Tris, 1 mM EDTA, 1 mM EGTA, Complete (1 pill per 50 ml buffer), pH 7.4). The cell suspension was passed through a 26-gauge needle three times, set aside on ice for 5 min, and centrifuged for 30 min at 70000 × g. The resulting pellet (crude membranes) was resuspended in PBS, and aliquots were frozen at −70°C until analysis.

Low-Km GTPase activity. For each crude membrane sample the basal and carbachol-induced low-Km GTPase was measured in duplicate or triplicate with 10 μg of protein per assay as previously described (14).

Immunoblot analysis. Each component required for muscarinic cholinergic low-Km GTPase activity was analyzed by immunoblotting with 3.65 μg of membrane protein solubilized and loaded on a gel for SDS-PAGE separation. After separation, proteins were transferred onto PVDF membranes, probed with antibodies directed against M1-AChR, $G_{\alpha q/11}$, or $G_{\beta\gamma}$ and the bands corresponding to these components were quantified.

The APP contents of the media (normalized to cellular content) and CHO cell membranes (normalized to protein content) were assayed by immunoblot analysis as described previously (20).

Lipid analysis. Membrane preparations were derivatized to fatty acid methyl esters and dimethylacetals (plasmalogens): 200 μl of acetyl chloride were added to 0.25 ml of aqueous sonicate of membranes in the presence of 1.0 ml of methanol:methylene chloride (3:1). The tubes were placed in a 75°C oven for 1 hour. After cooling, 4 ml of 7% potassium carbonate was added to each tube, followed by 2 ml of hexane, and the mixture was shaken vigorously. After a brief centrifugation, the hexane layer was collected, dried under nitrogen, and transferred in 100 μl of hexane for capillary gas chromatographic analysis. These samples were analyzed on two separate capillary columns, a 100-meter SP-2380 column (polar) (Supelco Inc., Bellefonte, PA) and a 50-meter Ultra1 column (nonpolar) (Hewlett Packard Co., San Fernando, CA). The tracings of each column were compared for fatty acid peak identification and averaged. Fifty-five different fatty acids and/or dimethylacetals were identified. Table 2 presents a subset of these identified fatty acids and dimethylacetals that are the most relevant lipids for this study.

TABLE 1

Basal Low-Km GTPase Activity and Percentage of Induction of the Low-Km GTPase Activity by Carbachol in Membranes of Normal and Peroxisomal Disease Human Skin Fibroblasts

	n	Basal low-Km GTPase act. ($pmol.min^{-1}.mg^{-1}_{prot}$)	Induction of basal GTPase by carbachol (in percent)
Control	5	23 ± 7.4	+11.4 ± 4.8
Zellweger	7	22.2 ± 8.0	−0.4 ± 6.9*
RCDP	7	22.3 ± 9.8	+0.9 ± 3.5**
BIF	3	23.4 ± 6.9	+6.3 ± 7.8
X-ALD	2	13.1	+10

Note. Values represent means ± standard deviation. Statistical analysis (bidirectional, unpaired t-test) compared peroxisomal disease values to controls: *p = 0.008, **p = 0.001. Carbachol induction in BIF samples was not significantly different from controls (p = 0.29).

Protein assay. Membrane protein content was determined by the Dc-protein assay kit (Bio-Rad, Hercules, CA) with albumin as a standard.

RESULTS

To determine whether peroxisomal disease cell lines, known to have defects in lipid metabolism and altered membrane lipid composition, had impaired muscarinic cholinergic signal transduction activity, we examined the carbachol-induced low-Km GTPase activity (Table 1) and found that ZW and RCDP cell lines had a statistically significant decrease in the carbachol induction of low-Km GTPase activity. BIF and X-ALD cell lines did not show any change. The X-ALD samples had a lower basal level of low-Km GTPase activity, the significance of which is currently unknown.

We checked whether this decrease in carbachol induction of the low-Km GTPase activity was due to a decrease in the components of the GTPase activity (M1-AChR, $G_{\alpha q/11}$, and $G_{\beta\gamma}$) in membranes (Fig. 1). None of the peroxisomal disease cell lines showed a decrease in any of these components.

Since muscarinic cholinergic signal transduction affects APP secretion we examined whether APP secretion was altered in peroxisomal disease cell lines (Fig. 2A). We found decreased APP secretion in the same cell lines (ZW and RCDP) that had a decreased carbachol-induced low-Km GTPase activity. Compared to normal (n = 3 or 4 samples), APP secretion was reduced in ZW and RCDP but not in BIF or X-ALD cell lines. Quantification of total APP-immunoreactivity showed a 35%

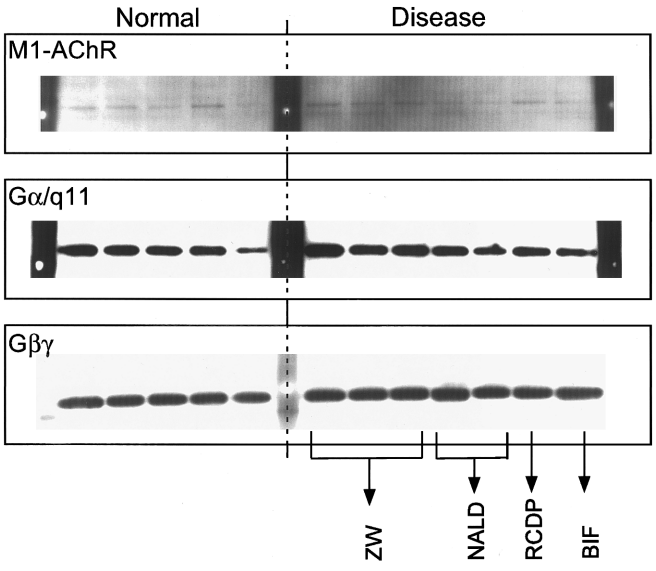

FIG. 1. Western-blot analysis of the components (M1-AChR, $G_{\alpha q/11}$, and $G_{\beta\gamma}$) involved in the low-Km GTPase activity in normal and peroxisomal disease human skin fibroblasts. ZW, Zellweger syndrome; NALD, neonatal adrenoleukodystrophy; RCDP, rhizomelic chondrodysplasia punctata; BIF, bifunctional enzyme deficiency.

reduction in secreted APP in Zellweger samples ($n = 4$, $p = 0.058$), a 50% decrease in RCDP samples ($n = 4$, $p = 0.038$), but no significant decrease in either BIF samples (50% increase, $n = 4$, $p = 0.10$) or X-ALD samples ($n = 3$, $p = 0.71$).

We next determined whether the changes in carbachol-induced low-Km GTPase activity and APP secretion were correlated with specific changes in lipid composition (Table 2). We found that only cell lines with lower plasmalogen levels (ZW, RCDP) had decreased muscarinic cholinergic signal transduction activity and APP secretion.

These diseases are characterized by multiple enzyme defects. In order to determine whether the changes in carbachol-induced low-Km GTPase activity and APP secretion were associated with a deficit in plasmalogens, we measured APP secretion in CHO-cell mutants with either a deficit in peroxisome biogenesis (CHO-ZR82) or an isolated deficit in plasmalogen synthesis (CHO-NZel-1) (Fig. 2B). Both CHO-ZR82 and CHO-NZel-1 had decreased levels of secreted APP (Figure 2B, upper panel), and decreased levels of plasmalogens (Table 2). We checked whether this decrease in APP secretion in plasmalogen-deficient cell lines was the result of decreased APP content in membranes (Fig. 2B). Whereas the plasmalogen-deficient CHO-NZel-1 had a membrane APP level similar to that in control (CHO-K1), the peroxisome-deficient CHO-KR82 had a higher membrane level than did the control CHO-K1 cell line.

DISCUSSION

This study supports the concept that alterations in membrane lipids influence membrane-receptor function (10). This influence appears to be lipid-specific since changes in VLCFA (in BIF and X-ALD samples) did not affect the carbachol induction of low-Km GTPase activity, whereas plasmalogen-deficient sam-

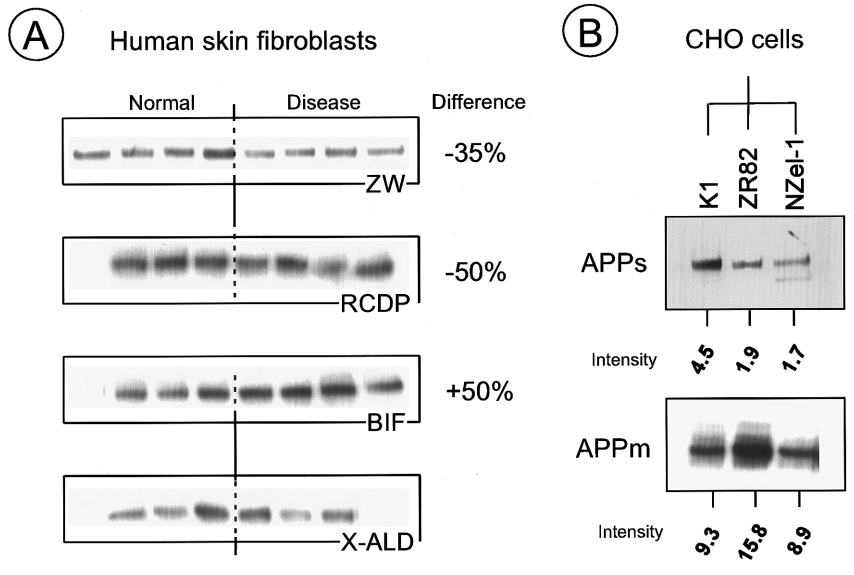

FIG. 2. Western-blot analysis of APP in human skin fibroblasts (A) and CHO cells (B). A, Analysis of the amount of APP secreted by human skin fibroblasts in normal (left) and diverse peroxisomal diseases (right). The difference in total APP immunoreactivity in disease samples is given as a percentage of increase/decrease compared to normal. B, Analysis of the amount of secreted-APP (APPs) and membrane-associated APP (APPm) in control (K1), peroxisome-deficient (ZR82), and plasmalogen-deficient (NZel-1) CHO cell lines.

TABLE 2

Membrane Lipid Composition in Normal and Peroxisomal Disease Human Skin Fibroblast Cell Lines and in Control (CHO-K1), Peroxisome-Deficient (CHO-ZR82), and Plasmalogen-Deficient Only (CHO-NZel-1) Chinese Hamster Ovary Cell lines (in Percent of Identified Fatty Acids ± Standard Deviation)

	Percent of identified fatty acids in cell lines							
	Human skin fibroblasts					Chinese hamster ovary cells		
	Control $n = 9$	Zellweger $n = 9$	RCDP $n = 4$	X-ALD $n = 4$	BIF $n = 4$	K1	ZR82	NZel-1
Plasmalogens								
16:0	2.15 ± 0.29	0.48 ± 0.21[c]	0.61 ± 0.13[c]	2.09 ± 0.38	2.22 ± 0.75	3.13	0.12	0.14
18:0	1.96 ± 0.31	0.29 ± 0.18[c]	0.43 ± 0.21[c]	1.97 ± 0.23	1.78 ± 0.55	1.62	0.06	0.07
18:1n-9	0.99 ± 0.31	0.21 ± 0.18[c]	0.30 ± 0.15[c]	1.13 ± 0.15	0.68 ± 0.32	0.44	0.02	0.04
Fatty acids								
22:0	1.05 ± 0.18	0.66 ± 0.20[b]	0.88 ± 0.23	0.95 ± 0.09	0.50 ± 0.15[b]	0.92	0.87	0.90
26:0	0.09 ± 0.02	0.50 ± 0.17[c]	0.05 ± 0.02[b]	0.31 ± 0.04[c]	0.51 ± 0.14[c]	0.03	0.37	0.09
20:4n-6	10.05 ± 2.44	6.70 ± 3.08[a]	10.35 ± 0.85	11.27 ± 0.90	9.40 ± 1.83	2.88	3.65	3.10
22:6n-3	3.32 ± 0.79	2.18 ± 0.72[b]	3.52 ± 0.48	3.36 ± 0.40	3.13 ± 0.74	0.94	1.15	1.04

* Significantly different from control values ([a], $p < 0.05$, [b], $p < 0.01$, [c], $p < 0.0001$, bidirectional, unpaired t-test).

ples (RCDP, ZW, CHO-ZR82, and CHO-NZel-1) showed no response to carbachol (Table 2). This change in low-Km GTPase activity in ZW and RCDP does not result from a decrease in the components of the mAChR signal transduction activity (M1-AChR, $G_{\alpha q/11}$, and $G_{\beta\gamma}$) since they were unaltered (Fig. 1).

The changes in lipid composition in peroxisomal disorders are disease-specific (Table 1). ZW affects DHA, plasmalogen, and VLCFA levels, RCDP decreases the plasmalogen level, and BIF and X-ALD show an increase in VLCFA only. The present results suggest that peroxisomal function is important for mAChR signal transduction and that plasmalogens significantly affect this process. APP processing is affected by muscarinic cholinergic signal transduction activity and stimulation by carbachol has been shown to enhance secretion of the physiological, neurotrophic soluble form of APP (21, 22). The present study shows that alterations in the muscarinic cholinergic signal transduction observed in plasmalogen-deficient cell lines (ZW and RCDP) are associated with a marked reduction in APP secretion (Fig. 2). Since ZW, RCDP and CHO-ZR82 cell lines are associated with multiple enzyme defects, we also tested APP secretion in the CHO-NZel-1 cell line, in which plasmalogen deficiency is the only defect. The finding that this cell line showed a reduction in APP secretion (Fig. 2B, upper panel) supports the hypothesis that the changes in carbachol induction of the low-Km GTPase activity and secreted APP in ZW and RCDP samples result from the decrease in cell plasmalogen levels. Moreover, the plasmalogen-deficient CHO-NZel-1 cell line showed a level of membrane-associated APP similar to that in control, suggesting that APP secretion in this cell line is also the result of altered membrane lipid composition. The findings in the CHO-ZR82 cell line are of interest since in addition to a decrease in APP secretion, APP accumulates within membranes. This could result from an additional alteration in intracellular APP processing secondary to the other alterations in lipid composition (VLCFA, DHA) associated with the absence of peroxisomes.

Plasmalogens are components of all cell membranes and are present at high concentration in the heart and nervous system (23). Their function is still not understood. It has been suggested that they protect cells against reactive oxygen species (24) and facilitate membrane fusion (25, 26). The multiple roles of one plasmalogen, platelet activating factor (PAF), have been reviewed extensively (27). Plasmalogens have been shown to be involved in signal transduction (28) and PAF has been shown to play a role in long-term potentiation (29) and neuronal migration (30). The present results suggest that plasmalogens affect muscarinic cholinergic signal transduction and APP processing. This is intriguing since a decrease in brain plasmalogen content was recently reported in patients with AD (8) where muscarinic cholinergic signal transduction and abnormal APP processing are altered (10, 11). A potential role for a membrane defect in AD has been proposed previously (31). The study of peroxisomal disorder cell lines may shed new light on the understanding of complex alterations affecting the nervous system in aging and in aging-related neurodegenerative diseases such as AD.

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