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Catalytic hydrogenation of fatty acyl chains in plasma membranes; effect on membrane lipid fluidity and expression of cell surface antigens

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Optimal reaction conditions were established for hydrogenation of plasma membranes of living murine GRSL leukemia cells, using the water-soluble catalyst $Pd(QS)_2$ (QS, sulphonated alizarine; $C_{14}H_6O_7NaS$). Under these conditions more than 80% of the cells remained viable. Analysis by gas chromatography revealed that hydrogenation occurred predominantly in the 18:2, 20:4 and 22:6 fatty acyl chains of the membrane phospholipids. Under the same conditions hydrogenation was also performed in purified plasma membranes from GRSL cells and from rat liver, and in liposomes prepared from the total lipid extracts of these membranes. Hydrogenation increased the lipid structural order parameter in the membranes, as measured by fluorescence polarization. This increase was more pronounced in the liposomes (46%) than in the plasma membranes (17–25%). Hydrogenation increased the expression of a 15 kDa antigen on the surface of viable GRSL cells, as measured in a Fluorescence Activated Cell Sorter, using monoclonal antibodies. The expression of four other antigens, among which H-2^k, was not or much less affected by this treatment.

Introduction

The lipid fluidity of biological membranes has been shown by many studies to influence a variety of membrane functions, such as the activity of membrane-associated enzymes, transport activities and the expression of cell-surface antigens and receptors (reviewed in Refs. 1–3). To demonstrate such effects at a physiological temperature, the lipid composition of membranes or cells has to be modulated, for instance by introducing alterations in the cholesterol content or in the degree of unsaturation of the phospholipid acyl chains [3,4]. The former type of alterations have often been brought about by treatment of cells with cholesteryl hemisuccinate, which has been reported to increase immunogenicity [5,6] of tumor cells and to decrease the expression of H-2 antigens on the cell surface [7]. We considered it worthwhile to examine whether similar effects can be obtained when the membrane fluidity is altered

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by another principle, that is, without introducing foreign molecules in the membrane. Homogeneous catalytic hydrogenation which was pioneered by Chapman and Quinn [8] is a fundamentally different approach to decrease membrane fluidity. By this technique double bonds of fatty acyl moieties are being removed, whereas otherwise the preexisting composition of the membrane is retained.

Recently a homogeneous catalytic hydrogenation procedure was developed, utilizing a water-soluble catalyst, a palladium(II) sulphonated alizarine complex ($Pd(QS)_2$) [9,10] which in principle should be less toxic to cells than the previous used rhodium catalysts [11], since it can be rapidly removed from the reaction mixture by washing. Our purpose in the present study was to test this new catalyst on lymphoid cells, finding conditions where maximal hydrogenation is obtained with minimal cell death, and to see whether this treatment affected the expression of various cell surface antigens. Furthermore, in view of the conflicting results that have been reported as to whether a certain change in the level of fatty acyl unsaturation may lead to a change in physically measured membrane fluidity [3,12], we have investigated the effect of hydrogenation of purified plasma membranes and liposomes prepared from them, by gas chromatographic analysis of the fatty acids in relation to the membrane fluidity as measured by fluorescence polarization with the probe 1,6-diphenyl-1,3,5-hexatriene [13].

Materials and Methods

Membrane preparation and lipid analysis

Plasma membranes were purified from rat (R/A) liver [15] and murine leukemic GRSL cells [14,16] according to standard methods in our laboratory. Briefly, livers were homogenized in 1 mM $NaHCO_3$ (pH 7.5), using a Potter-Elvehjem homogenizer. After several low-speed differential centrifugations, plasma membranes were purified by flotation in a discontinuous sucrose gradient at the *d* 1.16/1.18 interface [15]. GRSL ascites cells were disrupted by pumping a suspension of $5 \cdot 10^7$ cells/ml in Hanks' balanced salt solution (Oxoid, London, U.K.) through an air-driven cell disruptor (Stansted Fluid Power Limited, Stansted, Essex, U.K.; model AO 612, disrupting valve 516) using an air pressure of 45 lb/inch^2. The GRSL plasma membranes were purified from (1.95–945) $\cdot 10^4$ g $\cdot$ min pellets of the cell homogenate, utilizing a discontinuous sucrose gradient, as described in detail before [16]. The purity of the plasma membrane preparations was ascertained routinely by electron microscopy and by marker assays, as described previously [15,16]. Total lipids were extracted from these membrane preparations and from whole cells by chloroform/methanol (2:1, v/v), followed by Folch's partition [17]. Phospholipids were isolated by standard silicic acid column chromatography in the methanol eluate. The fatty acid composition of the total phospholipids was analysed, after transesterification with H_2SO_4/methanol, by capillary gas-liquid chromatography on an open tubular glass column, 25 m $\times$ 0.21 mm, wall-coated with Silar 5 CP, as described in detail [14].

Hydrogenation procedure

Aliquots (10–50 ml) of murine GRSL cells, adjusted to a concentration of $1 \cdot 10^6$ cells/ml in Hanks' medium containing 1.2% glucose and 3.5% polyvinylpyrrolidon (M_r 40 000; Sigma), were placed in high-pressure siliconized glass vessels and transferred to a thermostated (20°C) water bath. The reaction vessels were connected to a manifold and the gas phase was evacuated and replaced by 1.5 atm hydrogen or oxygen-free nitrogen (control). The catalyst used for the hydrogenation was palladium-di-(sodium alizarine monosulphonate), abbreviated as $Pd(QS)_2$. It was prepared by Dr F. Joó using methods described elsewhere [18]. A stock solution containing 5 mg catalyst per ml of glass distilled, deionized and degassed water was prepared and aliquots were injected into the cell suspension through a silicon rubber septum to initiate the reaction. The final catalyst concentration in the reaction medium was 100 μg/ml. The gas pressure was increased to 2 atm and the reaction vessels were rotated at 45 rev./min for the duration of the hydrogenation (45 min). At the end of the incubation period the gas pressure was reduced slowly over a period of 5 min to avoid the formation of gas bubbles in the medium. After having established the optimal hydrogenation conditions for living GRSL cells, isolated plasma membranes from GRSL cells and

from rat liver, and of liposomes of the extracted total lipids of these membranes were subjected to the same hydrogenation procedure, at a membrane concentration corresponding to 70 nmol phospholipid per ml reaction mixture. Finally, the cells, membranes or liposomes were spun down and washed twice with fresh assay medium to remove the catalyst.

Estimation of membrane lipid fluidity

The fluorescent hydrocarbon 1,6-diphenyl-1,3,5-hexatriene (DPH) was used as a probe for measuring the degree of lipid fluidity in plasma membrane preparations and liposomes prepared from them or from the whole cells, by steady-state fluorescence polarization (P_{DPH}). The measurements were performed at 25°C with an Elscint apparatus, model MV-1A (Elscint Ltd., Haifa, Israël) as described previously [16].

P_{DPH} values mainly reflect the orientational constraint of the motions of the probe, and these values, or rather the r_s values (steady-state fluorescence anisotropy) can be quantitatively converted into lipid order parameters, S_{DPH}, using semi-empirical relationships [13,19]. Membrane lipid fluidity may be defined as the reciprocal of the lipid order parameter [13]. In the present paper we used for calculation of S_{DPH} the equations: $r_\infty = (4\ r_s/3) - 0.10$ (valid for the region $0.13 < r_s < 0.28$ or $0.18 < P_{DPH} < 0.37$) and $(S_{DPH})^2 = r_\infty/r_0$, in which r_∞ represents the limiting hindered fluorescence anisotropy, and $r_0 = 0.4$ is the theoretically maximal fluorescence anisotropy [13].

Immunofluorescence of cell surface antigens

The following rat monoclonal antibodies were used (diluted 1/10) to measure the expression of antigens on the surface of murine GRSL cells: 30-H11, detecting a glycolipid antigen on GRSL cells [20], has been prepared and described by Ledbetter and Herzenberg [21]; I42/5 detects a glycoprotein of 95 kDa, denominated Pgp-1 by Trowbridge et al. [22]; 120C2, raised against GRSL plasma membranes, detects a 15 kDa protein which is also present on normal mouse lymphocytes and bone marrow cells (kindly provided by H. Haisma in our Institute); 78H3B5F12 (kindly provided by R. Nusse in our Institute) was raised against the mouse mammary tumor viral envelope component gp52 and detects the viral precursor molecule $Pr73^{env}$, also called MLr antigen [16,23], on the surface of GRSL cells. Monoclonal antibodies from hybridoma clone H100-5/28 against the $H\text{-}2^k$ haplotype (determinant H-2.m3; Ref. 24) were purchased from Camon Labor-Service GmbH (Wiesbaden, F.R.G.); it was used in a dilution of 1/100. As secondary antibodies we used fluoresceinated (FITC-conjugated) goat IgG against rat or mouse IgG, purchased from Nordic Immunological Labs. (Tilburg, The Netherlands), in a dilution of 1/20.

The cells were first incubated with the monoclonal antibodies for 30 min, then washed twice with Hanks' balanced salt solution, incubated with the second antibodies and washed again twice. All treatments were done at 4°C.

Flow microfluorometry was carried out with a Fluorescence-Activated Cell Sorter (FACS IV; Becton-Dickinson) in which the cells were excited by an argon laser (488 nm) and fluorescence emission was measured at 520 to 550 nm. The fluorescence signal is given in channel numbers, which are a logarithmic function of its intensity. Cells were light scatter-gated for live cells [7].

Results and Discussion

Optimal reaction conditions were established ror homogeneous catalytic hydrogenation of the fatty acids in living GRSL leukemia cells. Under these conditions, specified under Methods, at least 80% of the cells remained viable, as judged by trypan blue exclusion. The additions of 1.2% glucose and 3.5% polyvinylpyrrolidon to the hydrogenation medium ($Pd(QS)_2$ containing Hanks' balanced salt solution) were found to be beneficial for cell viability. Bovine serum albumin was also good in this respect, but was found to inhibit hydrogenation significantly. The extent of hydrogenation of double bonds in the fatty acids of the phospholipids was analyzed by gas-liquid chromatography. The results are shown in Table I. Hydrogenation predominantly occurred in the polyunsaturated fatty acids 18 : 2, 20 : 4 and 22 : 6, resulting in a reduction of the double bond index in the cellular phospholipids of about 40%.

Having thus established the optimal hydrogena-

tion conditions for living cells, we then subjected purified plasma membranes from these GRSL cells and liposomes prepared from their lipid extracts to the same hydrogenation procedure. Table I shows that also in these cases the polyunsaturated fatty acids become hydrogenated, to an even greater extent than in whole cells. This is understandable because the catalyst may have no or less access to the membranes of the interior of the cell, which contain the highest amounts of polyunsaturated fatty acids. In fact, evidence of selective catalytic hydrogenation of a peripherally located membrane has been reported for alga cells, where the cytoplasmic membrane lipids become hydrogenated immediately, but the inner thylakoid membranes only at much later stage [25].

Hydrogenation was also performed under the same conditions on a second type of plasma membrane, i.e. that purified from rat liver (Table II). Also in this case, hydrogenation occurred in the 18 : 2, 20 : 4 and 22 : 6 fatty acyl chains, and thereby the double bond index in these membranes was reduced 60%, somewhat less than in the GRSL plasma membranes (67%; see Table I). Liposomes prepared from liver membrane lipids exhibited the same susceptibility to hydrogenation as the native membranes, whereas liposomes from GRSL membranes were somewhat more susceptible. In the latter liposomes the double bond index was reduced by 75% upon catalytic hydrogenation. It appears that membranes or liposomes with a higher degree of fluidity (see below) are more susceptible to hydrogenation, possibly due to facilitated intercalation of the $Pd(QS)_2$ catalyst in membrane lipid chains that are more loosely packed (less ordered; more fluid). It should be noted that the higher fluidity of GRSL plasma membranes as compared to liver plasma membranes is mainly due to their lower cholesterol and sphingomyelin contents, rather than to a difference in fatty acid composition [13–16]. In a different experimental system, i.e. chloroplasts, the extent of catalytic hydrogenation of membrane lipids was found to increase in the order of the number of double bonds per lipid molecules present [26].

The structural order (reciprocal of membrane fluidity; Ref. 13) in dispersed lipid extracts of treated or untreated cells, and in treated or untreated plasma membranes or their liposomes, was determined by fluorescence polarization with the probe diphenylhexatriene (P_{DPH}) (Table III). In

TABLE I

EFFECT OF CATALYTIC HYDROGENATION OF THE PHOSPHOLIPIDS IN LIVING GRSL LEUKEMIA CELLS, THEIR ISOLATED PLASMA MEMBRANES, AND OF LIPOSOMES OF THE EXTRACTED TOTAL LIPIDS FROM THESE MEMBRANES

Data are representatives of three experiments with essentially similar results. d.b.i. (double bond index) = mean of double bonds per fatty acid.

Fatty acids	Whole GRSL cells		Plasma membranes		Liposomes	
	control	H_2 + cat.	control	H_2 + cat.	control	H_2 + cat.
16:0	20.8	20.5	22.5	22.0	19.3	20.4
16:1	1.7	1.4	3.8	4.5	3.2	3.4
18:0	24.5	33.1	17.5	28.2	19.4	32.7
18:1	17.9	16.5	15.8	14.3	15.5	10.9
18:2	19.9	14.3	13.0	3.2	12.8	3.0
20:0	–	3.4	–	6.4	–	8.6
20:1	–	1.2	–	3.2	–	2.6
20:4	10.0	4.8	8.5	1.1	10.0	0.5
22:0	–	1.6	–	2.4	–	2.8
22:6	3.5	1.5	2.6	–	2.7	–
d.b.i.	1.22	0.77	1.14	0.38	1.21	0.29

TABLE II

EFFECT OF CATALYTIC HYDROGENATION OF PURIFIED PLASMA MEMBRANES FROM RAT (R/A) LIVER AND LIPOSOMES OF THE EXTRACTED TOTAL LIPIDS OF THESE MEMBRANES [a]

Fatty acids	Rat liver plasma membranes			
	Native plasma membranes		Liposomes	
	control	H_2 + cat.	control	H_2 + cat.
16:0	24.5	26.0	22.0	22.1
16:1	4.9	5.1	5.2	4.1
18:0	18.4	21.0	17.4	24.4
18:1	13.1	17.5	14.0	15.7
18:2	14.3	4.2	15.1	7.9
20:0	–	6.8	–	7.4
20:1	–	2.6	–	2.5
20:4	9.6	1.0	9.2	1.2
22:0	–	3.0	–	2.7
22:6	4.5	1.1	4.4	0.7
d.b.i. [a]	1.25	0.50	1.29	0.53

[a] See legend of Table I.

TABLE III

CATALYTIC HYDROGENATION OF LIVING GRSL LEUKEMIA CELLS, ISOLATED PLASMA MEMBRANES FROM GRSL CELLS AND FROM RAT (R/A) LIVER, AND OF LIPOSOMES OF THE EXTRACTED TOTAL LIPIDS OF THESE MEMBRANES; EFFECT ON FLUORESCENCE POLARIZATION (P_{DPH}) AND LIPID ORDER PARAMETER (S_{DPH}) AT 25°C

Data for whole cells refer to a diphenylhexatriene (DPH)-labelled sonicate in phosphate-buffered saline of the total lipids extracted from the untreated or treated cells, respectively, thus eliminating effects of proteins and of residual traces of the catalyst itself (partitioning in the water phase). In the catalyst-treated membranes or liposomes some catalyst (coloured) still remained, after washing, intercalated in the phospholipids and as such affected the P_{DPH} value.

	P_{DPH}			S_{DPH}		
	untreated	cat. + N_2	cat. + H_2	untreated	cat. + N_2	cat. + H_2
GRSL leukemia cells						
whole cells	0.175	0.180	0.191	0.41	0.42	0.45
plasma membranes	0.276	0.289	0.387	0.65	0.68	0.85
liposomes	0.227	0.248	0.400	0.54	0.59	0.87
Rat (R/A) liver						
plasma membranes	0.291	0.309	0.382	0.68	0.72	0.84
liposomes	0.244	0.247	0.394	0.58	0.59	0.86

all membrane and liposome cases there was a small but clear effect of the catalyst by itself, with N_2 instead of H_2 (non-hydrogenating conditions), indicating that some catalyst remained intercalated in the membranes, even after extensive washing (see also table IV). However, the specific hydrogenation effect itself, by which extensive saturation of fatty acids occurred (Tables I, II), was much larger. The structural order parameters (S_{DPH}), calculated from the P_{DPH} values, were increased 17% and 25% in hydrogenated plasma membranes from liver and GRSL cells, respectively (Table III). Hydrogenation of the liposomes of these membranes resulted in 45% augmented S_{DPH} values. The difference in this hydrogenation effect between plasma membranes and their liposomes may be attributed to intrinsic membrane proteins, which by themselves may impose some structural order upon the apolar regions of the lipid bilayer [13,27]. The structural order parameter of membrane lipids has been shown to increase exponentially with rigid intrinsic membrane molecules, such as cholesterol or certain proteins, towards a maximal plateau value [27,28]. In the present study the values reached in the membranes and liposomes by catalytic hydrogenation are all very similar ($0.382 \leqslant P_{DPH} \leqslant 0.400$; $0.84 \leqslant S_{DPH} \leqslant 0.87$) and very close to the maximum values that can be obtained experimentally ($P_{DPH} = 0.425$, $S_{DPH} = 0.90$) [27,28].

The expression of a number of cell surface antigens was estimated by membrane immunofluorescence in the fluorescence-activated cell sorter (FACS), using monoclonal antibodies against the

TABLE IV

CATALYST EFFECT ON THE FLUORESCENCE POLARIZATION (P_{DPH}) VALUE AFTER CATALYTIC HYDROGENATION OF ISOLATED PLASMA MEMBRANES OF GRSL LEUKEMIA CELLS, DEMONSTRATED BY COMPARISON OF THE WHOLE MEMBRANES WITH LIPOSOMES OF THEIR EXTRACTED TOTAL LIPIDS [a]

Treatment of plasma membranes	P_{DPH} (at 25°C)	
	plasma membranes directly	liposomes of total lipids [b]
Untreated control	0.276	0.271
$Pd(QS)_2$ only	0.290	0.273
$Pd(QS)_2/N_2$ pressure	0.295	0.268
$Pd(QS)_2/H_2$ pressure	0.340	0.318

[a] In this particular experiment catalytic hydrogenation reduced the fatty acids 18:2 and 20:4 from 17% to 11.5%, and from 5.4% to 1.4%, respectively.

[b] Lipids were extracted from the membranes after the various catalyst treatments. The liposomes were devoid of catalyst (were uncoloured).

antigens and secondary ('sandwich') antibodies which were conjugated to a fluorescent label (FITC). Fig. 1 shows that the monoclonal reagent 120C2, detecting a 15 kDa surface protein (the function of which is unknown yet), is exposed at a higher level on hydrogenated cells. The increase in fluorescence intensity per cell was in this case estimated to be 4-fold relative to the non-hydrogenated cells. Whether this increase represents previously unexposed (hidden) antigens or whether it is due to some lateral rearrangement leading to more of less clustering of the antigens, is not clear. Fig. 1 further shows that the expression of two other antigens, i.e. a H-2^k antigen and a glycolipid antigen (reacting with 30H11) is hardly affected. Also two other surface antigens (see Methods) were not altered in expression by hydrogenation (not shown). It should be noted that the viability of the cells, being at least 80% after hydrogenation, could sometimes decrease to a level as low as 40% due to the subsequent manipulations (incubations with antibodies, washings etc.). The fluorescence measured in the fluorescence-activated cell sorter was, however, in all cases light scatter-gated for live cells [7].

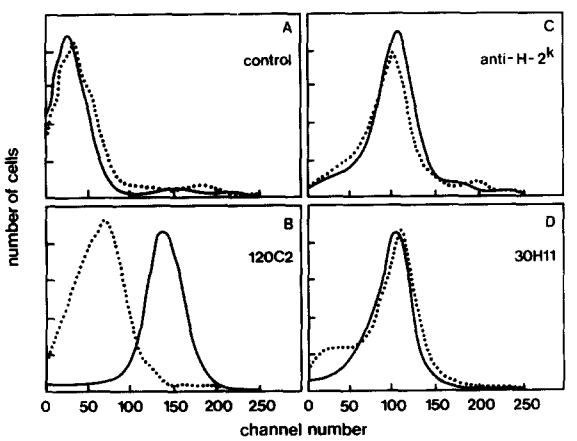

Fig. 1. Relative expression of cell surface antigens on GRSL leukemic cells as measured by the membrane immunofluorescence intensity (channel number) in the fluorescence-activated cell sorter (FACS IV). The profiles shown are representatives of three experiments with similar results. Cells were pretreated with $Pd(QS)_2$ under N_2 (dotted line) or under hydrogenation conditions (H_2; solid line). Primary monoclonal antibodies were (A) an immunoglobulin unrelated to the GRSL cell surface (control), (B) 120C2, (C) H100-5/28 against H-2^k, (D) 30H11.

In conclusion, we have demonstrated that it is feasible to reduce the lipid fluidity of cellular membranes to a great extent by homogeneous catalytic hydrogenation. This provides an alternative for the membrane rigidization by incorporation of cholesterol or its hydrophilic esters, a treatment that has been shown to increase immunogenicity [5,6,29] and to alter the exposition of various cell surface determinants [7,29]. However, in this respect the two approaches of membrane rigidization may not yield similar results, since cholesteryl hemisuccinate treatment reportedly decreased the expression of histocompatibility (H-2) determinants [7], whereas we have presently shown that catalytic hydrogenation leaves the H-2 expression unaltered. One should thus be cautious in proposing causal relationships between the expression of cell surface antigens and a bulk physical property such as membrane fluidity.

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