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Cultured human skin fibroblasts modify their plasma membrane lipid composition and fluidity according to growth temperature suggesting homeoviscous adaptation at hypothermic (30°C) but not at hyperthermic (40°C) temperatures *

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Mammalian cell metabolism is responding to changes in temperature. Body temperature is regulated around 37°C, but temperatures of exposed skin areas may vary between 20°C and 40°C for extended periods of time without apparent disturbance of adequate cellular functions. Cellular membrane functions are depending from temperatures but also from their lipid environment, which is a major component of membrane fluidity. Temperature-induced changes of membrane fluidity may be counterbalanced by adaptive modification of membrane lipids. Temperature-dependent changes of whole cell- and of purified membrane lipids and possible homeoviscous adaptation of membrane fluidity have been studied in human skin fibroblasts cultured at 30°C, 37°C and 40°C for ten days. Membrane anisotropy was measured by polarized fluorescence spectroscopy using TMA-DPH for superficial and DPH for deeper membrane layers. Human fibroblasts were able to adapt themselves to hypothermic temperatures (30°C) by modifying the fluidity of the deeper apolar regions of the plasma membranes as reported by changes of fluorescence anisotropy due to appropriate changes of their plasma membrane lipid composition. This could not be shown for the whole cells. At 40°C growth temperature, adaptive changes of the membrane lipid composition, except for some changes in fatty acid compositions, were not seen. Independent from the changes of the membrane lipid composition, the fluorescence anisotropy of the more superficial membrane layers (TMA-DPH) increased in cells growing at 30°C and decreased in cells growing at 40°C.

Introduction

Mammalian cell-metabolism is responding to environmental factors. A main factor of the physical influences is temperature. Body temperature is regulated within a rather narrow range [1], although in areas specially exposed to heat and cold, such as the skin, the temperature may vary to a greater extent. Skin temperatures between 20°C and 40°C may be tolerated for extended periods and are compatible with adequate cellular function [2].

Membrane enzyme activities are modulated both by their lipid environment and by temperature. Temperature-induced enhancement or reduction in enzyme activities may be counterbalanced by adaptive changes in the lipid environment of these enzymes. Changes may consist of variations in cholesterol (Chol) and total phospholipid (PL) content, in the relative distribution of individual PL and in the degree of saturation of fatty acids [3]. Membrane lipid composition defines membrane fluidity or microviscosity which in turn is responsible for complex cellular functions such as cell division, endocytosis or phagocytosis [4–9]. Fluidity is a biophysical property of the membrane expressing the

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amplitude and the rate of rotational motions of lipid molecules. It is influenced by intrinsic factors within the cell membranes such as the contents of cholesterol, phospholipids and proteins [10,11], as well as by extrinsic factors such as pH value, calcium and salt concentrations [12]. Temperature-dependent changes in membrane lipids and possible homeoviscous adaptation of membrane fluidity can be studied preferably in cultured cells using different growth temperatures. Adaptation to thermal stimuli has been demonstrated in hamster ovary cells grown at 40°C instead of 37°C [10,13].

In the present paper we investigated the effects of growth temperature on lipid composition of whole cultured human skin fibroblasts and of a purified plasma membrane fraction thereof as well as on fluorescence anisotropy as a measure for membrane fluidity. The markers used were TMA-DPH (trimethylaminodiphenylhexatriene) for the superficial layers and DPH (diphenylhexatriene) for the hydrophobic layers of the membranes [14]. The experiments showed that human fibroblasts may adapt, up to a certain degree, to lower ambient growth temperatures (30°C) by modifying plasma membrane lipid compositions and membrane fluidity as reported by fluorescence anisotropy (DPH), but only insufficiently to elevated growth temperatures (40°C).

Materials and Methods

Tissue culture

Fibroblast cultures used were from stocks of frozen cells of healthy donors. Cells were cultured as previously described [15]. After trypsinization cells were seeded at $5 \cdot 10^4$ cells/cm^2 and were grown at 37°C for 24 h to about confluence and then maintained for 9 days at 30°C, 37°C or 40°C, respectively. Temperatures were kept constant within 0.5°C. Cells were fed every 3 days by changing the culture medium. The culture medium consisted of Eagle's minimal essential medium (MEM) with 10% of fetal calf serum, the pH value was kept at 7.4 with 2 g/l of bicarbonate and 5% CO_2 in air. At day 10 cells were harvested by trypsinization, washed, pelleted and stored frozen at −20°C until analyses. Simultaneously living fibroblast cultures grown on glass coverslips under the same conditions were used for fluorescence anisotropy measurements [12].

Isolation of plasma membranes

The isolation of plasma membranes was achieved by a modification of the method of Scott [16]. Cells were grown to confluence in 175 cm^2 Falcon culture flasks. The cell monolayers of 10 flasks were washed with phosphate-buffered saline (PBS) and subsequently incubated with 25 mM formaldehyde and 2 mM dithiothreitol in Ca^{2+} and Mg^{2+} containing PBS at 37°C for 90 min [17]. The plasma membrane vesicles (PMV) formed were washed away from the cells three times with 10 ml of isotonic (140 mM NaCl) Tris-HCl buffer (10 mM), pH 7.4, containing 0.1% of bovine serum albumine (BSA). Detached cells and cellular debris were separated from the vesicles by centrifugation ($500 \times g$). The PMV were pelleted at $48000 \times g$, collected, washed, and recentrifuged in hypotonic Tris buffer to remove soluble proteins from the lysed PMV [18]. The final pellet was stored at −20°C prior to analyses.

Lipid analyses

Frozen cell pellets or PMV-pellets were suspended in 1 ml of saline solution and sonicated three times for 5 s at 50 W (Branson Sonic Power, Danbury, CT, USA). The following analyses were directly performed in the sonicated cell or membrane suspensions. Total PL were fluorimetrically determined according to the method of Jouanel et al. [19], using a lecithin standard from bovine brain. Total cholesterol was determined enzymatically with a test kit based on a colorimetric enzymatic method (Boehringer GmbH, Mannheim, FRG) and proteins were measured with the BCA protein assay reagent (Pierce Chemical Company, Rockford, IL, USA). DNA was analyzed according to Hinegardner et al. using bovine thymus DNA as a standard [20]. Total cellular lipids were extracted from whole cells or PMV homogenates according to Folch et al. [21]. Fatty acid methyl esters were prepared in a one-step transesterification reaction of all classes of lipids and analyzed by gas-liquid chromatography according to a method by Lepage and Roy [22]. Aliquots of the lipid extracts were applied to silica gel-HPTLC plates (No. 5641, Merck, Darmstadt, FRG). The plates were developed with a solvent mixture of chloroform, methyl acetate, propanol-2, methanol and KCl 0.74% in water (25:25:25:10:9, by vol.) for 30 min [23], dried (5 min at 180°C) and subsequently cooled for 5 min. Spots corresponding to individual PL were visualized by immersing the plates into Cu(II) acetate (0.5 g/100 ml ethanol) for 10 s and into H_3PO_4 (20.7 ml of 85% H_3PO_4 in 220 ml methanol), for another 10 s. After heating (6 min at 180°C) the PL-spots were quantified by reflection densitometry (Camag TLC scanner I, Muttenz, Switzerland) according to Kolarovic and Traitler (personal communication).

Fluorescence anisotropy of fibroblast monolayers

Cells were seeded in Petri dishes (60 mm diameter) containing two glass coverslips (40×11 mm) at a density of $5 \cdot 10^4$ cells/cm^2. Temperature adapted cultures were analyzed 10 days after subcultivation. Cultures were prepared for polarization measurements by washing the coverslips in Hank's solution (37°C and pH 7.4). The coverslips were placed in upright position into

quartz glass cuvettes (Hellma SA, Switzerland) containing 5 μM TMA-DPH or 2 μM DPH, respectively, in 3 ml Hank's solution (37°C and pH 7.4) as previously described [12]. Special coverslip holders allowing only one geometrical configuration positioned the cell containing surface towards the incident light at an angle near 30°. Fluorescence anisotropy was determined with a Shimadzu RF-540 spectrofluoro-photometer (L-format), equipped with a constant temperature four cell holder and two remote controlled rotating polarizers, which avoided the necessity to open the sample compartment. Fluorescence intensities were determined at four positions of the polarizers and anisotropy rG was calculated as follows (Eqn. 1).

$$rG = \frac{I_{vv} - G \cdot I_{vh}}{I_{vv} + 2 \cdot G \cdot I_{vh}} \quad (1)$$

$$G = \frac{I_{hv}}{I_{hh}}$$

rG, corrected fluorescence anisotropy; G, correction factor for the optical system (Shimadzu RF-540); $I_{vv(vh)}$, vertical excitation, vertical (horizontal) emission polarizer; $I_{hh(hv)}$, horizontal excitation, horizontal (vertical) emission polarizer; excitation wavelength, 360 nm; emission wavelength, 430 nm. Slits: excitation 5 nm; emission 20 nm.

Statistical analyses

Student's t-test was routinely performed. Significance levels used were $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***), not significant (ns.).

Materials and chemicals

Glass coverslips were obtained from Assistent (Altnau, Switzerland), MEM dry powdered media with Earle's salt were purchased from Seromed (Munich, FRG). Fetal calf serum was from Boehringer (Mannheim, FRG). Penicillin G was from Gist/Brokades (Delft, Holland). TMA-DPH was obtained from Molecular Probes, Inc. (Eugene, OR, USA) and kept as 2 mM ethanolic stock solution in the dark (at 4°C), DPH, also from Molecular Probes, was dissolved in tetrahydrofurane and also kept as 2 mM stock solution. All other chemicals, solutions and solvents were of analytical purity grade and were purchased from Merck (Darmstadt, FRG).

Results

Cell growth

At 30°C fibroblasts grew at a slightly lower rate than cells at 37°C while at 40°C growth was slightly higher than at 37°C. Cell growth was determined by measuring DNA and protein contents per plate (Figs. 1 and 2). At either temperature fibroblasts appeared morphologically normal.

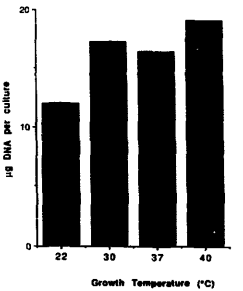

Fig. 1. Cell growth (DNA) at different growth temperatures.

Temperature-induced changes in the lipid compositions

(A) in whole cells

Table 1 shows the cholesterol and total PL contents and the PL compositions of whole cells grown at different temperatures. Compared to 37°C cholesterol contents per protein and Chol./PL ratios were reduced in cells grown at 30°C as well as in cells grown at 40°C. These reductions were statistically significant only in cells grown at 40°C ($P < 0.05$). PL-contents per protein were not changed. No significant changes were found among the individual PL, however, the relative contents of SP were decreased and thus the PC/SP ratios were significantly increased in cells grown at 30°C. In cells grown at 40°C the relative amount of PI was increased and that of PS was decreased.

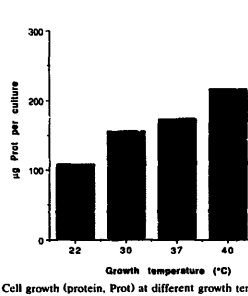

Fig. 2. Cell growth (protein, Prot) at different growth temperatures.

TABLE I

Lipid contents of whole cells and of purified plasma membrane vesicles

Results are from four different individual experiments. Significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (control 37°C) Student's *t*-test.

	Growth temperature					
	whole cells			plasma membranes		
	30°C	37°C	40°C	30°C	37°C	40°C
Chol/Prot (μg/mg)	29.2± 2.7	31.7± 1.2	28.3± 1.7 *	113 ± 16 *	168 ±40	164 ± 28
PL/Prot (μg/mg)	403 ±38	417 ±53	414 ±42	907 ±101 **	1044 ±70	1090 ±150
Chol/PL (μg/mg)	72.7± 8.8	77.3± 12.1	68.2± 8.0 *	125 ± 31 **	162 ±44	153 ± 40
SP (%)	7.0± 0.2 *	7.5± 0.9	7.0± 0.4	12.3± 2.2	13.8± 1.4	14.1± 1.0
PC (%)	45.6± 1.6	45.0± 1.7	45.4± 2.4	46.0± 3.2	43.7± 7.2	43.4± 3.0
PS (%)	5.6± 0.7	5.3± 0.7	4.7± 0.2 *	6.0± 0.4	5.9± 0.8	6.1± 1.2
PI (%)	4.0± 0.4	3.8± 0.4	4.3± 0.4 *	2.4± 0.1	2.5± 0.2	2.3± 0.2
PA (%)	1.9± 0.4	2.0± 0.7	1.8± 0.4	2.2± 0.4	2.4± 0.8	2.5± 0.6
PE (%)	36.1± 1.1	36.1± 1.5	36.8± 2.7	31.1± 1.6	31.8± 4.2	31.7± 0.6
PC/PE	1.26	1.25	1.22	1.49	1.41	1.37
PC/SP	6.7 *	6.0	6.5	3.74 *	3.17	3.01 *

(B) in plasma membranes

In plasma membranes of cells grown at 30°C PL ($P < 0.01$) and cholesterol contents ($P < 0.05$) were significantly lower than in membrane preparations of cells grown at 37°C and 40°C (Table I). The cholesterol/PL ratios were lower in cells grown at 30°C than in control cells ($P < 0.01$). Lipid contents and PL-composition were similar in cells grown at 37°C or 40°C. The relative increases in PC contents of cells grown at 30°C were accompanied by a decrease in the SP-contents (Table I) resulting in significant change of the PC/SP ratio at 30°C and 40°C.

(C) in comparison between plasma membranes and whole cells

Lipid contents and compositions of individual PL in purified plasma membranes prepared by vesiculation of intact cells greatly differed from those in whole cells. When compared to whole cells, plasma membranes showed a 3-fold enrichment of PL and a 6-fold increase in cholesterol. The relative proportions of SP, PS and PA were increased, PC was about the same whereas PI and PE were relatively decreased in plasma membranes. Table II summarizes the ratios between cholesterol, PL and individual PL of whole cells and of plasma membranes at the three growth temperatures. The ratios were generally smaller at 30°C, and somewhat higher at 40°C compared to 37°C growth temperature.

TABLE II

Ratios of lipid compositions (membrane vesicles / whole cells, w / w)

Values represent mean ratios of results from Table I of four different experiments, individual PL calculated as μg/mg protein

	Growth temperature		
	30°C	37°C	40°C
Chol/Prot	3.9	5.4	5.8
PL/Prot	2.3	2.5	2.6
SP	4.0	4.5	5.3
PC	2.3	2.4	2.5
PS	2.4	2.8	3.3
PI	1.4	1.5	1.4
PA	2.1	3.1	3.9
PE	1.9	2.2	2.3

(D) fatty acid composition of total cellular lipids

The major fatty acids of total lipids of whole cells grown at different temperatures are shown in Table

TABLE III

Relative proportions of fatty acids in whole cell lipids

The results of fatty acid analyses were normalized by setting the ratio between the internal standard (13:0) and 18.0 as 1.0. All other ratios were corrected according to 18:0.

Growth temperatures	Ratios of fatty acids				
	14:0	16:0	18:0	18:1	20:4
30°C	0.115 ± 0.015	0.780 ± 0.06	1.0	0.420 ± 0.055	0.170 ± 0.045
37°C	0.124 ± 0.003	0.850 ± 0.015	1.0	0.470 ± 0.002	0.140 ± 0.018
40°C	0.103 ± 0.003	0.745 ± 0.012	1.0	0.395 ± 0.008	0.101 ± 0.004

TABLE IV

Fluorescence anisotropy values of cell monolayers (DPH)

Cells were grown at 30, 37 or 40°C and measured at either temperature with 2 μM DPH in Hank's solution. Results shown were measured at the respective growth temperatures. Otherwise the results from different growth temperatures (30 and 40°C) were measured at 37°C and compared to those of cells grown at 37°C. Results represent the means ± SD of at least 14 different coverslips from four individual experiments. Significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (control 37°C).

Measurement	Growth temperature			
	30°C		37°C	40°C
30°C	*0.168 ± 0.019*	n.s.	0.180 ± 0.011	0.169 ± 0.013
37°C	0.159 ± 0.018 **		*0.176 ± 0.010*	0.170 ± 0.020 n.s.
40°C	0.154 ± 0.019		0.165 ± 0.010	*0.166 ± 0.013* **

III. The fatty acid compositions are expressed in relation to 18:0 which was arbitrarily set at 1.0. Compared with fatty acid composition in lipids of cells grown at 37°C there was an increase in the relative proportions of 20:4 and a slight decrease of 14:0 in lipids of cells grown at 30°C, while in cells grown at 40°C there was a relative decrease of 20:4 and of 18:1 but also of 14:0.

Fluorescence polarization measurements in whole cells

Fluorescence anisotropy values were measured with DPH as fluorescent probe in whole cells grown at different temperatures (Table IV). Cells grown at 30°C exhibited significantly lower fluorescence anisotropy values than those of cells grown at higher temperatures when measured at 37°C. The values measured at 30°C, however, were similar to those of cells grown and measured at 37°C. This indicated the existence of homeoviscous adaptation. Values in cells grown at 40°C and measured at 37°C were not significantly different from control cells, but when fluorescence anisotropy was measured at 40°C the cells showed a significant decrease in anisotropy compared to cells grown and measured at 37°C.

Fluorescence anisotropy values analyzed with TMA-DPH as the fluorescent probe in monolayers of cells grown at different temperatures were considerably higher than those measured with DPH. When measured at 37°C cells grown at 30°C were not different in their anisotropy values from cells grown at 37°C, while cells grown at 40°C exhibited statistically significant lower fluorescence anisotropy values. When fluorescence anisotropy was measured at the same temperature as the cells were previously grown, cells at 30°C showed a significantly higher than normal anisotropy, while cells grown at 40°C exhibited a significantly lower fluorescence anisotropy compared to cells grown and measured at 37°C but identical to that measured at 40°C (Table V).

TABLE V

Fluorescence anisotropy values of cell monolayers (TMA-DPH)

Cells were grown at 30, 37 or 40°C and measured at either temperature with 5 μM TMA-DPH in Hank's solution. Results printed in bold letters show the statistical analyses of fluorescence anisotropy measured at the respective growth temperatures. Otherwise the results from different growth temperatures (30 and 40°C) were measured at 37°C and compared to those of 37°C growth temperature. Results represent the means ± S.D. of at least 14 different coverslips from four individual experiments. Significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (control 37°C).

Measurement	Growth temperature		
	30°C	37°C	40°C
30°C	*0.373 ± 0.015* ***	0.354 ± 0.016	0.366 ± 0.016
37°C	0.337 ± 0.617 n.s.	*0.345 ± 0.012*	0.326 ± 0.010 ***
40°C	0.351 ± 0.014	0.334 ± 0.013	*0.334 ± 0.011* **

Discussion

Changes in fluidity of membranes in response to ambient temperatures of longer duration are known as homeoviscous cellular adaptations. Relevant changes in body temperature are found in hibernating animals [24], in fish adapting to ambient water temperature [25,26], in areas of human skin which are not covered and in male gonades which are only functioning at temperatures lower than 37°C. In procaryotes [25] and in some poikilotherm animals [27] such adaptation occurs in respect to changes in membrane lipid composition. Comparatively little is known about temperature adaptation of human cells in culture.

Cultures of human skin fibroblasts are usually grown at 37°C but continue to grow at temperatures between 30°C and 40°C and even higher. However, cells stop growing at temperatures below 28°C. Even at room temperature (22°C) these cells can stay alive for an extended period of time (unpublished results).

Between 28°C and 40°C cellular membrane lipids are in their liquid crystalline state. Some of the proteins embedded in that lipid environment undergo conformational changes both around 30°C and 40°C [28]. Above 30°C lipid transitions are stabilizing protein functions [29]. Temperatures higher than 40°C *in vitro* and *in vivo* will damage cells and organisms because of protein modifications (heat shock proteins). Cells may, however, acquire thermotolerance within a short period of about 12 h which may protect the cells against further heat exposures for a period of several days [30].

Living cells tend to keep their vital cell functions constant. It has been shown that cells were able to adapt to temperature-induced changes in cell membrane fluidity by altering the lipid composition [31].

Preliminary experiments with cultures had shown that temperature induced changes of fluorescence

anisotropy did not occur before 3 days at either temperature.

In this paper we studied whole cell and membrane lipid composition of human fibroblast monolayers after a growth period of 10 days at temperatures between 30°C and 40°C. In parallel, fluorescence anisotropy was determined, which is a measure for membrane fluidity to which it is inversely related.

Unsaturated fatty acids and increases in PC contents of the membranes are fluidizing, increases in SP, PE, cholesterol and protein contents are rigidifying membrane lipid bilayers. Thus changes in lipid fluidity can in part be expressed in terms of changes in PC/SP or Chol/PL ratios [6].

Expecting homeoviscous adaptation the fluidizing parameters should be prevalent in cells grown at 30°C. Indeed in whole cells cholesterol and SP-contents as well as the Chol/PL ratio decreased and concomitantly the PC/SP ratio increased when compared to cells grown at 37°C. In purified plasma membranes thereof cholesterol, PL/protein and Chol/PL ratios were decreased while PC contents, and PC/PE as well as PC/SP ratios increased. These findings are consistent with a fluidizing effect of the lipid composition. Anisotropy values (DPH) in cells grown at 30°C and measured at 37°C were significantly lower than those in cells grown and measured at 37°C. On the other hand anisotropy values in cells grown and measured at 30°C were similar to those in control cells (grown and measured at 37°C). These findings indicate a compensatory mechanism and are compatible with homeoviscous adaptation through changes in membrane lipid composition. Adaptive changes were also observed in the fatty acid composition of whole cell lipids insofar that the relative amounts of polyunsaturated fatty acids (20:4) were increasing at the expense of saturated fatty acids (C_{14}). Such changes have previously been found in cold temperature adapted goldfish [26].

Correspondingly cells grown at elevated temperatures might be expected to adapt by rigidifying their membranes. Such increases in fluorescence anisotropy could be brought about by increases in PE, cholesterol and SP contents or decreases in PC contents. Neither lipid compositions of whole cells and of isolated membranes nor anisotropy data (DPH) of cells grown at 40°C were in favour of such hypothesis. Fatty acid composition of whole cell lipids showed a relative reduction of both 14:0 saturated as well as of 18:1 and 20:4 nonsaturated fatty acids suggesting a relative increase in 18:0. This kind of change could at least in part be compatible with a rigidification of membranes of whole cells although lipid composition and fluorescence anisotropy measurements indicated a fluidizing rather than a rigidifying effect of increased growth temperature on cell membranes. Therefore, fibroblasts seem not to be able to compensate for the effects of exposure to higher temperatures, except by some changes in fatty acid composition. This is at variance with the findings of Anderson et al. in cultured CHO cells [32]. These cells showed a positive correlation between growth temperature, Chol/PL ratio and fluorescence anisotropy values (DPH) also at elevated growth temperature.

TMA-DPH is located in membranes near the polar head groups of the PL, close to the water/lipid interface. Thus it is a fluorescent probe for the outer and more superficial layers. Its anisotropy is always higher than that of DPH, indicating that it characterizes a membrane zone with a higher degree of order. Cholesterol content has little effect on fluorescence anisotropy measured by TMA-DPH, while it affects fluorescence anisotropy measured with DPH [33]. This would indicate that TMA-DPH is less sensitive to the degree of packing at the membrane surface than to changes in molecular organization. Our anisotropy data with TMA-DPH were rather unexpected. Cells grown and measured at 30°C exhibited even higher fluoresence anisotropies and those grown and measured at 40°C showed lower values than those grown and measured at 37°C (control cells). This is in contrast to the observed homeoviscous adaptation of cells grown at 30°C in the deeper membrane layers (DPH) although it could also be a compensatory mechanism.

Concluding from our results human skin fibroblasts are able to a homeoviscous adaptation of the deeper apolar regions of the plasma membranes when grown at 30°C but not when grown at 40°C. In cells growing at 40°C neither total cellular lipids (except for some changes in fatty acid composition) nor membrane lipids showed significant changes in respect to cells grown at 37°C.

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