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Changes of fatty acid composition of phospholipids and lipid structural order in rat liver mitochondrial membrane subsequent to galactosamine intoxication. Effect of clofibrate

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Since it has been earlier reported that D-galactosamine induces an inhibition of palmitoylcarnitine transferase I and a depletion of mitochondrial phospholipids which were both prevented by clofibrate, an evaluation of the effects of these drugs on mitochondrial fatty acid composition was made. Galactosamine does not alter the fatty acid pattern of these fatty acids whereas clofibrate induces a 2-fold increase in monounsaturated / saturated fatty acids ratio and a 10-fold decrease of the 20:4 ($n-6$) / 20:3 ($n-6$) ratio in phosphatidylcholine. These alterations suggest an increase of Δ^9-desaturation and a decrease of Δ^5-desaturation. To determine whether the drug-induced changes in mitochondrial phospholipids has an effect on the physical properties of the membrane, the lipid structural order of mitochondrial preparations was studied using the lipophilic probes DPH and TMA-DPH. Mitochondria isolated either from galactosamine- or clofibrate-treated rats showed a decrease in fluorescence polarization, indicating an overall decrease in lipid structural order. This alteration is more drastic when both drugs are administered. This phenomenon suggests drastic changes in the bulk phase of inner mitochondrial membrane lipids after treatments and could explain the altered kinetic properties of palmitoylcarnitine transferase I.

Introduction

We have previously reported that a D-galactosamine injection induces a decrease in membrane-bound palmitoylcarnitine transferase I activity correlated to a depletion of phospholipids in the mitochondrial membrane [1]. Clofibrate appeared able to prevent both the inhibition of palmitoylcarnitine transferase I activity and the depletion of phospholipids in the mitochondrial membrane. Besides, both treatments decrease the sensitivity of the enzyme to malonyl-CoA, its physiological inhibitor. This effect was particularly drastic when the two agents were administered since in these conditions the I_{50} of malonyl-CoA (the concentration required for 50% of maximal inhibition) was increased 4-fold as was the K_m of the enzyme for palmitoyl-CoA. This point is of interest since it has been reported elsewhere [2] that the sensitivity of the enzyme to its inhibitor required membrane integrity. Particular interest was devoted to the diphosphatidylglycerol (DPG), a specific phospholipid of the inner mitochondrial membrane [3]. Indeed, galactosa-

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mine induced a drastic decrease of this compound whereas clofibrate feeding resulted in an enhanced biosynthesis of DPG. Attempts were made to correlate the latter effect with the clofibrate-induced proliferation of mitochondria [4] since it has been proposed by Tzagoloff [5] that mitochondria increase in mass and number by a process of accretion and integration of newly synthesized material into preexisting organelles.

It has been reported for many membrane-bound enzymes, that not only polar head groups of phospholipids but also their fatty acid composition could alter the enzymatic activities via a modulation of physical properties of the membrane. Thus, it appears of interest to investigate whether galactosamine and clofibrate could alter the fatty acid composition of phospholipids and whether or not the variations of DPG levels in the membrane altered the membrane lipid structural order since a rigidifying effect of this compound has been evidenced in biological membranes [6].

Experimental procedure

Animals

The rats (200–230 g) were male Wistar rats. They received an ordinary laboratory diet ad libitum. They were starved overnight before killing and given water ad libitum. Clofibrate (0.2 mmol/100 g body wt. per day) was given orally as corn oil solution in 1 ml for 4 successive days (clofibrate group). Control rats received the vehicle only. D-Galactosamine-HCl neutralyzed at pH 7.4 was administered in 0.2 ml saline (0.28 mmol/100 g body wt.) intraperitoneally 3 h before killing either to corn oil-fed rats (galactosamine group) or to clofibrate-fed rats (clofibrate + galactosamine group). All experiments were started between 9.30 and 10.00 a.m.

Chemicals

Sources of chemicals were as described by Sire et al. [7]. Clofibrate (2-(4-chlorophenoxy)-2-methylpropionic acid ethyl ester) was a gift from I.C.I. Pharma, Pontoise, France. TMA-DPH was obtained from Molecular Probes, Junction City, OR. U.S.A. DPH from Aldrich, Beerse, Belgium.

Isolation of mitochondria

Liver mitochondria were isolated by differential centrifugation as described earlier [7]. Protein was measured by the method of Lowry et al. [8].

Isolation of inner mitochondrial membrane. The inner mitochondrial membrane plus matrix was isolated by treatment with digitonin followed by differential centrifugation according to Schnaitman and Greenawalt [9]. This method was recommended by Colbeau et al. [10] for studies focused on this membrane since the particle obtained by the digitonin method appear to give purer inner membrane fractions than Parson's method [11] by which the outer membrane is largely detached from the inner mitochondrial membrane by swelling in hypotonic phosphate buffer. Briefly, digitonin, in 0.25 M sucrose, is added to a cold suspension of mitochondria in 0.25 M sucrose (25 μg of digitonin per mg protein, final concentration 0.25%, w/v) and kept for 10 min at 0°C without stirring. The action of digitonin is stopped by dilution with 0.25 M sucrose and rapid centrifugation of the mitochondria.

Marker enzymes. The marker enzymes were succinate dehydrogenase (EC 1.3.99.1) for the inner mitochondrial membrane, kynurenine 3-hydroxylase (EC 1.14.1.2) for the outer mitochondrial membrane and glucose-6-phosphatase for endoplasmic reticulum. Succinate dehydrogenase was assayed according to Pennington [12] using *p*-iodonitroneotetrazolium violet (INT) as artificial acceptor of hydrogen. The reduction of INT is followed at 490 nm. Kynurenine 3-hydroxylase activity is determined spectrophotometrically by the method of Hayashi [13] in which the disappearance of NADPH in presence of L-kynurenine is measured at 340 nm. Glucose-6-phosphatase activity is measured by the method of Baginski et al. [14]. The inorganic phosphate liberated is determined with ammonium molybdate; ascorbic acid is used as the reducing agent. Excess molybdate is bound with an arsenic citrate solution, so that it can no longer react with other phosphate esters or with P_i formed by acid hydrolysis of the substrate. The specific activities and recovery of these markers are displayed in Table I. It appears that only 2% of outer membrane fraction is still present in mitoplasts as deduced from kynurenine 3-hydroxylase activity.

TABLE I

PURIFICATION OF INNER MITOCHONDRIAL MEMBRANE

Specific activities are expressed as nmol/min per mg protein for succinate dehydrogenase and kynurenine 3-hydroxylase and as μmol/min mg protein for glucose-6-phosphatase.

	Succinate dehydrogenase		Kynurenine 3-hydroxylase		Glucose 6-phosphatase	
	total act.	spec. act.	total act.	spec. act.	total act.	spec. act.
Homogenate	100%	57± 9	100%	1.8±0.3	100%	1.3±0.2
Mitochondria	20.1%	138± 7	35.0%	9.0±0.7	3.9%	0.7±0.1
Mitoplasts	17.7%	186±15	2.2%	0.6±0.1	2.6%	0.6±0.1

Fluorescence polarization

Mitochondria prepared from each group were labeled with either 1-(4-(trimethylamino)phenyl)-6-phenylhexa-1,3,5-triene (TMA-DPH) or 1,6-diphenylhexa-1,3,5-triene (DPH) according to the method of Hitzemann and Harris [15]. Mitochondrial samples were diluted with phosphate buffer (pH 7.4) to a concentration of 0.1–0.2 mg protein/ml and temperature was equilibrated at 37°C. DPH or TMA-DPH in tetrahydrofuran was then added in order to get a molar ratio of probe/phospholipids equal to 1 : 300. The incubations were performed under continuous stirring for 60 min. Fluorescence intensity was monitored using a T-format SLM 8000 (Urbana, U.S.A.) polarization fluorescence spectrophotometer. Fluorescence polarization measurements were performed at 25°C on the TMA-DPH- and DPH-labeled mitochondria according to the procedures of Shinitzky and Barenholz [16]. The excitation and emission wavelengths were 360 and 430 nm, respectively. The fluorescence polarization (P) was calculated as follows: $(I'' - I')/(I'' + I')$ where I'' and I' are the parallel and perpendicular components of the fluorescence intensity, respectively. For each sample, at least six successive determinations were made and the final value of P was the mean of the six determinations. P values reflect mainly the orientational constraint of the probe, and these values, or better the R_s values (steady-state fluorescence anisotropy calculated as follows: $R_s = 2P/(3 - P)$), can be quantitatively converted into lipid order parameter, S, using a semi-empirical relationship. In the present paper we used for calculation of S(DPH) the equation $R_\infty = (4R_s/3) - 0.10$ valid for the region $0.13 < R_s < 0.28$ [17] in which R_∞ represents the limiting hindered fluorescence anisotropy. Since this relation was quite specific of DPH, Van der Meer et al. [18] have determined an extended Perrin equation which applied not only to DPH, but to other fluorophores as well. Therefore the relation $R_\infty = R_0 R_s^2/(R_0 R_s + (R_0 - R_s)^2/m)$ was used for the calculation of S(TMA-DPH) with $R_0 = 0.390$ [19] and $m = 1.19$ [18]. The latter parameter expresses the difference between the rotational diffusion of the probe in the membrane and that in the isotropic reference oil. The lipid order parameter, S, was then calculated with the relation $S = \sqrt{R_\infty/R_0}$ where R_0 is the theoretically maximal fluorescence anisotropy.

Isolation and purification of phospholipids

The phospholipids were extracted according to Folch et al. [20]. Phospholipids were purified from the total lipid extract by separation from neutral lipids on fluorescent silica gel preadsorbent thin-layer chromatographic plates (20 cm × 20 cm) obtained from Whatman with acetone/light petroleum (1 : 3, by vol.). The phospholipids were separated from each other by a subsequent two-dimensional TLC with chloroform/methanol/25% aq. NH_3 (65 : 25 : 4, by vol.) followed by chloroform/acetone/methanol/acetic acid/water (30 : 40 : 10 : 10 : 1, by vol.) as solvents systems. After development of the plates, the spots were outlined under ultraviolet light and scrapped off. The phospholipids were eluted from the gel using 1,2-dichloromethane/methanol (1 : 2, by vol.). Inorganic phosphorus was assayed according to Chen et al.

[21] after the lipids were oxidized with 70% $HClO_4$ (w/v).

To obtain the fatty acid methyl esters, phospholipids were methanolyzed (0.5 M methanolic KOH, 5 min, 60°C) and the methylation was performed with methanol in presence of Boretrifluorite. The resulting methylesters were extracted with hexane.

Gas-liquid chromatography

The GLC was performed with a Girdel serie 30 gas chromatograph equipped with a flame ionization detector. The 25-m long capillary column was filled with 20 M carbowax and the carrier gas was helium. The fatty acid methyl esters were analysed isothermally at 200°C and identified by comparison of retention times with a mixture of standard methyl esters (Supelco).

Results

Phospholipid composition of rat liver mitochondria

The effects of clofibrate and galactosamine on the phospholipid composition of the mitochondrial membrane are displayed in Table II. Since phosphatidylcholine (PC), phosphatidylethanolamine (PE) and diphosphatidylglycerol (DPG) represent at least 90% of total phospholipid content of the mitochondrial membrane [3], the present study was focused on these three classes. These results confirm the drastic decrease of the phospholipid content which occur in this membrane after galactosamine administration [1]. The stimulating effect of clofibrate on DPG biosynthesis is also confirmed as well as its preventing effect on galactosamine-induced phospholipid depletion as it appears from total phosphorus assay. However, galactosamine-induced DPG decrease still occurs when clofibrate pretreatment is performed since the mitochondrial content of this phospholipid is decreased by approx. 50% when compared to the clofibrate group.

Fatty acid pattern of rat liver mitochondrial phospholipids

Effect of galactosamine and clofibrate on PC fatty acid pattern. The fatty acid pattern of rat liver mitochondrial PC is displayed in Table III. The major fatty acids in this class were palmitic (21%), stearic (21%), linoleic (13%) and arachidonic (31%) acids. It appears that galactosamine injection results in no change of PC fatty acid pattern whereas clofibrate feeding induces deep alterations in this pattern. Indeed, palmitic, oleic and dihomo-γ-linolenic acids amounts are all increased while those of stearic and arachidonic acids are decreased. These alterations result in a 2-fold increase of the monounsaturated/saturated fatty acids ratio which suggest that clofibrate feeding induces a stimulation of Δ^9 desaturase. Besides, the C20 : 4 $(n-6)$/C20 : 3$(n-6)$ ratio appears to be 10-fold decreased suggesting that a marked inhibition of the Δ^5 desaturase occurs. As a consequence of these alterations, the unsaturation index decreases from 189 for control to 146 for clofibrate-treated rats. Galactosamine failed to induce any modification in the mitochondrial fatty acid pattern of PC, the fatty acid pattern resulting

TABLE II

CHANGES IN PHOSPHOLIPIDS IN INTACT LIVER MITOCHONDRIA

PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol. Each value (nmol phosphorus per mg protein) represents the mean ± S.E. from three different preparations. [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$.

Phospholipid	Treatments			
	control	clofibrate	galactosamine	clofibrate + galactosamine
PC	56 ± 4	62 ± 2	45 ± 4 [a]	61 ± 3
PE	54 ± 3	47 ± 3	40 ± 2 [b]	54 ± 2
DPG	32 ± 2	50 ± 2 [c]	21 ± 2 [b]	24 ± 4 [a]
Total phosphorus	154 ± 7	167 ± 9	119 ± 12 [a]	157 ± 6

TABLE III

FATTY ACID COMPOSITION OF PHOSPHATIDYLCHOLINE IN INTACT RAT LIVER MITOCHONDRIA

Figures are mole percentages of fatty acid methyl esters and means from duplicate. M.I., monounsaturated fatty acids; Satd., saturated fatty acids.

Fatty acid	Treatments			
	control	galactosamine	clofibrate	clofibrate + galactosamine
14:0	0.4	0.4	0.5	0.2
16:0	21.0	22.1	30.9	28.2
16:1	0.5	0.7	0.5	2.1
18:0	21.3	20.6	10.8	9.6
18:1($n-9$)	4.2	5.1	12.6	12.0
18:1($n-7$)	2.4	2.1	1.7	1.8
18:2($n-6$)	12.8	15.1	21.5	20.1
18:3($n-6$)	0.2	0.2	–	1.0
20:3($n-9$)	0.3	0.3	1.0	0.3
20:3($n-6$)	0.7	0.6	3.6	3.9
20:4($n-6$)	30.9	28.4	15.2	18.0
22:6($n-3$)	4.9	4.3	2.3	3.0
M.I/Satd.	0.17	0.18	0.35	0.42
20:4/20:3	44.1	47.3	4.22	4.62

TABLE IV

FATTY ACID COMPOSITION OF PHOSPHATIDYLETHANOLAMINE IN INTACT RAT LIVER MITOCHONDRIA

Figures are mole percentages of fatty acid methyl esters and means from duplicate. M.I., monounsaturated fatty acids; Satd., saturated fatty acids.

Fatty acid	Treatments			
	control	galactosamine	clofibrate	clofibrate + galactosamine
14:0	0.5	0.3	0.3	0.2
16:0	14.5	13.4	16.7	15.4
16:1	0.6	2.1	0.7	0.3
18:0	27.1	27.2	23.5	22.6
18:1($n-9$)	3.1	2.7	4.5	4.8
18:1($n-7$)	2.1	2.4	2.0	1.4
18:2($n-6$)	6.3	9.4	13.2	10.0
18:3($n-6$)	–	–	–	–
20:3($n-9$)	–	–	–	–
20:3($n-6$)	0.4	0.3	1.3	1.5
20:4($n-6$)	31.1	28.6	30.0	33.7
22:6($n-6$)	13.2	12.4	7.2	9.5
M.I/Satd.	0.14	0.18	0.18	0.17
20:4/20:3	77.7	95.3	23.1	22.5

from the effect of both compounds reflects mainly the effect of clofibrate itself and the same alterations of the monounsaturated/saturated fatty acids and C20:4($n-6$)/C20:3($n-6$) ratios are observed.

Effect of galactosamine and clofibrate on PE fatty acid pattern. The fatty acid pattern of rat liver mitochondrial PE is displayed in Table IV. The major fatty acids in this class were palmitic (14%), stearic (27%), arachidonic (31%) and cervonic (13%) acids. As it was observed in PC, no change in the fatty acid pattern of PE occurs subsequent to galactosamine injection except a 3-fold increase in palmitoleic acid, a minor component of PE fatty acids. The effects of clofibrate on PE fatty acid pattern appear to be less drastic than in PC. However, the amount of linoleic acid is increased 2-fold whereas the docosahexaenoic acid level is decreased by 50%. No change occurs in the arachidonic acid level as it was the case in PC and the 70% decrease of the C20:4($n-6$)/C20:3($n-6$) ratio results mainly from the increase of homo-γ-linolenic acid amount. The effects resulting from the action of both compounds are weaker than in PC and, unsurprisingly, reflect those of clofibrate itself.

Effect of galactosamine and clofibrate on DPG fatty acid pattern. The fatty acid pattern of rat liver mitochondrial DPG is displayed in Table V. The fatty acid composition of this phospholipid appears to be quite different from that of PC or PE since DPG is mainly formed with linoleic acid (64%). Neither galactosamine nor clofibrate alters the amount of this major fatty acid. However, it appears from the data in Table V that clofibrate induces minor changes in other fatty acids which do not differ from the alterations which occur in PC. Indeed, palmitic, oleic and homo-γ-linolenic acids amounts are increased by +34%, +85% and +62%, respectively.

Fluorescence polarization

The steady-state fluorescence polarization, fluorescence anisotropy and lipid order parameter for DPH- and TMA-DPH-labeled mitochondrial preparations from control and treated animals are given in Tables VI and VII. Two different probes were used in this study since it appears that both clofibrate and galactosamine induce changes in phospholipids head groups and in their fatty acid

TABLE V

FATTY ACID COMPOSITION OF DIPHOSPHATIDYLGLYCEROL IN INTACT RAT LIVER MITOCHONDRIA

Figures are mole percentages of fatty acid methyl esters and means from duplicate. M.I., monounsaturated fatty acids; satd., saturated fatty acids.

Fatty acid	Treatments			
	control	galactosamine	clofibrate	clofibrate + galactosamine
14:0	1.1	1.6	0.9	1.0
16:0	7.9	6.0	8.2	10.6
16:1	3.4	4.2	5.5	2.3
18:0	6.0	6.3	5.0	6.3
18:1($n-9$)	4.1	4.0	7.6	7.6
18:1($n-7$)	7.1	6.7	6.9	6.3
18:2($n-6$)	63.9	64.8	61.2	57.9
18:3($n-6$)	–	–	–	–
20:3($n-9$)	2.4	1.6	1.1	1.1
20:3($n-6$)	2.1	1.7	3.2	3.4
20:4($n-6$)	3.2	3.2	1.7	3.6
22:6($n-3$)	–	–	–	–
M.I./Satd.	0.97	1.07	1.42	0.91
20:4/20:3	1.52	1.88	0.53	1.06

moieties. DPH is a probe of the hydrophobic core of the bilayer [22]. TMA-DPH dives into the membrane but it is anchored at the interface by a cationic group. It reflects therefore the degree of order of the outer part of the external leaflet [19] (the cationic charge results in a slow flip-flop motion). DPH is presumably distributed equally between the inner and outer leaflets. The data obtained with this probe reflect the averaged changes in lipid structural order of the two membrane fractions. Since the palmitoylcarnitine transferase I is localized in the inner membrane, we have also measured the fluorescence polarization on mitochondria whose outer membrane has been discarded by a smooth digitonin treatment. The fluorescence studies performed on these mitoplasts would give specific information about the physical state of the inner membrane.

The lipid order parameters monitored by DPH or TMA-DPH labelling appear to vary after digitonin treatment. The increased S(DPH) observed seems to reflect mainly the fact that the inner membrane core is more organized than the outer

TABLE VI

STEADY-STATE FLUORESCENCE POLARIZATION, FLUORESCENCE ANISOTROPY, AND LIPID ORDER PARAMETER OF INTACT HEPATIC MITOCHONDRIA

The values represent the means ± S.E. for four animals.
Fluorescence polarization: $P = (I'' - I')/(I'' + I')$
Fluorescence anisotropy: $R_s = 2P/(3-P)$
DPH limiting fluorescence anisotropy: $R_\infty = (4/3R_s) - 0.10$ (with $0.13 < R_s < 0.28$).
TMA-DPH limiting fluorescence anisotropy: $R_\infty = R_0 R_s^2/(R_0 R_s + (R_0 - R_s)^2/m)$ (with $m = 1.19$ and $R_0 = 0.39$).
Lipid order parameter: $S = \sqrt{R_\infty/R_0}$ (where R_0 (DPH) = 0.362 and R_0 (TMA-DPH) = 0.390 is the theoretically maximal fluorescence anisotropy).
[a] $p < 0.05$; [b] $p < 0.02$; [c] $p < 0.01$; [d] $p < 0.001$.

	Treatments			
	control	galactosamine	clofibrate	clofibrate + galactosamine
P(DPH)	0.231 ± 0.004	0.214 ± 0.006 [c]	0.212 ± 0.005 [d]	0.212 ± 0.004 [d]
R_s	0.167 ± 0.003	0.154 ± 0.004 [c]	0.152 ± 0.004 [d]	0.152 ± 0.003 [d]
R_∞	0.123 ± 0.004	0.105 ± 0.006 [c]	0.102 ± 0.005 [d]	0.103 ± 0.004 [d]
S(DPH)	0.582 ± 0.008	0.538 ± 0.013 [c]	0.531 ± 0.010 [d]	0.532 ± 0.009 [d]
P(TMA-DPH)	0.339 ± 0.002	0.339 ± 0.003	0.330 ± 0.002 [d]	0.334 ± 0.002 [a]
R_s	0.255 ± 0.002	0.255 ± 0.002	0.248 ± 0.002 [c]	0.250 ± 0.002 [b]
R_∞	0.221 ± 0.002	0.221 ± 0.003	0.210 ± 0.002 [d]	0.214 ± 0.003 [b]
S(TMA-DPH)	0.752 ± 0.005	0.752 ± 0.005	0.735 ± 0.004 [d]	0.741 ± 0.004 [b]

membrane. On the contrary, the decrease of S(TMA-DPH), after removing of the outer membrane, suggests that the polar region of the inner membrane is more fluid when compared to the outer membrane. As this sounds unlikely, this effect should rather be due to some disorganization by digitonin in the polar region of the inner membrane.

As can be seen from Tables VI and VII, on both mitochondria and mitoplasts, clofibrate induces a significant decrease in DPH ($p < 0.001$) and TMA-DPH ($p < 0.05$) lipid order parameters. Galactosamine appears to have no effect on the outer part of the outer membrane as it appears from TMA-DPH studies of intact mitochondria and induces only a weak fluidizing effect on the same region of the inner membrane. This point is apparently contradictory with the drastic effect of galactosamine on the phospholipid content and will be discussed later. On the contrary, this hepatotoxin induces larger alterations in the lipid bilayer core of the inner membrane since the DPH lipid order parameter of mitoplasts decreases from a mean of 0.605 ± 0.015 in hepatic mitochondria from control animals to a mean of 0.565 ± 0.018 in hepatic mitochondria from galactosamine-treated animals.

The most prominent effect resulting from the action of both compounds is a significant decrease (-11%, $p < 0.01$) of the DPH lipid order parameter of the inner membrane. It should be mentioned also, that the addition of the two compounds induce decrease of TMA-DPH lipid order parameter in the inner membrane which is more pronounced than the effect of each drug (approx. -3%, $p < 0.001$). These results suggest significant alterations in the physical state of the bulk phase of inner mitochondrial membrane lipids as a consequence of galactosamine and clofibrate administration.

Discussion

As we have earlier reported [1], clofibrate is able to prevent the galactosamine-induced depletion of mitochondrial phospholipids and inhibition of membrane-bound palmitoylcarnitine transferase I. In order to link the biochemical and ultrastructural perturbations induced by galactosamine and clofibrate in rat mitochondria, the present study was focused on the physico-chemical properties of this membrane after treatments by the two compounds.

The present study confirms our previous results concerning the effects of galactosamine on mitochondrial phospholipids and provides evidence that this hepatotoxin does not alter the fatty acid pattern of mitochondrial phospholipids. However, the fluorescence polarization studies show that galactosamine induces a decrease of

TABLE VII

STEADY-STATE FLUORESCENCE POLARIZATION, FLUORESCENCE ANISOTROPY, AND LIPID ORDER PARAMETER OF HEPATIC INNER MITOCHONDRIAL MEMBRANE

Details are the same as those in Table VII.

	Treatments			
	control	galactosamine	clofibrate	clofibrate + galactosamine
P(DPH)	0.241 ± 0.006	0.224 ± 0.003 [b]	0.217 ± 0.003 [d]	0.214 ± 0.005 [c]
R_s	0.174 ± 0.005	0.162 ± 0.005 [b]	0.156 ± 0.001 [d]	0.153 ± 0.008 [c]
R_∞	0.133 ± 0.006	0.116 ± 0.007 [b]	0.107 ± 0.002 [d]	0.104 ± 0.011 [c]
S(DPH)	0.605 ± 0.015	0.565 ± 0.018 [b]	0.545 ± 0.004 [d]	0.537 ± 0.029 [c]
P(TMA-DPH)	0.308 ± 0.002	0.303 ± 0.002 [a]	0.302 ± 0.004 [a]	0.298 ± 0.001 [d]
R_s	0.229 ± 0.002	0.224 ± 0.004	0.224 ± 0.003 [a]	0.221 ± 0.002 [d]
R_∞	0.184 ± 0.003	0.177 ± 0.005	0.177 ± 0.004 [a]	0.173 ± 0.003 [c]
S(TMA-DPH)	0.687 ± 0.005	0.674 ± 0.010	0.674 ± 0.008 [a]	0.665 ± 0.005 [d]

lipid order parameter in the inner membrane core. This decrease of lipid structural order would suggest that galactosamine should induce an increase in the content of unsaturated fatty acid but this is not the case. Indeed, it cannot be taken as axiomatic that there is a simple linear relationship between physical properties and the number of double bonds [23]. Moreover, the mitochondrial membrane exhibits particuliar behaviour when compared to other biological membranes since no correlation was observed by Yamauchi et al. [6] between the lipid order parameter and the unsaturated/saturated fatty acids ratio.

Indeed, it has been shown by Sjöstrand [24] that the inner mitochondrial membrane appears to be mainly formed with 'packed-proteins', the phospholipids representing only a small part of the whole membrane. This particular behaviour could be explained by the 17% increase of PC/DPG ratio which occurs after galactosamine treatment and, thus, it seems reasonable to hypothetize that the delipidation induces alterations in the protein-packing which confer a greater disorder into the membrane. The same effect with the same cause was observed by Rouslin et al. [25] in heart mitochondria from ischemic rats. This disorder should alter consequently the tightness of the lipid-protein binding and could warrant for the decreased palmitoylcarnitine transferase I activity and slight desensitization of the enzyme to malonyl-CoA which occurs after galactosamine injection.

The most prominent effect of clofibrate on the inner mitochondrial membrane is the large increase in DPG content which is the most abundant phospholipid of the palmitoylcarnitine transferase I lipid anulus [26]. As the amount of linoleic acid in DPG is not decreased by clofibrate, it can be assumed that the uptake of this essential fatty acid by the hepatocyte is increased as well to pace with the increased DPG synthesis. Contrarily to galactosamine, clofibrate alters deeply the fatty acid pattern of the mitochondrial phospholipids, the more drastic effect occurring in PC. In this class, we observe a 2-fold increase of monounsaturated/saturated fatty acids ratio suggesting that clofibrate stimulates the activity of the Δ^9 desaturase. This observation is supported by those of Kawashima and Kozuka [27,28] who have reported that clofibrate stimulates the elongation of palmitoyl-CoA and the desaturation of stearoyl-CoA. Besides, the present data suggest that clofibrate induces a marked inhibition of the Δ^5 desaturation as it appears from the 10-fold decrease of the $20:4(n-6)/20:3(n-6)$ ratio in PC. The question remains to know how these alterations are linked to the hypolipidemic effect of clofibrate. A very attractive hypothesis about the mechanism of action of clofibrate would be that the enhancement of DPG in the mitochondrial membrane induces phase transitions [29] which are known to facilitate the incorporation of cytosolic phospholipids and proteins in the membrane [30]. This early event could hence be one of the first steps of membrane biogenesis induced by clofibrate.

One of the main problems which prompted us to study the physico-chemical properties of the mitochondrial membrane was the understanding of the preventing action of clofibrate on galactosamine-induced palmitoylcarnitine transferase I inhibition. From the present study, it appears that phospholipid fatty acid composition is not involved in this preventing action. Moreover, clofibrate and galactosamine appear to induce additive effects on membrane lipid structural order. This fact agrees well with the alteration of the kinetic properties of palmitoylcarnitine transferase I, which are more important when both compounds are administered [1]. The present data bring evidence that the sensitivity of palmitoylcarnitine transferase I to malonyl-CoA is strongly dependent from the physical properties of the membrane. Besides, it seems interesting to point out that the PC/DPG ratio is increased from 1.8 (control) to 2.5 in the clofibrate + galactosamine group; since DPG exerts a rigidifying effect on biological membranes [6], this decrease of the relative content of DPG could warrant for the decreased lipid order parameter and the drastic alterations of the kinetic constants of palmitoylcarnitine transferase I in the same group. As these properties are very well and short-termed regulated in vivo, the different sensitivities of palmitoylcarnitine transferase I to malonyl-CoA observed in various physiological states [31] could be due to modulations involving qualitative modifications of membrane components.

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