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Modulation of rat liver lipid metabolism by prolactin

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Summary The effect of chronic hyperprolactinemia on the Δ6- and Δ5-desaturation activity, total lipid and fatty acid composition, as well as fluorescence anisotropy, was studied in liver microsomes from anterior pituitary-grafted rats. We observed a depression in Δ6-desaturation activity but no changes in the Δ5-desaturation activity in the grafted animals. The microsomal fraction from the hyperprolactinemic rats contained significantly less amount of linoleic acid and a higher content of 20:4 n-6, 22:5 n-6 and 22:6 n-3 acids. Lipid rotational mobility was increased in microsomes as well as in liposomes obtained from the microsomes of transplanted animals. The fluidifying effect induced by PRL was located in the deepest zone of the membrane. The results obtained indicate that high levels of prolactin induce changes in polyunsaturated fatty acid distribution in liver microsomes, which regulates the lipid rotational mobility and hence membrane fluidity.

INTRODUCTION

As fundamental components of major membrane-forming lipids, the polyunsaturated fatty acids (PUFAs) are essential elements of all biological membranes. They are responsible for relevant physical properties of the lipid bilayer, such as lipid packing and fluidity state. Besides their structural role, PUFAs also originate cellular second messengers like eicosanoids. Therefore, it is critical to find out how the cell governs both PUFA production and fate. The biosynthesis of polyenoic acids is largely modified by a wide variety of hormonal conditions.[1] Among the pituitary hormones, prolactin (PRL) was described to be involved in lipid metabolism. The inhibition of PRL secretion during lactation reduces lipid synthesis in rat mammary glands by decreasing the mRNA synthesis and catalytic activities of several lipogenic enzymes.[2] These authors also reported an enhanced lipogenesis and triglyceride storage in adipose tissue in the same experimental conditions. Cultured mouse mammary glands explants treated with PRL exhibited a greater incorporation of [^{14}C]acetate into both neutral and polar lipids.[3] In rat testis, treatment with PRL leads to a reduction in free cholesterol and phospholipids in plasma membrane, accompanied by a more elevated monounsaturated fatty acid content in the major polar lipid species.[4] However, hyperprolactinemic monkeys showed higher levels of circulating lipids than controls, as well as an increase in hepatic and adipose tissue triacylglycerol and certain phospholipids.[5]

On the other hand, it has been shown previously that essential fatty acid deficiency affects the maintenance and induction of PRL receptors in liver.[6] In this connection, a higher binding of PRL to its receptors in hepatic membranes was shown when the membrane lipid environment was less fluid.[7] The changes in the lipid surrounding were attributed to alterations in serum and liver lipid composition. The same authors previously reported that prostaglandins induced a higher PRL binding by decreasing membrane lipid fluidity.[8]

Since changes in the fatty acid profile are strongly correlated to membrane lipid fluidity, we considered of interest to investigate the relationship between chronic high levels of PRL and PUFA biosynthesis, focusing on the regulation of fatty acid desaturation and lipid rotational mobility.

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MATERIAL AND METHODS

Chemicals

[1-^{14}C]linoleic (59.0 Ci/mol) and [1-^{14}C]eicosa-8,11,14-trienoic (47.0 Ci/mol) acids were purchased from New England Nuclear Corp. (Boston, MA, USA). The following chemicals and cofactors were purchased from the indicated

suppliers in the USA: unlabeled acids, Nu-Chek Prep. (Elysian, MN); NADH, ATP and CoA, Sigma Chemical Co. (St Louis, MO), DPH (1,6-diphenyl-1,3,5 hexatriene, Aldrich Chemical Company (Milwaukee, WI) and n-AS (2-, 7- and 12- (9-anthroyloxy) stearic acid), Molecular Probes, Inc. (Junction City, OR). All other chemicals were of analytical grade.

Animals, endocrine manipulation and microsomal preparation

Wistar rats were housed in a temperature controlled room on a 14:10 h light:dark cycle with food and water ad libitum. Chronic hyperprolactinemia was achieved by grafting two rat anterior pituitary (AP) glands from young females under the kidney capsule of each of six recipient animals of the same sex and age.[9] Sham-operated rats were considered the control group. Twenty-two days after transplantation, the animals were decapitated, blood was drained off and plasma and serum were separated for hormone and lipid determinations. Livers were excised, weighed and homogenized in an ice-cold solution (1:3 v/v) containing 0.25 M sucrose, 62 mM phosphate buffer (pH 7.0), 0.15 M KCl, 5 mM $MgCl_2$ and 100 μM EDTA. The microsomal fraction was obtained by differential centrifugation at 105 000 × g as described previously.[10] Protein content was measured following the method of Lowry et al.[11]

Hormone assays

Pituitary hormone levels were assessed by specific radioimmunoassays (RIA) performed using the rat materials provided by the National Hormone and Pituitary Program NIDDK, NICHHD, USDA, USA. Hormones were iodinated by the Iodogen® method[12] and purified on PD-10 Sephadex G-25M columns (Pharmacia, Upssala, Sweden) equilibrated with 0.01 M phosphosaline, pH 7.6. A goat anti-rabbit IgG serum (1/30) diluted in 0.01 M PBS was used to separate bound from free hormones. Corticosterone was measured by RIA according to the method of Gómez-Sanchez et al.[13]

Measurement of fatty acid desaturation

The desaturation of fatty acids from liver microsomes was measured by estimation of the conversion percentage of [1-^{14}C]linoleic acid to γ-linolenic acid and [1-^{14}C]eicosa-8,11,14-trienoic acid to arachidonic acid. Four nmol of the labeled acid plus 96 nmol of the corresponding unlabeled acid were incubated with 5 mg of liver microsomal protein in a metabolic shaker at 37°C for 10 min. The incubation medium contained 4 μmol of ATP, 0.1 μmol of CoA, 1.25 μmol of NADH, 5 μmol of $MgCl_2$, 2.42 μmol of N-acetyl cysteine, 62.5 μmol of NaF, 0.5 μmol of nicotinamide and 62.5 μmol of phosphate buffer (pH 7) in a total volume of 1.5 ml of 0.15 M KCl, 0.25 M sucrose solution per tube. The reaction was stopped by the addition of 2 ml 10% (w/v) KOH in ethanol. The fatty acids were recovered by saponification of the incubation mixture (45 min at 85°C), followed by acidification and extraction with petroleum ether (boiling point 40–60°C). The free fatty acids were first separated by reverse phase high pressure liquid chromatography after the technique of Narce at al.[14] as modified by Garda et al.,[15] and finally quantitated in a liquid scintillation counter.

Lipid extraction and analysis

Lipids from liver microsomal suspensions were extracted according to the procedure described by Folch et al.[16] After conversion of the fatty acids to their corresponding methyl esters, the latter were analyzed in a Hewlett-Packard Model 5840-A gas–liquid chromatograph, equipped with a flame ionization detector, as described previously.[7] The number of double bonds per 100 acyl-chains was calculated from the total population of fatty acids analyzed by gas–liquid chromatography. Acyl chain shortening (50% of the single-bond axial length) for each double bond[18] was considered. Phospholipids were separated into their classes by thin layer chromatography (TLC) on Silicagel H with florisil, resolved using a one dimensional-double solvent system: chloroform-methanol-ammonium hydroxide-water (70:25:3.5:1.5, by vol) and chloroform-methanol-acetic acid-water (80:10:2:0.75, by vol). Phospholipid species were identified by comparison of their R_f with the corresponding standards run on the same plates. The distribution of the fractions was determined by optic densitometry in a Zeiss spectrophotometer after charring the plates with sulphochromic acid solution.[19]

The quantitative determination of the neutral lipids was carried out by TLC coupled to a flame ionization detector (FID) in an latroscan apparatus model TH 10, after their separation on chromarods type S-III, using a double-solvent system: hexane-benzene (70:30, by vol) and benzene-chloroform-formic acid (70:25:2, by vol). Lipidic species were quantified by comparison with known amount of standards run under the same conditions. The signals from the FID were registered on a Hewlett-Packard model HP-3396 A integrator.

Multilamellar liposome preparation and labeling

Aliquots of the corresponding microsomal lipid extract (100 μg of lipids) were evaporated under N_2. Liposomes were prepared adding 3 ml of 20 mM sodium phosphate pH 7.0 to the tubes, according to the procedure described by Kutchai et al.[20] One μM DPH or n-AS was added to the

tubes and the preparation was sonicated at room temperature for 20 min, in the dark and under N_2 atmosphere. Simultaneously, another group of samples was prepared identically without the addition of fluorophors. Fluorescent anisotropy was measured immediately, as indicated below.

Fluorescence anisotropy measurements

Steady state fluorescence anisotropy (r_S) was measured in whole microsomal membrane and in liposomes at 37°C following the procedure of Shinitzky and Barenholz[21] using a SLM 4800 C spectrofluorometer. The probes used were DPH, a rigid molecule that senses almost all the lipid matrix, 2-,7- and 12-AS, whose fluorescent anthroyl moieties, attached to a flexible acyl chain, are located at different depths from the surface to the center of the lipid bilayer respectively.[22]

Excitation wavelength was 360 nm for all the fluorophors and the emitted light was passed through a sharp cut-off filter. Light scattering of blanks represented less than 5%, and fluorescence values were corrected accordingly. The phospholipid:probe ratio was maintained at more than 200:1 (mol:mol) in order to reduce to a minimum possible probe–probe interactions. Results were calculated as described previously.[23]

Statistical analyses

The results were analyzed statistically through the Student's *t*-test.

RESULTS

As expected, serum PRL levels increased in those animals with ectopic pituitary tissue as compared to sham-operated controls (Table 1). No differences in the concentration of the other hormones studied were detected between control and experimental animals. In the grafted rats, hyperprolactinemia was associated with significantly high levels of circulating cholesterol (Table 1).

To investigate the effect of hyperprolactinemia on polyunsaturated fatty acid biosynthesis we assayed for the activity of liver microsomal Δ6- and Δ5-desaturases, as measured by the transformation of linoleic to γ-linolenic acid, and eicosa-8,11,14-trienoic acid to arachidonic acid, respectively (Fig. 1). The Δ6-desaturase activity decreased significantly in transplanted rats compared to controls, while the conversion of eicosatrienoic to arachidonic acid increased in a non significant manner.

Because the microsomal fatty acid composition could be a possible target for the action of PRL, we also performed the analysis of the fatty acid profile of plasma and liver microsomal fraction of control and AP-grafted rats (Table 2). Arachidonic acid and docosahexenoic acids increased in the serum of transplanted animals. These

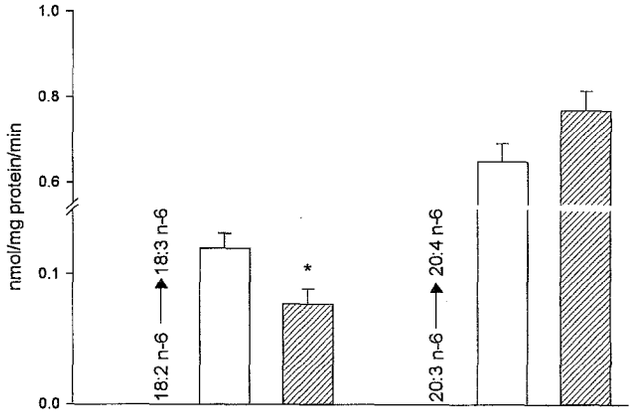

Fig. 1 Fatty acid desaturase activities in liver microsome of control (open bars) and AP-grafted rats (hatched bars). Results are the mean±SEM from six animals, analyzed in duplicate. Signifcant difference from control values at $^*P<0.05$.

Table 1 Serum hormone and total cholesterol levels in rats implanted with anterior pituitary (AP) glands and in non-implanted controls, at the time of sacrifice

Hormone ng/ml	Controls	AP grafted
PRL	11.5±2.1	32.5±3.5*
GH	15.7±2.4	16.7±3.4
FSH	11.6±1.9	14.0±1.7
TSH	4.2±0.2	4.4±0.5
Corticosterone	640±40	549±69
Cholesterol mg/dl	42.0±1.9	57.0±4.5**

Values are the mean±SEM of six analyses. PRL, prolactin; GH, growth hormone; FSH, follicle-stimulating hormone, TSH, thyrotropin-stimulating hormone. Significant difference from control values at: $^*P < 0.001$; $^{**}P < 0.02$.

Table 2 Fatty acid composition of total lipids of serum and liver microsomes in control and AP grafted rats

	Serum		Liver	
Fatty acid	Control	AP grafted	Control	AP grafted
16:0	16.8±0.6	14.8±0.8	14.4±0.5	12.4±1.2
16:1	1.9±0.4	1.5±0.2	0.6±0.1	0.6±0.1
18:0	16.3±0.6	17.0±0.9	25.9±0.7	25.2±0.6
18:1	11.2±0.3	10.9±0.2	7.6±0.3	7.2±0.4
18:2 n-6	22.0±0.9	19.8±0.8	13.0±0.2	12.1±0.1**
18:3 n-6	1.3±0.2	1.2±0.1	0.6±0.05	0.6±0.1
18:3 n-3	0.8±0.1	0.7±0.1	0.4±0.04	0.4±0.05
20:3 n-6	0.9±0.1	1.0±0.1	0.7±0.1	0.7±0.04
20:4 n-6	22.6±0.8	25.5±0.3*	25.6±0.4	27.3±0.4**
22:4 n-6	0.9±0.04	1.3±0.2	0.7±0.02	0.8±0.1
22:5 n-6	0.6±0.1	0.8±0.1	0.2±0.02	0.5±0.1***
22:5 n-3	1.2±0.2	0.9±0.2	1.4±0.04	1.6±0.1
22:6 n-3	3.5±0.2	5.1±0.5*	8.9±0.1	10.6±0.4**
U.I.	5.6	6.4	5.1	6.0

Results are expressed as μg% of the total fatty acids (means±SEM). Significant difference from control values at $^*P < 0.02$; $^{**}P < 0.01$; $^{***}P < 0.001$.

acids together with docosapentenoic acid of n-6 family were also enhanced in the liver microsomal membranes, while linoleic acid decreased in the hyperprolactinemic rats, compared to controls. The unsaturation index was, therefore, increased in those rats implanted with an anterior pituitary.

It is well known that the unsaturation level can alter the organization of the lipid bilayer.[24] However, as much as the double-bond number, the double-bond position may play a major role in modulating the lipid matrix physical properties.[25] For this reason, we also made a calculation of the transversal double-bond distribution within the membrane-lipid bilayer, at different depths from the outer polar zone to the inner most hydrophobic region. Figure 2 shows the distribution of fatty acid double-bonds across the liver microsomal membranes of the two experimental groups at different positions along the acyl carbons of fatty acid chains. The unsaturation was distributed all along the bilayer showing a significant increase at the levels of carbons 3-4, 6-7 and 9-10 and in the deepest region of the bilayer (16,17 position) in the AP-grafted rats compared to controls.

The amount of the different neutral and polar lipids as well as the distribution of phospholipid species in liver microsomes from both groups were similar (data not shown).

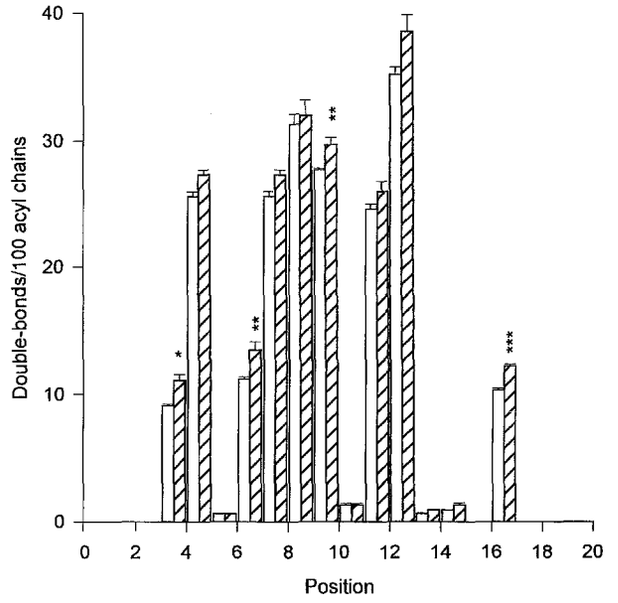

Fig. 2 Transverse double bond distribution from the interface to the deep interior of the lipid bilayer in liver microsomes of control and AP-grafted rats. Position corresponds to the carbon number of saturated acyl chain in *sn*-1. Polyunsaturated fatty acids were considered to be acylated to the phospholipid *sn*-2 position and chain bond was taken into account. Control rats open bars and AP-grafted rats hatched bars. Results are the mean±SEM from six animals. Significant difference from control values at $^{*}P<0.01$, $^{**}P<0.02$, $^{***}P<0.001$.

Table 3 Fatty acid composition of liver microsomal phosphatidylcholine and phosphatidylethanolamine

Fatty acid	Phosphatidylcholine Control	Phosphatidylcholine AP-grafted	Phosphatidylethanolamine Control	Phosphatidylethanolamine AP-grafted
16:0	16.0±0.7	13.3±1.4	17.2±1.5	16.8±0.4
18:0	28.9±0.8	30.9±1.3	26.9±1.0	26.3±0.4
18:1 n-9	7.0±0.2	6.0±0.4	8.1±0.5	7.5±0.4
18:2 n-6	12.5±1.0	10.6±0.7	9.9±0.8	9.6±0.6
20:3 n-6	0.9±0.2	0.9±0.2	1.1±0.3	0.9±0.1
20:4 n-6	25.9±0.3	28.8±0.7*	22.3±1.6	21.4±0.7
22:5 n-6	0.8±0.2	0.7±0.2	1.5±0.4	1.0±0.2
22:5 n-3	0.9±0.1	0.8±0.1	1.7±0.2	1.6±0.2
22:6 n-3	7.2±0.9	7.7±0.9	11.1±0.6	14.8±0.7*

Results are expressed as percentage of the total fatty acids (means±SEM). Significant difference from control values at $^{*}P < 0.01$.

In order to discriminate which polar lipid fraction was affected in its acyl composition, the fatty acid distribution of individual phospholipid species was determined in liver microsomal preparations (Table 3). The most important change observed in the fatty acid profile was an increase in arachidonic acid of the PC fraction and in docosahexenoic acid of the PE fraction, in AP-grafted rats compared to controls. No changes were observed in the other fractions.

The fluidity of the membrane environment is in part determined by the acyl composition of membrane phospholipids. Considering the modification in the fatty acid profile seen in the AP-grafted rats, we decided to explore the rotational lipid motion of liver microsomal and liposomal membranes, by measuring fluorescence anisotropy.

Figure 3 represents the rotational mobility of DPH in liver microsomal membranes and in liposomes derived from them (Panel A), in the samples from control and AP-grafted rats. Panel B shows the results obtained when multilamellar liposomes prepared from hepatic microsomes of both experimental groups were tested with n-AS fluorophors. DPH fluorescence anisotropy revealed a significant decrease in r_s values in AP-grafted rats compared to controls either in microsomes or in liposomes. The values of the rotational mobility of the anthroyl group showed no differences at the depth of carbon 2 and 7 between both experimental groups. At the deepest zone of the membrane (12-AS), the r_s values were significantly decreased in transplanted rats.

DISCUSSION

The present results confirm that AP grafting is an effective and specific method to induce hyperprolactinemia in the female rat.[9] The concomitant hypercholesterolemia present in AP-grafted animals suggests that PRL may alter either the rate of cholesterol synthesis in liver or the

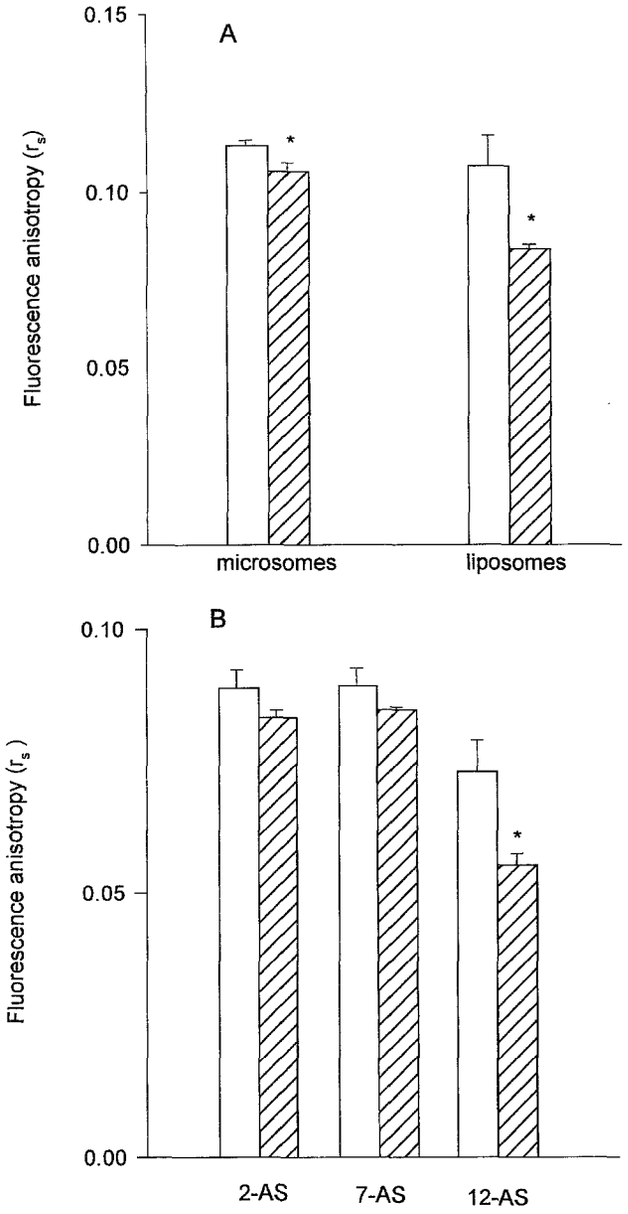

Fig. 3 Fluorescence anisotropy of DPH measured in rat liver microsomes and in multilamellar liposomes prepared from the protein-free lipids of the same cellular fraction (A). Fluorescence anisotropy of 2-, 7- or 12-AS probes in multilamellar liposomes prepared from the protein-free lipids of rat liver microsomes (B). The membrane samples were obtained from either control (open bars) or AP-grafted rats (hatched bars). Results are the mean±SEM from six animals. Significant difference from control values at $^*P<0.01$.

mobilization of cholesterol from hepatic stores. Although we are unaware of any documentation on the effects of PRL on blood cholesterol homeostasis, studies in our laboratory have shown that the activity of liver hydroxymethylglutaryl-coenzyme A reductase, the rate-limiting enzyme in the biosynthesis of cholesterol, is altered in 33-month-old rats that develop an hyperprolactinemic state.[26] On the other hand, the increase in plasma cholesterol levels did not affect the cholesterol content of liver microsomal membranes.

Concerning fatty acids, the excess of PRL also affects the membrane acyl composition. We observed higher contents of n-6 and n-3 polyunsaturated fatty acids in the microsomal membranes from AP-grafted rats. Since we also detected a lower Δ6-desaturase activity in the same subcellular fraction, we can therefore assume that the key enzyme of polyenoic acid biosynthesis is down regulated by the higher levels of its final products.[27] In addition, the changes in microsomal 20:4 n-6, 22:5 n-6 and 22:6 n-3, promoted by the hyperprolactinemia were of a phospholipid-specific type, which indicates that PRL could also modulate phospholipid acyl transferase activity.

It has been proved that *cis* unsaturated fatty acids are able to decrease the packing of the membranes making it more fluid.[28] Therefore, we measured the steady-state fluorescence polarization of DPH in the liver microsomal membranes from both groups of rats. In AP-grafted rats, a decrease in the membrane lipid microviscosity was observed. This observation agrees with what was reported by Dave et al.,[29] who detected a lower fluorescence polarization, as a measure of microviscosity level, in hepatic membranes from normal rats treated with PRL. Knowing that proteins also participate in the regulation of the physical properties of membranes,[30] we also determined the DPH fluorescence polarization in protein-free liposomes prepared from microsomal total lipid extracts. Under these conditions, the r_s values were again found to be significantly decreased in the vesicles obtained from livers of hyperprolactinemic rats. From this result it is possible to infer that any alteration in microsomal protein content or distribution owing to hyperprolactinemia not playing a significant role in determining the physical properties of the lipids within the membrane bilayer.

Changes in the rotational mobility of probes inserted into the membrane lipid domain might be induced by local modifications of the ratio of phospholipid to cholesterol,[31,32] the degree of unsaturation and length of fatty acid moieties within the phospholipids[33,34] or the ratio of lipid to protein.[31,35] Since no changes were found in the amount of either neutral lipids or phospholipid species, and considering that an increased fluidity was also observed in the absence of proteins (liposomes) in transplanted rats, we can assume that the higher degree of membrane lipid unsaturation might be responsible for the observed changes in rotational mobility of probes. A change in unsaturation index at this membrane cross sectional level could be one of the reasons for this phenomenon. By calculating the fatty acid double-bond density, we determined that the unsaturation increased at different levels in the membranes of hyperprolactinemic rats. However, the values of the rotational mobility of the anthroyl group increased only at the deepest zone of

the membrane where the distribution of the double bond reached a higher statistical significance.

We can, therefore, conclude that PRL appears to be involved in the regulation of the complex functions and physical properties of biological membranes by modulating the lipid composition and fluidity state of the membrane matrix.

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