The microviscosity of liver plasma membranes of rats fed with oleoylanilide

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1. Oleoylanilide was administered orally to groups of rats according to different patterns. 2. Oleoylanilide was perfused at different concentrations through rat liver. 3. Oleoylanilide was added to isolated hepatocytes. 4. Oleoylanilide was added to plasma-membrane preparations. 5. Membrane preparations were obtained after experiments performed *in vivo* and perfusion experiments and, by using 1,6-diphenylhexa-1,3,5-triene as fluorescence probe, the fluorescence polarization parameter was measured, from which the microviscosity (η) was calculated. In all cases the microviscosity decreased markedly. 6. Addition of oleoylanilide to hepatocyte preparations and to isolated membranes produced the same effect, increasing the fluidity of the membranes. 7. These data suggest that oleoylanilide partitions into the membrane, disordering some lipid interactions.

Long-chain fatty acid acylanilides, mainly oleoylanilide, are analytical markers of the oil mixtures containing denatured rape-seed oil, responsible for a recent epidemic of poisoning in Spain. Oleoylanilide has the capacity to alter markedly rat lipid metabolism in lung and adipose tissue with a high loss of adipose-tissue stores (Casals *et al.*, 1983). These metabolic alterations of the animal experimental model agree with some clinical observations of human patients suffering from the toxic epidemic syndrome, e.g. weight loss, the disappearance of fat tissue and pneumonic symptoms (Noriega, 1982). The clinical features in the course of the toxic disease have been varied, and among them pulmonary and neurological manifestations have been the most conspicuous. The neurological symptoms involve muscles, peripheral nerves and central nervous system. Muscle changes are characterized by inflammatory myopathy with lymphocytic-cell infiltration of nervous structures. As the disease proceeds, the inflammatory response becomes restricted to the blood vessels, mainly veins and capillaries. Perineuritis and myelin damage were also marked pathological features (Martinez-Tello *et al.*, 1982).

With regard to the multisystemic character of the disease (Noriega, 1982; Vicario *et al.*, 1982) and the surface properties of long-chain acylanilides (Murphy & Vodyanoy, 1982), the present

paper reports a study of the membrane-disordering effect of oleoylanilide, in which long-chain acylanilides are shown to exert their biological influence by the same basic mechanism. With this purpose in mind, the influence of oleoylanilide on membrane microviscosity was assayed in liver plasma membranes *in vitro* and *in vivo* after rats had been fed under various experimental conditions. Liver-perfusion experiments with oleoylanilide and ^{14}C -labelled oleoylanilide were also carried out.

Materials and methods

Materials

Collagenase and scintillation reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Minimum essential medium (MEM medium) was supplied by Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.) and the newborn-calf serum was from Gibco Europe (Paisley, Renfrewshire, Scotland, U.K.). DPH was a product of Aldrich Chemical Co. (Frankfurt, Germany). $[1\text{-}^{14}\text{C}]$ Oleic acid and $[U\text{-}^{14}\text{C}]$ aniline were supplied by Amersham International (Amersham, Bucks., U.K.).

Labelled oleoylanilide was synthesized from either labelled oleic acid and aniline or oleic acid and labelled aniline, yielding respectively $[^{14}\text{C}]$ oleoylanilide with specific radioactivity 2010 d.p.m./ μg and 2120 d.p.m./ μg , by the method of Casals *et al.* (1983).

Abbreviation used: DPH, 1,6-diphenylhexa-1,3,5-triene.

Other chemicals were purchased from Merck (Darmstadt, Germany).

Animals

Wistar rats (180–200 g) were fed on standard rat chow and water *ad libitum*. Animals were used for treatment with oleoylanilide and for perfusion experiments. Two groups of male rats received daily 5 mg of oleoylanilide dissolved in 0.5 ml of pure olive oil by an oesophageal tube for 30 days and were then left for 10–30 days without administration of the anilide before being killed. Control animals received 0.5 ml of pure olive oil alone.

Perfusion experiments

Perfusion experiments were carried out for either the preparation of isolated hepatocytes or recycling oleoylanilide through the liver.

Hepatocytes were isolated by the perfusion technique, by using collagenase in Krebs–Ringer bicarbonate solution (KRB medium), by the general method of Berry & Friend (1969) as described previously (Pagani *et al.*, 1981).

Before the perfusion experiments, animals were starved for 20 h with water *ad libitum* and anaesthetized by intraperitoneal pentothal injection (10 mg/100 g body wt.). Portal vein and inferior vena cava were cannulated for recycling of the perfusion medium. Liver was pre-perfused with 100 ml of KRB III medium, pH 7.4, and subsequently cycle-perfused for 15 min with 100 ml of MEM medium and 5 ml of neonatal serum that contained different amounts of oleoylanilide according to the experiments. Either 10 mg or 20 mg of oleoylanilide was dissolved in diethyl ether and added to the neonatal serum, and the mixture was sonicated until a homogeneous dispersion formed; the organic solvent was eliminated under vacuum and the oleoylanilide dispersion was added to MEM medium. At the end of the perfusion, the whole liver was washed with 0.9% NaCl and membrane preparations were obtained.

Standard perfusions without oleoylanilide for the obtention of control membranes were also performed.

Isolation of membranes

Liver plasma membranes were isolated from liver of control animals and oleoylanilide-fed animals as well as from liver perfused with oleoylanilide, according to the experiments. The membrane isolation procedure was that described by Emmelot *et al.* (1964) and 5'-nucleotidase activity was determined as an index of purity of the preparation (Coleman *et al.*, 1967).

Fluorescence labelling

DPH was incorporated into the hydrophobic portion of the membrane bilayers by incubating either the plasma membranes (in 5 mM-Tris/HCl buffer, pH 7.5, containing 0.25 M-sucrose; 90 µg of protein/ml) or the cells (10⁶ cells/ml of KRB III medium) with a stable dispersion of the probe (final concentration 9 µM) during 30 min at 37°C. After this treatment, fluorescence polarization in either cells or membranes was measured.

Effect in vitro of oleoylanilide

To either 10 mg or 20 mg of oleoylanilide dissolved in 0.5 ml of diethyl ether, 5 ml of MEM medium, KRB III medium or 5 mM-Tris/HCl buffer, pH 7.5, containing 0.25 M-sucrose was added. After sonication, diethyl ether was eliminated under vacuum. This suspension of oleoylanilide was stable for at least 3–4 h. Plasma membranes or cells, labelled with DPH, were incubated with different amounts of oleoylanilide at 37°C for 30 min, and fluorescence polarization was measured.

Fluorescence polarization

Fluorescence polarization was measured at 25°C in a Perkin–Elmer MPF-44E spectrofluorimeter equipped with a polarization attachment. All measurements were made in the ratio mode. Temperature in the cuvettes was controlled with a Lauda thermostatically controlled circulating water bath. Polarization, *P*, was calculated from the equation:

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}$$

where *I*_{VV} and *I*_{VH} are respectively the vertical and horizontal components of the fluorescent light when the sample is excited by the vertical component of the light. The emission grating factor was calculated as *G* = *I*_{HV}/*I*_{HH}. Excitation was at 365 nm and emission was measured at 425 nm. Slit widths of 6 nm and 5 nm were used respectively for excitation and emission channels.

P values were corrected for light-scattering as indicated by Lentz *et al.* (1979).

Microviscosity (*η*) was calculated from the *P* values through the relation *η* = 2*P*/(0.46 – *P*) (Shinitzky & Barenholz, 1978).

Lipid extraction

In some perfusion experiments with labelled oleoylanilides, the recycling media were extracted by a modification of the Folch–Lees method (Guarnieri *et al.*, 1976) and total lipids were fractionated by t.l.c. with as developing solvent hexane/diethyl ether/acetic acid (70:30:1, by vol.)

and pure oleoylanilide as reference standard on silica-gel G plates. Lipid spots were scraped off into counting vials containing 15 ml of toluene scintillation mixture [0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-(5-phenyloxazol-2-yl)benzene in scintillation-grade toluene/Triton X-100/water (10:5:1, by vol.)]. The radioactivities of samples were counted in a Packard 3255 scintillation spectrometer with external-standard correction for quenched samples.

Cell viability

Cell viability was routinely monitored by Trypan Blue exclusion; cell preparations had 85–90% viability. Maintenance of cell structure was checked by electron microscopy of thin sections of Epon-embedded cell suspensions, cut on a LKB Ultratome III ultramicrotome, and examined in a Jeol 100 electron microscope. The yield of viable hepatocytes was 90×10^6 – 110×10^6 cells per rat liver.

Results

The 5'-nucleotidase activity of the plasma-membrane preparations from control liver and liver treated with oleoylanilide ranged over $87.6 \pm 5.3 \mu\text{mol}$ of phosphate/h per mg of protein, whereas this value for liver homogenates was $3.2 \pm 0.5 \mu\text{mol/h}$ per mg; thus a 25-fold purification of the enzyme marker was obtained. When plasma-membrane preparations were assayed for glucose-6-phosphatase and cytochrome *c* oxidase activities, less than 2% of the total activity was found in both control livers and treated with oleoylanilide. The protein recovery was quite similar in both cases, 1 mg of membrane protein from 1 g of wet tissue. Also, the absence of any significant contamination by other organelles was demonstrated by phase-contrast microscopy. All these facts, in addition to a very similar cell viability of hepatocytes from both standard perfusions and with oleoylanilide, together confirm the similarity of biochemical properties of the plasma membranes from control liver, and liver of oleoylanilide-fed rats and liver perfused with oleoylanilide.

Fluorescence polarization in liver plasma membranes of rats was determined when oleoylanilide was supplied to the system under the following conditions: (1) experiments *in vivo* in which oleoylanilide was given to groups of rats by oesophageal tube at different time conditions; (2) perfusion experiments in which oleoylanilide was recycled through the liver; (3) experiments *in vitro* in which oleoylanilide was added to isolated hepatocytes. In each case the corresponding controls were obtained under the same experimental conditions.

Two groups, each containing four rats, were used for the treatments *in vivo*. Each animal received daily 5 mg of oleoylanilide for 30 days, and then one group (group 1) remained for 10 days without treatment before being killed, whereas the other (group 2) remained for 30 days without treatment. Table 1 shows the values of fluorescence polarization and microviscosity of liver plasma membranes and those of the control rats, with DPH as fluorescence probe. These values indicate clearly a more fluid environment for DPH in the groups of treated rats than in the control group; however, there was no significant difference between the results for the two treated groups.

Table 2 shows fluorescence polarization and microviscosity of plasma-membrane preparations obtained from liver that had been previously perfused with oleoylanilide dispersions. The control membrane preparations were obtained from liver perfused with MEM medium; however, perfusion itself had no effect on the microviscosity of membranes, since the values obtained for the perfused control liver are identical with those obtained for the non-perfused liver control. There is, then, a clear difference between control values and those obtained after perfusion of the liver with oleoylanilide; this effect is dependent on the concentration of oleoylanilide employed in the recycled perfusion experiment. Perfusion with both concentrations of oleoylanilide produces a decrease in the membrane microviscosity.

The fluorescence polarization was also measured in isolated hepatocyte suspensions of both control cells and cell suspensions to which oleoylanilide was added (Table 3). The low fluorescence polari-

Table 1. *Effect in vivo of oleoylanilide on the microviscosity ($\bar{\eta}$) of liver plasma membranes*

Rats were given oleoylanilide for 30 days followed by 10 days (group 1) or 30 days (group 2) without this treatment before being killed. The group of control animals received during the first 30 days the same amount of oil as the other two groups. Values are means $\pm$ s.d. Polarization measurements were carried out in triplicate in each of the animals of the group.

Animals	<i>P</i>	$\bar{\eta}$ (Pa·s) at 25°C
Control (<i>n</i> = 6)	0.295 ± 0.010	0.36 ± 0.03
Group 1 (<i>n</i> = 4)	0.269 ± 0.009	0.28 ± 0.01
Group 2 (<i>n</i> = 4)	0.276 ± 0.005	0.30 ± 0.01

Table 2. *Effect of liver perfusion with oleoylanilide on the microviscosity ($\bar{\eta}$) of liver plasma membranes*

A homogeneous dispersion of oleoylanilide (10 or 20 mg) was recycled through the liver for 20 min, after which membranes were isolated and the fluorescence polarization was determined. Values are means $\pm$ s.d. Polarization measurements were carried out in triplicate in each of the three different perfusion experiments. The control group of animals was also perfused in a cyclic way with MEM medium.

Experiment	<i>P</i>	$\bar{\eta}$ (Pa·s) at 25°C
Control (<i>n</i> = 3)	0.287 $\pm$ 0.004	0.33 $\pm$ 0.01
Perfusion, 10 mg	0.277 $\pm$ 0.003	0.30 $\pm$ 0.01
Perfusion, 20 mg	0.270 $\pm$ 0.004	0.28 $\pm$ 0.01

Table 3. *Effect of the addition of oleoylanilide on isolated hepatocytes*

Isolated hepatocytes (1.5×10^6 cells in 1.5 ml) were incubated with 500 μ g of oleoylanilide for 30 min at 37°C. The control experiments were carried out under the same experimental conditions in the absence of oleoylanilide. Values are means $\pm$ s.d. Polarization measurements were done in triplicate in each of three different controls and in experiments in the presence of oleoylanilide.

Experiment	<i>P</i>	$\bar{\eta}$ (Pa·s) at 25°C
Control	0.192 $\pm$ 0.005	0.14 $\pm$ 0.01
Oleoylanilide	0.172 $\pm$ 0.004	0.12 $\pm$ 0.01

zation value for the control cells in comparison with that of plasma membranes indicates the solubilization of DPH in the cell surface and also in some intracellular hydrophobic structures. However, when this value is compared with the polarization value of hepatocytes incubated with oleoylanilide a significant decrease is observed. This diminution is an index of the fluidity increase in the cell membranes.

The effect *in vitro* of oleoylanilide on the microviscosity of membrane preparations was determined comparatively at a series of concentrations of oleoylanilide and oleic acid. Fig. 1 shows clearly that oleoylanilide increases the fluidity of the membranes but oleic acid decreases the microviscosity to a greater extent.

Taking into account the possibility that oleoylanilide could be hydrolysed as a consequence of the metabolic activity of the liver, perfusion experiments were performed with either [1- 14 C]oleoylanilide or oleoyl[U- 14 C]anilide, previously synthesized by the method of Casals *et al.* (1983). After 15 min, the perfusion medium was extracted by a modification of the Folch-Lees procedure (Guarnieri *et al.*, 1976) and the lipid products were separated by t.l.c. on silica-gel G plates. Almost 100% of the radioactivity could be recovered from the area of the plate corresponding to the R_f of oleoylanilide. The radioactivity present in the final membrane preparations indicates the existence of approx. 13 μ g of oleoylanilide/mg of membrane protein. Thus oleoylanilide partitions into the hydrophobic portion of the membrane. The experiments carried out with oleoyl[U- 14 C]anilide indi-

Fig. 1. *Microviscosity of liver plasma membranes after treatment with oleoylanilide*

Liver plasma membranes were incubated with increasing concentrations of either oleic acid ($\blacktriangle$) or oleoylanilide ($\bullet$). Fluorescence polarization, with DPH, was measured at 25°C and the *P* values were corrected by the method of Lentz *et al.* (1979) with the use of an unlabelled solution of the same composition. Microviscosity values ($\bar{\eta}$) expressed as Pa·s, were calculated as described in the Materials and methods section. Each experimental point represents the mean $\pm$ s.d. of triplicate determinations of three different experiments.

cate that there is no radioactive product bound to the protein components of the plasma membranes, when tested by polyacrylamide-gel-electrophoretic analysis. In addition, previous kinetic studies in-

volving the feeding of rats with a single dose of oleoyl[U-³H]anilide showed its rapid uptake by liver ($t_{\frac{1}{2}}$ for $k_{in} = 0.66$ h) and its long hepatic presence ($t_{\frac{1}{2}}$ for $k_{out} = 58.8$ h) (Camón *et al.*, 1983).

Discussion

DPH has been used widely to investigate the dynamic properties of plasma membranes. This compound monitors the fluidity within the hydrophobic portion of the lipid bilayer (Shinitzky & Barenholz, 1974, 1978). From the above data it can be concluded that oleoylanilide *in vivo* and *in vitro* fluidizes the hydrophobic region of the membrane. This diminution of microviscosity can be attributed to the anilide and not to any of the hydrolysis products, thus indicating that oleoylanilide partitions into the membrane, altering its fluidity properties. The similar increases in membrane fluidity produced by oleoylanilide under different experimental conditions, in addition to the clear presence of oleoylanilide in plasma membranes after liver perfusion and its rapid uptake in experiments *in vivo*, allow us to attribute the increase in fluidity to the presence of the additive. This effect does not involve membrane proteins, since they are not modified in the present experimental conditions. These facts suggest that oleoylanilide modifies either lipid-lipid or lipid-protein interactions through a non-specific disordering effect on the packing of the cell membranes.

This model for the interaction of long-chain fatty acid acylanilides with cell membranes may be useful, at least partially, for the aetiopathogenic interpretation of the toxic syndrome described

above and should be extended to other cells more directly involved in the organ damage, such as fibroblasts, endothelial cells etc.

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