Peroxisome Enzyme Modification and Oxidative Stress in Rat by Hypolipidemic and Antiinflammatory Drugs

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ABSTRACT

Changes in the activities of two peroxisomal enzymes (catalase and thiolase), some parameters related to oxidative situations, such as conjugated dienes, zinc, iron, copper and superoxide dismutase after the administration of two known peroxisome proliferators (clofibrate and acetylsalicylic acid), and two drugs pharmacologically related to the former (probucol and diflunisal) have been studied in male Wistar rats. Administration of the drugs was made by p.o. for 15 days. After the treatment the rats were killed, their livers and brains were taken out, and their blood was collected. Peroxisomes were purified by differential centrifugation followed by ultracentrifugation. Total RNA was also extracted and the acyl CoA oxidase mRNA expression was studied. Clofibrate was inactive on both enzymes studied in liver and diflunisal in brain. However, the acyl CoA oxidase mRNA expression increased by clofibrate treatment. Results are justified by the liposolubility and protein-binding properties of the drugs. Otherwise, the present results show the existente of an increased lipid peroxidation, lower value of superoxide dismutase, and variable results for zinc, copper and iron trace elements. These data evidence an oxidative stress situation in plasma of rats treated with these drugs, probably as a consequence of an increase in some β-oxidation enzymes, which brings about an overproduction of H_2O_2.

KEY WORDS

Peroxisome, Hypolipidemic drugs, Antiinflammatory drugs, Oxidative stress.

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Peroxisomes are subcellular organelles that are universally found in animal cells, except mature erythrocytes, (15) and are characterized by their high content in certain enzymes (oxidases and catalase) (57) of the H_2O_2 metabolism. Hydrogen peroxide is produced by peroxisomal oxidases (15) and is highly toxic and can generate an important number of free radicals ($O_{2\cdot}$, ·OH, RO·, RO_2·, RS·). Oxidative stress can be induced by a large increase in the H_2O_2-producing peroxisomal β-oxidation (10 to 30 fold increase), which the H_2O_2-destroying enzyme catalase may not confront (46). This peroxisomal β-oxidation increase may be generated by the peroxisome proliferators (16, 30, 42).

Clofibrate causes hepatomegalia and hepatic peroxisome proliferation in rats, mice and hamsters (45). Other drugs structurally unrelated to clofibrate like acetylsalicylic acid (29) or tibric acid (45) were also identified as peroxisome proliferators. Some xenobiotics, including fibrate hypolipidemic drugs, phtalate plasticizers, phenoxy acid herbicides and perflurinated fatty acids, induce the proliferation of the peroxisomal population as well as the activity of its oxidases (47). The H_2O_2 accumulated inside the peroxisome can leave the organelle and generate an oxidative stress situation. Reactive free radicals can produce lipid peroxidation (2), oxidation of proteins and DNA (25), changes in enzyme activities and in redox status of NADPH (23) and carcinogenesis (9) in cells and tissues (52).

The main mechanism of hydrogen peroxide toxicity is the formation of a highly reactive species in the presence of suitable transition metal catalysts as iron and copper (22, 24). Zinc reduces free radical activity either by a direct inhibitory effect via protecting metalloenzymes or by interacting with polyunsatured fatty acids or other metal ions (51). Superoxide dismutase and H_2O_2 removing enzymes are important intracellular antioxidants (19).

The aim of this work was to study the effect of four drugs on some peroxisomal enzymes, at enzyme activity (catalase, thiolase) level and at molecular level (acyl CoA oxidase), as well as the evaluation of their effects on the peroxidative metabolism. Two drugs, clofibrate and acetylsalicylic acid, and two pharmacologically related to the former, diflunisal and probucol were chosen. The liver was selected for being a central-target organ of many metabolic pathways, and the brain by the neurological manifestations in patients affected by peroxisomal disorders with possible alterations in brain peroxisomes (49).

MATERIALS AND METHODS

Animals and Treatment

Male Wistar rats weighing about 200 g were distributed in five groups (n = 10) and received the following doses: 1) Control rats (Carboxymethylcellulose: CMC); 2) Clofibrate (CLO), 250 mg/kg; 3) Probucol (PRO), 250 mg/kg; 4) Acetylsalicylic acid (AAS), 500 mg/kg; and 5) Diflunisal (DIF), 50 mg/kg (11, 50).

Clofibrate, probucol, acetylsalicylic acid and diflunisal were suspended in 1 % (w/v) CMC (Panreac) with 0.1 % Tween 80 (Merck) and the suspensions were micronized by ultrasonication. Drugs were administered by p.o. once daily for 15 days. Control animals received only CMC 1 % + Tween 80 0.1 % (10 mL/kg).

Purification of peroxisomes from rat liver and brain

When treatment was finished all the animals were killed by decapitation and livers and brains were quickly removed and homogenized in ice-cold 0.25 M sucrose (Merck), pH = 7.4, containing 0.1 % ethanol to prevent the formation of the inactive catalase compound II (10).

Peroxisomes were purified from the light mitochondrial fraction (21) by sucrose density gradient (d = 1.04-1.24 g/mL) ultracentrifugation (31). About 2 mL of the light mitochondrial fraction was layered on the top of the gradient which was prepared as described by BAXTER-GABBARD (4). After ultracentrifugation, 1 mL fractions were collected from the top of the gradient and stored until analysis for density, proteins, catalase, thiolase, monoamine oxidase and cytochrome c oxidase.

Density, Protein and Enzyme Analysis

Density of fractions was determined by measuring the refraction index with an AO TSMeter (Goldberg). Proteins were measured by the method of LOWRY et al. (35) with human serum albumin as standard. Catalase was measured as described by PETERS and MÜLLER (43) based on the oxidant capacity of the H_2O_2. Thiolase activity was estimated by using acetoacetyl CoA as substrate and measuring its absorbance change at 303 nm (56). A molar extinction coefficient of 21400 M^{-1} cm^{-1} for acetoacetyl CoA (54) was used for the calculations. Cytochrome c oxidase and monoamine oxidase were determined according to SOTTOCASA et al (53) and WEISSBACH and SCITH (59), respectively. Superoxide dismutase (SOD) activity in plasma was measured at 30 °C by a modification of the method of ABBÉ and FISCHER (1) which uses xanthine/xanthine oxidase as source of superoxide radicals and the reduction of cytochrome c as the indicator reaction.

Due to the high number of samples to analyze, the enzymatic assays were automated in a COBAS FARA Centrifugal Analyzer (Hoffman-La Roche).

Lipid Peroxidation

Blood was collected from the neck by means of a funnel with heparin, and plasma was obtained by centrifugation and stored at —70 °C until subsequent analysis. Lipid peroxidation was determined by measuring the appearance of conjugated dienes at 240 nm in samples previously extracted with chloroform : methanol (2:1), according to the method of CAWOOD et al. (8).

Cations analysis

Zinc and copper were assayed by atomic absorption spectrophotometry with hollow cathode lamps specific for each metal. Plasma iron concentration was determined by the FerroZine (6) with the Biomerieux Kit (Marcy L'Etoile, France).

RNA

Some pieces of liver and brain for RNA extraction were stored at —80 °C until analysis.

Total RNA from livers and brains was prepared by using the guanidinium-thiocyanate technique (12). The concentration and purity of the RNA were determined by measuring the optical densities at 260, 280, and 310 nm of an aliquot of the final preparation. Electrophoresis of RNA in 1 % agarose-formaldehyde gels was performed according to SAMBROOK and FRITSCH (48). Equal quantities of RNA (50 µg) were loaded on each well and the ethidium bromide staining of the ribosomal bands was compared. After electrophoresis, RNA was blotted by capillarity to a nylon membrane (BioRad), and the membrane was baked at 80 °C under vacuum conditions. Filters were prehybridized and hybridized overnight with the radiolabelled probe of acyl-CoA oxidase (3.8 kb). Air-dried filters were exposed to Kodak X-Omat film at —70 °C using an intesifying screen. An oligonucleotide probe was synthesized on a 391 DNA Synthesizer (Applied Biosystem Inc.). The oligonucleotide probe used for acyl CoA oxidase was 5' ATT AAT TCG AAG GTA GGT CTC CTT CAT GTA CCT TCC TAC GAA GTG GAA 3' (36).

Statistics

Results were analysed using the non-parametric U Mann-Whitney test and the means, standard deviations and correlation coefficients were calculated.

Chemicals

The hypolipidemic drugs and diflunisal were purchased from Sigma, while acetylsalicylic acid was obtained from Merck. All other chemicals were obtained from sources listed as they appear.

RESULTS

Mitochondrial Enzymes

Table I shows the enzyme activities of the different fractions obtained after density-gradient centrifugation. Fractions with the highest activities of the mitochondrial marker enzymes were mainly in the middle of the sucrose-density gradient.

Peroxisomal Enzymes

Enzyme activities were determined in each fraction from sucrose density-gradient centrifugation. The richest fractions in catalase were 9 and 10, which are represented as mean enzyme activity in fraction number 10 (fig. 2). Figure 2a shows the enzyme distribution in the liver fractions. In brain, hypolipidemic drugs were more effective than antiinflammatory agents (fig. 2b).

As in liver catalase, antiinflammatory drugs produced a significantly higher increase of thiolase activity (fig. 3a), although probucol also caused a significant activity increase. Administration of the four drugs produced an increase in brain enzyme activity (fig. 3b), although the change produced by diflunisal was not significant. The most intense effects were produced by probucol.

Lipid Peroxidation

To determine the lipid peroxidation of samples the concentration of conjugated dienes was measured. Values are represented as absorbance units with respect to a blank value, since no standards are available for these substances (9). A minimal but significant increase may be observed in all treated groups with respect to control animals, which received only carboxymethylcellulose (table II).

SOD

Plasma SOD activity was significantly lowered after hypolipidemic and antiinflammatory drug administration (table II).

Trace Elements

A significant decrease in zinc levels was observed in the hypolipidemic treated rats (table II). Copper did not vary in any group and increased values of iron were found in all groups except in group 5.

RNA

Figure 4 shows the electrophoretic pattern of total liver RNA from normal, hypolipidemic and antiinflammatory treated rats stained with ethidium bromide. The expression of the acyl CoA oxidase mRNA is markedly stimulated by clofibrate (fig. 5). This increase is not so great in probucol and acetylsalicylic acid treated rats (positive results are marked with a vertical arrow).

DISCUSSION

Catalase is the marker enzyme of peroxisomes, which equilibrate at 1.2 g/mL (33 and fig. 1), as it represents 16 % of the peroxisome protein content (34). Our results show that fractions 9 and 10 were the richest in catalase, which seems to indicate that peroxisomes were in these fractions represented as mean activity enzyme in fraction 10 of the gradient. Catalase is distributed in a bimodal way, appearing in a greater degree at the top and at the bottom of the gradient (fig. 2a), similar results having been reported (26, 32). Catalase in the low density fractions can be citoplasmatic catalase (39) or it can more likely proceed from the peroxisome split in the homogenisation (18) as occurs with thiolase (fig. 3). Clofibrate does not provoke an increase in catalase in liver, as reported before (13, 35, 58), although results are contradictory (5). Furthermore,

clofibrate and allilisopropylacetamide (an inhibitor of the catalase synthesis) administration produces peroxisome proliferation, without or with a slight increase in catalase activity (37, 44). Antiinflammatory drugs cause a higher increase in the hepatic catalase and thiolase than hypolipidemic drugs, while in brain both hypolipidemic drugs are the most active ones. This phenomenon may be due to the liposolubility and plasma protein binding of the drugs, two limiting factors in hematoencephalic barrier crossing. On the other hand, both enzymes are present in the matrix organelle, being therefore equally exposed to drugs. Another possibility is that the proliferator administration might produce a peroxisomal β-oxidation increase, as well as in H_2O_2 concentration, which can inhibit the thiolase (27).

The results of the expression of the acyl CoA oxidase mRNA induction supports this last hypothesis. The increase of mRNA in some clofibrate treated rats is greater than the increase in probucol and acetylsalicylic treated rats (fig. 5). A peroxidative cellular situation that modulates directly-indirectly the DNA expression could be produced. Some data indicate that the carcinogenity of these drugs is due to their ability to induce oxidative DNA damage in liver as a result of increased levels of transcription of genes of H_2O_2 generating peroxisomal β-oxidation system (38). Also, a mechanism of nonmutagenic carcinogenesis has been discussed (7).

Our results show that there is a slight increase in lipid peroxidation in plasma in agreement with other reports in mice (3) or plants (40).

Current techniques for measuring lipid peroxidation end products are flawed. Application of the diene conjugation assay to human body fluids measures a UV absorbing product that may not be produced by lipid peroxidation (55). In spite of this contraindication, the conjugated diene determination is a useful routine technique for this purpose (17).

The antioxidant properties of probucol are worth noting (41) with lesser conjugated diene formation and iron concentration and higher levels of zinc and SOD, than those of clofibrate. HIRANO et al. (28) described a decreased malondialdehyde after probucol administration. All drugs except diflunisal produce a rise in iron levels which has a harmful role due to the participation in Fenton and Haber Weiss reactions (22). Zinc levels became lowered in the hypolipidemic treated rats but not in the antiinflammatory agent treated ones. These results are valid as zinc reduces free radicals (51). Nevertheless, copper concentration did not change, which indicates that copper performs a less important role in free radical formation (20, 22).

The damaging effect of these oxygen reactive species is prevented by the endogenous scavengers (SOD, GSHPx, catalase, transferrin, etc). However, when excess amounts of oxygen radicals and hydrogen peroxide are formed, the endogenous scavengers are insufficient to react with these active molecules. Decreases in the activity of liver SOD and GSHPx (14, 60) have been described. We have found a significant decrease of SOD, which could be partially justified with the simultaneous stimulation of the lipid peroxidation.

To summarize, the hypolipidemic and antiinflammatory drugs studied produce a modification of some peroxisomal enzymes related to the H_2O_2 metabolism, which could be indicative of an oxidative stress situation.

RESUMEN

Se estudia en ratas Wistar macho, la modificación de dos enzimas peroxisómicos (catalasa y tiolasa) y algunos parámetros relacionados con el estado oxidativo, como los dienos conjugados, zinc, hierro, cobre y superóxido dismutasa, tras la administración por v.o. de dos drogas conocidas como proliferadores peroxisómicos (clofibrato y ácido acetilsalicílico), y otras farmacológicamente relacionadas con ellas (probucol y diflunisal). Tras el tratamiento (15 días) se sacrifican los animales, se extraen hígado y cerebro y se recoge sangre para su análisis. Los peroxisomas se purifican tras ultracentrifugación en gradiente de densidad. En hígado, el clofibrato no modifica la actividad catalasa ni tiolasa, aunque produce un aumento en la expresión del mRNA de la acil CoA oxidasa. Las determinaciones plasmáticas indican un aumento de la peroxidación lipídica, disminución de la superóxido dismutasa, y resultados variables de los elementos traza. Los productos utilizados modifican las enzimas peroxisómicas estudiadas y producen estrés oxidativo.

PALABRAS CLAVE

Peroxisomas, Hipolipemiantes, Antiinflamatorios no esteroideos, Estrés oxidativo.

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Table 1. Mitochondrial enzymes (U) in each gradient fractions (n=10) collected after the sucrose density gradient centrifugation.

F	MAO		COX	
	Liver	**Brain**	**Liver**	**Brain**
1	28 ± 22	113 ± 66	0	0
2	18 ± 8	117 ± 58	0	0
3	22 ± 4	120 ± 42	0	0
4	40 ± 14	201 ± 124	2 ± 1	25 ± 11
5	142 ± 24	235 ± 78	11 ± 4	30 ± 9
6	267 ± 80	253 ± 61	14 ± 3	33 ± 14
7	150 ± 63	425 ± 173	24 ± 2	22 ± 13
8	21 ± 2	123 ± 71	33 ± 9	19 ± 9
9	25 ± 8	45 ± 12	7 ± 3	10 ± 9
10	17 ± 9	46 ± 10	7 ± 4	12 ± 3

MAO: monoamine oxidase (U: nmol kynurenine/min).
COX: cytochrome c oxidase (U: nmol cytochrome c/min).

Table 2. Plasma levels of conjugated dienes (absorbance units), SOD (U/dL), Zn, Fe and Cu (mg/dL).

	Dienes	**SOD**	**Zn**	**Fe**	**Cu**
CMC	0.22 ± 0.15	6±2	122 ± 15	217 ± 26	118 ± 31
CLO	0.39 ± 0.17**	2 ± 1**	109 ± 10**	252 ± 38**	115 ± 23
PRO	0.28 ± 0.1**	3 ± 2**	110 ± 10**	243 ± 32*	116 ± 22
AAS	0.38 ± 0.1**	2.2 ± 1**	121 ± 19	252 ± 45**	130 ± 25
DIF	0.35 ± 0.1***	2.8 ± 0.8***	119 ± 13	208 ± 29	116 ± 43

*$p < 0.1$; ** $p < 0.05$; *** $p < 0.01$.

CMC, CLO, PRO, AAS, DIF: control, clofibrate, probucol, acetylsalicylic acid and diflunisal treated rats, respectively. Values are Mean ± SD. The amount of SOD required to inhibit the rate of reduction of cytochrome c by 50 % is defined as 1 unit of activity.

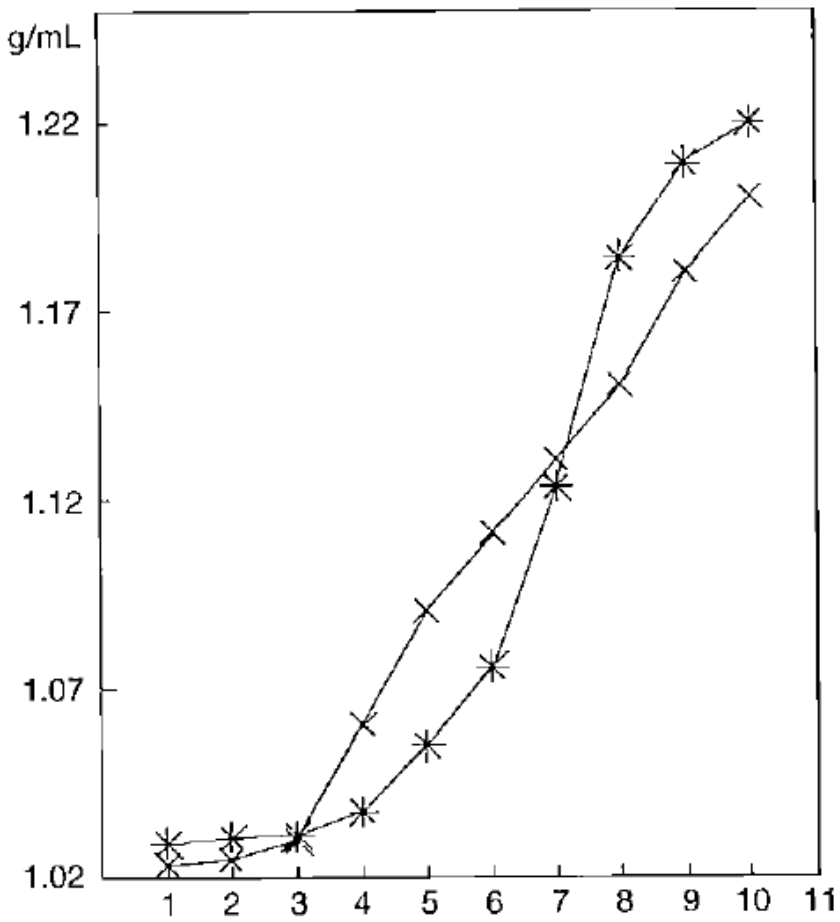

Figura 1. Density in each fraction from liver (*) and brain (x) collected after the sucrose gradient centrifugation.
Light mitochondrial fraction obtained was centrifuged to purify the peroxisomes.

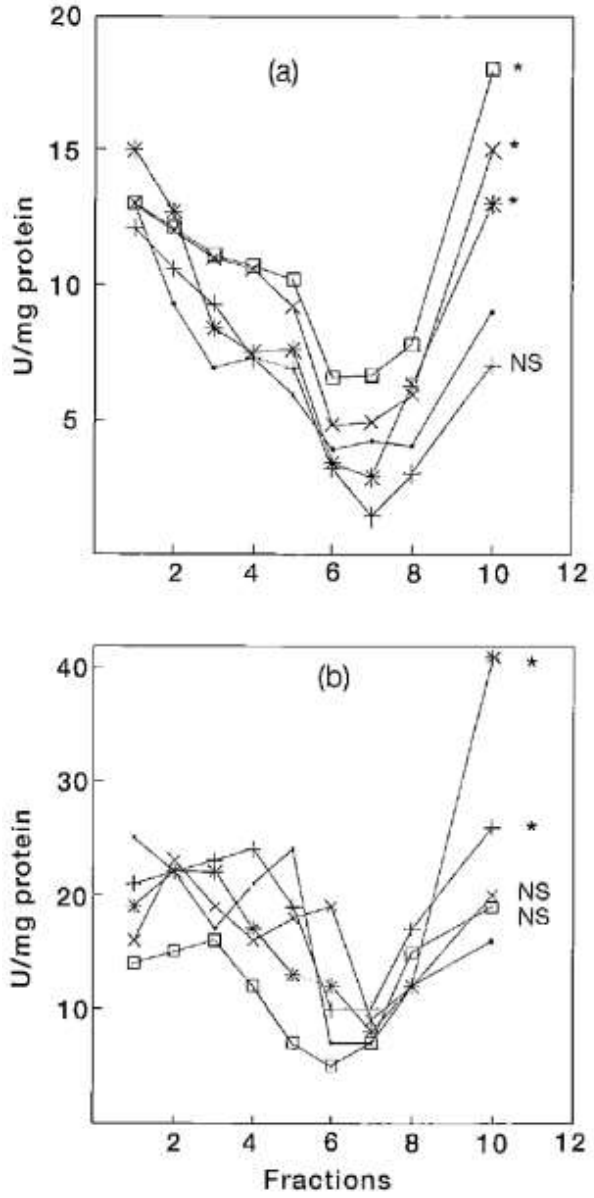

Figura 2. Liver (a) and brain (b) catalase activity (U: mmol H_2O_2/min) in each fraction collected after the sucrose gradient centrifugation.

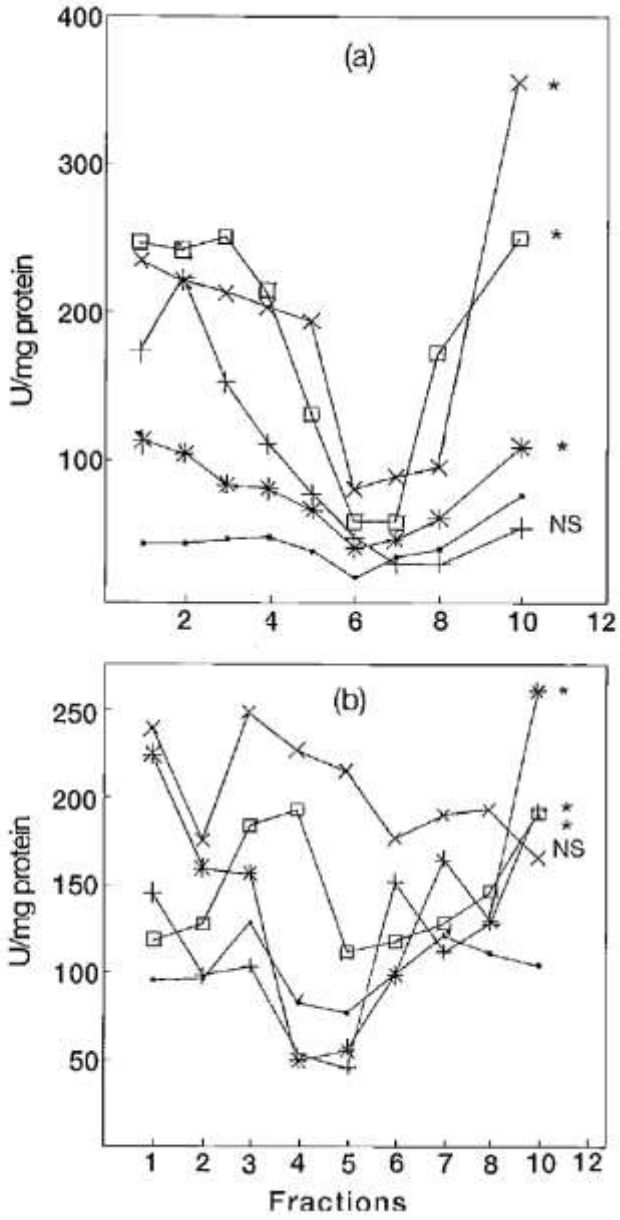

Figura 3. Liver (a) and brain (b) thiolase activity (U: µmol acetoacetyl CoA/min) in each fraction collected after the sucrose gradient centrifugation.
Legend as in fig. 2.

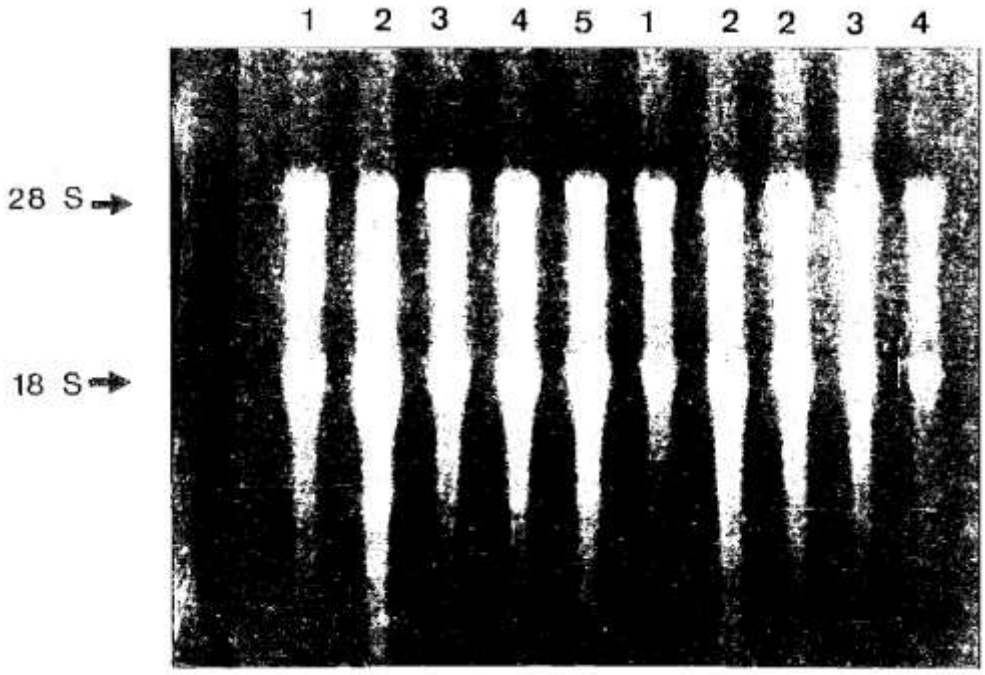

Figure 4. RNA (50 µg) electrophoresis of liver samples.
18 S and 28 S ribosomal RNA is shown. (1: Control Rats, 2: Clofibrate-treated rats, 3: Probucol-treated rats, 4: Acetylsalicylic acid-treated rats, 5: Diflunisal-treated rats).

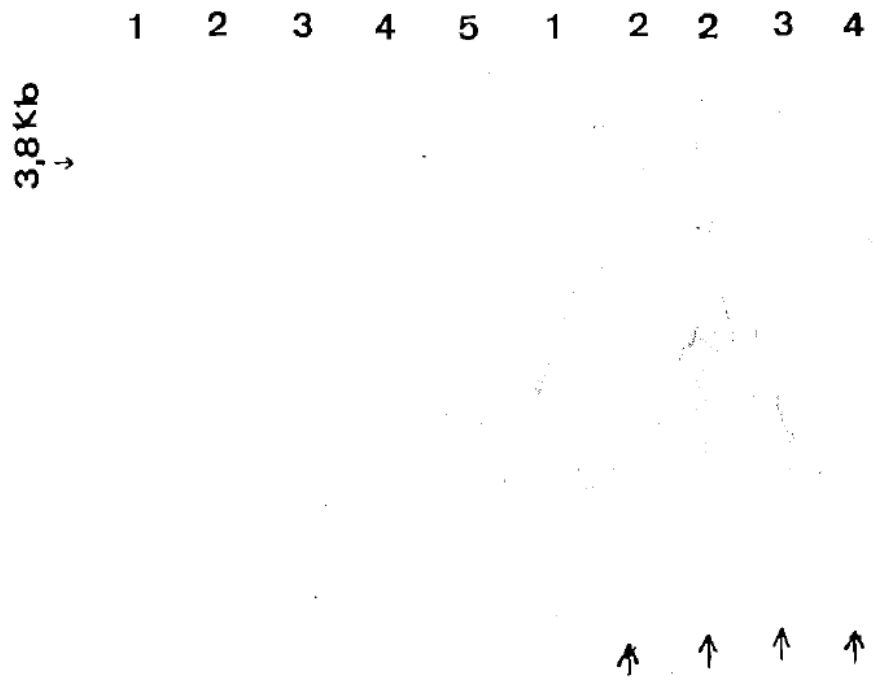

Figure 5. Expression of acyl CoA oxidase mRNA (3.8 kb) after drug treatment.
After electrophoresis RNA was blotted to a nylon membrane and hybridized with the radiolabelled probe of acyl CoA oxidase (3.8 kb). Legend as in fig.4. In clofibrate, probucol, and acetylsalicylic treated-rats 2 of 3, 1 of 2, and 1 of 2, respectively, were positive.