EXPERIMENTAL STUDY

Effect of clofibrate on the enzymes associated with oxidative stress in Wistar rat liver

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Abstract

Background: **A diversity of endogenous and exogenous compounds have been demonstrated to cause proliferation of peroxisomes and variety of associated effects in rodents.**
The aim **of our study was to see the effect of clofibrate on the enzymes associated with oxidative stress.**
Methods: **Male Wistar rats weighting 250–350 g were treated with clofibrate in a dose of 250 mg/1000 g/ /24 h for 12 days. Whole liver homogenates and subsequent subcellular fractions wrere proceeded for enzyme measurements by spectrophotometric methods.**
Results: **The activity of hydrogen producing enzymes D-aminoacid oxidase, Urate oxidase and Palmitoyl CoA oxidase was statistically significantly increased in the treated group in comparison with the control one.**
On the contrary to the massive increase of the Palmitoyl CoA oxidase – a marker enzyme for peroxisomal proliferation, there was only a limited increase of catalase (which inactivates hydrogen peroxide) activity. On the other hand superoxide dismutase and glutathione peroxidase activities in experimental group were down regulated in the treated group.
Conclusion: **Our results support the hypothesis that clofibrate (peroxisomal proliferators) application might produce oxidative stress in rat livers *(Tab. 2, Fig. 8, Ref. 56)* Full Text (Free, PDF) *www.bmj.sk*.**
Key words: Wistar rats, clofibrate, oxidative stress.

In the beginning of the 1960‘s it was found that clofibrate (ethylchlorophenoxybutirate) has hypolipidemic effects in rats and in men (1). Further studies on rats revealed that this compound, as well as several other structural analogues of clofibrate has been also shown to cause peroxisomal proliferation (2). In addition, it has been found that a large number of compounds structurally unrelated to clofibrate can cause the same effect (2). The class of xenobiotics referred to as peroxisome proliferators (PPs) encompasses a large number (>1000 at present) of structurally dissimilar and wide-spread foreign compounds. These compounds include numerous industrial chemicals, agrochemicals and important clinical drugs and exposure of humans to them is common (3). The effects of the so-called peroxisomal proliferators are numerous and not always correlated and may occur at different time-points. These effects are usually sustained throughout the treatment and the withdrawal of the compound results with abolishment of the effects and a return to the control state. Effects associated with peroxisomal proliferation in mouse and rat liver are decrease in body weight; liver weight enlargement; morphological changes: increased number and size of peroxisomes, increased number of mitochondria, increased amount of smooth endoplasmic reticulum; and biochemical changes (4, 5). Biochemical changes include: selective induction of peroxisomal enzymes-marked up-regulation of fatty acid ß-oxidation enzymes, but only a small increase in catalase activity, up-regulation of microsomal fatty acid enzyme activities, up-regulation of carnitine acetyltransferase and soluble epoxide hydrolase activities and down-regulations of glutathione peroxidase, superoxide dismutase, glutathione transferase and peroxisomal oxidases other than acyl-CoA oxidase (6–11). Sensitivity to peroxi-

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somal induction by peroxisomal proliferators varies greatly among animal species and tissues. Rats and mice are highly sensitive hamsters are intermediate and guinea pigs, primates and humans are relatively insensitive (7, 12). Effects associated with peroxisome proliferation in rodent liver depend on the test compound, dose and duration of treatment (12). Long-term treatment of rodents with PPs has been found to cause hepatocarcinogenesis and there are also reports of tumor formation in other organs, such as the pancreas and testis (13–15). The different mechanisms for this carcinogenicity include oxidative stress, increased cell proliferation, inhibition of apoptosis and promotion of spontaneously formed preneoplastic lesions in the liver (16–19). Oxidative stress occurs when there is disturbance in the cellular balance of prooxidants (that can produce an reactive oxidant specieses) (ROS) and antioxidants in favor of the prooxidants leading to potential damage. ROS are produced the sequential addition of electrons to molecular oxygen to produce superoxide, hydrogen peroxide, hydroxyl radical, peroxinitrite superoxide and nitric oxide and singlet oxygen (20). Protection against ROS is afforded by specific degradation enzymes, antioxidant vitamins and non-enzymatic substances (ascorbic acid, vitamin E, carotenoids, ubiquinone) and other radical scavengers, as well as by cellular repair mechanisms (20–25). Even under physiological conditions oxidative phosphorylation in mitochondria and number of peroxisomal processes are associated with production of superoxide radical, hydrogen peroxide and related metabolites. Many cellular defense mechanisms against ROS are enzymatic. Superoxide is converted to hydrogen peroxide by superoxide dismutase (SOD) in the cytosol, mitochondria and peroxisomes, and hydrogen peroxide is in turn converted to water and oxygen, primarily by catalase in peroxisomes and glutathione peroxidase (GPX) in the cytoplasm. The excessive production of H_2O_2 (ROS) by oxidases and by cytochrome P450 enzyme system, along with the observed loss of antioxidant protection by loss of activities of catalase in peroxisomes and CuZn-SOD in cytoplasm, may be the critical factors in peroxisomal proliferator-induced oxidative stress.

The objective of this study was to see:

– Whether antioxidant enzymes that facilitate the removal of ROS were modulated following short-term administration of clofibrate,

– Whether the modulation was in the same pattern as the pro-oxidant one,

– And whether there was difference of the modulation of enzyme activity in different sub cellular fractions of Wistar rat liver.

Material and methods

Chemicals

Clofibrate was purchased from Sigma Chemicals. In order to prepare the dose of 250 mg/1000 g/24 h we have weighted 5 g of Clofibrate and dissolved it in 80 ml of 0.2 mol/L NaOH, than neutralized with HCl to pH 7–8 and filled up with sterile water till 100 ml.

Experimental design

The study was performed in male Wistar rats weighting 250–350 g divided in two groups: control and experimental one; n=14 for each group. Animals were obtained from the Animal Facility of the Department of Preclinical and Clinical Pharmacology and toxicology, Medical Faculty, Skopje. Animals were kept 5 in cages, maintained under controlled light (12-h light/dark) cycle and temperature conditions (25°C), fed a normal rat chow and had a free access to tap water. The clofibrate was applied in a dose of 250 mg/1000 g/ 24 h as a single dose by intrapeitoneal (i.p.) injection for 12 days. Control groups were injected i.p. with sterile saline. The weight of the animals was monitored every day.

At the end of the experiment rats were starved over night and sacrificed by cervical decapitation. The livers were dissected out, weighted and homogenized in 0.25 M sucrose pH 7.4 using three times up-and-down strokes of a Potter-Elvehjem homogenizer at 440 rpm. For subcellular fractionation 20 % whole liver homogenates were used. Homogenates were processed for differential centrifugation using high-speed centrifuge Janezki K 24 and nuclear, heavy mitochondrial (enriched in mitochondria); light mitochondrial (enriched in peroxisomes) and supernatant (mycrosomal and cytosolic fraction) were obtained at the end of subcellular fractionation. All the preparations of the samples were performed on +4 °C. Samples were frozen for enzyme measurement on -70 °C.

Enzyme assays

The activity of the enzymes was measured on a PU 8630 UV-VIS spectrophotometer.

Assay *of D-aminoacid oxidase (EC.1.4.3.3)* was done according to Baudhin et al (26).

After the oxidation of D-alanine in aerobic conditions hydrogen peroxide and pyruvic acid are released. The last one reacts with 2,4-dinythrophenolhydrasone (DNFH) producing hydrasone. Adding of NaOH stops the reaction. Absorption of the colored product is proportional with the activity of the enzyme.

The activity was expressed as μmol/ml/min/g wet tissue.

Urate oxidase (EC.1.7.3.3) was measured employing spectrophotometric method by Bergmeyer (27).

Decomposition of uric acid to alantoin by urate oxidase was monitored at the length weight of 292 nm. The decrease of the absorbency is proportional with the activity of urate oxidase and was expressed as μmol/ml/min/g wet tissue.

Palmitoyl CoA oxidase was measured according to spectrophotometric method of Hryb and Hogg (28).

In the process of α-oxidation with Palmitoyl CoA oxidase NAD+ is reduced to NADH that is recorded on 340 nm. The enzyme activity was expressed in μmol/ml/min/g wet tissue.

Assay of *catalase (CAT; EC.1.11.1.6) activity*:

Catalase activity was measured at 25 °C by the method of Aebi (29). The decomposition of substrate H_2O_2 was monitored spectrophotometrically at 240 nm for 3 minutes. Activity was

expressed as k (rate constant of the first order reaction as defined by Aebi) in mmol/ml/min/g wet tissue.

Assay of superoxide dismutase (SOD; EC.1.15.1.1) activity:

SOD activity was measured with RANSOD kits (cat. No. SD 125; Randox Labs., Crumlin, North Ireland) (30). This method employs xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)/5-phenilthetrazolium chloride (INT) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. All diluted sample rates were converted into percentages of the sample diluents rate, and substracted from 100 % to give a percentage of inhibition. The activity was measured at 37 °C, and absorbency was monitored at 505 nm for 3 minutes. The unit of activity is defined as the amount of enzyme that inhibits the rate of the formazan dye formation by 50 %.

SOD unites were obtained from standard curve using percentage inhibition of the samples and the activity was expressed as mmol/ml/minunits/g wet tissue. Standards were prepared by diluting a commercial SOD preparation (cat. No. SD 125) in order to obtain a standard curve.

Assay of *glutathione peroxidase (GPX; EC.1.11.1.9) activity*:

GPX activity was determined with Ransel kits (Cat. No. RS 505; Randox Labs.; Crumlin, North Ireland) at 37 °C at 340 nm for 3 minutes. This assay based on the method of Paglia and Valentine, requires cumene hydroperoxide as a substrate (31). The final concentration of the reagents in the assay was those recommended by the manufacturer. The activity of GPX was expressed in mmol/min/g-wet tissue.

Enzyme assays of each sample were performed in duplicate and each result represents the results from the three independent experiments.

Data analyses

Statistical analyses were performed using the commercial statistical package Statistical for Windows Version 5.0. Results were expressed as means x±SD. Comparisons were made using the Student t test. The level of significance was $p<0.05$.

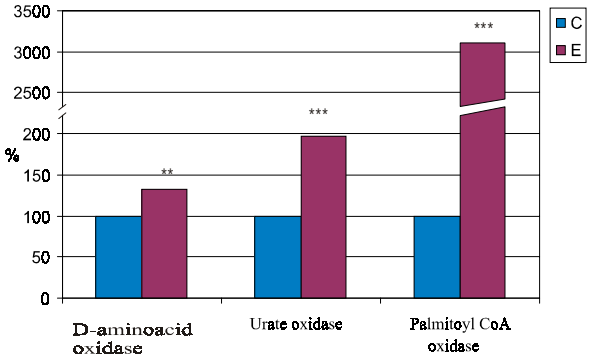


Fig. 1. Percentage ratio of total activity of the hydrogen peroxide producing enzymes in homogenate of experimental group treated with clofibrate (E) compared to control group (C) (100 %) ** p<0.01; *** p<0.001.

Results

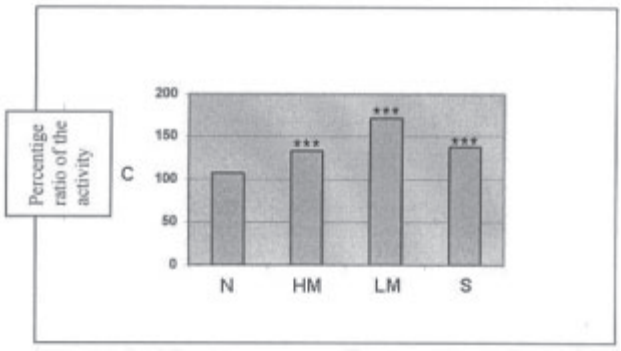
Total activities of the hydrogen peroxide producing enzymes: D-aminoacid oxidase, urate oxidase and palmitoyl CoA oxidase in homogenate and in the subcellular fractions are presented in the Table 1 and Figure 1.

The data from the Figure 1 show that activities of D-aminoacid oxidase, urate oxidase and palmitoyl CoA in homogenate were increased for 31 %, 97 %, and 3002 % respectively in comparison with the control group. The increase of the activity was present in all subcellular fractions: in heavy mitochondrial fraction (33 % for D-amino acid oxidase, 125 % for urate oxidase and 3900 % for palmitoyl CoA oxidase) in light mitochondrial fraction (70 % for D-amino acid oxidase, 90 % for urate oxidase and 2600 % for palmitoyl CoA oxidase) and in supernatant (cy-

Tab. 1. Hydrogen peroxide producing enzymes activities in Wistar rats liver treated with clofibrate (experimental group) (n=14) expressed as µmol/ml/min/g liver tissue (x̄±SD) and percentage ratio of the activities compared with the control group (100 %).

H and SF	Enzymes								
	D-amino acid oxidase			Urate oxidase			Palmytoil CoA oxidase		
	Control	Experimental	Percentage ratio from the control (100%)	Control	Experimental	Percentage ratio from the control (100%)	Control	Experimental	Percentage ratio from the control (100%)
H	0.91±0.05	1.21±0.25**	+31.9	1.27±0.36	2.51±0.9***	+97.6	0.118±0.013	3.66±1.14***	+3002
N	0.20±0.02	0.21±0.03	+7.1	0.16±0.05	0.34±0.11***	+112.5	0.018±0.011	0.73±0.2**	+3972
HMF	0.20±0.01	0.27±0.04***	+33.3	0.33±0.16	0.75±0.32*	+125.2	0.023±0.008	0.94±0.2***	+3986
LMF	0.15±0.01	0.26±0.054x***	+71.7	0.43±0.26	0.83±0.4**	+92.1	0.059±0006	1.63±0.5***	+2663
S	0.37±0.03	0.51±0.04***	+34.67	0.31±0.04	0.45±0.06***	+45.2	0.017±0.03	0.29±0.05**	+1606

H – homogenate; N – nuclear fraction; HMF – heavy mitochondrial fraction; LMF light mitochondrial fraction; S – supernatant; SF – subcelular fractions; ** p<0.01; *** p<0.001



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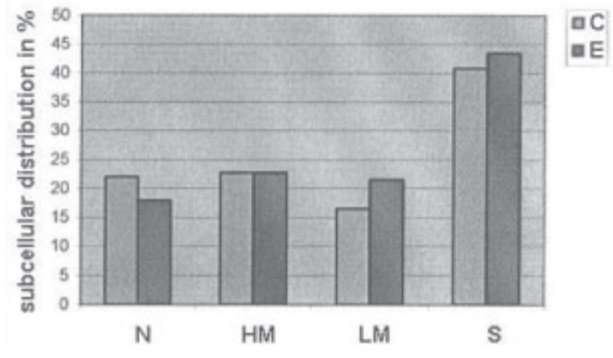

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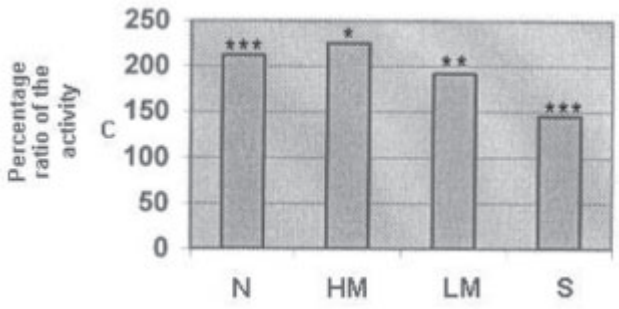
Fig. 2. Percentage ratio of total activity of D-amino acid oxidase in the subcellular fractions of the experimental group treated with clofibrate (E) compared to control group (C) (100%) (A) and subcellular distribution expressed as a % of the homogenate (100 %) (B); N – nuclear fraction; HM – heavy mitochondrial fraction; LM – light mitochondrial fraction; S – supernatant; *** p<0.001.



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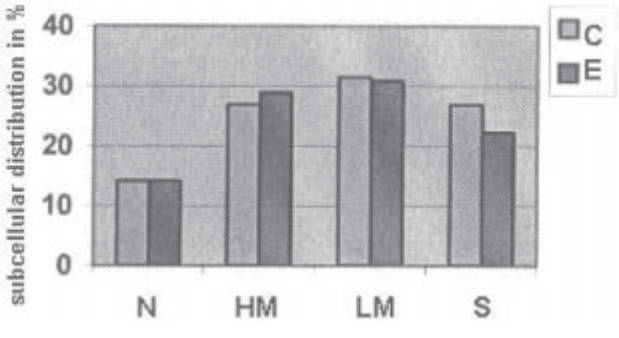

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Fig. 3. Percentage ratio of total activity of Urate oxidase in the subcellular fractions of the experimental group treated with clofibrate (E) compared to contrrol group (C) (100 %) (A) and subcellular distribution expressed as a % of the homogenate (100 %) (B); N – nuclear fraction; HM – heavy mitochondrial fraction; LM – light mitochondrial fraction; S – supernatant; * p<0.05; ** p<0.01; *** p<0.001.

tosol and mycrosomal fraction) (30 % for D-amino acid oxidase, 45 % for urate oxidase and 1600 % for palmitoyl CoA oxidase) (Tab. 1).

The activity and the distribution of D-amino acid oxidase in subcellular fractions in treated group in comparison with the control group are presented on Figure 2 (A, B). D-amonoacid oxidase showed up-regulation of the activity in all subcellular fractions, the most prominent in light mitochondrial fraction (enriched with peroxisomes) (70 %) in comparison with the control group (Fig. 2A). Changes in the distribution upon the treatment were observed in light mitochondrial fraction and in supernatant and were in the positive directions (Fig. 2B).

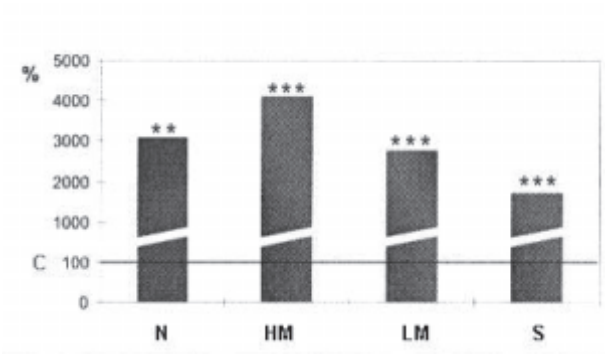
The activity and the distribution of urate oxidase in the subcellular fractions in the treated group in comparison with the control group are presented on Figure 3 (A, B). The activity was increased in all subcellular fractions in the treated group in comparison with the control one (Fig. 3A). Subcellular distribution showed maximum distribution in light mitochondrial fraction (enriched in peroxisomes) with no distribution changes in this fraction upon the clofibrate treatment in comparison with the control group (Fig. 3B).

The activity and subcellular distribution of Palmitoyl CoA oxidase are presented on Figure 4 (A, B). The activity showed statistically significant massive increase (up to 400 times) in all fractions of clofibrate treated group in comparison with the control group (Fig. 4A). Subcellular distribution showed maximum distribution in light mitochondrial fraction in both control and treated group. Redistribution after the treatment with clofibrate in a positive direction was observed in heavy mitochondria fraction and in a negative direction in the light mitochondrial fraction and in the supernatant (Fig. 4B).

Total activity of antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) is presented on Table 2 and Figure 5.

The data in the Table 2 and Figure 5 showed that only catalase activity has been statistically significant increased in clofibrate treated group in comparison with the control one ($p<0.001$). There was statistically significant decrease of SOD and GPX activity ($p<0.05$) in the homogenate of clofibrate treated group in comparison with the control one.

The data of the activity of catalase in subcellular fractions and distribution are presented in Figure 6 (A, B). Catalase activ-



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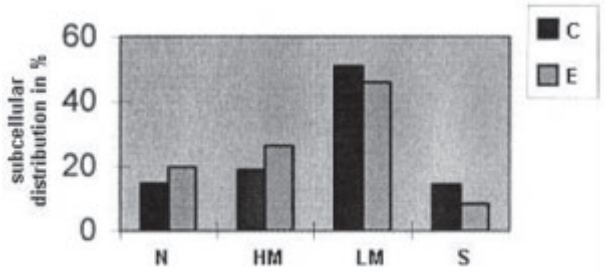

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Fig. 4. Percentage ratio of total activity of Palmitoyl CoA oxidase in the subcellular fractions of the experimental group treated with clofibrate (E) compared to contrrol group (C) (100 %) (A) and subcellular distribution expressed as a % of the homogenate (100 %) (B); N — nuclear fraction; HM – heavy mitochondrial fraction; LM – light mitochondrial fraction; S – supernatant; ** p<0.01; *** p<0.001.

ity was statistically significant increased in all fractions in the experimental group in comparison with the control one ($p<0.001$) (Fig. 6A). There was a positive redistribution of the

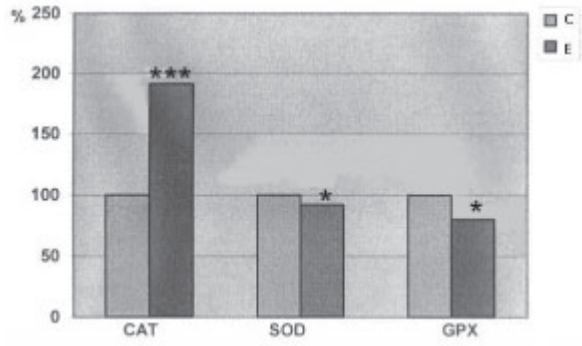


Fig. 5. Percentage ratio of total activity of antioxidant enzymes in homogenate of experimental group treated with clofibrate (E) compared to control group (C) (100 %); CAT – catalase; SOD – superoxide dismutase; GPX – glutathione peroxidase; * p<0.05; *** p<0.001.

activity in the supernatant and a negative redistribution in heavy and light mitochondrial fraction after the treatment with clofibrate (Fig. 6B).

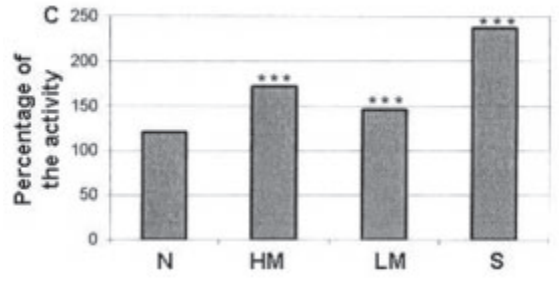
The data of the activity of superoxide dismutase in the subcellular fractions and the distribution are presented in Figure 7 (A, B). SOD activity was statistically significant decreased in all fractions except in supernatant ($p<0.01$ and $p<0.001$) (Fig. 7A). Superoxide dismutase activity showed maximum distribution in supernatant both in the control and in the clofibrate treated group. No changes in the distribution of the SOD activity were observed after the treatment with clofibrate (Fig. 7B).

The data of the activity of glutathione peroxidase presented in Figure 8 (A, B) showed slight statistically non-significant decrease of the activity in all sub-cellular fractions except in nuclear fraction (Fig. 8A). GPX showed maximum distribution in supernatant (Fig. 8B). There were redistributions of the activity in heavy mitochondrial fraction and supernatant in comparison with the control group that were in the positive directions (Fig. 8B).

Tab. 2. Total activity of antioxidant enzymes in Wistar rat livers treated with clofibrate in a dose of 250 mg/100 g/24 h (E) expressed as mmol/ml/min/g tissue (x±SD, n̄=14) and percentage ratio (%) compared to the control group (100 %) (C).

H and SF	Enzymes								
	CAT			SOD			GPX		
	Control	Experimental	Percentage ratio from the control (100%)	Control	Experimental	Percentage ratio from the control (100%)	Control	Experimental	Percentage ratio from the control (100%)
H	104.6±16.4	200.1±16.7***	+91.3	2.3±0.18	2.089±0.17*	-8	17.86±1.71	14.3±3.7*	-19.9
N	7.98±2.5	9.6±4.3	+20.3	0.035±0.005	0.028±2.7***	-21.5	3.3.2±1.7	3.2±1.7**	-3.4
HMF	24.6±2.28	42.2±7.6***	+71.6	0.142±0.013	0.121±0.01***	-14.9	2.52±0.8	2.44±0.5	-3.2
LMF	28.9±4..5	42.2±8.4***	+45.9	0.176±0.017	0.141±0.02**	-19.5	0.4±0.06	0.36±0.1	-4.2
S	43.1±4.93	102.0±23.6***	+137.2	1.71±0.2	1.64±0.16	-6.5	11.0±1.3	9.77±1.6	-11.2

H – homogenate; N – nuclear fraction; HMF – heavy mitochondrial fraction; LMF light mitochondrial fraction; S – supernatant; SF – subcelular fractions; CAT – catalase; SOD – superoxide dismutase; GPX – glutathione peroxidase; * p<0•05; ** p<0.01; *** p<0.001



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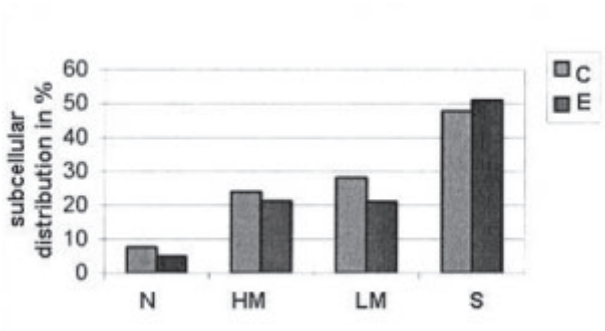

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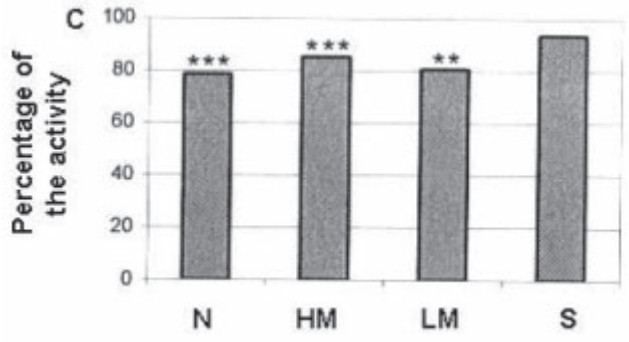
Fig. 6. Percentage ratio of total activity of catalase in the subcellular fractions of the experimental group treated with clofibrate (E) compared to contrrol group (C) (100 %) (A) and subcellular distribution expressed as a % of the homogenate (100 %) (B); N — nuclear fraction; HM – heavy mitochondrial fraction; LM – light mitochondrial fraction; S – supernatant; *** p<0.001.



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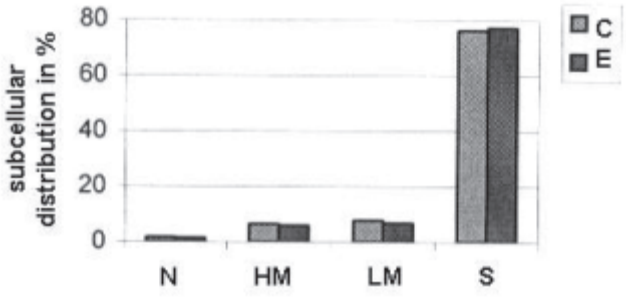

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Fig. 7. Percentage ratio of total activity of Superoxide dismutase in the subcellular fractions of the experimental group treated with clofibrate (E) compared to contrrol group (C) (100 %) (A) and subcellular distribution expressed as a % of the homogenate (100 %) (B); HM – heavy mitochondrial fraction; LM – light mitochondrial fraction; S – supernatant; ** p<0.01; *** p<0.001.

Discussion

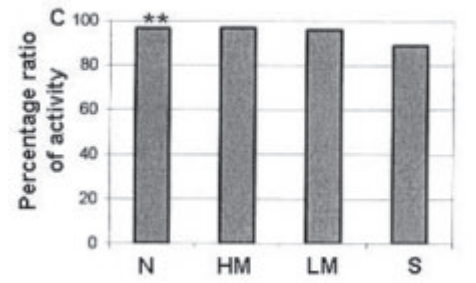
The antitriglycedemic effect of clofibrate in human is related to the increased catabolism of serum triglycerides (TG)-rich proteins (VLDL and VLDL remnants), but not to any effect on hepatic TG or VLDL synthesis and release from liver. The action of clofibrate is related to an increase in adipose tissue or muscle LPL activity that accelerates the rate of intravascular catabolism of VLDL to IDL and LDL and is used in the treatment of hyperlipidemia type III and severe hypertriglyceridemia (32).

It well known that clofibrate, as well as other hypolipidemic drugs cause peroxisomal proliferation and variety of associated effects in rodents.

Long-term treatment of rodents with peroxisomal proliferators has been found to cause hepatocarcinogenesis and there are also reports of tumor formation in other organs, such as pancreas and testis (13–15, 33–36).

One of the proposed possible mechanisms underlying the hepatocarcinogenicity of peroxisomal proliferators is the hypothesis of oxidative stress, which does not exclude the other two proposed hypotheses of increased cell proliferation and inhibition of apoptosis (37, 38). Oxidative stress occurs when there is a disturbance in the cellular balance of prooxidants-oxidants and antioxidants. A unique feature of peroxisomes is their high content of several hydrogen-producing oxidases (39). Treatment with peroxisome proliferators results in a massive increase in maximal capacity to produce hydrogen peroxide, primary through up-regulation of the peroxisomal β-oxidation of fatty acids, as well as through up-regulation of peroxisomal oxidases other than acyil CoA oxidase (3). Our results showed statistically significant massive up-regulation of Palmitoyl CoA oxidase (300 % up-regulation of the activity in homogenate and in heavy and light mitochondrial fraction) wit no changes in the subcellular distribution upon the treatment with clofibrate, that is consistent to the findings of Lazarow, Moody, Watanabe, Hemesley and Kozuka (40–45).

The other two hydrogen peroxide producing enzymes D-aminoacid oxidase and urate oxidase have also shown statistically significant up-regulation of the activity in homogenate, as well as in light and heavy mitochondrial fraction with no changes in the subcellular distribution under the treatment with clofibrate. The increase of the activities of these oxidases is not as prominent as the up-regulation of plamitoyl CoA oxidase (30 % and 100 % respectively versus marked up-regulation of Palmitoyl



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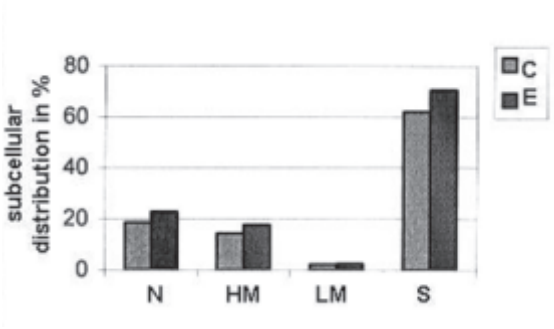

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Fig. 8. Percentage ratio of total activity of Glutathione peroxidase in the subcellular fractions of the experimental group treated with clofibrate (E) compared to contrrol group (C) (100 %) (A) and subcellular distribution expressed as a % of the homogenate (100 %) (B); HM – heavy mitochondrial fraction; LM – light mitochondrial fraction; S – supernatant; ** p<0.01.

CoA oxidase). Our results are consistent with the findings of Angermuller, Moody, Hemsley and Chandoga Schon, Stanko (39, 42, 46–48).

On the contrary to up-regulation of peroxisomal oxidases, antioxidant enzymes superoxide dismutase and glutathione peroxidase have shown statistically significant decrease of the activity (10 % and 20 % respectively) in the homogenate of the treated group. Only catalase showed statistically significant increase of the activity in the homogenate of the treated group in comparison with the control group (90 %). Catalase has shown statistically significant 0.5 to 1 fold increase of the activity in light mitochondrial fraction and in the supernatant respectively, with a redistribution of the activity in the supernatant. Although there is an increase of the catalase activity, still it is not in the same extent as the increase of the Palmitoyl CoA oxidase. On the other hand, SOD has shown statistically significant decrease of the activity in the light mitochondrial fraction. The decrease of the activity in light mitochondrial fraction with redistribution of the activity in the supernatant was noticed for GPX. Our findings are consistent with the results reported by Diez Fernandez, Dobashi, Qu O'Brien (49–52).

Hydrogen peroxide produced by oxidases in peroxisomes is converted to water and oxygen primarily by catalase in the peroxisomes and glutathione peroxidase in the cytoplasm. Superoxide dismutase is converting superoxide to hydrogen peroxide in the cytosol, mitochondria and peroxisomes. Catalase and superoxide dismutase activities have been suggested to act synergistically, since superoxide radicals have been suggested to cause inhibition of catalase and hydrogen peroxide inhibits superoxide dismutase (53–56). Neither catalase, nor glutathione peroxidase, the major hydrogen peroxide-metabolizing enzymes, were up-regulated to the same extent as it was acyl-CoA oxidase upon clofibrate treatment. On the other hand GPX, as well as superoxide dismutase were down regulated. Thus, there would seem to be an imbalance between the production and further metabolism of hydrogen peroxide after the treatment with clofibrate, probably resulting in an increased oxidative stress.

In the conclusion, our study supports the hypothesis that clofibrate treatment might lead to oxidative stress in Wistar rat liver.

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