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# THE EFFECT OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS α (PPARα) AGONIST, FENOFIBRATE, ON LIPID PEROXIDATION, TOTAL ANTIOXIDANT CAPACITY, AND PLASMA PARAOXONASE 1 (PON1) ACTIVITY

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The aim of this study was to investigate the effect of peroxisome proliferator activated receptors α agonist, fenofibrate, on the level of oxidative stress, total antioxidant capacity, and plasma paraoxonase 1 (PON1) activity in the rat. The adult male Wistar rats received fenofibrate for 7 days. The drug was added to food at concentrations 0.005%, 0.05% and 0.5%, which corresponded to doses of 3, 30 and 300 mg/kg/day, respectively. Fenofibrate treatment dose-dependently reduced plasma concentration of malonyldialdehyde and 4-hydroxydialkenals. The level of these lipid peroxidation products in animals treated with 0.005%, 0.05% and 0.5% fenofibrate was lower than in control group by 52.8%, 62.7% and 87.1%, respectively. Lipid hydroperoxides in plasma decreased by 29.7%, 23.4% and 27.5% in these groups, respectively. The drug had no significant effect on total antioxidant capacity measured as ferric reducing ability of plasma (FRAP). Paraoxon-hydrolyzing activity (PON) of plasma paraoxonase was 81.5% lower in animals receiving 0.05% fenofibrate and 69.2% lower in rats treated with 0.5% fenofibrate than in control. Phenyl acetate hydrolyzing activity (arylesterase, AE) was reduced by 15.2%, 49.6% and 55.8% in rats receiving 0.005%, 0.05% and 0.5% fenofibrate, respectively. PON/AE ratio decreased following 0.05% and 0.5% fenofibrate by 64.9% and 30.4%, respectively. The drug had no significant effect on total plasma triglycerides and cholesterol concentrations. The results indicate that fenofibrate treatment favourably modulates oxidant-antioxidant balance and unfavourably affects plasma PON1 activity in normolipidemic rats. These effects can contribute to the influence of PPARα agonists on pathological processes involved in atherogenesis.

Key words: *peroxisome proliferator-activated receptors, fenofibrate, oxidative stress, lipid peroxidation, paraoxonase*

## INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to nuclear hormone receptors superfamily. Upon ligand binding, PPARs heterodimerize with 9-*cis*-retinoic acid RXR receptors and regulate transcription of target genes by binding to peroxisome proliferator response element (PPRE) within their promoters. Three types of PPARs have been identified: PPARα, PPARβ (PPARδ) and PPARγ. (1, 2). PPARα are involved in the regulation of the lipids metabolism in liver, heart, kidney and muscles. Their exogenous agonists, fibrates, effectively lower plasma triglycerides and are widely used in the treatment of hyperlipidemia (3, 4). Although fibrates have been shown to slow the progression of atherosclerosis and to reduce cardiovascular mortality (5-8), the results of some trials are ambiguous demonstrating increased incidence of arrhythmias, myositis, cerebral hemorhages, deterioration of renal function, cancer, and noncardiovascular mortality in patients receiving these drugs (9-13). Therefore, the effect of fibrates on other processes involved in atherogenesis needs to be considered.

Oxidative modification of low density lipoproteins (LDLs) following their exposure to reactive oxygen species (ROS) plays an important role in atherogenesis. Oxidatively modified LDLs impair endothelial function, decrease NO availability, stimulate inflammatory response of monocytes/macrophages, activate migration and proliferation of vascular smooth muscle cells and induce immune response (14). Oxidative stress, i.e. increased ROS production and/or impaired antioxidant defence, may lead to excessive peroxidation of polyunsaturated fatty acids contained in LDL particles and thus can promote atherogenesis. The results of studies concerning the effect of fibrates on oxidative stress are extremely controversial. Some of them demonstrated antioxidant activity of these drugs (15-21), whereas others showed no effect (22-25) or even prooxidant properties of fibrates (26-30). However, most *in vivo* studies were performed in animals (17) or humans (18, 19, 21, 22) with hyperlipidemia, which *per se* modulates oxidant-antioxidant balance and thus could confound the results.

The purpose of this study was to reevaluate the effect of PPARα agonist, fenofibrate, on overall oxidative stress in normolipidemic rats by measuring plasma concentration of lipid peroxidation products and total antioxidant capacity. In addition, we investigated the effect of this drug on plasma paraoxonase 1 (PON1) - the enzyme contained in high density lipoproteins and protecting LDL particles from oxidative damage.

## MATERIALS AND METHODS

### *Experimental protocol*

All studies were performed on adult male Wistar rats weighing 249±3 g. The animals were kept at a temperature of 20±2°C and had free access to food and tap water. The study protocol was reviewed and approved by the Animal Care and Use Committee of the Medical University in Lublin.

After two weeks of acclimation, the animals were randomized into 4 groups. Control group received standard laboratory chow and water *ad libitum*. The remaining groups were treated with different doses of fenofibrate for 7 days. The drug was added to food at concentrations 0.005%, 0.05% and 0.5%. Chow consumption was monitored and recorded daily and the dose of drug was calculated on the basis of food intake and averaged 3, 30 and 300 mg/kg/day, respectively.

After 7 days of drug administration the animals were anaesthetized with pentobarbital (50 mg/kg i.p.) and the blood from abdominal aorta was collected into EDTA-containing tubes (for lipid peroxidation products, antioxidant capacity, cholesterol and triglycerides) and into heparinized tubes (for paraoxonase assay). The animals were sacrificed by a lethal dose of pentobarbital. Blood was centrifuged for 5 minutes at 3 000 rpm at 4°C, plasma was separated, frozen and stored at -25°C until analysis.

### *Biochemical studies*

The concentration of malonyldialdehyde (MDA) and 4-hydroxydialkenals (4-HDA) was measured by the spectrophotometric method using Bioxytech LPO-586 assay kit (31). Lipid hydroperoxides in plasma were assayed by the method of Naurooz-Zadeh et al. (32). Total plasma antioxidant capacity was measured according to the FRAP method (ferric reducing ability of plasma) (33). This test measures total antioxidant capacity determined by nonenzymatic antioxidants; the main contributors in this test are ascorbic acid and uric acid whereas plasma proteins and low molecular weight SH groups containing compounds, such as glutathione, have very low activity in this method (34).

Paraoxonase activity was measured using two synthetic substrates: paraoxon (diethyl-p-nitrophenyl phosphate) and phenyl acetate. The activity toward paraoxon (PON activity) was determined by measuring the initial rate of liberation of p-nitrophenol, which absorbance was monitored at 412 nm at 25°C in the assay mixture (800 µl) containing 1.0 mM paraoxon, 1.0 mM $CaCl_2$ and 20 µl of heparinized plasma in 50 mM glycine/NaOH buffer (pH 10.5). The blank sample (incubation mixture without plasma) was run simultaneously to correct for spontaneous substrate breakdown. The activity was calculated from $E_{412}$ of p-nitrophenol (18 290 $M^{-1}$ $cm^{-1}$) and expressed in U/ml, 1U of enzyme hydrolyzes 1 nmol of paraoxon/minute. Arylesterase (AE), the activity toward phenyl acetate, was assayed on the basis of initial rate of hydrolysis in the assay mixture (3 ml) containing 1 mM substrate, 1 mM $CaCl_2$ in 20 mM Tris HCl (pH 8.0) and 30 µl of plasma diluted 1:8. The absorbance was monitored at 270 nm. Blank sample prepared as described above but without plasma, representing nonenzymatic hydrolysis, was subtracted and the activity was calculated assuming $E_{270}$=1310 $M^{-1}$ $cm^{-1}$. The results are expressed in U/ml; 1 U hydrolyzes 1 mmol of phenyl acetate/min (35).

Plasma triglycerides and total cholesterol were assayed using Sigma-Aldrich kits (Infinity Triglycerides Reagent, Cat. No 343 and Infinity Cholesterol Reagent, Cat. No 401, respectively). Fenofibrate, paraoxon, phenyl acetate and the reagents used in MDA+4-HDA, hydroperoxides and FRAP assay were also obtained from Sigma-Aldrich.

*Statistical analysis*

Data are presented as mean ± SEM from 8 animals in each group. Statistical significance was evaluated by ANOVA followed by Duncan's multiple range test for comparisons of different means. A p value less than 0.05 was considered significant.

## RESULTS

Fenofibrate treatment had no effect on food intake (control group: 19 ± 3 g/rat/day, 0.005% fenofibrate: 20 ± 4 g/rat/day, 0.05% fenofibrate: 21 ± 3 g/rat/day, 0.5% fenofibrate: 19 ± 4 g/rat/day). Food intake, and thus the dose of drug remained stable throughout the experiment. After 7 days of fenofibrate administration body weight was similar in all groups (control: 244 ± 3 g, 0.005% fenofibrate: 246 ± 9 g, 0.05% fenofibrate: 239 ± 6 g, 0.5% fenofibrate: 238 ± 4 g).

Fenofibrate treatment dose-dependently reduced plasma concentration of MDA + 4-HDA. The level of these lipids peroxidation products in animals treated with 0.005%, 0.05% and 0.5% fenofibrate was lower than in control group by 52.8%, 62.7% and 87.1%, respectively (*Fig. 1*). The difference between 0.005%

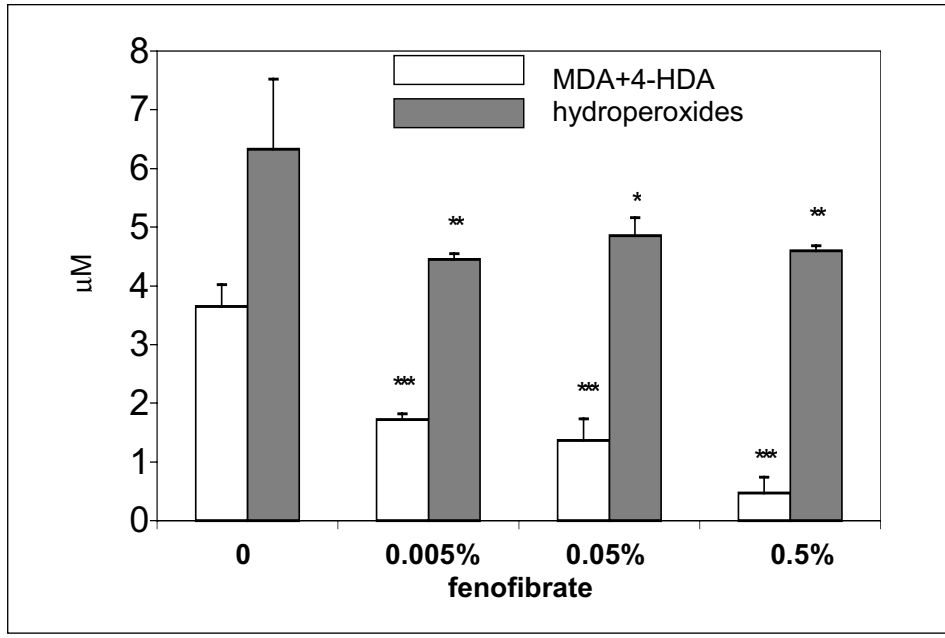


*Fig. 1* The effect of fenofibrate administration on lipid peroxidation products in plasma. Fenofibrate was added to food at concentrations 0 - 0.5% and administered for 7 days and the concentrations of malonyl dialdehyde + 4-hydroxydialkenals (MDA+4-HDA) and lipid hydroperoxides in plasma were determined. Values are expressed in mmol/l and presented as mean ± SEM from n=8 animals in each group. *p<0.05, **p<0.01, ***p<0.001, compared to control by ANOVA and Duncan's test.

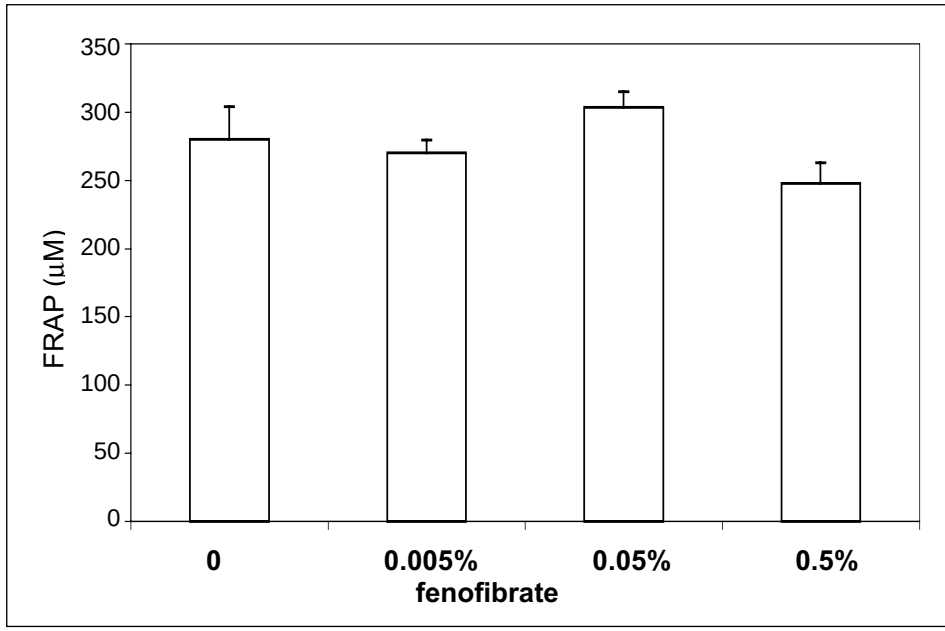


*Fig. 2* The effect of fenofibrate administration on total plasma antioxidant capacity assayed as ferric reducing ability of plasma (FRAP).

and 0.05% groups was not significant but MDA+4-HDA concentration in rats receiving 0.5% fenofibrate was significantly lower than in 0.005% group (-72.7%, $p<0.001$) and than in 0.05% group (-65.4%, $p<0.001$). Fenofibrate administration also decreased plasma concentration of lipids hydroperoxides. However, in this case the effect was not dose-dependent. Maximal reduction (-29.7%) was observed following the lowest dose of drug (0.005%). Higher doses of fenofibrate caused similar decrease in hydroperoxides concentration (-23.4% and -27.5% for 0.05% and 0.5% fenofibrate, respectively). There was no significant difference between groups receiving different doses of PPARα agonist (*Fig. 1*). Taken together, these data indicate that fenofibrate treatment reduces the level of oxidative stress in healthy rats. Fenofibrate had no significant effect on plasma antioxidant capacity (*Fig. 2*).

Fenofibrate administration dose-dependently decreased plasma PON1 activity. The activity toward paraoxon decreased significantly in groups treated with 0.05% and 0.5% fenofibrate by 81.5% and 69.2%, respectively (*Fig. 3*). The difference between both these groups was not significant. The activity in animals receiving 0.005% fenofibrate also tended to be lower than in control, but the difference did not reach the level of significance. PON1 activity assayed using phenyl acetate as a substrate was also reduced following fenofibrate treatment. The reduction was significant even after the lowest dose of PPARα agonist (-15.2%). Higher doses of fenofibrate caused more marked arylesterase inhibition

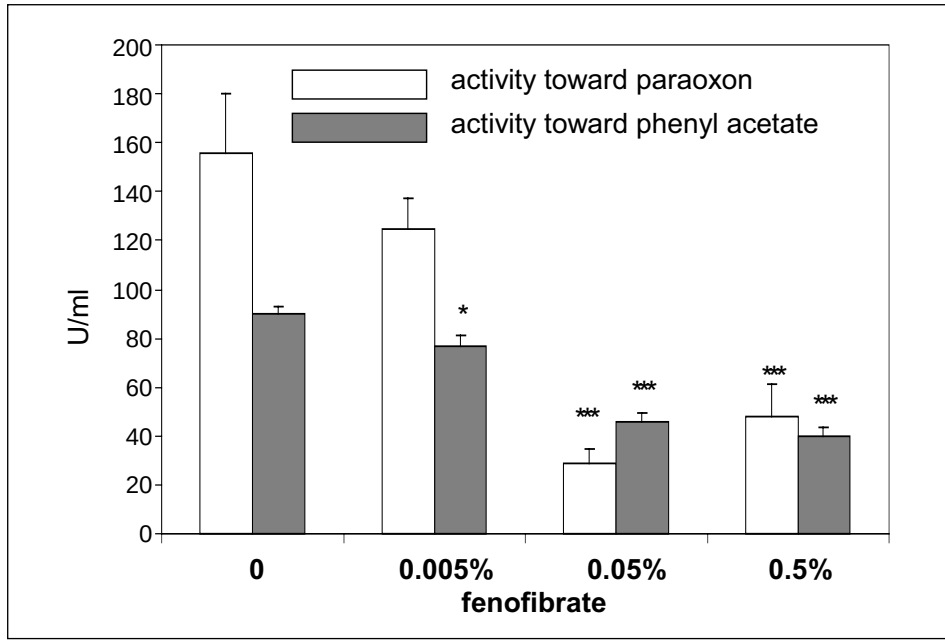


*Fig. 3* The effect of fenofibrate on plasma paraoxonase 1 (PON1) activity. The enzyme activity was assayed using two synthetic substrates: paraoxon (paraoxonase activity, PON) and phenyl acetate (arylesterase activity, AE). Values expressed in U/ml, 1U of PON hydrolyzes 1 nmol paraoxon/minute, 1U of AE hydrolyzes 1 mmol phenyl acetate/minute. *p<0.05, ***p<0.001, compared to control by ANOVA and Duncan's test.

(-49.6% and -55.8% in 0.05% and 0.5% groups, respectively). According to some studies (36, 37) arylesterase activity is less sensitive to modulating factors, such as genetic polymorphism, diet, oxidative stress and acute phase response, and better represents the amount of circulating enzyme than the activity toward paraoxon. Therefore, we calculated PON//AE ratio which can be regarded as a measure of enzyme specific activity. 0.005% fenofibrate did not change PON/AE ratio significantly. In experimental group treated with 0.05% fenofibrate PON/AE ratio was lower than in control by 64.9%. In group receiving 0.5% of this drug PON/AE ratio was also decreased in comparison to control by 30.4%. This difference was marginally significant (p=0.059). PON/AE ratio in this group was, however, 98.3% higher than in 0.05% (*Fig. 4*). These data suggest that fenofibrate most likely decreases not only plasma paraoxonase concentration but also modulates its catalytic activity.

Fenofibrate administration had no significant effect on plasma triglycerides and total cholesterol concentrations (data not shown). Because lipid peroxidation products originate partially from peroxidation of plasma lipoproteins, we calculated the ratios between MDA+4-HDA or hydroperoxides and plasma lipids concentrations (*Fig. 5*). (MDA+4-HDA)/triglycerides ratio decreased following

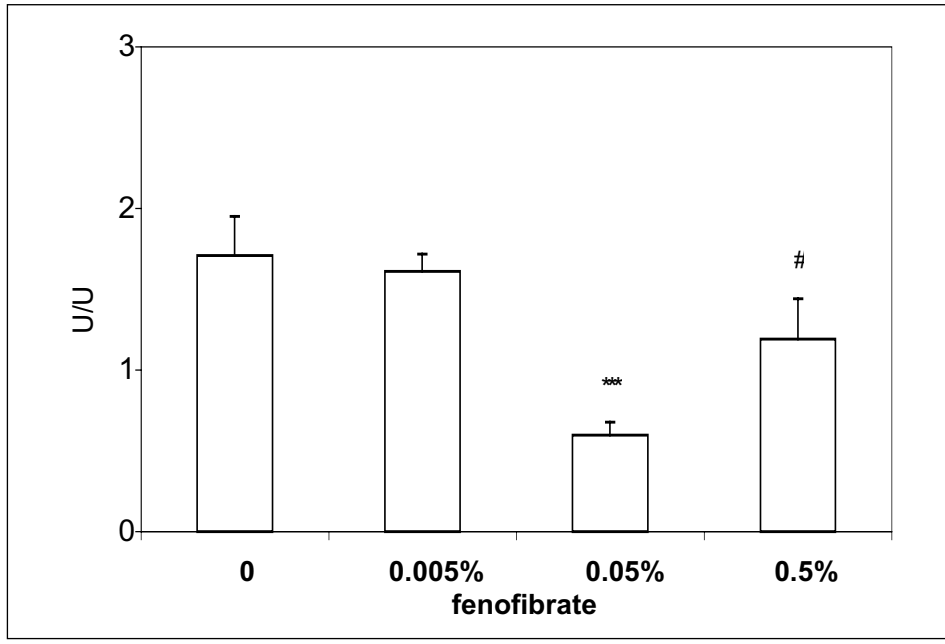


*Fig. 4* The ratio between PON1 activity assayed toward paraoxon and measured toward phenyl acetate (PON/AE ratio) in control and fenofibrate-treated rats. Values expressed in units paraoxonase/units arylesterase. #p=0.059, ***p<0.001, compared to control by ANOVA and Duncan's test.

0.005%, 0.05% and 0.5% fenofibrate by 54.0%, 66.9% and 89.7%, respectively. Also the ratio between hydroperoxides and triglycerides was reduced in all fenofibrate-treated groups (-23.1%, -28.7% and -31.2% compared to control for 0.005%, 0.05% and 0.5% fenofibrate, respectively). (MDA+4-HDA)/cholesterol ratio decreased by 42.0%, 28.7% and 31.2% following 0.005%, 0.05% and 0.5% fenofibrate, respectively. Finally, hydroperoxides/cholesterol ratio was reduced by 26.7% and 33.5% in 0.05% and 0.5% fenofibrate groups, respectively (the difference for 0.005% group was insignificant). Taken together, reduced peroxidation products/plasma lipids ratios suggest that fenofibrate inhibits the peroxidation process through the mechanisms other than reduced availability of substrates.

## DISCUSSION

The results of this study indicate that fenofibrate administered to normolipidemic rats demonstrates antioxidant activity at broad range of doses without affecting plasma lipids concentration. The antioxidant activity may contribute to antiatherosclerotic effect of fibrates observed in some clinical trials,

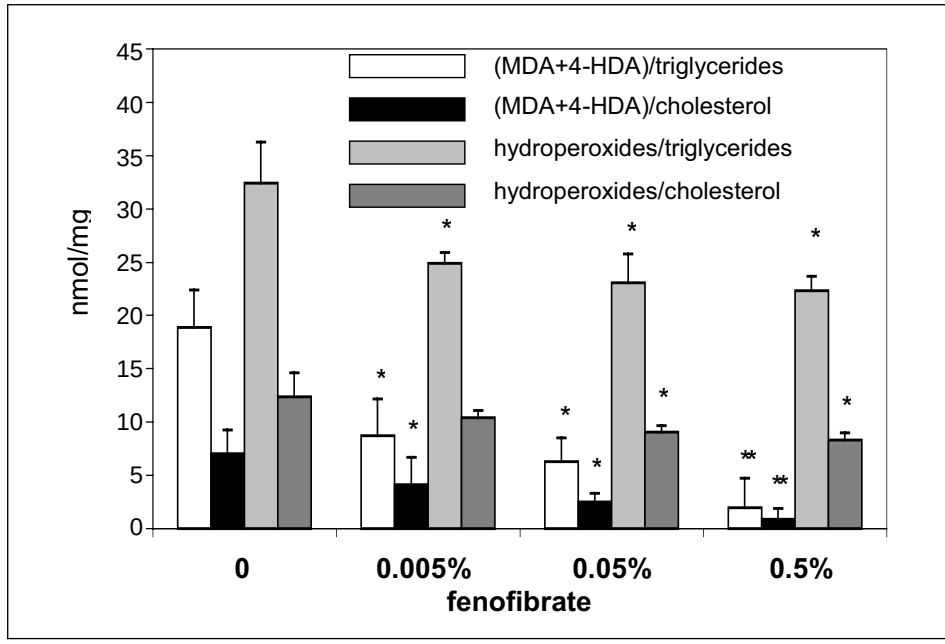


*Fig. 5* The ratio between lipid peroxidation products and plasma lipids in control and fenofibrate-treated rats. Values expressed in nmol malonyl dialdehyde + 4-hydroxydialkenals or lipid hydroperoxides per mg of plasma triglycerides or cholesterol. *p<0.05, **p<0.01, compared to control by ANOVA and Duncan's test.

also in patients with normal triglycerides level (8). Moreover, the present results suggest that oxidative stress is not involved in adverse effects of fibrates observed in some studies (9-13).

It should be noted that, although it was not investigated by us, the highest dose of fenofibrate used in the present study induces marked peroxisome proliferation in rodents (16, 27, 38). Peroxisomal fatty acids β-oxidation is considered the main source of PPARα-mediated reactive oxygen species, in particular $H_2O_2$, production (16, 27). Although we can not exclude the possibility that fenofibrate increases oxidative stress locally within the liver, the significance of this for plasma lipids peroxidation and thus for vascular pathology seems negligible. It is possible that increased $H_2O_2$ synthesis is effectively counterbalanced by upregulation of antioxidant enzymes, especially catalase and glutathione peroxidase (16, 39), or that local intrahepatic oxidative stress does not result in elevation of plasma lipid peroxidation products. This eventual prooxidative effect should be even less important in humans in whom fibrates do not induce peroxisomes proliferation. Thus we suggest that one should not be afraid of prooxidant effect of fibrates, at least as long as plasma lipids oxidation is considered.

Our results are consistent with some (15-21) but not all (22-30) studies concerning the effect of fibrates on oxidant-antioxidant balance. In particular, bezafibrate reduces plasma thiobarbituric acid-reactive substances (TBARS), the marker of lipids peroxidation, in rats with dietary-induced hyperlipidemia (17). In rats kept on different diets plasma TBARS inversely correlate with hepatic PPARα expression (40). Age-related increase in TBARS concentration is accelerated in PPARα knockout mice and PPARα agonists attenuate age-dependent TBARS elevation in wild-type but not in PPARα-/- animals (41).

The mechanism of antioxidant effect of fenofibrate is not clear, but several possibilities can be proposed. First, several fibrates metabolites (but not fibrates themselves) possess direct radicals-scavenging properties (18). Second, some studies demonstrate that treatment with fibrates reduces the susceptibility of plasma lipoproteins, especially LDLs, to oxidation (15, 18, 19, 20). This is most likely the effect of modified composition of fatty acids contained in LDL particles, i.e. decreased polyunsaturated/(monounsaturated+saturated) fatty acids ratio, which renders LDL more resistant to oxidative modification (15). Third, fibrates can improve antioxidant defence mechanisms. We found no effect of fenofibrate treatment on plasma antioxidant capacity. This is consistent with previous studies in which fibrate treatment did not change plasma levels of nonenzymatic antioxidants such as α-tocopherol and β-carothene (17, 19). However, fibrates could affect FRAP (or any other test of plasma antioxidant potential) by increasing renal uric acid excretion and reducing its plasma concentration (42, 43). Although this effect has not been described in the rat, we can not exclude that it masked increased concentration of other antioxidants resulting in unchanged FRAP. The effect of fenofibrate on plasma uric acid in the rat has to be studied to test this possibility. Fourth, fibrates have potent antiinflammatory properties (44-47). Inflammatory cells are an important source of ROS generated by phagocytes' plasma membrane NAD(P)H oxidase (48). However, data about the effect of fibrates on ROS production by inflammatory cells are controversial. Fenofibrate inhibits superoxide anion generation by leukocytes after high-fat meal in patients with ischemic heart diseases (49) and ciprofibrate does so in individuals with type 2 diabetes (21). In contrast, Scatena et al. (28) observed stimulation of leukocytes and monocytes respiratory burst by gemfibrozil both *in vivo* and *in vitro* and Rose *et al.* (30) found the same in hepatic Kupfer cells. Finally, PPARα agonists stimulate the expression of cytochrome P450, which catabolizes some lipid peroxidation products, including 4-hydroxynonenal (50). It is noteworthy that in the present study fenofibrate dose-dependently reduced MDA+4-HDA concentration by up to ~90%, whereas hydroperoxides concentration decreased maximally by only ~30% and this effect was obtained even after the lowest dose of drug. It is possible that higher doses of fenofibrate stimulated cytochrome P450 to metabolise hydroxyalkenals, which resulted in more marked effect of PPARα agonist on 4-HDA than on hydroperoxides concentration.

Paraoxonase 1 contained in high density lipoproteins protects against atherosclerosis by preventing oxidative modification of plasma lipoproteins. PON1 activity is negatively modulated by atherosclerosis risk factors, such as smoking, diabetes and hyperlipidemia, as well as by oxidative stress and acute phase response (51). The present study indicates that fenofibrate treatment reduces both paraoxon and phenyl acetate-hydrolyzing activity of plasma paraoxonase. In contrast, Durrington et al. (52) found that treatment of hyperlipidemic patients with bezafibrate or gemfibrozil had no effect on plasma PON1 activity. However, hyperlipidemia *per se* decreases PON1 activity (51) and the effect of fibrates in that study could be masked by beneficial changes in plasma lipids profile. Our results suggest that fenofibrate decrease PON1 activity in normolipidemic subjects and this seems especially noteworthy when fibrates are used in patients with normal triglycerides concentration (8). It is unclear at present whether this potentially adverse effect is mediated by PPARα and is characteristic for all fibrates but if so, it will be expected to modulate atheroprotective activity of these drugs. Further studies, using other fibrates as well as non-fibrate PPARα agonists, such as Wy 14643, are needed to clarify these issues

In conclusion, the results of this study demonstrate that fenofibrate administration in normolipidemic rats decreases plasma level of lipid peroxidation products without changing triglycerides and cholesterol concentrations. Fenofibrate has no effect on total plasma antioxidant capacity but significantly reduces plasma paraoxonase 1 activity. The beneficial, antioxidant, and potentially not beneficial, PON1-lowering action can contribute to the effect of fenofibrate on pathological processes involved in atherogenesis.

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