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EFFECTS OF THE PEROXISOME PROLIFERATORS CIPROFIBRATE AND PERFLUORODECANOIC ACID ON HEPATIC CELLULAR ANTIOXIDANTS AND LIPID PEROXIDATION IN RATS

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Abstract—The purpose of this study was to determine if hepatic cellular antioxidants and indices of oxidative damage are altered by administration of the peroxisome proliferators ciprofibrate and perfluorodecanoic acid (PFDA). Rats were fed 0.01% ciprofibrate in the diet or were injected with PFDA (0.5 or 5.0 mg/kg, i.p.) every 4 weeks for 6, 14, 30, 54, and 78 weeks. Peroxisomal fatty acyl-CoA oxidase and catalase activities were increased by both ciprofibrate and PFDA throughout the study. Neither ciprofibrate nor PFDA increased the levels of malonaldehyde or conjugated dienes, but ciprofibrate decreased these indices at early time points. Ciprofibrate decreased the following cellular antioxidants or antioxidant enzymes: vitamin C, vitamin D, DT-diaphorase, glutathione peroxidase, glutathione-*S*-transferase, and glutathione reductase; superoxide dismutase and glutathione were not affected. PFDA decreased DT-diaphorase and increased superoxide dismutase, but did not affect other cellular antioxidants. This study shows that administration of the peroxisome proliferators ciprofibrate and PFDA did not increase indices of lipid peroxidation, but that cellular antioxidant defenses were inhibited for a prolonged period of time by the peroxisome proliferator ciprofibrate.

Several hypolipidemic drugs, plasticizers, and other chemicals induce hepatic peroxisome proliferation and hepatocellular carcinoma when administered to rodents [1–4]. The mechanisms by which these agents cause hepatocellular carcinomas are not known, but are likely to be related to biochemical changes induced by peroxisomal proliferators, since they have never been conclusively shown to be genotoxic. Peroxisome proliferators induce increased cell proliferation and increased expression of several proteins, including the enzymes of the peroxisomal β-oxidation pathway, the carnitine acyltransferases, cytosolic epoxide hydrolase, and the cytochrome P450IVA family [1]. One of the enzymes of the peroxisomal β-oxidation pathway, fatty acyl-CoA oxidase, produces hydrogen peroxide as an end product. The activity of this enzyme is increased greater than 10-fold by certain potent peroxisome proliferators, whereas the activity of the peroxisomal enzyme catalase, which detoxifies hydrogen peroxide, is only increased about 2-fold. This increased production of hydrogen peroxide has been hypothesized to be important in peroxisome proliferator-induced carcinogenesis by increasing cellular oxidative damage [2].

We have found that a unique peroxisome proliferator, perfluorodecanoic acid (PFDA¶), produces different biochemical effects than other peroxisome proliferators. Although PFDA induces the enzymes of peroxisomal β-oxidation it, unlike other peroxisome proliferators, inhibits total peroxisome β-oxidation (measured as flux through the pathway) [5, 6]. This inhibition of peroxisomal β-oxidation is caused by noncompetitive inhibition of the peroxisomal bifunctional enzyme by PFDA [7]. PFDA also induces selenium-dependent glutathione peroxidase activity at certain dietary selenium concentrations, unlike other peroxisome proliferators [8]. Finally, PFDA lacks hepatic tumor-promoting activity, unlike most other peroxisome proliferators [1–4, 9].

The purpose of this study was to determine if PFDA and a conventional peroxisome proliferator, ciprofibrate, induce lipid peroxidation and alter cellular antioxidant concentrations in rat liver. Rats were administered ciprofibrate or PFDA for up to 78 weeks, and the levels of malonaldehyde, conjugated dienes, vitamin C, and glutathione, and the activities of glutathione peroxidase, glutathione-*S*-transferase, DT-diaphorase, catalase, superoxide dismutase, and glutathione reductase were determined.

MATERIALS AND METHODS

Materials. Ciprofibrate was a gift from Sterling

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¶ Abbreviation: PFDA, perfluorodecanoic acid.

Drug Inc., Rensselaer, NY. Perfluorodecanoic acid was a gift from 3M Inc., Minneapolis, MN. High performance liquid chromatography grade chloroform and methanol were from Fisher Scientific, Cincinnati, OH. All other chemicals were from the Sigma Chemical Co., St. Louis, MO.

Animal treatment. One hundred and forty female Sprague–Dawley rats (130–150 g, Harlan Sprague Dawley, Indianapolis, IN) were housed three per plastic cage in a temperature- and light-controlled room. Rats were fed an unrefined diet (Purina Rodent Laboratory Chow No. 5001, Purina Mills, St. Louis, MO) *ad lib*. After 1 week of acclimatization, the rats were divided into five groups. Rats were fed 0.01% ciprofibrate or injected with PFDA (0.5 or 5.0 mg/kg body weight in corn oil) once every 4 weeks. Control rats received corn oil injections or no treatment (no difference was seen between control rats receiving corn oil and those not receiving corn oil for any endpoint). Rats were weighed once every 2 weeks. Six rats from each group were killed by decapitation at 6, 14, 30, 54 and 78 weeks. Rats were fasted for 12 hr before being killed. Livers were perfused *in situ* with ice-cold 1.15% KCl containing 0.1 mM EDTA. Whole liver homogenates were prepared by mincing and then homogenizing with an Ultra-Turrax homogenizer (Tekmar Co., Cincinnati, OH). Liver homogenates were used immediately (for glutathione, malondialdehyde, catalase, and conjugated diene measurements) or frozen at −80° until time of assay. The 100,000 *g* cytosolic fraction was obtained by differential centrifugation [10].

Biochemical determinations. The following biochemical variables were analyzed in the liver homogenates. Protein was determined by the method of Lowry *et al.* [11] using bovine serum albumin as the standard. Total vitamin C (both ascorbic acid and dehydroascorbic acid) was determined by the method of Omaye *et al.* [12]. Vitamin E was analyzed as described by Srinivasan *et al.* [13]. Reduced glutathione was quantified spectrophotometrically using the method of Sedlack and Lindsay [14]. Malondialdehyde was determined by the method of Tatum *et al.* [15], by high performance liquid chromatography. The activity of fatty acyl-CoA oxidase was measured according to the method of Poosch and Yamazaki [16]. Catalase activity was determined by the method of Beers and Sizer [17]. Conjugated dienes were determined by the method of Recknagel and Ghoshal [18]. Total lipids were extracted from the liver and quantified as described by Folch *et al.* [19]. Cytosol was used for the following enzyme assays. Selenium-dependent glutathione peroxidase activity was determined by the method of Lawrence and Burk [20] using hydrogen peroxide as the substrate. Superoxide dismutase activity was estimated by the method of McCord and Fridovich [21]. DT-Diaphorase activity was assessed using the method of Ernster [22] with *p*-benzoquinone as the substrate. Glutathione-*S*-transferase activity was quantified by the method of Habig and Jakoby [23] with 1-chloro-2,4-dinitrobenzene as the substrate. Glutathione reductase was assayed by the method of Racker [24].

Statistical analysis. Data were analyzed using one-way analysis of variance. If significant effects were seen, data were further analyzed by Dunnett's test [25]. For vitamin E, data were analyzed by Student's *t*-test. The level of significance for all tests was $\alpha = 0.05$.

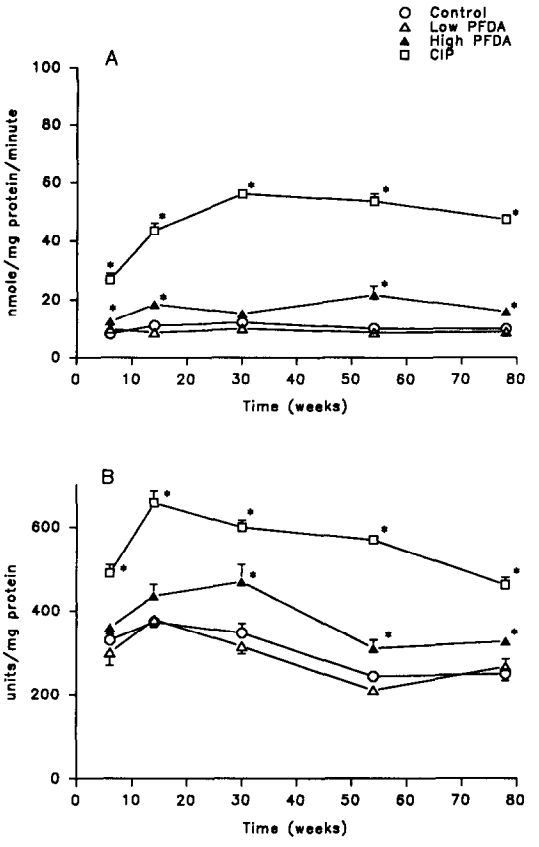

Fig. 1. Effects of ciprofibrate and perfluorodecanoic acid (PFDA) on peroxisomal enzyme activities: fatty acyl-CoA oxidase activity (A) and catalase (B). Rats were fed 0.01% ciprofibrate or were injected every 4 weeks with PFDA (0.5 or 5.0 mg/kg/injection). Values are means ± SEM, N = 6. Key: (*) significantly different from control values ($P < 0.05$).

RESULTS

In this study we examined the ability of the peroxisome proliferators ciprofibrate and PFDA to induce lipid peroxidation and alter the levels of cellular antioxidants in the liver. Rats were administered ciprofibrate and PFDA for up to 78 weeks, and five different time points were examined. Body weights of the rats were not affected by any of the treatments (data not shown). Tumors were not seen at any time point.

Peroxisomal proliferation was verified by quantifying the activity of the peroxisomal β-oxidation enzyme fatty acyl-CoA oxidase (Fig. 1A). Ciprofibrate treatment greatly increased the activity at every time point. The high PFDA dose significantly increased enzyme activity at all time points except

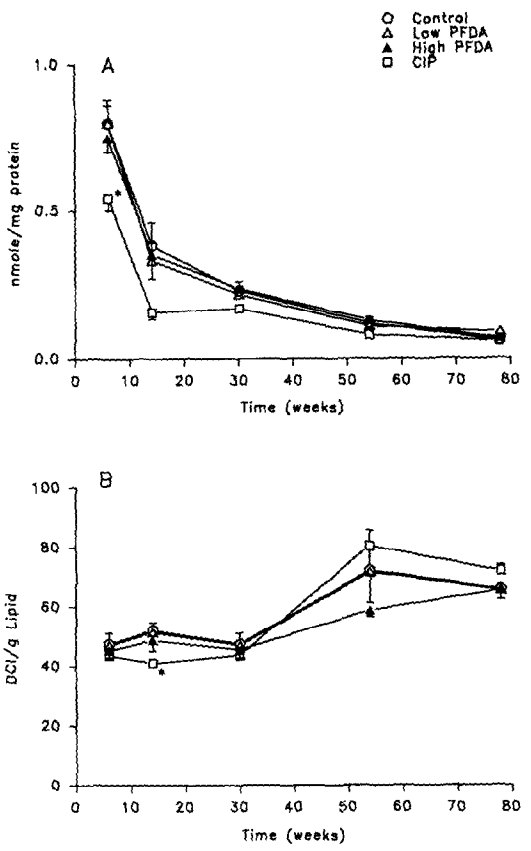

Fig. 2. Effects of ciprofibrate and PFDA on indices of lipid peroxidation: malondialdehyde (A) and conjugated diene (B) concentrations. Rats were fed 0.01% ciprofibrate or were injected every 4 weeks with PFDA (0.5 or 5.0 mg/kg/injection). Values are means ± SEM, N = 6. Key: (*) significantly different from control values ($P < 0.05$).

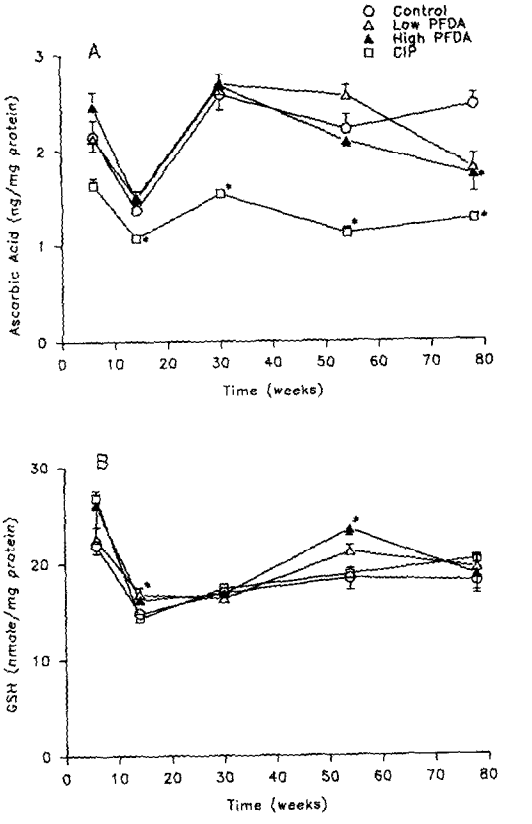

Fig. 3. Effects of ciprofibrate and PFDA on hepatic concentrations of the antioxidants ascorbic acid (A) and glutathione (B). Rats were fed 0.01% ciprofibrate or were injected every 4 weeks with PFDA (0.5 or 5.0 mg/kg/injection). Values are means + SEM, N = 6. Key: (*) significantly different from control values ($P < 0.05$).

30 weeks; the low PFDA dose had no effect. Catalase activity was also increased by ciprofibrate at all time points and by the high dose of PFDA at 30, 54, and 78 weeks (Fig. 1B). The magnitude of the catalase increase was much less than that of fatty acyl-CoA oxidase, as has been reported previously [1, 2].

The amount of lipid peroxidation was estimated by quantifying malondialdehyde and conjugated dienes (Fig. 2). For all groups, malondialdehyde decreased over time, whereas conjugated dienes increased slightly. Ciprofibrate significantly decreased malondialdehyde concentrations at 6 weeks and the diene conjugation index at 14 weeks, but did not alter either end point significantly at other times. PFDA did not affect either malondialdehyde or conjugated dienes at any times.

Three cellular antioxidants—ascorbic acid, vitamin E, and glutathione—were quantified (Fig. 3, Table 1). Ascorbic acid was decreased significantly by ciprofibrate at all but the 6-week time point. PFDA only reduced it at 78 weeks, at the higher dose. Vitamin E was similarly reduced by ciprofibrate throughout the study (effect of PFDA not studied). Glutathione was not affected consistently: ciprofibrate had no effect, and one of the doses of PFDA increased it at two separate times.

Table 1. Effect of ciprofibrate on hepatic vitamin E levels

Duration of treatment (weeks)	Vitamin E (μg/g liver)	
	Control	Ciprofibrate
6	18.9 ± 1.0	10.4 ± 1.0*
14	24.2 ± 2.3	13.7 ± 0.8*
30	24.2 ± 3.5	10.0 ± 1.0*
54	30.9 ± 4.8	19.4 ± 4.7
78	39.2 ± 11.9	18.2 ± 2.3

Values are means ± SEM, N = 6.
* Significantly different from control values ($P < 0.05$).

Several enzymes that are part of the cellular antioxidant defense system were affected by ciprofibrate or PFDA. DT-Diaphorase activity was decreased significantly by ciprofibrate and one or both doses of PFDA at all time points (Fig. 4). Superoxide dismutase activity was not affected by

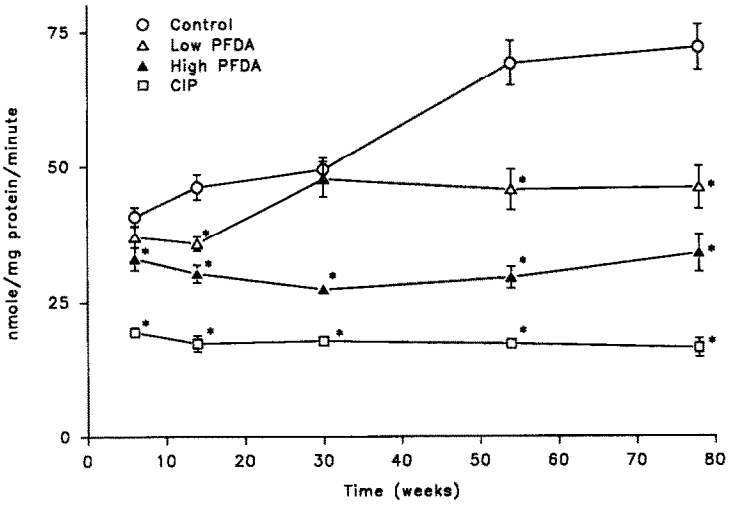

Fig. 4. Effects of ciprofibrate and PFDA on hepatic DT-diaphorase activity. Rats were fed 0.01% ciprofibrate or were injected every 4 weeks with PFDA (0.5 or 5.0 mg/kg/injection). Values are means ± SEM, N = 6. Key: (*) significantly different from control values ($P < 0.05$).

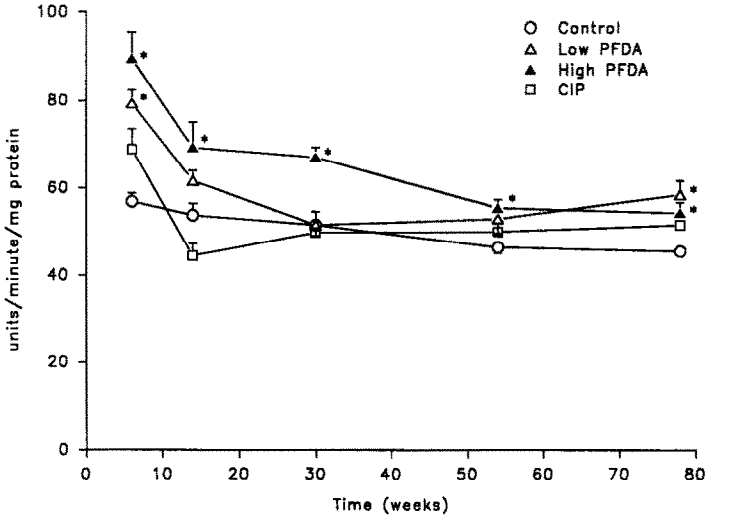

Fig. 5. Effects of ciprofibrate and PFDA on hepatic superoxide dismutase activity. Rats were fed 0.01% ciprofibrate or were injected every 4 weeks with PFDA (0.5 or 5.0 mg/kg/injection). Values are means ± SEM, N = 6. Key: (*) significantly different from control values ($P < 0.05$).

ciprofibrate, but was increased by one or both doses of PFDA at all times (Fig. 5). Enzymes of glutathione metabolism—glutathione peroxidase, glutathione-*S*-transferase, and glutathione reductase—were all generally decreased by ciprofibrate, but were not affected by PFDA at most times (Fig. 6).

DISCUSSION

In this study we found that the administration of the peroxisome proliferator ciprofibrate did not greatly alter the levels of hepatic lipid peroxidation as measured by malondialdehyde and conjugated diene formation. However, the concentrations of hepatic vitamin C and vitamin E, and the activities of hepatic glutathione peroxidase, glutathione-*S*-transferase, glutathione reductase, and DT-diaphorase were generally lowered by ciprofibrate over the entire course of the study. PFDA lowered DT-diaphorase activity and increased superoxide dismutase activity but did not affect the other cellular antioxidants or lipid peroxidation. Because PFDA only induced fatty acyl-CoA oxidase activity about 2-fold at these doses, it is possible that PFDA may have an effect at higher doses.

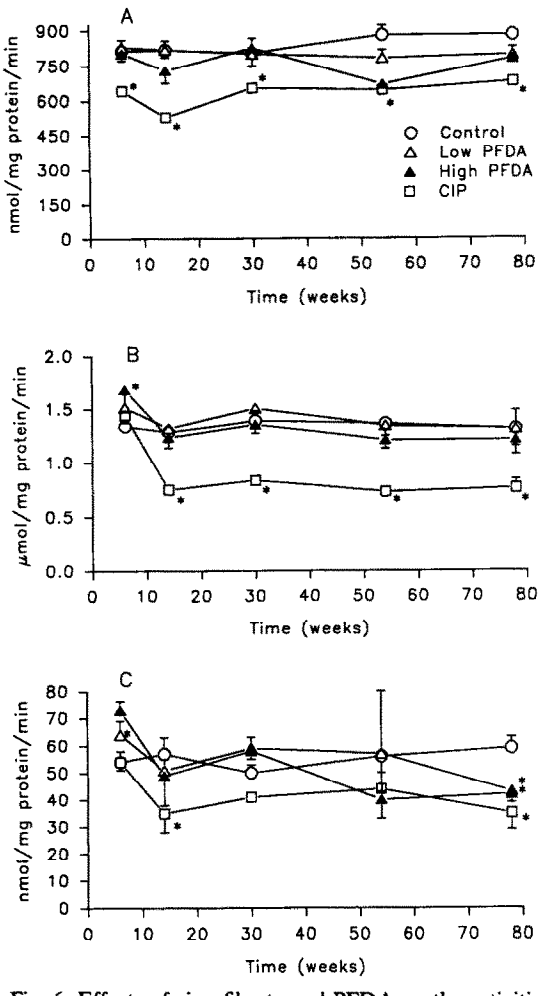

Fig. 6. Effects of ciprofibrate and PFDA on the activities of enzymes of glutathione metabolism: glutathione peroxidase (A), glutathione-*S*-transferase (B), and glutathione reductase (C). Rats were fed 0.01% ciprofibrate or were injected every 4 weeks with PFDA (0.5 or 5.0 mg/kg/injection). Values are means ± SEM, N = 6. Key: (*) significantly different from control values ($P < 0.05$).

Previous studies have also found cellular antioxidants to be lowered by peroxisome proliferators. The antioxidant enzymes selenium-dependent glutathione peroxidase [26–37], glutathione-*S*-transferase [28–31, 36–39], glutathione reductase [28, 36], and superoxide dismutase [26, 33] all have been found previously to be decreased by the administration of peroxisome proliferators for varying periods of time, ranging from 1 week to 2 years. The concentration of the cellular antioxidant vitamin E [32, 36] also has been found to be lowered, whereas that of glutathione was found to be increased [28, 29, 36, 38] or unaffected [32]. No consistent effect of peroxisome proliferators on hepatic ascorbic acid concentrations has been demonstrated previously [36], and the decrease in DT-diaphorase has not been observed previously. The results of previous studies and the present study demonstrate that hypolipidemic drug peroxisome proliferators decrease a large number of cellular antioxidants and antioxidant enzymes for a prolonged period.

The effect of peroxisome proliferators on the induction of lipid peroxidation in previous studies is less clear. After short-term administration of peroxisome proliferators (1–4 weeks), conjugated diene or malondialdehyde levels are either unchanged or decreased [26, 27, 29, 32, 40], as was seen in the present study. Peroxisome proliferators also inhibit lipid peroxidation induced by choline deficiency, iron/ascorbic acid, and *t*-butyl hydroperoxide [29, 40]. But after longer periods of time (150 days–2 years), conjugated diene concentrations in previous studies were increased [28, 30, 32], whereas the concentration of malondialdehyde was not affected [30] or decreased [35]. In the present study, no significant effect on either of these indicies was observed after 14 weeks. The reason for the different results for conjugated dienes in our study is not known, but may relate to the strain and sex of rat used, or the use of different peroxisome proliferators. The increased deposition of lipofuscin after long-term administration of peroxisome proliferators has been demonstrated in numerous studies [28, 30, 32, 34, 41–43].

Although the decrease in antioxidant defenses by ciprofibrate did not result in pronounced lipid peroxidation in this study, other deleterious changes in hepatocytes may be related to this decrease. Ciprofibrate or other peroxisome proliferators have been found to induce the oxidative DNA adducts hydroxymethyldeoxyuridine and 8-hydroxydeoxyguanosine [44–47], although Hegi *et al.* [48] found that nafenopin does not influence 8-hydroxydeoxyguanosine levels. Hegi *et al.* additionally isolated nuclei before isolating DNA, indicating that 8-hydroxydeoxyguanosine adducts in other studies may be derived from mitochondrial DNA. The induction of the enzymes fatty acyl-CoA oxidase in peroxisomes or the cytochrome P450IVA family, both of which produce hydrogen peroxide or other active oxygen species, and the concurrent lowering of cellular antioxidant defenses may allow hydrogen peroxide or other active oxygen species to diffuse into the nucleus and cause oxidative DNA damage.

The administration of PFDA produced very few changes: only a decrease in DT-diaphorase activity and an increase in superoxide dismutase activity. The lack of changes in cellular antioxidants may be related to the marginal increases in peroxisomal enzyme activities (fatty acyl-CoA oxidase, catalase) that were seen. Multiple injections of PFDA, at higher doses, have been shown to produce much higher activities for β-oxidation enzymes such as fatty acyl-CoA oxidase—up to 20-fold higher [6]. High doses of PFDA in short-term studies also decrease the activity of glutathione-*S*-transferase but increase glutathione peroxidase activity and the concentration of glutathione [8, 39], all of which were largely unaffected by PFDA in the present study. PFDA, however, is excreted very slowly, is quite toxic and can produce marked weight loss and either decreased food intake or decreased feed efficiency [5, 6, 49, 50]. Therefore, choosing a dose

in a long-term experiment that both is non-toxic and produces large increases in enzyme activities is difficult. In the present study, it is not known if PFDA was without effect or whether higher doses would be necessary in order to see effects similar to those seen with ciprofibrate.

In conclusion, in this study we have demonstrated that the peroxisomal proliferator ciprofibrate induces a sustained decrease in a large number of cellular antioxidants and antioxidant enzymes. Indices of lipid peroxidation, however, were either not affected or actually decreased; therefore, the decrease in cellular antioxidant defenses did not appear to influence lipid peroxidation. Nevertheless, the results from this study and other studies indicate that decreases in cellular antioxidant status are correlated with carcinogenesis by peroxisome proliferators. Whether these changes bring about oxidative DNA damage and the formation of liver tumors will be an important subject for future research.

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