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HYPOLIPIDEMIA AND PEROXISOME PROLIFERATION INDUCED BY PHENOXYACETIC ACID HERBICIDES IN RATS

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Abstract—Male Wistar rats were treated daily by gavage with two phenoxy herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D)(100–200 mg/kg body wt) and 4-chloro-2-methylphenoxyacetic acid (MCPA) (100–200 mg/kg body wt), and with the chemically different glyphosate *N*-phosphonomethyl glycine (300 mg/kg body wt) 5 days per week for 2 weeks. A hypolipidemic drug, clofibrate [ethyl-2-(4-chlorophenoxy)-2-methylpropionate], which is structurally related to phenoxy acids, was used as a positive control (200 mg/kg body wt). 2,4-D and MCPA had several effects similar to those of clofibrate: all three compounds induced proliferation of hepatic peroxisomes, decreased serum lipid levels, and increased hepatic carnitine acetyltransferase and catalase activities. 2,4-D and MCPA, but not clofibrate, decreased lipoprotein lipase activity in the adipose tissue to about a third of the control value but did not change the lipoprotein lipase activity in the heart muscle. The data suggest that these compounds cause hypolipidemia not by enhancing the storage of peripheral lipids in adipose tissue but by preferentially increasing lipid utilization in the liver. Glyphosate caused no peroxisome proliferation or hypolipidemia, suggesting that these effects are associated with the structural similarity between phenoxy acid herbicides and clofibrate.

The phenoxy acid herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA), and glyphosate (*N*-phosphonomethyl glycine) are used widely as agricultural chemicals. Epidemiological surveys in Sweden have suggested a possible carcinogenic action of phenoxy herbicides [1]. We demonstrated recently that 2,4-D and MCPA induce peroxisome proliferation in the liver of Chinese hamsters [2], in a manner similar to that of the hypolipidemic drug, clofibrate [ethyl 2-(4-chlorophenoxy)-2-methylpropionate]. Clofibrate is closely related structurally to phenoxy acids, and it is also a suspected carcinogen in animals [3].

It has been suggested that clofibrate elicits its lipid-lowering effects by activating extrahepatic lipoprotein lipase [4–8]. However, recent data indicate that proliferation of peroxisomes and mitochondria takes place in the livers of patients receiving clofibrate therapy [9–11]. Such proliferation can lead to increased β-oxidation of fatty acids, which may play an important role in the hypolipidemic mechanism of action of clofibrate [12].

The structural similarities between phenoxyacetic acid herbicides and therapeutically used clofibrate led us to compare their biological effects and to compare them further with the structurally unrelated herbicide glyphosate. To this aim, we have studied the effects of 2,4-D, MCPA, clofibrate and glyphosate on blood lipids, adipose and muscle tissue lipoprotein lipases and hepatic enzymes, and on peroxisome proliferation.

MATERIALS AND METHODS

Chemicals. 2,4-D and MCPA were kindly provided by the manufacturer (Kemira Oy, Finland) as commercial herbicide products bearing the trade names 'Vesakontuho tasku' (aqueous solution of the amine salt of 2,4-D) and 'Vesakontuho MCPA' (solution of the iso-octyl ester of MCPA in a petroleum fraction). The concentrations of 2,4-D and MCPA in the solutions were 550 and 500 g/kg, respectively. Each preparation contained only one effective compound. A commercial preparation of glyphosate (Roundup®) was kindly provided to us by Farmos Oy, Finland. Clofibrate (Klofiran®) was obtained from Remeda, Finland.

Test procedure. Male Wistar rats weighing 358–495 g were treated for 2 weeks (5 days/week) with a single daily dose of one of the test compounds given intragastrically by gavage. The volumes of solution with which the animals were treated varied from 0.60 to 1.80 ml, depending on the dose: dilutions were made from stock solutions, in physiological saline for the phenoxy acids and glyphosate, and in olive oil for clofibrate. Treatment solutions were prepared daily immediately before use. Each treatment group consisted of five rats housed in the same cage. The rats were weighed on the day before the first treatment and subsequently every other day during treatment. Two dose levels of 2,4-D and

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MCPA were selected, 100 and 200 mg/kg; however, after the first treatment week, the higher dose was lowered to 150 mg/kg due to drowsiness produced in rats by the original level. One animal receiving 200 mg/kg 2,4-D died on the fourth day of treatment. The daily doses of clofibrate and glyphosate were 200 and 300 mg/kg, respectively. All animals were killed 24 hr after the last treatment.

Enzyme assays. A mitochondrial fraction of liver was used to determine carnitine acetyltransferase and catalase activities. This fraction (which also contained most of the peroxisomes) was isolated as described by Myers and Slater [13] in 0.25 M sucrose, 5 mM Tris–HCl and 1 mM EDTA at pH 7.4.

The activity of carnitine acetyltransferase was measured according to the method of Bieber *et al.* [14] by following the release of coenzyme A-SH from acetyl-coenzyme A in the presence and absence of L-(−)-carnitine. Dithionitrobenzene (0.1 mM) was used as the thio reagent. Catalase was assayed by following the disappearance of the H_2O_2 substrate at 240 nm [15]. Protein was estimated by the method of Lowry *et al.* [16].

Blood samples for lipid determinations were drawn from rats by cardiac puncture 24 hr after administration of the last dose. Plasma was separated by centrifugation at 340 *g* for 15 min. Serum triglyceride and cholesterol concentrations were determined enzymatically as described by Wahlefeld [17] and Röschlau *et al.* [18]. The epididymal fat pads and heart were excised for determination of lipoprotein lipase activity, which was done according to the method of Schotz *et al.* [19] as modified by Hietanen and Greenwood [20] and Green *et al.* [21], and by eluting the enzyme with heparin, essentially as described by Nikkilä *et al.* [22].

Histochemistry. Biopsies were taken from the livers of 33 animals immediately after killing and were fixed by immersion in 2% phosphate-buffered (pH 7.4) glutaraldehyde for 2.5 hr. Minced tissue samples were washed for 0.5 hr in phosphate buffer (pH 7.5). The specimens were then incubated for 90 min at 38° in a catalase localization medium (alkaline diaminobenzidine) [23] and washed in phosphate buffer (pH 7.4) overnight. The specimens were post-fixed in 1% phosphate-buffered (pH 7.4) osmium tetroxide, dehydrated and embedded in Epon. Ultrathin sections were stained with lead citrate and examined under a JEM-100 CX electron microscope.

The frequency of peroxisomes per unit area of hepatocyte was calculated by counting the number of peroxisomes in 700–900 μm^2 cytoplasm per animal. The areas were selected without knowledge of the type of the specimen (total number of peroxisomes, 7085).

The areas of the hepatocytic peroxisomes in tissue samples from 23 animals (those receiving 100 mg/kg 2,4-D or MCPA were excluded) were determined from electron micrographs (6600 ×) projected onto a drawing board (final enlargement, 100,000 ×). The peroxisomes were outlined, and these were used later for morphometric evaluations. The areas of 345 peroxisomes (between 60 and 75 in each group) were analysed with the aid of a semiautomatic measuring device (Hipad digitalizer and ABC-80 data processing unit).

Statistical analyses. Cochran's [24] approximate *t*-test was used to calculate statistical significances. Values significantly different from those of controls are indicated in the tables by asterisks, as follows: * $P < 0.05$, ** $P < 0101$, *** $P < 0.001$. NS = not significant.

Table 1. Effects of 4-chloro-2-methylphenoxyacetic acid (MCPA), 2,4-dichlorophenoxyacetic acid (2,4-D), clofibrate and glyphosate on the density of peroxisomes per 100 μm^2 of hepatocytic cytoplasm

Exposure	No. of animals	Number of peroxisomes/100 μm^2 (mean ± S.D.)
Controls	5	14.9 ± 5.2
MCPA (100 mg/kg)	5	29.7 ± 2.9 ***
MCPA (150–200 mg/kg)	5	32.7 ± 8.4 **
2,4-D (100 mg/kg)	5	20.7 ± 2.7 *
2,4-D (150–200 mg/kg)	4	39.2 ± 3.6 ***
Clofibrate (200 mg/kg)	5	35.6 ± 4.3 ***
Glyphosate (300 mg/kg)	4	11.8 ± 2.5 NS

Table 2. Effect of 4-chloro-2-methylphenoxyacetic acid (MCPA), 2,4-dichlorophenoxyacetic acid (2,4-D), clofibrate and glyphosate on the mean size of peroxisomes, in hepatocytic cytoplasm

Exposure	No. of animals	Peroxisome size (μm^2) (mean ± S.D.)
Controls	5	0.249 ± 0.032
MCPA (150–200 mg/kg)	5	0.362 ± 0.021 **
2,4-D (150–200 mg/kg)	4	0.424 ± 0.115 *
Clofibrate (200 mg/kg)	5	0.371 ± 0.045 **
Glyphosate (300 mg/kg)	5	0.258 ± 0.064 NS

RESULTS

The body weights of the rats in the group treated with clofibrate increased by an average of 1% during the treatment period, as did the body weights of the control rats. In the groups treated with the higher dose of 2,4-D and MCPA (150–200 mg/kg) and with glyphosate, average decreases of about 5% were observed. Corresponding decreases of about 1% were observed in the body weights of rats treated with the lower dose of 2,4-D and MCPA (100 mg/kg).

The mean frequencies of peroxisomes per 100 μm^2 of hepatocytic cytoplasm (Table 1) were 14.9 in the controls, 20.7–39.2 in rats treated with 2,4-D, 29.7–32.7 in rats given MCPA, and 35.6 in animals treated with clofibrate. Glyphosate treatment caused no increase in the number of peroxisomes in hepatocytes.

The mean sizes of the peroxisomes in the hepatocytes (Table 2) were 0.249 μm^2 in control rats, 0.424 μm^2 in rats given 150 mg/kg 2,4-D, 0.362 μm^2 in animals given the same dose of MCPA, and 0.371 μm^2 in rats receiving clofibrate. Glyphosate caused no increase in the size of peroxisomes. The proliferation and increase in size of liver peroxisomes are also illustrated in Fig. 1.

Of all the agents studied, only clofibrate increased markedly the relative liver weight of rats (Table 3).

Serum triglyceride concentrations were decreased in rats receiving 2,4-D, MCPA or clofibrate; no change in serum triglycerides was found in rats treated with glyphosate (Table 3). A trend to hypocholesterolemia, similar to that caused by clofibrate, was seen in animals treated with 2,4-D and MCPA (Table 3).

Clofibrate caused a slight, but statistically insignificant, decrease in lipoprotein lipase activity in adipose tissue (Table 4). Glyphosate had no effect. Both 2,4-D and MCPA caused a drastic decrease in

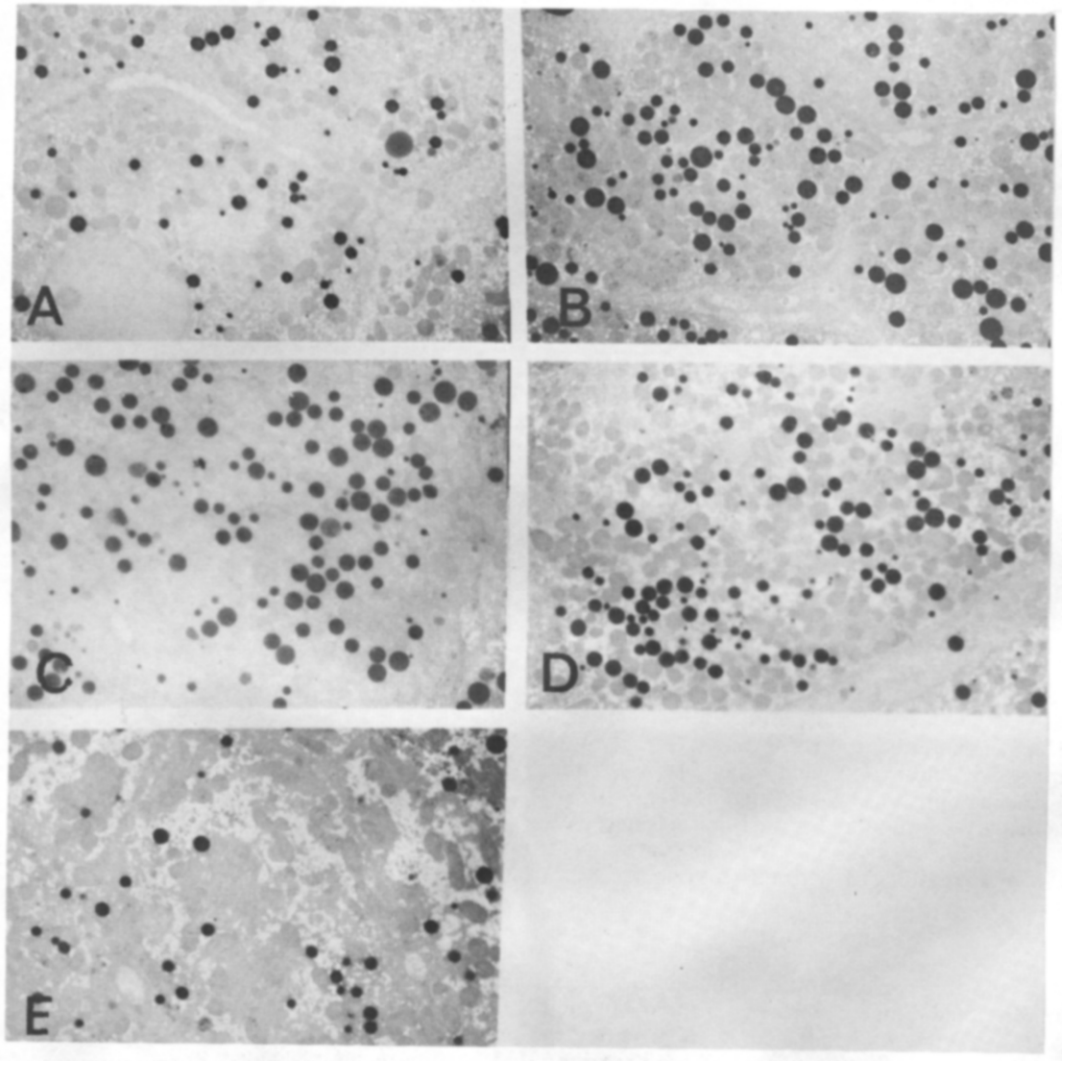

Fig. 1. Rat hepatocytes, alkaline diaminobenzidine incubation (× 5800). (A) Control: normal number of dark-staining peroxisomes; (B) 2,4-dichlorophenoxyacetic acid (2,4-D) (150 mg/kg) treatment: enlargement and proliferation of peroxisomes; (C) 4-chloro-2-methylphenoxyacetic acid (MCPA) (150 mg/kg) treatment: enlargement and proliferation of peroxisomes; (D) clofibrate (200 mg/kg) treatment: enlargement and proliferation of peroxisomes; (E) glyphosate (300 mg/kg) treatment: normal number of peroxisomes.

Table 3. Effects of 4-chloro-2-methylphenoxyacetic acid (MCPA), 2,4-dichlorophenoxyacetic acid (2,4-D), clofibrate and glyphosate on liver weight and serum lipid levels (mean ± S.D.)

Exposure	No. of animals	Liver weight (% of body wt)	Cholesterol (mmol/l)	Triglycerides (mmol/l)
Controls	5	3.29 ± 0.05 (5)	1.29 ± 0.18	1.82 ± 0.63
2,4-D (100 mg/kg)	5	3.29 ± 0.25 (5)	0.90 ± 0.18 *	1.58 ± 0.58 NS
2,4-D (150–200 mg/kg)	4	3.27 ± 0.11 (4)	1.08 ± 0.26 NS	0.89 (2)
MCPA (100 mg/kg)	5	3.22 ± 0.22 (5)	1.03 ± 0.31 NS	0.67 ± 0.18 **
MCPA (150–200 mg/kg)	5	3.44 ± 0.19 (5)	1.26 ± 0.20 NS	0.88 ± 0.16*
Clofibrate (200 mg/kg)	5	4.50 ± 0.19 (5)***	0.88 ± 0.14 *	1.11 ± 0.25*
Glyphosate (300 mg/kg)	4	3.50 ± 0.27 (4)	1.5 ± 0.10 NS	2.18 (2)

Table 4. Effects of 4-chloro-2-methylphenoxyacetic acid (MCPA), 2,4-dichlorophenoxyacetic acid (2,4-D), clofibrate and glyphosate on lipoprotein lipase (LPL) activities in muscle (heart) and adipose tissue (mean ± S.D.)

Exposure	No. of animals	LPL activity (μmol FFA h × g wet wt)	
		Adipose tissue	Muscle (heart)
Controls	5	15.4 ± 4.9	10.1 ± 5.4
2,4-D (100 mg/kg)	5	7.9 ± 4.9 *	9.1 ± 2.7 NS
2,4-D (150–200 mg/kg)	4	5.1 ± 1.6 **	7.9 ± 1.8 NS
MCPA (100 mg/kg)	5	5.1 ± 2.5 **	7.0 ± 1.8 NS
MCPA (150–200 mg/kg)	5	4.9 ± 1.8 **	15.2 ± 3.1 NS
Clofibrate (200 mg/kg)	5	10.5 ± 5.1 NS	11.9 ± 3.8 NS
Glyphosate (300 mg/kg)	4	11.8 ± 6.5 NS	9.7 ± 3.6 NS

Table 5. Effects of 4-chloro-2-methylphenoxyacetic acid (MCPA), 2,4-dichlorophenoxyacetic acid (2,4-D), clofibrate and glyphosate on the activities of liver catalase and carnitine acetyltransferase (mean ± S.D.)

Exposure	No. of animals	Catalase (nmol/min) mg protein)	Carnitine acetyltransferase (μmol/min/mg protein)
Controls	5	330 ± 60	5.0 ± 3.5
2,4-D (150–200 mg/kg)	4	572 ± 84 *	66.1 ± 14.8 **
MCPA (150–200 mg/kg)	5	493 ± 64 *	43.1 ± 13.8 **
Glyphosate (300 mg/kg)	4	507 ± 58 *	9.0 ± 4.4 NS
Clofibrate (200 mg/kg)	5	366 ± 33 NS	113.5 ± 13.0***

lipoprotein lipase activity in the adipose tissue. None of the treatments significantly affected lipoprotein lipase activity in heart muscle (Table 4).

Hepatic carnitine acetyltransferase activity was increased in those treatment groups in which the peroxisome number also increased (Table 5). Catalase activity was doubled in rats that had received 2,4-D, MCPA or glyphosate, but clofibrate caused no increase in the activity of this enzyme.

DISCUSSION

Phenoxyacetic acid herbicides induce peroxisome proliferation in Chinese hamsters [2] and in rats, and cause hypotriglyceridemia and large increases in hepatic carnitine acetyltransferase and catalase activities. It has been suggested that these herbicides cause mesenchymal tumours in man [25]; however, unlike many DNA-binding carcinogens they are not mutagenic in point-mutation assays [26, 27]. The possibility has been raised that peroxisome proliferators may be carcinogenic due to an indirect genotoxic action [28–30]. Since in our study the specific catalase activities were less than doubled and the total cellular proportion (number × volume) of peroxisomes was quadrupled, it is possible that these proliferators reduced the catalase content of peroxisomes. Thus, peroxisome proliferation may initiate the malignant transformation of cells by increasing the intracellular production of oxygen radicals [28, 29]. A similar, disproportionally small increase in catalase activity has been observed with other hypolipidemic peroxisome proliferators [12, 28]. However, there is as yet no direct evidence that the amount of oxygen radicals generated is sufficient to produce genetic lesions. No increase in the number of sister chromatid exchanges in the peripheral blood lymphocytes of rats was detected in a similar experiment [31].

The phenoxyacetic acid herbicides tested were found, like clofibrate, to be potent hypotriglyceridemic agents and to have somewhat lesser effects on

serum cholesterol. Treatment with 2,4-D and MCPA decreased the body weights of rats during the treatment period, but clofibrate did not. Although the body weights of animals treated with glyphosate were markedly decreased, however, there was no decrease in serum lipid levels compared to controls. Thus, the effects of 2,4-D and MCPA on serum lipid levels are unlikely to be due to changes in dietary intake.

The mechanism by which clofibrate and phenoxyacetic acid herbicides decrease blood lipids are far from clear. Clofibrate was thought previously to affect serum lipids by increasing tissue lipoprotein lipase activity, thus facilitating the removal of VLDL and chylomicron triglycerides from the circulation to adipose tissues, especially in hypertriglyceridemic men [6, 7]. In our study, no such increase in lipoprotein lipase activity was found. Furthermore, a correlation has been found between peroxisomal proliferation and a decrease in serum triglyceride concentration [11]. The lipid-lowering effect of clofibrate may well be related to peroxisomal (and mitochondrial) proliferation, thus increasing the β-oxidation capacity of fatty acids. Increased activity of carnitine acetyltransferase, which has been identified in mammalian peroxisomes [32, 33], would facilitate the transport of the two-carbon end-product of fatty acid β-oxidation between subcellular fractions. We found a 20-fold increase in carnitine acetyltransferase activity in the mitochondrial fraction of livers (containing peroxisomes) of 2,4-D- and MCPA-treated rats, but not in glyphosate-treated animals. Furthermore, recent results from our laboratory (Hietanen *et al.*, unpublished data) indicate that the rate of β-oxidation of fatty acids is increased in the mitochondria and peroxisomes, causing increased consumption of serum triglyceride fatty acids. The changes in serum cholesterol levels might be caused by the presence of an increased amount of specific cytochrome P-450 isozyme, which catalyses cholesterol oxidation [34].

Our results support further the conclusion that the hypotriglyceridemic effect involves peroxisomes. They also substantiate the hypothesis that phenoxy acid herbicides belong to a novel class of carcinogens which exert their action indirectly via peroxisome proliferation. However, there is as yet little direct mechanistic evidence to link peroxisome proliferation with the development of neoplasia. Whether peroxisome proliferation is of practical concern to humans deserves careful consideration and further study.

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