Toxicology, 34 (1985) 103—111
Elsevier Scientific Publishers Ireland Ltd.

ENHANCED PEROXISOMAL β-OXIDATION OF FATTY ACIDS AND GLUTATHIONE METABOLISM IN RATS EXPOSED TO PHENOXY-ACETIC ACIDS

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(Received May 28th, 1984)
(Accepted September 28th, 1984)

SUMMARY

Peroxisomal β-oxidation of fatty acids and the activities of glutathione-metabolizing enzymes in rat liver were measured after administration of 2,4-dichlorophenoxyacetic acid (2,4-D), 4-chloro-2-methylphenoxyacetic acid (MCPA), clofibrate [ethyl], 2-(*p*-chlorophenoxy)-2-methylpropionate], glyphosate (*N*-phosphonomethyl glycine, a herbicide not structurally related to phenoxy acids) or saline for 14 days. β-Oxidation increased by 6-fold in the group given clofibrate, 3-fold in the 2,4-D-treated group, and 2-fold in the MCPA-treated group over the level in the controls (saline-treated). Glyphosate did not increase β-oxidation. No significant change in reduced glutathione content from that in controls was found in any of the treated groups. Glutathione reductase activity increased by about 40% after administration of either 2,4-D or MCPA, and glutathione peroxidase activity increased by 30 % in animals given MCPA. A slight decrease in glutathione *S*-transferase activity was found in the group treated with clofibrate. The marked increases in peroxisomal β-oxidation of fatty acids were accompanied by only minor changes in the activities of enzymes involved in glutathione-dependent inactivation of organic hydroperoxides and other oxygen-centred reactive agents.

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Abbreviations· 2,4-D, 2,4-dichlorophenoxyacetic acid, EGTA, ethyleneglycolbis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; MCPA, 4-chloro-2-methylphenoxyacetic acid, MOPS, 4-morpholinepropanesulphonic acid, 2,4,5-T, 2,4,5-trichlorophenoxy acetic acid, TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

0300-483X/85/$03.30

Key words Clofibrate; Glutathione; Glutathione peroxidase; Glutathione reductase; Glutathione *S*-transferase; Herbicides; Liver; β-Oxidation

INTRODUCTION

Although phenoxyacetic acids and glyphosate are herbicides that are used widely in agriculture and forestry, few experimental studies have been done on their biological effects in animals. Human exposure to phenoxyacetic acid herbicides has been associated with an increased incidence of soft-tissue sarcomas in epidemiological studies [1,2]; these herbicides have not been found to be mutagenic in short-term mutagenicity assays [3,4].

The manufacture of 2,4-dichlorophenoxyacetic acid (2,4-D) starts with a phenol; bis(2,4-dichlorophenoxy)methane is found as an impurity [5]. 4-Chloro-2-methylphenoxyacetic acid (MCPA), another commonly used phenoxyacetic acid, is produced from a cresol and contains 4-chloro-*o*-cresol as an impurity [6]. In addition, small amounts of polychlorodibenzo-*p*-dioxins and polychlorodibenzo-*p*-furans, which are less toxic isomers than 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) may be formed during the production of both compounds [7,8].

Phenoxyacetic acid herbicides can also cause peroxisomal proliferation and may alter lipid metabolism [9,10]. The increased peroxisomal activity is due to enhanced accumulation of hydrogen peroxide and other oxygen-derived compounds [10,11], as shown in a study [10], in which peroxisomal proliferation greatly exceeded the increase in catalase activity after exposure of rats to phenoxyacetic acid herbicides or clofibrate [ethyl 2-(*p*-chlorophenoxy)-2-methylpropianate], a hypolipidaemic drug which also induces β-oxidation of fatty acids in peroxisomes and causes peroxisomal proliferation [9,10,12—14]. The peroxisomal proliferation may subsequently enhance the production of oxygen-centred reactive agents, and this might be one mechanism for the carcinogenic effects of peroxisomal proliferators such as clofibrate and phenoxyacetic acid herbicides [11,15,16]. The glutathione peroxidase activity may also be of importance, in that this enzyme catalyses the oxidation of reduced glutathine and results in consumption of the hydrogen peroxide and organic hydroperoxides that normally protect the cell [17].

The purpose of the present study was to investigate how phenoxyacetic acid herbicides affect peroxisomal β-oxidation of fatty acids in the liver. Since glutathione and glutatione-metabolizing enzymes may inactivate the resulting products, the concentration of glutathione and the activities of glutathione peroxidase, reductase and *S*-transferase were determined.

MATERIALS AND METHODS

Six-week-old male Wistar rats were used and given free access to food and drinking-water. 2,4-D and MCPA, obtained from Kemira Company, Finland, were given at doses of 1 mmol/kg body weight in solutions containing 550 g/l 2,4-D (as the amine salt) and 500 g/l MCPA (as the isooctyl ester) intragastrically every morning for 14 days.

The dose of MCPA was reduced to 0.5 mmol/kg after a 4-day treatment because of excessive toxicity. Glyphosate (*N*-phosphonomethylglycine; Roundup®, Monsanto Co, U.S.A.) was given intragastrically as the isopropylamine salt in a solution containing 360 g/l at a dose of 1 mmol/kg. As a positive control, clofibrate (Klofiran®; Remeda Ltd, Finland) was given intragastrically at a dose of 1 mmol/kg. Negative controls received equivalent volumes of saline.

The rats were decapitated, and the liver of each rat was dissected and placed in ice-cold sucrose (0.25 mol/l). The liver was homogenized in 4 volumes of sucrose with a Potter-Elvehjem-type glass-Teflon homogenizer and centrifuged at 800 *g* for 10 min in a refrigerated centrifuge peroxisomes were then isolated identically to the method of Neat and Osmundsen [18]. The supernatant was further centrifuged (+4°C) at 31 700 *g* for 10 min. The pellet, containing peroxisomes and mitochondria, was resuspended in buffer containing 0.25 mol/l sucrose, 2 mmol/l 4-morpholinepropanesulphonic acid (MOPS), 5 mmol/l ethyleneglycolbis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid (EGTA) and 0.1% ethanol (pH 7.2) [17,18]. A Percoll gradient for enrichment of the peroxisomes was prepared by pipetting 1.56 ml of 60% (1.75 mol/l) sucrose onto the bottom of an ultracentrifuge tube and adding 10.97 ml of Percoll solution (50% v/v) and an equal amount of buffer containing 0.5 mol/l sucrose, 4 mmol/l MOPS, 10 mmol/l EGTA and 0.2% ethanol (pH 7.2). A 1.5-ml sample was pipetted onto the top. After centrifugation at 83 000 *g* for 30 min in a refrigerated centrifuge, the peroxisomes that had collected in the top 10 ml were visible as a brown collar. This portion was pipetted into another ultracentrifuge tube, which was then filled with 0.25 mol/l sucrose and centrifuged at 126 000 *g* for 30 min; the peroxisomes sedimented to the bottom [17,18]. The supernatant fraction was discarded, and the peroxisomes were harvested in 2 ml volumes for enzyme assays. The supernatant of the centrifugation at 31 700 *g*, which contained the soluble fraction and microsomes, was further centrifuged at 105 000 *g* for 60 min in a refrigerated centrifuge. The microsomes sedimented to the bottom, and the soluble fraction was poured into another test tube and saved for determinations of glutathione and of glutathione reductase, peroxidase and transferase.

Peroxisomal β-oxidation of fatty acids was determined as described by Lazarow [19]. The reaction is based on the oxidation of palmitoyl CoA in the presence of NAD^+, which is then reduced to NADH and monitored in a thermostatted double-beam spectrophotometer. The assay mixture contained 940 µl 50 mmol/l Tris—HCl buffer (pH 8.0), 10 µl 20 mmol/l NAD^+, 3 µl 0.33 mol/l dithiothreitol, 5 µl of 1.5% bovine serum albumin (fraction V), 5 µl of 2% Triton X-100, 10 µl of 10 mmol/l CoA, 10 µl of 1 mmol/l FAD and 10 µl of 100 mmol/l potassium cyanide; after these solutions had been mixed, 20 µl of the peroxisomal suspension was added. The reaction was started with 10 µl of 1 mmol/l palmitoyl-CoA and followed for 5 min against a reference cuvette without palmitoyl-CoA.

The concentrations of glutathione and the activities of the glutathione metabolizing enzymes were measured in the postmicrosomal supernatant

fraction. The contents of non-protein sulfhydryl compounds were determined according to the method of Ellman [20]; since reduced glutathione accounts for more than 90% of these compounds [21], the results are expressed as GSH. The activity of glutathione reductase was recorded by measuring the rate of NADPH oxidation at 340 nm using a thermostatted cuvette (37°C), as described by Heinonen and Vainio [22]. Glutathione peroxidase activity was measured as described by Tappel [23]. Glutathione *S*-transferase activity was measured using 1-chloro-2,4-dinitrobenzene as the substrate; the reaction was monitored at 340 nm (37°C), as described by Habig et al. [24], except that the reaction mixture contained 0.02% Triton X-100 as detergent. Protein content was determined by the biuret reaction, according to the method of Gornall et al. [25]. Student's *t*-test was used for the statistical analyses.

RESULTS

No significant change in body or liver weights of rats receiving the herbicides or clofibrate was found in comparison with the controls, and there was no significant difference in peroxisomal protein content (Table I). However, β-oxidation measured in isolated peroxisomes was found to increase by 6-fold in rats treated with clofibrate, by 2-fold after MCPA administration and by 3-fold after administration of 2,4-D, in comparison with the level in controls (Fig. 1). Glyphosate caused no increase in β-oxidation activity. In preliminary experiments (data not shown), 4- and 3-fold increases in β-oxidation were found after intragastric administration of 2,4-D and MCPA, respectively, for 7 days at the same doses as in the 14-day study.

The content of reduced glutathione was not affected by administration of any of the compounds tested. Glutathione peroxidase activity was elevated in rats treated with MCPA, and hepatic glutathione reductase activity was

TABLE I

BODY AND LIVER WEIGHTS OF RATS RECEIVING VARIOUS HERBICIDES OR CLOFIBRATE FOR 2 WEEKS, AND PEROXISOMAL PROTEIN CONTENTS OF LIVER

Mean ± S E M are given Numbers of animals in parentheses No statistically significant difference was found for any of the parameters between the exposed groups and the controls

Treatment	Body wt (g)	Liver wt (g)	Liver/body wt ratio (%)	Peroxisomal protein (mg/g liver)
2,4-D	261 ± 8 (4)	10.89 ± 0 65	4 16 ± 0 16	6.80 ± 0.30
MCPA	225 ± 12 (6)	10 43 ± 0 86	4 78 ± 0.66	8.10 ± 0.85
Glyphosate	243 ± 5 (5)	9 05 ± 0 46	3.72 ± 0.17	8 44 ± 0.48
Clofibrate	258 ± 6 (7)	12 24 ± 0.37	4 76 ± 0 18	7 47 ± 0.56
Controls	274 ± 25 (5)	10 66 ± 0 99	4.18 ± 0 23	7 37 ± 0.51

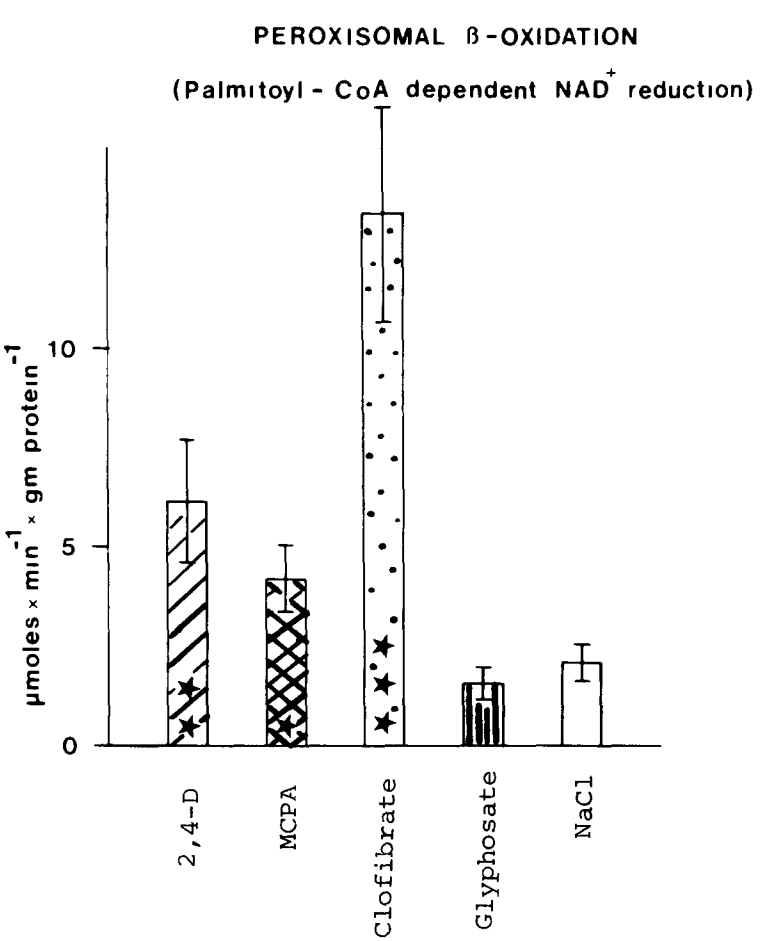

Fig. 1. Peroxisomal β-oxidation rate, expressed as μmol NAD^+ reduced equivalent to acetyl CoA formed per min and per g of peroxisomal protein. Means ± S.E.M. are given. Statistical significance in comparison with controls: $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$

elevated in rats receiving either MCPA or 2,4-D. Clofibrate, but none of the herbicides, decreased glutathione *S*-transferase activity (Table II).

DISCUSSION

Previous studies have shown that MCPA and 2,4-D increase the number and size of peroxisomes in rodents and that clofibrate causes peroxisomal proliferation both in experimental animals and in man [8,9,13,26,27]. Increased peroxisomal and mitochondrial oxidation may be primary causes of the lipid-lowering effect of clofibrate in man [13,26,27]. The relation of increased β-oxidation of fatty acids to increased production of hydrogen peroxide has been suggested as a possible mechanism for the hepatocarcinogenicity of peroxisome proliferators [28,29,35].

TABLE II

HEPATIC NON-PROTEIN SULFYDRYL CONTENT (EXPRESSED AS GSH) AND GLUTATHIONE PEROXIDASE (GSH-Px), REDUCTASE (GSSG-Red) AND S-TRANSFERASE (GSH-S-Trans) ACTIVITIES AFTER A 14-DAY EXPOSURE OF RATS TO HERBICIDES OR CLOFIBRATE

Treatment	GSH (μmol/g)	GSH-Px (μmol/min per g)	GSSG-Red (μmol/min per g)	GSH-S-Trans (μmol/min per g)
2,4-D	4 88 ± 0 45	26.5 ± 3.1	3 33 ± 0 51*	63 9 ± 7 5
MCPA	6 24 ± 0 30	33 4 ± 2 6**	3 19 ± 0 20**	71 4 ± 9.0
Glyphosate	5 27 ± 0 51	21 8 ± 4 4	2 59 ± 0 17	68 4 ± 7 5
Clofibrate	6 31 ± 0 30	25 0 ± 2.6	1 80 ± 0.21	45 3 ± 3 8*
Control	5 64 ± 0 32	21.4 ± 0.4	2 17 ± 0 18	61 6 ± 6 0

Means ± S E M are given GSH contents and enzyme activities are calculated per original liver wet weight. Statistical significance in comparison with controls. *$P < 0\ 05$, **$P < 0\ 01$

An increase in the amount of peroxisomes is usually accompanied by an increase in β-oxidation activity [17]. Sometimes, however, the qualitative properties of peroxisomes may change, resulting in enhanced enzyme activities with no apparent increase in protein content [14]: in the present study, the protein cotent of peroxisomes was not significantly altered by administration of the peroxisome proliferators. Purification of peroxisomes is essential in order to obtain reproducible results on β-oxidation even when potassium cyanide is used to inhibit the mitochondrial activity [18]. In the peroxisome purification process used here, the yield of peroxisomal protein was of the same magnitude as that reported by Neat et al. [17]. According to the data reported in the original method mitochondria contributed only 1% and other subcellular fractions 10% to the protein in the peroxisomal preparation [18].

The dose of clofibrate used in our study was relatively low (about 60 mg/250 g) and caused only a slight increase (10%) in the relative liver weight, in accordance with previous findings [27]. This low dose was chosen in order to compare the effects of clofibrate with those of herbicides on a molar basis but to avoid toxic levels of the herbicide. Our data on β-oxidation are, however, in accordance with those reported earlier in clofibrate-treated rats: 23 nmol/min per mg peroxisomal protein and, after different dietary modifications, 5.4—24.3 nmol/min per mg peroxisomal protein [17,18]. Data derived by a radiochemical method are also in agreement with those we obtained [30]. β-Oxidation is often measured in crude homogenates, despite the drawbacks inherent in the multistep involvement of enzymes [19]; nevertheless, our data, expressed on a liver wet-weight basis, are also in accordance with those published previously [18,31,32] although also different activity levels have been reported depending on the method and enzyme source used [19,29].

In a recent publication, Kawashima et al. [33] reported an approximately 10-fold increase in β-oxidation after administration of 2,4,5-trichlorophenoxy acetic acid (2,4,5-T) and a 5-fold increase after treatment with 2,4-D for 7 days; a somewhat smaller increase was seen after 14 days feeding of the compounds to rats in their diet at a concentration of 0.25% and when the activities were measured in a crude postnuclear supernatant. They also reported a decrease in blood lipids after treatment with 2,4-D and 2,4,5-T and a modest increase in catalase activity but a large increase in carnitine acetyltransferase activity. These data are well in agreement with our findings and with data we reported earlier [10,34], despite the fact that Kawashima et al. only partially purified the peroxisomes and only those from 2,4,5-T-treated rats.

Peroxisomal β-oxidation may result in increased production of intracellular oxygen-derived radicals, organic peroxides, hydroperoxides and hydrogen peroxide [27,28,32]. The predominant enzymes in the decomposition of endogenously produced hydrogen peroxide in the liver are catalase and glutathione peroxidase. It has been suggested that catalase plays a major role in the metabolism of hydrogen peroxide generated in peroxisomes, whereas the glutathione peroxidase system is important in the decomposition of hydrogen peroxide in cytosol [36].

No change was found in the content of non-protein sulfhydryl groups after administration of any of the herbicides or clofibrate. The 30% increase in glutathione peroxidase found after administration of MCPA and the approximately 40% increase in the activity of glutathione reductase after administration of both 2,4-D and MCPA may suggest an adaptive response to the increased turnover of glutathione due to the production of organic hydroperoxides.

The data presented here support the hypothesis that phenoxyacetic acid herbicides facilitate fatty acid oxidation in peroxisomes and may also enhance the production of hydrogen peroxide and organic hydroperoxides, reflected as a weak stimulation of glutathione peroxidase activity.

ACKNOWLEDGEMENTS

This study was supported in part by a grant from the National Institutes of Health, USA (RO1 ES 01684), by the J. Vainio Foundation and by the Academy of Finland. We thank Ms E. Heseltine for editorial assistance and Ms M. Wrisez for typing the manuscript.

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