Evidence for two enzymatic pathways for ω-oxidation of docosanoic acid in rat liver microsomes

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Abstract We studied the ω-oxidation of docosanoic acid (C22:0) in rat liver microsomes. C22:0 and 22-hydroxy-docosanoic acid (ω-hydroxy-C22:0) were used as substrates, and the reaction products were analyzed by electrospray ionization mass spectrometry. In the presence of NADPH, ω-oxidation of C22:0 produced not only the hydroxylated product, ω-hydroxy-C22:0, but also the dicarboxylic acid of C22:0, docosanedioic acid (C22:0-DCA). When rat liver microsomes were incubated with ω-hydroxy-C22:0 in the presence of either NAD^+ or NADPH, C22:0-DCA was formed readily. Formation of C22:0-DCA from either C22:0 or ω-hydroxy-C22:0 with NADPH as cofactor was inhibited strongly by miconazole and disulfiram, whereas no inhibition was found with NAD^+ as cofactor. Furthermore, ω-oxidation of C22:0 was reduced significantly when molecular oxygen was depleted. The high sensitivity toward the more specific cytochrome P450 inhibitors ketoconazole and 17-octadecynoic acid suggests that hydroxylation of C22:0 and ω-hydroxy-C22:0 may be catalyzed by one or more cytochrome P450 hydroxylases belonging to the CYP4A and/or CYP4F subfamily. This study demonstrates that C22:0 is a substrate for the ω-oxidation system in rat liver microsomes and that the product of the first hydroxylation step, ω-hydroxy-C22:0, may undergo further oxidation via two distinct pathways driven by NAD^+ or NADPH.—Sanders, R-J., R. Ofman, F. Valianpour, S. Kemp, and R. J. A. Wanders. **Evidence for two enzymatic pathways for ω-oxidation of docosanoic acid in rat liver microsomes. *J. Lipid Res.* 2005.** 46: **1001–1008.**

Supplementary key words hydroxylase • cytochrome P450 • ω-hydroxy-fatty acids • dicarboxylic acids

Fatty acid oxidation plays a major role in the production of energy, notably in the heart and skeletal muscle, and is the main energy source during periods of fasting. The primary degradation route of fatty acids is via β-oxidation that takes place in mitochondria and peroxisomes. Other mechanisms for the oxidation of fatty acids involve α-oxidation in peroxisomes or ω-oxidation in the smooth endoplasmic reticulum.

It is generally accepted that under normal physiological conditions, fatty acid ω-oxidation is a minor pathway that accounts for ~5–10% of total fatty acid oxidation in the liver (1). During periods of fasting or starvation, intracellular levels of free fatty acids increase and subsequently become available as substrates for ω-oxidation (2–4). The ω-oxidation of fatty acids consists of three sequential steps in which the terminal methyl group is converted into a carboxyl group. The initial step, hydroxylation of the methyl group, requires NADPH and molecular oxygen and is catalyzed by microsomal enzymes belonging to the cytochrome P450 4A (CYP4A) family (5–7). The ω-hydroxy fatty acid that is produced can be oxidized further by an alcohol dehydrogenase into an ω-oxo-fatty acid (8). Finally, this product can be converted into a dicarboxylic acid by an aldehyde dehydrogenase. Dehydrogenation of ω-hydroxy- and ω-oxo-fatty acids requires NAD^+ and has been identified in the cytosol (5, 9, 10). The dicarboxylic acids that are produced via the ω-oxidation pathway can be either excreted into the urine or β-oxidized in mitochondria or peroxisomes (11–13).

Several rat cytochrome P450 4A isoforms (CYP4A1, CYP4A2, CYP4A3, and CYP4A8) and two human isoforms (CYP4A11 and CYP4A22) have been characterized (7, 14, 15). All of these enzymes were demonstrated to have highest activity for medium-chain fatty acids, and the hydroxylation rate was found to decline with increasing chain length. However, studies of the ω-oxidation of long-chain fatty acids in brain suggest the existence of additional hydroxylation enzymes with different chain length specificities (16, 17). At present, little is known about the ω-oxidation of fatty acids with a chain length of more than 20 carbons.

Very long-chain fatty acids (VLCFAs; >22 carbons) are exclusively β-oxidized in peroxisomes. In several inherited peroxisomal diseases, including X-linked adrenoleukodystrophy, peroxisomal biogenesis disorders, and two single

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Abbreviations: C22:0, docosanoic acid; C22:0-DCA, docosanedioic acid; ω-hydroxy-C22:0, 22-hydroxy-docosanoic acid; 17-ODYA, 17-octadecynoic acid; VLCFA, very long-chain fatty acid.

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peroxisomal enzyme deficiencies, acyl-CoA oxidase deficiency and peroxisomal bifunctional protein deficiency, VLCFAs are increased in plasma as a result of reduced β-oxidation of these fatty acids. In contrast, plasma levels of docosanoic acid (C22:0) are decreased in these patients (18). An excess of long-chain hydroxylated fatty acids and dicarboxylic acids was found in urine of patients with peroxisomal biogenesis disorders (19). Based on these data, we postulate that ω-oxidation of (very) long-chain fatty acids may provide an alternative route for fatty acid metabolism. The aim of this study was to investigate whether C22:0 is a substrate for the microsomal ω-oxidation system.

MATERIALS AND METHODS

Materials

NAD^+, NADPH, and glucose-6-phosphate dehydrogenase were obtained from Roche Applied Science. 22-Hydroxy-docosanoic acid (ω-hydroxy-C22:0) and hexacosanedioic acid were purchased from Larodan Fine Chemicals (Malmö, Sweden). 22,22,22-D_3-Docosanoic acid was obtained from CDN Isotopes (Québec, Canada). Omeprazole, sulfaphenazole, quinidine, ketoconazole, furafylline, trimethoprim, diethyldithiocarbamate, 17-octadecynoic acid (17-ODYA), *N*-ethylmaleimide, miconazole, disulfiram, and L-lactate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). 4-Chloromercuribenzoate was obtained from Janssen Chemica. All other chemicals used were of analytical grade.

Purification of rat liver microsomes

Microsomes were isolated from rat livers by differential centrifugation essentially as described by Baudhuin et al. (20). To this end, male Wistar rats (200–250 g) were fed ad libitum with standard rodent chow and fasted overnight before being killed. The livers were removed immediately and washed with homogenization buffer containing 250 mM sucrose, 5 mM MOPS, pH 7.4, and 2 mM EDTA, minced, and homogenized (five strokes at 500 rpm) using a Wheaton homogenizer with a Teflon pestle. The homogenate was centrifuged at 550 *g* for 10 min to remove the nuclei and cell debris. Subsequently, the supernatant was centrifuged at 3,200 *g* for 10 min, followed by centrifugation at 22,500 *g* for 10 min to remove the bulk of the mitochondria, lysosomes, and peroxisomes. To isolate enriched microsomal membranes, the supernatant was sonicated three times for 10 s with an interval of 1 min on ice and centrifuged for 1 h at 100,000 *g*. The complete isolation procedure was carried out at 4°C. The enriched

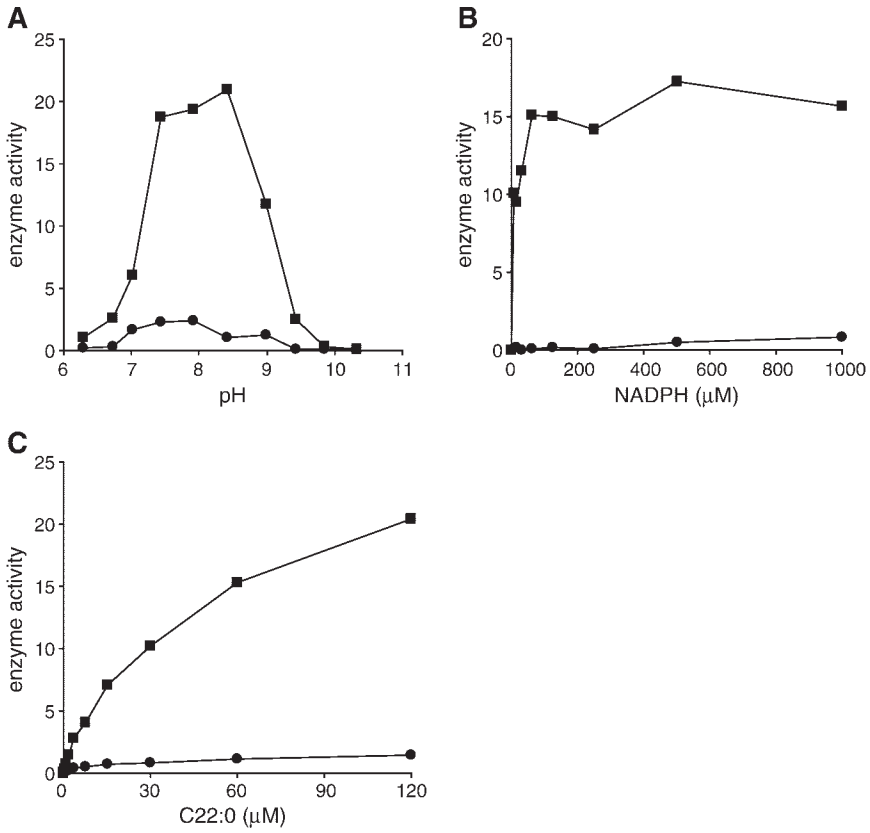

Fig. 1. ω-Oxidation of docosanoic acid (C22:0) in rat liver microsomes. A: Rat liver microsomes were incubated in the standard reaction mixture, which contained 40 μg of protein and 120 μM C22:0 at different pH values. Reactions were allowed to proceed for 15 min at 37°C, and after termination the amount of 22-hydroxy-docosanoic acid (ω-hydroxy-C22:0) produced was analyzed as described in Materials and Methods. B: Microsomal protein was incubated using the standard reaction mixture containing different concentrations of NADPH in the presence of a cofactor regenerating system as described in Materials and Methods. C: Microsomal protein was incubated at different concentrations of C22:0. Enzyme activity is expressed as nanomoles per milligram per 15 min. Closed squares, ω-hydroxy-C22:0; closed circles, docosanedioic acid (C22:0-DCA). Each point represents the mean of two independent experiments.

microsomal membrane fraction was resuspended in phosphate-buffered saline, pH 7.4, and divided into small aliquots that were stored at −80°C until further use. Protein was measured according to the method described by Bradford (21).

Enzyme assay

Incubations were carried out in a reaction mixture containing glycine (100 mM)/Hepes (100 mM) buffer, α-cyclodextrin (1 mg/ml), and NAD^+ or NADPH (1 mM) in a total volume of 200 μl for 15–30 min at 37°C. Incubations were carried out at pH 8.5 using NADPH as cofactor or at pH 9.5 using NAD^+ as cofactor. After preincubation at 37°C for 10 min, the reaction was initiated by addition of the substrate at a final concentration of 120 μM and terminated by addition of 1 ml of hydrochloric acid to a final concentration of 1.7 M. C22:0 and ω-hydroxy-C22:0 were dissolved in dimethyl sulfoxide to a final concentration of 2.8 mM. Depletion of molecular oxygen was accomplished by gently flushing the reaction mixture with nitrogen for 20 min on ice.

Experiments to determine the cofactor dependence were carried out in the standard reaction mixture supplemented with a NAD^+ or NADPH regenerating system. With the NAD^+ regenerating system, the reaction mixture contained pyruvate (1 mM) and L-lactate dehydrogenase (4 U/ml). To regenerate NADPH, $MgCl_2$ (3 mM), glucose-6-phosphate (10 mM), and glucose-6-phosphate dehydrogenase (20 U/ml) were added to the reaction mixture.

Analysis of ω-oxidation intermediates

After termination of the reactions, the mixture was transferred to a 4 ml glass vial followed by addition of 100 μl of internal standard solution containing D_4-C22:0 and D_4-C24:0 in toluene and 2 ml of hexane. The samples were vortex mixed thoroughly for 30 s and centrifuged at 3,000 rpm for 1 min. Approximately 1.5 ml of the upper phase was transferred to a 16 × 125 mm glass tube, and hexane was evaporated at 37°C under a constant stream of nitrogen. Finally, the residue was dissolved in a 100 μl chloroform-methanol-water (50:45:5, v/v/v) mixture containing 0.01% aqueous ammonia and transferred to autosampler vials (Gilson, Middleton, WI). The samples were analyzed according to the method described by Valianpour et al. (22). For the calculation of fatty acid concentrations, a five-point calibration curve was made for ω-hydroxy-C22:0 and hexacosanedioic acid. The calibration curve of hexacosanedioic acid was used to determine the concentration of docosanedioic acid (C22:0-DCA) because the latter compound is not commercially available. Samples were extracted

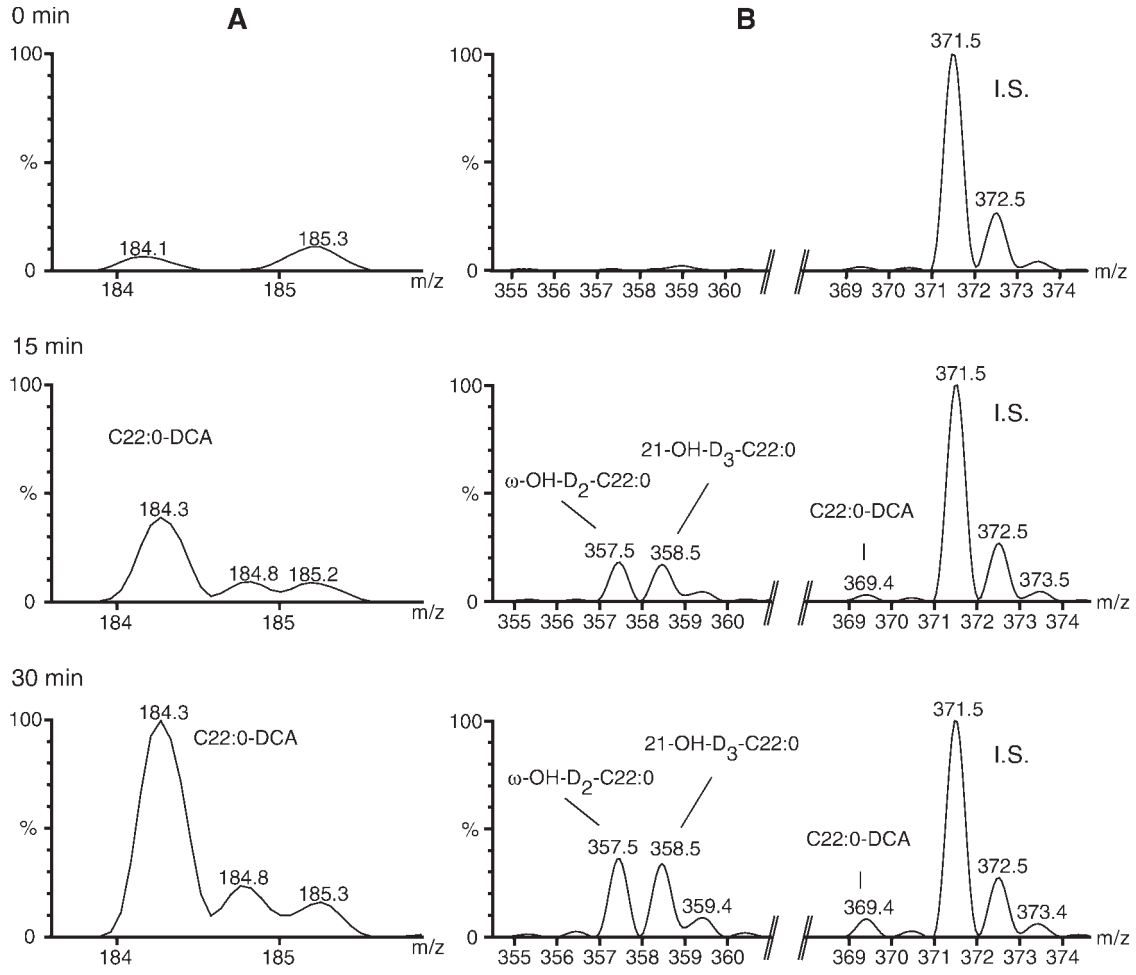

Fig. 2. Time course study of ω- and ω-1-oxidation of C22:0 in rat liver microsomes. The standard reaction mixture contained 40 μg of protein, 1 mM NADPH, and 120 μM 22,22,22-D_3-docosanoic acid. Reactions were terminated at the indicated times. A: The spectra of the double negatively charged ion of C22:0-DCA. The signals with an *m/z* value of 184.3 and 184.8 represent the double charged ion and its first isotope peak, respectively. B: The single ion spectra. The signals with *m/z* values of 357.5, 358.5, and 369.4 represent the negatively charged ions of ω-hydroxy-D_2-C22:0, 21-hydroxy-D_3-C22:0, and C22:0-DCA, respectively. D_4-C24:0 was used as an internal standard (I.S.) and has a signal with an *m/z* value of 371.5.

and analyzed as described above. For each analyte, the input concentration was plotted against the ratio of the peak height of the analyte to the peak height of its corresponding internal standard. The trend line and the intercept were used to calculate the concentrations of the analytes in the samples.

RESULTS

ω-Oxidation of C22:0

ω-Oxidation of saturated fatty acids longer than 20 carbon atoms was examined in rat liver microsomes. We started with determination of the optimal assay conditions using C22:0 as substrate. To this end, rat liver microsomes were incubated in a buffered medium at different pH values containing 1 mM NADPH and α-cyclodextrin to solubilize C22:0. The results, depicted in **Fig. 1A**, show a broad pH optimum between 7.5 and 8.5. The greatest activity was found at pH 8.5, and this pH was selected for further experiments. Figure 1B shows the results of an experiment in which the NADPH concentration was varied between 0 and 1 mM. In the presence of NADPH, a product was formed with an *m/z* value of 355.5 corresponding to ω-hydroxy-C22:0. Interestingly, at concentrations of NADPH exceeding 250 μM, a second product was formed with an *m/z* value of 369.5 that corresponds to C22:0-DCA. The identity of this compound as C22:0-DCA was supported by the fact that its formation was accompanied by the coappearance of a signal at *m/z* 184.3, which represents the double negatively charged ion of C22:0-DCA (**Fig. 2**). Fig. 1C shows the results of the ω-oxidation of C22:0 at different substrate concentrations. Calculated from the corresponding Lineweaver-Burk plot, the apparent K_m value for C22:0 was 30 μM. Formation of C22:0-DCA was detected already at the lowest substrate concentration used and increased upon higher substrate concentrations. To test whether the production of C22:0-DCA was attributable to contamination of NADPH with NAD^+, the purity of NADPH was examined using HPLC analysis. This revealed that NADPH was not contaminated with NAD^+ (data not shown) and indicates that C22:0 can be converted to C22:0-DCA using NADPH as cofactor.

To discriminate between ω- and ω-1-hydroxylation of C22:0, the substrate was replaced by 22,22,22-D_3-C22:0. In-

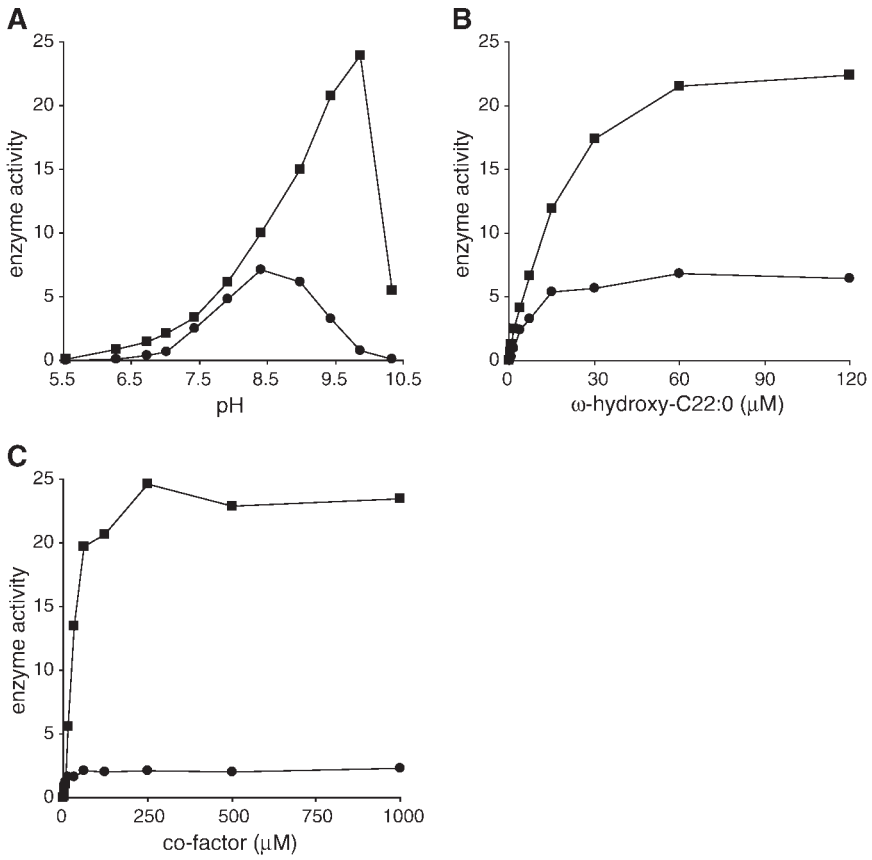

Fig. 3. Characterization of the NADPH- and NAD^+-driven ω-oxidation system using ω-hydroxy-C22:0 as substrate. A: Rat liver microsomes were incubated in the standard reaction mixture with 1 mM NAD^+ (closed squares) or 1 mM NADPH (closed circles) at different pH values. B: Formation of C22:0-DCA was studied at different concentrations of ω-hydroxy-C22:0 in the NAD^+-containing (closed squares) or NADPH-containing (closed circles) medium. C: Microsomes were incubated in the standard reaction mixture with NAD^+ and NADPH concentrations ranging from 0 to 1 mM in the presence of a cofactor regenerating system as described in Materials and Methods. Each point represents the mean of two independent experiments. Enzyme activity is expressed as nanomoles per milligram per 15 min.

cubation of rat liver microsomes with this substrate resulted in the formation of the two hydroxylated products (Fig. 2). Hydroxylation of the substrate at the terminal carbon atom resulted in ω-hydroxy-22-D_2-C22:0, with an *m/z* value of 357.5, attributable to the loss of one deuterium. In addition, a signal with an *m/z* value of 358.5 was detected that corresponds to 21-hydroxy-22-D_3-C22:0, the ω-1-hydroxylation product of C22:0. The intensity of the peak with *m/z* 358.5 was corrected for the contribution of the first isotope peak (24.2%) from ω-hydroxy-22-D_2-C22:0. From these results, we calculated that the ratio of ω- to ω-1-hydroxylation was ~1.5.

ω-Oxidation of ω-hydroxy-C22:0

To study the enzymatic characteristics of the conversion of ω-hydroxy acid to its dicarboxylic acid, we replaced C22:0 by ω-hydroxy-C22:0 in subsequent experiments. In the presence of NADPH as well as NAD^+ as cofactor, C22:0-DCA was formed readily (**Fig. 3C**), whereas no product was detected when NADH or $NADP^+$ was used as cofactor (data not shown).

ω-Oxidation of ω-hydroxy-C22:0 to C22:0-DCA was maximal at pH 8.5 with NADPH as cofactor (Fig. 3A). At this pH, the kinetic constants for NADPH and ω-hydroxy-C22:0 were determined and calculated from their corresponding Lineweaver-Burk plots. The apparent K_m values were 7 μM for ω-hydroxy-C22:0 and 4 μM for NADPH (Fig. 3B, C). In the presence of NAD^+, ω-oxidation of ω-hydroxy-C22:0 was maximal at pH 9.5 (Fig. 3A). The apparent K_m values were 13 and 23 μM for ω-hydroxy-C22:0 and NAD^+, respectively (Fig. 3B, C).

To provide further substantiation for the existence of two enzymatic pathways for ω-oxidation of C22:0, we studied the oxygen dependence of the NAD^+- and NADPH-driven routes. Depletion of molecular oxygen resulted in a marked decrease in the formation of C22:0-DCA in the presence of NADPH, whereas in the presence of NAD^+ ω-oxidation activity was not affected significantly (**Table 1**). To distinguish further between these two ω-oxidation pathways, several inhibitors were tested (Table 1). Formation of C22:0-DCA via either the NADPH- or the NAD^+-dependent system was inhibited strongly by the sulfhydryl reagents 4-chloromercuribenzoate and *N*-ethylmaleimide. Miconazole, an inhibitor of a broad spectrum of cytochrome P450s (23), strongly inhibited the NADPH-dependent pathway but not the NAD^+-driven system. Similar results were observed with disulfiram, which is an inhibitor of the microsomal fatty aldehyde dehydrogenase (FALDH) (24) and cytochrome P450 2E1 (25).

Cytochrome P450 inhibition studies

The contribution of different cytochrome P450 enzymes was studied using CYP family-specific inhibitors: sulfaphenazole (CYP2C6/9), quinidine (CYP2D6), ketoconazole (CYP2A1 and CYP3A1/2/4), furafylline (CYP1A2) (26, 27), diethyldithiocarbamate (CYP2E1/3A4) (26), omeprazole (CYP2C19) (28), trimethoprim (CYP2C8) (29), and 17-ODYA (CYP4A/F) (30, 31). Hydroxylation of C22:0 and C22:0-OH was inhibited markedly by ketoconazole and 17-ODYA, whereas the other inhibitors had no effect on the hydroxylation rates of the two substrates (**Fig. 4**).

DISCUSSION

In this study, we demonstrate that C22:0 is a substrate for the ω-oxidation system in rat liver microsomes. The first reaction step, the hydroxylation of the terminal methyl group, is NADPH- and molecular oxygen-dependent. This reaction is affected by miconazole, a cytochrome P450 inhibitor, indicating that one or more members of the cytochrome P450 family are involved. Both the terminal and the penultimate carbon atom can be hydroxylated. The hydroxylation of the carbon atom at the ω or ω-1 end occurs with a ratio ω/ω-1 of 1.5, indicating some preference for the ω-carbon.

TABLE 1. Effect of molecular oxygen depletion and different inhibitors on the ω-oxidation of C22:0 and ω-hydroxy-C22:0

Inhibitor	Concentration	C22:0 NADPH Activity	ω-Hydroxy-C22:0	
			NADPH Activity	NAD^+ Activity
			%	
Oxygen depletion		20 ± 5.0	4 ± 0.8	71 ± 6.8
4-Chloromercuribenzoate	10 μM	6 ± 0.8	4 ± 0.3	2 ± 0.6
	100 μM	2 ± 0.2	3 ± 0.7	1 ± 0.2
N-Ethylmaleimide	1 mM	10 ± 0.2	12 ± 0.8	57 ± 3.1
	10 mM	2 ± 0.1	3 ± 0.1	3 ± 0.3
Miconazole	6.3 μM	25 ± 1.9	21 ± 0.7	95 ± 9.2
	12.5 μM	4 ± 0.3	10 ± 2.4	88 ± 3.0
	25 μM	1 ± 0.1	6 ± 0.3	76 ± 2.1
Disulfiram	10 μM	90 ± 8.7	86 ± 0.1	86 ± 7.6
	100 μM	41 ± 7.9	20 ± 0.6	99 ± 3.6

C22:0, docosanoic acid; ω-hydroxy-C22:0, 22-hydroxy-docosanoic acid. Rat liver microsomes (40 μg) were preincubated in the standard reaction mixture containing inhibitors at the indicated concentrations for 10 min. After preincubation, reactions were initiated by adding the substrate and were allowed to proceed for 15 min at 37°C. The results are means ± SD of three to four independent experiments and are expressed relative to the activity observed in the absence of inhibitors.

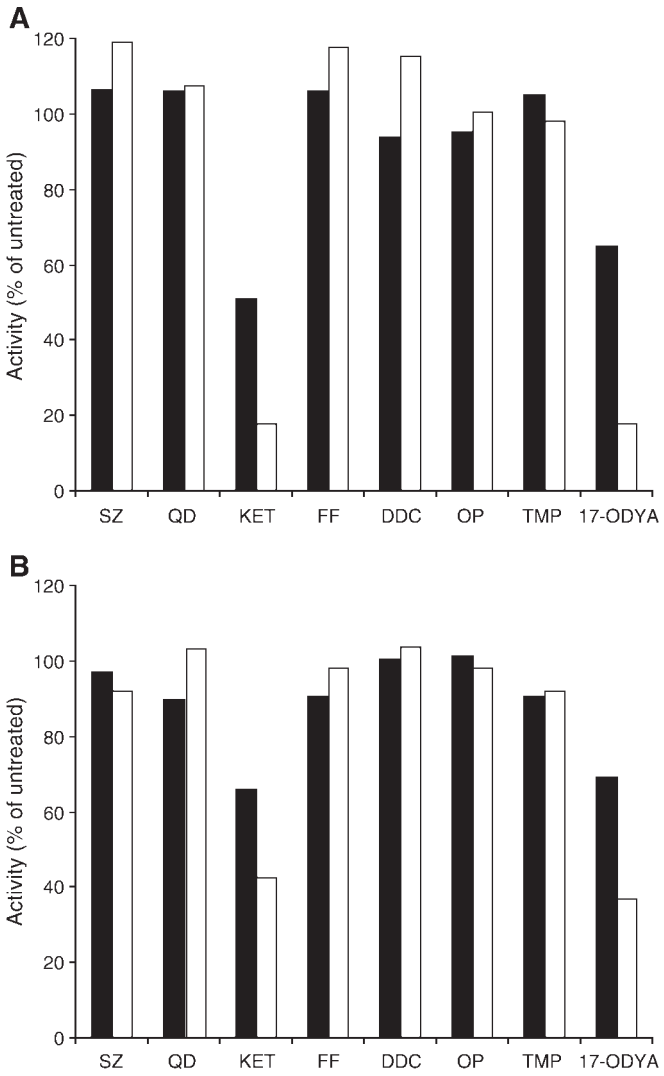

Fig. 4. Rat liver microsomes (40 μg) were preincubated for 15 min in the standard reaction mixture containing 1 mM NADPH and inhibitors at the indicated concentrations. After preincubation, the reaction was initiated by addition of the substrate, either C22:0 (A) or ω-hydroxy-C22:0 (B). Further procedures are described in Materials and Methods. The results are expressed as the percentage of residual activity compared with the control. The results are mean values of two independent experiments and do not vary by more than 10%. Closed bars, inhibitor at a final concentration of 1 μM; open bars, inhibitor at a final concentration of 10 μM. SZ, sulfaphenazole; QD, quinidine; KET, ketoconazole; FF, furafylline; DDC, diethyldithiocarbamate; OP, omeprazole; TMP, trimethoprim; 17-ODYA, 17-octadecynoic acid.

Interestingly, we found that incubation of microsomes with C22:0 in the presence of NADPH not only produced ω-hydroxy-C22:0 but also resulted in the formation of C22:0-DCA. Incubation of microsomes with ω-hydroxy-C22:0 clearly demonstrated the formation of C22:0-DCA in the presence of either NADPH or NAD^+. Based on the differences in pH optimum, substrate affinity, and cofactor dependence, we conclude the existence of two different ω-oxidation systems for ω-hydroxy-C22:0 in rat liver microsomes.

In the presence of NADPH, the formation of C22:0-DCA from ω-hydroxy-C22:0 was decreased markedly when molecular oxygen was depleted, whereas little effect was observed with NAD^+ as cofactor. Disulfiram is known for its strong inhibition of FALDH, a membrane-associated microsomal fatty aldehyde dehydrogenase (24), and CYP2E1 hydroxylase (25). FALDH is encoded by the ALDH10 gene and is deficient in patients with Sjörgen-Larson syndrome (32). In contrast to incubations with NADPH, disulfiram had no considerable inhibitory effect on the formation of C22:0-DCA in the presence of NAD^+. This suggests that FALDH is unlikely to be involved in the NAD^+-dependent ω-oxidation of ω-hydroxy-C22:0. Therefore, it is most likely that another aldehyde dehydrogenase(s) is present in rat liver microsomes, which catalyzes the oxidation of ω-oxo-C22:0 into its dicarboxylic acid.

Miconazole, an inhibitor of cytochrome P450 enzymes, only inhibited C22:0-DCA production when NADPH was used as cofactor. This result strongly suggests that cytochrome P450 hydroxylases are involved not only in the conversion of C22:0 into ω-hydroxy-C22:0 but also in the subsequent oxidation of ω-hydroxy-C22:0 into C22:0-DCA. A possible mechanism for the two enzymatic pathways is depicted in **Fig. 5**. In the first step (path A), the conversion of C22:0 into ω-hydroxy-C22:0 is catalyzed by a NADPH- and molecular oxygen-dependent cytochrome P450 hydroxylase. In the NAD^+-dependent route, ω-hydroxy-C22:0 can be converted into an aldehyde by an alcohol dehydrogenase producing ω-oxo-C22:0 (path B), which is oxidized further by an aldehyde dehydrogenase, resulting in C22:0-DCA (path C). Alternatively, ω-hydroxy-C22:0 can also be oxidized into C22:0-DCA via a NADPH- and molecular oxygen-dependent pathway. According to this scheme, ω-hydroxy-C22:0 would be hydroxylated to 22,22-dihydroxy-C22:0 (path D), which can be catalyzed either by the same cytochrome P450 hydroxylase that is involved in the initial step or by some other cytochrome P450. Elimination of water from ω-dihydroxy-C22:0 would yield ω-oxo-C22:0 (path E), which can be hydroxylated to produce C22:0-DCA (path F). This mechanism has also been proposed for sterol hydroxylation in rabbit liver mitochondria as catalyzed by CYP27 (33), hydroxylation of alkanes in yeast (34), and oxidation of ethanol to acetaldehyde in rabbit liver microsomes (35). Hydroxylation of C22:0 and C22:0-OH was inhibited considerably by ketoconazole and 17-ODYA (Fig. 4). These findings demonstrate that one or more cytochrome P450 enzymes belonging to the CYP2, CYP3, and/or CYP4A/F families are involved. The inhibition by 17-ODYA, in particular, strongly suggests to the participation of cytochrome P450 enzymes belonging to the CYP4A and CYP4F families (31, 30, 27).

The results described in this paper show that C22:0 is a substrate for the microsomal ω-oxidation pathway. We hypothesize that this alternative fatty acid oxidation route may be important in several inherited peroxisomal diseases in which plasma levels of VLCFAs are increased but C22:0 levels are markedly decreased (18). Patients with a peroxisomal biogenesis disorder who have a deficiency in VLCFA β-oxidation show increased levels of ω-hydroxylated fatty acids and dicarboxylic acids in urine (19). This

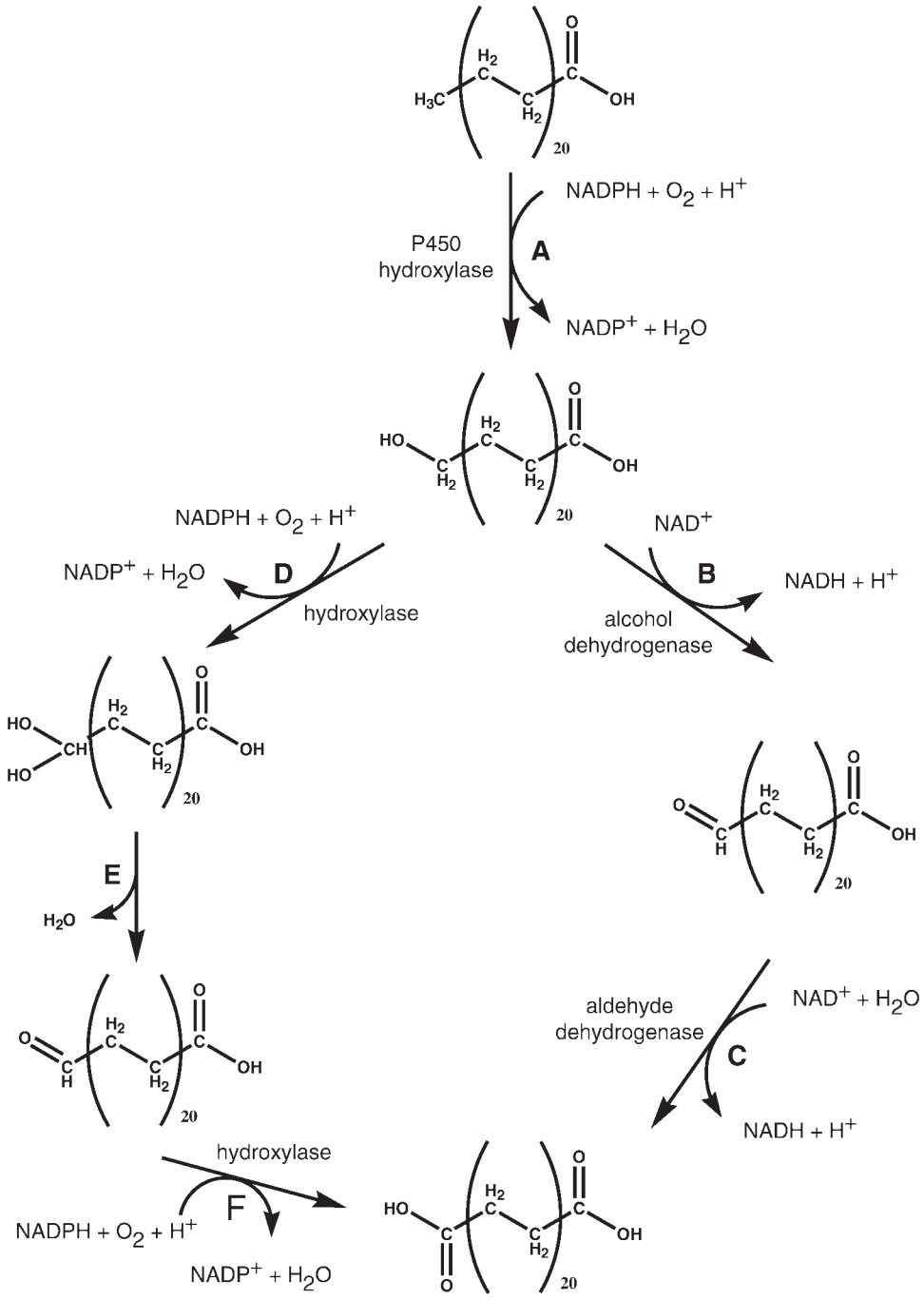

Fig. 5. Two possible routes for ω-oxidation of C22:0 in rat liver microsomes. See Discussion for details.

indicates that microsomal ω-oxidation of VLCFAs is also operational in vivo.

In summary, our results indicate that C22:0 may undergo ω-oxidation to C22:0-DCA via two distinct routes that share the first step (i.e., the cytochrome P450-mediated formation of ω-hydroxy-C22:0 from C22:0). Next, ω-hydroxy-C22:0 can undergo two subsequent oxidation reactions driven by NAD^+ to produce C22:0-DCA. Alternatively, ω-hydroxy-C22:0 may also undergo two subsequent cytochrome P450-mediated hydroxylase reactions to give the same end product, C22:0-DCA. It remains to be established whether all of these cytochrome P450 hydroxylase-dependent steps involve one or more members of this enzyme family.

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