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INHIBITION OF *p*-NITROANISOLE O-DEMETHYLATION IN PERFUSED RAT LIVER BY OLEATE*

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Abstract—*p*-Nitroanisole O-demethylation in perfused livers from fasted, phenobarbital-treated rats was rapidly and reversibly inhibited by sodium oleate (0.3 to 0.6 mM). Xylitol partially reversed this inhibitory effect. The inhibition was not mediated by a direct effect of oleate on microsomal components since concentrations of oleate ranging up to 1.0 mM did not affect *p*-nitroanisole O-demethylation by isolated microsomes. Infusion of 0.6 mM oleate did not alter the measured intracellular NAD^+/NADH ratio but did cause a significant increase in the intracellular $NADP^+$/NADPH ratio. A significant decrease in the ATP/ADP ratio was also observed. Oleoyl CoA inhibited *p*-nitroanisole O-demethylation in microsomes (K_i about 30 μM), and both oleoyl CoA and palmitoyl CoA inhibited the energy-linked nicotinamide nucleotide transhydrogenase in submitochondrial particles (K_i about 1 μM). Thus, inhibition of mixed-function oxidation in the intact liver by oleate is most likely mediated by oleoyl CoA. Oleoyl CoA inhibits mixed-function oxidation in the intact liver by acting directly on cytochrome P-450 and by decreasing generation of NADPH via inhibition of key enzymes of the citric acid cycle and the energy-linked transhydrogenase.

Free fatty acids and phospholipids have many influences upon mixed-function oxidation. For example, a chronic fat-free diet decreases cytochrome P-450 content and rates of microsomal oxidation of several substrates [1]. Depletion of dietary fat also decreases the ability of phenobarbital to induce cytochrome P-450 [2]. Reconstituted mixed-function oxidation systems require phosphatidyl choline for maximum activity [3]. Moreover, free fatty acids are endogenous substrates for cytochrome P-450 and cause a shift from low to high spin states in the heme iron [4]. At high concentrations (3.5 mM), free fatty acids act as detergents and inhibit mixed-function oxidation directly [5].

In whole cells, fatty acids may further influence mixed-function oxidation as a consequence of their metabolism. Long chain acyl CoA compounds have been shown to be potent inhibitors of cytochrome P-450 in microsomal preparations [6]. Since acyl CoA compounds are known to inhibit several enzymes in the tricarboxylic acid cycle and in the pentose phosphate shunt [7, 8], they may decrease the generation of NADPH required for drug oxidation. In contrast, fatty acids may also enhance the rate of mixed-function oxidation by serving as a major source of reducing equivalents necessary for mixed-function oxidation in the fasted state [9]. When oxidation of fatty acids is inhibited by 2-bromooctanoate, an inhibitor of thiolase I [10], the rate of mixed-function oxidation in livers of fasted rats decreases by 80% [9].

Since fatty acids have multiple effects on both NADPH-generating processes and the microsomal P-450 system, it is difficult to predict their net action on mixed-function oxidation in intact cells. Effects of fatty acids that are mediated through their metabolites or through their effects upon pyridine nucleotides cannot be studied in microsomal preparations or in reconstituted systems. Therefore, the present study was designed to examine the influence of sodium oleate upon rates of mixed-function oxidation in the isolated perfused liver. The data indicate that long-chain fatty acids inhibit mixed-function oxidation in the perfused liver at concentrations that fail to inhibit microsomal activity *in vitro*. This inhibition is most likely mediated by direct effects of oleoyl CoA on both microsomal components and the supply of NADPH.

MATERIALS AND METHODS

Animals. Female Sprague–Dawley rats, 100–250 g, were treated for 5 days to 2 weeks with phenobarbital (1 mg/ml) in their drinking water [11] and were fasted for 24 hr prior to perfusion experiments.

Liver perfusion. Liver perfusion was carried out in a nonrecirculating system as described previously [12] except that the perfusate was oxygenated with fritted gas disperison discs. Krebs–Henseleit buffer [13] was warmed to 37° and saturated with an oxygen–carbon dioxide mixture (95:5). Bovine serum albumin (fraction V) dissolved in buffer was infused at a final concentration of 0.3%. Effluent from the

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liver was pumped via a cannula placed in the vena cava past a Teflon-shielded, Clark-type oxygen electrode for continuous measurement of venous oxygen tension. Rates of oxygen consumption were calculated from the liver weight, the flow rate and the influent–effluent oxygen concentration differences and were used to assess tissue viability. *p*-Nitroanisole (0.2 mM, Kodak) was dissolved in Krebs–Henseleit buffer and infused as indicated in the figure and table legends. Formation of free *p*-nitrophenolate ion was monitored continuously as described previously [14]. Total *p*-nitrophenolate concentration in perfusate was measured after incubation of 1.0-ml samples with 25 units of sulfatase and 250 Fishman units of β-glucuronidase (Sigma Chemical Co., St. Louis, MO) for 90 min [15]. The sodium salts of oleic acid (Sigma) and 2-bromooctanoic acid (Tridom Co., Hauppauge, NY) were prepared by combining equimolar amounts of fatty acid and NaOH in 95% ethanol and evaporating to dryness. The salt was then dissolved in normal saline containing 1% albumin and the pH was adjusted to 7.4 [9].

Rates of ketogenesis were calculated from acetoacetate and β-hydroxybutyrate concentrations measured in the effluent perfusate enzymatically, the liver wet weight and the flow rate [16].

Microsomal p-*nitroanisole* O-*demethylase activity*. Rates of hepatic microsomal O-demethylation were determined essentially as described previously [17]. Assays performed in the presence of oleyl CoA contained excess NADPH (1 mM) rather than an NADPH-generating system. *O*-Demethylase activity was expressed as the sum of *p*-nitrophenol and 4-nitrocatechol formed per minute per milligram of microsomal protein. Protein was determined by the biuret method [18].

Surface fluorescence of pyridine nucleotides and flavoproteins. The oxidation–reduction state of pyridine nucleotides and flavoproteins was monitored fluorometrically from the liver surface [19]. Flavoproteins were excited at 460 nm and fluorescence of the oxidized form was monitored at 520 nm. Pyridine nucleotides were excited at 366 nm and their fluorescence was monitored at 450 nm. Flavoprotein fluorescence reflects the oxidation–reduction state of mitochondrial flavoproteins whereas pyridine nucleotide fluorescence reflects mitochondrial and extramitochondrial NADH and NADPH [20].

Metabolite measurements. Metabolites were measured in $HClO_4$ extracts of livers that were freeze-clamped with tongs chilled in liquid nitrogen [21] as described previously [22]. Total pyridine nucleotides and their reduced forms were measured by enzymatic cycling techniques [23]. $NADP^+$/NADPH ratios were also calculated from substrates assumed to be near equilibrium with malic enzyme and isocitrate dehydrogenase as described previously [24].

Energy-linked nicotinamide nucleotide transhydrogenase activity. Liver mitochondria were isolated from adult rats following the procedure of Myers and Slater [25] except that the isolation medium contained 70 mM sucrose, 230 mM mannitol and 0.2 mM EDTA. The final preparation was stored at −80°. Submitochondrial particles were prepared from thawed mitochondria by resuspending them in 1–2 ml of 20 mM Tris–acetate, pH 7.5, containing 1.0 mM EDTA, followed by sonication (six times for 20 sec each) under an atmosphere of N_2 in an ice-cooled, bath-type sonicator (Laboratory Supplies, Hicksville, NY). The sonicate was centrifuged for 10 min at 12,000 *g* at 4°, and the resulting supernatant fraction was centrifuged at 125,000 *g* for 30 min at 4°. The pellet was resuspended in a small volume of the initial isolation medium. The energy-linked transhydrogenase was assayed according to the procedure of Rydstrom [26] by monitoring the formation of NADPH continuously at 340 nm. The reaction was linear for at least 30 min with protein ranging up to 0.2 mg/ml. Protein was determined by the method of Lowry *et al.* [27] using bovine serum albumin as a standard.

RESULTS

Effect of oleate on p-*nitroanisole O-demethylation in the perfused liver*. The influence of oleate upon *p*-nitroanisole O-demethylation was studied by infusing oleate for 6 min prior to *p*-nitroanisole infusion. Control livers and livers infused with 0.6 mM oleate had maximal rates of *p*-nitrophenolate production of 10.5 ± 1.8 and 5.7 ± 0.4 μmoles/g/hr respectively (Fig. 1, $P < 0.05$).

To determine whether the inhibition of mixed-function oxidation by oleate was reversible, an alternate source of NADPH was supplied by simultaneous infusion of xylitol (2 mM). Maximal rates of

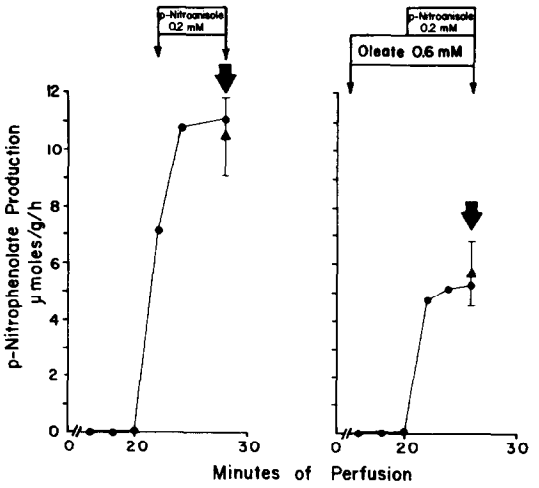

Fig. 1. p-Nitroanisole O-demethylation in the presence and absence of oleate. Left panel: Illustration of a typical experiment in which the liver of a fasted, phenobarbital-treated rat was perfused with Krebs–Henseleit bicarbonate buffer containing 0.3% albumin. *p*-Nitroanisole (0.2 mM) was infused as indicated by the horizontal bar. Rates of *p*-nitrophenolate production (●—●) were measured as described in Materials and Methods. The liver was freeze-clamped with liquid nitrogen, as indicated by the heavy vertical arrow, for measurement of metabolites. Triangle (▲) and error bar represent the mean ± S.E.M. for all livers in this group. Right panel: Rates of *p*-nitrophenolate production with conditions as in the left panel except that oleate was infused for 6 min prior to addition of *p*-nitroanisole.

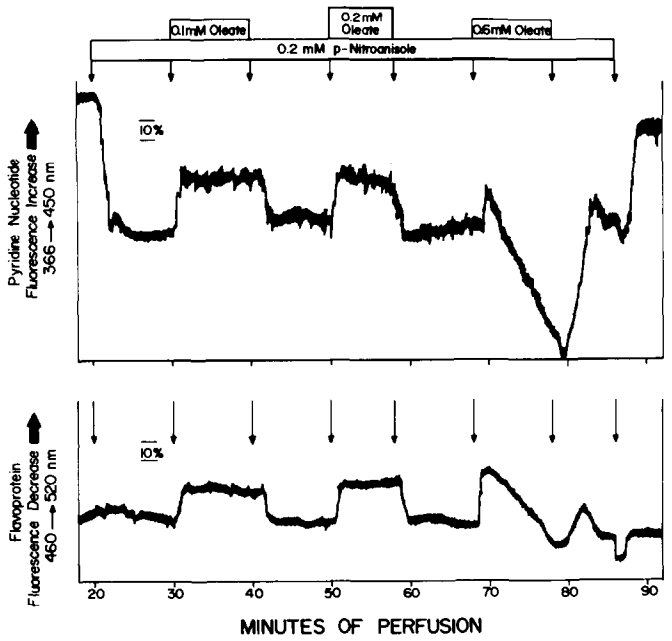

Fig. 2. Influence of oleate upon surface fluorescence of pyridine nucleotides and flavoproteins. The liver of a phenobarbital-treated, fasted rat was perfused with Krebs–Henseleit bicarbonate buffer. *p*-Nitroanisole (0.2 mM) and oleate additions are indicated by the horizontal bars. Fluorescence was detected as described in Materials and Methods.

p-nitroanisole O-demethylation were 70% greater in livers infused with xylitol plus oleate (N = 5) than in livers given oleate alone (N = 5) ($P < 0.01$).

Effect of oleate on pyridine nucleotide and flavin fluorescence and ketogenesis. The infusion of *p*-nitroanisole caused a marked decrease in pyridine nucleotide fluorescence due to fluorescence quenching by *p*-nitroanisole and *p*-nitrophenol [28]. Infusion of 0.1 and 0.2 mM oleate converted both pyridine nucleotides (Fig. 2, upper panel) and flavoproteins (Fig. 2, lower panel) to their reduced form. With the infusion of 0.6 mM oleate, a transient reduction of pyridine nucleotides and flavins occurred which was followed rapidly by a large oxidation (Fig. 2). Oleate (0.6 mM) increased ketogenesis from a basal rate of 25 μmoles/g/hr to about 45 μmoles/g/hr (not shown); however, the rate of ketogenesis returned towards the baseline over a 10-min period. The kinetic changes in ketogenesis paralleled the changes in pyridine nucleotide and flavin fluorescence observed during infusion of 0.6 mM oleate (Fig. 2).

p-Nitroanisole O-demethylation in hepatic microsomes. At concentrations used in the perfused liver, oleate did not inhibit *p*-nitroanisole O-demethylation in microsomes (data not shown). In contrast, incubation of microsomes with oleoyl CoA (48.6 μM) inhibited *p*-nitrophenolate production by 69% (Table 1).

Effect of oleate on hepatic adenine and pyridine nucleotide content. Perfusion of *p*-nitroanisole alone had no significant effect on concentrations of ATP (Table 2) or pyridine nucleotides (Table 3 and 4).

Table 1. Effect of oleoyl CoA on *p*-nitroanisole O-demethylation in microsomes*

Oleoyl CoA (μM)	Rate of *p*-nitroanisole O-demethylation (nmoles/min/mg protein)	% Inhibition
0	2.67	0
8.3	2.59	3
48.6	0.82	69

* Hepatic microsomes (2.4 mg) from phenobarbital-treated rats were incubated for 10 min at 30° in 500 μl of reagent containing 50 μM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 7.4, 200 μM *p*-nitroanisole, 1.0 mM NADPH, and 100 μM dithiothreitol. NADPH was used, rather than an NADPH-generating system, to avoid any inhibitory effect of the CoA compound upon isocitrate dehydrogenase. *p*-Nitrophenol absorbance was measured at 400 nm. Data are the mean of triplicate samples, with a coefficient of variation of less than 1%.

Table 2. Effect of oleate on adenine nucleotides in the perfused liver*

	Adenine nucleotides (μmoles/kg wet liver wt)				
	ATP	ADP	AMP	ATP + ADP + AMP	ATP/ADP
I Control	2467 ± 242	974 ± 74	266 ± 45	3707 ± 325	2.52 ± 0.14
II *p*-Nitroanisole	2070 ± 152	983 ± 47	286 ± 58	3339 ± 162	2.10 ± 0.09
III Oleate	872 ± 113†	682 ± 39	737 ± 137	2291 ± 242	1.27 ± 0.13†
IV Oleate + *p*-nitroanisole	592 ± 206‡	534 ± 69	745 ± 141	2187 ± 571	1.04 ± 0.35†

* Livers from fasted, phenobarbital-treated animals were perfused for 26 min with Krebs–Henseleit buffer containing 0.3% albumin prior to clamping with aluminum tongs chilled in liquid nitrogen as shown in Fig. 1. Oleate (0.6 mM) was added at 14 min and *p*-nitroanisole was added at 20 min. Adenine nucleotides were measured in acid extracts as described in Materials and Methods. Values are means ± S.E.M. from five livers per group. Data for control and *p*-nitroanisole infused livers (lines I and II) are those published previously [9] from simultaneous experiments. Ratios are the sum of ratios from individual livers. P values represent comparisons between lines I and III and between lines II and IV.
† $P < 0.001$.
‡ $P < 0.01$.

On the other hand, oleate (0.6 mM) in both the presence and absence of *p*-nitroanisole reduced ATP concentrations and ATP/ADP ratios significantly (Table 2).

Oleate (0.6 mM) and oleate plus *p*-nitroanisole produced a significant increase in the $NAD^+/NADH$ ratio calculated from substrates assumed to be in near equilibrium with lactate dehydrogenase (Table 3) but had no effect on the $NAD^+/NADH$ ratio calculated from substrates assumed to be in near equilibrium with β-hydroxybutyrate dehydrogenase. Ratios determined from measured pyridine nucleotides were also not altered. Oleate plus *p*-nitroanisole significantly increased $NADP^+/NADPH$ ratios calculated from measured pyridine nucleotides as well as values calculated from substrates assumed to be in near equilibrium with malic enzyme and isocitrate dehydrogenase (Table 4).

Effect of oleate on cellular intermediates. The infusion of oleate caused a significant decrease in the concentrations of several citric acid cycle intermediates including α-ketoglutarate and isocitrate (Table 5). Glutamate and aspartate, which are synthesized from citric acid cycle intermediates, were

Table 3. Effect of oleate on nicotinamide adenine dinucleotides*

	NAD^+ (μmoles/kg wet liver wt)	NADH (μmoles/kg wet liver wt)	$NAD^+/NADH$	$NAD^+/NADH$ calculated from lactate dehydrogenase	$NAD^+/NADH$ calculated from β-hydroxy butyrate dehydrogenase
I Control	278 ± 60	146 ± 35	2.15 ± 0.06	591 ± 175	63 ± 7
II *p*-Nitroanisole	259 ± 54	80 ± 7	3.38 ± 0.80	340 ± 94	52 ± 4
III Oleate	223 ± 23	61 ± 20	6.16 ± 3.13	1424 ± 242†	48 ± 11
IV Oleate + *p*-nitroanisole	208 ± 15	93 ± 27	4.05 ± 1.29	1605 ± 604†	88 ± 63

* Conditions as in Table 2. Pyridine nucleotides were measured in alkaline extracts by enzymatic cycling techniques. Values are mean ± S.E.M. from four livers in each group. Ratios represent the sum of ratios from individual livers. P values represent comparisons between lines I and III and between lines II and IV. As in Table 2, data for control and *p*-nitroanisole-infused livers are those published previously [9].
† $P < 0.05$.

Table 4. Effect of oleate on nicotinamide adenine dinucleotide phosphates*

	$NADP^+$ (μmoles/kg wet liver wt)	NADPH (μmoles/kg wet liver wt)	$NADP^+/NADPH \times 100$	$NADP^+/NADPH \times 100$ calculated from malic enzyme	$NADP^+/NADPH \times 100$ calculated from isocitrate dehydrogenase
I Control	40 ± 14	199 ± 74	39 ± 26	2.28 ± 0.75	3.30 ± 0.85
II *p*-Nitroanisole	156 ± 42	149 ± 9	107 ± 28	2.02 ± 0.57	2.62 ± 0.54
III Oleate	149 ± 10	45 ± 15	261 ± 75	4.00 ± 0.36	7.45 ± 1.92
IV Oleate + *p*-nitroanisole	244 ± 12	73 ± 20	445 ± 159†	16.23 ± 7.54	6.49 ± 1.10†

* All conditions as in Table 2.
† $P < 0.05$.

Table 5. Effect of oleate on cellular intermediates in perfused liver*

	Cellular intermediates (μmoles/kg wet liver wt)					
	Glutamate	α-Ketoglutarate	Pyruvate	Malate	Aspartate	Isocitrate
I Control	1185 ± 74	127 ± 15	21 ± 6	23 ± 2	451 ± 51	4.7 ± 1.0
II *p*-Nitroanisole	1195 ± 128	89 ± 8	14 ± 5	21 ± 4	409 ± 42	3.0 ± 0.5
III Oleate	703 ± 129†	44 ± 5‡	52 ± 4‡	45 ± 7†	228 ± 15‡	0.7 ± 0.2‡
IV Oleate + *p*-Nitroanisole	462 ± 182†	41 ± 4‡	52 ± 5‡	31 ± 18	302 ± 26	0.7 ± 0.1‡

* Conditions as in Table 2. Intermediates of the Embden–Myerhoff pathway were measured enzymatically by fluorometric procedures [23], and intermediates of the tricarboxylic acid cycle were measured by methods of Goldberg *et al.* [29]. Values are the means ± S.E.M. for five livers in each group. P values are for comparisons between lines I and III and between lines II and IV.

† $P < 0.05$.

‡ $P < 0.01$.

decreased in the presence of both oleate and *p*-nitroanisole. In contrast, pyruvate and malate were increased significantly by oleate (Table 5).

Effect of fatty acyl CoA on energy-linked nicotinamide nucleotide transhydrogenase activity. The effect of palmitoyl CoA and oleoyl CoA on the energy-linked transhydrogenase was investigated by measuring enzymatic activity in the presence of various amounts of CoA esters at several concentrations of $NADP^+$. Palmitoyl and oleoyl CoA inhibited both the ATP- and succinate-stimulated transhydrogenase activities competitively with K_i values of 1 μM or less (Fig. 3). The succinate-stimulated reaction was nonlinear at high CoA ester concentrations possibly due to effects upon the respiratory chain.

DISCUSSION

Effect of oleate on mixed-function oxidation. The experiments reported here demonstrate that oleate inhibited mixed-function oxidation by more than 50% (Fig. 1) in the perfused liver at near physiological concentrations (0.6 mM) that have no effect in microsomal preparations. Direct inhibition of

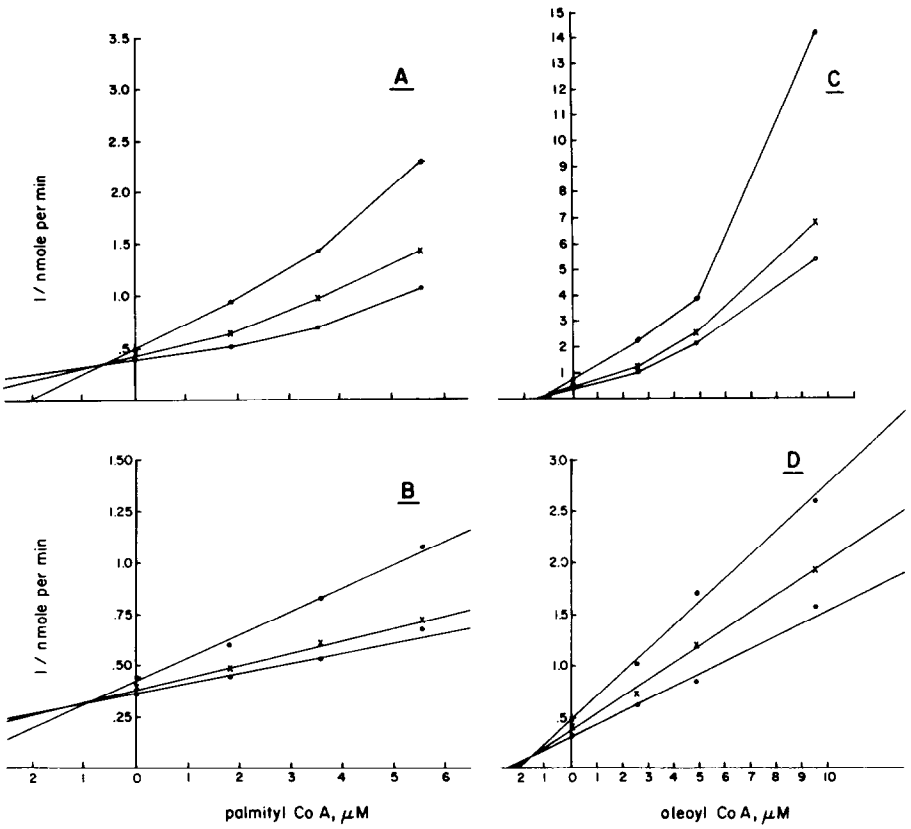

Fig. 3. Effect of palmitoyl CoA and oleoyl CoA on succinate-stimulated (B and D) and ATP-stimulated (A and C) transhydrogenase activity. Transhydrogenase activity was measured as described in Materials and Methods. The succinate concentration was 3.0 mM and the ATP concentration 1 mM (+ 2 mM $MgCl_2$). The protein concentration was 49 μg; Key: (●) 18 μM NADP; (×) 36 μM NADP; and (○) 61 μM NADP. Each point is the average of three replicate samples.

microsomal mixed-function oxidation of type 1 substrates by unsaturated fatty acids was observed by Augustine and Fouts [5], but only at unphysiologically high concentrations (3.5 mM). Abbott and Mannering [6] showed that oleoyl CoA (50 μM) inhibited ethylmorphine *N*-demethylase activity by stimulating lipid peroxidation and by detergent actions. Similarly, we observed an inhibition of *p*-nitroanisole *O*-demethylase activity in microsomes by oleoyl CoA (Table 1). Thus, one mechanism for inhibition of mixed-function oxidation in whole cells by low concentrations of free fatty acids is inhibition of monooxygenases by CoA esters.

It is also possible that oleate inhibits mixed-function oxidation in the intact hepatocyte by diminishing the supply of the cofactor, NADPH. This possibility is supported strongly by two observations. First, the $NADP^+/NADPH$ ratio was elevated significantly by oleate in the presence of *p*-nitroanisole (Table 4). Second, xylitol, which generates NADPH when metabolized, partially reversed the inhibition of mixed-function oxidation by oleate. Thus, it is concluded that diminished supply of reducing equivalents plays a role in the ability of oleate to inhibit mixed-function oxidation in whole cells.

The hypothesis that oleate inhibits mixed-function oxidation to some extent by interfering with the generation of reducing equivalents might seem in conflict with the conclusion reached previously that β-oxidation of acyl CoA compounds is obligatory for the generation of reducing equivalents for mixed-function oxidation in the fasted state [9]. This conclusion rests on the observation that the metabolism of *p*-nitroanisole is diminished markedly by inhibition of β-oxidation by either 2-bromooctanoate, an inhibitor of thiolase I [9], or by tetradecylglycidic acid, an inhibitor of carnitine acyl transferase I [30]. How can fatty acids be a major source of reducing equivalents for mixed-function oxidation in livers from fasted rats [9] and yet inhibit mixed-function oxidation (Fig. 1) when added exogenously to the perfused liver?

The mechanism by which fatty acid metabolism supports mixed-function oxidation in the fasted state is thought to be as follows. Fatty acyl CoA compounds are synthesized in the cytosol (Fig. 4) and

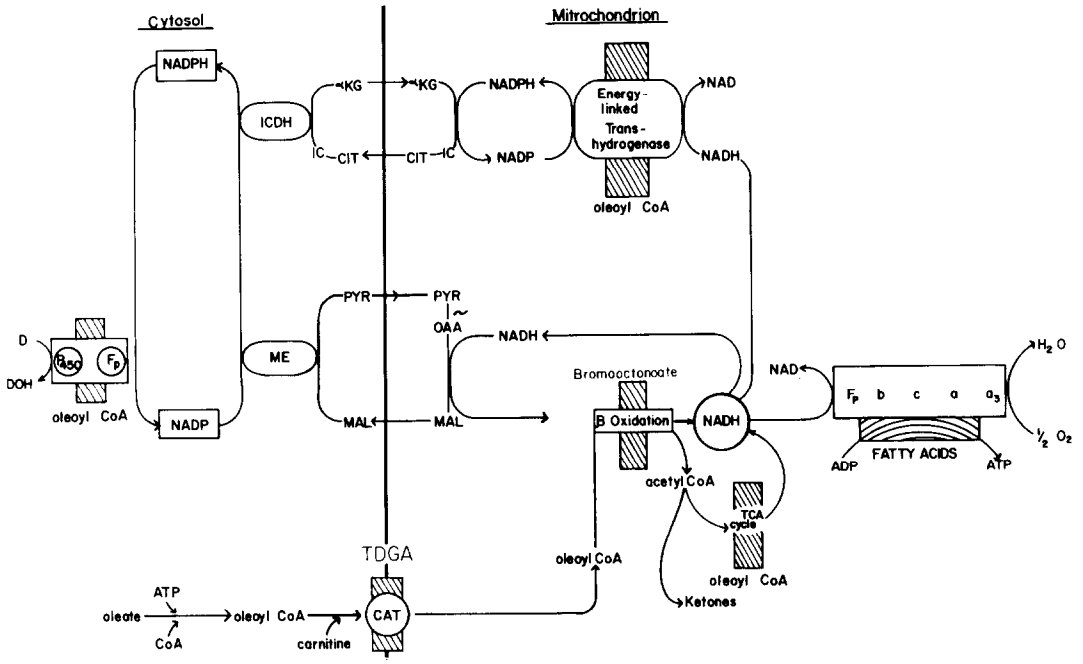

Fig. 4. Scheme depicting the mechanism of oleate inhibition of mixed-function oxidation. NADH and NADPH yield cytosolic $NADP^+$ by the malate shuttle and the isocitrate shuttle respectively. The malate (MAL) shuttle involves the carboxylation of pyruvate (PYR) via pyruvate carboxylase to form oxaloacetate (OAA), reduction to malate, movement of malate into the cytosol, and generation of NADPH via malic enzyme (ME). The isocitrate shuttle involves reductive carboxylation of α-ketoglutarate (αKG) to isocitrate (IC) by an NADPH specific isocitrate dehydrogenase (ICDH); conversion of isocitrate to citrate (CIT) through the aconitase reaction; transport of CIT and IC into the cytosol where NADPH may be generated by the reverse reactions. αKG is then moved back into the mitochondria either directly or as glutamate after transamination (not shown). NADPH may be generated in the mitochondrial space by an energy-linked transhydrogenase. Fatty acids may uncouple oxidative phosphorylation [31], or may decrease the P : O ratio by futile cycling as discussed in the text [32]. Long chain acyl CoA compounds also directly inhibit microsomal activity and energy-linked transhydrogenase activity as well as enzymes involved in the citric acid cycle [7]. β-Oxidation of fatty acids generates NADH and acetyl CoA. 2-Bromooctanoate inhibits thiolase I [10]. Tetradecylglycidic acid (TDGA) inhibits carnitine acyl transferase (CAT) [33]. Other abbreviations include D (drug); DOH (oxidized drug); Fp (flavoprotein reductases for cytochrome P-450); and TCA (tricarboxylic acid cycle).

transferred into the mitochondrial space via the carnitine acyl transferase enzymes where they generate NADH via β-oxidation and the citric acid cycle (Fig. 4). Reducing equivalents generated in this manner move back into the cytosol via a substrate shuttle mechanism involving malate dehydrogenase and malic enzyme or are converted into NADPH via the energy-linked transhydrogenase and moved into the cytosol via a substrate shuttle mechanism involving mitochondrial and cytosolic $NADP^+$-dependent isocitrate dehydrogenases, isocitrate and α-ketoglutarate. The NADPH generated in this fashion is then available for mixed-function oxidation (Fig. 4).

Oleate (0.6 mM) inhibited mixed-function oxidation in the perfused liver without having any effect on the oxidation : reduction state of mitochondrial NAD^+, as calculated from substrates assumed to be in equilibrium with β-hydroxybutyrate dehydrogenase (Table 3). While low concentrations of infused oleate (0.1 and 0.2 mM) caused the predicted reduction in pyridine nucleotide fluorescence, higher concentrations of oleate (0.6 mM) did not cause a sustained increase in pyridine nucleotide fluorescence (Fig. 2). Under these conditions, NAD^+/NADH ratios calculated from measured pyridine nucleotides were not different from control values (Table 3). Thus, oleate inhibits mixed-function oxidation at least in one unique experimental condition in the absence of a change in oxidation–reduction state of mitochondrial NAD^+. Infusion of oleate caused a significant decrease of ATP/ADP ratios as might be expected since long-chain fatty acids are known to uncouple oxidative phosphorylation [31] and to increase futile cycling between long-chain fatty acids and triglycerides and between pyruvate and phosphoenolpyruvate [32]. This decrease in ATP may have contributed to the inhibition of mixed-function oxidation due to oleate because of the energy requirement of the transhydrogenase.

The inhibitory effect of oleate could be mediated by several effects of its coenzyme A ester. One site may involve cytoplasmic dehydrogenases. Long chain acyl CoA compounds have been shown to inhibit glucose-6-phosphate dehydrogenase [7, 9, 34]. The K_i is an inverse function of fatty acid chain length ($K_i = 10\ \mu M$ for stearyl CoA). While inhibition of glucose-6-phosphate dehydrogenase could block the generation of NADPH via the pentose phosphate shunt and thus inhibit mixed-function oxidation in the fed state, it probably does not explain the inhibition in these studies with fasted animals in which pentose phosphate shunt activity is diminished markedly by substrate depletion.

A second site of inhibition by oleoyl CoA involves mitochondrial enzymes. Palmitoyl CoA has been reported to inhibit fumarase ($K_i < 0.1\ \mu M$) [7]. Thus, inhibition of the citric acid cycle may be involved in the mechanism by which acyl CoA compounds inhibit mixed-function oxidation, both through decreased formation of NADH and through decreased availability of shuttle intermediates. Decreased concentrations of α-ketoglutarate and isocitrate in livers perfused with oleate are consistent with this possibility (Table 5). Furthermore, palmitoyl CoA has been reported to be a potent inhibitor of the energy-linked transhydrogenase in heart ($K_i = 0.15\ \mu M$) [26]. The marked inhibition in the liver (Fig. 3) of both ATP- and succinate-stimulated transhydrogenase activities by very low concentrations of both palmitoyl and oleoyl CoA strongly supports the possibility that oxidation of NADPH is at least partially due to direct inhibition of the nicotinamide nucleotide transhydrogenase. Thus, mitochondrial reducing equivalents normally available through substrate shuttle mechanisms for the generation of cytosolic NADPH are unavailable to support mixed-function oxidation in the presence of relatively low concentrations of oleoyl CoA.

The observations made here raise interesting possibilities with regard to the influence of acute nutritional alterations upon mixed-function oxidation. In clinical studies, plasma free fatty acid levels vary with meals and in the ketotic state and lie in the range (0.1 to 1 mM) used in the studies reported here [35, 36]. Liver perfusions have shown that the uptake of fatty acids is proportional to their concentration in the medium [37] and that increasing fatty acid uptake leads to increasing fatty acid oxidation and triglyceride synthesis, whereas the hepatic fatty acid concentration remains independent of the concentration in the medium [37]. Thus, endogenous free fatty acid concentration in the liver remains stable with increasing fatty acid infusion whereas acyl coenzyme A concentration increases from control levels of approximately 12–16 μM to 25–35 μM [38] with infusion of oleate into the perfused liver. Thus, it is possible that changes in plasma fatty acid concentration that occur with changes in nutritional state or with illness may modulate the rate of mixed-function oxidation *in vivo* through the effects of their acyl coenzyme A derivatives.

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