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EFFECTS OF SODIUM 2-[5-(4-CHLOROPHENYL)PENTYL]-OXIRANE-2-CARBOXYLATE (POCA) ON INTERMEDIARY METABOLISM IN ISOLATED RAT-LIVER CELLS

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Abstract—In hepatocytes isolated from meal-fed rats, sodium 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate (POCA) decreased the rate of lipogenesis measured as incorporation of ^3H from ^3H_2O into glycerolipids and cholesterol. Moreover, POCA inhibited the oxidation of added oleate, whereas oleate esterification was stimulated. In hepatocytes from 24-hr-starved rats, inhibition of gluconeogenesis by POCA was observed only with gluconeogenic precursors which require pyruvate carboxylation. This inhibition was secondary to impaired oxidation of long-chain fatty acids by POCA. It is concluded that, in addition to its inhibition of long-chain fatty acid oxidation, POCA interferes with *de novo* synthesis of cholesterol and fatty acids. On the other hand, neither fatty acid esterification nor the conversion of oxaloacetate into glucose are affected by POCA.

Inhibitors of fatty acid oxidation may act *in vivo* as hypoglycemic drugs by promoting increased use of glucose as an energy source, thus providing a rationale for new approaches in the chemotherapy of diabetes [1]. The continuing search for more specific and effective inhibitors of fatty acid oxidation led to the development of 2-tetradecylglycidate (MCN-3802) by Tutwiler *et al.* [2] and, more recently, of 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate (B 807-27, POCA) by Wolf *et al.* [3]. In the course of our own studies on hepatic lipogenesis we became interested in employing these new inhibitors which are potentially useful tools in studies concerning metabolic regulation.

POCA had already been demonstrated to lower blood levels of glucose and ketone bodies in fasted rats and guinea pigs [3]. Chronic administration of high concentrations of POCA to normal rats did not cause toxic effects, although some accumulation of lipid in the liver occurred [4]. Moreover, POCA partially restored the insulin sensitivity of perfused hearts from streptozotocin-diabetic rats [5]. In other *in vitro* studies, POCA was shown to inhibit gluconeogenesis from lactate and pyruvate in rat hepatocytes [6] and perfused rat liver [7], whereas it stimulated glucose utilization in rat diaphragms [6] and perfused rat hearts [8].

The major effect of POCA underlying all these observations is its inhibition of long-chain fatty acid oxidation shown to occur in isolated rat hepatocytes [6, 9, 10] and diaphragms [6], in isolated perfused rat livers [7] and rat hearts [8], and in cultured human fibroblasts [11]. POCA exerts this inhibitory effect at the level of carnitine palmitoyltransferase I (EC 2.3.1.21), an enzyme located at the outer side of the inner mitochondrial membrane that is involved in the transport of long-chain acyl-CoA into the mitochondria [12]. A specific inhibition of carnitine palmitoyltransferase I by POCA has been shown to occur in isolated mitochondria of rat heart [5] and liver [7, 11], the active form being POCA-CoA rather than POCA itself [7, 11].

In this report we show that POCA also inhibits incorporation of ^3H_2O into cholesterol and glycerolipids of isolated rat hepatocytes, whereas esterification of exogenous oleate is enhanced. Inhibition of gluconeogenesis by POCA was observed only with carbon precursors requiring the participation of pyruvate carboxylase (EC 6.4.1.1) in the gluconeogenic sequence.

Part of this study has been presented in abstract form [13].

MATERIALS AND METHODS

Bovine serum albumin and collagenase Type I were purchased from Sigma; other enzymes, biochemicals and GOD-Perid kits were from Boehringer Mannheim. Radioactive compounds were obtained from the Radiochemical Centre, Amersham. Sodium 2-[5-(4-chlorophenyl)pentyl]-oxirane-2-carboxylate (POCA) was a gift from Byk Gulden Lomberg GmbH, Konstanz (F.R.G.).

Freshly isolated hepatocytes [14] from male Wistar rats (200–250 g), meal-fed a standard pelleted diet, were used in suspension. In experiments on gluconeogenesis, meal-fed rats were starved for 24 hr prior to hepatocyte preparation. The basic reaction mixture consisted of Krebs–Ringer bicarbonate buffer (pH 7.4) with 2.5 mM $CaCl_2$, 10 mM glucose and 3.5% (w/v) defatted and dialysed bovine serum albumin present; cell concentration, 3–6 mg protein/ml. Glucose was not included in the medium when hepatocytes from starved rats were used. Triplicate incu-

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bations (final vol. 2.0 ml) were carried out for 60 min at 37° in a metabolic shaker (90 osc/min) in 25-ml Erlenmeyer flasks under an atmosphere of O_2/CO_2 (19:1). Incubations were terminated with 0.5 ml of ice-cold 3 N $HClO_4$ or, in case of lipid extractions, with 13 ml $CH_3OH/CHCl_3$ (1:1, v/v).

To determine *de novo* rates of cholesterol and glycerolipid synthesis, ^3H_2O (1.5 mCi/ml) was included in the reaction mixtures. Oleate esterification was measured as incorporation of [1-^{14}C]oleate (0.2 and 0.5 mM oleate, 0.5 Ci/mol; 2.0 mM oleate, 0.05 Ci/mol) into glycerolipids. Standard methods were used for extraction and separation of free cholesterol and glycerolipids [15] and for enzymatic assays of ketone bodies [16], lactate [17] and pyruvate [18]. Glucose was assayed by the GOD-Perid method.

Oxidation of [1-^{14}C]oleate (0.5 Ci/mol) and [1-^{14}C]octanoate (0.01 Ci/mol) was monitored as $^{14}CO_2$ production and formation of acid-soluble radioactive products. The $^{14}CO_2$ released was trapped in 0.1 ml 6 N KOH absorbed on filter paper in center wells.

RESULTS

Synthesis of glycerolipids and cholesterol in hepatocytes from meal-fed rats

Bicarbonate-buffered suspensions of hepatocytes were incubated in the presence of ^3H_2O, exogenous carbon precursor and POCA at various concentrations. About 80–90% of the ^3H label recovered afterwards in glycerolipids originated from their fatty acyl moieties (not shown here). In this connection it should be noted that incorporation of ^3H_2O into fatty acids and cholesterol is considered the most reliable method to estimate rates of *de novo* fatty acid [19] and cholesterol [20, 21] synthesis, respectively.

As shown in Fig. 1, POCA at low concentrations (≤ 1 μM) was slightly stimulatory. This stimulation of lipogenesis was significant only in case of triacylglycerol and cholesterol synthesis (cf. ref. 10). However, at higher concentrations POCA consistently lowered the rate of lipogenesis in a dose-dependent manner. In the standard reaction medium 10 mM glucose served as the sole exogenous carbon source (Fig. 1). Inclusion of 10 mM lactate in the medium to provide an additional and perhaps more physiological carbon precursor for fatty acid synthesis (cf. ref. 22) did not significantly alter the results (not shown). The degree of inhibition of ^3H_2O incorporation into lipids was roughly the same for various lipid classes (Fig. 1).

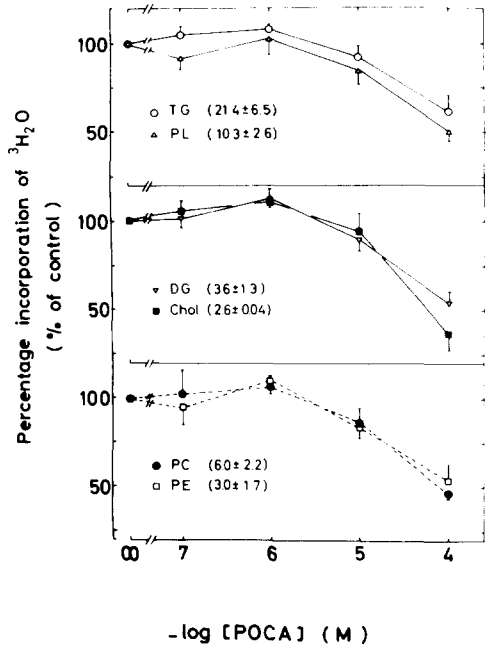

Fig. 1. Dose-dependent inhibition of *de novo* lipid synthesis by POCA. Data represent mean ± SEM of four different hepatocyte preparations. The 100% values (nmoles ^3H_2O incorporated/mg protein per hr) are shown in parentheses. TG, triacylglycerols; PL, total phospholipids; DG, diacylglycerols; Chol, cholesterol; PC, phosphatidylcholines; PE, phosphatidylethanolamines.

Fatty acid oxidation

In agreement with other studies employing hepatocytes from starved [6] or *ad libitum* fed [9] rats, we observed a profound inhibition of [1-^{14}C]oleate oxidation by POCA (Fig. 2). Medium-chain fatty acids like octanoate are converted into acyl-CoA esters in the mitochondrial matrix [23], thus bypassing the carnitine transferase step. If POCA inhibits fatty acid oxidation only at the level of carnitine palmitoyltransferase I [7, 11], the rate of [1-^{14}C]octanoate oxidation should not be influenced by the drug, as was indeed the case (Fig. 2; cf. ref. 7). In our experiments POCA merely caused a small shift in the relative production of acid-soluble radioactive compounds, and of $^{14}CO_2$ from [1-^{14}C]octanoate (not shown here).

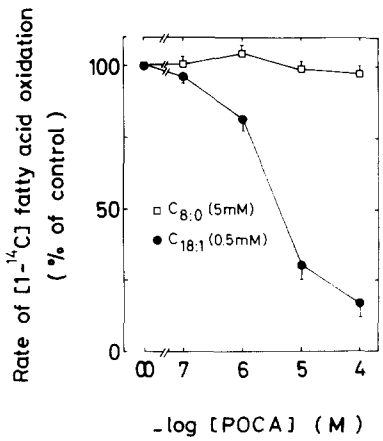

Fig. 2. Effect of POCA on hepatic fatty acid oxidation. [1-^{14}C]Fatty acid oxidation is presented as the sum of $^{14}CO_2$ formation and production of acid-soluble radioactive compounds (mainly ketone bodies). Acid-soluble compounds constituted 65–75% and 90–96% of total oxidation products in the case of oleate ($C_{18:1}$, 0.5 mM) and of octanoate ($C_{8:0}$, 5.0 mM), respectively. The 100% values (nmoles fatty acid oxidized/mg protein per hr) were 11.1 ± 5.3 (oleate) and 189 ± 54 (octanoate). Data represent mean ± SEM of 6 (oleate) and 2 (octanoate) different hepatocyte preparations.

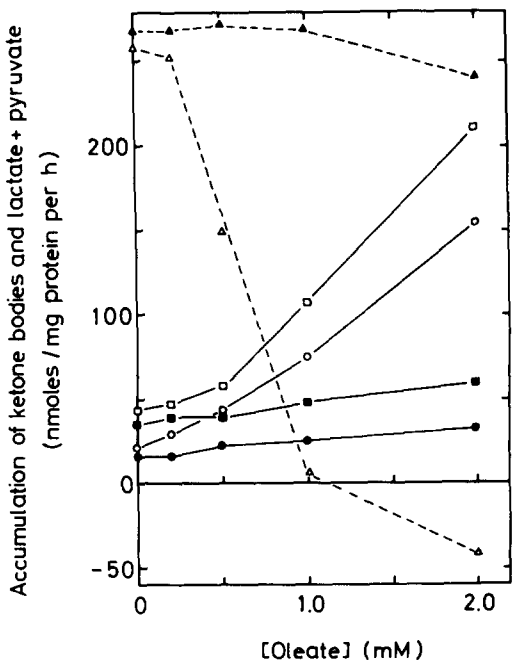

Fig. 3. Accumulation of ketone bodies, lactate and pyruvate as a function of oleate concentration. Shown are means of triplicate determinations from one hepatocyte preparation representative of three independent experiments. ○---○, 3-hydroxybutyrate; □---□, acetoacetate; △---△, lactate plus pyruvate. Open symbols, in the absence of POCA; closed symbols, 10 μM POCA present.

Figure 3 documents the influence of 10 μM POCA on ketogenesis from oleate as a function of substrate concentration. Irrespective of the oleate concentration applied, the amount of ketone bodies accumulated in the 0–60 min incubation period remained low and nearly constant. Therefore, 10 μM POCA almost completely blocked ketogenesis from oleate, even at the highest oleate concentration (2 mM) tested. In line with other reports [6, 10] the mitochondrial redox state, as judged from the 3-hydroxybutyrate/acetoacetate ratio, was slightly decreased by POCA.

In addition, Fig. 3 demonstrates that, in the presence of 10 μM POCA, oleate at concentrations up to 1 mM was unable to decrease the accumulation of lactate plus pyruvate during the incubations.

Gluconeogenesis in hepatocytes from fasted rats

Fatty acids are known to promote hepatic glucose synthesis from lactate, pyruvate and alanine, that is, from precursors which include pyruvate carboxylase in the gluconeogenic pathway. As the activity of this mitochondrial enzyme is strongly dependent on the concentration of the allosteric activator acetyl-CoA, the stimulatory effect of fatty acids may be caused by elevated acetyl-CoA levels, secondary to increased β-oxidation [24]. Alternatively, fatty acid oxidation may promote pyruvate carboxylation via increased mitochondrial pyruvate uptake [25].

This offers an additional possibility to answer the question whether or not POCA interferes with fatty acid oxidation only at the level of carnitine palmitoyltransferase I. POCA should diminish the oleate-induced stimulation of gluconeogenesis from, for example, lactate plus pyruvate. On the other hand, stimulation of gluconeogenesis by octanoate should not be affected by POCA. This expectation was borne out, as shown in Table 1.

Moreover, we investigated the effect of POCA on gluconeogenesis from proline and dihydroxyacetone. Isolated hepatocytes readily convert these substrates to glutamate and dihydroxyacetone phosphate, respectively, thereby obviating the need for pyruvate carboxylation. The results of Table 1 (lines 7 and 8) demonstrate that POCA does not affect the gluconeogenic process to any appreciable extent (cf. ref. 6).

Esterification of exogenous oleate in hepatocytes from meal-fed rats

Whereas hepatic lipid synthesis from endogenously synthesized fatty acids was inhibited by POCA

Table 1. Effect of POCA on hepatic gluconeogenesis from various precursors

Additions	Glucose synthesis (nmoles/mg protein per hr)		
	Control	10 μM POCA	% Inhibition by POCA
Lac/Pyr	236 ± 4	208 ± 4	11.7 ± 0.8
+ oleate	458 ± 9	236 ± 5	48.3 ± 3.5
+ octanoate	349 ± 6	330 ± 6	5.5 ± 1.3
Alanine	91 ± 3	77 ± 1	15.9 ± 2.1
+ oleate	195 ± 4	115 ± 3	40.7 ± 2.1
+ octanoate	118 ± 4	117 ± 4	0.9 ± 1.2
Proline	46 ± 3	44 ± 3	3.9 ± 2.3
Dihydroxyacetone	425 ± 6	400 ± 5	5.9 ± 2.0

Hepatocytes from 24-hr-starved rats were incubated in the absence (control) or presence of 10 μM POCA, with further additions as indicated: Lac/Pyr, 10 mM L-lactate + 1.5 mM pyruvate; oleate, 2 mM; octanoate, 5 mM; L-alanine, 10 mM; L-proline, 10 mM; dihydroxyacetone, 10 mM. Data represent mean ± SEM of three different hepatocyte preparations.

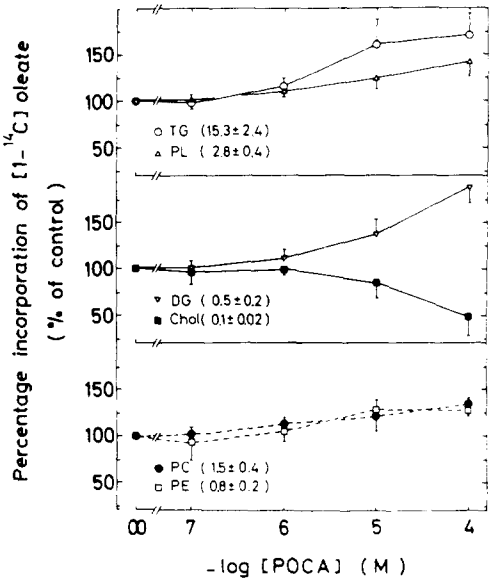

Fig. 4. Effect of POCA on incorporation of exogenous oleate as a function of POCA concentration. Oleate concentration, 0.5 mM. Data represent mean ± SEM of five different hepatocyte preparations. The 100% values (nmoles [1-^{14}C]oleate incorporated/mg protein per hr) are shown in parentheses: TG, triacylglycerols; PL, total phospholipids; DG, diacylglycerols; Chol, cholesterol; PC, phosphatidylcholines; PE, phosphatidylethanolamines.

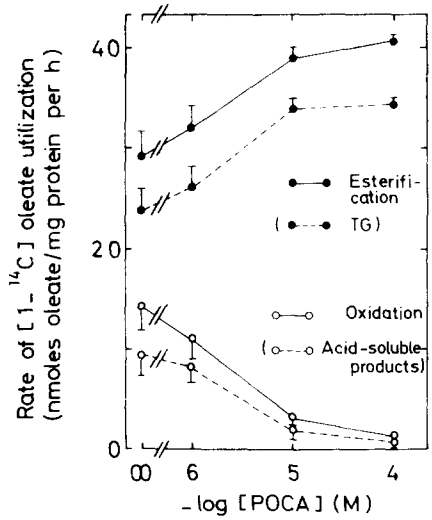

Fig. 5. The balance between oxidation and esterification of exogenous oleate. Oleate concentration, 0.5 mM. Data represent mean ± SEM of four different hepatocyte preparations. Contribution to total oleate utilization: solid lines, esterification (triacylglycerols + diacylglycerols + total phospholipids) vs oxidation ($^{14}CO_2$ + acid-soluble radioactive products); dashed lines, triacylglycerol (TG) synthesis vs formation of acid-soluble radioactive products.

(Fig. 1), esterification of added [1-^{14}C]oleate in various glycerolipid classes was enhanced by POCA in a dose-dependent fashion (Fig. 4). This stimulation was most pronounced in the case of the diacylglycerols and triacylglycerols. When using two different oleate concentrations (see Table 2) the stimulatory effect of POCA (100 μM) was relatively higher at the higher substrate concentration. Oleate incorporation into cholesterol was inhibited rather than stimulated by POCA (Fig. 4 and Table 2). This is primarily due to inhibition by POCA of carnitine palmitoyltransferase I, as the use of oleate for cholesterol synthesis implicates oleate oxidation [26, 27]. Finally, as can be seen from Fig. 5, the increase in oleate esterification caused by POCA fairly matched the decrease in oleate oxidation. In other words, total oleate utilization remained almost constant (total oleate utilized in the presence of 100 μM POCA, 94.4 ± 2.7% (mean ± SD; N = 4) of the control value in the absence of POCA).

DISCUSSION

The present study explicitly demonstrates that hepatocytes isolated from meal-fed rats are able to oxidize octanoate, but not oleate, in the presence of POCA. Similar results were reported by other groups for isolated hepatocytes or perfused livers from 24-hr-starved rats [6, 7]. The lack of inhibition of octanoate oxidation by POCA indicates that this

Table 2. Effect of POCA on incorporation of exogenous oleate as a function of oleate concentration

	Oleate incorporation (nmoles/mg protein per hr)			
	0.2 mM oleate		2.0 mM oleate	
	−	+	−	+
Triacylglycerols	8.77 ± 0.61	9.94 ± 0.58 (113)	71.2 ± 5.5	105.3 ± 1.2 (148)
Diacylglycerols	0.27 ± 0.04	0.43 ± 0.04 (159)	0.97 ± 0.28	2.67 ± 0.19 (275)
Phospholipids	1.78 ± 0.09	2.18 ± 0.14 (122)	8.85 ± 1.0	13.5 ± 1.1 (153)
Cholesterol	0.044 ± 0.007	0.006 ± 0.004 (14)	0.22 ± 0.05	0 0 (0)

Hepatocytes from meal-fed rats were incubated with [1-^{14}C]oleate at the concentrations indicated, with (+) or without (−) 100 μM POCA. Data represent mean ± SEM of three different hepatocyte preparations. In parentheses, oleate incorporation in the presence of POCA as percentage of oleate incorporation in its absence.

drug does not affect enzymes involved in β-oxidation, ketone body formation and Krebs cycle activity.

In our experimental conditions the concentration of POCA required for half-maximal inhibition of oleate oxidation was about 3 μM. No attempts were made to compare hepatocytes from meal-fed and starved rats in this respect. However, it has been observed by others [7, 11] that POCA-CoA, the metabolically active form of POCA, is a less effective inhibitor in liver cells from fasted animals. A similar observation [28, 29] was made for malonyl-CoA, the physiological inhibitor of carnitine palmitoyltransferase I [30]. A recent report on the cAMP-dependent phosphorylation and concomitant activation of carnitine palmitoyltransferase I [31] may serve to explain the influence of the nutritional state of the animal. Phosphorylated carnitine palmitoyltransferase I, in view of the elevated glucagon levels likely to be the predominant form in the starved liver, is less sensitive to malonyl-CoA than the dephosphorylated form of the enzyme [31]. This may also pertain to the inhibition of carnitine palmitoyltransferase I by POCA-CoA. If so, one would expect that the enzyme from skeletal muscle behaves differently towards POCA-CoA, as malonyl-CoA inhibits fatty acid oxidation equally strong in skeletal muscle mitochondria from fed and starved rats [32].

In this study we have also shown that accumulation of lactate and pyruvate is diminished in incubations with added oleate (Fig. 3). As hepatic oleate oxidation tends to decrease flux through pyruvate dehydrogenase [33] due to end-product inhibition, the reduced accumulation of C_3 bodies was presumably caused by decreased pyruvate production (i.e. inhibition of glycolysis) rather than increased pyruvate consumption (cf. ref. 9). This interpretation is in line with the well-known inhibition of hepatic glycolytic enzymes by acetyl-CoA [34]. Fatty acid oxidation is obviously required for the oleate-induced inhibition of glycolysis, because POCA suppressed this action of oleate (Fig. 3).

In agreement with other studies [6, 7] POCA did not prevent the octanoate-induced stimulation of gluconeogenesis (Table 1). The small inhibition of glucose synthesis by POCA observed with octanoate and lactate plus pyruvate (Table 1, line 3) may reflect a slight effect of POCA on mitochondrial pyruvate uptake. No such inhibition was seen with octanoate and alanine (Table 1, line 6), a precursor which can be transaminated to pyruvate both extra- and intramitochondrially [35]. Incidentally, it may be noted that octanoate was much less effective than oleate in enhancing gluconeogenesis from alanine (Table 1, compare lines 4–6). The reason for this is not yet clear.

Gluconeogenic precursors like proline and dihydroxyacetone do not require pyruvate carboxylation. It follows that glucose formation from proline or dihydroxyacetone is not promoted by fatty acids [24]. Table 1 shows that POCA exerted no major effects on gluconeogenesis from these precursors, thereby indicating a lack of inhibition by POCA of the enzymatic steps leading from mitochondrial oxaloacetate towards glucose formation. The small percentage inhibition by POCA actually observed (Table 1, lines 7 and 8) can easily be explained by inhibition of the oxidation of endogenous long-chain fatty acids, thus preventing the increase in cytosolic redox state which favours the glyceraldehyde-3-phosphate dehydrogenase equilibrium [36] and/or limiting the supply of ATP for the energy-requiring process of gluconeogenesis.

A novel finding in our studies is the inhibition by POCA of *de novo* glycerolipid synthesis and cholesterol synthesis. The degree of inhibition of ^3H_2O incorporation into glycerolipids was approximately the same for triacylglycerols, diacylglycerols and phospholipids (Fig. 1), pointing to interference of POCA with an early step in lipid synthesis. This interpretation is corroborated by the fact that POCA stimulated esterification of exogenous oleate (Fig. 4 and Table 2). The latter observation also rules out extensive depletion of the cytosolic CoA pool, due to activation of POCA to POCA-CoA, as an explanation for impaired lipogenesis (cf. ref. 2). As pointed out earlier [13], the most likely candidate for the inhibitory effect of POCA is acetyl-CoA carboxylase (EC 6.4.1.2), generally assumed to be the rate-limiting enzyme of *de novo* fatty acid synthesis. Data from the literature indicate that CoA esters of agents such as 5-(tetradecyloxy)-2-furoic acid [37] and 2-tetradecylglycidic acid [38] are potent inhibitors of this enzyme. On the other hand, a direct inhibition of fatty acid synthase by POCA-CoA has been suggested as well [10]. Obviously, further studies with POCA-CoA and purified preparations of acetyl-CoA carboxylase and fatty acid synthase are needed to clarify this point.

As to the inhibition of *de novo* cholesterol synthesis by POCA (Fig. 1), an inhibitory effect of POCA-CoA on the rate-limiting enzyme 3-hydroxy-3-methylglutaryl CoA reductase (EC 1.1.1.34) may be surmised. However, since ketone bodies are excellent cholesterogenic precursors in the liver [26], inhibition of the oxidation of endogenous fatty acids by POCA could contribute to the observed inhibition of cholesterol formation as well (cf. Fig. 4 and Table 2).

In conclusion, POCA is a specific inhibitor of hepatic lipid metabolism which has no direct effect on either carbohydrate metabolism, β-oxidation or Krebs cycle activity. In its active form as POCA-CoA, it inhibits at least two enzymatic steps, i.e. the carnitine palmitoyltransferase I and, probably, the acetyl-CoA carboxylase reaction. As a consequence, exogenous long-chain fatty acids are diverted from oxidation towards esterification (Fig. 5), whereas *de novo* synthesis of glycerolipids and cholesterol is inhibited (Fig. 1). Whether or not POCA-CoA itself is also esterified, thereby creating abnormal triacylglycerols (cf. ref. 4), remains to be elucidated.

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