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EFFECTS OF SODIUM 2-[5-(4-CHLOROPHENYL)PENTYL]-OXIRANE-2-CARBOXYLATE (POCA) ON CARBOHYDRATE AND FATTY ACID METABOLISM IN LIVER AND MUSCLE

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Abstract—In isolated rat hepatocytes, rates of gluconeogenesis, ketogenesis and oleate oxidation to CO_2 were measured at various concentrations of lactate, pyruvate and oleate in the presence or absence of sodium 2-[5-(4-chlorophenyl)-pentyl]oxirane-2-carboxylate (POCA). With increasing lactate and pyruvate concentrations, but constant oleate concentration, oleate oxidation to CO_2, concomitantly to gluconeogenesis, was accelerated, whereas ketogenesis was decreased. In the presence of POCA, rates of gluconeogenesis, ketogenesis and oleate oxidation to CO_2 were diminished; the concentrations for half-maximal inhibition were in the micromolar range for all metabolic processes studied. When octanoate was present instead of oleate, the inhibitory effect of POCA on gluconeogenesis was reduced and that of ketogenesis was nearly abolished, suggesting that POCA specifically inhibits the oxidation of long-chain fatty acids.

In addition, the oxidation of glucose and oleate was studied in isolated rat diaphragms. POCA inhibits the oxidation to CO_2 of long-chain fatty acids also in muscle tissue; the concentration for half-maximal effect, however, was about one order of magnitude higher than in liver. Concomitantly, glucose oxidation was enhanced by POCA indicating a shift in the substrate preference of energy-yielding metabolism.

Recently, several substituted derivatives of 2-(phenylalkyl)-oxirane-2-carboxylic acids have been shown to lower blood levels of glucose and ketone bodies in fasted rats or guinea pigs [1, 2]. These effects are not secondary to a stimulation of insulin secretion [2]. Furthermore, a diminished increase in blood glucose levels after a glucose load could be demonstrated in rats and mice as well as in genetically diabetic mice pretreated with POCA [2]. From these phenomena it was suggested that 2-(phenylalkyl)-oxirane-2-carboxylic acids might offer a new concept in therapy of diabetes distinct from the action of sulfonylureas or biguanides and independent of insulin [2]. Another class of compounds with similar biological effects was presented by Tutwiler *et al.* [3, 4].

In order to study the mechanism of the POCA effects on the cellular level, experiments with isolated hepatocytes and isolated rat hemidiaphragms have been performed. The data indicate that POCA affects fatty acid oxidation and gluconeogenesis in liver in a similar way as was reported for the tetradecyl derivative of oxirane-2-carboxylic acid, suggesting an inhibition of the transport of long-chain acyl derivatives into the mitochondria [3, 4]. Moreover, the data indicate that the energy-yielding metabolism in muscle tissue is shifted from fatty acid to glucose oxidation when POCA is present. This together with a suppression of hepatic gluconeogenesis could provide the basis for a new approach in the therapy of diabetes.

MATERIALS AND METHODS

Enzymes and coenzymes were from Boehringer, Mannheim (F.R.G.). All chemicals were of reagent grade and were purchased from Sigma, München (F.R.G.). Radiochemicals were from Amersham-Buchler, Braunschweig (F.R.G.). Three derivatives of phenylalkyl-oxirane carboxylic acids were employed: sodium 2-(5-phenylpentyl)-oxirane-2-carboxylate (B 807-26), sodium 2-[5-(4-chlorophenyl)-pentyl]oxirane-2-carboxylate (POCA) and sodium 2-[3-(3-trifluoromethylphenyl)propyl]oxirane-2-carboxylate (B 807-01). The synthesis of these compounds has been described recently [1].

Hepatocytes and hemidiaphragms were isolated from 24-hr starved rats (male Wistar II strain, 200–220 g and 80–100 g, respectively), according to the procedures described by Berry and Friend [5] and Goldberg *et al.* [6], but modified as described previously [7]. The incubation medium contained Na^+ 140, K^+ 6, Ca^{2+} 1.4, Cl^- 127, phosphate 2.5, bicarbonate 20 and oleate 0.2 mmole/l, albumin 5 mg/l. In experiments with hepatocytes, the medium contained lactate 10 mmole/l and 1 mmole/l pyruvate, if not stated otherwise; the content of cellular protein was about 8 mg/ml. In experiments with hemidiaphragms, the medium was supplemented with glucose (5 mmole/l). In order to study fatty acid or glucose oxidation, trace amounts of [1-^{14}C]-oleate or [U-^{14}C]-glucose were added.

The incubations of hepatocytes or hemidiaphragms were stopped after various intervals by addition of perchloric acid. Glucose, lactate, pyruvate, 3-hydroxybutyrate and acetoacetate were measured enzymatically in the neutralized perchloric acid extracts [8]. $^{14}CO_2$ was trapped in hyamine and the radioactivity was determined by liquid scintillation spectroscopy.

RESULTS

Gluconeogenesis, ketogenesis and oleate oxidation in hepatocytes from fasted rats

When hepatocytes from fasted rats were incubated in the absence of gluconeogenic substrates, rates of glucose production were extremely low. On the other hand, high rates of acetoacetate plus 3-hydroxybutyrate production from endogenous substrates and added oleate as well as $^{14}CO_2$-production from ^{14}C-oleate were observed (Fig. 1, open symbols). Ketone body, glucose and CO_2-production from oleate had been observed to be linear under these conditions. Addition of lactate plus pyruvate with increasing concentrations increased the rates of glucose production and oleate oxidation to CO_2 but decreased the rate of ketogenesis. Striking differences between the two concentrations with molar ratios 10:1 or 1:1 (lactate:pyruvate; Fig. 1, left and right, respectively) were not observed. Half-maximal gluconeogenesis was observed with a total substrate concentration of about 2 mmol/l. The concentration ratio of 3-hydroxybutyrate/acetoacetate present at the end of a 60 min incubation period was around 0.4 (Fig. 2) irrespective of the cytosolic redox state enforced by the lactate and pyruvate concentrations added to the incubation medium.

Inhibition by POCA

When the experiments described above were performed in the presence of POCA, rates of gluconeogenesis, ketogenesis and oleate oxidation to CO_2 were considerably lower (Fig. 1, closed symbols) than in the control experiments. The inhibition was dependent upon the concentrations and ratios of added lactate and pyruvate; it appeared to be greater with the high than with the low lactate/pyruvate ratio.

In the presence of POCA, the mitochondrial redox state as indicated by the 3-hydroxybutyrate/acetoacetate ratio was lowered. With increasing lactate plus pyruvate concentrations, however, the values

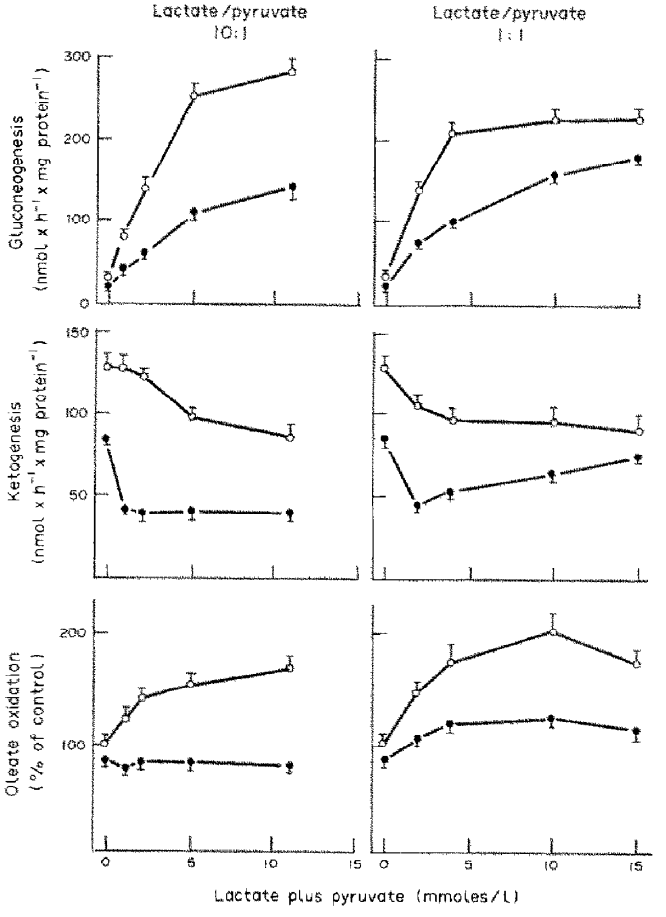

Fig. 1. Rates of gluconeogenesis from lactate plus pyruvate, ketogenesis and $^{14}CO_2$-production from [1-^{14}C]-oleate in hepatocytes. Hepatocytes isolated from 24-hr fasted rats were incubated (see Materials and Methods) in the absence (open symbols) or presence (closed symbols) of POCA (10 μmole/l). The concentrations of gluconeogenetic substrates, lactate plus pyruvate, were varied between 0 and 15 mmole/l; the molar concentration ratios lactate/pyruvate were either 10:1 (left) or 1:1 (right). Oleate (0.2 mmole/l) was present under all substrate conditions. Rates of gluconeogenesis (glucose production, upper panel), ketogenesis (production of acetoacetate plus 3-hydroxybutyrate, middle panel) and oleate oxidation ($^{14}CO_2$ production from [1-^{14}C]oleate, lower panel) were calculated from the products formed during the 60 min incubation period. The data are means ± S.E.M. from eight experiments.

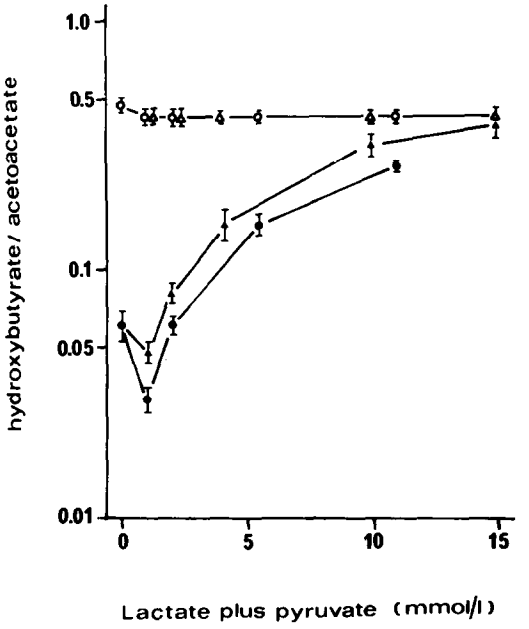

Fig. 2. 3-Hydroxybutyrate/acetoacetate ratios in hepatocytes from fasted rats. Data from the experiments with isolated hepatocytes shown in Fig. 1 (control: open symbols, presence of 10 μmole/l POCA: closed symbols). The lactate/pyruvate ratios were either 10:1 (○—○) or 1:1 (△—△).

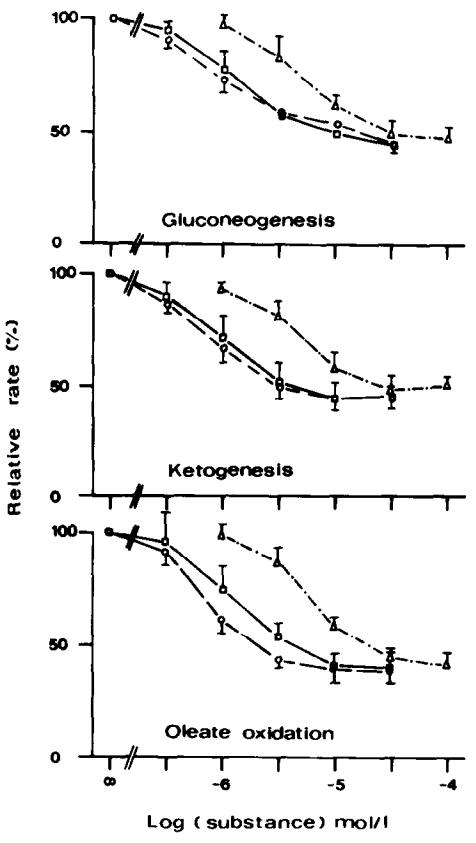

Fig. 3. Dose-dependent inhibition of gluconeogenesis, ketogenesis and fatty acid oxidation by phenyl-alkyl-oxirane carboxylic acids. Gluconeogenesis from lactate (10 mmole/l) plus pyruvate (1 mmole/l), ketogenesis and $^{14}CO_2$ production from [1-^{14}C]oleate (0.2 mmole/l) were studied in hepatocytes isolated from fasted rats (see Materials and Methods). Three derivatives of phenylalkyl-oxirane carboxylic acids (○—○ B 807-26, □—□ POCA, △—△ B 807-01) were present throughout the incubation period. Metabolic rates were calculated from the products formed in a 60 min incubation period. The data are presented as percentage of control (means ± S.E.M., N = 8).

were close to those of the control (Fig. 2). This effect was more pronounced with substrate ratios of 1:1 than 10:1.

The dose-dependence of the inhibitory effects of POCA on gluconeogenesis, ketogenesis and oleate oxidation to CO_2 was studied employing three different derivatives (POCA, B 807-26, B 801-01). Hepatocytes were incubated with lactate, pyruvate and oleate (10, 1 and 0.2 mmole/l, respectively) corresponding to one of the conditions in Fig. 1 (i.e. highest concentrations in the diagrams on the left side). As shown in Fig. 3, maximal inhibition of gluconeogenesis, ketogenesis and $^{14}CO_2$-production from labelled oleate was between 60 and 70%. The concentrations required for half-maximal inhibition of the three processes were similar with each compound. The most effective inhibitors were B 807-26 and POCA (half-maximal effects with *ca.* 1 μmole/l), whereas B 807-01 was significantly less effective (half-maximal effects with 50 μmole/l).

When after 30 min of incubation in the presence of POCA hepatocytes were washed twice and then incubated in the absence of the inhibitor, no significant increase of the metabolic rates—measured as time course of glucose and ketone body production—could be observed. Thus, the inhibitory effects appeared to be irreversible under these conditions.

Differential inhibitory effects of POCA in the presence of oleate and octanoate

Similar experiments were performed with octanoate instead of oleate. In Table 1, the inhibitory effects of POCA on gluconeogenesis and ketogenesis in the presence of long and medium chain length fatty acids are compared. The concentrations employed were either 0.4 mmole/l octanoate or 0.2 mmole/l oleate, in order to provide a nearly equimolar carbon source. Rates of gluconeogenesis were higher in the presence of oleate, whereas octanoate was the better substrate for ketogenesis. POCA (10 μmole/l) inhibited ketogenesis from oleate by more than 50%, but did not alter that of octanoate. On the other hand, gluconeogenesis was inhibited in the presence of both oleate or octanoate, although the inhibitory effect of POCA was less pronounced when octanoate was present.

It should be noted that similar data were obtained with perfused rat livers (Wolf *et al.* and Scholz *et al.*, manuscripts in preparation), although in some parameters the data differed quantitatively. For example, gluconeogenesis from lactate plus pyruvate and ketogenesis from oleate could be totally inhibited by POCA; ketogenesis was considerably more sensitive to inhibition by POCA than gluconeogenesis.

Table 1. Inhibition of hepatic gluconeogenesis and ketogenesis by POCA in the presence of oleate and octanoate

Lactate + pyruvate mmole/l	Oleate	Octanoate	POCA	Gluconeogenesis nmole × hr^{-1} × mg $protein^{-1}$	Ketogenesis nmole × hr^{-1} × mg $protein^{-1}$
0	0.2	0	0	30 ± 5	133 ± 8
10 + 1	0.2	0	0	285 ± 7 (100)	85 ± 8 (100)
10 + 1	0.2	0	0.001	230 ± 11 (81)	66 ± 7 (78)
10 + 1	0.2	0	0.010	127 ± 6 (44)	39 ± 6 (46)
0	0	0.4	0	28 ± 7	152 ± 16
10 + 1	0	0.4	0	225 ± 10 (100)	114 ± 11 (100)
10 + 1	0	0.4	0.001	197 ± 15 (88)	111 ± 8 (98)
10 + 1	0	0.4	0.010	157 ± 8 (70)	104 ± 9 (92)

Hepatocytes isolated from fasted rats were incubated with various concentrations of lactate, oleate, octanoate and POCA (for further details see Materials and Methods). Rates of gluconeogenesis and ketogenesis were calculated from changes in the concentrations of glucose, acetoacetate and 3-hydroxybutyrate before and at the end of a 60 min incubation period. The data are means ± S.E.M. from six experiments in each group; percentage of the respective control in parentheses.

Effects of POCA on rates of glucose and oleate oxidation to CO_2 in isolated rat hemidiaphragms

Isolated hemidiaphragms from fasted rats were incubated with 5 mmole/l glucose and 0.5 mmole/l oleate. They produced $^{14}CO_2$ from either [U-^{14}C] glucose or [1-^{14}C]oleate at a constant rate for at least 2 hr. Based on the specific radioactivities of the extracellular precursors, the rates of $^{14}CO_2$-production were nearly equal from either substrate (Fig. 4). In the presence of POCA, $^{14}CO_2$ production from glucose was increased whereas that from oleate was decreased (Fig. 4). The concentrations required for these effects, however, were at least one order of magnitude higher than those required for the inhibition of gluconeogenesis and ketogenesis in liver (Table 2, Fig. 2). Furthermore, in experiments with hemidiaphragms where glucose was replaced by ketone bodies, $^{14}CO_2$ production from [1-^{14}C]3-hydroxybutyrate was increased when oleate oxidation was inhibited by POCA (data not shown).

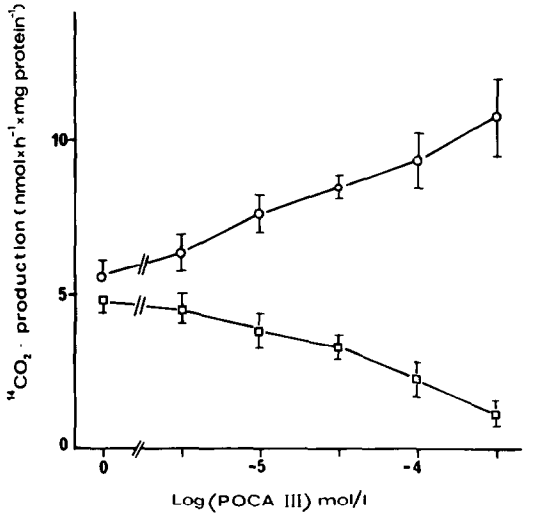

Fig. 4. Effects of POCA on rates of glucose oxidation and oleate oxidation in isolated rat hemidiaphragm. Isolated rat hemidiaphragms were incubated as described in Materials and Methods. The medium contained glucose (5 mmole/l) and oleate (0.5 mmole/l). Two sets of experiments were performed; one with [U-^{14}C]glucose and the other with [1-^{14}C]oleate. $^{14}CO_2$ production from either glucose (○—○) or oleate (□—□) was determined over a 60 min incubation period. Rates were calculated on the basis of the average specific radioactivity of the C-atoms in glucose or oleate. The intracellular dilution of radioactivity was not accounted for. In experiments with different concentrations of POCA (0–300 μmole/l; abscissa: base 10, logarithm of POCA concentration), the inhibitor was present throughout the 60 min incubation period. Data are means ± S.E.M. from eight experiments in each group.

DISCUSSION

Fatty acid oxidation and gluconeogenesis

In the liver, fatty acids are oxidized to CO_2 and ketone bodies to provide either ATP for hepatic energy-consuming anabolic processes, such as gluconeogenesis, or water-soluble and easily oxidizable nutritives for extrahepatic tissues. When gluconeogenesis is gradually accelerated due to increasing substrate supply, ketogenesis is suppressed, while

Table 2. Enhancement of glucose oxidation by POCA in muscle

μmole/l	Glucose oxidation percentage of control B 807-26	POCA	B 807-01
3	101 ± 1	103 ± 6	105 ± 10
10	126 ± 8	120 ± 20	125 ± 13
30	128 ± 17	140 ± 20	152 ± 19
100	146 ± 18	159 ± 18	188 ± 27

Isolated diaphragms from fasted rats were incubated as described in Materials and Methods. The medium contained glucose (5 mmole/l) and oleate (0.5 mmole/l). $^{14}CO_2$-production from U-^{14}C-glucose was determined over a 60 min incubation period. Rates of glucose oxidation in the presence of different concentrations of POCA, B 807-26, B 807-01 were expressed as percentage of the control values (absence of inhibitor). The data are means ± S.E.M. from eight experiments in each group.

fatty acid oxidation to CO_2 is increased concomitantly (Fig. 1). This phenomenon is well known from *in vivo* measurements as the 'antiketogenic effect' of gluconeogenic precursors [9, 10]; it is demonstrated also for *ex vivo* conditions by our data.

Inhibition of ketogenesis and fatty acid oxidation by POCA

Previously, it was shown that α-substituted fatty acid analogues, such as 2-bromopalmitic acid [11] or oxirane carboxylic acids [4], are capable of inhibiting the transfer of long chain acyl-CoA across the mitochondrial membrane. A prerequisite seems to be that these compounds are converted into their coenzyme A derivatives, which then act as inhibitors of carnitine-acyl-transferase (CAT I). This was first demonstrated for oxirane-carboxylic acids by Tutwiler and Dellevigne [4].

Recently, Bartlett *et al.* [12] have shown that POCA acts in a similar way. Our data are consistent with their conclusion, since ketogenesis and CO_2 production from oleate, but not from octanoate, were suppressed in the presence of POCA. Ketogenesis appears to be highly susceptible to inhibition by POCA at low lactate and pyruvate concentrations; the "antiketogenic effect" of gluconeogenetic precursors, therefore, appears to be enhanced. Obviously, the two mechanisms by which ketogenesis is affected (i.e. decreased β-oxidation of long chain fatty acids due to inhibition of carnitine-acyltransferase and increased citrate cycle activity due to the ATP demand for gluconeogenesis) are additive. With higher lactate plus pyruvate concentrations the exaggeration of the antiketonic effect by POCA is gradually diminished, most likely due to the fact that now acetyl-CoA and reducing equivalents are provided sufficiently by pyruvate oxidation. The restoration of the mitochondrial redox state (i.e. 3-hydroxybutyrate/acetoacetate, see Fig. 2) towards the control conditions is consistent with this view.

Inhibition of gluconeogenesis by POCA

The observation that gluconeogenesis from lactate plus pyruvate is inhibited by POCA is not easy to explain. One possibility is that this energy consuming process is affected due to a limited supply of ATP when fatty acid oxidation is suppressed. Consistent with this explanation are the observations that the inhibitory effect of POCA on gluconeogenesis was less pronounced in the presence of octanoate which can be oxidized without a carrier-dependent transport and, furthermore, in the presence of high pyruvate concentrations which can serve as a mitochondrial fuel. It is unlikely, therefore, that gluconeogenesis is directly affected by POCA. Another possibility could be a deactivation of pyruvate carboxylase due to a lowering of the mitochondrial acetyl-CoA level. Finally, gluconeogenesis could also be inhibited by a secondary effect of POCA on the transport of reducing equivalents across the mitochondrial membrane, since gluconeogenesis from lactate seemed to be more sensitive towards an inhibition by POCA than that from pyruvate. The inhibitory mechanism of POCA on gluconeogenesis, therefore, awaits further studies.

Shift from fatty acid oxidation to glucose oxidation in muscle due to POCA

In muscle tissue, the inhibition of oleate oxidation was accompanied by an increase in glucose oxidation (Fig. 4). Similar phenomena were observed with heart muscle [13]. Rough calculations indicate that the extra ATP produced by the extra glucose oxidation compensates for the diminished ATP generation from oleate oxidation. In experiments with octanoate, on the other hand, POCA did not stimulate glucose oxidation, indicating that the POCA effect on the oxidative metabolism of the glucose is not a direct one. Thus, the enhancement of glucose oxidation reflects the metabolic response of muscle tissue in order to meet its energetic demand when the oxidation of long-chain fatty acids is inhibited. In this respect, glucose can be replaced by ketone bodies. The oxidation of 3-hydroxybutyrate was also stimulated when oleate oxidation was inhibited by POCA.

Perspectives towards a therapeutical application of POCA

With regard to the metabolic disorders which are characteristic for the diabetic state, POCA exhibits interesting capabilities. In liver, POCA affects fatty acid oxidation and ketogenesis; secondarily, synthesis and release of glucose are diminished, similar changes in metabolism are observed with increasing blood levels of insulin. In muscle, the inhibition of fatty acid oxidation by POCA results in a reactive enhancement of glucose utilization. Thus, POCA imitates one important function from the spectrum of insulin actions: it shifts the energy-yielding metabolism from fatty acid oxidation to glucose oxidation. Metabolism in liver and muscle is switched from a state corresponding to diabetes or starvation to a state which is characterized by a high insulin/glucagon ratio. Consequently, blood glucose levels are lowered. One should expect a simultaneous increase of free fatty acid blood levels. However, it has been observed that, after a short transitory enhancement of fatty acids and triglycerides in blood, a hypolipidemic effect occurred [14]. Direct influences of POCA on lipogenesis and reesterification await further investigation. POCA cannot replace insulin; in cases of absolute or relative insulin deficiency, however, it may support therapeutical intentions to normalize the metabolic disorders in diabetes.

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REFERENCES

1. K. Eistetter and H. P. O. Wolf, *J. med. Chem.* **25**, 109 (1982).
2. H. P. O. Wolf, K. Eistetter and G. Ludwig, *Diabetologia* **22**, 1 (1982).
3. G. F. Tutwiler, Th. Kirsch, R. J. Mohrbacher and W. Ho, *Metabolism* **27**, 1539 (1978).
4. G. F. Tutwiler and P. Dellevigne, *J. biol. Chem.* **254**, 2935 (1979).
5. M. N. Berry and D. S. Friend, *J. Cell Biol.* **43**, 506 (1969).

6. A. L. Goldberg, S. B. Martel and M. J. Kushmerick, *Meth. Enzym.* **39**, 82 (1975).
7. Ch. Schudt, *Biochim. biophys. Acta* **629**, 499 (1980).
8. H. U. Bergmeyer, *Methoden der enzymatischen Analyse*, pp. 1241–1246, 1491–1496, 1510–1514, 1883–1890. Verlag Chemie, Weinheim (1974).
9. J. D. McGarry and D. W. Foster, *J. biol. Chem.* **246**, 6247 (1971).
10. G. Dietze, M. Wicklmayr, K. D. Hepp, W. Bogner, H. Mehnert, H. Czempiel and H. G. Henftling, *Diabetologia* **12**, 555 (1976).
11. J. F. A. Chase and P. K. Tubbs, *Biochem. J.* **129**, 55 (1972).
12. K. Bartlett, A. J. Bone, P. P. Koundakjian, E. Meredith, D. M. Turnbull and H. St. A. Sherratt, *Biochem. Soc. Trans.* **9**, 574 (1981).
13. P. Rösen and H. Reinauer, *Metabolism* **33**, 177 (1984).
14. P. P. Koundakjian, T. M. Turnbull, A. J. Bone, M. P. Rogers, S. I. M. Younan and A. S. H. Sherratt, *Biochem. Pharmac.* **33**, 465 (1984).