Inhibition of Gluconeogenic Enzymes by Free Fatty Acids and Palmitoyl Coenzyme A*

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SUMMARY

The effects of free fatty acids on rat liver phosphoenolpyruvate carboxykinase (EC 4.1.1.32), pyruvate carboxylase (EC 6.4.1.1), and glucose 6-phosphatase (EC 3.1.3.9) were determined to evaluate the suggestion that free fatty acid inhibition of key glycolytic enzymes constitutes a specific inhibitory effect. Activities of all of these gluconeogenic enzymes were found to be markedly inhibited by long chain free fatty acids as well as by palmitoyl coenzyme A. The manner of the inhibition of gluconeogenic enzymes was indistinguishable from that reported for the glycolytic enzymes. It is therefore concluded that such effects in vitro of free fatty acids or long chain acyl-CoA esters on glycolytic and gluconeogenic enzymes may not be relevant for the regulation of metabolism in vivo.

There is good evidence that conditions of enhanced fatty acid mobilization and hepatic fatty acid oxidation are associated with increased gluconeogenesis (for references, see 1–5). This has led to the suggestion that the former may in some manner regulate the latter process. In this connection Weber et al. (6, 7), Weber, Lea, and Stamm (8) and Lea and Weber (9) have reported that free fatty acids inhibited several glycolytic enzymes selectively without affecting either the bifunctional or the gluconeogenic enzymes. They have suggested that free fatty acids regulate gluconeogenesis by suppressing flux through glycolysis. Recently Tsutsumi and Takenaka (10) observed that, similar to lipolysis, heart muscle pyruvate kinase was also inhibited by free fatty acids. Thus it was inferred that glycolysis in heart could also be regulated by the free fatty acid inhibition of this glycolytic enzyme. Ferdinandus and Clark (11) reported that octanoic acid selectively inhibited the enzymes of Arthrobacter crystallopoietes involved in glycolysis and lipogenesis without affecting two of the bifunctional enzymes or a gluconeogenic enzyme, fructose 1,6-diphosphatase. They concluded that free fatty acids did not inhibit gluconeogenesis and that their inhibition of glycolytic and lipogenic enzymes could be regulatory for metabolism, hence relevant for bacterial morphogenesis. To evaluate the suggestion that inhibition of key glycolytic enzymes by free fatty acids could regulate gluconeogenesis by suppressing glycolysis, we have investigated the specificity of free fatty acid inhibition by determining the effects of free fatty acids on key gluconeogenic enzymes. It was found that long chain fatty acids, as well as palmitoyl coenzyme A, inhibited all of the gluconeogenic enzymes tested, in a manner analogous to the reported inhibition of glycolytic enzymes. This lack of selectivity in the inhibitory effects suggests that such effects of free fatty acids in vitro or long chain acyl-CoA esters on enzyme activities need not be significant for metabolic regulation in vivo.

EXPERIMENTAL PROCEDURE

Materials—Phosphoenolpyruvate, NADH, pyruvic acid (potassium salt), ATP, palmitoyl-CoA, oxaloacetic acid, ADP, dithiothreitol, lactate dehydrogenase, Tris (Sigma-I21), malate dehydrogenase, bovine serum albumin, and glucose 6-phosphate were obtained from Sigma Chemical Company. Acetyl-CoA and ITP were P L Biochemical products. Pyruvate kinase was obtained from Boehringer (Germany), NaH14CO3 from New England Nuclear Corporation, and oleate from Serdary Research Laboratories (Canada). (−)-Palmitoylcarnitine was a gift from Dr. S. V. Pande.

Enzyme Preparations—Supernatant and microsomes from rat liver were prepared as described before (12) and kept frozen when not in use. The supernatant and the microsomal fractions were used, respectively, as sources of phosphoenolpyruvate carboxykinase and glucose 6-phosphatase. Mitochondrial fraction from rat liver was prepared as described by Johnson and Lardy (13). The mitochondria were disintegrated, using a Bronwill Biosonic Probe (20 kc, delivering 120 watts), by four successive sonic disruptions of 15 sec each, with 25-sec cooling intervals in between.

Protein Estimation—Protein was determined by the differential absorbance biuret method of Parvin, Pande, and Venkitasubramanian (14).

Phosphoenolpyruvate Carboxykinase Assay—This enzyme was assayed in the direction of phosphoenolpyruvate formation by coupling with pyruvate kinase and lactate dehydrogenase. This assay procedure was similar to that of Seubert and Huth (15) in that KBH4 was used to terminate the phosphoenolpyruvate carboxykinase-catalysed reaction. However, to eliminate the interference due to pyruvate kinase of the rat liver supernatant, KCI was omitted from the assay system at this step and sodium salts were used. The assays were initiated with a freshly pre-

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Fig. 1. Effect of palmitoyl-CoA and palmitoylcarnitine on phosphoenolpyruvate carboxykinase activity at different protein concentrations. The preliminary incubation mixture, in a final volume of 0.3 ml, contained 50 μmole of Tris-chloride (pH 7.5), 0.8 μmole of dithiothreitol, and protein from rat liver supernatant (O—O). In addition, curves △——△ and ●——● had 0.32 mm of either palmitoylcarnitine or palmitoyl-CoA, respectively. After a 10-min preliminary incubation at 37° 5 μmole of MgCl₂, 6 μmole of ITP, and 4 μmole of oxalacetate were added to start the enzyme reaction. Final volume was 0.5 ml. After a 5-min incubation at 37°, the reaction was stopped and phosphoenolpyruvate (PEP) was determined as described under "Experimental Procedure."

Fig. 2. Inhibition of phosphoenolpyruvate (PEP) carboxykinase activity with varying palmitoyl-CoA concentration. Preliminary incubation and assay conditions were as described for Fig 1, except that 0.185 mg of supernatant protein was present and the amount of palmitoyl-CoA was varied.

Inhibition of Pyruvate Carboxylase Reaction by Acidification (21), we have employed the EDTA-NADH-malate dehydrogenase mixture which eliminated prior acidification and allowed oxalacetate to be converted to acid-stable malate without delay in manipulations. The ability of EDTA to arrest pyruvate carboxylase reaction was ascertained in separate experiments, where it was found that presence of EDTA completely prevented bicarbonate fixation and that this effect of EDTA was instant. Control sets lacking pyruvate were always included along with the experimental sets. The incorporation of bicarbonate in the absence of added pyruvate or acetyl-CoA was insignificant.

Glucose 6-Phosphatase Assay—Glucose 6-phosphatase was assayed as described previously (22) except that orthophosphate was determined by the method of Bartlett (23).

RESULTS

Inhibition of Phosphoenolpyruvate Carboxykinase by Palmitoyl-CoA and Olate—The effect of the preliminary incubation of rat liver supernatant with (+)-palmitoylcarnitine and palmitoyl-CoA on its phosphoenolpyruvate carboxykinase activity is presented in Fig. 1. The initial rate of enzyme activity was linear with the amount of enzyme protein, palmitoyl-CoA strongly inhibited its activity. However, (+)-palmitoylcarnitine, under comparable conditions, was without any effect. Fig. 2 shows the effect of varying concentrations of palmitoyl-CoA on the phosphoenolpyruvate carboxykinase activity. The concentration of palmitoyl-CoA giving 50% inhibition under the experimental conditions employed was 64.5 μM during preliminary incubation (40 μM during assay). Preliminary incubation with olate also resulted in a marked loss of phosphoenolpyruvate carboxykinase activity and the results of varying olate concentrations on enzyme activity showed (Fig. 3) that a sigmoidal type of curve was obtained, similar to that seen with palmitoyl-CoA (Fig. 2). The concentration of olate giving 50% inhibition of the phosphoenolpyruvate carboxykinase was 1.20 mM during preliminary incubation (0.8 mM during assay), which was considerably higher than the corresponding palmitoyl-CoA concentration described above.

Inhibition of Pyruvate Carboxylase by Olate and Palmitoyl-CoA—The rate of the pyruvate carboxylase reaction was linear
FIG. 3. Inhibition of phosphoenolpyruvate (PEP) carboxykinase activity with varying oleate concentration. Preliminary incubation and assay conditions were as for Fig. 1, except that 0.138 mg of supernatant protein was used and the amount of oleate was varied.

FIG. 4. The effect of varying protein concentration on pyruvate carboxylase activity as modified by oleate. Varying amounts of mitochondrial protein and 100 μmoles of Tris-chloride (pH 7.4) were incubated for 10 min at 37°, either with 3.3 mM oleate (△—△) or without oleate (●—●), in a final volume of 0.3 ml. The enzyme assay was initiated by the addition of a freshly prepared mixture containing 10 μmoles of pyruvate, 2 μmoles of ATP, 5 μmoles of Mg2+, and 0.4 μmole of acetyl-CoA followed by 15 μmoles of K2HCO3 (about 10⁶ cpm per μmole). Final volume was 0.5 ml. After incubation for 5 min at 37°, the reaction was terminated and radioactivity determined as described under "Experimental Procedure."

Fig. 5. Effect of varying palmitoyl-CoA concentration on pyruvate carboxylase activity. Preliminary incubation and assay conditions were described as for Fig. 4, except that the amount of oleate was varied and 0.5 mg of sonically disrupted mitochondrial protein was present.

FIG. 6. Effect of varying oleate concentration on pyruvate carboxylase activity. Preliminary incubation and assay conditions were as described for Fig. 4, except that the amount of oleate was varied and 0.5 mg of sonically disrupted mitochondrial protein was present.

with the amount of mitochondrial protein when the enzyme preparation was incubated without oleate (Fig. 4). Inclusion of oleate during preliminary incubation markedly inhibited the pyruvate carboxylase activity and the rate of reaction was no longer linear with the amount of mitochondrial protein. As the protein concentration was increased from 0.25 to 0.75 mg, the percentage of inhibition decreased from 94 to 78%, showing that the extent of the inhibition was related to the amount of enzyme protein (Fig. 4). The effect of palmitoyl-CoA on pyruvate carboxylase activity was also studied. The enzyme preparation was previously incubated with varying amounts of palmitoyl-CoA and then assayed. The results showed (Fig. 5) that palmitoyl-CoA was also markedly inhibitory; its concentration

for 50% inhibition under these conditions was 250 μM during preliminary incubation (150 μM during assay). Similar experiments with oleate showed (Fig. 6) that this was less inhibitory; the concentration required for 50% inhibition was 0.665 mM during preliminary incubation (0.4 mM during assay).

In these experiments, the specific activity of the mitochondrial pyruvate carboxylase was found to be considerably lower even in controls in which the enzyme preparation was previously incubated without any added oleate or palmitoyl-CoA as compared with the specific activity obtained when no preliminary incubation was performed. It seems that the loss of pyruvate carboxylase in the absence of inhibitor caused by low protein concentrations, as there was no loss in activity when either bovine serum albumin (5 mg) was included during preliminary incubation or when concentrated mitochondrial preparation (25 mg of protein per ml) was previously incubated. Inclusion of acetyl-CoA during preliminary incubation gave considerable protection...
Fig. 7. Effect of preliminary incubation on pyruvate carboxylase activity as modified by either acetyl-CoA or oleate, or both. Assay conditions were as described for Fig. 4. The preliminary incubation time was varied from 0 to 20 min as indicated. The preliminary incubation system in 0.3 ml contained 100 μmoles of Tris-chloride (pH 7.4) and 0.08 mg of sonically disrupted mitochondrial protein (○—○). In addition, curves △—△, ●—●, and △—△ had, respectively, 0.33 mM of oleate, 0.33 mM of acetyl-CoA or 0.33 mM of oleate plus 0.33 mM of acetyl-CoA.

Fig. 8. Effect of varying amounts of palmitate on glucose 6-phosphatase activity at two different levels of microsomal protein. Varying amounts of palmitate as indicated, 100 μmoles of Tris-chloride (pH 7.5), and 0.11 mg (▲—▲) or 0.28 mg (●—●) of microsomal protein in 0.3 ml were incubated for 10 min at 37°C. After this, the assay was started by the addition of 20 μmoles (0.2 ml) of glucose 6-phosphatase and 10 min later 0.1 ml of 10% trichloracetic acid was added to stop the reaction.

For the reversal of glycolysis, it is well known that the energy barrier (25) at the pyruvate kinase step is overcome by the participation of pyruvate carboxylase and phosphoenolpyruvate carboxykinase (26-29). The activities of these two enzymes have been shown to be closely related to gluconeogenesis (26, 30-33). It was therefore to be expected that these enzymes would not be subject to inhibition by free fatty acids if the contention (6-9, 11) that free fatty acids selectively inhibited key glycolytic enzymes without affecting gluconeogenic enzymes were to be valid. However, there is no doubt from the present work that such is not the case, since oleate inhibited the activities of both pyruvate carboxylase as well as phosphoenolpyruvate carboxykinase. Similar to the free fatty acid inhibition of glycolytic enzymes, which is increased by increasing the time of preliminary incubation of enzymes with free fatty acids (6, 7), the inhibition of pyruvate carboxylase by oleate was also a time-dependent effect. It has been shown by other investigators
stimulation of gluconeogenesis under these conditions is related to the amount of the enzyme protein which binds free fatty acids or fatty acyl-CoA (16-18). In agreement with this, we found that the inhibition of all the three gluconeogenic enzymes was lowered as the amount of enzyme protein was increased (Figs. 1, 4, and 8).

It was pointed out by Pande and Mead (18) that those enzymes which are subject to inhibition by free fatty acids are also inhibited by long chain fatty acyl-CoA esters in a comparable way. Similarly, present results show that pyruvate carboxylase, glucose 6-phosphatase, and phosphoenolpyruvate carboxykinase were also inhibited by palmitoyl-CoA in a way analogous to that of the fatty acid inhibition, except that much lower palmitoyl-CoA concentrations were required for comparable inhibitions. Likewise, Dawson and Hales (34) reported for glucokinase activity that under conditions in which palmitate inhibition was not apparent, palmitoyl-CoA was strongly inhibitory. Pyruvate kinase is another enzyme that has been reported to be inhibited by free fatty acids (6-8, 10, 11) and we have found that palmitoyl-CoA was a more effective inhibitor of this enzyme than oleate. These findings show that long chain acyl-CoA esters are more inhibitory than long chain free fatty acids (19). Inasmuch as long chain acyl-CoA esters are better surface-active compounds than free fatty acids, the presently studied inhibitions appear to be related to the detergent action of the inhibitory compounds (16, 18).

The suggestion that free fatty acid inhibition of key glycolytic enzyme activities is specific (6, 7, 11) and regulatory for gluconeogenesis (6-9, 11) is based on the observations that free fatty acids, even at a high concentration as 20 mM, inhibited neither the bifunctional enzymes that are involved in both glycolysis and gluconeogenesis nor two of the enzymes of the gluconeogenic pathway, namely, glucose 6-phosphatase and fructose 1,6-diphosphatase (6, 7, 11). However, present and other results (18) show that 50% inhibition of glucose 6-phosphatase is obtained with 0.12 mM palmitate. Lea and Weber (9) have shown a 50% inhibition of glucokinase with 1 mM palmitate. The same extent of inhibition of two gluconeogenic enzymes, pyruvate carboxylase and phosphoenolpyruvate carboxykinase, was obtained with the same or much smaller (0.67 mM) concentrations of oleate. Thus fatty acid inhibitions are not specific for key glycolytic enzymes alone. This is supported by the recent observations of Reshef and Heller (35) that oleate inhibited the activity of fructose diphosphate aldolase, showing that this bifunctional enzyme is also subject to similar inhibition. These considerations, together with the fact that such inhibitory effects in vitro involve irreversible enzyme inactivations (16, 17), being related to the detergent action of inhibitory compounds, render it unlikely that these effects would be involved in metabolic regulation in vivo. Although Weber et al. (7) and Lea and Weber (9) have shown with rat liver supernatant system that the presence of free fatty acid in the incubation medium inhibited glycolysis as judged by decreased lactate production from glucose or glucose 6-phosphate, it has not been ascertained whether it also increased gluconeogenesis under similar conditions. Increased gluconeogenesis occurs when fatty acids are made available to gluconeogenic tissues (for a review see Reference 5). However, it has been shown that the stimulation of gluconeogenesis under these conditions is related to the process of fatty acid oxidation rather than resulting from the presence of free fatty acids per se (5, 36, 37).

As discussed by Weber (7), gluconeogenic conditions are associated with a rise in plasma free fatty acid levels. It is significant in this connection, however, that varying rates of free acid uptake by liver (38) and tumor cells (39) are not accompanied by increase in intracellular free fatty acid levels. This makes it more doubtful that intrahepatic fatty acid concentration as such could be involved in enzyme regulation in vivo. On the other hand, hepatic fatty acyl-CoA concentrations do increase with increased free fatty acid availability (40-43). However, since acyl-CoA esters are more effective inhibitors of key gluconeogenic enzymes, and their concentrations are increased under conditions of enhanced gluconeogenesis (40-43), it is evident that the inhibitions in vivo due to these compounds as well may not be of any physiological relevance.

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