A Possible Physiological Role for Glyceroneogenesis in Rat Adipose Tissue*

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SUMMARY

Pyruvate decreased the rate of free fatty acid release from rat epididymal adipose tissue incubated in vitro by increasing free fatty acid esterification. The magnitude of the pyruvate effect, tested in tissues from fasted, adrenalectomized, or diabetic rats, closely correlated both with the rate of glyceride-glycerol labeling from radioactive pyruvate and with the activity of P-enolpyruvate carboxykinase. The addition of 25 mM pyruvate to adipose tissue from 24-hour fasted rats produced a 2-fold increase in free fatty acid esterification. At a lower concentration (0.25 mM) pyruvate had no effect on free fatty acid esterification but butyrate added in equimolar concentrations caused a 50% increase in esterification of free fatty acids in tissue from fasted animals. These findings support the role of P-enolpyruvate carboxykinase as a regulatory enzyme in the glyceroneogenic sequence and suggest that glyceroneogenesis is important in adipose tissue for the maintenance of free fatty acid esterification. The role of the adrenals and of insulin in regulating the activity of rat adipose tissue P-enolpyruvate carboxykinase is also presented. Diabetes increased the activity of this enzyme in adipose tissue and liver but adrenalectomy superimposed upon diabetes caused a further increase in adipose tissue P-enolpyruvate carboxykinase activity and a reduction in the activity of the hepatic enzyme. Both insulin and triamcinolone reduced the activity of adipose tissue P-enolpyruvate carboxykinase in adipose tissue from diabetic-adrenalectomized rats but only when the two hormones were administered simultaneously was the level of the enzyme reduced to normal.

The rate of free fatty acid release from adipose tissue is determined by the difference between lipolysis and esterification (1), either of which may be affected independently (2-4). During fasting there is a fall in the concentration of plasma insulin (5) with a concomitant depression of glucose uptake by peripheral tissues. These lead to an increased release of FFA. Recently, we have shown (6-8) that in adipose tissue from 24-hour fasted rats the synthesis of glyceride-glycerol from pyruvate was increased, presumably indicating that more α-glycerophosphate had been formed. Although the physiological role of this pathway is as yet not clear it is possible that the ability of adipose tissue to synthesize α-glycerophosphate from sources other than glucose could restrain FFA output by increasing the rate of esterification. It has been found that quantitative differences in the rate of FFA release were caused by glucose deprivation in vivo (9). For example, both fasting for 48 hours and pancreatectomy caused an FFA output from adipose tissue which was 2- to 3-fold higher than that found after 24 hours of fasting. It is suggested that these differences are not caused by changes in the rate of lipolysis alone, but by alterations in the capacity of the adipose tissue to re-esterify FFA.

The purpose of the present study was to determine whether the addition of pyruvate, used as a model substrate, to adipose tissue in vitro would reduce FFA release under conditions of enhanced fat mobilization. On the basis of these experiments we hoped to determine the potential of adipose tissue to regulate FFA output by altering the rate of re-esterification, as well as to relate FFA esterification with the activity of P-enolpyruvate carboxykinase.

EXPERIMENTAL PROCEDURES

Animals—Male albino rats, 6 to 7 weeks of age, maintained on standard lab chow were used throughout these experiments. Alloxan diabetes was produced by giving the animals 19 mg of alloxan per 100 g of body weight subcutaneously. Those rats with a urine glucose concentration of at least 500 mg/100 ml 3 days after alloxan treatment were used for further study. Adrenalectomy was performed on the 3rd day after alloxan treatment and the animals given 0.9% NaCl to drink. Adrenalectomized diabetic animals were used on the 3rd postoperative day as long as the high degree of glucosuria was maintained. Triamcinolone and trypsin-treated insulin were given subcutaneously. Protamine zinc insulin was given intramuscularly, while actinomycin D and cycloheximide were given intraperi-

1 The abbreviation used is: FFA, free fatty acid.
Dr. O. K. Behrens of Eli Lilly and Company, Indianapolis, used in this study was a pharmaceutical preparation from Squibb. Trypsin-treated bovine insulin (glucagon-free) was a gift of the protamine zinc insulin preparation from Eli Lilly. Actinomycin D (Lyovac Cosmogen) was from Merck Sharp and Dohme; and P-enolpyruvate, IDP, malate dehydrogenase (EC 1.1.1.37), NADH, and cycloheximide were purchased from Sigma.

**Materials**—Radioactive compounds were purchased from the Radiochemical Centre, Amersham, England. The trypsinolone used in this study was a pharmaceutical preparation from Squibb. Tryptsin-treated bovine insulin (glucagon-free) was a gift of Dr. O. K. Behrens of Eli Lilly and Company, Indianapolis, Indiana whereas the porcine zinc insulin was a pharmaceutical preparation from Eli Lilly. Actinomycin D (Lyovac Cosmogen) was from Merck Sharp and Dohme; and P-enolpyruvate, IDP, malate dehydrogenase (EC 1.1.1.37), NADH, and cycloheximide were purchased from Sigma.

**FFA Release and Glyceroneogenesis**—Portions of epididymal fat pad weighing about 100 mg were incubated at 37°C in 2 ml of Krebs-Ringer phosphate buffer, pH 7.4, containing 2% bovine serum albumin (fatty acid-free) (10), and 25 mM pyruvate-3-14C (1.0 μCi). After 2-hours incubation, the medium was extracted with the acidic isopropanol-heptane extraction mixture described by Dole (11) and FFA determined by the method of Duncombe (12). Radioactive glyceride-glycerol was isolated and counted as previously described (13).

**FFA Esterification**—The method used for the measurement of FFA esterification by rat adipose tissue in vitro was essentially that described by Vaughan (1). In this procedure FFA and glycerol are measured in both the tissue and the incubation medium before and after incubation. Total lipolysis is calculated by multiplying by three the net glycerol released, while the rate of FFA esterification is considered to be the difference between this value and the net FFA accumulated during the incubation. As discussed in detail by Vaughan (1) this method is correct only if (a) adipose tissue lipid is predominately triglyceride, (b) the glycerol released by the fat pad is not phosphorylated, and (c) the FFA resulting from lipolysis is not oxidized to CO₂ to an appreciable extent by the tissue. Epididymal fat pads from a single animal were bisected to give four sections. One piece was homogenized directly in 3 ml of water and 0.2 to 0.3 ml of 0.1 N metaphosphoric acid added to precipitate protein, and the tissue glycerol (14) and FFA (13) were determined. The remaining portion of tissue was incubated in Krebs-Ringer bicarbonate containing various additions. Tissue from the same animal was used for a comparison of the effects of pyruvate and butyrate on FFA esterification. Segments of adipose tissue were incubated for 3 hours at 37°C in 3 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 3% defatted (10) bovine serum albumin and appropriate substrates. After incubation, the tissue was removed, rinsed twice in 0.9% NaCl and homogenized in 3 volumes of water. A 1-ml aliquot of the incubation medium was taken for the determination of FFA and the remainder of the incubation medium deproteinized by the addition of 0.2 to 0.3 ml of 1 N metaphosphoric acid (1). After centrifugation at 1000 x g for 5 min followed by neutralization, the glycerol content of the extracted incubation medium was measured by the method of Wieland (14). FFA was determined as described by Duncombe (12).

**P-Enolpyruvate Carboxykinase** (EC 1.1.1.37)—Adipose tissue was homogenized in 3 volumes, and liver in 9 volumes of 0.25 M buffered sucrose (15). Supernatant fractions prepared by centrifugation at 105,000 x g for 30 min were used for assay of P-enolpyruvate carboxykinase.

The assay was a minor modification (7) of the method of Chang and Lane (16) which involves the fixation of 14C-labeled bicarbonate in the presence of P-enolpyruvate, IDP, MnCl₂, GSH, NADH, malate dehydrogenase, and tissue extract. One unit of activity is defined as the amount of extract that will catalyze the fixation of 1 μmol of KH₁₄CO₃ per min at 30°C.

Urine glucose was determined by test tape (Ames Laboratories, Elkhart, Indiana), and protein by the method of Lowry et al. (17).

**RESULTS**

Effect of Pyruvate on FFA Release and Glyceride-Glycerol Synthesis—Studies in vitro, in which one of the two epididymal fat pads was incubated in the absence and the other in the presence of pyruvate (25 mM) indicated that the amounts of the FFA...
released from the tissues, either with or without added pyruvate, varied with the treatment of the animals. Thus fasting and alloxan diabetes enhanced FFA release, whereas adrenalectomy lowered it. However, the addition of pyruvate reduced the output of FFA in all tissues tested, irrespective of the treatment in vivo given to the animals (Table I).

In the absence of pyruvate no difference in the rate of the FFA output between tissues from fasted and diabetic animals was noted. With added pyruvate, on the other hand, the output of FFA was higher in tissues from diabetic than in tissues from fasted animals (Table I). This difference between diabetes and fasting was further increased if the adrenals were removed. As shown in Table I, tissues from adrenalectomized-diabetic rats still released FFA in the presence of added pyruvate, whereas tissues from adrenalectomized-fasted or adrenalectomized-fed rats took up FFA from the medium. The net pyruvate effect refers to the additional amount of FFA retained by the tissue when pyruvate was added. The negative values indicate an uptake of palmitate from the incubation medium. The magnitude of this effect correlated closely with the rate of $^{14}$C-glyceride-glycerol formation. Both parameters increased under all conditions tested when compared to intact, fed animals. Adrenalectomy, which increased both the pyruvate effect and the rate of glyceride-glycerol synthesis in tissue of fasted animals, had no effect on FFA retention in adipose tissue from diabetic rats.

Effect of Pyruvate and Butyrate on FFA Esterification in Vitro—

The possibility that the decreased FFA release caused by pyruvate might be caused by an increased rate of FFA esterification was tested with adipose tissue from fed and 24-hour fasted rats. As shown in Table II, FFA esterification was evident in adipose tissue from both the fed and fasted animals in the absence of added substrate. In agreement with Vaughan (1) the rate of esterification without substrate was markedly increased by fasting the animals. The addition of 25 mM pyruvate to the medium caused an increase in FFA esterification in adipose tissue from both fed and fasted rats. At a lower and presumably more physiological concentration, the addition of an equimolar concentration of butyrate was required for the effect. It should be pointed out that whenever pyruvate increased FFA esterification it did not inhibit lipolysis. This clearly shows that the effect of pyruvate on FFA release is the result of an increased esterification rather than a diminished rate of lipolysis.

Effect of Diabetes and Adrenalectomy on Activities of Liver and Adipose Tissue P-Enolpyruvate Carboxykinase—As shown in Table III, both diabetes and adrenalectomy caused a rise in the activity of adipose tissue P-enolpyruvate carboxykinase. The combined treatment of adrenalectomy and diabetes yielded an additive effect. If diabetic-adrenalectomized rats were treated with either triamcinolone or insulin a decline in the activity of P-enolpyruvate carboxykinase was evident. Giving insulin together with triamcinolone resulted in an additive decay in the activity of the enzyme to the level found in adipose tissue from intact rats.

Unlike the adipose tissue enzyme, liver P-enolpyruvate carboxykinase was dependent on the presence of the adrenals for its induction by diabetes (Table III). In agreement with Shargo et al. (18) the removal of the adrenals markedly reduced, whereas triamcinolone treatment fully restored, the effect of diabetes on the liver enzyme. On the other hand, fasting the diabetic adrenalectomized rat for 24 hours induced a rise in the activity of liver P-enolpyruvate carboxykinase, indicating a difference between the effects of fasting and diabetes in the adrenalectomized animal. In adipose tissue, on the other hand, only a slight additional effect was evident by fasting the diabetic-adrenalectomized rat. Insulin decreased the activity of the hepatic enzyme in a manner similar to its effect on adipose tissue P-enolpyruvate carboxykinase. The time course following insulin treatment (Fig. 1) as well as the dose response to insulin (Table IV) were similar in both tissues. The decay in the activity of the enzyme proceeded until 10 hours after the injection of insulin. The $t_{1/2}$ of P-enolpyruvate carboxykinase decay caused

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Table II

<table>
<thead>
<tr>
<th>Dietary status</th>
<th>Additions</th>
<th>Glycerol</th>
<th>FFA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\Delta$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\Delta$ Initial</td>
<td>3 hrs</td>
<td>3 hrs</td>
</tr>
<tr>
<td></td>
<td>mm</td>
<td>Tissue</td>
<td>Tissue</td>
</tr>
<tr>
<td>Fed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate, 25</td>
<td>0.10</td>
<td>0.08</td>
<td>2.79</td>
</tr>
<tr>
<td>Pyruvate, 0.25 + butyrate &amp; 0.25</td>
<td>0.15</td>
<td>0.07</td>
<td>0.55</td>
</tr>
<tr>
<td>Fasted 24 hours</td>
<td>0.68</td>
<td>0.66</td>
<td>2.50</td>
</tr>
<tr>
<td>Pyruvate, 25</td>
<td>0.66</td>
<td>4.66</td>
<td>2.95</td>
</tr>
<tr>
<td>Pyruvate, 0.25 + butyrate, 0.25</td>
<td>0.66</td>
<td>4.66</td>
<td>2.95</td>
</tr>
</tbody>
</table>

Glycerol and indicated substrates. Values are expressed as micromoles per g with means $\pm$ S.E.M. for five animals are given. The values for $\Delta$ glyceraldehyde and FFA were determined by finding the difference in net glycerol or FFA for each individual animal. The S.E.M. is, therefore, also calculated on this basis. Further details are given under “Experimental Procedures.”
by insulin in both tissues depended on the route of hormone administration, with a t½ of 7 hours if insulin was given subcutaneously. In several experiments in which insulin was injected intraperitoneally in repeated doses, the t½ was 5½ hours. On the other hand, the very fast rise in the activity of P-enolpyruvate carboxykinase (Fig. 1) between 10 and 16 hours after insulin injection is difficult to explain by a mere inhibition of enzyme degradation, since the rate of enzyme induction by fasting was far slower (8).

The rise in P-enolpyruvate carboxykinase activity caused by diabetes depended on the synthesis of enzyme protein, since cycloheximide treatment reduced the activity of both the adipose and liver enzyme (Table III). At the dose used this drug was quantitatively as effective as insulin in causing a relatively fast decay in enzyme activity which was much more rapid than noted after injection of actinomycin D (t½ = 16 hours, Table V). Also, 24 hours after actinomycin D administration the level of the adipose tissue enzyme remained high compared to that found in liver (22-26). Consequently a lower output of FFA from adipose tissue would result in a diminished supply of FFA to the liver and subsequently to a lower rate of ketogenesis. The experimental evidence presented in this report clearly shows that the addition of pyruvate to adipose tissue incubated in vitro de-
increased the output of FFA. Since, as shown in Table I, the pyruvate effect was caused by increased esterification of FFA, it was anticipated that the magnitude of this effect would be determined by the capacity of the tissue to synthesize glycercide-glycerol from pyruvate. This was indeed evident since the effect of pyruvate on FFA output closely reflected the rate of glycercide-glycerol synthesis. Consequently it seems probable that in restraining FFA mobilization the physiological role of the glycerconeogenic pathway may also be antiketogenic. However, the actual amount of the FFA released in the presence of pyruvate (Table I) depends not only on glycercide-glycerol synthesis but also on lipolysis. Despite the presence of pyruvate, the output of FFA will still proceed if lipolysis exceeds the rate of glycercide-glycerol synthesis.

This is clearly seen by comparing adipose tissue from diabetic and fasted animals. Both possess a similar glycerconeogenic activity and the effect of pyruvate on FFA retention is also the same, therefore the higher output of FFA from adipose tissue from diabetic rats must reflect a higher rate of lipolysis. If such a comparison is extended to tissues from diabetic and adrenalectomized-diabetic animals we can suggest that adrenalectomy decreases the rate of lipolysis. Consequently it may be inferred that the lack of insulin increases both lipolysis and glycerconeogenesis whereas adrenalectomy superimposed upon diabetes decreases lipolysis. This would indicate that glycercide-glycerol synthesis is regulated independently from lipolysis. It should also be pointed out that although the glycerconeogenic process in adipose tissue is similar to gluconeogenesis in liver, adrenalectomy has an opposite effect on the two pathways, gluconeogenesis being reduced and glycerconeogenesis increased. This observation agrees with the changes in activity of P-enolpyruvate carboxykinase in the two tissues. The activity of the adipose tissue enzyme increases following the removal of the adrenals in fed, fasted, or diabetic animals, whereas in the liver the enzyme activity is either not affected by adrenalectomy as in the fed animal, or decreases when diabetic rats are adrenalectomized.

The fact that FFA esterification proceeds at a rapid rate in adipose tissue from fasted rats incubated without substrate indicates that some source of precursor must be available within the fat pad to provide the necessary γ-glycerophosphate. It is unlikely that glycogen, present at relatively low levels in adipose tissue from fasted rats, could provide enough γ-glycerophosphate to account for the 8.63 μmoles of FFA esterified. This finding substantiates the earlier studies of Vaughan (1) and underlines further the potentially important role of glycerconeogenesis in counteracting FFA release. This pathway may, therefore, be providing the necessary γ-glycerophosphate by converting precursors such as amino acids into triglycerides. In the present study we have employed pyruvate as a tool to measure the capacity of the glycerconeogenic sequence to affect FFA esterification. Pyruvate markedly increases the esterification of FFA by adipose tissue of fasted rats when added at a concentration of 25 mM. Although this concentration is unphysiological, the fact that it can produce a remarkably high rate of esterification of FFA in adipose tissue of fasted, but not of fed animals is consistent with our earlier studies which show a 5-fold induction of P-enolpyruvate carboxykinase during fasting. At lower pyruvate concentrations, the addition of low concentrations of butyrate (0.25 mM) also stimulates FFA re-esterification in fat pads from fasted rats. Reshef, Niv, and Shapiro (6) have reported that butyrate in low concentrations, causes a 3 to 4 fold increase in the conversion of pyruvate-2-14C to glycercide-glycerol. It is possible that butyrate oxidation by adipose tissue mitochondria shifts the oxidation-reduction potential of the mitochondrial pyridine nucleotides to a more reduced state. If so, the normally high rate of citrate formation that occurs when pyruvate is the substrate (27), may be shifted to favor malate synthesis. This mechanism would be similar to that proposed for liver (98) and would provide a common regulatory site in both tissues. Direct studies with isolated rat adipose tissue mitochondria are currently in progress to test such a hypothesis. An alternative but complementary explanation of the butyrate effect is that the fatty acid acts by stimulating pyruvate carboxylation. Unlike P-enolpyruvate carboxykinase, the activity of adipose tissue pyruvate carboxylase is not changed substantially by dietary or hormonal manipulation (8). This lack of adaptivity suggests that the regulation of oxalacetate synthesis in adipose tissue mitochondria is dependent on factors such as the availability of acetyl-CoA to activate pyruvate carboxylase.

The increase in adipose tissue P-enolpyruvate carboxykinase activity produced by a variety of treatments, may be explained by the removal of the adrenals or of insulin, both of which act as depressors of the activity of this enzyme. The independency of these depressors is inferred by the data that in diabetic-adrenalectomized rat either triamcinolone or insulin when given alone had only a partial effect in restoring the normally low activity of P-enolpyruvate carboxykinase. A maximum effect was found when both hormones were administered together. Also, the rate of enzyme depression caused by insulin (t½ = 58 hours) was considerably faster than found for triamcinolone (t½ = 96 hours) when optimal amounts of each hormone were injected (13). We note that a comparison between the depression of enzyme activity caused by actinomycin D and that caused by cycloheximide indicates a more rapid decay with cycloheximide. Thus insulin and cycloheximide cause a rapid fall in P-enolpyruvate carboxykinase activity as compared to the effects of either actinomycin D or triamcinolone. We assume, therefore, that the depression of P-enolpyruvate carboxykinase activity in adipose tissue may occur either at the level of protein synthesis as with insulin or cycloheximide, or alternatively at the level of RNA synthesis as in the case of the triamcinolone or actinomycin D. Since these two systems are additive, their independence is therefore implied. However, verification of these proposals by use of immunological techniques will be required.

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A Possible Physiological Role for Glyceroneogenesis in Rat Adipose Tissue
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