Role of carnitine in hepatic ketogenesis

[carnitine palmitoyltransferase/carnitine acyltransferase]/fatty acid oxidation/glucagon/insulin

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ABSTRACT The enhancement of long-chain fatty acid oxidation and ketogenesis in the perfused rat liver, whether induced acutely by treatment of fed animals with anti-insulin serum or glucagon, or over the longer term by starvation or the induction of alloxan diabetes, was found to be accompanied by a proportional elevation in the tissue carnitine content. Moreover, when added to the medium perfusing livers from fed rats, carnitine stimulated ketogenesis from oleic acid. The findings suggest that the increased fatty acid flux through the carnitine acyltransferase (carnitine palmitoyltransferase; palmitoyl-CoA:carnitine O-palmitoyltransferase; EC 2.3.1.21) has been brought about by glucagon excess, with or without insulin deficiency, is mediated, at least in part, by elevation in the liver carnitine concentration.

The marked enhancement in hepatic fatty acid oxidation and ketogenic capacity characteristic of states such as starvation and diabetes appears to result primarily from an increased ability of the liver to transport activated fatty acids from the cytosol into the mitochondrion (1–4), an event that is catalyzed by the carnitine acyltransferase* system of enzymes (7). Moreover, we have recently shown (4) that the initiating event responsible for this alteration in liver metabolism is almost certainly the elevation in plasma glucagon concentration that invariably occurs under circumstances of insulin deficiency (8). This follows from the observation that the low ketogenic capacity of livers from fed rats (as defined by the ability of the perfused liver to convert oleic acid into acetooacetate and β-hydroxybutyric acids) could be increased abruptly by prior treatment of the animals with anti-insulin serum or glucagon for periods as short as 1 hr (4). A major problem, however, has been the fact that neither (4, 9) nor others (10) have been able to activate ketogenesis from oleate in perfused livers from fed rats when anti-insulin serum or glucagon was included directly in the perfusion fluid. The possibility was raised, therefore, that the effects of these agents in vivo were mediated through some extrahepatic factor that acted to stimulate the carnitine acyltransferase reaction in liver (4).

On theoretical grounds it seemed reasonable to suppose that the flux of fatty acid through the β-oxidation sequence might be governed, at least in part, by the concentration of carnitine. This notion was based on two considerations. First, carnitine is a substrate for the initial step in the oxidation of fatty acids, namely the carnitine acyltransferase I reaction. Second, studies by Fritz some twenty years ago (11) established that carnitine had the capacity to stimulate fatty acid oxidation when added to incubations of rat liver slices. However, since that time no systematic study has been made in which the carnitine content of the liver has been examined in relation to the ketogenic capacity of the intact organ. The experiments to be described demonstrate that there is a remarkable correlation between these two parameters and raise the possibility that one of the effects of an elevated glucagon-to-insulin ratio in the blood is to "turn on" hepatic ketogenic capacity via an increase in the carnitine concentration of the liver.

MATERIALS AND METHODS

Animals and Treatment. Male Sprague-Dawley rats weighing between 100 and 130 g were used in all experiments. They were fed a diet containing 58.5% sucrose, 21% casein and less than 1% fat, by weight, together with all necessary vitamins and minerals (Teklad, Madison, Wisc.) and were used for experiments between 7:00 and 8:00 a.m. Fasted rats were deprived of food for 24 hr before use. Alloxan diabetes was induced by the intravenous injection of alloxan monohydrate at a dose of 70 mg/kg of body weight. Experiments with these animals were carried out 42 hr after administration of the drug, at which time values for plasma glucose and ketone bodies (acetacetate plus β-hydroxybutyrate) were generally in the region of 50 and 20 mM, respectively.

In some experiments animals were lightly anesthetized with ether, fitted with a femoral vein catheter, and placed in individual restraining cages until they had recovered from the anesthesia. They then received 100 units of heparin intravenously, followed by an infusion of guinea pig serum containing insulin antibody (neutralizing capacity 1.6 units/ml) or glucagon (100 μg/ml) at a rate of 200 μl/min for 5 min followed by 10 μl/min until the 3 hr time point (4). Except as indicated animals were anesthetized with pentobarbital prior to further manipulations. Tissues were rapidly frozen in liquid nitrogen and analyzed for carnitine and its derivatives as described below.

Liver Perfusion. Livers were perfused in a recirculating or nonrecirculating fashion with medium containing 0.7 mM oleic acid using the apparatus and techniques described elsewhere (1, 2).

Analytical Procedures. Carnitine and its short-chain acyl derivatives were extracted from tissues with a slight modification of the method described by Behmer (12). The frozen tissue (generally 2 g), together with a trace quantity of (D,L)-[methyl-14C]carnitine, was extracted with 20 ml of chloroform:methanol (1:1, vol/vol) in a ground-glass, motor-driven homogenizer. After centrifugation to remove denatured material the supernatant fluid was shaken with 5 ml of water. Following removal of the upper aqueous layer the lower phase was washed with 5 ml of Folch's theoretical

* Unless otherwise specified the general term "carnitine acyltransferase" will be used to denote the coupled enzyme system comprising carnitine acyltransferase I and II. According to current concepts the I is believed to catalyze the conversion of long chain acyl-CoA and carnitine into acylcarnitine and CoASH on the outer aspect of the inner mitochondrial membrane. This is followed by the reverse reaction which utilizes an intramitochondrial pool of CoASH and is catalyzed by transferase II on the inner aspect of the inner mitochondrial membrane (5, 6). The Enzyme Commission has recommended the name carnitine palmitoyltransferase and assigned the number 2.3.1.21.
Table 1. Effect of various treatments on the carnitine content of rat liver

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Treatment</th>
<th>Liver wt. g</th>
<th>Free carnitine*</th>
<th>Total carnitine†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>nmol/total liver</td>
<td>nmol/g liver</td>
</tr>
<tr>
<td>I</td>
<td>Fed (6)</td>
<td>—</td>
<td>64 ± 7</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Fasted (6)</td>
<td>—</td>
<td>220 ± 17</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Alloxan diabetic (6)</td>
<td>—</td>
<td>355 ± 33</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Fed, AIS 3 hr (3)</td>
<td>—</td>
<td>130 ± 13</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Fed, glucagon 3 hr (6)</td>
<td>—</td>
<td>130 ± 13</td>
<td>—</td>
</tr>
<tr>
<td>II</td>
<td>Fed (6)</td>
<td>5.99 ± 0.32</td>
<td>374 ± 60</td>
<td>61 ± 8</td>
</tr>
<tr>
<td></td>
<td>Fasted (6)</td>
<td>3.67 ± 0.15</td>
<td>721 ± 76</td>
<td>198 ± 22</td>
</tr>
<tr>
<td></td>
<td>Alloxan diabetic (7)</td>
<td>4.32 ± 0.15</td>
<td>1471 ± 61</td>
<td>343 ± 19</td>
</tr>
</tbody>
</table>

In Exp. I carnitine determinations were made after extraction of 2 g of liver as described under Materials and Methods. In Exp. II the entire liver was processed. Values refer to means ± SEM for the number of animals shown in parentheses.

* Based on assay for carnitine before hydrolysis of samples with KOH (3).
† Based on assay for carnitine after hydrolysis of samples with KOH (3).
‡ The abbreviation "AIS" refers to anti-insulin serum.

"upper phase" (13). The combined polar phases were evaporated to dryness under air and the residue was dissolved in 1-2 ml of water. Aliquots of the final extract were assayed for free carnitine, esterified carnitine, and acetylcarnitine as described previously (3). Basically the assay for carnitine utilizes the coupling of acetylcarnitine transferase, citrate cleavage enzyme, and malate dehydrogenase such that a stoichiometric relationship exists between the quantity of carnitine present and the disappearance of NADH as detected spectrophotometrically at 340 nm. This procedure circumvents the frequent blank values encountered in the more conventional colorimetric assay (14). Values were corrected for loss of material on the basis of the quantity of 14C recovered, which was generally in the region of 85-90%.

All other analytical procedures have been described in previous reports (1-4). The term "ketone bodies" refers to the sum of acetoacetate and beta-hydroxybutyrate.

Materials. Guinea pig serum containing antibody to pork insulin was kindly provided by Dr. P. H. Wright, Indianapolis, Ind. Glucagon was from Sigma Chemical Co., St. Louis, Mo.

RESULTS

The first series of experiments was designed to test the effects of a variety of experimental manipulations, known from previous studies to produce marked increases in hepatic ketogenic capacity, on the concentration of carnitine and its derivatives in rat liver. Table 1, Exp. I, shows that all four treatments, namely, infusion of anti-insulin serum or glucagon for 3 hr, starvation for 24 hr, and the induction of severe diabetic ketoacidosis with alloxan, resulted in pronounced elevations in hepatic carnitine levels when expressed on a per gram wet weight basis. Since liver mass is known to be altered by fasting and diabetes, a similar experiment (II) was carried out in which the entire liver was extracted and analyzed for carnitine content. Two points should be emphasized. First, despite the reduction in liver weight seen in the ketotic groups, total tissue carnitine levels were approximately doubled after 24 hr of starvation and quadrupled in the alloxan diabetic animals. Second, carnitine values expressed per g of tissue were almost identical with those of Exp. I, lending confidence to the reproducibility of the techniques employed. Direct assay of the tissue extracts for acetylcarnitine revealed that in most instances this compound accounted for the bulk of the esterified carnitine (data not shown). The striking effect of alloxan treatment seen here is consistent with observations made by Snoswell and Koundakjian in sheep (15).

To determine the correlation between rates of ketone synthesis and carnitine content in individual livers, rats were treated in the same way as those referred to in Table 1. Livers were then perfused with oleic acid to measure ketone body production and at the end of the experiment carnitine levels were determined. A striking correlation was noted between the two variables (Table 2), the significance of which is better appreciated when the rate of ketone production is plotted against the tissue concentration of free or total carnitine for each liver tested (Fig. 1). While the carnitine concentrations shown are based on total tissue measurements and, as such, give no indication as to its possible intracellular compartmentation, it is nevertheless of interest that they fall in a range that is generally below the Km value for carnitine (0.45 mM) reported by Kopec and Fritz (16) for purified carnitine palmitoyl transferase.

Since all of our previous attempts to activate the ketogenic machinery of the liver in vivo had failed it was of interest, in light of these new observations, to test the effect of carnitine when added to the medium perfusing livers from fed rats. To this end, livers were perfused in nonrecirculating fashion with 0.7 mM oleic acid and the quantity of acetoacetate and beta-hydroxybutyrate in the effluent medium was determined every 5 min. As shown in Fig. 2B, ketogenesis in the fed livers was characteristically low compared with the rate seen in the fasted group. However, immediately upon the infusion of carnitine, ketone production by the livers from fed animals began to accelerate such that by the 70-min time point the rate had increased approximately 2.5-fold over that seen without the addition of carnitine. This ef-

‡ Table 1 shows no overlap between free carnitine levels in the fed versus treated groups. After perfusion (Fig. 1) the distribution of free and total carnitine is altered as a result of the high rates of fatty acid oxidation in the fasted and hormonally manipulated groups. Consequently the free carnitine levels in the fed livers (Fig. 1A) appear to be disproportionately high for the observed rate of ketogenesis.
and analyzed. Each liver was then perfused with recirculating medium containing 0.7 mM oleic acid to determine rates of ketogenesis and were subsequently analyzed for their content of free and total carnitine. The best fit line for the regression was determined by the method of least squares.

The effect of carnitine appeared to be independent of its concentration in the perfusion medium over the range tested (0.5-3.0 mM). In experiments not shown, the high rate of ketone production in livers from fasted animals was not further enhanced by the infusion of carnitine. That the carnitine-stimulated rate of ketogenesis observed in the fed group resulted from enhanced β-oxidation of fatty acids was indicated by the simultaneous increase in the ratio of β-hydroxybutyrate to acetoacetate leaving the liver (Fig. 2A). In experiments utilizing [1-14C]oleic acid as substrate the specific activity of the ketone bodies formed was unchanged after the infusion of carnitine, indicating that the oxidation of both endogenous and added fatty acids was stimulated to a comparable extent (data not shown).

**DISCUSSION**

The evidence that the primary control site for the regulation of hepatic ketogenesis resides at the carnitine acyltransferase reaction is now quite persuasive. Furthermore, it is clear that activation-inactivation of the sequence can occur rapidly. In the rat as little as six hours of fasting will initiate the process (2) and the same change can be produced in only one hour with glucagon or anti-insulin serum (4). However, neither agent is effective in vitro, suggesting that they act not directly but through some chemical mediator produced in vivo. The thought that carnitine might be the putative mediator was intriguing for several reasons. First, carnitine is a substrate for the initial reaction specific for β-oxidation of fatty acids. second, there is ample precedent for interactions between peripheral tissues and liver under circumstances of insulin deficiency and glucagon excess; e.g., muscle amino acids are known to be mobilized for gluconeogenic purposes and adipose tissue fatty acids are the substrate for acetoacetate and β-hydroxybutyrate synthesis. A similar situation involving mobilization of carnitine to liver seemed entirely possible (7). Third, Lundsgaard had shown that when muscle and liver were linked in a perfusion system oxygen uptake by the latter was stimulated (17). Finally, Fritz had demonstrated that of many muscle constituents tested only carnitine stimulated fatty acid oxidation in liver slices (11).

The results of the present study show clearly that ketotic states in the rat are accompanied by an increased carnitine content in liver and that the ketogenic capacity of hepatic tissue correlates directly with the carnitine concentration. Furthermore, carnitine alone, of all the many compounds we have tested, has the capacity to activate directly the ketogenic machinery in the isolated perfused liver taken from nonketotic (fed) rats.

The mechanism by which the carnitine concentration of the liver is increased by elevation of plasma glucagon (with or without insulin deficiency (4)) is not known. Carnitine concentration in skeletal muscle was not found to be decreased in the ketotic states studied here (data not shown). However, carnitine concentrations are high in this tissue (18, 19) and, since the muscle mass is large, relatively small changes might be sufficient to produce the increase seen in

**Table 2. Relationship between ketogenic capacity and carnitine content in the perfused rat liver**

| Treatment          | Ketone production from oleate, µmol/100 g body wt per 30 min | Free carnitine nmol/g wet wt of liver | Total carnitine carnitine | risk | 4.42 | 0.000  \\
|--------------------|---------------------------------------------------------------|-------------------------------------|---------------------------|-----|------|-------- \\
| Fed (8)            | 26 ± 3                                                       | 40 ± 5                              | 102 ± 10                  | 0.05| 0.02| 0.003  \\
| Fed, glucagon 3 hr (6) | 87 ± 5                                                      | 68 ± 8                              | 220 ± 13                  | 0.05| 0.02| 0.003  \\
| Fasted (6)         | 118 ± 8                                                     | 70 ± 8                              | 228 ± 13                  | 0.05| 0.02| 0.003  \\
| Alloxan diabetic (6) | 192 ± 10                                                   | 172 ± 12                            | 416 ± 6                   | 0.05| 0.02| 0.003  \\

Animals were treated as described under Materials and Methods. Each liver was then perfused with recirculating medium containing 0.7 mM oleic acid to determine its rate of ketogenesis, after which a portion of the tissue was rapidly frozen in liquid nitrogen and analyzed for its carnitine content.
liver. Also, the hepatic uptake of carnitine might be increased by glucagon in an analogous fashion to the situation with amino acids (20). A third possibility would be that hepatic synthesis of carnitine is increased in ketotic states, perhaps as a consequence of the mobilization of a precursor from peripheral tissues. Regardless of the mechanism, it seems reasonable to conclude that the sharp increase in liver carnitine concentration caused by elevation in the blood glucagon level represents a central feature of the role of this hormone in activating the liver's ketogenic machinery.

Finally, while the present studies have focused on the importance of carnitine acyltransferase I in the regulation of hepatic fatty acid oxidation, additional evidence exists that implicates carnitine acyltransferase II as an equally important site in the overall control of ketogenesis. This conclusion is based on the fact that whether induced acutely with insulin antibodies or glucagon, or over the longer term by starvation, the resultant acceleration in ketogenesis from long chain fatty acids is accompanied by a proportional stimulation in the oxidation of (−)-octanoylcarnitine (3) while free octanoic acid is oxidized at similar rates in livers from all experimental groups (21). It thus appears likely that overall activation of long-chain fatty acid oxidation in liver requires that both transferase I and transferase II be activated simultaneously. How these two events are coordinated remains an intriguing question.

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