Regulation of Carnitine Palmitoyltransferase by Insulin Results in Decreased Activity and Decreased Apparent $K_i$ Values for Malonyl-CoA*

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Administration to normal rats of 100 mg of streptozotocin/kg body weight produced ketotic diabetic rats in which the affinity of carnitine palmitoyltransferase for malonyl-CoA was decreased by 10-fold and its activity was increased by 30%, but the injection of insulin brought the affinity and the activity back to normal within 4 h. Administration of 60 mg of streptozotocin/kg produced non-ketotic diabetic rats and caused a less substantial change in the affinity of carnitine palmitoyltransferase for malonyl-CoA. In the BB Wistar diabetic rat, the onset of diabetes also increased the activity of carnitine palmitoyltransferase and decreased its affinity for malonyl-CoA. Injection of insulin brought both of these values back to normal within 2 h. The total activity of mitochondrial carnitine palmitoyltransferase (outer + inner activities) was 40% greater in the BB Wistar diabetic rat, but treatment with insulin did not decrease the total activity to normal values within 2 h. The elevated activity and decreased affinity for malonyl-CoA found in fasting rats did not respond to short-term insulin treatment. The evaluation of a previous report that cycloheximide blocks the effects of starvation indicated that cycloheximide did not act by inhibiting protein synthesis, but produced its effect by preventing gastric emptying. Current data suggest that diabetes increases the activity of carnitine palmitoyltransferase and greatly diminishes the affinity of the enzyme for malonyl-CoA and that the severity of diabetes is associated with differences in the affinity of the enzyme for its inhibitor. Insulin acts on the outer carnitine palmitoyltransferase to reverse these effects very rapidly, but diabetes produces some change in the total activity that is not reversed by short-term treatment with insulin.

Carnitine palmitoyltransferase (EC 2.3.1.21) is a membrane-bound enzyme whose activity in rat liver is expressed in two locations, the mitochondrial matrix and the mitochondrial intermembrane space, i.e. activity is expressed both inside and the mitochondrial inner membrane to which the enzyme is bound (1, 2). The outer activity of this enzyme plays a key role in the regulation of hepatic fatty acid oxidation, being located at the point in the pathway immediately preceding transport into the mitochondrial matrix for oxidation by the enzymes of $\beta$-oxidation (1, 2). A very important aspect of regulation of hepatic fatty acid oxidation by the outer carnitine palmitoyltransferase is its inhibition by physiological concentrations of malonyl-CoA, which is an intermediate in the biosynthesis of fatty acids (see Ref. 1 for a review). The expressed activity of this enzyme in livers of fed rats is not low enough to be rate-controlling in the absence of malonyl-CoA, but the presence of malonyl-CoA confines a rate-controlling ability (3). Another equally important aspect in the control of fatty acid oxidation by carnitine palmitoyltransferase is the change in the sensitivity of the enzyme to inhibition by malonyl-CoA (3-6). The change in sensitivity results from a change in the apparent $K_i$ value for malonyl-CoA which occurs during the feeding-fasting transition (7). Because malonyl-CoA is a competitive inhibitor of carnitine palmitoyltransferase, its $K_i$ value is actually the reciprocal of the binding constant of the enzyme for its inhibitor, and thus the $K_i$ value is a measure of the affinity of carnitine palmitoyltransferase for malonyl-CoA (7), i.e. the smaller the $K_i$ value, the greater the affinity of the enzyme for malonyl-CoA.

A third important aspect of the regulation of carnitine palmitoyltransferase is that malonyl-CoA apparently induces cooperativity in carnitine palmitoyltransferase so that inhibition by the very low concentrations of malonyl-CoA found in vivo produce inhibition very effectively in the fed animal, but very large differences in the $K_i$ values between fed and fasting animals cause little cooperativity to be demonstrated in the fasting animal (7). The cooperative nature of inhibition thus conveys a type of amplification system to the control of fatty acid oxidation so that the difference in inhibition of carnitine palmitoyltransferase by malonyl-CoA between fasting and fed animals is even more exaggerated.

In a recent communication (8), we reported that a change in the affinity of carnitine palmitoyltransferase for malonyl-CoA also occurs with the onset of streptozotocin-induced diabetes and that treatment of diabetic rats with insulin over a period of 5 days was able to reverse the effects of diabetes on the enzyme. The purpose of the studies reported here was to determine whether diabetes and subsequent insulin treatment had other effects on carnitine palmitoyltransferase, such as changing its activity. It has been reported previously (9) that diabetes increases the activity of carnitine palmitoyltransferase, so we have examined the effects of insulin on activity as well as the affinity for malonyl-CoA. We also wanted to examine the similarity of changes in carnitine palmitoyltransferase that occur at the onset of diabetes with those occurring during fasting and to ascertain whether insulin had similar effects in the diabetic and the fasting animal.

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It has been previously reported that the effects of fasting on carnitine palmitoyltransferase can be blocked by cycloheximide, suggesting that a different form of the enzyme, one that is not sensitive to the inhibitory effects of malonyl-CoA, is synthesized during fasting (10). Since our previous data had suggested the presence of multiple forms of carnitine palmitoyltransferase (8), we have also examined this possibility in the studies reported here.

EXPERIMENTAL PROCEDURES

Animal Care—Male Wistar rats weighing 180–250 g were fed ad libitum (Purina Lab Chow, Ralston Purina Co., Richmond, IN) or fasted for 24–72 h and were allowed free access to water. Ketotic diabetic rats were produced by injecting rats with 100 mg of streptozotocin/kg of body weight into the tail vein (11). Two other groups of diabetic rats (not having ketone bodies in their urine) were produced by injecting 60 mg/kg of streptozotocin either intravenously (tail vein) or subcutaneously. Streptozotocin was injected immediately after dissolving the drug in citrate buffer, pH 7.2. Animals injected with 100 mg/kg of streptozotocin were used for the isolation of mitochondria 48 h after injection. Animals injected with 60 mg/kg of streptozotocin were used 7 days after injection. Only animals having urine ketone bodies ≥80 ng/dl (determined with Bill-Labstix; Miles Laboratories, Elkhart, IN) were considered diabetic. The final group of diabetic rats were the BB Wistar genetically diabetic rats. A portion of each litter of these rats became diabetic within the first 60 days of life; other individuals in each litter never became diabetic. Both the diabetic and nondiabetic littermates were used in these studies, and care was taken to compare experiments with the diabetic BB Wistar rats only with their nondiabetic littermates and not with the control rats of the normal Wistar strain. Diabetic BB Wistar rats also were not used unless they had blood ketone bodies ≥80 mg/dl. These rats were maintained on Lilly NPH insulin at ~1 unit/day until 48 h before use for experiment. The nondiabetic BB Wistar rats had no urine ketone bodies or glucose. Stomachs and intestines were checked to be certain that fed or diabetic rats were well fed and that fasting animals had not inadvertently received food.

Insulin Treatment of Animals—One group of animals was treated with 3 units of proamine/zinc insulin (Lilly) daily for 5 days before use in experiments. In other experiments animals were injected with 4 units of Regular Insulin for Injection (Lilly) at short time periods (0.5, 1, 2, or 4 h) before isolation of mitochondria.

Isolation of Mitochondria and Assay of Carnitine Palmitoyltransferase—Mitochondria were isolated from rat livers by the method of Johnson and Lardy (12) with the modifications previously published (7). The final mitochondrial pellet was resuspended to a concentration of 10 mg/ml in 0.25 M sucrose, 1 mM EDTA, 3 mM Tris, pH 7.2. Protein was determined by a biuret procedure (13). Respiratory control ratios (14) were 5 or greater for all preparations used when determined with 10 mM glutamate and 0.5 mM malate. The outer carnitine palmitoyltransferase was assayed as described previously (8). Each assay contained, in a total volume of 1 ml, 82 mM sucrose, 70 mM KCl, 35 mM Hepes, 1 mM imidazole, 5 mM reduced glutathione, 2 mg of bovine serum albumin, 0.5 mM L-carnitine (0.4 μCi of L-[methyl-14C]carnitine), 1 μg of antimony A, 3 mM ATP, and 2 mM MgCl2, and palmitoyl-CoA or oleoyl-CoA and malonyl-CoA at the concentrations indicated. Because of problems in obtaining oleoyl-CoA from our supplier we have used palmitoyl-CoA in several experiments. We have examined the effects of both coenzyme-A esters and have found no difference in the activity of carnitine palmitoyltransferase as observed in the inhibition caused by malonyl-CoA at concentrations tested (up to 250 μM). ATP and magnesium were added to the assay because of a previous report that acyl-CoA concentrations were maintained better in their presence (15). Reactions were carried out at pH 7 and 30°C for 5 min following a 5-min preincubation of the presence of all components except carnitine, which was added to initiate the reaction. K, values were determined by the graphical method of Dixon (16) using three concentrations of palmitoyl-CoA or oleoyl-CoA and 6 concentrations of malonyl-CoA. K, values derived from the curve plots obtained from fed and insulin-treated animals were determined by drawing a tangent to the curve at the point where the curve crossed the ordinate. Total carnitine palmitoyltransferase activity (inner + outer activity) was determined by mixing equal volumes of 1% Triton X-100 and final mitochondrial suspensions after freezing, and then sonicating for 30 s in a Branson ultrasonic cleaning bath, giving a final Triton X-100 concentration of 0.5% and a final mitochondrial concentration of 5 mg of protein/ml of medium. Assays were performed under the same conditions as for the outer carnitine palmitoyltransferase with the exceptions that 0.1 μg of protein was used in each assay, and the final concentrations of carnitine and palmitoyl-CoA were 2.5 mM and 150 μM, respectively. These concentrations were determined to be optimal for measuring the total activity of carnitine palmitoyltransferase even though they were much higher than the concentrations that were optimal for the assay of the outer enzyme (0.5 mM carnitine and 80–100 μM palmitoyl-CoA). These observations indicate that great care must be taken to use optimal conditions for comparing activities in intact mitochondria and in sonicated mitochondria.

Cycloheximide Treatment of Rats—Cycloheximide was dissolved in 0.15 M NaCl. Normal fed rats were injected intravenously with 2 mg of cycloheximide/kg body weight. Control rats were injected with an equal volume of 0.15 M NaCl. Food was removed from rats for 24 h, and mitochondria were isolated for the assay of the outer carnitine palmitoyltransferase at the end of the 24-h period of fasting.

Materials—Malonyl-CoA, oleoyl-CoA, palmitoyl-CoA, reduced glutathione, imidazole, Hepes, L-carnitine hydrochloride, EDTA, ATP, cycloheximide, and essentially fatty acid-free bovine serum albumin were purchased from Sigma. L-[methyl-14C]Carnitine hydrochloride was obtained from Amersham Corp. Normal rats were obtained from Harlan Industries (Indianapolis, IN). BB Wistar spontaneously diabetic rats were provided by Dr. Solomon S. Solomon of the University of Tennessee, Memphis and the Veterans Administration.

RESULTS

Data from our recent communication indicated that streptozotocin-induced diabetes diminished the affinity of carnitine palmitoyltransferase for malonyl-CoA and apparently abolished the cooperativity seen in the normal fed state (8) just as fasting does (6). Those data further indicated that long-term treatment (5 days) of rats with a heavy dose of insulin was able to reverse the effects of diabetes on changes in affinity of enzyme for malonyl-CoA and also to restore the cooperative nature of inhibition by malonyl-CoA. Fig. 1 illustrates further data from a study identical to the initial study in which we have examined the activity of carnitine palmitoyltransferase in each of the original groups of animals at various concentrations of oleoyl-CoA. Streptozotocin diabetes caused an increase of ~30% in the activity of carnitine palmitoyltransferase (measured at 100 μM oleoyl-CoA). The 5-day treatment with insulin decreased the elevated activity of the streptozotocin diabetic animal by ~40% so that the insulin-treated rats had significantly lower activity (at 100 μM oleoyl-CoA) than either the diabetic rats or the normal control rats.

In an attempt to determine whether the severity of diabetes (ketotic versus non-ketotic) was related to changes in the characteristics of carnitine palmitoyltransferase, we injected rats with 60 mg of streptozotocin/kg of body weight rather than 100 mg/kg, since it was previously reported that differences in rates of ketone body formation had been found with differences in the dose of streptozotocin (11). The data of Fig. 2 indicate that all of the groups receiving the lower dose of streptozotocin responded identically to inhibition by malonyl-CoA, indicating that the manner of administration of streptozotocin had no effect on response and that the time from injection to isolation of mitochondria was not critical.

Animals receiving 100 mg of streptozotocin/kg body weight had approximately the same enzyme activity as those receiving 60 mg/kg, but the apparent K, for malonyl-CoA was increased to a much greater extent in animals receiving the higher dose (Table I). None of the animals receiving the lower dose of streptozotocin were found to have measurable ketone bodies in their urine (limit of detection = 5 mg/dl), but we rarely found an animal that had been injected with the higher

1 The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
dose of streptozotocin that had less than 80 mg/dl of ketone bodies in the urine, indicating that the higher dose produced a severely ketotic diabetic animal while the lower dose did not. We have observed that animals treated with the higher dose of streptozotocin do not live more than 3 days, but animals given the lower dose will live much longer (all animals were used within 2 weeks).

Fig. 3 illustrates the rapid effect of insulin on the activity of hepatic carnitine palmitoyltransferase in streptozotocin diabetic rats. Activity was depressed by insulin at both 30 min and 4 h. The apparent $K_i$ for malonyl-CoA was also decreased rapidly by insulin, apparently being completed within 4 h (8). As we have noted previously, however, the cooperativity of inhibition was not evident within the first 4 h after insulin treatment (8).

In order to evaluate more thoroughly the changes occurring in the kinetic characteristics of carnitine palmitoyltransferase as a result of the diabetic state, we have used the BB Wistar strain of rats in further studies. The use of the BB Wistar strain circumvents questions regarding the possibility of toxic side effects that might arise with the use of streptozotocin-induced diabetic animals. BB Wistar rats in our colony became diabetic spontaneously at ~50–60 days of age, but only about two-thirds of each litter became diabetic. This result is typical of the genetic background of the BB Wistar rat (17) and, because all animals do not become diabetic, nondiabetic rats of the same litter are available as control animals.

BB Wistar rats that did not become diabetic appeared to have a normal carnitine palmitoyltransferase (Fig. 4A). The
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FIG. 4. Activity of the outer carnitine palmitoyltransferase and inhibition by malonyl-CoA in nondiabetic BB Wistar rats (panel A), diabetic BB Wistar rats (panel B), and diabetic BB Wistar rats injected with insulin (panel C). Assays were conducted as described under “Experimental Procedures” in the absence (O) or the presence (●) of 10 μM malonyl-CoA. Points are means ± S.E. (n = 4). Insulin-treated diabetic rats were injected intraperitoneally with 3 units of insulin and were used 2 h after injection.

TABLE II
Characteristics of carnitine palmitoyltransferase in diabetic and nondiabetic BB Wistar rats and the effects of insulin treatment
Mitochondria were isolated from diabetic or nondiabetic BB Wistar rats or from diabetic BB Wistar rats that had been injected 2 h previously with 3 units of Regular Insulin. The outer carnitine palmitoyltransferase activity, the total activity (outer + inner), and the apparent K_i were measured as described under “Experimental Procedures.” Data are presented as means ± S.E. (n = 4 for nondiabetic and insulin-treated diabetic rats; n = 6 for diabetic rats).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apparent K_i</th>
<th>Outer activity</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondiabetic</td>
<td>0.6 ± 0.06</td>
<td>7.6 ± 0.5</td>
<td>36 ± 1</td>
</tr>
<tr>
<td>Diabetic</td>
<td>4.1 ± 0.3^a</td>
<td>13.3 ± 0.6^a</td>
<td>50 ± 2^a</td>
</tr>
<tr>
<td>Diabetic + insulin</td>
<td>1.5 ± 0.2^a</td>
<td>8.0 ± 1.1</td>
<td>46 ± 5^a</td>
</tr>
</tbody>
</table>

*Significantly different from nondiabetic control group (p < 0.05, Student’s t test).

Palmitoyl-CoA saturation curve was very similar to the curve previously published for normal, fed Wistar rats (7), except, of course, for differences in acyl-CoA concentrations that resulted from the different albumin concentrations used in the two studies. Also, inhibition by malonyl-CoA demonstrated the very strong cooperativity that was seen previously with the normal, fed Wistar rat. BB Wistar diabetic rats, however, demonstrated palmitoyl-CoA saturation curves that were very similar to the normal fasting Wistar rats with increased activity and decreased sensitivity to inhibition by malonyl-CoA (Fig. 4B). Insulin treatment apparently reversed, within 2 h, the changes caused by the onset of diabetes (Fig. 4C), decreasing the activity of carnitine palmitoyltransferase and increasing the sensitivity to inhibition by malonyl-CoA.

Data presented in Table II indicate that the outer mitochondrial carnitine palmitoyltransferase in the BB Wistar diabetic rats had apparent K_i values for malonyl-CoA that were 7 times greater than in the nondiabetic littersmates. Insulin treatment for 2 h decreased the apparent K_i for malonyl-CoA almost to the values found in the nondiabetic rats. The activity of the outer enzyme measured at 100 μM palmitoyl-CoA was increased by 75% by the diabetic state and was returned to normal by insulin treatment. The outer carnitine palmitoyltransferase activity was about one-fifth of the total enzyme activity measured in disrupted mitochondria. This was somewhat less than the percentage reported by

Bremer (5) for normal Wistar rats. The total enzyme activity was increased by 40% in the diabetic rats, but even though the outer activity of diabetic rats was returned to normal by insulin, the total activity was not significantly decreased (p > .05, Students t test).

Fasting caused a large increase in the apparent K_i of carnitine palmitoyltransferase for malonyl-CoA which seemed to be even more elevated after 3 days of fasting than after 1 or 2 days (Fig. 5). There is also an increase in the outer activity of carnitine palmitoyltransferase during fasting (7). It has been reported recently that cycloheximide can block the changes in carnitine palmitoyltransferase that occur during fasting (10). The effects of cycloheximide were attributed to an inhibition of protein synthesis, suggesting that fasting induces the synthesis of a form of carnitine palmitoyltransferase that is not inhibited by malonyl-CoA (10). Because some of our previous results had also suggested the presence of two forms of the outer carnitine palmitoyltransferase, we reinvestigated the effects of cycloheximide. We found that 24 h of fasting in the cycloheximide-injected rats caused no change in either the activity of carnitine palmitoyltransferase or its affinity for malonyl-CoA (results not shown), whereas the control rats showed changes identical to those previously seen with fasting (7). We found also that the effect of cycloheximide was probably not caused by an inhibition of protein synthesis. We found that all of the animals injected with cycloheximide had abundant stomach content, even though they had been fasting for 24 h, while the stomachs and intestines of the saline-injected, fasting rats were entirely empty. Our results indicate that the actual effect of cycloheximide is to inhibit gastric emptying. This means that even though the animals were not eating, food was released so slowly from the stomach that the animals remained in the fed state. This effect has been reported previously for several protein synthesis inhibitors (18–20).

In the normal, 72-h-fasting rat, insulin had no effect on the activity of the outer carnitine palmitoyltransferase (Fig. 6), and the apparent K_i values were identical. These experiments were repeated using 24-h-fasting rats with similar results (results not shown).
DISCUSSION

In previous work on the effects of diabetes on carnitine palmitoyltransferase, we examined only the changes in the affinity of the enzyme for malonyl-CoA, but here we have studied other effects of diabetes on the enzyme such as the outer activity as expressed in intact mitochondria and the total activity recoverable in disrupted mitochondria. The outer activity of carnitine palmitoyltransferase increased 30-70% in the diabetic models we have studied, similar to the increase in activity occurring in the fasting rat (5,7). Insulin treatment in the diabetic models we tested brought the outer activity back to normal very rapidly. The total activity of the enzyme was increased by diabetes by a very similar percentage (40%). Our results for the increase of the total activity caused by diabetes are in contrast to those of Bremer (5), who reported a 55% increase in the outer activity during fasting but the total activity was not significantly increased. The results of Bremer (5) led him to postulate that the total activity was not changed, but that there had possibly been a transfer during fasting of carnitine palmitoyltransferase activity from the inside of the mitochondria to the outside. Our results suggest that the inner and outer activities increase in concert during diabetes. However, short-term insulin treatment decreased to normal only the outer activity; the total activity was only partially decreased, suggesting that differences may exist in the regulation of the outer and inner carnitine palmitoyltransferase activities.

We have previously described the curved nature of Dixon plots for inhibition of carnitine palmitoyltransferase by malonyl-CoA and the sigmoid nature of v versus S plots in the presence of malonyl-CoA to the induction of cooperativity by malonyl-CoA. This would imply that there are at least two binding domains in the native enzyme, either on the same or on different polypeptide chains. Although we did not see sigmoid behavior in v versus S plots in the absence of malonyl-CoA (7), such behavior has been reported with hepatic carnitine palmitoyltransferase (6). The results of these investigators would suggest that there are multiple binding domains for the acyl-CoA substrate. The differences in our data may be explained by differences in assay conditions since we obtain sigmoid curves in the absence of malonyl-CoA when we use the exact conditions used by these other investigators (results not shown). One difference in our assay conditions is the concentration of albumin used. On theoretical grounds, it has been suggested that the binding of acyl-CoA to albumin and the substrate inhibition caused by acyl-CoA prevents us from obtaining meaningful data on the $K_m$ for acyl-CoA.

A previous report that cycloheximide prevented the changes in malonyl-CoA inhibition of carnitine palmitoyltransferase that occur during fasting suggested that regulation of protein synthesis may be involved in the fasting-feeding effects (10). Our finding that the effects of cycloheximide are due to its effects in inhibiting gastric emptying does not rule out the possibility that protein synthesis may be involved somehow in the regulation of carnitine palmitoyltransferase, but only serves to point out that when working with carnitine palmitoyltransferase the investigator must always observe very carefully the nutritional state of the animals used. Our observations on changes occurring in activity of this enzyme and its affinity for malonyl-CoA might be explained either by regulation of protein synthesis or a modification of the existing enzyme, although the latter possibility seems more likely.

The data currently available on the changes in carnitine palmitoyltransferase that occur during fasting or with the onset of the diabetic state indicate that the most quantitatively significant change is in the affinity of the enzyme for its physiological inhibitor, malonyl-CoA, but changes in the hepatic content of malonyl-CoA, changes in the inner and outer activity of the enzyme, and changes in cooperativity may all contribute to an amplification mechanism for regulation of fatty acid oxidation in these different physiological and pathophysiological states.

Acknowledgment—Grateful acknowledgment is due to Jeanine Weskey, who provided excellent technical assistance on this project.

Note Added in Proof — The colony of spontaneously diabetic rats at the University of Tennessee, Memphis, which was a source of animals used in this paper has recently been officially designated BB/WorUmm by the Institute of Laboratory Animals of the National Science Foundation. The designation indicates the source of the substrain (Umm for the University of Tennessee, Memphis, and Wor for the University of Massachusetts at Worcester) of the original colony of spontaneously diabetic rats (BB for the BioBreeding Laboratories of Ottawa, Canada). This colony was developed and maintained by funds provided in part by the Tennessee Affiliate and the Memphis Chapter of the American Diabetes Association.

REFERENCES

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