Insulin regulates enzyme activity, malonyl-CoA sensitivity and mRNA abundance of hepatic carnitine palmitoyltransferase-I

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The regulation of hepatic mitochondrial carnitine palmitoyltransferase-I (CPT-I) was studied in rats during starvation and insulin-dependent diabetes and in rat H4IIE cells. The $V_{max.}$ for CPT-I in hepatic mitochondrial outer membranes isolated from starved and diabetic rats increased 2- and 3-fold respectively over fed control values with no change in K_m values for substrates. Regulation of malonyl-CoA sensitivity of CPT-I in isolated mitochondrial outer membranes was indicated by an 8-fold increase in K_i during starvation and by a 50-fold increase in K_i in the diabetic state. Peroxisomal and microsomal CPT also had decreased sensitivity to inhibition by malonyl-CoA during starvation. CPT-I mRNA abundance was 7.5 times greater in livers of 48-h-starved rats and 14.6 times greater in livers of insulin-dependent diabetic rats compared with livers of fed rats. In H4IIE cells, insulin increased CPT-I sensitivity to inhibition by malonyl-CoA in 4 h, and sensitivity continued to increase up to 24 h after insulin addition. CPT-I mRNA levels in H4IIE cells were decreased by insulin after 4 h and continued to decrease so that at 24 h there was a 10-fold difference. The half-life of CPT-I mRNA was 4 h in the presence of actinomycin D or with actinomycin D plus insulin. These results suggest that insulin regulates CPT-I by inhibiting transcription of the CPT-I gene.

INTRODUCTION

It is generally believed that carnitine palmitoyltransferase-I (CPT-I) is the most important enzyme for controlling the availability of long-chain acyl-CoAs for hepatic mitochondrial β-oxidation [1–3]. Hepatic mitochondria contain two CPT activities. CPT-I is located in the mitochondrial outer membrane and is inhibited by malonyl-CoA [4]. Carnitine palmitoyltransferase-II (CPT-II) is located on the inside of the mitochondrial inner membrane and cannot be inhibited by malonyl-CoA [5]. CPT-I and CPT-II have been shown to be distinct proteins that are encoded by different genes [6,7]. A complicating factor in the study of hepatic mitochondrial CPT-I is the fact that hepatic peroxisomal [8] and microsomal [9,10] CPT activities exist that are sensitive to inhibition by malonyl-CoA. So far only the peroxisomal CPT has been shown to exhibit changes in sensitivity to malonyl-CoA inhibition [11], but a recent report suggests that microsomal vesicles are involved in part of the sensitivity change of CPT-I [12].

Hepatic mitochondrial CPT-I regulation occurs through modest changes in enzyme activity and alterations in the sensitivity of the enzyme to its physiological inhibitor, malonyl-CoA, as well as changes in hepatic malonyl-CoA concentrations. We have previously shown that in states of increased fatty acid oxidation such as fasting and diabetes, CPT-I activity measured under optimum assay conditions increases by approximately 50% while sensitivity to inhibition by malonyl-CoA decreases by a factor of 10, as measured by the K_i for malonyl-CoA [13–15]. The IC_{50} value for malonyl-CoA inhibition of CPT-I has generally been accepted as an indication of malonyl-CoA sensitivity even though the K_i value (the equilibrium constant for dissociation of the enzyme–inhibitor complex) for CPT-I is the actual factor that changes in starvation and diabetes [13–15]. The K_i value for CPT-I is a more precise estimate of sensitivity when comparing different conditions because the IC_{50} value is a theoretical function of not only the K_i value for the inhibitor but also the concentration and the K_m of the competitive substrate [16]. Insulin is able to reverse the effects of diabetes on CPT-I activity and its malonyl-CoA sensitivity [14,15]. It has been proposed that hepatic CPT activity is regulated by glucagon through protein phosphorylation [17,18], but there is no direct support for such a mechanism. Two reports have appeared suggesting that okadaic acid and agents that cause increased levels of cyclic AMP (cAMP) increase CPT-I activity in isolated hepatocytes and decrease its malonyl-CoA sensitivity [19,20]; however, recent studies have determined that phosphorylation is not directly involved in activation of CPT-I but that cAMP decreases malonyl-CoA levels by inhibiting acetyl-CoA carboxylase [21].

Fatty acid oxidation and resultant ketogenesis are regulated in isolated rat hepatocytes in primary culture by dibutyryl-cAMP and insulin [22]. cAMP stimulates fatty acid oxidation in 24-h cultured hepatocytes [21], while both insulin and proinsulin inhibit fatty acid oxidation in this system [22,23]. Insulin reportedly inhibits ketogenesis by increasing the apparent K_m of CPT-I for palmitoyl-CoA without affecting the $V_{max.}$ of the enzyme [23].

Hepatoma cells in culture have also been used for the study of mechanisms involved in the regulation of hepatic fatty acid oxidation. Rates of fatty acid oxidation in rat Fao cells and H4IIE cells [24] and in human HepG2 cells [25] are severely limited; however, this difference from normal rat hepatocytes in culture is not due to a deficiency of the mitochondrial CPT

Abbreviations used: CPT-I, carnitine palmitoyltransferase-I of the mitochondrial outer membrane; CPT-II, carnitine palmitoyltransferase-II of the mitochondrial inner membrane; CPT, any enzyme having carnitine palmitoyltransferase activity; cAMP, cyclic AMP; $I_{max.}$, maximum inhibition at theoretically infinite inhibitor concentration {obtained from plots of 1/(% inhibition) versus 1/[I]}; DMEM, Dulbecco's modified Eagle's medium.

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system [24,25]. The lower rates of fatty acid oxidation, at least in Fao cells, are due to higher than normal malonyl-CoA concentrations and to a CPT-I that has an inherently greater sensitivity to inhibition by malonyl-CoA [24].

Currently very little is known of transcriptional control for the CPT-I gene. Rat hepatic CPT-I mRNA has been reported to increase at birth, to remain elevated during the suckling period and to decrease after weaning on a high-carbohydrate diet [26]. CPT-I mRNA was also elevated in adult rat liver after 48 h of starvation [26]. Hormonal mechanisms responsible for these changes in mRNA are not known. It was speculated that high plasma glucagon or low plasma insulin might be responsible, but no direct effect of the pancreatic hormones was demonstrated [26]. We have recently reported that CPT-I mRNA abundance varied over a 40-fold range in response to thyroid hormone, which suggests regulation at the level of transcription [27]. In this paper, we have examined the regulation of CPT-I during starvation and diabetes and studied the regulatory properties of the peroxisomal and microsomal CPT activities during starvation. In addition, we have directly examined the effects of insulin on CPT-I activity, malonyl-CoA sensitivity and mRNA abundance in H4IIE cells.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats, 150–250 g (Harlan Industries, Inc., Indianapolis, IN, U.S.A.), were fed Purina rodent chow (Ralston Purina Co., Richmond, IN, U.S.A.) *ad libitum* or were starved for 48 h before livers were removed for isolating mitochondria or RNA. Another group of rats was injected with streptozotocin by tail vein (150 mg per kg body weight) to induce diabetes. Urine samples from these animals were checked with Ames Multistix (Miles, Inc., Elkhart, IN, U.S.A.) to ensure that a highly ketotic diabetic state was induced [13,14]. All diabetic animals used for these studies had urine glucose > 200 mg/ml and urine ketone bodies $\geqslant 8$ mg/ml, while fed control and starved animals had no trace of glucose or ketone bodies in their urine. Starved and diabetic animals were examined when livers were removed to ensure that stomachs of starved animals were empty and that diabetic animals were very well-fed.

Isolation of subcellular membranes and assay of CPT-I

Hepatic mitochondria were isolated by the method of Johnson and Lardy [28] with modifications as previously described [14]. The post-mitochondrial supernatant suspension was centrifuged at 20000 g for 20 min to remove light mitochondria and peroxisomes, and the resulting supernatant suspension was centrifuged at 100000 g for 60 min to collect microsomes. Mitochondria were purified further by centrifugation (50000 g for 60 min) in a swinging-bucket rotor through a self-generating density gradient formed by layering the mitochondrial suspensions over a 50% Percoll solution. The mitochondrial fraction, which was collected from the gradient, was found to band between 12 and 18 mm from the bottom of the tube (top of tube = 50 mm), corresponding to densities of between 1.098 and 1.110 g/ml. Microsomes and peroxisomes band near the top of this gradient (25–45 mm from the bottom). Catalase [29], UDP-glucuronosyltransferase [30] and monoamine oxidase [31] were measured as peroxisomal, microsomal and mitochondrial outer membrane markers respectively to determine the extent of purification. The Percoll gradient reduced peroxisomal contamination in the mitochondrial fraction by 90% and reduced microsomal contamination by 60% based on specific activity of the marker enzymes. Monoamine oxidase-specific activity was increased 50% by Percoll treatment. Mitochondrial outer membranes were then isolated by hypotonic swelling of mitochondria followed by sucrose density gradient separation [32]. A modified procedure of Bremer [33] described earlier [34] was employed to measure CPT activity. Final concentrations in a total volume of 1 ml at 37 °C were: 80 mM sucrose, 70 mM KCl, 70 mM imidazole (pH 7.0), 1 mM EGTA, 1 μg of antimycin A, 2 mg of BSA. A 5 min preincubation period was initiated by the addition of myristoyl-CoA. The reaction was started with L-carnitine (0.4 mCi/mmol L-[*methyl*-^{3}H]carnitine) and stopped after 5 min by adding 4 ml of 1.0 M perchloric acid. CPT-I activity was determined in mitochondrial outer membranes at several concentrations of L-carnitine and myristoyl-CoA for the construction of Lineweaver–Burk plots [35] from which $V_{max.}$ values were determined. K_i values for the inhibition of CPT-I by malonyl-CoA were estimated by constructing Dixon plots [36] using five malonyl-CoA concentrations at each of three myristoyl-CoA concentrations.

Culture of H4IIE cells

Rat H4IIE hepatoma cells were grown on 100 cm plates in Dulbecco's modified Eagle's medium (DMEM)/nutrient mixture F-12 (DMEM/F-12) containing 5% fetal calf serum and 5% calf serum. Cells were grown to 60–70% confluence, then the medium was replaced with DMEM/F-12 plus dexamethasone (100 nM) overnight (16 h). Subsequently the medium was changed to either DMEM/F-12 plus dexamethasone (control cells) or DMEM/F-12 containing 100 nM insulin, 10 nM glucagon, 500 μM 8-chlorophenylthio-cAMP and 100 nM dexamethasone or combinations of these hormones. After additional time periods, as indicated in the Figures, cells were harvested by scraping from plates with a rubber spatula and used for measurement of CPT activity or for determination of mRNA abundance. For measurement of CPT activity, cells were homogenized in mitochondrial isolation medium [14] with three passes of a Teflon–glass homogenizer before centrifugation at 10000 g for 10 min at 4 °C. Approximately 95 ± 6% of monoamine oxidase activity was found in this pellet, which was used for assay of CPT-I after resuspension in mitochondrial isolation medium using four passes of a Teflon–glass homogenizer. Different plates, from which cells were removed by scraping and homogenized in guanidinium thiocyanate/phenol/chloroform, were used for determinations of mRNA abundance (below). Initial cell-culture experiments were conducted without an overnight period in the absence of serum and showed little or no effects of insulin on CPT-I mRNA levels when insulin was added for 18 h compared with 18-h controls containing only plain medium (results not shown).

Synthesis of cDNA and cRNA probes

Total RNA was isolated from fresh rat liver using the guanidinium thiocyanate/phenol/chloroform extraction procedure (RNAzol B from TEL-TEST Inc., Friendswood, TX, U.S.A.). The CPT-I cDNA was obtained by PCR amplification of rat liver mRNA. First-strand cDNA was synthesized from the rat liver RNA using random primers and avian-myelobastosis-virus reverse transcriptase (cDNA Cycle Kit, Invitrogen Corp., San Diego, CA, U.S.A.). This cDNA was used as a template for PCR amplification. The forward and reverse primers were complementary to bases 1–27 and 340–366 respectively of the open reading frame for CPT-I [7]. The primers for PCR amplification were synthesized at the Molecular Resource Center at the University of Tennessee, Memphis, TN, U.S.A. After 35

cycles of amplification, the major band of approximately 366 bp was gel-isolated and inserted into the pGEM-T vector (Promega, Madison, WI, U.S.A.). JM109 cells were transformed and positive colonies were screened for the appropriate-size insert. The orientation of the inserted CPT-I cDNA was checked by sequence analysis (Sequenase-II Kit, United States Biochemical) and found to be in the reverse orientation with respect to the T7 promoter of pGEM-T. A ^{32}P-labelled antisense RNA probe, complementary to CPT-I mRNA, was synthesized using T7 RNA polymerase (MAXIscript Transcription Kit, Ambion, Austin, TX, U.S.A.). Another ribonucleotide probe, complementary to human 18 S ribosomal RNA was synthesized using pT7 RNA 18 S template, supplied with the MAXIscript kit. These cRNA probes were gel-purified and used in a ribonuclease protection assay.

Isolation of RNA and measurement of CPT-I mRNA

After isolation of total RNA from fresh rat liver by the guanidinium thiocyanate/phenol/chloroform extraction procedure, CPT-I mRNA abundance was measured by ribonuclease protection or Northern blot analysis. The ribonuclease protection assay (RPA II Kit; Ambion) was performed by diluting 21 μg of rat liver RNA to 1 μg/μl. A 1 μl aliquot was removed and hybridized with a 5 M excess of the human 18 S RNA antisense probe, assuming 18 S RNA constituted 20 % of the total RNA. The remaining 20 μg of RNA was hybridized with a 5 M excess of CPT-I cRNA, assuming that CPT-I mRNA constituted 0.1 % of the total mRNA. After the overnight hybridization at 45 °C, single-stranded RNA was digested with a mixture of RNase A and RNase T1 and protected fragments were analysed by denaturing 5% PAGE followed by autoradiography. Autoradiographs were analysed using a scanning densitometer (model DNA 45; PDI, Huntington Station, NY, U.S.A.).

Northern analyses were conducted as described elsewhere [37]. Briefly, the RNA was resolved on an agarose gel and transferred to a Gene Screen Plus membrane (Dupont-NEN, Boston, MA, U.S.A.). The CPT-I DNA fragment was labelled using the Prime-it II random primer labelling kit (Stratagene, La Jolla, CA, U.S.A.) and [α-^{32}P]dCTP. Hybridization of the probe to the membrane-bound mRNA was conducted overnight at 42 °C. The membranes were washed to remove non-specifically bound probe and exposed to XAR-5 film (Kodak, Rochester, NY, U.S.A.). Autoradiographs were analysed as described above.

Materials

L-[*methyl*-^{3}H]Carnitine hydrochloride was purchased from Amersham Corporation (Arlington Heights, IL, U.S.A.). [^{32}P]UTP and [α-^{32}P]dCTP were purchased from DuPont–NEN. Unless otherwise stated, all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Protein was determined using the BCA Protein Kit (Pierce, Rockford, IL, U.S.A.).

RESULTS AND DISCUSSION

Starvation and diabetes led to larger differences in activity (Table 1) than we previously reported using whole, intact mitochondria [13,15]. Part of the apparent difference may be due to calculation of $V_{max.}$ values, but purification of mitochondria and separation of outer membranes obviously led to much greater specific activity. $V_{max.}$ values indicated a 2-fold increase in activity in 48-h starved rats and a 3-fold increase caused by diabetes. The values for starved animals are in agreement with data published previously by Kolodziej and Zammit [38] using purified mitochondrial outer membrane preparations, but we report here that $V_{max.}$ values are even more elevated in the diabetic rat, strongly suggesting an effect of insulin. A high dose of streptozotocin was used in these studies to obtain diabetic animals that were insulin-dependent. We have previously shown that while CPT-I responds to decreased levels of insulin produced by a lower dose of streptozotocin at both 2 and 7 days, the enzyme is more responsive to the higher dose [15]. Furthermore, ketone bodies are not found in the urine unless the high dose is used [15]. The animals were monitored carefully to be certain that they were well fed and that kidney failure was avoided. All the diabetic animals in our studies possessed distended stomachs which contained more food than those of control animals. Urine samples (urination was not forced) were taken a few minutes before killing the animals. The urine contained high levels of glucose and ketone bodies but no protein.

Table 1 The effects of starvation and diabetes on the activity of CPT-I and mRNA abundance

The activity of CPT-I was measured in isolated mitochondrial outer membranes of livers of rats that were fed, starved for 48 h or made diabetic by injection of streptozotocin. Enzyme activity measurements were used to construct Lineweaver–Burk plots from which $V_{max.}$ values were derived. K_i values are taken from Dixon plots like those in Figure 1. CPT-I mRNA and 18 S ribosomal RNA were measured by solution hybridization using total RNA isolated from rat liver as described in the Materials and methods section. RNA abundance was determined from densitometric measurements of autoradiographs of protected fragments of RNA derived from ribonuclease protection assays (illustrated in Figure 2). Relative abundance of CPT-I mRNA was calculated with respect to the 18 S rRNA. All values are means ± S.E.M. for four animals. * Values significantly different from the respective fed control value as determined by the Mann–Whitney U-test ($P < 0.02$).

	Fed	Starved	Diabetic
CPT-I activity ($V_{max.}$) (nmol/min per mg of protein)	75 ± 4	158 ± 12*	206 ± 16*
CPT-I K_i for malonyl-CoA (μM)	0.5 ± 0.1	4.0 ± 0.5*	25 ± 6*
CPT-I mRNA (arbitrary units)	0.55 ± 0.03	4.1 ± 1.6*	8.1 ± 1.4*
18 S rRNA (arbitrary units)	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.1
CPT-I mRNA (relative abundance)	1.0	7.5 ± 2.9*	14.6 ± 2.6*

The sensitivity of CPT-I to inhibition by malonyl-CoA was diminished in mitochondrial outer membranes by starvation and diabetes (Figure 1), as previously reported in experiments using whole mitochondria [13–15]. Positive co-operative inhibition was demonstrated in outer membrane preparations from fed and starved animals by the upward curvature of the Dixon plots, but this was not seen in outer membranes from diabetic animals (Figure 1). This effect was seen previously only in fed animals when whole mitochondria were used [13]. K_i values were all somewhat lower in the present study using isolated outer membranes, suggesting that purification of mitochondria may have removed contaminating peroxisomal or microsomal CPT activities (Table 1); however, differences among the fed, starved and diabetic groups were similar to previous results with the K_i value increasing 8-fold in starved animals and 50-fold in diabetic animals. The theoretical extent of inhibition by malonyl-CoA was also examined to be certain that CPT-II activity of the mitochondrial inner membrane had been eliminated as a possible contaminant of the outer membrane preparations. Maximum inhibition values by malonyl-CoA ($I_{max.}$) [39] were 102 ± 5%, indicating that all CPT in the outer membrane preparations was sensitive to malonyl-CoA inhibition and therefore no detectable contamination by CPT-II or any other non-inhibitable CPT was present.

In these studies with mitochondrial outer membranes, contamination from peroxisomal and microsomal enzymes was

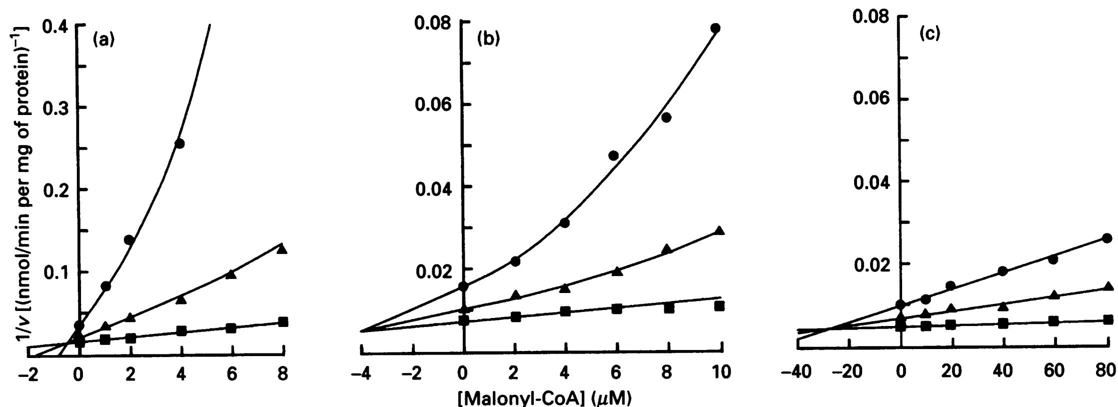

Figure 1 Changes in K_i values for hepatic CPT-I with starvation and diabetes

Rats fed a chow diet *ad libitum* were used as controls for one group of rats that were starved for 48 h and another group of rats that were made diabetic by the injection of streptozotocin as indicated in the Materials and methods section. Activity measurements were carried out using outer membranes of mitochondria purified on Percoll gradients after separation from microsomes. Dixon plots were constructed from activity measurements as indicated in the Materials and methods section. The Figure depicts inhibition data obtained from membranes from one fed (**a**), one starved (**b**) and one diabetic (**c**) animal. Myristoyl-CoA was present at 15 (●), 30 (▲) or 60 μM (■). K_i data from four animals in each group are presented in Table 1 as means ± S.E.M.

Table 2 The effects of starvation on CPT of hepatic peroxisomes and microsomes

The activity of CPT was measured in peroxisomes and microsomes isolated from livers of rats that were fed or starved for 48 h. Enzyme activity measurements and IC_{50} values for malonyl-CoA inhibition of CPT were obtained as described in the Materials and methods section. All values are means ± S.E.M. for three animals. * Values significantly different from the respective fed control value ($P < 0.05$).

	Peroxisomes		Microsomes	
	Fed	Starved	Fed	Starved
CPT activity (nmol/min per mg of protein)	1.7 ± 0.3	7.5 ± 1.5*	2.4 ± 0.3	8.0 ± 0.7*
IC_{50} (μM)	10 ± 2	28 ± 7*	6 ± 1	25 ± 4*

minimized by utilizing Percoll gradients to remove peroxisomes and microsomes before subjecting mitochondria to hypotonic treatment for separation of outer membranes. We examined whether the hepatic peroxisomal and microsomal CPT activities were affected by physiological changes that alter CPT-I. The specific activities of these enzymes were approximately equal. Following 48 h of starvation, there was an increase in activity of approximately 3- to 4-fold (Table 2). This induction was greater than the effect on the mitochondrial enzyme. The sensitivity of these enzymes to inhibition by malonyl-CoA was diminished 3–4-fold by starvation. We have previously observed an effect of starvation on the peroxisomal enzyme [11], but this is the first report of such an effect on the microsomal enzyme.

Changes in CPT-I activity might be explained by either increased synthesis of the CPT-I protein, a post-translational modification of CPT-I or a change in the phospholipid composition of the mitochondrial outer membrane. Evidence has been presented to indicate that there is no direct involvement of phosphorylation in the regulation of CPT-I activity [21]. We have shown that the addition of cardiolipin and some other phospholipids to mitochondria and mitochondrial outer membranes increases sensitivity to inhibition by malonyl-CoA and also increases CPT-I activity [40]. However, starvation and diabetes produce increased enzyme activity while having the opposite effect on sensitivity to inhibition by malonyl-CoA [13–15]. These observations suggest that changes in membrane phospholipid composition may not act by the same mechanism as starvation and diabetes to increase CPT-I activity.

Insulin regulates the synthesis of a number of liver proteins by altering the transcription of their genes (see [41,42] for reviews). Although many studies have shown increased rates of transcription with insulin treatment, there are also examples of insulin inhibiting transcription [43,44]. Initially we examined the effects of starvation and diabetes on the relative abundance of CPT-I mRNA in rat liver. A cDNA for CPT-I was generated by PCR amplification of rat liver RNA following reverse transcription. The region of CPT-I selected for PCR amplification was the 122-amino-acid N-terminus of CPT-I that had no sequence similarity to CPT-II and other proteins, as determined by screening through GenBank. The CPT-I cDNA probe was tested against RNA prepared from rat liver in a Northern blot analysis and was found to hybridize to a band of 4.7 kb [27]. This mRNA was identical with the size reported by Esser et al. for CPT-I [7].

To quantify the changes in CPT-I mRNA abundance in livers of fed, starved and diabetic rats, we used the ribonuclease protection assay. The full-length CPT-I RNA probe (generated from the T7 promoter) is 425 bp long. After hybridization and ribonuclease digestion, the protected fragment was found to be between 360 and 370 bp, corresponding to the predicted size of the protected fragment of 366 bp. Figure 2 shows the results of a protection assay using liver RNA isolated from fed, starved and diabetic rats. Densitometric quantification of the bands from autoradiographs indicated a greater than 7-fold increase in CPT-I mRNA levels in starved rats and a 14-fold increase in diabetic rats (Table 1). These data demonstrate a clear correlation between CPT-I mRNA concentrations and measured CPT-I activity and suggest that CPT-I is regulated at the transcriptional level during starvation and diabetes. Although starvation and diabetes produce very large changes in CPT-I mRNA abundance, these data cannot rule out the possibility that other factors, such as changes in mRNA stability, may contribute to the overall effect.

Rat H4IIE hepatoma cells were used to examine the effects of insulin and its counter-regulatory hormone glucagon on CPT-I

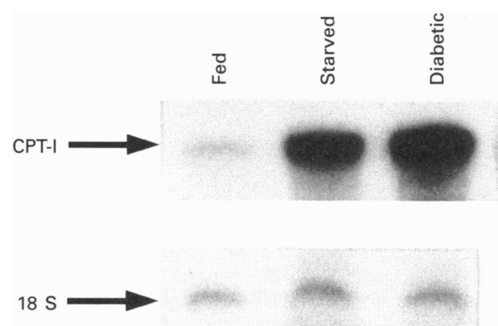

Figure 2 Changes in hepatic CPT-I mRNA with starvation and diabetes

Rats fed a chow diet *ad libitum* were used as controls for one group of rats that were starved for 48 h and another group of rats that were made diabetic by the injection of streptozotocin as indicated in the Materials and methods section. CPT-I mRNA was measured by solution hybridization of a ^{32}P-labelled CPT-I RNA probe, corresponding to bases 1–366 of the open reading frame of CPT-I, and 20 μg of rat liver RNA, followed by ribonuclease digestion, separation of protected fragments by PAGE and exposure to X-ray film. 18 S ribosomal RNA content was determined with a probe for human 18 S ribosomal RNA and was used to normalize the data in each lane to a constant amount of RNA. Relative mRNA abundance from densitometric analysis of autoradiographs from ribonuclease protection assays are compared in Table 1.

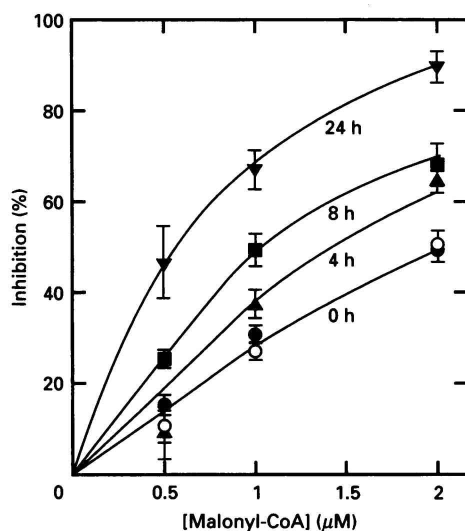

Figure 3 Effects of insulin on the sensitivity of CPT-I to inhibition by malonyl-CoA in rat H4IIE cells

Cells were incubated overnight in serum-free DMEM/F-12 containing 100 nM dexamethasone. At time zero, fresh media containing 100 nM dexamethasone, with or without 100 nM insulin, were added to the cells. Cells were harvested at the times indicated and used for assays of CPT-I activity in the absence or presence of the concentrations of malonyl-CoA indicated. Percentage inhibition data plotted in the Figure are mean ± range calculated from two experiments. Times of incubation with insulin are zero (○), 2 h (●), 4 h (▲), 8 h (■) and 24 h (▼). CPT-I activity measured in the presence of 40 μM myristoyl-CoA without malonyl-CoA was 2.7 ± 0.5, 2.1 ± 0.6, 1.9 ± 0.5, 1.5 ± 0.5 and 1.4 ± 0.3 nmol/min per mg of protein at 0, 2, 4, 8 and 24 h respectively after insulin addition.

activity and malonyl-CoA sensitivity. To determine a time course for insulin effects on CPT-I in these cells, we measured malonyl-CoA inhibition of CPT activity over a period of 24 h. We found that the sensitizing effects of insulin on CPT activity could be seen at 4 h after insulin addition and that sensitivity to inhibition by malonyl-CoA continued to increase up to 24 h

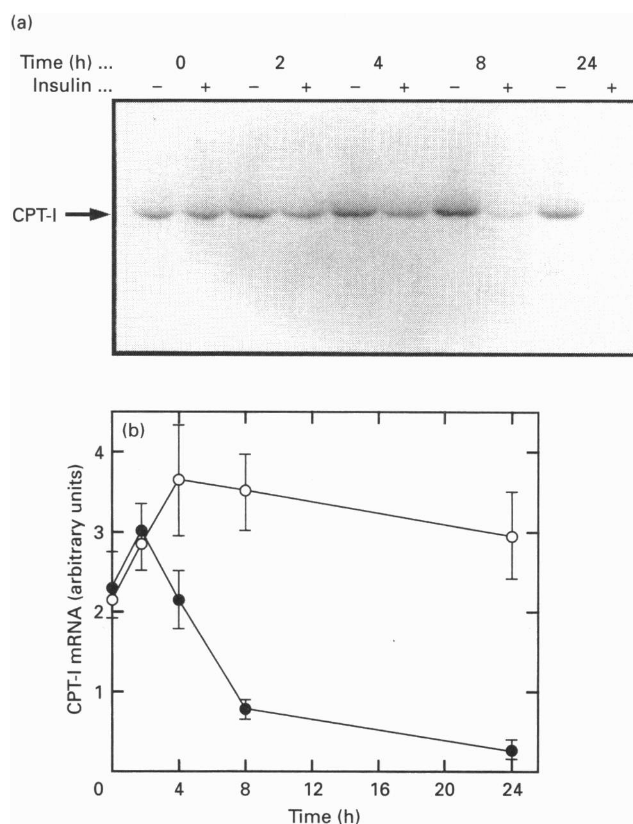

Figure 4 Effects of insulin on the CPT-I mRNA content of rat H4IIE cells

Cells were cultured as indicated in the Materials and methods section. Hormone treatments were conducted as described in the legend to Figure 3. RNA was harvested from the cells at the indicated times. (**a**) CPT-I mRNA abundance was determined by Northern analysis. Each lane contains 20 μg of total RNA. (**b**) Results of at least three independent experiments in control (○) and insulin-treated (●) cells are shown for each time point in the plot (means ± S.E.M.).

(Figure 3). The IC_{50} for malonyl-CoA inhibition was decreased from 2 to 0.6 μM, not only demonstrating an effect of insulin on these hepatoma cells, but also showing the greater sensitivity to malonyl-CoA in these cells than in normal liver, as previously indicated [24]. It has been reported that glucagon and cAMP increase oleate oxidation and decrease malonyl-CoA sensitivity of CPT-I in cultured fetal rabbit hepatocytes following a lag period of 12 h, but there was no change in activity of CPT-I with these agents [45]. Insulin decreased oleate oxidation and increased malonyl-CoA sensitivity in rabbit hepatocytes at 24 and 48 h, but no change in the activity of CPT-I was found. Our results may have differed because the rabbit hepatocytes were derived from fetal animals of a different species examined in primary cultures, whereas our studies were conducted using a hepatoma cell line. Another previous report has indicated that cultured Fao hepatoma cells oxidized oleate at a slow rate that was not affected by cAMP or insulin and that insulin increased malonyl-CoA sensitivity without affecting CPT-I activity [24]. Two differences between the latter study and ours were the lack of dexamethasone in the Fao cell cultures and the fact that a relatively short incubation time with insulin (4 h) was used for the Fao cells.

Insulin treatment of H4IIE cells produced a decrease in CPT-I mRNA abundance that approximated the same time course as that seen for malonyl-CoA sensitivity (Figure 4). CPT-I mRNA

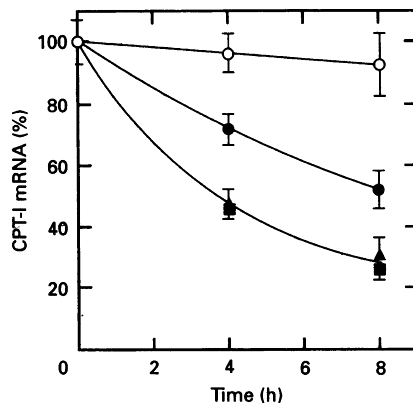

Figure 5 Inhibition of CPT-I mRNA synthesis in H4IIE cells by actinomycin D

H4IIE rat hepatoma cells were incubated in DMEM/F-12 media in the absence of serum for 16 h. At zero time, the media were changed to media containing either 5 μg per ml of actinomycin D or 100 nM insulin or both. The RNA was harvested from the cells at either 0, 4 or 8 h as indicated. Treatments are: no addition (○); insulin (●); actinomycin D (▲); insulin plus actinomycin D (■).

in insulin-treated cells was significantly different from that in control cells at 4 h and continued to decrease up to 24 h when it varied by a factor of 10. In parallel experiments, CPT-I activity measured in identically treated cells in the presence of 100 μM myristoyl-CoA was 6.8 ± 0.9 (mean $\pm$ S.E.M., $n = 3$) and 2.6 ± 0.6 nmol/min per mg of protein after 24 h in the absence or presence respectively of insulin. Control cells showed a slight increase in CPT-I mRNA up to 4 h with slightly decreasing levels from 4 to 24 h. This small effect was due to the addition of dexamethasone to both sets of cultured cells at zero time. Neither glucagon nor cAMP was found to have any effect on CPT-I mRNA abundance in these cells (results not shown).

When transcription in H4IIE cells was blocked by addition of actinomycin D, the abundance of CPT-I mRNA was dramatically reduced (Figure 5). H4IIE cells were maintained in serum-free DMEM/F-12 before the addition of either actinomycin D or insulin. The half-life of CPT-I mRNA in the presence of actinomycin D was approximately 4 h. The CPT-I mRNA abundance decreased in a similar manner, but not as rapidly, in the presence of insulin. Insulin did not alter the time course of mRNA disappearance seen in the presence of actinomycin D, indicating that both agents were probably inhibiting transcription.

CPT-I is the primary regulatory enzyme allowing long-chain fatty acids to be oxidized in mitochondria. Its importance as a regulatory enzyme in starvation and diabetes is demonstrated by the multiple levels of regulation that have been observed to influence its activity, including substrate-level inhibition, allosteric inhibition by malonyl-CoA, modulation of its sensitivity to inhibition by malonyl-CoA and changes in its intrinsic activity. Data presented here represent the first demonstration that insulin plays a major role in regulating CPT-I mRNA abundance in liver, and that insulin exerts its effect on CPT-I primarily by decreasing gene transcription.

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