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Effect of starvation and diabetes on the sensitivity of carnitine palmitoyltransferase I to inhibition by 4-hydroxyphenylglyoxylate

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The sensitivity of carnitine palmitoyltransferase I to inhibition by 4-hydroxyphenylglyoxylate was decreased markedly in liver mitochondria isolated from either 48 h-starved or streptozotocin-diabetic rats. These treatments of the rat also decreased the sensitivity of fatty acid oxidation by isolated hepatocytes to inhibition by this compound. Furthermore, incubation of hepatocytes prepared from fed rats with $N^6O^{2'}$-dibutyryl cyclic AMP also decreased the sensitivity, whereas incubation of hepatocytes prepared from starved rats with lactate plus pyruvate had the opposite effect on 4-hydroxyphenylglyoxylate inhibition of fatty acid oxidation. The sensitivity of carnitine palmitoyltransferase I of mitochondria to 4-hydroxyphenylglyoxylate increased in a time-dependent manner, as previously reported for malonyl-CoA. Likewise, oleoyl-CoA activated carnitine palmitoyltransferase I in a time-dependent manner and prevented the sensitization by 4-hydroxyphenylglyoxylate. Increased exogenous carnitine caused a moderate increase in fatty acid oxidation by hepatocytes under some conditions and a decreased 4-hydroxyphenylglyoxylate inhibition of fatty acid oxidation at low oleate concentration, without decreasing the difference in 4-hydroxyphenylglyoxylate inhibition between fed- and starved-rat hepatocytes. Time-dependent changes in the conformation of carnitine palmitoyltransferase I or the membrane environment may be involved in differences among nutritional states in 4-hydroxyphenylglyoxylate-sensitivity of carnitine palmitoyltransferase I.

INTRODUCTION

Carnitine palmitoyltransferase I (CPT I), an important regulatory enzyme of fatty acid oxidation (McGarry & Foster, 1980), is inhibited by malonyl-CoA, an intermediate of fatty acid synthesis in the liver. However, malonyl-CoA concentration may not be the sole regulatory factor for fatty acid oxidation. Indeed, changes in sensitivity of CPT I to inhibition by malonyl-CoA may also be important in starvation and diabetes (Cook *et al.*, 1984). The mechanism responsible for this change in sensitivity to malonyl-CoA inhibition is unknown. Physiological conditions have been observed where variations in malonyl-CoA concentration were insufficient to explain fatty acid oxidation capacity (Benito & Williamson, 1978), and a wide range of fatty acid oxidation rates has been observed over a narrow range of malonyl-CoA concentrations (Boyd *et al.*, 1981). A major obstacle to such investigations is the inability to vary intracellular malonyl-CoA concentrations readily and specifically. Additionally, rate limitation or high control strength (Kacser & Burns, 1973) of liver fatty acid oxidation by CPT I has been confirmed by some (McGarry & Foster, 1973; Cook *et al.*, 1980; Harano *et al.*, 1982) and questioned by others (Brass & Hoppel, 1980).

With respect to mechanism, Zammit (1984) has proposed that CPT I of mitochondria isolated from rats in various nutritional states may differ in sensitivity to malonyl-CoA inhibition, owing to 'memory' of the malonyl-CoA concentration *in vivo*. Bremer *et al.* (1985) have verified the slow sensitization process and demonstrated that KCl and palmitoyl-CoA enhance a desensitization/activation process. The possiblity that conditions during the isolation of mitochondria (malonyl-CoA concentration, acyl-CoA concentration, ionic strength and temperature) may alter the sensitivity of CPT I to malonyl-CoA inhibition makes a method where sensitivity can be studied in intact cells desirable. Covalent phosphorylation of a catalytic subunit of CPT I, resulting in a lower K_m for acyl-CoA and consequently a higher K_i for malonyl-CoA, is another possible mechanism for sensitivity changes (Harano *et al.*, 1985). Changes in K_i for malonyl-CoA have been concluded to be responsible for changes in sensitivity to malonyl-CoA inhibition (Cook, 1984*b*; Gamble & Cook, 1985).

The current studies use 4-hydroxyphenylglyoxylate (HPG) as an inhibitor of CPT I to study sensitivity changes in CPT I of liver mitochondria and fatty acid oxidation by isolated hepatocytes. This inhibitor was chosen because it has properties similar to those of malonyl-CoA (Stephens *et al.*, 1985). In contrast with malonyl-CoA, which cannot penetrate the plasma membrane, HPG inhibits fatty acid oxidation when added to the external medium of isolated hepatocytes. With this compound, known modulators of fatty acid oxidation can be incubated with hepatocyte preparations and the sensitivity of fatty acid oxidation to HPG inhibition monitored. HPG was also used to investigate the mechanism of time-dependent changes in sensitivity

Abbreviations used: CPT I, carnitine palmitoyltransferase I (EC 2.3.1.21); HPG, 4-hydroxyphenylglyoxylate; Bt_2cAMP, $N^6O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate.

of CPT I of liver mitochondria, by using known effectors of this process.

EXPERIMENTAL

Materials

HPG was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). [1-^{14}C]Oleate was obtained from Amersham (Arlington Heights, IL, U.S.A.). Sources of other materials have been given previously (Stephens *et al.*, 1985). Wistar rats (chow-fed or 48 h-starved; 200–250 g) were obtained from Harlan Industries (Indianapolis, IN, U.S.A.). Streptozotocin-diabetes was induced by injection of 150 mg of streptozotocin/kg body wt. 48 h before animals were killed (Schein *et al.*, 1971). Only very ketotic (urine ketone bodies $\geqslant$ 40 mg/dl) animals were used for these studies.

Preparation of mitochondria and hepatocytes

Liver mitochondria were prepared essentially as described by Johnson & Lardy (1967) and suspended in 0.25 M-sucrose (adjusted to pH 7.4 with Tris base), except where the final wash was performed in KCl medium, which was 150 mM-KCl, 5 mM-Tris/HCl and 1 mM-EGTA, pH 7.4 (indicated in Figures). Respiratory control ratios were determined by measuring O_2 consumption with a Clark oxygen electrode, and averaged 9.5, 5.1 and 4.5 for mitochondria isolated from fed, starved and diabetic rats with 10 mM-glutamate plus 0.5 mM-malate as substrates.

Hepatocytes were isolated by the method of Berry & Friend (1969) with modifications described previously (Harris, 1975).

Hepatocytes were permeabilized with filipin essentially as described by Gankema *et al.* (1981), except that filipin was added as a solution in dimethyl sulphoxide to a final concentration of 100 μM and cells were incubated at 37 °C for 2 min before cooling to ice temperature.

Assay of CPT I

CPT I activity of liver mitochondria was assayed by the radioisotope method described previously with a 5 min preincubation and 5 min incubation (Stephens *et al.*, 1985). The complete system (1 ml) contained 0.1 mg of mitochondrial protein, 80 mM-sucrose, 70 mM-KCl, 50 mM-imidazole/HCl (pH 7.0 at 30 °C), 1 mM-EGTA, 4 mg of bovine serum albumin, 5 mM-glutathione, 1 μg of antimycin A, 0.5 mM-phenylpyruvate, 50 μM-oleoyl-CoA, 0.2 mM-L-carnitine (2 μCi of L-[*methyl*-^{3}H]carnitine/μmol). The incubation and extraction procedures were modified for studies of the time course for HPG inhibition. Mitochondria suspended in the KCl medium at 20 mg/ml were added to prewarmed preincubation mixture in a 25 ml Erlenmeyer flask. At the desired preincubation times, a sample (1.8 ml) of this mixture was transferred to prewarmed tubes containing 0.2 ml of substrates to initiate the enzymic reaction. Concentrations of components in this reaction mixture were the same as above, except that mitochondrial protein was 1 mg/ml. The reaction was terminated after 15 s by the addition of 4 ml of 1 M-$HClO_4$. The extraction of acid-insoluble acylcarnitines was performed essentially as described by Bremer *et al.* (1985), except that the protein pellet was resuspended in 3.5 ml of 75 mM-Na_2HPO_4 saturated with butanol and extracted with 1.0 ml of butanol. The use of $(NH_4)_2SO_4$ was eliminated in this procedure without significant effect. Results by this extraction procedure were identical with those obtained by the procedure described previously (Stephens *et al.*, 1985), except that blank radioactivity was decreased.

Assay of CPT I with filipin-permeabilized hepatocytes was performed as described above, except that 0.1–0.2 mg of hepatocyte protein was used per assay.

Incubation of hepatocytes and measurement of fatty acid oxidation

Each hepatocyte incubation (2.5 ml) contained 45–65 mg wet wt. of hepatocytes, 2% (w/v) dialysed fatty acid-free bovine serum albumin, 0.5 mM- or 2 mM-oleate (0.07 μCi of [1-^{14}C]oleate/μmol) in Krebs–Henseleit medium plus additions as indicated. Hepatocytes were preincubated at 37 °C under O_2/CO_2 (19:1) for either 5 or 30 min, and then fatty acid oxidation was initiated with oleate, followed by a 30 min incubation. Termination of the incubation, collection of $^{14}CO_2$ and determination of radioactive acid-soluble products were performed as described previously (McCune & Harris, 1979). Oleate was omitted, and hepatocytes were incubated with or without Bt_2cAMP or glucagon for 30 min, and centrifuged to harvest the cells for filipin treatment and measurement of CPT I activity. The fatty acid oxidation rate was measured as the sum of $^{14}CO_2$ and acid-soluble radioactive-product formation from [1-^{14}C]oleate (McCune & Harris, 1979). The rate of fatty acid oxidation is expressed in terms of nmol of oleate converted into $^{14}CO_2$ plus acid-soluble products/min per mg wet wt of hepatocytes.

Statistical analysis

Data in Table 1 were evaluated for heterogeneity of variance by Bartlett's Chi Square test (Sokal & Rohlf, 1969). Three-way analysis of variance was performed by the General Linear Model program of the SAS Statistical Package (SAS Institute, Cary, NC, U.S.A.) with reciprocal cell error variances as weighting factors. Individual comparisons of means were made by using the linear contrast option of this program.

RESULTS

Effect of starvation and diabetes on HPG inhibition of CPT I activity of liver mitochondria and fatty acid oxidation by hepatocytes

CPT I of liver mitochondria from starved or ketotic-diabetic animals was less sensitive to HPG inhibition than was CPT I of liver mitochondria from normal fed animals (Fig. 1). Analogous results are obtained with malonyl-CoA as inhibitor (Cook *et al.*, 1984). The ability to detect sensitivity changes in CPT I of liver mitochondria with HPG as inhibitor, and the effectiveness of HPG as an inhibitor of fatty acid oxidation by hepatocytes (Stephens *et al.*, 1985), suggested that HPG could be used to detect changes in CPT I sensitivity within intact hepatocytes.

The same change in inhibitory sensitivity seen with mitochondrial CPT I activity was found when fatty acid oxidation by hepatocytes derived from fed, starved and ketotic-diabetic animals was titrated with HPG (Fig. 2), i.e. fatty acid oxidation by hepatocytes from starved or

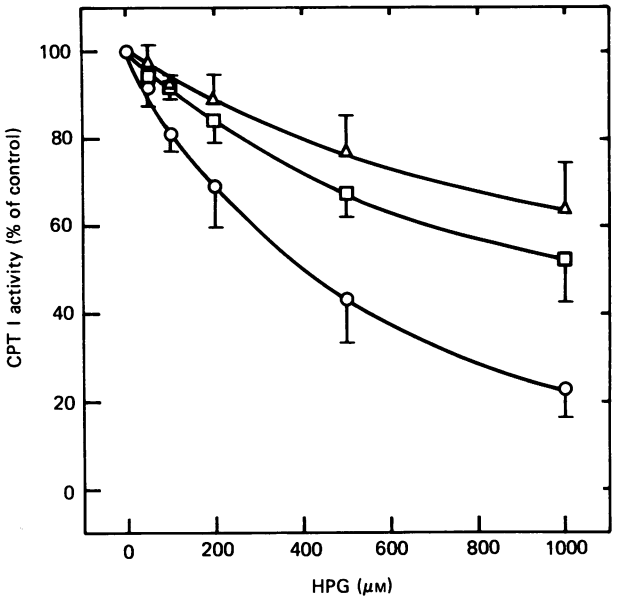

Fig. 1. Effect of starvation and diabetes on HPG inhibition of CPT I of liver mitochondria

Liver mitochondria were isolated from fed (○), starved (□) or diabetic (△) rats. Each point represents the mean ± S.E.M. for three mitochondrial preparations. Activity in the absence of HPG averaged 5.9, 5.1 and 5.7 nmol/min per mg for mitochondria isolated from fed, starved and diabetic rats respectively.

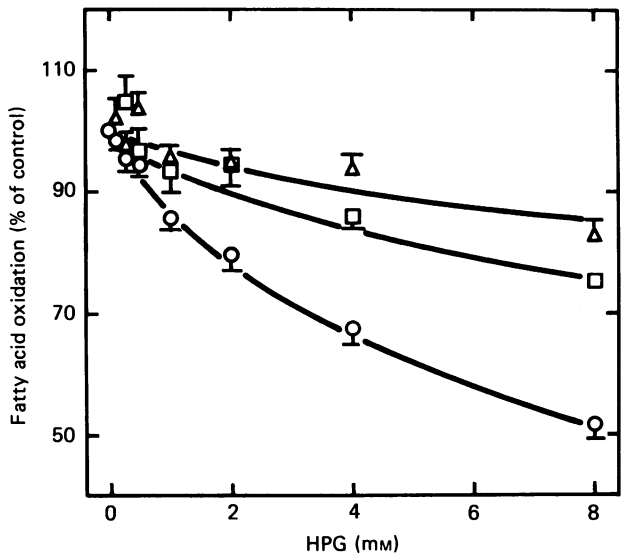

Fig. 2. Effect of starvation and diabetes on HPG inhibition of fatty acid oxidation by hepatocytes

Hepatocytes were isolated from fed (○), starved (□) or diabetic (△) rats. Hepatocytes were preincubated with HPG and other components for 15 min before initiation of the incubation with oleate. Each point represents the mean ± S.E.M. for three (starved and diabetic) or six (fed) hepatocyte preparations. Fatty acid oxidation averaged 0.30, 0.46 and 0.57 nmol/min per mg wet wt. for hepatocytes isolated from fed, starved and diabetic rats in the absence of HPG.

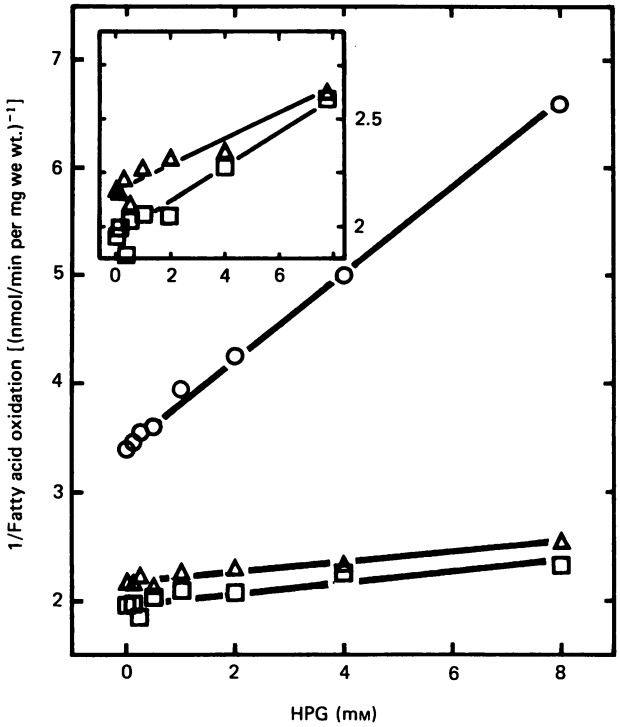

Fig. 3. Dixon plot of reciprocal fatty acid oxidation rate by hepatocytes versus HPG concentration

The reciprocal of the mean fatty acid oxidation rate for hepatocytes from fed (○), starved (□) or diabetic (△) rats from the experiment in Fig. 2 is plotted versus HPG concentration in the incubation medium. The inset is a replot of the starved- and diabetic-rat hepatocyte data with an expanded vertical scale. Units in the inset are the same as in the main Figure.

ketotic-diabetic animals was much less sensitive to inhibition by HPG than was that by hepatocytes from fed rats. Inhibition of fatty acid oxidation was mainly due to inhibition of radioactive acid-soluble product formation (results not shown). HPG inhibition of fatty acid oxidation by hepatocytes from fed animals caused an increase in $^{14}CO_2$ production, whereas no change or a small inhibition was seen with hepatocytes from starved or ketotic-diabetic animals (results not shown).

Dixon plots produced by plotting the reciprocal of fatty acid oxidation rate versus inhibitor concentration were linear (Fig. 3). This is indicative of a rate-limiting step of CPT I under these conditions (Rognstadt, 1979). Plots of data from starved and ketotic-diabetic rats had a minimal slope, making it difficult to determine if curvature existed. Data from starved and diabetic rats, plotted in the inset with an expanded scale, also appeared linear. The agreement of data obtained with isolated mitochondria with data obtained with hepatocytes coupled with the linear Dixon plots, suggest that changes in CPT I sensitivity can be detected in intact hepatocytes by determination of the effectiveness of HPG as an inhibitor of fatty acid oxidation.

Effects of modulators of fatty acid oxidation on HPG inhibition of fatty acid oxidation by hepatocytes

Both Bt_2cAMP and glucagon increased fatty acid oxidation rate and decreased HPG inhibition of fatty

Table 1. Effect of Bt_2cAMP, glucagon, lactate plus pyruvate, glucose and oleate on the sensitivity of fatty acid oxidation to inhibition by HPG

The results are given as means ± S.E.M. for the numbers of hepatocyte preparations in parentheses. Hepatocytes were preincubated for 30 min in the presence of additions before initiation of the incubation with oleate. Concentrations of additions were 20 μM-HPG, 50 μM-Bt_2cAMP, 1.0 μM-glucagon, 5 mM-lactate plus 1 mM-pyruvate, and 20 mM-glucose. The main effects were signficant by weighted three-way analysis of variance ($P < 0.05$; see the Experimental section for details). Variance between groups was significantly heterogeneous by Bartlett's Chi-square test ($P < 0.05$); $*P < 0.05$ for individual mean comparisons against the no-additive mean for the same concentration of oleate and nutritional status by linear contrast (see the Experimental section).

[Oleate]	Hepatocytes	Additions	Fatty acid oxidation (nmol/min per mg wet wt.)	HPG inhibition (%)
0.5 mM	Fed rat	None	0.099 ± 0.005 (5)	71.7 ± 2.4 (5)
		Bt_2cAMP	0.120 ± 0.006 (5)*	43.1 ± 5.3 (5)*
		Glucagon	0.126 ± 0.008 (3)*	41.2 ± 3.4 (3)*
		Lactate + pyruvate	0.090 ± 0.006 (5)	74.1 ± 1.7 (5)
	Starved rat	None	0.216 ± 0.010 (5)	23.9 ± 3.1 (4)
		Bt_2cAMP	0.232 ± 0.008 (4)	16.9 ± 4.7 (4)
		Lactate + pyruvate	0.214 ± 0.011 (5)	44.1 ± 4.6 (5)*
		Glucose	0.205 ± 0.019 (3)	43.0 ± 2.2 (3)*
2.0 mM	Fed rat	None	0.231 ± 0.014 (5)	46.2 ± 1.0 (5)
		Bt_2cAMP	0.255 ± 0.016 (5)	35.2 ± 1.6 (5)*
		Lactate + pyruvate	0.228 ± 0.012 (5)	48.5 ± 0.8 (5)
	Starved rat	None	0.405 ± 0.014 (5)	28.6 ± 3.8 (5)
		Bt_2cAMP	0.463 ± 0.016 (5)*	25.3 ± 2.5 (5)
		Lactate + pyruvate	0.403 ± 0.022 (5)	39.5 ± 2.9 (5)*

Table 2. Effect of carnitine on the sensitivity of fatty acid oxidation to inhibition by HPG

Results are given as means ± S.E.M. for the numbers of hepatocyte preparations in parentheses. Hepatocytes were preincubated with or without 4 mM-carnitine for 30 min before initiation of the incubation with oleate. The main effects were significant by weighted three-way analysis of variance ($P < 0.05$); $*P < 0.05$ for individual mean comparisons against the mean without carnitine for the same concentration of oleate and nutritional status.

[Oleate]	Hepatocytes	Additions	Fatty acid oxidation (nmol/min per mg wet wt.)	HPG inhibition (%)
0.5 mM	Fed rat	None	0.099 ± 0.005 (5)	71.7 ± 2.4 (5)
		Carnitine	0.117 ± 0.004 (3)*	36.1 ± 5.5 (3)*
	Starved rat	None	0.216 ± 0.010 (5)	23.9 ± 3.1 (5)
		Carnitine	0.227 ± 0.018 (3)	−8.2 ± 2.1 (3)*
2.0 mM	Fed rat	None	0.231 ± 0.014 (5)	46.2 ± 1.0 (5)
		Carnitine	0.276 ± 0.026 (3)	44.3 ± 2.5 (3)
	Starved rat	None	0.405 ± 0.015 (5)	28.6 ± 4.2 (5)
		Carnitine	0.558 ± 0.024 (2)*	19.6 ± 6.7 (2)

acid oxidation by hepatocytes isolated from fed rats (Table 1). The increase in acid-soluble product formation by glucagon addition was more pronounced than the increase in total fatty acid oxidation (60% versus 27% increase in acid-soluble product formation and total fatty acid oxidation respectively).

Lactate plus pyruvate or glucose increased HPG inhibition of fatty acid oxidation without affecting rate of fatty acid oxidation by hepatocytes isolated from starved rats (Table 1).

The effects of Bt_2cAMP and lactate plus pyruvate were decreased by increasing the oleate concentration (Table 1). Increasing oleate from 0.5 to 2.0 mM increased fatty acid oxidation by 230% ($P < 0.02$) and 190% ($P < 0.01$) with hepatocytes isolated from fed and starved rats respectively. With increasing oleate concentration, HPG inhibition of fatty acid oxidation decreased with hepatocytes from fed rats, but did not change with hepatocytes from starved rats. These effects may be due to increased long-chain acyl-CoA concentration, expected when hepatocytes are incubated with oleate (McGarry & Foster, 1981). The degree of HPG inhibition and concentration of oleate correlated significantly with fatty acid oxidation rate by hepatocytes isolated from

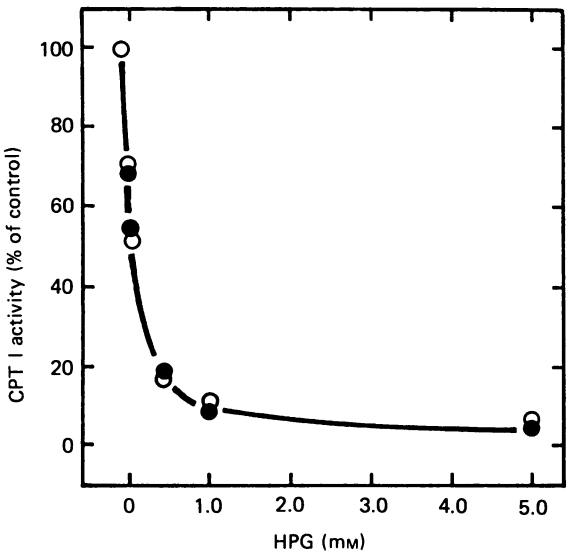

Fig. 4. Effect of Bt_2cAMP on HPG inhibition of CPT I activity of filipin-treated hepatocytes

Hepatocytes were isolated from fed rats and incubated in the absence (●) or presence (○) of Bt_2cAMP. Hepatocytes were then collected by centrifugation and treated with filipin as described in the Experimental section. Filipin-treated hepatocytes were assayed for CPT I activity in the presence of the indicated concentration of HPG. Each point represents the average of closely agreeing values for two filipin-treated hepatocyte preparations. Activity in the absence of HPG averaged 2.09 and 2.52 nmol/min per mg of hepatocyte protein for hepatocyte incubated in the absence and presence of Bt_2cAMP respectively.

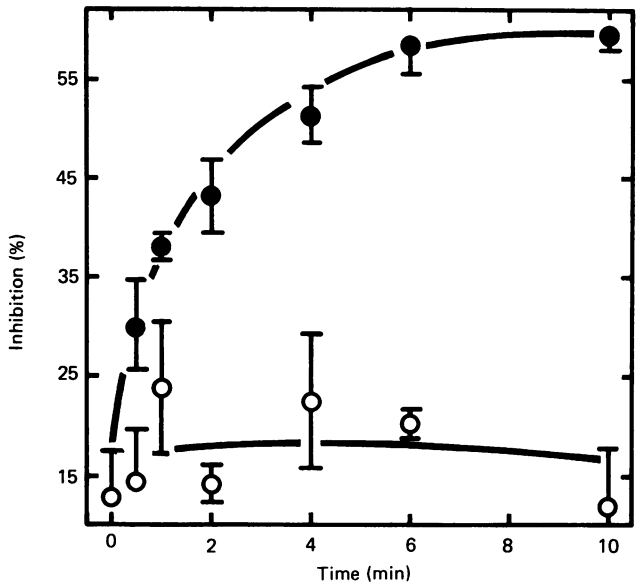

Fig. 5. Time course of HPG inhibition of liver mitochondrial CPT I

Liver mitochondria were isolated from fed rats. The final wash was with the KCl medium. Mitochondria, used in the CPT I assay within 1 h of isolation, were preincubated in complete reaction mixture, but without substrates and HPG, for 8 min. Mitochondria were incubated subsequently in either the absence (○) or the presence (●) of HPG, and samples were transferred at the times indicated to prewarmed tubes containing substrate (●) or substrate plus HPG (○). Reactions were after 15 s with $HClO_4$ as described in the Experimental section. Percentage inhibition was calculated on the basis of controls run in an analogous manner without HPG. Each point represents the mean ± S.E.M. for three mitochondrial preparations. HPG concentration was 20 μM.

fed, starved and diabetic rats ($t = -5.16$, $P < 0.001$, and $t = 5.23$, $P < 0.001$ respectively). In 22 out of 24 experiments, the addition of Bt_2cAMP, glucagon or carnitine resulted in both a decrease in HPG inhibition and an increase in fatty acid oxidation rate. The addition of lactate plus pyruvate or glucose did not have this consistent concomitant effect on both HPG inhibition and fatty acid oxidation rate. HPG inhibition was increased by lactate plus pyruvate or glucose (starved-rat hepatocytes), whereas fatty acid oxidation rate was increased, decreased or not changed.

Incubation with 4 mM-carnitine at 0.5 mM-oleate increased fatty acid oxidation by 18% ($P < 0.01$) with hepatocytes from fed rats, but had no significant effect with hepatocytes from starved rats (Table 2). In contrast, a significant increase (38%, $P < 0.01$) in fatty acid oxidation rate was seen with the addition of carnitine at 2.0 mM-oleate for starved-, but not for fed-, rat hepatocytes. At 0.5 mM-oleate, carnitine decreased HPG inhibition with hepatocytes from fed rats and totally reversed HPG inhibition with those from starved rats. In spite of this significant decrease in HPG inhibition caused by high concentrations of carnitine, HPG inhibition was still significantly higher with fed-rat hepatocytes than with starved-rat hepatocytes without carnitine supplementation. At 2.0 mM-oleate, carnitine was not effective in decreasing HPG inhibition with hepatocytes from either fed or starved rats.

Effect of Bt_2cAMP on HPG inhibition of CPT I activity of filipin-permeabilized hepatocytes

Filipin treatment of hepatocytes results in cells which are permeable to low-M_r compounds (Gankema *et al.*, 1981), making it possible to measure CPT I activity of isolated hepatocytes without isolating mitochondria. Hepatocytes were incubated with and without Bt_2cAMP under standard incubation conditions (omitting oleate) and then treated with filipin. CPT I activity was then measured directly without isolation of mitochondria. Bt_2cAMP did not affect HPG inhibition of CPT I activity by filipin-treated hepatocytes (Fig. 4). Filipin treatment led to 100% Trypan Blue permeability and gave acceptable CPT I activity (2 nmol/min per mg of protein for filipin-treated hepatocytes versus 6 nmol/min per mg of protein for isolated mitochondria), which was 95% inhibitable by HPG. These results suggest that filipin-treated hepatocytes were permeable to substrates and that the mitochondria must have remained intact. In spite of the lack of change in sensitivity of HPG inhibition, Bt_2cAMP increased CPT I activity by 24%, which is similar to the 27% increase in fatty acid oxidation seen with intact hepatocytes. Glucagon similarly increased CPT I activity without altering HPG inhibition (results not shown).

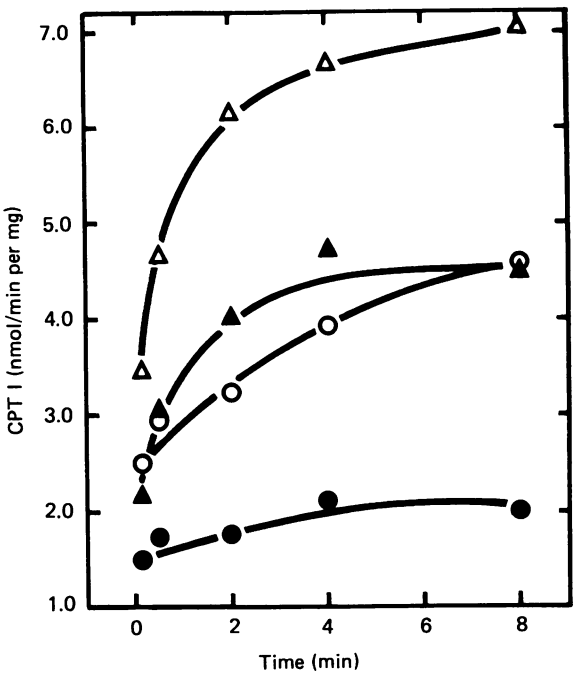

Fig. 6. Effect of preincubation time on liver mitochondrial CPT I in the presence or absence of oleoyl-CoA and HPG

Mitochondria were preincubated in the complete reaction mixture in the absence (△, ▲) or presence (○, ●) of 20 μM-HPG and the absence (●, ▲) or presence (○, △) of oleoyl-CoA for the times indicated above. Incubations were performed in prewarmed tubes containing the missing substrates. Each point represents the mean of two mitochondria preparations.

Time-dependency of HPG inhibition and CPT I activity of isolated liver mitochondria

HPG inhibition of isolated liver mitochondrial CPT I increased from 12.9±4.6% at zero time to 59.7±1.6% after preincubation for 10 min in the presence of 20 μM-HPG (Fig. 5). Preincubation in the absence of HPG during the same period had no effect on HPG inhibition. Preincubation in the presence of HPG and the absence of oleoyl-CoA is most likely responsible for the significantly higher HPG inhibition observed in Fig. 5 than in Fig. 1 (see the Discussion section).

Preincubation of isolated liver mitochondria in the presence of KCl led to an activation of CPT I (Fig. 6). KCl plus oleoyl-CoA led to an even greater activation. The addition of HPG prevented activation by KCl in the absence, but not in the presence, of oleoyl-CoA. When data from Fig. 6 were plotted as percentage inhibition by HPG (not shown), oleoyl-CoA, but not carnitine, appeared to prevent the sensitization caused by preincubation with HPG.

DISCUSSION

The decrease in HPG inhibition of liver mitochondrial CPT I resulting from starvation and ketotic diabetes is very similar to that shown previously for malonyl-CoA inhibition (Cook *et al.*, 1984). HPG inhibition depends on many of the same factors as malonyl-CoA inhibition (Stephens *et al.*, 1983, 1985). These similarities suggest that sensitivity changes in HPG inhibition of CPT I are analogous to changes in sensitivity to inhibition by malonyl-CoA. On the other hand, HPG inhibition appears competitive with carnitine (Stephens *et al.*, 1985), whereas malonyl-CoA is competitive with long-chain acyl-CoA. More recently, the K_m for carnitine has been reported to be increased by malonyl-CoA and the $K_{0.5}$ for malonyl-CoA increased by carnitine (Bird & Saggerson, 1985). Unlike malonyl-CoA, the intracellular concentration of HPG can be readily varied (by adding exogenous HPG) to determine effects on fatty acid oxidation. The observation that starvation and ketotic diabetes also decreased the sensitivity to HPG inhibition of fatty acid oxidation by isolated hepatocytes suggests that a common mechanism determines the degree of inhibition in the two experimental systems (mitochondria and hepatocytes) and argues that HPG can be used to titrate CPT I activity in intact hepatocytes.

The linearity of the Dixon plot for HPG inhibition of fatty acid oxidation by hepatocytes from fed rats suggests that CPT I is rate-limiting for fatty acid oxidation. The observation that inhibition of CPT I by HPG causes a proportional inhibition in fatty acid oxidation suggests a control strength of 1 for this enzymic step (Kacser & Burns, 1973). Brass & Hoppel (1980) argued that CPT could not be rate-limiting, on the basis of increased acylcarnitine concentrations in rats injected with carnitine without a concomitant change in blood ketone bodies. McGarry & Foster (1973), Cook *et al.* (1980) and Harano *et al.* (1982), in agreement with our results, found CPT I to catalyse the apparent rate-limiting step under the conditions studied.

Bt_2cAMP and glucagon decreased HPG inhibition of fatty acid oxidation by fed-rat hepatocytes while increasing the overall rate of fatty acid oxidation. Harano *et al.* (1985) found that Bt_2cAMP and glucagon caused a decrease in K_m of CPT I for palmitoyl-CoA, an increase in K_i of CPT I for malonyl-CoA, and the phosphorylation of an M_r-69000 anti-CPT immunoprecipitated protein. However, in the present study the decrease in sensitivity of hepatocyte fatty acid oxidation to HPG inhibition by incubation with Bt_2cAMP was not found to be accompanied by a decrease in HPG-sensitivity of CPT I activity, as assayed in filipin-treated hepatocytes. Total CPT I activity increased to the same extent as fatty acid oxidation increased with intact hepatocytes, suggesting that fatty acid oxidation can be stimulated by a mechanism that does not directly affect HPG sensitivity of CPT I. Saggerson *et al.* (1981) have reported that mitochondrial fatty acid oxidation can be significantly increased in homogenates of liver perfused with glucagon without affecting malonyl-CoA-sensitivity of CPT I.

Addition of lactate plus pyruvate or glucose to the incubation medium increased HPG inhibition of fatty acid oxidation by starved-rat hepatocytes without affecting the fatty acid oxidation rate. These oxidizable substrates are good precursors for malonyl-CoA formation and have been shown to inhibit fatty acid oxidation as well as to increase malonyl-CoA concentrations in hepatocytes (McGarry & Foster, 1979). The lack of effect of lactate plus pyruvate or glucose on fatty acid oxidation rate by hepatocytes from starved rats in the present study suggests that malonyl-CoA concentrations were not increased sufficiently by metabolism of these

substrates to cause inhibition of fatty acid oxidation. The addition of lactate plus pyruvate may decrease long-chain acyl-CoA by increasing fatty acid esterification (Stakkestad & Lund, 1984), perhaps resulting in higher sensitivity of CPT I to HPG inhibition. This is supported by the finding that mercaptopicolinate blocks the increase in sensitivity caused by lactate plus pyruvate (results not shown), probably by blocking the formation of glycerol 3-phosphate. Redox effects cannot be ruled out, but are not expected to be a direct factor at a 5:1 ratio of lactate to pyruvate. Addition of lactate plus pyruvate to the incubation of hepatocytes isolated from fed rats resulted in a small increase in HPG inhibition and a decrease in fatty acid oxidation rate at an oleate concentration of 0.5 mM (Table 1).

Incubation with carnitine resulted in a small increase in fatty acid oxidation rate under certain conditions. HPG inhibition was decreased when 0.5 mM-oleate, but not 2.0 mM-oleate, was used as substrate. HPG inhibition is competitive with carnitine (Stephens *et al.*, 1985), and carnitine concentrations have been found either to increase in starvation and diabetes (McGarry *et al.*, 1975) or not to change (Brass & Hoppel, 1980). By incubating hepatocytes with 4 mM-carnitine during the 30 min preincubation, intracellular concentrations as high as 2 mM can be obtained (Christiansen *et al.*, 1976). This value is considerably higher than found *in vivo* in livers of fed, starved or diabetic rats (McGarry *et al.*, 1975). HPG inhibition remained significantly higher with fed-rat than with starved-rat hepatocytes incubated with carnitine, suggesting that variation in carnitine concentration is not the explanation for the difference in sensitivity of HPG inhibition caused by nutritional state. Reversal of HPG inhibition of hepatocyte fatty acid oxidation by carnitine at low, but not high, oleate concentrations is similar to observed effects of carnitine on malonyl-CoA inhibition of CPT I (Bird & Saggerson, 1985).

Fatty acid oxidation was more than doubled and HPG inhibition decreased almost to half by increasing oleate concentration from 0.5 to 2.0 mM. Fatty acid oxidation was significantly correlated with HPG inhibition and oleate concentration when varied by starvation, diabetes and chow feeding, suggesting that sensitivity of CPT I to inhibition is a factor in determining fatty acid oxidation rate under these conditions.

Inhibition of CPT I activity of isolated mitochondria increased from 15 to 60% after 10 min of incubation in the presence of 20 μM-HPG. Malonyl-CoA inhibition of mitochondrial CPT I activity is also time-dependent (Zammit, 1984). Oleoyl-CoA caused a rapid activation of mitochondrial CPT I and a decrease in HPG inhibition in the current studies. Bremer *et al.* (1985) reported that palmitoyl-CoA desensitizes CPT I to malonyl-CoA inhibition. It is important to note here that HPG inhibition was unaffected when oleoyl-CoA concentrations were varied between 25 and 100 μM (Stephens *et al.*, 1985). For the latter studies, mitochondria had been preincubated with oleoyl-CoA and HPG in a KCl-containing medium for 5 min at 30 °C before starting the reaction with carnitine. Therefore, any sensitivity change may have been complete before assay of CPT I activity. Carnitine did not activate CPT I or affect HPG inhibition in a time-dependent manner in the current studies. Cook (1984*a*) has concluded that changes in sensitivity of CPT I owing to apparent hysteretic inhibition by malonyl-CoA are of insufficient duration and magnitude to explain the changes seen in sensitivity of mitochondria isolated from fed, starved and ketotic-diabetic animals. However, the degree of time-dependent change in HPG inhibition seen in the present study could account for the observed differences in HPG sensitivity of CPT I activity of mitochondria isolated from fed, starved and diabetic rats (60, 26 and 24% respectively). Phosphorylation of CPT I might result in some conformational change in the enzyme which is ionic-strength-, temperature- or time-dependent and which results in changes in affinity for substrate or inhibitors. Under the appropriate conditions, any of these factors might account for changes in sensitivity to inhibition by promoting the same conformational change in CPT I.

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