Phosphorylation of carnitine palmitoyltransferase and activation by glucagon in isolated rat hepatocytes

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Effects of glucagon and forskolin on the phosphorylation and changes of activity of carnitine palmitoyltransferase (CPT) have been studied in isolated rat hepatocytes using anti-CPT immunoglobulin. When the activity was determined in lysed hepatocytes after glucagon or forskolin treatment, it was found to be stimulated 30–80% mainly through increased affinity for palmitoyl-CoA. By SDS electrophoresis of the immunoprecipitates, CPT subunit (M_r 69000) was noted to be phosphorylated 4–5-fold with glucagon (1.2×10^{-7} M) and forskolin (0.1 mM) over control. These results indicate that hepatic ketogenesis is regulated with glucagon by phosphorylation of CPT through cAMP-dependent protein kinase.

Ketogenesis *Carnitine palmitoyltransferase* *Glucagon* *Forskolin* *Phosphorylation*

1. INTRODUCTION

Glucagon has been shown to stimulate hepatic ketogenesis probably through the stimulation of the carnitine palmitoyl-transferase (CPT) step [1–3]. A lowered malonyl-CoA level following glucagon has been advocated to stimulate the CPT step since malonyl-CoA is an inhibitor for CPT [4]. Although reciprocal changes of ketogenesis and fatty acid synthesis (malonyl-CoA level) are the basis for this theory, direct proof has not yet been provided. The mode of inhibition of CPT with malonyl-CoA is competitive with palmitoyl-CoA and therefore it is unlikely that the small changes of malonyl-CoA level regulate ketogenesis from exogenous palmitate (0.3–0.5 mM).

We have shown the direct activation of CPT in mitochondria isolated from hepatocytes after treatment with glucagon for 30 min, and proposed a direct regulatory mechanism of CPT by glucagon and insulin [5]. Cook et al. [6] also reported that the inhibitor concentration giving 50% inhibition for malonyl-CoA inhibition of CPT was much greater in mitochondria from starved or ketotic diabetic rats.

Here, the possibility of covalent modification of CPT through phosphorylation was studied in lysed hepatocytes after incubation with glucagon or forskolin for 15–30 min. In addition to the activities of CPT, ^{32}P incorporation into CPT was simultaneously determined using anti-CPT immunoglobulin which was obtained in rabbit by injecting purified rat liver CPT [7].

2. METHODS

2.1. *Isolation of hepatocytes*

Hepatocytes were isolated as described before with a cell viability over 95% from rats fed on laboratory chow [5]. The usual cell suspension ($400–600 \times 10^4$/ml) in Krebs-Ringer-bicarbonate buffer (pH 7.4) contains 30–40 mg/ml protein concentration.

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2.2. *Hormonal treatment and preparation of cell lysate*

Hepatocytes corresponding to 80–120 mg protein were preincubated with ^{32}P in phosphate-free Krebs-Ringer-Hepes buffer (pH 7.4) containing 2% bovine albumin in 2.6 ml for 30 min at 37°C in a conical flask. Then glucagon, insulin or forskolin was added and the mixture was further incubated for 15 and 30 min for study of ^{32}P incorporation and assay of CPT activity, respectively. Cells were washed with ice-cold 0.25 M sucrose containing 5 mM Hepes buffer (pH 7.4), 1 mM NaF, 0.1 mM EDTA 4 times by centrifugation at 1000 rpm for 3 min. 0.5 ml of 0.5% Triton X-100 was added to the cell precipitate, and the mixture frozen at −20°C for cell lysis. Then the lysed cell suspension was centrifuged at $1 \times 10^5 \times g$ for 60 min. CPT activity was determined using an aliquot of the supernatant by measuring carnitine-dependent CoA release from palmitoyl-CoA using a Shimazu UV 300 dual wave-length spectrophotometer [5].

2.3. *Immunoprecipitation of CPT*

To an aliquot of the supernate (0.2 ml), a sufficient amount of anti-CPT immunoglobulin (4 mg) was mixed and left overnight at 4°C. No activity was detectable in the supernate after centrifugation. The precipitate was washed 3 times with 0.15 M NaCl, 50 mM Tris-HCl buffer (pH 7.4), 2% Triton X-100, and once with 10 mM NaCl, 0.1% (w/v) SDS, 2 mM EDTA, 0.1% (v/v) Triton X-100. Finally, the washed precipitate weas mixed with 75 μl of the previous buffer containing DTT, and heated at 100°C for 10 min. The properties of the antibody against purified CPT from rat liver

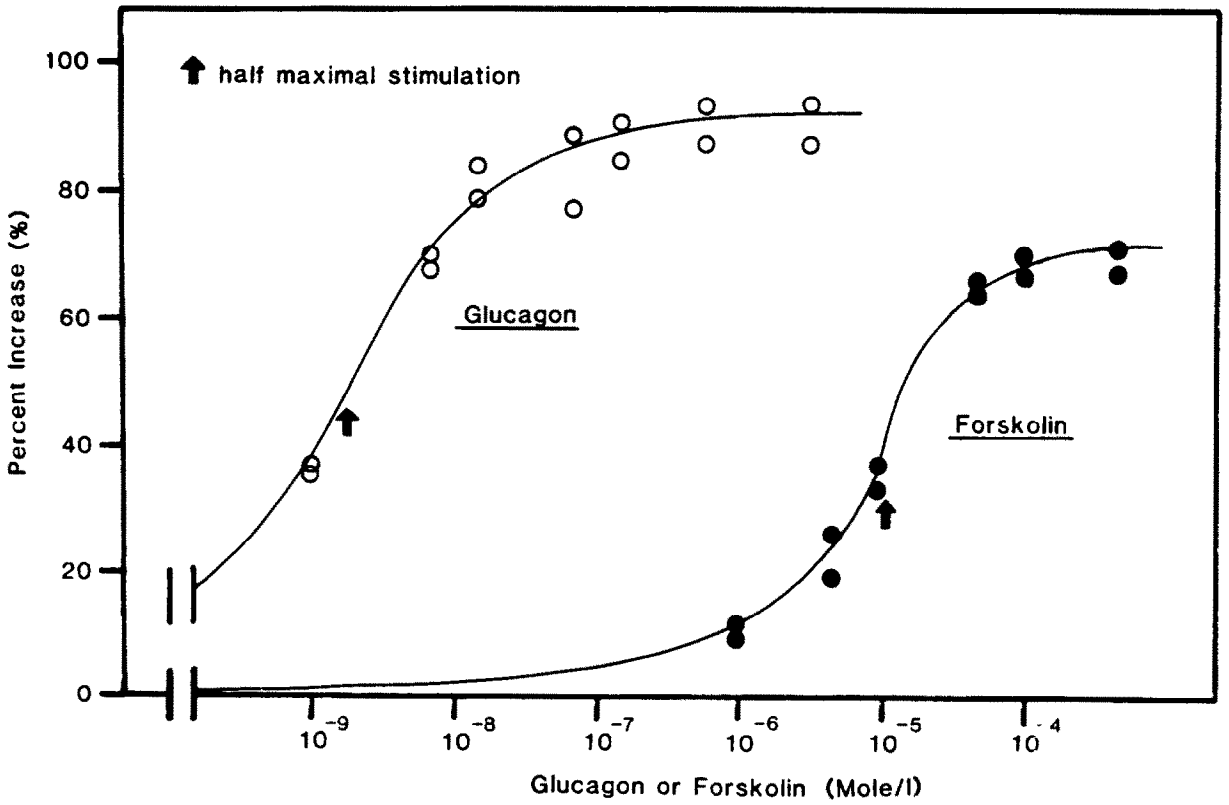

Fig.1. Dose-responsive stimulation of hepatic ketogenesis by glucagon and forskolin in isolated rat hepatocytes. Hepatocytes were incubated with 0.3 mM [U-^{14}C]palmitate bound to 0.3 mM albumin in the presence or absence of various concentrations of glucagon or forskolin [5]. The reaction was terminated by 3% PCA and the radioactivity determined in the supernate as described [5].

mitochondria have been described [7]. 1 mg IgG precipitated CPT corresponding to an activity of 1.7 nmol/min in the presence of 0.5% Triton.

2.4. *SDS-polyacrylamide gel electrophoresis*

Electrophoresis was carried out by the method of Weber and Osborn [8] using 10% polyacrylamide gel. The markers used were phosphorylase *b*, 97.4 kDa; bovine serum albumin, 67 kDa; GDH, 55.4 kDa and LDH, 36.5 kDa. The peptide was stained by Coomassie blue R-250. The density was determined by the transmission of light at 600 nm against 750 nm as a reference using a Shimazu Densitometer.

2.5. *Autoradiography of CPT immunoprecipitate obtained from cell lysate prelabelled with ^{32}P*

After staining for protein, the gel was covered with film (AR film Kodak X-Omat), and kept at −20°C for 2 weeks. After development, the ^{32}P-labelled band was quantified by reflection of light at 600 nm using the Shimazu Densitometer.

3. RESULTS

3.1. *Dose-responsive stimulation of ketogenesis by glucagon and forskolin in isolated rat hepatocytes*

Ketogenesis from [U-^{14}C]palmitate was stimulated by glucagon and forskolin in a dose-responsive manner as shown in fig.1. Maximum stimulation (90% over control) was observed at 10^{-7} M glucagon. A 70% stimulation was observed at 0.1 mM forskolin.

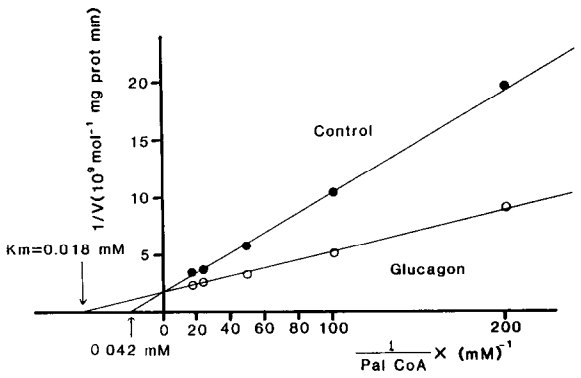

Fig.2. Lineweaver-Burk plots of CPT activity in relation to palmitoyl-CoA concentrations. Freshly isolated hepatocytes were incubated with or without 6.0×10^{-8} M glucagon for 30 min. Cells were centrifuged down, lysed with 0.5% Triton, then centrifuged at $1\times10^{5}\times g$ for 60 min. CPT activity was determined in the presence of 1 mM NaF using an aliquot of the supernate [5].

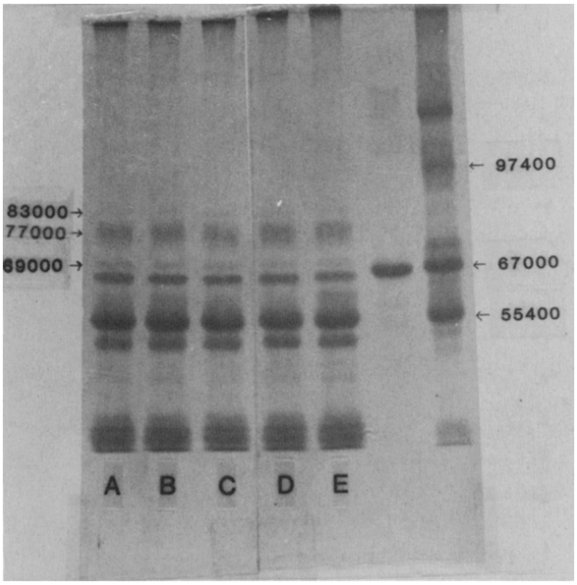

Fig.3. Peptide staining after SDS-polyacrylamide gel electrophoresis of CPT immunoprecipitate. Hepatocytes were preincubated with ^{32}P for 30 min. 15 min after the hormonal addition, the cells were lysed by 0.5% Triton and anti-CPT IgG was added to the lysed cell supernate. The precipitate was washed as described in section 2 and the peptide stained with Coomassie blue. (A) Control, (B) 6.9×10^{-8} M insulin, (C) 5.8×10^{-9} M glucagon, (D) 1.2×10^{-7} M glucagon, (E) 0.1 mM forskolin.

3.2. *Activation of carnitine palmitoyltransferase by glucagon and forskolin*

Mean CPT activity at 20 and 80 μM palmitoyl-CoA for the control was 3.4 ± 0.56 and 12.6 ± 0.89 nmol/mg protein per min (mean ± SE, $n=12$), respectively. Glucagon (6.0×10^{-8} M) or forskolin (0.1 mM) treatment for 30 min enhanced CPT activity by 70–80% at 20 μM palmitoyl-CoA and by 30–50% at 80 μM palmitoyl-CoA over normal. Lineweaver-Burk plots revealed that K_m for palmitoyl-CoA in the control lysed hepatocytes was 42 μmol/ml, which decreased to 18 μmol/ml in the cell lysate after glucagon treatment (fig.2). A similar increase of the affinity for palmitoyl-CoA was observed for forskolin.

3.3. *SDS-polyacrylamide gel electrophoresis of the immuno-precipitate after incubation of the cell supernatant with anti-CPT IgG*

There are at least 4 bands (M_r 83 000, 77 000, 69 000, 67 000) other than those derived from the heavy or light chains of IgG (fig.3). A protein band corresponding to M_r 69 000 was identical with the CPT subunit (M_r 69 200), which has been demonstrated by Miyazawa et al. [7]. The other 3 protein bands were not identified, but seemed to be polypeptides derived from coprecipitate with CPT antibody.

3.4. *Effect of glucagon, insulin or forskolin on ^{32}P labelling of the CPT subunit*

Fig.4 shows an autoradiogram taken after SDS gel electrophoresis of CPT precipitated with anti-CPT IgG. The CPT subunit (M_r 69 000) was darkly stained in samples obtained from glucagon (1.2×10^{-7} M) or forskolin (0.1 mM) treated hepatocytes, and only vague lines were visible in samples from the control, insulin or low glucagon (5.8×10^{-9} M)/1-treated hepatocytes. The other distinct staining which was as dark as the CPT subunit, was observed in the larger polypeptide band (M_r 83 000). However, the nature of this peptide has not yet been identified. The other 2 polypeptide bands (M_r 77 000 and 67 000) seen in fig.3 were not phosphorylated under the incubation conditions.

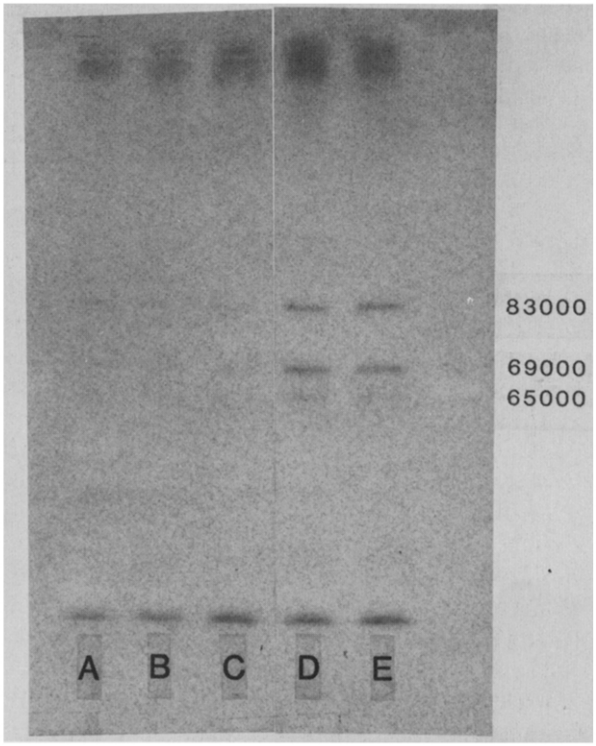

Fig.4. Autoradiography of CPT immunoprecipitate obtained from cell lysate after various hormonal treatments. An autoradiogram was obtained as described in section 2. Sample application from A to E was the same as in fig.3.

Table 1

Specific ^{32}P incorporation into immunoprecipitable CPT separated by polyacrylamide gel electrophoresis

Treatment	^{32}P incorporation to CPT band/protein concentration of CPT band	
	%	Fold over control
Control	6.8	1
Insulin (6.9×10^{-8} M)	8.2	1.2
Glucagon (5.8×10^{-9} M)	10.6	1.6
Glucagon (1.2×10^{-7} M)	32.1	4.7
Forskolin (1×10^{-4} M)	25.3	3.7

The ratio of ^{32}P incorporation into the CPT subunit to the amount of peptide determined by densitometry is shown in table 1. While insulin treatment had no effect on the ^{32}P incorporation into CPT, the low dose of glucagon stimulated 1.6-fold, and 1.2×10^{-7} M glucagon and forskolin stimulated ^{32}P incorporation into CPT subunit by 4.7- and 3.7-times, respectively, over the control (table 1).

4. DISCUSSION

Carnitine palmitoyltransferase activity was determined in lysed hepatocytes with 0.5% Triton rather than in isolated mitochondria after incubation with hormones for 30 min. This method omitted the mitochondrial isolation step and enabled estimation of total CPT activity without any loss in recovery. In our previous report, CPT 1 was shown to be increased in mitochondria isolated from hepatocytes treated with glucagon for 30 min [5]. Here, we have not attempted to quantify CPT 1 and 2, but aimed to determine the changes in

total CPT activity following the hormonal treatment. During the purification step of CPT in rat liver, Miyazawa et al. [7] were unable to find heterogeneous CPT in solubilized rat liver mitochondria, and only a single precipitation line was observed in the immuno-diffusion plates between liver extract and anti-CPT IgG. Thus, CPT 1 and 2 may be the same protein, although it is localized both inside or outside the barrier for acyl-CoA transport as shown by Hoppel and Tomex [9]. The identical properties of both CPT 1 and 2 have been reported by Bergstrom and Reitz [10]. The CTP activity was found to increase in lysed hepatocytes after glucagon or forskolin treatment. The increased CPT activity was mainly attributed to the increase in affinity for palmitoyl-CoA rather than V_{max}, when analyzed by Lineweaver-Burk plots. This is consistent with the observation that in isolated hepatocytes, enhanced palmitate oxidation was more significant at lower concentrations (0.1–0.5 mM) rather than at higher concentrations (0.5–1.0 mM) of palmitate.

When ^{32}P incorporation into the CPT subunit was studied in lysed hepatocytes, the obvious ^{32}P incorporation was demonstrated by radioautography in the CPT subunit obtained from glucagon or forskolin treated hepatocytes. The weaker stimulation of phosphorylation with lower concentrations of glucagon is consistent with the observation that stimulation of palmitate oxidation with glucagon is not maximal at lower glucagon levels because of the considerable degradation occurring during incubation. The nature of the other polypeptide (M_r 83 000) which was also highly phosphorylated with glucagon or forskolin was not identified. This polypeptide is larger than the precursor of CPT(71 kDa) [11]. Since the antibody was found to be unreactive with carnitine octanoyltransferase or acetylcarnitine transferase, these bands are most likely to be coprecipitates of the proteins which tightly bound to CPT and coprecipitated with anti-CPT. Carnitine translocase seemed to be tightly bound to CPT and this might be one of the possibilities.

This study has presented suggestive evidence for the phosphorylation of CPT. An activation of CPT was also observed 15 min after the hormonal treatment, indicating that the activation is most likely due to the consequence of enzyme phosphorylation. A dose-response stimulation of ketogenesis from exogenous palmitate (0.3–0.5 mM) with glucagon or forskolin is better explained by covalent modification of CPT itself rather than the small changes in endogenous malonyl-CoA level as proposed by McGarry et al. [4]. Palmitate oxidation has been shown by our laboratory [12] to be stimulated by epinephrine through α_1-adrenergic receptor. Since esterification of palmitate was not affected by epinephrine, the stimulation of palmitate oxidation may be mediated through Ca-calmodulin dependent protein kinase. The phosphorylation of CPT with epinephrine has not been investigated here.

In this study, CPT was determined in hepatocytes treated with glucagon or forskolin for 30 min in contrast to 15 min in the case of ^{32}P-labelling experiments. However, activation of CPT was also noted at 15 min after glucagon or forskolin addition. Activated CPT seemed to be less sensitive to malonyl-CoA inhibition, since 20 μmol/l malonyl-CoA inhibited CPT by 50–60 or 20–30% in lysed cells treated with saline or glucagon, respectively.

In this study, CPT activity was determined in a lysed cell supernate. However, the K_m for palmitoyl-CoA was similar to that in isolated intact mitochondria from control or glucagon-treated hepatocytes (K_m, 25 and 13 μM for control and glucagon-treated mitochondria). Also, inhibition of malonyl-CoA (K_i 25 μM) in intact mitochondria from control hepatocytes was similar to that in lysed hepatocytes. The K_i value was 10-times higher than that reported by McGarry et al. They reported that in a lysed mitochondria, malonyl-CoA became less inhibitory, although the mechansim was not shown. Hepatocytes which were freshly isolated from meal-fed rats were reported to contain 2.5 ± 0.2 nmol malonyl-CoA per g cells [13]. It is questionable that CPT activity is only regulated by changes in this low level of malonyl-CoA. Under certain conditions, malonyl-CoA may play a role in the regulation of hepatic fatty acid oxidation, but covalent modification of CPT through phosphorylation may play a more important role in the mechanism of hormonal control of hepatic fatty acid oxidation.

REFERENCES

[1] Harano, Y., Kowal, J., Yamazaki, R., Lavine, L. and Miller, M. (1972) Biochem. J. 153, 426–437.

[2] Norman, P.T., Ingerestsen, O.C. and Flatmark, T. (1978) Biochim. Biophys. Acta 501, 286–295.
[3] McGarry, J.D., Mannaerts, G.P. and Foster, D.W. (1978) Biochim. Biophys. Acta 530, 305–313.
[4] McGarry, J.D., Takabayashi, Y. and Foster, D.W.J (1978) J. Biol. Chem. 253, 8294–8300.
[5] Harano, Y., Kosugi, K., Kashiwagi, A., Nakano, T., Hidaka, H. and Shigeta, Y, (1982) J. Biochem. 91, 1739–1748.
[6] Cook, G.A., Stephens, T.W. and Harris, R.A. (1984) Biochem. J. 219, 337–339.
[7] Miyazawa, S., Ozawa, H., Osumi, T. and Hashimoto, T. (1983) J. Biochem. 94, 529–542.
[8] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[9] Hoppel, C.L. and Tomex, R. (1972) J. Biol. Chem. 247, 832–841.
[10] Bergstrom, J.D. and Reitz, R.C. (1980) Arch. Biochem. Biophys. 204, 71–79.
[11] Ozasa, H., Miyazawa, S. and Osumi, T. (1983) J. Biochem. 94, 543–549.
[12] Kosugi, K., Harano, Y., Nakano, T., Suzuki, M., Kashiwagi, A. and Shigeta, Y. (1983) Metabolism 32, 1081–1087.
[13] Cook, G.A., King, M.T. and Veeck, A.R. (1978) J. Biol. Chem. 253, 2529–2531.