A suppressive role of c-kinase for the stimulation of hepatic ketogenesis by glucagon and epinephrine

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The regulatory mechanism of hepatic palmitate oxidation into ketone bodies by c-kinase has been studied in isolated hepatocytes. Glucagon and epinephrine stimulated [U-^{14}C]palmitate oxidation to ketone bodies by 60 and 25% as early as at 1 h. The stimulatory effects were almost totally prevented by the simultaneous presence of vasopressin, phorbol 12-tetradecanoate 13-acetate (TPA), or diacylglycerol (1-oleoyl-2-acetylglycerol). When hepatocytes were treated with glucagon or epinephrine, carnitine palmitoyltransferase (CPT), a key regulatory enzyme of palmitate oxidation, was activated. This hormone-induced activation of CPT was not observed in the presence of TPA. These observations suggest that c-kinase inhibits glucagon- or epinephrine-stimulated palmitate oxidation to ketone bodies, and that this inhibition may be mediated through a covalent modification of CPT.

Long-chain fatty acid oxidation *Ketogenesis* *c-Kinase* *Glucagon* *Epinephrine* *Carnitine palmitoyltransferase*

1. INTRODUCTION

Hepatic ketogenesis has been reported to be stimulated by glucagon and catecholamines [1,2]. We have recently proposed a new regulatory mechanism by covalent modification of carnitine palmitoyltransferase (CPT) [3,4]. Glucagon probably stimulates ketogenesis by the activation of cAMP-dependent protein kinase (A-kinase) [3] while epinephrine or norepinephrine may similarly stimulate it through α_1-adrenergic receptors and probably via Ca-calmodulin-dependent protein kinase [2]. Recently, a third protein kinase (c-kinase) has been discovered by Nishizuka, and new regulatory roles in hepatic glucose metabolism have been suggested [5]. Hems and Whitton [6] have reported that vasopressin, which activates c-kinase, stimulates hepatic glycogenolysis through activation of phosphorylase [7], the data suggesting an involvement of c-kinase. In contrast, vasopressin seems to inhibit glucagon-stimulated ketogenesis in isolated hepatocytes [7]. The effect of vasopressin on catecholamine-stimulated ketogenesis has not yet been studied.

Here, the effect of vasopressin, TPA and diacylglycerol (a second messenger of the c-kinase system) on hepatic ketogenesis and CPT activity has been investigated in attempting to show a possible interaction of 3 protein kinases (A-kinase, Ca-calmodulin-dependent protein kinase, c-kinase).

2. MATERIALS AND METHODS

2.1. *Materials*

[U-^{14}C]Palmitate (500 mCi/mmol) and aqueous counting scintillant (ACS-II) were obtained from the Research Centre, Amersham, England. Collagenase class 2 (133 U/mg) was obtained from Worthington, Freehold, NJ. Bovine serum albumin (fraction V), essentially FFA-free bovine albumin, vasopressin and TPA were purchased from Sigma, St. Louis, MO. Diacylglycerol (1-oleoyl-2-acetylglycerol) was kindly provided by

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Professor Y. Nishizuka, Kobe University, Kobe, Japan. Insulin-free glucagon was a gift from Dr J.A. Galloway, Eli Lilly Research Laboratories, Indianapolis, IN. TPA was freshly dissolved in DMSO at 5 mg/ml and diacylglycerol in DMSO at 10 mg/ml so that the DMSO concentration in the assay was always <0.1%. Each control also contained the corresponding amount of DMSO. Other reagents were analytical grade and were obtained from Nakarai Chemicals, Kyoto, Japan.

2.2. *Isolation of hepatocytes*

Male Sprague-Dawley rats weighing 250–300 g each were fed ad libitum on regular laboratory chow (CLEA-CEII, CLEA Japan, Osaka) until the time of the experiment. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (5 mg/100 g).

Hepatocytes were isolated by a modification of Seglen's method as described [2,8]. After being washed twice by centrifugation, cells were suspended in Krebs-Ringer-bicarbonate buffer at a concentration of 6–8 $\times$ 10^6 cells per ml (10.3 $\pm$ 1.89 mg protein/ml, mean $\pm$ SD). The isolated hepatocytes were more than 95% viable as judged by trypan blue exclusion. Protein was determined using the Bio-Rad assay after being solubilized in 0.1% SDS.

2.3. *Study of palmitate oxidation and ketogenesis*

The assay system consisted of 0.3 mM [U-^{14}C]palmitate bound to 0.3 mM albumin, 100 mg/dl glucose, and 11.5 mM Hepes in Krebs-Ringer-phosphate buffer (pH 7.4) in a total volume of 2.0 ml in 25 ml siliconized Erlenmeyer flasks filled with 95% O_2 and 5% CO_2 (v/v). The reaction was started by the addition of hepatocytes (300–400 $\times$ 10^4 cells corresponding to 4.2–5.6 mg protein). The flasks were sealed with rubber stoppers with hanging small caps and incubated for 1 h at 37°C. At the end of incubation, 1 ml of 10% perchloric acid (PCA) was added to the main flask, and the liberated CO_2 was absorbed in 0.3 ml scintilamine-OH which was placed in the small cap for 1 h. This mixture was centrifuged at 700 $\times$ g for 10 min. ^{14}C counts of 0.5 ml of the supernatant and 0.3 ml of scintilamine-OH were measured respectively by mixing with 5 ml ACS-II. The radioactivity measured in the acid-soluble fraction represents the β-oxidation products and almost exclusively ketone body formation as reported [2,3,9].

Glucagon and epinephrine stimulated ketogenesis in a dose-dependent manner and the dose which exhibited maximal response was used in this experiment. [U-^{14}C]Palmitate oxidation to acid-soluble products (mainly ^{14}C-ketone bodies) and $^{14}CO_2$ for 1 h were 12.2 $\pm$ 0.19 and 2.4 $\pm$ 0.33 nmol palmitate/mg protein, respectively. Thus, under the above assay conditions, the majority of the oxidation products were ketone bodies and only 20% was CO_2. Therefore, in most experiments, [U-^{14}C]palmitate oxidation was measured by determining acid-soluble ketone body production.

2.4. *Determination of CPT activity*

Isolated hepatocytes were incubated with hormones for 20 min at 37°C as described above without labelled palmitate. Cells were centrifuged at 700 $\times$ g for 3 min. 0.5 ml buffer containing 0.25 M sucrose, 5 mM Hepes, 1 mM NaF, 0.1 mM EDTA and 0.5% Triton X-100 was added to the cell precipitate, and the mixture frozen at -20°C. The lysed cell suspension was then centrifuged at 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 UV300 dual-wavelength spectrophotometer [3]. Activity was determined at low (20 μM) and high (80 μM) concentrations of palmitoyl-CoA.

3. RESULTS

3.1. *Effect of glucagon, epinephrine and c-kinase stimulants on hepatic ketogenesis*

Maximal stimulation of ketogenesis was observed at 200 ng/ml glucagon and 1 μg/ml epinephrine, amounting to 60 and 25% over control, respectively (table 1). Vasopressin (10 ng/ml), TPA (100 nM and 5 μM) or diacylglycerol (10 μg/ml) alone did not affect [U-^{14}C]palmitate oxidation.

3.2. *Effect of c-kinase stimulants on glucagon- or epinephrine-stimulated ketogenesis*

Fig.1 shows dose-responsive inhibition of glucagon-stimulated ketogenesis and CO_2 production by TPA. TPA totally inhibited the glucagon-

Table 1

Effect of glucagon, epinephrine and c-kinase stimulants on ketogenesis in freshly isolated hepatocytes

Additions	No. of experiments	[U-^{14}C]Palmitate oxidized into acid-soluble fraction (nmol palmitate/mg protein per h)
Control	23	12.2 ± 0.19
Glucagon (200 ng/ml)	9	19.0 ± 0.14[a]
Epinephrine (1 μg/ml)	17	15.3 ± 0.45[a]
Vasopressin (10 ng/ml)	16	11.9 ± 0.20
TPA (100 nmol/l)	5	11.6 ± 0.32
TPA (5 μM)	10	11.4 ± 0.21
Diacylglycerol (10 μg/ml)	6	12.0 ± 0.10

[a] $p < 0.001$ vs control

Each value is the mean ± SE. Results were evaluated using Student's *t*-test. Freshly isolated hepatocytes were incubated with 0.3 mmol/l [U-^{14}C]palmitate bound to 0.3 mmol/l albumin in the presence or absence of the indicated compounds. The reaction was terminated with 3% PCA and the radioactivity determined in the supernatant as in [4]

induced effects at 10^{-8}–10^{-5} M. In epinephrine-stimulated ketogenesis and CO_2 production, TPA also shows inhibition in a dose-responsive manner (fig.2).

In addition to TPA (100 nM and 5 μM), vasopressin (10 ng/ml), and diacylglycerol (10 μg/ml) almost totally prevented the glucagon-induced stimulation of ketogenesis (fig.3). Similarly, vasopressin, diacylglycerol, and TPA reversed the epinephrine-induced stimulation of hepatic ketogenesis.

3.3. *Effects of glucagon, epinephrine, and TPA on CPT activity*

Mean CPT activity at 20 and 80 μM palmitoyl-CoA for the control was 5.8 ± 0.6 and 11.9 ± 1.4 nmol/mg protein per min (mean ± SE, $n = 5$), respectively (table 2). Glucagon (200 ng/ml) treatment for 20 min enhanced CPT activity by 78% in lysed cell supernatant at 20 μM palmitoyl-CoA,

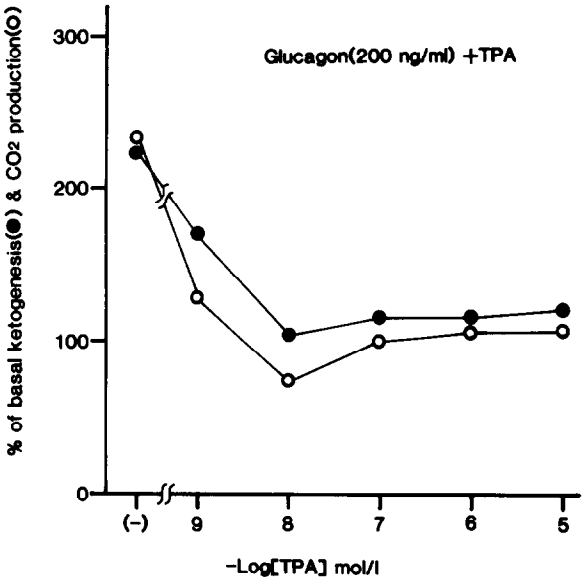

Fig.1. Inhibitory effect of TPA on glucagon-stimulated palmitate oxidation. Hepatocytes were incubated for 1 h with 0.3 mM [U-^{14}C]palmitate bound to 0.3 mM albumin in the presence of glucagon (200 ng/ml) and various concentrations of TPA. Each value is the mean of 2 experiments. Percent oxidation into ketone bodies (●) and CO_2 production (○) over control are shown on the ordinate. Mean value of control ($n = 8$) was 12.2 ± 0.19 and 2.4 ± 0.33 (nmol palmitate/mg protein) for the rate of ketone body and CO_2 production, respectively. (−) Experiments without TPA.

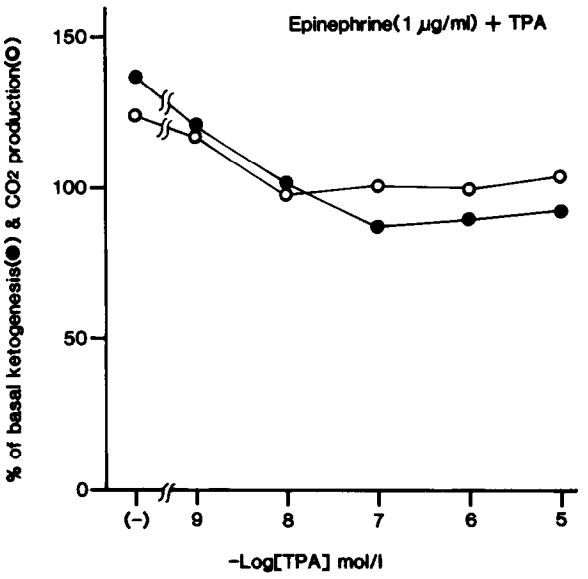

Fig.2. Inhibitory effect of TPA on epinephrine-stimulated palmitate oxidation. Hepatocytes were incubated under the same conditions as in fig.1.

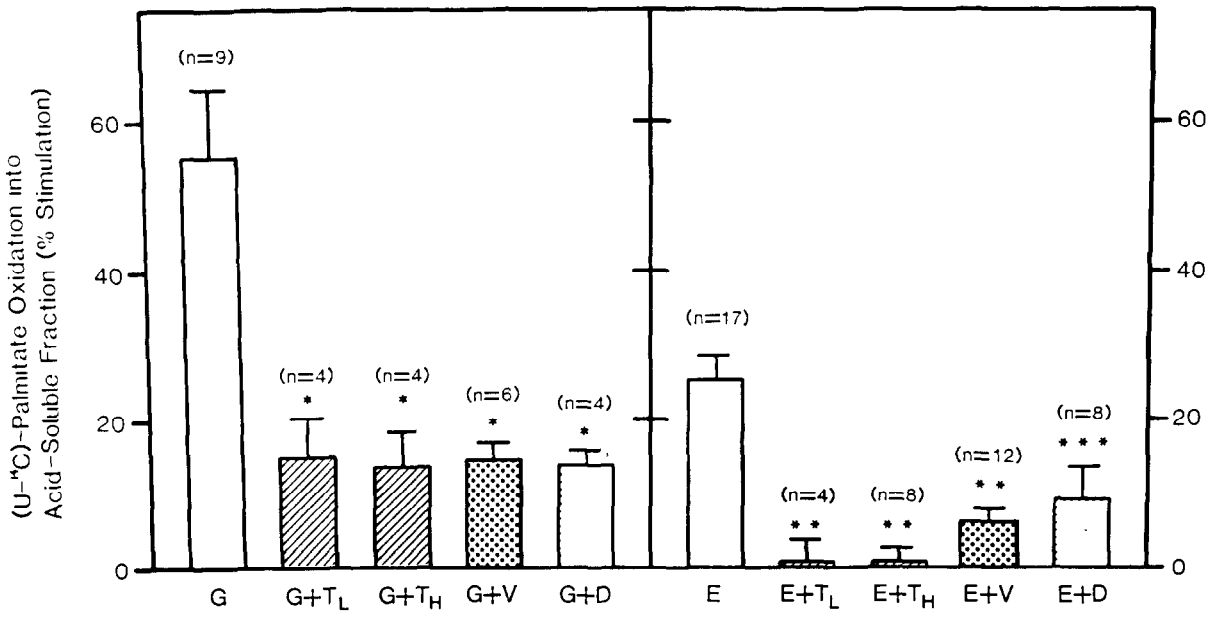

Fig.3. Effect of c-kinase stimulants of glucagon- or epinephrine-stimulated ketogenesis in isolated hepatocytes. Hepatocytes were incubated with 0.3 mM [U-^{14}C]palmitate bound to 0.3 mM albumin in the presence of glucagon (200 ng/ml) (G), glucagon (200 ng/ml) and TPA (100 nM) (G + T_L), glucagon (200 ng/ml) and TPA (5 μM) (G + T_H), glucagon (200 ng/ml) and vasopressin (10 ng/ml) (G + V), or glucagon (200 ng/ml) and diacylglycerol (10 μg/ml) (G + D) (left panel). The same experiments were performed with epinephrine (1 μg/ml) (E), epinephrine (1 μg/ml) and TPA (100 nM) (E + T_L), epinephrine (1 μg/ml) and TPA (5 μM) (E + T_H), epinephrine (1 μg/ml) and vasopressin (10 ng/ml) (E + V), or epinephrine (1 μg/ml) and diacylglycerol (10 μg/ml) (E + D) (right panel). Each value is the mean ± SE. Results were evaluated using Student's t-test. * $p < 0.005$ vs glucagon. ** $p < 0.025$ vs epinephrine. *** $p < 0.05$ vs epinephrine.

and by 68% at 80 μM palmitoyl-CoA over control. Epinephrine (1 μg/ml) treatment enhanced CPT activity by 79% at 20 μM palmitoyl-CoA and by 69% at 80 μM palmitoyl-CoA over control. Addition of TPA (5 μM) to the incubation mixture reversed the activation of CPT by both glucagon and epinephrine.

4. DISCUSSION

The present studies demonstrate that vasopressin (10 ng/ml), TPA (100 nM) and diacylglycerol (10 μg/ml) all inhibited glucagon- as well as epinephrine-stimulated hepatic ketogenesis. Vasopressin, TPA, or diacylglycerol alone showed no effect on basal ketogenesis. Furthermore, TPA inhibited the increase in CPT activity induced by glucagon or epinephrine in cell lysates, suggesting that TPA may regulate CPT activity through possible involvement of c-kinase. c-Kinase, which is activated by Ca^{2+}, phospholipid and diacylglycerol, is widely present in various tissues [10]. In the liver, much attention has been paid to the possible role of c-kinase in the regulation of glucose metabolism. In isolated rat hepatocytes, TPA antagonizes both glucagon-stimulated adenylate cyclase activity [11] and the epinephrine-induced stimulation of glycogenolysis [12]. Regarding ketogenesis, inhibition of glucagon-stimulated ketogenesis by vasopressin has been reported by Williamson et al. [8], but the precise mechanism remains unclear. Vasopressin generates two intracellular messengers, diacylglycerol and Ca^{2+} [13]. Recently, Morgan et al. [14] reported that vasopressin inhibited glucagon-induced cAMP accumulation, which was not dependent on cellular Ca^{2+} mobilization. Therefore the c-kinase system might involve direct inhibition of adenylate cyclase. Thus, c-kinase stimulants (TPA, vasopressin, diacylglycerol) may inhibit glucagon-

Table 2

Early effect of glucagon, epinephrine and TPA on CPT activity in lysed hepatocytes

Additions	CPT activity (nmol/mg protein per min)	
	Palmitoyl-CoA (20 μmol/l)	Palmitoyl-CoA (80 μmol/l)
Control	5.8 ± 0.6	11.9 ± 1.4
Glucagon (200 ng/ml)	10.3 ± 1.3[a]	20.6 ± 1.5[a]
Epinephrine (1 μg/ml)	10.4 ± 0.9[a]	20.1 ± 2.2[a]
TPA (5 μM)	6.2 ± 1.2	11.0 ± 2.7
Glucagon (200 ng/ml) + TPA (5 μM)	7.6 ± 0.6[b]	12.3 ± 0.3[b]
Epinephrine (1 μg/ml) + TPA (5 μM)	6.0 ± 0.9[c]	10.1 ± 0.6[c]

[a] $p < 0.025$ vs control
[b] $p < 0.025$ vs glucagon
[c] $p < 0.025$ vs epinephrine

Each value is the mean ± SE of 5 experiments. Results were evaluated by the use of paired *t*-test. Freshly isolated hepatocytes were incubated with or without the indicated compounds for 20 min. Cells were centrifuged down, lysed with 0.5% Triton X-100, then centrifuged at $1 \times 10^5 \times g$ for 60 min. CPT activity was determined using an aliquot of supernatant

stimulated ketogenesis either through direct inhibition of adenylate cyclase or via the inhibition of CPT.

Recently, Cooper et al. [13] have reported that pretreatment with phorbol ester or synthetic diacylglycerol inhibits phenylephrine-induced elevation of cytosolic Ca^{2+} concentration in isolated hepatocytes. This observation is consistent with the hypothesis that c-kinase modulates CPT activity through the inhibition of Ca^{2+} mobilization. However, there is still a possibility that different hormones display site-specific phosphorylation as a regulatory mechanism of CPT activity [4,15].

As for the activation of ketogenesis by glucagon, two mechanisms have been proposed. McGarry et al. [16] reported that glucagon decreased acetyl-CoA carboxylase activity and that the decreased formation of malonyl-CoA levels might stimulate hepatic ketogenesis. Recently, a new regulatory mechanism of ketogenesis has been proposed from our laboratory; glucagon stimulates hepatic ketogenesis via phosphorylation of CPT through cAMP-dependent protein kinase [4]. The increased CPT activity was mainly attributed to the increased affinity for palmitoyl-CoA rather than the enhancement of maximal velocity (V_{max}). This was confirmed at the two different concentrations of palmitoyl-CoA (20 and 80 μM) in this study.

The stimulatory effect of catecholamine on hepatic palmitate oxidation was reversed by prazosine (α_1-blocker) and W7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; a calmodulin antagonist] but not by yohimbine (α_2-blocker) [17]. Therefore, epinephrine-stimulated palmitate oxidation is most likely mediated by α_1-adrenergic receptors through the activation of Ca-calmodulin-dependent protein kinase. Oberhaensli et al. [18] have postulated that stimulation of ketogenesis by norepinephrine may be due to the decreased formation of malonyl-CoA, but our data suggest that epinephrine stimulates CPT possibly through phosphorylation as in the case of stimulation with glucagon, although direct evidence for the phosphorylation of CPT has not been obtained.

Since comparable changes of CPT activity in relation to palmitate oxidation were observed, direct activation of CPT by A-kinase or Ca-calmodulin-dependent protein kinase is thought to take place with glucagon and epinephrine, respectively, and c-kinase seems to antagonize the stimulation of hepatic ketogenesis stimulated by glucagon and epinephrine at the level of carnitine palmitoyltransferase.

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