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Okadaic acid stimulates carnitine palmitoyltransferase I activity and palmitate oxidation in isolated rat hepatocytes

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Okadaic acid increased in parallel with carnitine palmitoyltransferase I activity and the rate of palmitate oxidation in isolated rat hepatocytes. Nevertheless, okadaic acid had no significant effect on the rate of octanoate oxidation. Maximal effects of okadaic acid were similar and non-additive to those of dibutyryl-cAMP, forskolin and glucagon. Results indicate that carnitine palmitoyltransferase I activity may be controlled by a mechanism of phosphorylation/dephosphorylation.

Okadaic acid; Fatty acid oxidation; Carnitine palmitoyltransferase I; Hepatocyte

1. INTRODUCTION

Carnitine palmitoyltransferase I (CPT-I), the outer form of carnitine palmitoyltransferase (CPT), plays a key role in controlling the rate of long-chain fatty acid oxidation in the liver [1–3]. Together with a carnitine:acylcarnitine translocase and the inner form of CPT, carnitine palmitoyltransferase II (CPT-II), CPT-I mediates the entry of long-chain fatty acids into mitochondrial oxidative metabolism [1–3]. Both the kinetic and the regulatory properties of CPT-I change with the nutritional and hormonal status of the animal [1–4]. However, the molecular bases underlaying these alterations of the CPT-I enzyme are still a matter of controversy [3,5–7].

Short-term adaptive changes of hepatic CPT-I have thus far not received as much attention as long-term adaptive changes. This may be due to the fact that short-term modulation of CPT-I activity is difficult to preserve during the procedure of cell disruption and isolation of mitochondria for enzyme assay. This problem may be circumvented by assaying CPT-I activity in a permeabilized-cell system [8]. The use of this procedure has shown that rat liver CPT-I is regulated on the short term by different types of agonists, including some which are putative modulators of the phosphorylation state of the enzyme [8,9]. These observations thus suggest that a mechanism of phosphorylation–dephosphorylation might be involved in the control of hepatic CPT-I activity. In fact, Harano et al. [10] have observed that glucagon increases both the activity and the phosphorylation state of CPT in short-term hepatocyte incubations. Nevertheless, these authors only determined the activity of the total CPT system. In addition, their 69-kDa immunoprecipitated CPT protein is now believed to be the CPT-II enzyme (see e.g. [7,11,12]).

Very recently, an extremely useful probe for the study of protein phosphorylation in intact cells has become available. This is okadaic acid, a powerful and specific inhibitor of protein phosphatases 1 and 2A [13,14], which are the major protein phosphatases acting on many phosphoproteins in vivo [13–15]. Enzymes whose phosphorylation state is increased in hepatocytes after cell incubation with okadaic acid include the rate-limiting enzymes of glycogen synthesis and degradation , glycolysis, gluconeogenesis and fatty acid synthesis [13,14]. In other tissues, okadaic acid affects a great number of metabolic processes which may thus be supposed to be controlled by a mechanism of phosphorylation-dephosphorylation [14,16–18]. Therefore, in the present study we examined the effects of okadaic acid on hepatic CPT-I activity and fatty acid oxidation.

2. MATERIALS A[illegible] [illegible]ETHODS

2.1. *Hepatocyte isolation and [illegible]ation*

Male Wistar rats (225–275 g) were used throughout this study. Hepatocytes from the whole liver were isolated as described in [19], whereas periportal and perivenous hepatocytes were isolated by the method of Chen and Katz [20] and further characterized on the basis of the distribution pattern of several marker enzymes [21]. Hepatocytes were incubated in Krebs–Henseleit bicarbonate buffer supplemented with 10 mM glucose and 2% (w/v) defatted and dialysed bovine serum albumin as described before [21].

Abbreviations: CPT, carnitine palmitoyltransferase; CPT-I and CPT-II, outer and inner form of CPT, respectively

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2.2. *Rates of fatty acid oxidation*

In order to monitor rates of palmitate and octanoate oxidation, hepatocytes were preincubated for 20 min in the presence of the additions indicated. Then, reactions were started by the addition of 0.4 mM albumin- bound [1-^{14}C]fatty acid (either palmitate or octanoate, 0.5 Ci/mol) and carried on for 20 min. Reactions were stopped with 0.5 ml of 2 M perchloric acid and oxidation products were quantified as described before [21]. Results show the rates of fatty acid oxidation to total oxidation products, which were calculated as the sum of acid-soluble products plus CO_2. Acid-soluble products, which mainly represent ketone bodies, routinely accounted for more than 85% of total oxidation products (results not shown).

2.3. *CPT-I assay*

CPT-I activity was determined in digitonin-permeabilized hepatocytes as described in [8]. The final assay mixture contained 12.5 mM Tris–HCl (pH 7.4), 70 mM sucrose, 5 mM glucose, 32.5 mM KCl, 12.5 mM $NaHCO_3$, 60 mM NaCl, 0.6 mM KH_2PO_4, 0.6 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1 mM EDTA, 1 mM dithioerythritol, 50 μM palmitoyl-CoA, 0.5 mM L-[*Me*-^{14}C] carnitine (1 Ci/mol), 0.5% bovine serum albumin (defatted and dialysed) and 40 μg of digitonin/mg of cellular protein. Reactions were stopped by the addition of 0.4 ml of 1 M HCl and [^{14}C]palmitoylcarnitine product was extracted with butan-1-ol [8]. Malonyl-CoA- insensitive CPT activity, representing CTP-II activity [3,22,23], was always substracted from the CPT activity experimentally determined. CPT-II activity always accounted for less than 15% of the total CPT activity experimentally determined. Okadaic acid had no significant effect on CPT-II activity (results not shown).

2.4. *Other methods*

Acetyl-CoA carboxylase and fatty acid synthase activities were determined in a permeabilized-hepatocyte system as described before [21,24]. Rates of fatty acid synthesis de novo were monitored as the incorporation of ^3H_2O into total fatty acids [24].

3. RESULTS AND DISCUSSION

The effects of okadaic acid on fatty acid oxidation and CPT-I activity were studied in short-term hepatocyte incubations. As a control, we always determined in parallel the effects of okadaic acid on acetyl-CoA carboxylase activity and on the rate of fatty acid synthesis de novo. In agreement with published data [13], both acetyl-CoA carboxylase activity and the rate of fatty acid synthesis de novo were strongly reduced (more than 85% inhibition in all experiments) after hepatocyte incubation with 1 μM okadaic acid for 20 min. In addition, 1 μM okadaic acid had no effect on the activity of fatty acid synthase, an enzyme which is not believed to be regulated by a mechanism of phosphorylation–dephosphorylation (results not shown).

Addition of okadaic acid to the incubation medium increased the rate of palmitate oxidation in a dose-dependent manner, maximal effects observed with 1 μM okadaic acid (Fig. 1). Nevertheless, okadaic acid had no significant effect on hepatic octanoate oxidation (Fig. 1). It is well established that palmitate is transported into mitochondria by a carnitine-dependent process, whereas octanoate may enter mitochondria independently of carnitine [3]. Hence these results suggest that the target for okadaic acid action might be CPT-I, the key regulatory enzyme in the transport of long-chain

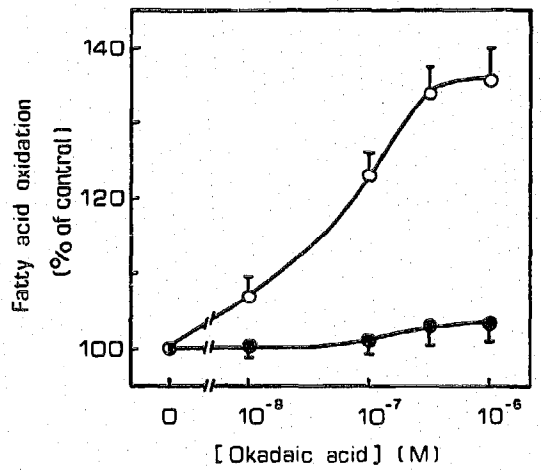

Fig. 1. Effect of okadaic acid on palmitate and octanoate oxidation by isolated hepatocytes. Hepatocytes were preincubated for 20 min in the presence of increasing concentrations of okadaic acid and then the rates of [1-^{14}C]palmitate (o) and [1-^{14}C]octanoate (●) oxidation were determined. Results represent the means ± SD of 6 (palmitate) or 5 (octanoate) different cell preparations with cell incubations performed in triplicate. Control rates of [1-^{14}C]fatty acid oxidation (in nmol of fatty acid into total oxidation products/h × mg protein) were 43.6±5.7 for palmitate and 102.2±28.0 for octanoate.

fatty acids into mitochondrial oxidative metabolism [1–3].

As may be seen in Fig. 2, CPT-I activity (as measured in a permeabilized-cell assay) was increased after hepatocyte incubation with okadaic acid. The dose-dependence of this effect was similar to the okadaic acid-induced stimulation of palmitate oxidation (Fig. 1). The stimulation of CPT-I activity by okadaic acid was evident both in the presence and in the absence of 10 μM malonyl-CoA in the assay, though this stimulation was slightly higher in the latter condition (Fig. 2). Okadaic acid thus seems to stimulate hepatic palmitate oxidation by enhancing CPT-I activity. A direct effect of okadaic acid on the CPT-I enzyme may be ruled out in view that enzyme activity in isolated rat-liver mitochondria exposed to 1 μM okadaic acid was unaffected. Similarly, no effect of okadaic acid on CPT-I activity was observed when directly added to the permeabilized-cell assay instead of being added to the cell preincubation (results not shown). In addition, the effects of okadaic acid on CPT-I activity and palmitate oxidation were not significantly different between periportal and perivenous hepatocytes, supporting the notion that both liver zones equally respond to short-term modulators of intermediary metabolism (see [21]).

Since CPT-I activity was determined as the hepatocellular CPT activity which is sensitive to inhibition by malonyl-CoA, it might be argued that rat liver contains other malonyl-CoA-inhibitable carnitine acyltransferase activities. Thus, it has been shown that the microsomal medium-chain carnitine acyltransferase is sensitive to malonyl-CoA [23]. Just like mitochondria, microsomes remain inside the permeabilized cells (cf. [24]).

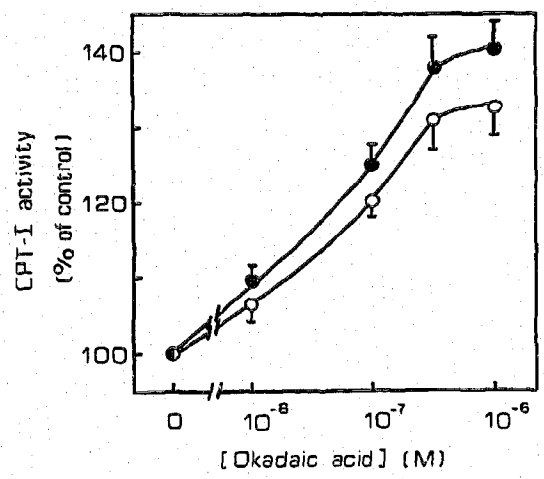

Fig. 2. Effect of okadaic acid on CPT-I activity in permeabilized hepatocytes. Hepatocytes were preincubated for 20 min in the presence of increasing concentrations of okadaic acid and then CPT-I activity was determined in permeabilized cells either in the absence (o) or in the presence (●) of 10 μM malonyl-CoA in the assay. Results represent the means ± SD of 6 different cell preparations with cell incubations and enzyme assays performed in triplicate. Control values of CPT-I activity (in nmol of palmitoylcarnitine/min × mg protein) were 5.48±0.76 and 1.87±0.44 for assays performed in the absence and in the presence of 10 μM malonyl-CoA respectively.

However, this enzyme activity should be strongly inhibited at the concentrations of palmitoyl-CoA used in our assay [23]. In addition, CPT activity in peroxisomes is also sensitive to malonyl-CoA and may account for up to 20% of total CPT activity in livers from fed rats [22]. Peroxisomes also remain inside the permeabilized cells. (cf. [24]), and in this case the substrate specificity of peroxisomal CPT seems to be very similar to that of mitochondrial CPT-I [22]. Thus, we are aware that malonyl-CoA-sensitive CPT activity in permeabilized cells mostly (though not exactly) represents CPT-I activity. It is well-established that the effects of okadaic acid on cellular metabolism are mediated by the inhibition of protein phosphatases 1 and 2A and thus by an increase in the phosphorylation state of target enzymes [13,14]. In order to ascertain whether CPT-I activity may be subjected to control by a mechanism of phosphorylation–dephosphorylation, we next studied the effects of other cell agonists which are supposed to increase the phosphorylation state of the enzyme. Similarly to okadaic acid, agents such as dibutyryl-cAMP, forskolin and glucagon stimulated palmitate oxidation as well as CPT-I activity (Table I). In all cases, variations in enzyme activity were paralleled by changes in the rate of palmitate oxidation, whereas no significant effect was evident on octanoate oxidation (Table 1). Data shown in Table 1 represent maximal effects of the different cell agonists under study. It is noteworthy that these maximal effects exerted on CPT-I activity or palmitate oxidation were in all cases (i) quantitatively similar and (ii) non-additive, suggesting a common modification of the CPT-I enzyme.

Table I

Effects of okadaic acid, dibutyryl-cAMP, forskolin and glucagon on hepatic fatty acid oxidation and CPT-I activity.

Additions	Percentage of incubations with no additions		
	CPT-I activity (n=8)	Palmitate oxidation(n=8)	Octanoate oxidation (n=6)
1 μM okadaic acid	134.6±4.7[a]	135.4±5.2[a]	104.3±2.6
50 μM dibutyryl-cAMP	131.3±2.8[a]	136.5±3.2[a]	107.4±4.3
50 μM forskolin	137.8±5.3[a]	140.1±4.7[a]	106.6±5.2
20 nM glucagon	127.7±7.0[a]	133.5±5.1[a]	108.3±5.7
1 μM okadaic acid + 50 μM dibutyryl-cAMP	135.1±3.9[a]	134.3±4.3[a]	106.9±3.5
1 μM okadaic acid + 50 μM forskolin	140.4±5.5[a]	139.7±2.6[a]	107.5±5.2
1 μM okadaic acid + 20 nM glucagon	132.9±4.1[a]	135.8±3.3[a]	107.4±4.3

Hepatocytes were preincubated in the presence of the additions indicated. After 20 min, part of the cells were used for measurement of CPT-I activity. The rest of the cells were used for determination of the rate of [1-^{14}C]palmitate and [1-^{14}C]octanoate oxidation. Results represent the means ± SD of the number of cell preparations indicated in every case. Cell incubations and enzyme assays were always performed in triplicate. As determined by the Student's t-test: [a]significantly different (P<0.01) from incubations with no additions.

Taken together, all these data support the notion that enhanced phosphorylation of CPT-I may lead to a stimulation of enzyme activity and, in turn, to an activation of palmitate oxidation. In addition, our results indicate that CPT-I seems to be a key regulatory enzyme in the control of the hepatic fatty-acid-oxidative process, at least in the experimental system presented herein.

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