Fatty acyl-CoA esters inhibit glucose-6-phosphatase in rat liver microsomes

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In native rat liver microsomes glucose 6-phosphatase activity is dependent not only on the activity of the glucose-6-phosphatase enzyme (which is lumenal) but also on the transport of glucose-6-phosphate, phosphate and glucose through the respective translocases T1, T2 and T3. By using enzymic assay techniques, palmitoyl-CoA or CoA was found to inhibit glucose-6-phosphatase activity in intact microsomes. The effect of CoA required ATP and fatty acids to form fatty acyl esters. Increasing concentrations (2–50 μM) of CoA (plus ATP and 20 μM added palmitic acid) or of palmitoyl-CoA progressively decreased glucose-6-phosphatase activity to 50% of the control value. The inhibition lowered the $V_{max.}$ without significantly changing the K_m. A non-hydrolysable analogue of palmitoyl-CoA also inhibited, demonstrating that binding of palmitoyl-CoA rather than hydrolysis produces the inhibition. Light-scattering measurements of osmotically induced changes in the size of rat liver microsomal vesicles pre-equilibrated in a low-osmolality buffer demonstrated that palmitoyl-CoA alone or CoA plus ATP and palmitic acid altered the microsomal permeability to glucose 6-phosphate, but not to glucose or phosphate, indicating that T1 is the site of palmitoyl-CoA binding and inhibition of glucose-6-phosphatase activity in native microsomes. The type of inhibition found suggests that liver microsomes may comprise vesicles heterogeneous with respect to glucose-6-phosphate translocase(s), i.e. sensitive or insensitive to fatty acid ester inhibition.

INTRODUCTION

Fatty acyl-CoA esters, which are obligatory intermediates in such fatty-acid-metabolizing pathways as oxidation and esterification, have also been reported to affect, and possibly to modulate, a variety of enzymes, transporters, receptors and cellular functions ([1–9]; see [8] for additional references).

Liver glucose-6-phosphatase (G-6-Pase; EC 3.1.3.9) catalyses the terminal reaction of gluconeogenesis and glycogenolysis [10]. It also plays a major role in the maintenance of blood glucose homoeostasis [11]. Recognized since the 1950s [12], the enzyme is intimately associated with the endoplasmic reticulum (ER) and appears to be a multicomponent system [13]. The catalytic subunit of the enzyme is located within the ER lumen and at least three reticular transport proteins are required [14]. T1 mediates the entry of the substrate glucose 6-phosphate into the ER lumen, whereas T2 and T3 allow the exit to the cytosol of the hydrolysis products P_i and glucose respectively.

In the liver ER membrane a long-chain fatty acyl-CoA synthetase (EC 6.2.1.3) is present, and it catalyses the biosynthesis of the appropriate acyl-CoA ester in the presence of MgATP, CoA and the respective fatty acid [15,16]. Fatty acyl-CoA synthesis, in turn, depends on cellular fatty acid availability. Blood levels of non-esterified fatty acids and hepatic levels of fatty-acyl CoA esters (and presumably also the ER levels) can vary under pathophysiological and/or nutritional conditions which affect hepatic glucose production, for example diabetes and fasting. It therefore seemed logical to elucidate the effects of these lipid intermediates on the liver G-6-Pase system.

In this paper we show that CoA and fatty acyl-CoAs inhibit G-6-Pase activity in rat liver microsomes. CoA appears to act by allowing the microsomal ATP-dependent synthesis of its esters with fatty acids.

EXPERIMENTAL

Materials

Glucose 6-phosphate, mannose 6-phosphate, ATP, phosphocreatine, creatine kinase (type III), CoA, acyl-CoA esters, alamethicin and streptozotocin were from Sigma, St. Louis, MO, U.S.A. Fatty-acid-free BSA was obtained from Boehringer, Mannheim, Germany. Heptadecan-2-onyldethio-CoA, the non-hydrolysable analogue of palmitoyl-CoA (PCoA), was kindly given by Professor T. Wieland. All other chemicals were of analytical grade.

Preparation of liver microsomes

Male Sprague–Dawley rats (180–230 g) were used. Animals were allowed free access to food and water (fed), or denied food but not water for 16 h before death (starved), or had diabetes induced by streptozotocin (75 mg/kg body wt. in buffered citrate, pH 4.5, given intravenously 3 days before animals were killed). Liver microsomes were prepared as reported [17]. The microsomal fractions were resuspended (approx. 80 mg of protein/ml) in a medium of the following composition (mM): KCl, 100; NaCl, 20; $MgCl_2$, 3.5; Mops, 20, pH 7.2. The suspensions were rapidly frozen and maintained under liquid N_2 until required. Intactness of native microsomal membrane preparations was ascertained by measuring the latency of mannose-6-phosphatase activity [18,19]. Mannose-6-phosphatase activity of native microsomes was less than 10% of the activity found in fully disrupted microsomal vesicles in all the preparations employed.

In some experiments, microsomes (20 mg of protein/ml) were incubated in the resuspension medium and in the presence of 0.5% (w/v) sodium deoxycholate (adjusted to pH 7.2) for 20 min

Abbreviations used: G-6-Pase, glucose-6-phosphatase; PCoA, palmitoyl-CoA; ER, endoplasmic reticulum.

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at 0–4 °C to disrupt the microsomal membrane before assay of G-6-Pase activity.

G-6-Pase assay

G-6-Pase activity was measured by incubating microsomes (200 μg of protein/ml) in 100 mM KCl, 20 mM NaCl, 3.5 mM $MgCl_2$, 20 mM Mops (pH 7.2) and 3 mM glucose 6-phosphate. Where indicated in individual experiments, 3 mM ATP plus an ATP-regenerating system (5 mM phosphocreatine and 10 units of creatine kinase/ml) was included in the incubation mixture to allow the synthesis of fatty acyl-CoA esters. The ATP-regenerating system allowed a constant ATP concentration in the incubation system over the reaction times (0–30 min), thus mimicking the cytosolic condition; in its absence, [ATP] might presumably decrease because of the relatively high microsomal ATPase activities [20]. In these experiments hydrolysis of glucose 6-phosphate was evaluated by measuring glucose production [21].

In some experiments, G-6-Pase activity was assayed under optimal 'standard' assay conditions for the enzyme system, i.e. at 30 °C in 20 mM sodium cacodylate buffer at pH 6.5 [19,22], including also 3 mM $MgCl_2$, to measure the kinetic constants $V_{max.}$ and K_m. The concentrations of the substrate glucose 6-phosphate used to calculate the kinetic constants were 1, 1.4, 2, 2.6, 5 and 30 mM. Kinetic constants were calculated by using a BBC computer program for non-linear multiple regression analysis based on that of Colquhoun [23].

In all experiments, microsomes were preincubated in the complete incubation systems (also including CoA or PCoA where indicated) for 1–2 min, and the G-6-Pase assay was started by adding the substrate glucose 6-phosphate.

Light-scattering measurements

Osmotically induced changes in microsomal vesicle size and shape were monitored at 400 nm at right angles to the incoming light beam (whose intensity was decreased by 80 % with the aid of a copper grid) by using a fluorimeter (Perkin-Elmer model LS-3B) equipped with a temperature-controlled cuvette holder (22 °C) and a magnetic stirrer, as described elsewhere [24]. Microsomal suspensions (80 mg of protein/ml) were diluted 1500-fold in a low-osmolality buffer (4 mM K-Pipes/1 mM EGTA, pH 7.0). Diluted microsomal suspension (2 ml) was placed in the fluorimeter cuvette, and vesicles were equilibrated and stirred until a stable baseline was obtained. Osmotically induced changes in the vesicles were initiated by increasing the osmolality of the vesicle medium. To this end, 0.1 ml of concentrated solutions of the different solutes (0.6–1.0 M in 4 mM K-Pipes/1 mM EGTA adjusted to pH 7.0 with KOH or HCl) were added to the vesicle suspension.

Other assays

Microsomal non-esterified fatty acids were measured colorimetrically [25] after t.l.c. separation [26] of the lipids extracted from microsomal membranes [27]. Fatty acyl-CoA esters were measured as reported elsewhere [28]. Protein determination was performed as reported [29], with BSA as standard. P_i was measured as reported [19].

RESULTS AND DISCUSSION

CoA (via formation of fatty acyl-CoAs), and fatty acyl-CoA esters, inhibit liver microsomal G-6-Pase activity

As shown in Figures 1(a) and 1(b), both CoA (50 μM, in the presence of MgATP plus an ATP-regenerating system and 20 μM palmitic acid) or PCoA (50 μM) inhibited (by approx. 40–50 %) the G-6-Pase activity in native fed-rat liver microsomes. The incubation medium had an ionic composition and pH resembling those of cytosol.

In the CoA experiments, ATP and palmitic acid were included in the medium to permit the synthesis of fatty acyl esters by microsomal long-chain fatty acyl CoA synthetase [16]. The inhibitory effect of CoA was already present at the earliest time point of incubation investigated (7.5 min) and increased linearly up to 30 min of incubation (Figure 1a). Consistent with these results, we found that the maximal formation of fatty acyl-CoA esters was already accomplished at 3 min of incubation, and that constant levels of fatty acyl-CoAs were maintained in the system up to 30 min of incubation (Table 1). It should be also noted that CoA was currently added to the microsomal system 1–2 min before adding glucose 6-phosphate and starting the G-6-Pase assay (see the Experimental section).

ATP (plus an ATP-regenerating system) by itself did not affect G-6-Pase activity (Table 2). In the absence of ATP, concentrations of CoA and palmitic acid higher than 50 and 20 μM, respectively, were inhibitory by themselves (Table 2). The inhibitory effect of CoA by itself at high concentrations was possibly due to the thiol group, as glutathione and other thiol compounds are known to inhibit microsomal G-6-Pase activity [30,31]. There have been several other previous reports of non-esterified fatty acids affecting G-6-Pase activity (e.g. [32–34]), but different effects have been found, e.g. that palmitic acid is inhibitory [32] or not inhibitory [34]. To investigate the effect of non-esterified fatty acids on the G-6-Pase system was out of the scope of the present paper. However, since fatty acids are lipophilic and tend to accumulate in and/or bind to membranes, relatively high ratios of [fatty acid] to [microsomal membrane] (e.g. 1 mmol to 1 mg of membrane protein in the case of 100 μM added palmitic acid; the incubation system contained 0.2 mg of microsomal protein/ml) would presumably result in unphysiologically high membrane concentrations of the fatty acid, which may unspecifically affect membrane protein activities.

Even in the absence of added palmitic acid (but in the presence of ATP), CoA inhibited G-6-Pase activity, although to a lesser extent (Table 2). CoA synthetase activity was probably made possible by non-esterified fatty acids already present in the microsomal fraction. Consistent with this, we found 35 ± 10 nmol of non-esterified fatty acids/mg of protein (mean $\pm$ S.D., $n = 4$) in the microsomal preparations. Moreover, even in the absence of added palmitic acid, measurable amounts of fatty acyl-CoAs were produced by microsomes incubated with ATP and 50 μM CoA (Table 1). To prove further that CoA indeed inhibited G-6-Pase via formation of fatty acyl-CoAs, fatty-acid-free albumin (0.5 mg/ml), which exhibits several high-affinity binding sites for non-esterified fatty acids [35], was added to incubations to decrease the availability of non-esterified fatty acids. Under this condition, 100 μM CoA was slightly inhibitory, but independently of ATP (see Figure 1c and Table 2). The addition of excess palmitic acid (200 μM) restored the inhibitory effect of CoA only when ATP was also present (Figure 1c).

The ability of fatty acyl-CoA derivatives of different chain lengths (50 μM), to inhibit microsomal G-6-Pase activity is shown in Table 3. Palmitoyl-, arachidonyl- and oleoyl-CoA were the most inhibitory. CoA derivatives of saturated fatty acids whose acyl chain was shorter or longer than 16 carbon atoms were relatively less inhibitory. Other acyl-CoA derivatives tested with acyl carbon number lower than 10 (namely acetyl-CoA, valeryl-CoA, isovaleryl-CoA and octanoyl-CoA) had little or no inhibitory effect on microsomal G-6-Pase activity.

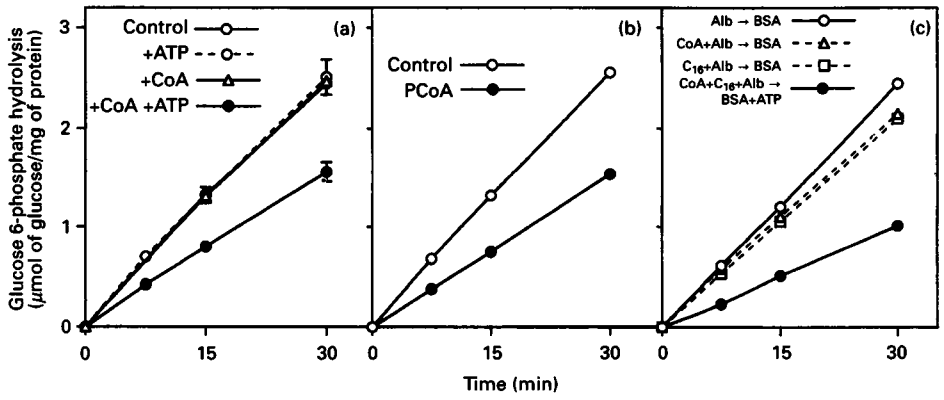

Figure 1 Inhibitory effect of CoA plus ATP (a) or PCoA (b) on glucose 6-phosphate hydrolysis by native rat liver microsomes, and suppression of the inhibitory effect of CoA by fatty-acid-free albumin (c)

Native rat liver microsomes (200 μg of protein/ml) were incubated at 37 °C in a medium composed of (mM): KCl, 100; NaCl, 20; $MgCl_2$, 3.5; Mops, 20, pH 7.2; and 3 mM glucose 6-phosphate. Where 'ATP' is indicated, 3 mM ATP (plus an ATP-regenerating system) and 20 μM palmitic acid were also included in the incubation mixture. Other additions were: panel (**a**), 50 μM CoA; panel (**b**), 50 μM PCoA; panel (**c**), 100 μM CoA, 0.5 mg/ml fatty-acid-free BSA and 200 μM palmitic acid (C_{16}) to saturate the fatty-acid-free BSA. Data are means ± S.D. of three or means of two independent experiments.

Table 1 Formation of fatty acyl-CoA esters in the microsomal system for the assay of G-6-Pase activity

The incubation system was as reported in the legend to Figure 1. 'ATP' indicates ATP plus its regenerating system. Fatty acyl-CoAs were measured as reported in [28]. Abbreviations: C_{16}, palmitic acid; n.d., not detectable; n.m., not measured. Data are means ± S.D. of three or means of two independent experiments.

Additions	Fatty acyl-CoA in the incubation system (nmol/ml)			
	0 min	3 min	15 min	30 min
ATP	n.d.	n.d.	n.d.	n.d.
ATP + 50 μM CoA	n.d.	2.4	3.2	2.6
ATP + 50 μM CoA + 20 μM C_{16}	n.d.	9.3 ± 0.9	9.1 ± 1.6	9.4 ± 2.3
ATP + 100 μM CoA + 20 μM C_{16}	n.d.	n.m.	9.8 ± 2.0	n.m.

Table 2 Effect of CoA and palmitic acid on G-6-Pase activity in liver native microsomes incubated in the presence and in the absence of ATP

The incubation system was as reported in the legend to Figure 1. 'ATP' indicates ATP plus its regenerating system. G-6-Pase activity was measured at 15 min of incubation and its rate was linear over 30 min (see also Figure 1). Data are means ± S.D. for the numbers of experiments reported in parentheses: $^*P < 0.005$, $^{**}P < 0.001$.

ATP	Palmitic acid (μM)	CoA (μM)	G-6-Pase activity (nmol of glucose/min per mg of protein)
−	0	0	96 ± 4.1 (4)
−	20	0	92 ± 4.2 (3)
−	50	0	86 ± 5.3 (3)
−	100	0	69 ± 7.4 (3)*
−	0	50	99 ± 2.8 (3)
−	0	100	90 ± 3.9 (3)
−	0	200	80 ± 4.6 (3)*
+	0	0	98 ± 3.3 (6)
+	0	50	65 ± 12.2 (4)*
+	20	50	55 ± 5.0 (5)**

Table 3 Effect of various fatty acyl-CoA derivatives on G-6-Pase activity of liver microsomes

The incubation system was as reported in the legend to Figure 1: Fatty acyl esters were added to 50 μM. G-6-Pase activity was measured at 15 min of incubation; the control value for G-6-Pase activity (nmol of glucose/min per mg of protein) was 99 ± 4.0. Data are means ± S.D. of three independent experiments: $^*P < 0.005$, $^{**}P < 0.001$.

Fatty acyl ester	G-6-Pase activity (% of control)
Control	100
Lauroyl-CoA	67 ± 3.7*
Palmitoyl-CoA	53 ± 4.5**
Stearoyl-CoA	60 ± 3.1**
Oleoyl-CoA	54 ± 4.0**
Arachidoyl-CoA	73 ± 3.9*
Arachidonyl-CoA	50 ± 5.1**
Behenoyl-CoA	90 ± 3.7*
Non-hydrolysable analogue of palmitoyl-CoA (10 μM)	81 ± 3.8*
Non-hydrolysable analogue of palmitoyl-CoA (50 μM)	49 ± 4.9**

To determine whether binding or hydrolysis of PCoA is required to inhibit microsomal G-6-Pase activity, a non-hydrolysable analogue of PCoA (heptadecan-2-onyldethio-CoA [36]) was employed. Should PCoA act directly, a non-hydrolysable analogue of PCoA would substitute for PCoA in inhibiting the enzyme. Table 3 shows that the non-hydrolysable analogue of PCoA (10 and 50 μM) also inhibited G-6-Pase activity, indicating that it is binding of PCoA that produces the inhibition.

The effect of varying the concentration of CoA (in the presence of ATP and palmitic acid) or PCoA on G-6-Pase activity was investigated in native, alamethicin-permeabilized and detergent-disrupted microsomes. The pore-forming oligopeptide alamethicin [37] was employed to allow the free access of glucose 6-phosphate to the intraluminal G-6-Pase enzyme, but without destroying the vesicular structure of microsomes [38]. In native microsomes, CoA (Figure 2a) showed the inhibitory effect even at the concentration of 2 μM; its effect was near maximal at

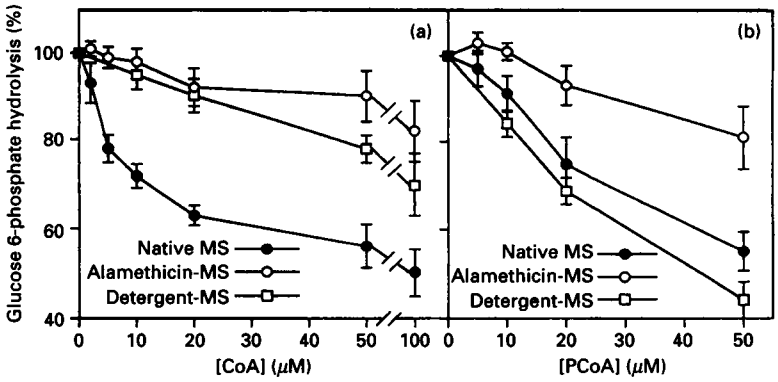

Figure 2 Dose-dependence of CoA- (a) and PCoA- (b) induced inhibition of G-6-Pase activity in native, alamethicin-permeabilized or detergent-disrupted liver microsomes

The incubation system was as reported in the legend to Figure 1. In the experiments in (**a**), 3 mM ATP (plus an ATP-regenerating system) and 20 μM palmitic acid were also included in the incubation mixture. Where indicated, microsomes were permeabilized (Alamethicin-MS) by adding 20 μg/ml alamethicin to the system, or disrupted (Detergent-MS) by a preincubation step in the presence of sodium deoxycholate (see the Experimental section). Glucose 6-phosphate hydrolysis was measured after 15 min of incubation. In some experiments it was verified that the rate of glucose 6-phosphate hydrolysis was linear over 30 min of incubation in all the experimental conditions shown. Control values (100% G-6-Pase activities) were (nmol of glucose/min per mg of protein): 101 ± 5, 179 ± 9 and 185 ± 7 (**a**), or 98 ± 3, 173 ± 6 and 191 ± 6 (**b**), in native, alamethicin-permeabilized and detergent-disrupted microsomes respectively. Data are means $\pm$ S.D. of three to five independent experiments.

50 μM, and half-maximal [CoA] was 7–10 μM. The higher concentration of CoA investigated (100 μM) gave some inhibition of G-6-Pase activity also in the absence of ATP (see Table 1); thus, taking into account this effect, the net inhibition by 100 μM CoA plus ATP and 20 μM palmitic acid did not exceed 50%. Consistently, in the presence of 100 μM CoA and 20 μM palmitic acid (plus ATP), the amount of fatty acyl esters formed did not exceed that measured in the presence of 50 μM CoA (see Table 1), likely because of the limited availability of substrate fatty acids. Concentrations of palmitic acid higher than 20 μM were not investigated, since they caused (in the presence of CoA and ATP) some membrane disruption, as revealed by the decrease in the latency of hydrolysis of mannose 6-phosphate. This effect was possibly due to the synthesis by microsomes of relatively high concentrations of fatty esters, which may result in detergent effects (as we discuss below in the case of PCoA experiments).

In alamethicin-permeabilized microsomes, only the higher concentration of CoA (50 μM) was slightly inhibitory to the G-6-Pase enzyme activity. A progressive, but minor, inhibition of G-6-Pase enzyme activity was also found in detergent-disrupted microsomal membranes with increasing [CoA] (Figure 2a). Increasing [PCoA] (0–50 μM) resulted in a progressive inhibition of G-6-Pase activity of both native and detergent-disrupted microsomes (Figure 2b). In alamethicin-permeabilized microsomes, a significant but relatively minor inhibition of G-6-Pase enzyme activity was present only at the highest [PCoA] investigated (50 μM); this inhibitory effect was lower than in native or detergent-disrupted microsomes (18% versus 45% and 56% respectively).

Concentrations of PCoA higher than 50 μM were not used, as they can result in artefacts. These are caused by the fact that relatively high concentrations of fatty-acyl CoA esters can behave like detergents and/or form micelles [2,4]. In ancillary experiments, we observed that the inhibitory effect of PCoA on G-6-Pase of native microsomes was dependent on the ratio of [fatty acyl ester] to [microsomal membrane], rather than on the absolute concentration of the ester. Therefore, in the experiments in this paper a constant concentration of microsomal membranes was used in the incubation systems (i.e. 0.2 mg of protein/ml). We also observed that by increasing the [PCoA] (up to 200 μM), or by decreasing the microsomal concentration (down to 50 μg of protein/ml), the latency of hydrolysis of mannose 6-phosphate (higher than 90% in the native microsomal fractions employed) decreased to values $\leqslant$ 40–30%. This is consistent with a detergent effect of PCoA causing microsomal disruption at ratios of [PCoA]/[microsomes] higher than 250 nmol/mg of microsomal protein.

An early report in 1967 [39] suggested that PCoA both inhibited and activated G-6-Pase activity, depending on both its concentration and the pH. However, unfortunately those data are of limited value, since the experiments were carried out before it was proposed that G-6-Pase was a multicomponent enzyme system [40] and before the importance of microsomal intactness was realized [41]. Therefore, it is not clear how PCoA was actually affecting G-6-Pase, nor what component of the system was being affected, or whether the effect of PCoA was a result merely of microsomal disruption, i.e. micellar- or detergent-like effects.

Data obtained in alamethicin-permeabilized microsomes indicate that fatty acyl-CoAs do not inhibit the intralumenal catalytic component of the G-6-Pase system. However, in detergent-solubilized microsomes, CoA, and most evidently PCoA (Figures 2a and 2b), appear also to inhibit this component. This effect could be due to exposure of inhibitory/binding sites for fatty acyl-CoAs in solubilized microsomes, or to complex interactions between the detergent and the amphipathic fatty acyl esters (which can also behave as detergents; see above). However, alamethicin permeabilization is a better reflection of the situation *in vivo* than is detergent disruption, as alamethicin preserves the vesicular structure of microsomes [38].

The effect of PCoA on the kinetic parameters of G-6-Pase activity was studied in native liver microsomes isolated from fed, starved and diabetic rats under optimal 'standard' assay conditions for the enzyme system (see the Experimental section). However, $MgCl_2$ (3.5 mM) was also included in the standard medium, since in its absence PCoA, even at concentrations lower than 50 μM (see above), resulted in a marked loss of microsomal intactness, as measured by mannose-6-phosphatase activity (re-

Table 4 Effect of PCoA and BSA on kinetic parameters of G-6-Pase measured in native microsomes from livers of fed, starved and diabetic rats and incubated under standard assay conditions

G-6-Pase activity was assayed and kinetic constants were measured as reported in the Experimental section. $V_{max.}$ is expressed as nmol/min per mg of microsomal protein; K_m is given in mM. PCoA was 50 μM and BSA was 10 mg/ml. Data are means ± S.D. of three independent experiments: $^*P < 0.005$, $^{**}P < 0.001$; differences were calculated from appropriate control values.

	Fed		Starved		Diabetic	
	$V_{max.}$	K_m	$V_{max.}$	K_m	$V_{max.}$	K_m
Control	122 ± 10	1.0 ± 0.2	185 ± 35	1.1 ± 0.2	703 ± 29	2.0 ± 0.5
PCoA	67 ± 9*	1.4 ± 0.2	129 ± 17	2.3 ± 0.4	316 ± 10**	1.9 ± 0.2
PCoA and BSA	131 ± 28	1.0 ± 0.5	235 ± 60	1.1 ± 0.8	720 ± 67	2.1 ± 0.2

sults not shown). Mg^{2+} ions have previously been reported to counteract micelle formation [2]. Starvation and diabetes resulted in increased microsomal G-6-Pase system activity (Table 4) as previously reported (e.g. [18,42,43]). In liver microsomes from fed and diabetic rats, PCoA (50 μM) caused a significant decrease by approx. 50% in the $V_{max.}$ of the G-6-Pase system (Table 4); in starved-rat liver microsomes, PCoA also caused a 30% decrease (although not statistically significant) in $V_{max.}$ of the G-6-Pase system. The effect of PCoA was reversible in all cases, as the addition of BSA (10 mg/ml) to PCoA-treated microsomes returned the $V_{max.}$ of the system to control values (Table 4). PCoA caused no significant change in the K_m value of the G-6-Pase system in microsomes isolated from fed, starved or diabetic rats (Table 4), although there was also a small non-significant increase in the K_m of the G-6-Pase system in starved-rat microsomes. Very similar results were obtained by replacing PCoA with arachidonyl-CoA (results not shown), and the inhibition by arachidonyl-CoA was also reversed by BSA.

The results above indicate that PCoA (and arachidonyl-CoA) inhibits G-6-Pase activity in native microsomes by binding to one or more of the transport proteins (T1, T2 and/or T3). The effects of PCoA (50 μM) addition on the kinetic parameters of G-6-Pase activity with PP_i as substrate were therefore also determined in intact microsomes isolated from fed, starved and diabetic rats to determine whether or not PCoA inhibits the T2 transport protein. T2 not only allows the efflux of P_i from microsomes, but also mediates the entry of PP_i as a substrate for the lumenal G-6-Pase enzyme [14,22]. No significant inhibition by PCoA of PP_i hydrolysis was present in any microsomal fraction [e.g. the kinetic parameters of PP_i hydrolysis in control intact diabetic-rat liver microsomes were $V_{max.} = 383 \pm 70$ nmol/min per mg of protein; $K_m = 3.3 \pm 0.5$ mM (means ± S.D., $n = 3$). In microsomes treated with PCoA as in Table 3, $V_{max.}$ was 356 ± 42, with a K_m of 2.7 ± 0.5 mM (means ± S.D., $n = 3$)]. This shows that the effect of PCoA on microsomal G-6-Pase activity is not mediated via T2. When glucose 6-phosphate is the substrate for G-6-Pase in native microsomes, it has been reported that glucose 6-phosphate transport across the microsomal membrane is rate-limiting [39], which suggests that T1 rather than T3 would be the most logical site of PCoA binding.

Fatty acyl-CoA esters decrease microsomal permeability to glucose 6-phosphate

The possibility that fatty acyl-CoAs inhibit the entry of glucose 6-phosphate into the microsomal lumen, via the putative translocase T1 [11,13,14,24], was evaluated by analysing the osmotic behaviour of microsomal vesicles by a light-scattering technique [24,44]. Compounds not entering the microsomal lumen, e.g. sucrose [24,44] or mannose 6-phosphate ([24]; see Figure 3b), cause a sustained shrinking of vesicles, as revealed by the increase in light-scattering. On the other hand, permeant compounds, e.g. glucose 6-phosphate ([24]; see Figure 3a) or KCl [24,44], cause a transient shrinking, followed by a swelling phase (decrease in light-scattering), whose rate reflects the rate of entry of these compounds into the native microsomal vesicles.

In initial experiments, native microsomal vesicles were preincubated with or without 50 μM CoA (10 min, at 37 °C, in the presence of ATP and 20 μM palmitic acid), recovered by ultracentrifugation, and resuspended in a hypo-osmotic medium for the light-scattering assay. After this procedure, microsomes preincubated with CoA had G-6-Pase activity which was 52 ± 4% ($n = 3$, mean ± S.D.) of that measured in control microsomes (preincubated, but without CoA), indicating that the inhibitory effect of CoA was retained. Moreover, microsomes still contained 86 ± 3% (mean ± S.D., $n = 3$) of the fatty acyl-CoAs formed in the preincubation step (see Table 1). On addition of glucose 6-phosphate (45 mM final concn.), both CoA-treated and control vesicles rapidly shrank (Figure 3a); however, the swelling phase was slower and only partial in CoA-treated microsomes as compared with control ones (Figure 3a). In contrast, the pattern of shrinking/swelling caused by glucose and P_i was very similar in both CoA-treated and control microsomes (Figures 3c and 3d), indicating no change in microsomal permeability to these compounds. The non-permeant phosphoester mannose 6-phosphate [24] caused sustained shrinking of vesicles independently of the CoA treatment (Figure 3b). Addition of alamethicin to any system resulted in a fast decrease of the signal to the basal value. Alamethicin added before the glucose 6-phosphate completely prevented any shrinking effect in control and CoA-treated microsomes (Figure 3e), indicating that it had completely permeabilized the membrane to glucose 6-phosphate. Alamethicin by itself caused a moderate decrease in the light-scattering signal (Figure 3e), which probably reflects a minor vesicle swelling due to the entry of Pipes (and EGTA) into vesicles (4 mM K-Pipes and 1 mM EGTA, pH 7.0, were present in the hypo-osmotic medium). The organic anion Pipes itself does not easily permeate the microsomal membrane [38]. These results show that the translocases T2 and T3 are not affected by PCoA, again indicating that the only site of inhibition of G-6-Pase activity in native microsomes is the microsomal glucose 6-phosphate-transport system.

In experiments to elucidate further the inhibition of glucose 6-phosphate entry, PCoA was added to the light-scattering assay system. Figure 3(f) shows that PCoA also decreased the rate and the extent of glucose 6-phosphate entry into microsomal vesicles. The effect was more evident on increasing the [PCoA] (2–5 μM). Palmitic acid (up to 20 μM) did not affect the microsomal

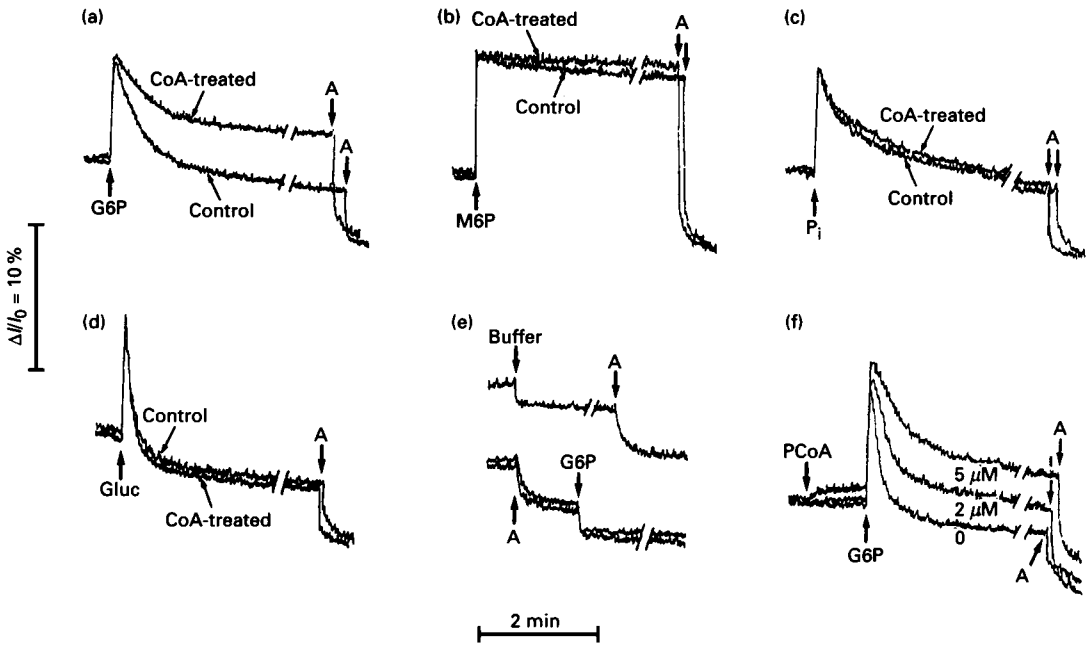

Figure 3 Osmotically induced changes in light-scattering intensity of rat liver microsomal vesicles induced by various solutes

Rat liver microsomes were preincubated with CoA (CoA-treated) or without CoA (Control) as described in the text. Light-scattering measurements were performed as described in the Experimental section. Concentrated solutions (0.6–1 M) of glucose 6-phosphate (G6P), mannose 6-phosphate (M6P), potassium phosphate buffer, pH 7.0 (P_i), or glucose (Gluc) were added to the microsomal suspension where indicated by arrows. The resulting final concentrations of added solutes were: 45 mM in (**a**), (**b**), (**c**), and (**e**), 100 mM in (**d**) or 30 mM in (**f**). As indicated, alamethicin (10 μg/ml; A) or PCoA (at the indicated concentrations) was added. A typical set of experiments out of three to five is shown.

permeability to glucose 6-phosphate. It should be noted that in the light-scattering assay the microsomal protein concentration was lower than in the G-6-Pase measurements (50 instead of 200 μg/ml). Thus the effect on glucose 6-phosphate entry was evident at ratios of [PCoA] to [microsomes] comparable with those affecting G-6-Pase activity (see Figure 2b). At [PCoA] higher than 5 μM, the light-scattering assay gave unclear results, as PCoA alone increased the signal, possibly due to aggregation of vesicles, and the increase in the signal was only partially decreased by alamethicin. Qualitatively similar results were observed on addition of arachidonyl-CoA and the non-hydrolysable analogue of PCoA (results not shown).

The swelling phase after glucose 6-phosphate addition to CoA- or PCoA-treated microsomes was decreased not only in rate but also in extent (see Figures 3a and 3f), thus indicating that the esters had possibly only made a portion of vesicles impermeable to the glucose 6-phosphate. Moreover, CoA (and PCoA) treatment resulted only in partial (maximum 50%) inhibition of G-6-Pase activity in native microsomes (see Figure 2 and Table 3). A logical explanation is that liver microsomes may comprise vesicles heterogeneous with respect to glucose 6-phosphate translocase(s), i.e. sensitive or insensitive by inhibition to fatty esters. This point requires further investigation, but unfortunately T1, the putative translocase(s) for glucose 6-phosphate, has not yet been identified, purified or characterized.

Effect of liver cytosolic fraction on the inhibition of microsomal G-6-Pase by CoA

The effects of CoA and PCoA on G-6-Pase activity were also studied in microsomes incubated in the presence of liver cytosolic fraction. This fraction has been reported to contain soluble high-affinity fatty-acyl-CoA-binding proteins [45], which may affect

Table 5 Effect of CoA on glucose 6-phosphate hydrolysis by liver microsomes incubated in the presence of liver cytosolic fraction

The incubation mixture was as detailed in the legend to Figure 1, except that it also included 100000 *g* rat liver supernatant equivalent to 50 mg of liver (approx. 2.7 mg of protein) per ml. 'ATP' indicates ATP plus its regenerating system. CoA and PCoA were 50 μM. Palmitic acid was 100 μM. Glucose production was measured at 15 min of incubation, and its rate was linear over 30 min of incubation. Data are means ± S.D. for the numbers of experiments reported in parentheses: $^{**}P < 0.001$.

Addition			Glucose production
ATP	CoA and palmitic acid	PCoA	(nmol/min per mg of protein)
−	−	−	95 (2)
−	+	−	90 (2)
−	−	+	99 (2)
+	−	−	44 ± 5.0 (3)
+	+	−	23 ± 5.4 (3)**
+	−	+	43 ± 9.3 (3)

(suppress) the activity of fatty acyl esters on cellular enzymes *in vivo* [46]. As shown in Table 5, under suitable conditions to allow the microsomal synthesis of fatty acyl-CoA derivatives, the G-6-Pase activity of microsomes incubated in the presence of liver cytosolic fraction was decreased (by at least 50%). In contrast, exogenously added PCoA alone failed to inhibit microsomal G-6-Pase, possibly because the cytosolic fatty-acyl-CoA-binding proteins prevented the interaction of the ester with the microsomal membrane. It should be noted that glucose production by microsomes in the presence of cytosolic fraction plus ATP was lower than that observed in the absence of liver cytosol (minus ATP, Table 5) or in the presence of ATP (but in the absence of

cytosolic fraction; see Table 2). In the former case, glucose production presumably reflected a balance between microsomal G-6-Pase and cytosolic glucokinase. Indeed, under the same experimental conditions, but without glucose 6-phosphate and in the presence of 2 mM glucose, a glucokinase activity of 23 ± 7 nmol/min per ml ($n = 3$, mean $\pm$ S.D.) was measured. This activity was decreased (approx. 20%) by CoA and by PCoA, but these effects were not further investigated, as they were out of the scope of the present study.

Conclusions

Taken together the data contained in this paper suggest that, in the liver, the synthesis in ER of fatty acyl-CoA esters (and presumably their ER concentrations) can affect the G-6-Pase enzyme system.

The addition of preformed fatty acyl-CoAs to liver microsomes is helpful in unravelling the inhibitory effect of the esters, as their actual concentration in ER of the intact hepatocytes is at present unknown (see [8]). However, it is reasonable to assume that the concentration of fatty acyl-CoAs within the ER membrane can vary, depending on the non-esterified fatty acid supply to the reticular fatty acyl synthetase. Other factors, such as cytosolic acyl-CoA-binding proteins [45,46] and microsomal long-chain acyl-CoA hydrolase(s) [47], may also influence ER levels of fatty acyl-CoAs. The total liver concentration of fatty acyl-CoAs has been shown to increase under conditions such as fasting [48] or oleate feeding [49], which are associated with high levels of blood non-esterified fatty acids. Under these conditions, higher levels of liver (reticular) fatty acyl-CoAs might well decrease G-6-Pase activity. Noticeably, liver glucokinase, the opposing enzyme to G-6-Pase, has also been shown to be inhibited by μM concentrations of long-chain acyl-CoAs [1,2]. To relate to the situation *in vivo*, one can speculate that physiological variations in the rate of ER acylation of fatty acids by CoA could have some control over the release (and storage) of glucose through the glucose/glucose 6-phosphate substrate cycle when lipolysis and lipid metabolism are increasing, such as during times of fasting or diabetes.

From the present results, another aspect related to liver pathology also arises. A number of patients have been found to have uncharacterized inhibitors of glucose 6-phosphate entry into microsomes (glycogen storage disease, named pseudo type 1b [50]), and the addition of albumin to the assay for G-6-Pase reversed the inhibitory affect (e.g. [50,51]). Although different inhibitors are probably present in individual patients, the present results suggest that abnormally high levels of fatty acyl-CoAs have the potential to cause pathological inhibition of the hepatic G-6-Pase system. This point needs further investigation when more fresh liver biopsy samples from such patients become available.

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