Fatty acid and amino acid modulation of glucose cycling in isolated rat hepatocytes

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We studied the influence of glucose/glucose 6-phosphate cycling on glycogen deposition from glucose in fasted-rat hepatocytes using S4048 and CP320626, specific inhibitors of glucose-6-phosphate translocase and glycogen phosphorylase respectively. The effect of amino acids and oleate was also examined. The following observations were made: (1) with glucose alone, net glycogen production was low. Inhibition of glucose-6-phosphate translocase increased intracellular glucose 6-phosphate (3-fold), glycogen accumulation (5-fold) without change in active (dephosphorylated) glycogen synthase (GS*a*) activity, and lactate production (4-fold). With both glucose 6-phosphate translocase and glycogen phosphorylase inhibited, glycogen deposition increased 8-fold and approached reported *in vivo* rates of glycogen deposition during the fasted → fed transition. Addition of a physiological mixture of amino acids in the presence of glucose increased glycogen accumulation (4-fold) through activation of GS and inhibition of glucose-6-phosphatase flux. Addition of oleate with glucose present decreased glycolytic flux and increased the flux through glucose 6-phosphatase with no change in glycogen deposition. With glucose 6-phosphate translocase inhibited by S4048, oleate increased intracellular glucose 6-phosphate (3-fold) and net glycogen production (1.5-fold), without a major change in GS*a* activity. It is concluded that glucose cycling in hepatocytes prevents the net accumulation of glycogen from glucose. Amino acids activate GS and inhibit flux through glucose-6-phosphatase, while oleate inhibits glycolysis and stimulates glucose-6-phosphatase flux. Variation in glucose 6-phosphate does not always result in activity changes of GS*a*. Activation of glucose 6-phosphatase flux by fatty acids may contribute to the increased hepatic glucose production as seen in Type 2 diabetes.

Key words: CP320626, glucose 6-phosphate, oleate, S4048.

INTRODUCTION

In isolated rat hepatocytes, physiological concentrations of glucose cause only slight increases in glycogen synthesis (for reviews, see [1,2]). This is in contrast with the *in vivo* situation, where oral administration of glucose results in initial glycogen synthetic rates of approx. 200 μmol/h per g dry mass [3]. However, even *in vivo*, glucose is a relatively poor substrate for hepatic glycogen synthesis. Part of the glucose is first converted into lactate in extrahepatic tissues before its carbon can be used for hepatic glycogen synthesis via the gluconeogenic pathway, a phenomenon known as the 'glucose paradox' [2]. This indirect pathway of glycogen synthesis would not be operational in the *in vitro* situation and could thus explain low glycogen synthetic rates from glucose. However, lactate does not induce high rates of glycogen accumulation in isolated hepatocytes [4].

In principle, cycling of glycogen via glycogen synthase (GS) and glycogen phosphorylase (GP) and of glucose between glucokinase and glucose 6-phosphatase could explain, at least in part, why glucose is a relatively poor substrate for net glycogen production *in vitro*. The difference between the *in vitro* and *in vivo* rate of glycogen accumulation has been a point of much discussion [1,2], and elucidation of mechanisms responsible for this difference may provide important information on the regulation of carbohydrate utilization and metabolism under normal and pathophysiological conditions.

A considerable amount of data obtained with techniques employing isotopes indicates that glycogen cycling, as well as glucose/glucose 6-phosphate cycling, does indeed occur *in vivo* and in *in vitro* preparations because of the co-existence of appreciable amounts of both active GP (phosphorylated GP; GP*a*) and GS (dephosphorylated GS; GS*a*), and of glucokinase and glucose 6-phosphatase. *In vivo*, ^{13}C NMR studies in the rat clearly indicated that, in both the fasted and in the fed state there is ongoing hepatic glycogen cycling at appreciable rates, with cycling rates in the fed state (40 %/h) exceeding those in the fasted state (20 %/h) [5]. *In vitro*, in the isolated liver from fasted rats perfused with 20 mM glucose and 10 mM alanine, Shulman et al. [6] found the rate of hepatic glycogen breakdown to be 60 % of the glycogen synthetic rate. In hepatocytes isolated from fasted rats, incubated with 20 mM glucose, the flux through GP was 50 % of the synthase flux [7]. High rates of glycogen cycling were also observed in hepatocytes from fed rats [8]. Glucose/glucose 6-phosphate cycling values of 20 % have been measured in starved rats *in vivo* [9], while values of 80 % cycling have been observed in isolated hepatocytes incubated with 10 mM glucose alone [1].

It was our hypothesis that the degree of glucose or glycogen cycling which occurs in isolated rat hepatocytes is influenced by the addition of substrates such as amino acids or fatty acids. We primarily examined the role of the fatty acid oleate, since long-chain fatty acids are implicated in the pathophysiological reaction

Abbreviations used: GS, glycogen synthase; GS*a*, active (dephosphorylated) GS; GS*b*, inactive (phosphorylated) GS; GS*a* + *b*, total GS; GP, glycogen phosphorylase; GP*a*, active (phosphorylated) GP; GP*a* + *b*, total GP; DHA, dihydroxyacetone; ER, endoplasmic reticulum.

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of the liver to produce glucose under hyperglycaemic conditions in Type 2 diabetes [10,11]. We also examined the role of amino acids, which are potent stimulators of glycogen accumulation [4,12]. While much of the previous work done to characterize the degree of glucose and glycogen cycling has made use of radioactive isotope labelling techniques, we have applied two novel and well-characterized inhibitors: S4048, a chlorogenic acid derivative which is a specific inhibitor of glucose 6-phosphate translocase [13,14] and CP320626, a specific inhibitor of GP [15,16]. This compound is related to CP-91149, which has been described previously [17].

Our results indicate that amino acids stimulate GS and inhibit glucose 6-phosphatase flux, while oleate inhibits glycolysis and stimulates flux through glucose 6-phosphatase, thereby promoting glucose production. With glucose 6-phosphate translocase pharmacologically suppressed, oleate stimulates glycogen deposition. The data show that extensive glucose/glucose 6-phosphate cycling in isolated rat hepatocytes prevents the accumulation of glycogen and that nutrients such as amino acids and fatty acids influence the degree of glucose/glucose 6-phosphate cycling.

MATERIALS AND METHODS

Materials

S4048 and CP320626 [16] were synthesized by the Chemistry Department at AVENTIS Pharma Deutschland G.m.b.H.

Hepatocyte isolation and incubation

Hepatocytes were isolated from 24-h-fasted male Wistar rats (200–250g body weight) by *ex situ* liver perfusion in the presence of collagenase [18]. Cells ($\pm$12 mg dry mass) were incubated at 37 °C in plastic scintillation vials in a volume of 2 ml in Krebs–Henseleit bicarbonate buffer, supplemented with 2% defatted BSA and 10 mM sodium Hepes, pH 7.4, in an atmosphere of O_2/CO_2 (19:1) for 1 h. Unless otherwise indicated, the standard incubation medium contained 20 mM glucose. This concentration is only slightly higher than the concentration of glucose we observed in the portal vein of rats fed a diet containing 60% (of total energy intake) carbohydrate, 18% fat and 22% protein (12.5$\pm$0.3 mM; $n = 4$, results not shown) and similar to the value of 17.8$\pm$1.0 mM ($n = 7$) in the liver of these rats (results not shown; cf. [3]). To examine the effect of amino acids on glycogen metabolism, a physiological mixture of amino acids (4$\times$AA), i.e. the postprandial concentration found in the portal vein of a rat, was added to some of the incubations. The composition of the 1$\times$ mixture is described in [18]. In order to examine glucose production, it was necessary to avoid the high glucose background with incubation with 20 mM glucose; therefore 20 mM dihydroxyacetone (DHA) was substituted for glucose in some incubations.

For measurement of the localization of glucokinase activity, hepatocytes were rapidly separated from their incubation medium at the end of the incubations by spinning in a microcentrifuge (1 s), followed by extraction of the hepatocytes with a medium containing 300 mM sucrose, 3 mM potassium Hepes, 5 mM $MgCl_2$, 2 mM dithiothreitol and 50 μg/ml digitonin as recommended by Agius and Peak [19].

Inhibitors (S4048 or CP320626) were dissolved in DMSO (final concentration, 0.7%, v/v) and were added apart or in combination. DMSO (0.7%) was added as vehicle control to incubations which were not exposed to either S4048 or CP320626. [2-^{3}H]Glucose was obtained from Amersham Corp.

Metabolite assays

Samples for the determination of glycogen were obtained by mixing 0.4 ml of the cell suspension with 1.6 ml of ice-cold 0.9% NaCl/10 mM sodium Hepes, pH 7.4, followed by rapid centrifugation (1 s) in a microcentrifuge and the cell pellet was extracted with 0.4 ml of 0.1 M KOH. After 45 min at 95 °C to destroy residual glucose, the samples were adjusted to pH 4.5 by the addition of 3 M acetic acid. A sample (100 μl) was incubated at 40 °C for 2 h with amyloglucosidase to degrade glycogen to glucose and then a standard fluorimetric glucose assay was performed. Glucose, lactate and ATP concentrations were measured in neutralized (pH 6.5–7.0) supernatants from $HClO_4$ (4%, w/v)-precipitated extracts of the cell suspension. For determination of glucose, samples were incubated with hexokinase, glucose-6-phosphate dehydrogenase, $NADP^+$ and ATP; lactate was measured spectrophotometrically with NAD^+, and lactate dehydrogenase and intracellular ATP concentrations were determined fluorimetrically, using glucose, hexokinase, glucose-6-phosphate dehydrogenase and $NADP^+$ [20].

[^{3}H]Water release from [2-^{3}H]glucose (1 μCi/ml) was measured as described in [21]. Protein was measured by the method of Lowry et al. [22].

Enzymic assays

GS and GP activities were measured in cell extracts that had been washed with 4 vol. of ice-cold 0.9% NaCl/10 mM sodium Hepes, pH 7.4, quickly pelleted for 1 s with a microcentrifuge and the pellet was then dissolved with buffer containing 50 mM glycylglycine, pH 7.0, 75 mM NaF, 3 mM EDTA, 0.5% glycogen and 0.1% Triton X-100. GS activity was measured with UDP-[U-^{14}C]glucose and glycogen (pH 7.2) for 20 min at 37 °C as previously described by Lavoinne et al. [21], in the presence of 6 mM glucose 6-phosphate (for total GS; GS$a+b$) or 7.5 mM Na_2SO_4 (for GSa). GPa activity was measured with [^{14}C]glucose-1-phosphate and glycogen (pH 6.8) for 20 min at 37 °C as previously described by Hue et al. [8], in the presence of caffeine for GPa and with AMP plus 1,2-dimethoxyethane for total GP (GP$a+b$) [23]. Glucokinase activity was measured with glucose, ATP, NAD^+ and NAD^+-specific glucose 6-phosphate dehydrogenase as described in [19]. Glucose 6-phosphatase activity in microsomes was measured exactly as described by Parker et al. [14]. Liver microsomes were prepared by differential centrifugation [14].

Statistics

All values are expressed per g dry mass of liver cells and as means$\pm$S.E.M. for the various conditions examined with the number of separate hepatocyte preparations in parentheses. The statistical significance of differences of the means was calculated using Student's t test.

RESULTS

Inhibition by S4048 of glucose 6-phosphate translocation in microsomes

The ability of S4048 as an inhibitor of glucose 6-phosphate translocation across the endoplasmic-reticulum (ER) membrane was tested in isolated liver microsomes. In intact microsomes, 10 μM S4048 inhibited glucose 6-phosphatase activity by 90%, from a control value of 59$\pm$7 (3) to 6$\pm$2 (3) nmol of P_i/min per

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Table 1 Effect of S4048 on glucose and glycogen production from gluconeogenic substrates

Hepatocytes were incubated for 1 h with the substrates indicated, in the absence or presence of 10 μM S4048. 4 × AA represents the concentration of amino acids found in the portal vein of fed rats, and the combination of 10 mM lactate and 1 mM pyruvate was used. Data are means ± S.E.M. for three to four different hepatocyte preparations. *, Significantly different from the corresponding control in the absence of S4048 ($P < 0.05$).

	Glucose production (μmol/g dry mass)		Glycogen synthesis (μmol/g dry mass)	
Substrate	Control	S4048	Control	S4048
None	26 ± 9	8 ± 5*	2 ± 1	1 ± 1
4 × AA	84 ± 12	9 ± 3*	5 ± 1	16 ± 4*
Lactate/pyruvate	243 ± 18	15 ± 2*	2 ± 1	38 ± 6*
DHA				
10 mM	381 ± 17	22 ± 9*	4 ± 2	122 ± 12*
20 mM	496 ± 46	91 ± 33*	15 ± 5	160 ± 15*

mg of protein ($P < 0.05$). However, no inhibition was observed when the microsomal membrane was disrupted with 0.5 % (w/v) cholate [control, 62 ± 11 (3) versus 69 ± 12 (3) nmol of P_i/min per mg of protein in the presence of S4048; results not shown].

Inhibition of gluconeogenesis by S4048

The efficacy of S4048 as an inhibitor of glucose 6-phosphate translocase in hepatocytes was tested by its ability to inhibit gluconeogenesis from various substrates, in the absence of glucose (Table 1). The concentration of 10 μM was found by titration to be the minimal concentration of S4048 that was maximally effective (results not shown). Inhibition of glucose production from amino acids (at physiological concentrations), 10 mM lactate plus 1 mM pyruvate or 10 mM DHA was almost complete (> 96 %). Inhibition by S4048 was incomplete, although still high (85 %) with 20 mM DHA, when the rate of glucose production (and thus flux through glucose 6-phosphate translocase) was very high. With each of the substrates, synthesis of glycogen was negligible in the absence of S4048, but increased considerably in the presence of S4048, with the highest values observed with DHA as the substrate (Table 1).

Effect of amino acids on glycogen production from glucose

In the presence of 20 mM glucose as the sole glycogenic substrate, basal glycogen accumulation rate was 16.9 ± 3.8 ($n = 8$) μmol/h per g (Figure 1A). Glycogen accumulation increased almost 5-fold when glucose 6-phosphate translocase was inhibited by S4048, and also when glycogen cycling was prevented by the inhibition of GP with CP320626. In the presence of both inhibitors, glycogen accumulation increased 8-fold. Addition of a physiological mixture of amino acids (4 × AA) increased the basal rate of glycogen accumulation 4-fold, while in the presence of either S4048 or CP320626, amino acid stimulation of glycogen accumulation was 1.5-fold. In the presence of both inhibitors amino acids did not significantly further increase glycogen accumulation.

The effect of amino acids, in combination with the two inhibitors, on the glycogen-metabolizing enzymes, is shown in Figure 1(B). GS*a* activity (which was 24 ± 4 % of the total activity of GS*a* + *b* under basal conditions; results not shown) increased 2-fold when amino acids were added to the incubation mixture. Incubation with amino acids and CP320626 resulted in

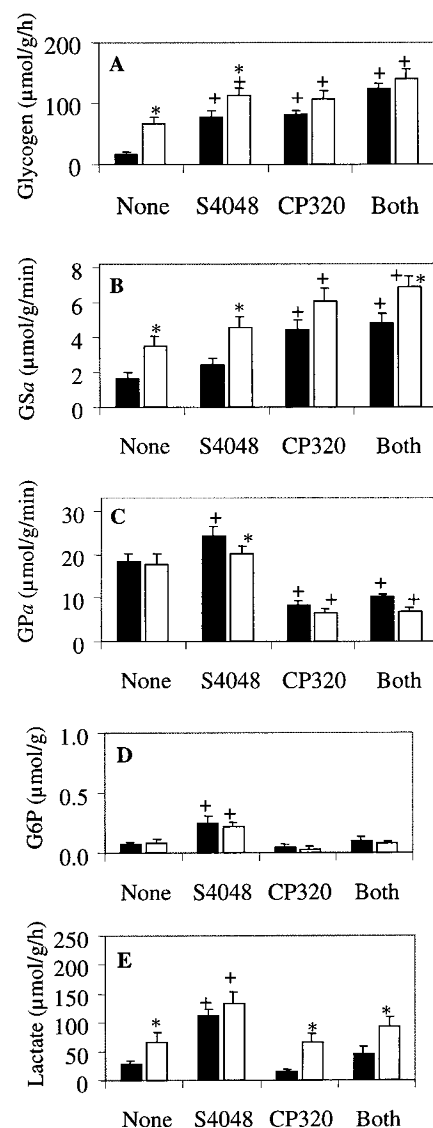

Figure 1 Influence of inhibition of glucose 6-phosphate (G6P) translocase and of GP on glucose metabolism

The influence of glucose only (black bars) or of glucose + 4 × AA mixture (open bars) was studied on glycogen synthesis (**A**), GS*a* activity (**B**), GP*a* activity (**C**), the intracellular concentration of glucose 6-phosphate (**D**) and lactate production (**E**) in the presence of: no inhibitors, 10 μM S4048, 10 μM CP320626 and both 10 μM S4048 and 10 μM CP320626. Data are means ± S.E.M. for five to eight experiments performed with separate hepatocyte preparations. *, Statistically different ($P < 0.05$) from own control (i.e. black bars); +, statistically different ($P < 0.05$) from the corresponding 'None' bars (i.e. with or without amino acids). CP320, CP320626.

the greatest extent of GS activation. The stimulation of GS by amino acids was also seen in the presence of the inhibitors.

Basal GP*a* activity (which was 31 ± 5 % of the total activity GP*a* + *b*; results not shown) was not affected by amino acids (Figure 1C). Incubation with CP320626 strongly decreased GP*a* activity, which was to be expected.

Effect of amino acids on intracellular glucose 6-phosphate levels and lactate production

In the presence of glucose alone, the level of intracellular glucose 6-phosphate was 0.07 ± 0.02 μmol/g dry mass (Figure 1D).

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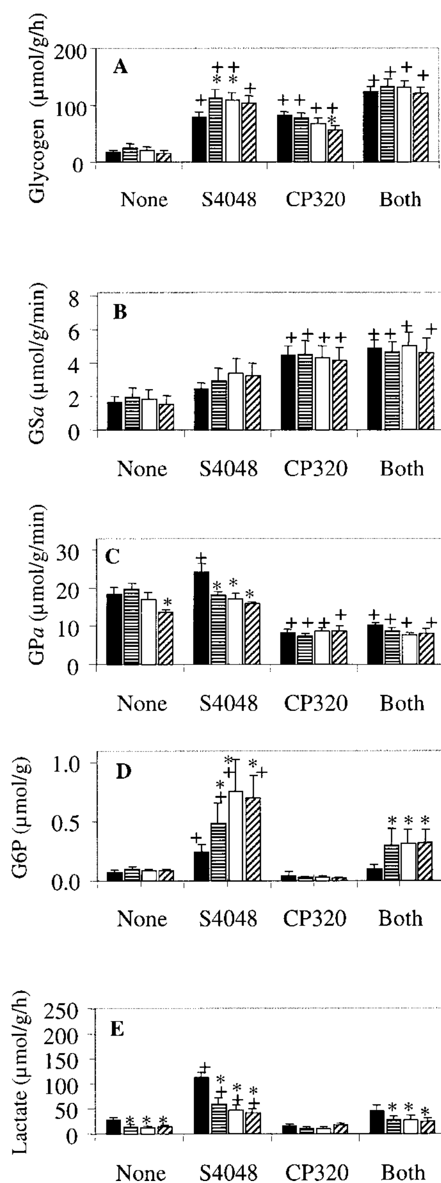

Figure 2 Influence of oleate on glucose metabolism

The influence of oleate (black bars, no oleate; horizontally hatched bars, 0.5 mM oleate; open bars, 1.0 mM oleate; cross-hatched bars, 1.5 mM oleate) was studied on glycogen synthesis from glucose (**A**), GS*a* activity (**B**), GP*a* activity (**C**), the intracellular concentration of glucose-6-phosphate (G6P) (**D**) and lactate production (**E**) in the presence of: no inhibitors, 10 μM S4048, 10 μM CP320626 and both 10 μM S4048 and 10 μM CP320626. Data are means $\pm$ S.E.M. for five to eight experiments performed with separate hepatocyte preparations. *, Statistically different ($P < 0.05$) from own control (i.e. black bars); +, statistically different ($P < 0.05$) from the corresponding 'None' bars (i.e. with or without oleate). CP320, CP320626.

Glucose 6-phosphate levels rose 3-fold in the presence of S4048, but remained low in the presence of CP320626 alone or CP320626 + S4048 respectively. Similar results were obtained in the presence of amino acids (Figure 1D).

Basal lactate production during the 60 min incubation period of the hepatocytes was 28.7 ± 4.9 μmol/h per g dry mass (Figure 1E). This value increased 4-fold when glucose 6-phosphate cycling to glucose was prevented with S4048. Lactate production was not significantly decreased ($P > 0.05$) by GP*a* inhibition, but was significantly decreased by GP*a* inhibition in the presence of S4048, as compared with S4048 alone. Amino acid incubation increased lactate production under all conditions. Presumably this extra lactate was derived from amino acid metabolism. It is noteworthy that the combination of both CP320626 and S4048 also caused a decrease in lactate production as compared with S4048 alone, even in the presence of amino acids.

Effect of oleate on glycogen metabolism

In order to study the effect of oleate on glycogen synthesis, three concentrations of oleate (0.5, 1.0 and 1.5 mM) were examined. Concentrations of oleate higher than 1.5 mM gave rise to considerable mitochondrial uncoupling (cf. [24]) and thus caused intracellular ATP to decline (results not shown). Basal glycogen synthesis was not influenced by oleate addition (Figure 2A). When glucose 6-phosphate translocase was being inhibited by S4048, however, oleate addition was stimulatory. By contrast, in the presence of CP320626 alone, the addition of a high concentration of oleate was inhibitory. In the presence of both inhibitors, oleate was neither stimulatory nor inhibitory. When oleate (0.5 mM) was added in the presence of amino acids, but in the absence of inhibitors, glycogen synthesis increased from 71.1 ± 4.9 (11) to 90.2 ± 4.0 (10) μmol/h per g dry mass (results not shown; see the Discussion).

In contrast with the stimulation of glycogen synthesis *per se* by oleate during exposure to S4048, GS*a* activity was not significantly influenced by oleate incubation (Figure 2B). GP*a* activity was decreased by 1.5 mM oleate, while in the presence of S4048, all three concentrations of oleate were inhibitory (Figure 2C).

Effect of oleate on intracellular glucose 6-phosphate levels and lactate production

Basal intracellular glucose 6-phosphate concentrations were not influenced by oleate addition (Figure 2D). In the presence of S4048, however, glucose 6-phosphate levels rose 2-fold with 0.5 mM oleate and 3-fold with 1.0 and 1.5 mM oleate. Glucose 6-phosphate levels remained low in the presence of CP320626 and oleate, while oleate increased the glucose 6-phosphate concentration in the presence of CP320626 + S4048. The difference between the glucose 6-phosphate levels with S4048 only and CP320626 + S4048 emphasizes the influence of glycogenolysis on intracellular glucose 6-phosphate levels.

Under most conditions, production of lactate decreased on the addition of oleate. Only in the presence of CP320626 alone, when lactate was already very low, was no effect of oleate observed (Figure 2E).

Effect of oleate on metabolism of DHA

The effect of oleate was also studied in the presence of DHA, instead of glucose, as substrate for glucose 6-phosphate synthesis (Figure 3). As already noted in Table 1, DHA is an efficient gluconeogenic substrate, and production of glucose in its presence is a direct measure of net flux through glucose 6-phosphatase. As in the experiment described in Table 1, glucose production decreased strongly by glucose 6-phosphate translocase inhibition with S4048 (Figure 3A). GP*a* inhibition also decreased glucose production, while the combination of CP320626 + S4048 resulted in the lowest amount of glucose produced. The addition of 1 mM oleate slightly, but significantly, stimulated glucose production, also in the presence of CP320626. In comparison with incubation

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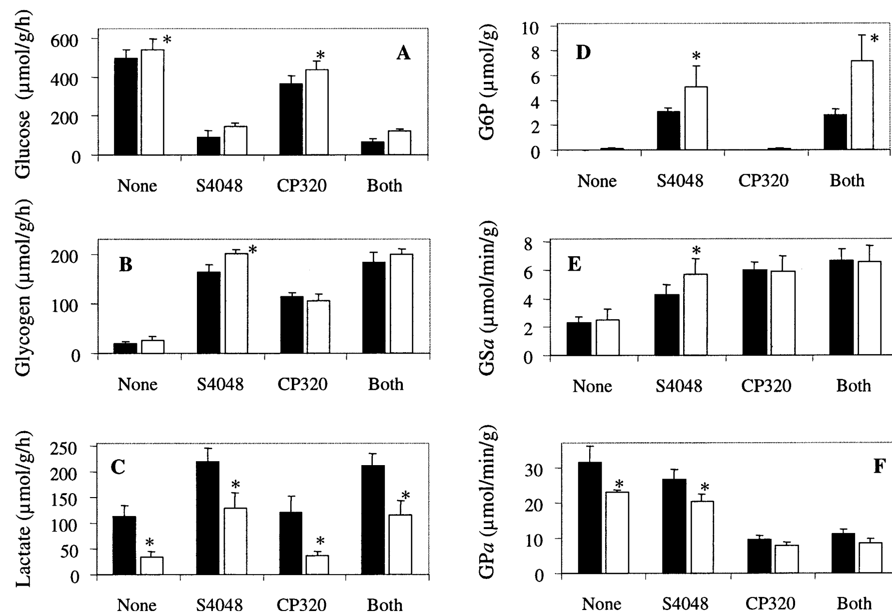

Figure 3 Effect of oleate on metabolism of DHA

Hepatocytes were incubated in the presence of 20 mM DHA in the absence (black bars) or presence of 1mM oleate (open bars). (**A**) Glucose production; (**B**) glycogen synthesis; (**C**) lactate production; (**D**) intracellular glucose 6-phosphate (G6P); (**E**) GS*a* activity; (**F**), GP*a* activity. Data are means $\pm$ S.E.M. for five to eight experiments performed with separate hepatocyte preparations. *, Statistically different ($P < 0.05$) from the control (without added oleate; black bars).

with 20 mM glucose, 20 mM DHA stimulated glycogen synthesis to a greater extent, but only in the presence of inhibitors (Figure 3B; cf Figure 1A). Oleate inhibited production of lactate from DHA under all conditions (Figure 3C) and increased intracellular glucose 6-phosphate (Figure 3D). Oleate stimulated production of glycogen only in the presence of S4048, but not in the presence of CP320626 (Figure 3B). This stimulation was accompanied by an increase in GS*a* (Figure 3E) and a decrease in GP*a* (Figure 3F). The very high levels of glucose 6-phosphate and the high rates of glycogen and lactate production after incubation with DHA and S4048, as compared with glucose as the substrate (cf. Figure 2; note the difference in scales for glucose 6-phosphate in Figures 2 and 3), are indicative of the rapid metabolism of DHA (Figures 3B, 3C and 3D).

Activity of glucokinase and detritiation of [2-^{3}H]glucose

Activity of glucokinase in hepatocyte extracts after incubation with glucose was not affected by any of the conditions studied. Neither its activity at the end of the incubations (which varied between 1.1 and 1.2 μmol/min per g dry mass, measured at 20 °C) nor its distribution (with about 90 % of glucokinase being cytosolic) were affected (results not shown). The cytosolic localization of glucokinase in the presence of 20 mM glucose is in agreement with the results obtained by Toyoda et al. [25]. Because the activity of glucokinase measured *in vitro* in hepatocyte extracts may not reflect its activity in the intact hepatocyte, glucokinase activity was also estimated by measurement of the production of [^{3}H]water from 20 mM [2-^{3}H]glucose [21]. No activity change was observed under any of the conditions examined. The observed rates of detritiation (in μmol/h per g dry mass) were: control, 171 $\pm$ 9 (9); +1 mM oleate, 172 $\pm$ 12 (8); +4 $\times$ AA, 168 $\pm$ 11 (9); +10 μM S4048, 185 $\pm$ 12 (9); +1 mM oleate +10 μM S4048, 187 $\pm$ 14 (7) (results not shown). The lack of effect of amino acids and of oleate is in agreement with previously obtained data [21,26].

Effect of inhibitors and oleate on ATP

In order to ascertain whether the effects of the inhibitors, amino acids or oleate were due to variations in cellular energetics, intracellular ATP was measured after each incubation. The mean value for the conditions containing glucose ranged from 12.1 to 13.7 μmol ATP/g dry mass (results not shown), and none of the conditions was found to be significantly different, thus indicating that cellular energetics were not compromised by any of the additions. Only in the condition where DHA and S4048 were both present did intracellular ATP decrease by approx. 20 % (results not shown), due to trapping of intracellular phosphate in the large pool of intracellular glucose 6-phosphate under these conditions (Figure 3D).

DISCUSSION

The purpose of the present study was to delineate the influence of glucose/glucose 6-phosphate cycling on glycogen deposition in isolated rat hepatocytes. This was achieved by making use of two novel inhibitors of glucose-6-phosphate translocase and GP. In this way it was possible to compare the rates of glycogen synthesis with or without glucose cycling and/or glycogen cycling. An important finding was that both glycogen and lactate accumulation greatly increased just through pharmacological inhibition of glucose 6-phosphate translocase. This result is a direct indication that glucose/glucose 6-phosphate cycling prevents glycogen deposition in fasted-rat hepatocytes incubated with glucose alone. Flux through glucokinase was not sensitive to inhibition by S4048. This is in agreement with the notion that the enzyme is not sensitive to product inhibition by glucose 6-

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phosphate [27,28]. Thus the increment in glycogen and lactate accumulation (in glucose equivalents) on addition of S4048 is a measure of flux through glucose 6-phosphatase. Our data are in agreement with previous estimates of glucose 6-phosphate cycling determined by isotopic analysis [1,9,26].

Foster et al. [29] have used high concentrations of 3-mercaptopicolinate to inhibit glucose 6-phosphatase and also observed increased glycogen deposition. However, 3-mercaptopicolinate is not specific and strongly inhibits phosphoenolpyruvate carboxykinase [30]. Inhibition of the latter enzyme results in increased cell volume by intracellular accumulation of impermeant metabolites connected to oxaloacetate [31,32], and cell swelling *per se* is a stimulus for glycogen synthesis [12] (see also below).

Glycogen accumulation also greatly increased with pharmacological inhibition of GP. CP320626 binds to the purine inhibitory site (I-site) of GP [15,17]. Apparently, inhibition of GP by this compound activates glycogen synthesis, possibly through a decreased interaction of GS phosphatase with inhibitory GP*a*, since activation of GS by CP320626 was observed (Figure 1B). Application of CP320626 thus creates the limitation that this compound simultaneously inhibits GP and activates GS. However, the compound is extremely useful for assessment of the capacity of glycogen deposition. Indeed, when both glucose 6-phosphate translocase and GP were inhibited, the rate of glycogen deposition from glucose was 140 μmol/h per g dry mass and approached the activity of glucokinase. This value is similar to the initial rate of glycogen synthesis (about 200 μmol/h per g dry mass of liver), observed in rat liver *in vivo* on glucose feeding after a 24-h fasting period [3], if one considers that *in vivo* part of the glycogen synthesized is not directly derived from glucose, but rather is produced via the indirect pathway [33,34].

High rates of glycogen synthesis were also observed in the presence of glucose and physiological concentrations of amino acids. Katz and co-workers [4] were the first to demonstrate that amino acids can stimulate glycogen production from glucose. Amino acid-induced cell swelling is, at least in part, responsible for activation of GS [13,35,36]. In response to cell swelling, hepatocytes undergo regulatory volume decrease by releasing KCl and the fall in intracellular chloride is responsible, at least in part, for stimulation of GS phosphatase which is inhibited by chloride [35]. The present data indicate that amino acids must also have decreased flux through glucose 6-phosphatase, because the increase in glycogen and lactate production by S4048 was less in the presence of amino acids than in their absence (Figure 1A). The activity of GP was not significantly affected by amino acids, in agreement with previous findings [7,12,21] and amino acids also do not affect flux through glucokinase (cf. [21]). It must be pointed out that the inhibition by amino acids of flux through glucose 6-phosphatase was not complete, because addition of S4048 further increased glycogen accumulation in the presence of amino acids (Figure 1A).

One explanation for the partial inhibition of glucose 6-phosphatase flux by amino acids may be that activation of GS diverts glucose 6-phosphate away from the glucose 6-phosphatase reaction. Another explanation is that amino acids, in conjunction with activation of GS, directly inhibit glucose 6-phosphatase or the translocase. We have tested this last possibility in isolated microsomes and also in amino acid-treated hepatocytes permeabilized with digitonin, but the results (not shown) were negative. However, the effect of amino acids on glucose 6-phosphatase may be indirect and also related to changes in intracellular chloride [37].

The effects of oleate on glycogen deposition can be explained on the basis of the known ability of fatty acids to inhibit glycolytic flux in hepatocytes [26,38,39]. The fate of accumulating glucose 6-phosphate depends on the activity of glucose 6-phosphatase relative to that of GS. In the presence of glucose and of S4048 (or of amino acids) to inhibit flux through glucose 6-phosphate translocase, inhibition of glycolysis by oleate resulted in accumulation of glucose 6-phosphate and repartitioning of this metabolite towards glycogen accumulation (Figure 2A). The high concentration of glucose 6-phosphate may have been responsible for the decrease in GP*a* activity under these conditions, as high glucose 6-phosphate concentrations inhibit phosphorylase kinase [27,40]. In the absence of S4048 (or of amino acids), glycogen accumulation did not increase upon oleate addition and glucose 6-phosphate was directed back to glucose. Figure 2(A) shows that oleate must in fact have stimulated flux through glucose 6-phosphatase, because oleate decreased glycogen formation with CP320626, but not when S4048 was also present. Indeed, when DHA was used to examine glucose production, 1mM oleate significantly elevated glucose production (Figure 3A; cf. [41]). These data are consistent with the fatty acid-induced increase in glucose 6-phosphatase flux as isotopically measured previously in hepatocytes incubated with glucose alone [26].

With glucose as the substrate and with amino acids present, the increase in glycogen deposition upon addition of S4048 was not accompanied by increased GS*a* activity (Figure 1B) (nor was GP*a* affected; Figure 1C), even though intracellular glucose 6-phosphate increased 3-fold under these conditions (Figure 1D). It is generally assumed that an increase in intracellular glucose 6-phosphate activates GS phosphatase and thus increases GS*a* [27]. In order to explain these apparently contradictory results, one must assume that the intracellular pool of glucose 6-phosphate is not homogeneous and that glucose 6-phosphate in the vicinity of the ER (when its access to the ER is prevented) has no direct access to GS phosphatase. Indeed, there is ample evidence in the literature supporting the idea of glucose 6-phosphate compartmentation in hepatocytes [42–44]. Only when the concentration of glucose 6-phosphate becomes very high, such as observed in the presence of DHA and S4048, may glucose 6-phosphate compartmentation be overcome and is activation of GS observed (Figure 3).

Since GS*a* did not increase, the mechanism responsible for the increase in glycogen deposition from glucose upon addition of S4048 remains to be explained. GP*a* activity was not decreased (Figure 1C). The increase in glycogen synthesis may simply have been due to an increased local substrate supply. Another explanation is that local glucose 6-phosphate does not stimulate the dephosphorylation of GS, but rather stimulates inactive (phosphorylated) GS (GS*b*) activity. A third possibility is that the increase in glucose 6-phosphate may have promoted translocation of GS to the plasma membrane [45].

In summary, our data show that in isolated hepatocytes incubated with glucose alone, cycling of glucose 6-phosphate prevents net glycogen deposition. Physiological concentrations of amino acids stimulate GS activity and slow down flux through glucose 6-phosphatase. This mechanism may contribute to the phenomenon of autoregulation in which hepatic glucose output is kept constant when the supply of gluconeogenic precursors to the liver varies [46]. Oleate, on the other hand, inhibits glycolysis, and promotes flux through glucose 6-phosphatase. This mechanism may contribute to hepatic glucose overproduction as seen in Type 2 diabetes.

After submission of the original manuscript a paper [47] came to our attention in which the GP inhibitor CP-91149 was used to demonstrate the importance of this enzyme in the control of net glycogen deposition in cultured hepatocytes. The results obtained in that study are similar to ours.

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