Inhibition of autophagic proteolysis by inhibitors of phosphoinositide 3-kinase can interfere with the regulation of glycogen synthesis in isolated hepatocytes

Peter F. DUBBELHUIS*, Daphne A. VAN SLUIJTERS*, Edward F. C. BLOMMAART*, Lori A. GUSTAFSON*, George M. VAN WOERKOM*, Andreas W. HERLING†, Hans-Joerg BURGER† and Alfred J. MEIJER*¹

*Department of Biochemistry, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands, and †AVENTIS Pharma Deutschland G.m.b.h., Frankfurt-am-Main, Germany

Amino acid-induced cell swelling stimulates conversion of glucose into glycogen in isolated hepatocytes. Activation of glycogen synthase (GS) phosphatase, caused by the fall in intracellular chloride accompanying regulatory volume decrease, and activation of phosphoinositide 3-kinase (PI 3-kinase), induced by cell swelling, have been proposed as underlying mechanisms. Because PI 3-kinase controls autophagic proteolysis, we examined the possibility that PI 3-kinase inhibitors interfere with glycogen production due to their anti-proteolytic action. The PI 3-kinase inhibitor wortmannin inhibited endogenous proteolysis, the production of glycogen from glucose and the activity of active (dephosphorylated) GS (GS_a) in the absence of added amino acids. The stimulation by amino acids of glycogen production and of GS_a was only slightly affected by wortmannin. These effects of wortmannin could be mimicked by proteinase inhibitors. A combination of leucine, phenylalanine and tyrosine,

which we showed previously to stimulate PI 3-kinase-dependent phosphorylation of ribosomal protein S6, did not stimulate glycogen production from glucose. In contrast with wortmannin, LY294002, another PI 3-kinase inhibitor, strongly inhibited both glycogen synthesis and GS_a activity, irrespective of the presence of amino acids. Inhibition of glycogen synthesis by LY294002 could be ascribed in part to increased glycogenolysis and glycolysis. It is concluded that, in hepatocytes, activation of PI 3-kinase may not be responsible for the stimulation of glycogen synthesis by amino acids; LY294002 inhibits glycogen synthesis and stimulates glycogen breakdown by a mechanism that is unrelated to its action as an inhibitor of PI 3-kinase.

Key words: cellular swelling, CP320626, LY294002, S4048, wortmannin.

INTRODUCTION

In hepatocytes, amino acids are potent stimulators of glycogen synthesis from glucose [1–3]. This is largely the consequence of the ability of certain amino acids (e.g. glutamine and proline) to cause cell swelling following their concentrative Na⁺-dependent transport across the plasma membrane and the rapid production of (impermeant) glutamate and aspartate in the course of their intracellular metabolism [4,5]. In response to the initial cell swelling, hepatocytes undergo ‘regulatory volume decrease’ and release KCl to the extracellular medium in an attempt to restore their original volume [6]. We have been able to demonstrate that the fall in intracellular chloride results in de-inhibition of glycogen synthase (GS) phosphatase and contributes, at least in part, to the mechanism responsible for the amino acid-induced increase in glycogen production [7].

An additional mechanism, accounting for the swelling-induced increase in glycogen synthesis, was on the basis of the observation [8] that cell swelling activates a protein kinase signal-transduction pathway containing phosphoinositide 3-kinase (PI 3-kinase) and p70S6k as components. Inhibition of PI 3-kinase by wortmannin and LY294002, but not the inhibition of p70S6k activation by rapamycin, was found to inhibit the swelling-induced activation

GS. It was concluded that PI 3-kinase, but not p70S6k, may be involved in the mechanism of activation of GS by cell swelling [8].

A complicating factor with regard to PI 3-kinase inhibitors is that these compounds also inhibit autophagic proteolysis [9,10] because of the requirement of PI 3-kinase class III in this process [10]. Therefore inhibition of glycogen synthesis by PI 3-kinase inhibitors may have been indirect, i.e. by inhibition of the production of amino acids from intracellular proteins. In addition, there is the observation that leucine does not affect synthesis of glycogen [1,4,11], even though it activates p70S6k [12] and stimulates phosphorylation of ribosomal protein S6 in a wortmannin- and LY294002-sensitive manner [9]. For these reasons, we analysed the interaction of the PI 3-kinase inhibitors with glycogen metabolism in more detail.

EXPERIMENTAL

Materials

Wortmannin and leupeptin (*N*-acetyl-Leu-Leu-Arg-al) were obtained from Sigma (St Louis, MO, U.S.A.), LY294002 [2-(4-morpholinyl)-8-phenylchromone] was purchased from Biomol

Abbreviations used: DHA(P), dihydroxyacetone (phosphate); GP, glycogen phosphorylase; GP_a, active (phosphorylated) GP; GS, glycogen synthase; GS_a, active (dephosphorylated) GS; G6P, glucose 6-phosphate; G6Pase, glucose-6-phosphatase; PEP, phosphoenolpyruvate; PI 3-kinase, phosphoinositide 3-kinase.

¹ To whom correspondence should be addressed (e-mail a.j.meijer@amc.uva.nl).

(Plymouth Meeting, PA, U.S.A.) and calpain inhibitor I (N-acetyl-Leu-Leu-norleucinal) was from Roche Diagnostics (Almere, The Netherlands). [$6\text{-}^3\text{H}$]Glucose was obtained from Amersham Pharmacia (Roosendaal, The Netherlands). CP320626 and S4048 were synthesized by the Chemistry Department of AVENTIS Pharma Deutschland G.m.b.h. Wortmannin, LY294002, calpain inhibitor I, CP320626 and S4048 were dissolved in Me_2SO ; the final concentration of Me_2SO in the incubations did not exceed 0.5% (v/v). Controls were carried out with Me_2SO alone. All other chemicals and enzymes were obtained from either Roche Diagnostics or Sigma. Rabbit polyclonal anti-(protein kinase B) (directed against Thr³⁰⁸) antibody was obtained from Cell Signaling Technology (Beverly, MA, U.S.A.).

Male Wistar rats (approx. 250 g) were obtained from T.N.O. (Zeist, The Netherlands), and were maintained on standard laboratory chow and water *ad libitum*, until initiation of the fasting period.

Preparation of hepatocytes

Hepatocytes from rats fasted for 16–20 h were isolated by collagenase perfusion, as described previously [9].

Hepatocyte incubation and sample preparation

Hepatocytes ($5\text{--}10\text{ mg of dry mass} \cdot \text{ml}^{-1}$) were incubated for 1 h at 37°C in Krebs–Henseleit bicarbonate buffer containing 10 mM sodium-Hepes, pH 7.4, 20 mM glucose and the components indicated in the legends to Figures and Tables (final volume, 2–3 ml). The gas atmosphere was O_2/CO_2 (19:1, v/v). When hepatocytes were incubated in a hypo-osmotic medium, the NaCl concentration in the Krebs–Henseleit bicarbonate buffer was decreased from 120 to 70 mM.

A complete mixture of amino acids in which each of the amino acids was present at a concentration 4 times that found in the portal vein of a fasted rat ($4 \times \text{AA}$) was used in some of the experiments. The composition of the $1 \times$ mixture was exactly as described in [13], except that the leucine concentration was $250\text{ }\mu\text{M}$.

At the end of the incubations, samples of the hepatocyte suspension were taken and prepared for metabolite and enzyme analyses. For the determination of lactate, glucose and ATP, 0.5 ml samples were quenched with HClO_4 (final concentration of 3.5%, w/v). The precipitated protein was removed by rapid centrifugation at 4°C in a microcentrifuge, and the supernatants were neutralized to pH 7 with a mixture of 2 M KOH plus 0.3 M Mops.

Samples for the determination of intracellular glucose 6-phosphate (G6P), dihydroxyacetone phosphate (DHAP) and phosphoenolpyruvate (PEP) were obtained by centrifugation of the cells through a layer of silicone oil (AR 200; Wacker Chemie, Munich, Germany) into a layer of HClO_4 (14%, w/v). The acid cell extracts were neutralized to pH 7 with 2 M KOH plus 0.3 M Mops.

Proteolysis was measured as the production of valine [14]. For the determination of valine, 0.5 ml samples were quenched with sulphosalicylic acid [final concentration, 4% (w/v)]. After removal of the protein by rapid centrifugation at 4°C in a microcentrifuge, the supernatant was brought to pH 2.2 with LiOH. Valine content was then determined with the aid of an amino acid analyser, using a lithium citrate buffer system (Alpha-plus; Pharmacia-LKB).

For the determination of glycogen, 0.5 ml of cell suspension was added to 1.5 ml of ice-cold 150 mM NaCl containing 10 mM sodium-Hepes, pH 7.4, and the cells were pelleted by centri-

fugation in a microcentrifuge for 1 s. After removal of the supernatant, the cell pellet was extracted with 0.5 ml of 0.1 M KOH, incubated for 30 min at 90°C , cooled and brought to pH 4.5 by addition of 3 M acetic acid. Precipitated protein was removed by centrifugation in a microcentrifuge for 1 min.

Samples for the determination of GS and glycogen phosphorylase (GP) were prepared as follows: 0.5 ml of the cell suspension was added to 1.5 ml of ice-cold 150 mM NaCl containing 10 mM sodium-Hepes, pH 7.4, and the cells were pelleted by centrifugation in a microcentrifuge for 1 s. After removal of the supernatant, cells were extracted with 0.3 ml of a medium containing 50 mM glycylglycine, pH 7.4, 75 mM NaF, 3 mM EDTA, 0.5% (w/v) glycogen and 0.1% (v/v) Triton X-100, and were immediately frozen in liquid nitrogen [15].

Glycolytic flux was measured with [$6\text{-}^3\text{H}$]glucose ($0.5\text{ }\mu\text{Ci/ml}$) [16].

Intracellular chloride was measured by incubation of the cells with $^{36}\text{Cl}^-$, exactly as described in [7].

Metabolite and enzyme assays

Glycogen, glucose, lactate, ATP and the activities of active (dephosphorylated) GS (GS_a), GS_a + b [total GS, where GS_b is inactive (phosphorylated) GS], active (phosphorylated) GP (GP_a) and GP_a + b [total GP, where GP_b is inactive (dephosphorylated) GP] at 37°C were measured as described previously [17]. Pyruvate kinase in hepatocyte extracts was measured at concentrations of PEP of 0.5 and 5 mM, as described by Felú et al. [18]. G6P, DHAP and PEP were measured fluorimetrically by methods described in [19]. Enzyme activities are expressed in units/g of dry mass of cells, in which 1 unit is equivalent to $1\text{ }\mu\text{mol} \cdot \text{min}^{-1}$. Cell protein was determined by the method of Lowry et al. [20].

Protein kinase B

At the end of the incubations, hepatocytes were diluted with 3 volumes of an ice-cold medium containing 150 mM NaCl and 10 mM sodium-Hepes, pH 7.4; cells were collected by rapid centrifugation (1 s) in a microcentrifuge. The cell pellet was lysed with Laemmli sample buffer and incubated at 95°C for 5 min. About $30\text{ }\mu\text{g}$ of cell protein per sample was separated using SDS/PAGE (10% polyacrylamide gels). After separation, a standard Western blotting procedure was performed, and the PVDF blot was incubated with anti-(phosphoprotein kinase B) antibody (directed against Thr³⁰⁸). The protein was visualized by enhanced chemiluminescence.

Statistical analysis

The data were summarized as means $\pm$ S.E.M., with the number of different hepatocyte preparations in parentheses. The statistical significance of differences of the means was calculated using Student's *t* test.

RESULTS

In order to test the ability of wortmannin and LY294002 to inhibit class I PI 3-kinase activity [the enzyme which produces $\text{PtdIns}(3,4)\text{P}_2$ and $\text{PtdIns}(3,4,5)\text{P}_3$] in hepatocytes, we first studied their effect on insulin-dependent phosphorylation of protein kinase B, which is known to depend on activity of class I PI 3-kinase [21]. Insulin-stimulated protein kinase B phosphorylation proved to be progressively inhibited by increasing concentrations of wortmannin and LY294002. Complete inhibition was obtained at concentrations of 300 nM wortmannin and 100 μM LY294002

Figure 1 Titration of insulin-stimulated phosphorylation of protein kinase B with the PI 3-kinase inhibitors LY294002 and wortmannin

Hepatocytes were pre-incubated for 18 min with 20 mM glucose; this was followed by addition of LY294002 or wortmannin at the concentrations indicated. After 2 min, insulin (0.1 μ M) was added and samples were taken after a further 4 min. Protein kinase B was probed with anti-(phospho-Thr³⁰⁸) antibody. Similar results were obtained with two other experiments carried out with different hepatocyte preparations.

(Figure 1), concentrations that were used in subsequent experiments.

Next, we studied the effect of both compounds on glycogen synthesis from glucose. Basal glycogen production was relatively low, but was greatly stimulated by a complete mixture of all 20 amino acids at physiological concentrations. In agreement with our previous observations, high concentrations of glutamine (in the absence and presence of ornithine to prevent ammonia accumulation), proline alone, and hypo-osmolarity also stimu-

lated glycogen production (Table 1) [4,5]. A combination of phenylalanine, tyrosine and leucine, which is effective in stimulating wortmannin-sensitive and LY294002-sensitive phosphorylation of ribosomal protein S6 in hepatocytes [9], was unable to stimulate glycogen production (Table 1). Wortmannin partially inhibited glycogen production, both in the absence and in the presence of amino acids or under hypo-osmotic incubation conditions. However, wortmannin only slightly affected the increment in glycogen production caused by amino acid addition or by hypo-osmolarity. By contrast, and unexpectedly, LY294002 strongly inhibited glycogen production under all conditions, and largely prevented the stimulation by amino acids or hypo-osmolarity. It must be stressed that LY294002 did not affect cellular ATP levels (Table 1).

Wortmannin and LY294002 also inhibit class III PI 3-kinase, which produces PtdIns3P. This lipid product is essential for autophagy, and both wortmannin and LY294002 inhibit autophagic proteolysis [9,10]. When amino acids are not present in the incubation medium, they can be formed by accelerated autophagic proteolysis and proteolytically formed amino acids, even when present in low amounts, may stimulate glycogen synthesis from glucose. As shown in Figures 2(A) and 2(C), increasing concentrations of either wortmannin or LY294002 did indeed progressively inhibit proteolysis under these conditions. Wortmannin also strongly inhibited glycogen synthesis from glucose in the absence of amino acids, at concentrations similar to those required for inhibition of proteolysis (Figure 2B). In the presence of amino acids, the same concentrations of wortmannin inhibited glycogen synthesis only slightly, and the extent of stimulation of glycogen production by amino acids was similar at

Table 1 Effect of wortmannin and LY294002 on glycogen production, on the activities of GS and GP, and on proteolysis and intracellular ATP

Hepatocytes were incubated with 20 mM glucose and the additions as indicated in the Table. Concentrations: wortmannin, 300 nM; LY294002, 100 μ M; calpain inhibitor I (CI), 0.15 mM; leupeptin, 0.2 mM; glutamine (Gln), 10 mM; ornithine (Orn), 3 mM; proline (Pro), 10 mM; leucine (Leu), 1 mM; phenylalanine (Phe), 0.2 mM; and tyrosine (Tyr), 0.3 mM. In the control ('None'), GSa was $29 \pm 3\%$ of total GS (GSa + b; see the Experimental section for further details), and GPa was $37 \pm 4\%$ of GPa + b. Hypo, hypo-osmolarity.

Additions	Glycogen (μ mol/gdm* per h)	GSa (μ mol/gdm per min)	GPa (μ mol/gdm per min)	Proteolysis (μ mol valine/gdm per h)	ATP (μ mol/gdm)
None	24.8 $\pm$ 2.0 (57)	1.5 $\pm$ 0.1 (9)	38.3 $\pm$ 5.5 (8)	10.6 $\pm$ 0.5 (25)	15.0 $\pm$ 0.8 (25)
Wortmannin	10.0 $\pm$ 1.3 (49)†	1.0 $\pm$ 0.1 (6)†	47.9 $\pm$ 9.1 (7)	4.1 $\pm$ 0.4 (21)†	13.7 $\pm$ 0.3 (17)
LY294002	3.5 $\pm$ 1.0 (28)†	0.9 $\pm$ 0.1 (9)†	36.6 $\pm$ 5.4 (9)	3.6 $\pm$ 0.1 (6)†	13.3 $\pm$ 0.3 (16)
CI	8.5 $\pm$ 1.7 (16)†	0.9 $\pm$ 0.1 (5)†	61.2 $\pm$ 9.9 (6)†	1.7 $\pm$ 0.2 (7)†	13.0 $\pm$ 1.0 (5)
Leupeptin	13.1 $\pm$ 2.4 (10)†	—	—	5.2 $\pm$ 0.6 (7)†	—
4 $\times$ AA	79.5 $\pm$ 3.0 (40)	3.5 $\pm$ 0.4 (8)	30.4 $\pm$ 5.9 (8)	4.4 $\pm$ 0.3 (11)	14.1 $\pm$ 0.4 (16)
4 $\times$ AA + wortmannin	65.0 $\pm$ 4.2 (31)†	2.3 $\pm$ 0.4 (7)†	35.3 $\pm$ 5.4 (7)	3.3 $\pm$ 0.3 (6)	14.4 $\pm$ 0.5 (9)
4 $\times$ AA + LY294002	22.2 $\pm$ 3.3 (24)†	1.5 $\pm$ 0.2 (8)†	44.7 $\pm$ 2.9 (6)	4.3 $\pm$ 0.1 (4)	13.4 $\pm$ 0.5 (12)
4 $\times$ AA + CI	59.9 $\pm$ 4.3 (11)†	2.4 $\pm$ 0.3 (6)†	39.9 $\pm$ 4.2 (6)	1.7 (2)	11.7 $\pm$ 1.4 (3)
4 $\times$ AA + leupeptin	73.7 $\pm$ 4.0 (5)	—	—	2.3 $\pm$ 0.2 (4)	—
Hypo	59.8 $\pm$ 5.8 (23)	—	—	9.6 $\pm$ 0.5 (12)	14.8 $\pm$ 0.5 (6)
Hypo + wortmannin	38.2 $\pm$ 4.7 (19)†	—	—	4.1 $\pm$ 0.3 (9)†	14.5 $\pm$ 0.5 (4)
Hypo + LY294002	14.2 $\pm$ 5.6 (6)†	—	—	3.8 $\pm$ 0.3 (4)†	14.2 $\pm$ 0.3 (3)
Hypo + CI	33.9 $\pm$ 1.6 (5)†	—	—	1.4 $\pm$ 0.3 (4)†	13.9 $\pm$ 0.3 (2)
Hypo + leupeptin	42.4 $\pm$ 7.5 (7)†	—	—	4.3 $\pm$ 0.5	—
Gln	86.3 $\pm$ 6.0 (20)	—	—	—	—
Gln + wortmannin	74.1 $\pm$ 6.1 (19)†	—	—	—	—
Gln + LY294002	51.8 $\pm$ 7.8 (3)†	—	—	—	—
Gln + Orn	91.5 $\pm$ 3.5 (15)	—	—	—	—
Gln + Orn + wortmannin	67.4 $\pm$ 4.5 (12)†	—	—	—	—
Gln + Orn + LY294002	34.0 $\pm$ 5.0 (5)†	—	—	—	—
Pro	99.7 $\pm$ 6.2 (16)	—	—	—	—
Proline + wortmannin	67.1 $\pm$ 10.0 (12)†	—	—	—	—
Proline + LY294002	35.8 $\pm$ 12.7 (3)†	—	—	—	—
Leu + Phe + Tyr	20.7 $\pm$ 5.6 (5)	—	—	—	—

* gdm, g of dry mass per cells.

† Significantly different from the corresponding control ($P < 0.05$).

Figure 2 Titration of proteolysis and glycogen synthesis with wortmannin and LY294002

Hepatocytes were incubated in the absence and presence of wortmannin and LY294002 at the concentrations indicated. In (A) and (C), proteolysis in the absence of added amino acids was measured as the production of valine. Synthesis of glycogen (B and D) was analysed in the absence (closed circles) or presence (open circles) of the complete mixture of amino acids ($4 \times \text{AA}$ medium). Data are the means $\pm$ S.E.M. for experiments carried out with three different hepatocyte preparations. In some cases, the S.E.M. is too small to be seen on the graphs. gdm, g of dry mass of cells.

all wortmannin concentrations (cf. Table 1). By contrast, increasing concentrations of LY294002 strongly inhibited glycogen synthesis from glucose in both the absence and presence of amino acids, and the extent of the stimulation by amino acids continued to decrease at concentrations of LY294002 above those required for maximal inhibition of proteolysis (Figure 2D). Apparently, LY294002 can also inhibit glycogen synthesis by a mechanism that is unrelated to its ability to inhibit endogenous proteolysis. This will be discussed below.

In order to obtain further support for the view that the inhibition of glycogen synthesis by wortmannin might have been due to inhibition of proteolysis, at least in a large part, we compared its effect with that of the cysteine proteinase inhibitors calpain inhibitor I and leupeptin. Leupeptin has previously been shown to be an effective inhibitor of autophagic proteolysis in hepatocytes [22]. As shown in Table 1, both calpain inhibitor I and leupeptin were at least as effective as wortmannin (and LY294002) in blocking protein breakdown. The inhibition was also similar to that caused by a physiological mixture of amino acids at concentrations known to inhibit autophagy (Table 1; also see [23,24]). Like wortmannin, calpain inhibitor I and leupeptin inhibited glycogen synthesis from glucose under basal conditions by 50–65%, but had little effect on the stimulation of glycogen production by the amino acid mixture (or by hypo-osmolarity; cf. Table 1). When glycogen synthesis in the absence

Figure 3 Rate of glycogen synthesis from glucose as a function of endogenous proteolysis

Hepatocytes were incubated in the absence of added amino acids with no further additions (1), 0.2 mM leupeptin (2), 300 nM wortmannin (3), 0.15 mM calpain inhibitor I (4) or 100 μM LY294002 (5). The data are extracted from Table 1. gdm, g of dry mass of cells.

Table 2 Effect of wortmannin and LY294002 on glycogenolysis in hepatocytes from fed rats

Hepatocytes from rats fed *ad libitum* were incubated in the absence or presence of 20 mM glucose, and in the absence or presence of either 300 nM wortmannin or 100 μM LY294002. The no. of independent experiments is shown in parentheses. gdm, g of dry mass of cells.

Addition	Glycogen disappearance (μmol/gdm per 90 min)	
	– Glucose	+ Glucose
None	323 $\pm$ 14 (4)	92 $\pm$ 4 (4)
Wortmannin	359 $\pm$ 12 (4)*	138 $\pm$ 4 (4)*
LY294002	422 $\pm$ 16 (3)*	207 $\pm$ 13 (3)*

* Significantly different from the corresponding control in the absence of wortmannin or LY294002 ($P < 0.05$).

of added amino acids was plotted against proteolysis in the absence and presence of the inhibitors, an approximately straight-line relationship between the two variables was obtained (Figure 3). LY294002 did not obey this relationship (Figure 3), again indicating that this compound also inhibited glycogen production independent of its ability to inhibit proteolysis. As with glycogen synthesis, wortmannin and calpain inhibitor I (leupeptin was not investigated) were equally effective in decreasing GSA activity in the absence and presence of amino acids, but had relatively little effect on the stimulation of GSA by the amino acid mixture (Table 1). These data suggested that a decreased rate of proteolysis by the PI 3-kinase inhibitor wortmannin must have contributed to its inhibition of glycogen production.

The question that remained to be answered was why LY294002 was more potent than wortmannin as an inhibitor of glycogen synthesis. Both wortmannin and LY294002 decreased GSA activity in the absence or presence of amino acids, LY294002 being the more effective (Table 1). GPa was affected neither by amino acids (cf. [3]) nor by wortmannin, but increased significantly when LY294002 was added in the presence of amino acids, but not in their absence (Table 1). Another indication that LY294002 may increase flux through GP was found in a series of experiments in which glycogen degradation was measured in hepatocytes obtained from fed rats (Table 2). Breakdown of glycogen was monitored in the absence or presence of glucose to

Table 3 Effect of LY294002 on glycogen production from glucose in the presence of CP320626 or S4048

Hepatocytes were incubated with 20 mM glucose (in the absence or presence of amino acids) and LY294002 (100 μ M), S4048 (10 μ M) or CP320626 (10 μ M), as indicated. The no. of independent experiments is shown in parentheses.

	Glycogen (μ mol/g of dry mass per h)	
	– LY294002	+ LY294002
None	21.5 $\pm$ 4.2 (10)	2.3 $\pm$ 0.4 (10) [†]
CP320626	79.7 $\pm$ 7.2 (10)*	53.6 $\pm$ 6.9 (10)* [†]
S4048	89.9 $\pm$ 11.9 (6)*	27.0 $\pm$ 4.5 (6)* [†]
4 $\times$ AA	65.3 $\pm$ 9.0 (5)	14.3 $\pm$ 2.6 (5)* [†]
4 $\times$ AA + CP320626	100.8 $\pm$ 10.1 (5)*	81.5 $\pm$ 9.3 (5)* [†]
4 $\times$ AA + S4048	110.4 $\pm$ 17.1 (3)*	54.8 $\pm$ 3.0 (3)* [†]

* Significantly different from the corresponding control in the absence of CP320626 or S4048 ($P < 0.05$); [†] significantly different from the corresponding control in the absence of LY294002 ($P < 0.05$).

Table 4 Effect of LY294002 on glycolytic flux

Hepatocytes were incubated with 20 mM [$6\text{-}^3\text{H}$]glucose, in the absence or presence of LY294002, wortmannin or S4048 at the concentrations indicated. Data are the means $\pm$ S.E.M. for three experiments carried out with different hepatocyte preparations.

Additions	Glycolytic flux (μ mol of glucose equivalents/g of dry mass of cells per h)
None	54.6 $\pm$ 5.7
LY294002 (5 μ M)	60.5 $\pm$ 5.0
LY294002 (50 μ M)	69.5 $\pm$ 5.0*
LY294002 (100 μ M)	67.7 $\pm$ 1.9*
S4048 (10 μ M)	98.3 $\pm$ 8.2 [†]
S4048 + LY294002 (5 μ M)	97.2 $\pm$ 9.1
S4048 + LY294002 (50 μ M)	117.8 $\pm$ 10.8*
S4048 + LY294002 (100 μ M)	128.2 $\pm$ 12.2*
Wortmannin (300 nM)	46.7 $\pm$ 3.8

* Significantly different from the control in the absence of LY294002 ($P < 0.05$);

[†] significantly different from the corresponding control in the absence of S4048 ($P < 0.05$).

cause inactivation of GP [25]; under both conditions, glycogen degradation was significantly increased by LY294002. Wortmannin slightly stimulated glycogen degradation, but the effect was much smaller than that observed with LY294002.

Recently, S4048 and CP320626, specific and powerful inhibitors of G6P translocase [26,27] and of GP [28,29] respectively, have become available. These compounds allowed us to obtain additional information on the mechanism by which LY294002 interferes with glycogen metabolism in hepatocytes. Hepatocytes from fasted rats were incubated with glucose in the absence or presence of the inhibitors, and in the absence or presence of amino acids to stimulate GS (Table 3). Inhibition of either GP or of G6P translocase greatly increased net glycogen deposition, especially when amino acids were absent, which stresses the importance of the two enzymes in the control of glycogen production in hepatocytes (cf. [30]). Inhibition of glycogen synthesis by LY294002 persisted in the presence of S4048, but was largely, albeit not completely, prevented by CP320626.

From these experiments, it is concluded that LY294002 decreased net glycogen production from glucose by promoting flux through GP, not by enhancing flux through glucose-6-phosphatase (G6Pase).

When hepatocytes were incubated with glucose and S4048, intracellular levels of G6P greatly increased [30], and addition of

Table 5 Effect of wortmannin and LY294002 on the production of glucose and lactate from DHA

Hepatocytes were incubated with either 15 mM DHA or 10 mM lactate plus 1 mM pyruvate (Lac/pyr), in the absence or presence of either 300 nM wortmannin or 100 μ M LY294002. Data are the means $\pm$ S.E.M. with the number of different hepatocyte preparations in parentheses.

Addition	Glucose (μ mol/g of dry mass per h)	Lactate (μ mol/g of dry mass per h)
None	16 $\pm$ 6 (3)	1 $\pm$ 5 (3)
Wortmannin	21 $\pm$ 5 (3)	8 $\pm$ 3 (3)
LY294002	24 $\pm$ 8 (3)	7 $\pm$ 4 (3)
DHA	385 $\pm$ 23 (5)	122 $\pm$ 13 (4)
DHA + wortmannin	360 $\pm$ 31 (5)	175 $\pm$ 6 (4)*
DHA + LY294002	311 $\pm$ 25 (5)*	209 $\pm$ 10 (4)*
Lac/pyr	291 $\pm$ 16 (3)	–
Lac/pyr + wortmannin	215 $\pm$ 11 (3)*	–
Lac/pyr + LY294002	180 $\pm$ 14 (3)*	–

* Significantly different from the corresponding control in the absence of wortmannin or LY294002 ($P < 0.05$).

LY294002 under these conditions decreased intracellular G6P from 1.21 ± 0.18 to 0.66 ± 0.06 μ mol/g dry mass of cells ($n = 7$; $P < 0.05$; results not shown). This is only possible if LY294002 has additional effects and that it stimulates glycolysis. Glycolytic flux, measured with [$6\text{-}^3\text{H}$]glucose, was, indeed, stimulated by LY294002, but not by wortmannin (Table 4). In the presence of S4048, with G6P translocase fully inhibited [30], glycolytic flux was greatly stimulated, as expected. In the presence of S4048, LY294002 enhanced further glycolytic flux.

Further evidence in support of the possibility that glycolysis was activated by LY294002 was obtained in experiments in which glucose was replaced with DHA. Under these conditions, glycogen deposition is negligible (results not shown; however, see [30]) and production of lactate increased upon addition of LY294002, with the simultaneous inhibition of glucose synthesis (Table 5). Addition of wortmannin had a smaller effect on lactate production, with no effect on gluconeogenesis. The inhibition of gluconeogenesis and stimulation of lactate production by 100 μ M LY294002 was accompanied by decreases in intracellular DHAP (from 817 ± 19 to 561 ± 72 nmol/g of dry mass of cells; $n = 3$; $P < 0.05$) and PEP (from 1141 ± 89 to 509 ± 29 nmol/g of dry mass of cells; $n = 3$; $P < 0.05$) (results not shown).

The effect of LY294002 on glucose production from lactate (plus pyruvate) was also studied. In this case, however, glucose production was not only substantially inhibited by LY294002 but also by wortmannin (Table 5; see the Discussion below).

DISCUSSION

An increase in hepatocyte volume, whether induced by certain amino acids or by hypo-osmolarity of the extracellular fluid, activates GS without affecting GP; as a result, net glycogen deposition from glucose increases [4]. Part of the underlying mechanism is activation of GS phosphatase caused by the fall in intracellular chloride accompanying regulatory volume decrease [7,8]. Production of intracellular glutamate by amino acid catabolism [5] is another stimulus of GS phosphatase [7]. Krause et al. [8] proposed that swelling-induced activation of total PI 3-kinase {but not insulin receptor substrate 1 ('IRS-1')-associated PI 3-kinase [31]} may be an additional mechanism contributing to GS activation. Our data with wortmannin as an inhibitor of PI 3-kinase confirm that wortmannin inhibits GS activity in hepatocytes [8]. Wortmannin decreased GS activity both in the

presence and absence of amino acids, but the increment caused by amino acid addition was only inhibited by 35% by the PI 3-kinase inhibitor (Table 1). This is analogous to the data of Krause et al. [8]. Similarly, glycogen deposition (not measured in [8]) was inhibited by wortmannin in both the absence and presence of amino acids, but in this case the increment caused by amino acid addition (or hypotonicity) remained largely unaffected by the PI 3-kinase inhibitor (Table 1 and Figure 2). This discrepancy between the effect of wortmannin on amino acid stimulation of GS activity and on net glycogen deposition may be due to the fact that amino acids not only increase flux through GS but simultaneously decrease G6Pase flux [30].

A complicating factor is that wortmannin inhibits endogenous amino acid production by autophagy, and that this may account for the inhibition of glycogen production in the absence of added amino acids (Table 1). In support of this, we also noted a similar inhibition of glycogen deposition by the protein degradation inhibitors leupeptin and calpain inhibitor I under these conditions.

It is important to stress that a combination of leucine, phenylalanine and tyrosine was not able to activate glycogen synthesis. High concentrations of leucine alone were also ineffective [4] and did not affect GS activity [31]. Among the various amino acids, leucine in particular is effective in stimulating wortmannin- and LY294002-sensitive phosphorylation of ribosomal protein S6 [9]. Activation of PI 3-kinase by leucine has also been demonstrated in muscle cells [32]. If activation of PI 3-kinase had contributed to the mechanism of activation of glycogen synthesis by amino acids, it is difficult to understand why leucine would not be effective. In our experiments, we were also unable to observe stimulation by insulin of glycogen production from glucose (results not shown; also see [31]), even though insulin was able to stimulate protein kinase B phosphorylation in a wortmannin- and LY294002-dependent manner (Figure 1; see [31]). Apparently, activation of PI 3-kinase was not sufficient to stimulate glycogen production under our experimental conditions.

Surprisingly, LY294002 strongly interfered with glycogen deposition and GS activity independently of its anti-proteolytic effect (Table 1, and Figures 2 and 3). A difference between LY294002 and wortmannin in their ability to inhibit hepatocytic glycogen production has been noted previously [33,34]. Although the existence of an LY294002-sensitive, wortmannin-insensitive PI 3-kinase cannot be excluded [33,34], most, if not all, classes of PI 3-kinases known today are sensitive to both compounds [21,35]. Moreover, cell swelling promotes the activation of class I PI 3-kinase [8,36], and this class can be completely inhibited by both wortmannin and LY294002 in hepatocyte preparations [9]. Therefore an alternative explanation for the aberrant behaviour of LY294002 was sought after, and we concluded that LY294002 must have enhanced flux through GP and also enhanced glycolysis, apparently by a mechanism that is not related to its ability to inhibit PI 3-kinase.

The specificity of the PI 3-kinase inhibitors is a source of continuous concern [21,37]. Wortmannin inhibits phospholipase A_2 at low concentrations [38]. Whether LY294002 has the same effect is not known. Arachidonic acid, a product of phospholipase A_2 activity, increases glycogenolysis [39], and inhibition of phospholipase A_2 would increase, rather than decrease, net glycogen production. In any case, wortmannin had relatively little effect on amino acid-stimulated glycogen production from glucose, and inhibition of phospholipase A_2 seems irrelevant under our experimental conditions. LY294002, but not wortmannin, has been shown to inhibit casein kinase II [37]. This cannot explain our results either, because inhibition of this

enzyme would increase rather than decrease glycogen production [40].

The reason for the difference in behaviour between LY294002 and wortmannin under our experimental conditions is not entirely clear. Initially, the possibility was considered that LY294002 enhanced intracellular chloride concentration, because chloride ions inhibit GS phosphatase [7]. However, this turned out not to be the case (results not shown). LY294002 (but not wortmannin) significantly increased activity of GP α , but only in the presence of amino acids (Table 1). The increase in GP α may explain the greater ability of LY294002, as opposed to wortmannin (Table 1), to inhibit activity of GS α under these conditions, because GP α is known to inhibit GS phosphatase [25]. An increase in cytosolic Ca^{2+} concentration cannot explain the increase in GP α by LY294002, because cytosolic Ca^{2+} decreases in the presence of LY294002 (and wortmannin) [41]. An increase in cAMP is also unlikely, because glycolytic flux increased, rather than decreased. A plausible explanation for the increased activity of GP is that the primary effect of LY294002 is activation of glycolysis. Activation of glycolysis results in a decrease in G6P (as was, indeed, observed), which may de-inhibit GP kinase [25]. If this mechanism is correct, the question of how LY294002 activates glycolysis remains to be solved. Evidence in support of activation of pyruvate kinase could not be obtained, because LY294002 treatment of hepatocytes, even when insulin is absent, has been reported to decrease pyruvate kinase activity in cell extracts by increasing its degree of phosphorylation [42]. We have confirmed this (results not shown). Notwithstanding, intracellular PEP decreased in the presence of LY294002, which does suggest activation of flux through pyruvate kinase. An attractive explanation would be that LY294002 stimulates flux through phosphofructokinase, because fructose 1,6-bisphosphate is a powerful activator of pyruvate kinase [43]. However, fructose 1,6-bisphosphate decreased, rather than increased, in the presence of LY294002 (results not shown). An alternative explanation for the increased flux through pyruvate kinase could be a fall in the cytosolic ATP/ADP ratio in the presence of LY294002. The precise mechanism of action of LY294002 on glycolysis was not explored further, however.

Because of the possible occurrence of cycling between glucose and lactate in hepatocytes [16], it cannot be excluded that, in addition to activation of glycolysis, gluconeogenesis from lactate was inhibited by LY294002. Although this was indeed the case, gluconeogenesis under these conditions was also sensitive to wortmannin (Table 5). This is to be expected, however, because gluconeogenesis from lactate requires mitochondrial efflux of oxaloacetate as aspartate, and the aspartate cycle in isolated hepatocytes is known to be stimulated by aspartate (and glutamate) produced by proteolysis [44].

In conclusion, the present data suggest that activation of PI 3-kinase may not be responsible for the stimulation of glycogen synthesis by amino acids. The inhibition of glycogen synthesis by wortmannin can be ascribed, at least to a large extent, to its anti-proteolytic effect. It must be stressed that this is not due to lack of specificity of the compound as an inhibitor of PI 3-kinase, because PI 3-kinase class III is essential for autophagy, as we showed previously [10]. LY294002 stimulates glycogen breakdown by a mechanism that is unrelated to its action as an inhibitor of PI 3-kinase.

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