Hormone and substrate regulation of glycogen accumulation in primary cultures of rat hepatocytes

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Hormonal and substrate regulation of hepatic glycogen accumulation was evaluated in primary cultures of hepatocytes prepared from 1-day-fasted rats. Hepatocytes were cultured in media containing 5 mM-glucose and 10 mM-lactate and then exposed to 100 nM-dexamethasone for 4 h before an increase in glucose concentration and the addition of insulin. When this protocol was used to mimic the post-prandial state *in vivo*, net glycogen accumulation (over 2 h) and insulin (10 nM) effects were linear at physiological (5–10 mM) and supraphysiological (20–30 mM) glucose concentrations. To define the role of substrates in glycogen accumulation, hepatocytes were incubated in a buffered salt solution containing 10 mM-glucose and either 10 mM-lactate or 5 mM-glutamine, or both. In the absence of hormones, net glycogen accumulation was increased by 59 %, 83 %, and 127 % by the addition of lactate, glutamine, and lactate plus glutamine respectively, compared with incubations with glucose alone, and 6-fold in the presence of substrates, insulin and dexamethasone. Labelling with [3-³H]glucose and [U-¹⁴C]glucose showed that in the absence of hormones approx. 50 % of glycogen formation came from glucose via the direct pathway and the remainder from glucose via the indirect pathway or from non-glucose precursors, or both. Insulin-dependent enhancement of glycogen formation is through stimulation of both the direct and indirect pathways, and dexamethasone-dependent stimulation occurs through stimulation of both these pathways of glycogen formation from glucose as well as from non-glucose precursors. Lactate serves as a gluconeogenic C₃ precursor for the observed enhanced glycogen formation, whereas glutamine-dependent enhancement of glycogen accumulation occurs primarily through a stimulation of the direct and indirect pathways of glycogen formation from glucose.

INTRODUCTION

The regulation of glycogen synthesis in the liver has been the subject of some controversy. It has been shown by several investigators (reviewed by Katz & McGarry, 1984; Katz *et al.*, 1986), in studies using perfused livers or isolated hepatocytes, that glucose is a poor precursor for glycogen synthesis. Concentrations of 30 mM or higher are required to achieve net glycogen accumulation. With the addition of mixtures of non-glucose substrates (e.g. glutamine, alanine, pyruvic acid, lactic acid), net glycogen synthesis can be achieved *in vitro* (Boyd *et al.*, 1981; Hems *et al.*, 1972; Katz *et al.*, 1976).

Previous studies (Newgard *et al.*, 1983; Parniak & Kalant, 1985; Spence & Koudelka, 1985) have employed labelled glucose in order to evaluate the non-glucose contribution to hepatic glycogenesis. In administering labelled glucose to rats, Newgard *et al.* (1983) reported 72–88 % decreases in the specific radioactivity of hepatic ³H-labelled glycogen glucose and 50–65 % decreases in that of hepatic ¹⁴C-labelled glycogen glucose. It was suggested (Newgard *et al.*, 1983) that most of the administered glucose is metabolized to C₃ substrates, including lactate. In studies coincidental with the present investigation, Parniak & Kalant (1985) and Spence & Koudelka (1985) demonstrated that the capacity to

synthesize glycogen via glucose by first converting glucose into gluconeogenic substrates is demonstrable in isolated rat hepatocytes maintained in primary culture.

With regard to the hormonal regulation of glycogen deposition, numerous studies have been reported (reviewed by Stalmans *et al.*, 1987; Van de Werve & Jeanrenaud, 1987) evaluating the regulation of glycogen synthesis by insulin and glucocorticoids. Although neither hormone is absolutely required for glycogen synthesis, the relationships between hormone and substrate effects on glycogen synthesis have been difficult to study, because of the lack of hormonal responsiveness of many liver preparations *in vitro* and the supraphysiological levels of glucose needed to achieve net glycogen accumulation.

In the present study, we employed primary cultures of rat hepatocytes to evaluate the incorporation of [3-³H]glucose and [¹⁴C]glucose into glycogen in order to follow the metabolic channelling of glucose, a gluconeogenic precursor (lactate) and an amino acid (glutamine) in our cells, and the effects of insulin and dexamethasone (DEX) on these events. Using a defined serum-free model *in vitro* mimicking the physiological state *in vivo*, we demonstrate that physiological concentrations of glucose can promote net glycogen deposition, that glycogen deposition is acutely responsive

Abbreviations used: PBS, phosphate-buffered saline [10 mM-sodium phosphate (pH 7.4)/0.15 M-NaCl]; DMEM, Dulbecco's Modified Eagle Medium; HBSS, Hanks' Balanced Salt Solution; DEX, dexamethasone.

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to insulin and a glucocorticoid, and that lactate and glutamine can each enhance glycogen deposition through different mechanisms.

METHODS

Male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.) weighing 200–300 g and fed *ad libitum* were used for all studies. Unless indicated, rats were fasted for 20 h before use, but allowed free access to water. Hepatocytes were isolated by the method of Feldhoff *et al.* (1977) as previously described (Cech *et al.*, 1980). Primary cultures of isolated hepatocytes in serum-free medium were performed by the method of Kletzien *et al.* (1976), as previously described (Caro & Amatruda, 1980a,b; Salhanick *et al.*, 1983) in Waymouth's MB 752/1 medium (GIBCO, Grand Island, N.Y., U.S.A., cat. no. 330-1440). Approx. 3 h after plating, the cells were washed repeatedly at room temperature (first medium change) with PBS, containing bovine serum albumin (1 mg/ml). The adherent cells were subsequently cultured for 18 h in a defined culture medium [either DMEM (GIBCO no. 320-1885) or Medium 199 (GIBCO no. 330-1180)] containing albumin (2 mg/ml) and 10 mM-lactate. This concentration of lactate (10 mM) approximates to hepatic portal-vein values (8.7 mM) reported in refed fasted rats (Newgard *et al.*, 1983). After being maintained in culture for 18 h, the cells were then washed (second medium change) and incubated in (a) culture media containing albumin (2 mg/ml), 10 mM-lactate and a final glucose concentration, unless indicated otherwise, of 10 mM, or in (b) a modified Hanks' Balanced Salt Solution (HBSS) (Hanks & Wallace, 1949), pH 7.4, containing 25 mM- NaHCO_3 , 2 mM-Hepes, 137 mM- NaCl , 5.4 mM- KCl , 0.43 mM- KH_2PO_4 , 0.80 mM- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.35 mM- Na_2HPO_4 , 1.26 mM- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mM-glucose, albumin (1 mg/ml) and, where present, 10 mM-lactate and 5 mM-glutamine. This concentration of glutamine (5 mM) is comparable with that found in widely used commercial culture media (e.g. DMEM).

To study the ability of DEX to modulate insulin-dependent glycogen accumulation, an aqueous stock solution of the steroid was prepared as previously described (Amatruda *et al.*, 1983) and added (final concn. 100 nM) 0–6 h before and at the time of the second medium change. Insulin (0.1–100 nM) was added at the time of the second medium change.

Glycogen content

Glycogen content in cultured hepatocytes was assessed by using the cold- HClO_4 procedure as described by Katz *et al.* (1976). The cells were washed repeatedly with ice-cold PBS and then stored frozen until assay. The extracted glycogen was then incubated with amyloglucosidase and the liberated glucose measured by standard spectrophotometric procedures. Net glycogen accumulation was calculated by subtracting the glycogen content at the beginning of the incubation (zero time). The data are expressed as nmol of glycogen glucose/mg of protein per unit time. Protein was measured as described by Miller (1959).

Glycogen synthesis

In studies to assess glycogen synthesis, hepatocytes were incubated for 2 h in HBSS containing 10 mM

unlabelled glucose and either D-[U- ^{14}C]glucose (0.75 $\mu\text{Ci/ml}$) or D-[3- ^3H]glucose (0.75 $\mu\text{Ci/ml}$). Previous investigators (Newgard *et al.*, 1983; Parniak & Kalant, 1985; Spence & Koudelka, 1985) have employed these labelled forms of glucose to quantify hepatic glycogen formation from glucose via the direct (glucose $\rightarrow$ glucose 6-phosphate $\rightarrow$ glycogen) and the indirect glucose (glucose $\rightarrow \text{C}_3 \rightarrow$ glucose 6-phosphate $\rightarrow$ glycogen) pathways. After termination of the incubation by repeated washes with ice-cold PBS, the labelled glycogen was extracted with cold 3% (v/v) HClO_4 and then precipitated on Whatman no. 3 filter-paper discs by using cold ethanol as described by Thomas *et al.* (1968), with unlabelled glycogen as carrier. The data are expressed as nmol of glucose incorporated into glycogen glucose/2 h per mg of cell protein, and are calculated from the specific radioactivity of glucose in the medium at the start of the incubation. In our studies, under optimal conditions of glycogen accumulation, < 1% of the medium glucose is utilized by the cultured hepatocytes.

Materials

D-[U- ^{14}C]Glucose (270 mCi/mmol) and D-[3- ^3H]glucose (5 Ci/mmol) were purchased from Amersham, Arlington Heights, NY, U.S.A.; crude collagenase (CLS II) was from Worthington Biochemicals, Freehold, NJ, U.S.A.; dexamethasone, lactic acid, glutamine, Hepes, amyloglucosidase and bovine serum albumin (fraction V) were from Sigma Chemical Co., St. Louis, MO, U.S.A.; and crystalline pig insulin was kindly provided by Eli Lilly and Co., Indianapolis, IN, U.S.A.

RESULTS

Effects of glucose and hormones on glycogen accumulation

In initial studies, primary cultures of hepatocytes from fed rats were incubated for 18 h in DMEM containing 10 mM-glucose and 10 mM-lactate in the absence and presence of 10 nM-insulin, 100 nM-DEX, and DEX plus insulin. After the second medium change, the cells were incubated in DMEM containing 10 mM-lactate and various concentrations of glucose in the absence and presence of hormones. As shown in Fig. 1, there is no net glycogen accumulation after 5 h at glucose concentrations of 5 and 10 mM; however, at 20 mM-glucose, increases in net glycogen content are found in all groups (no hormones, 56, insulin, 62, DEX, 36, and DEX plus insulin, 51 nmol of glycogen/mg of protein). Since all groups had glycogen contents less (69–80% decrease) than at the beginning of the culture period (at the time of the first medium change, equal to 535 nmol of glycogen/mg of protein), this would suggest that chronic hormone effects serve partially to prevent glycogen depletion in hepatocytes isolated from fed rats during an 18 h culture.

In order to examine the effect of hormones and substrates on glycogen accumulation, hepatic glycogen stores were depleted by a combination of preparing hepatocytes from fasted rats and subsequent incubation for 18 h in DMEM containing 5 mM-glucose and 10 mM-lactate but lacking hormones. After the second medium change, the hepatocytes were incubated (0–5 h) in the presence of 10 mM-glucose in the absence and presence of 10 nM-insulin. Where present, 100 nM-DEX was added 4 h before adding insulin. Preliminary experiments established that a 4 h preincubation period with DEX

Fig. 1. Effect of hormones and glucose concentrations on the absolute glycogen content of primary cultures of hepatocytes isolated from fed rats

Hepatocytes were isolated from fed rats and cultured for 18 h in DMEM containing 10 mM-glucose and 10 mM-lactate in the absence (○) and presence of 10 mM-insulin (●), 100 nM-DEX (□) and DEX plus insulin (■). After a medium change, the cells were then cultured for an additional 5 h in the same medium, washed, and then assayed for glycogen content. On the abscissa, the '0 h' and '18 h' arrows indicate the glycogen contents in hepatocytes at the beginning and end of the initial 18 h culture period respectively. The results represent mean values from two replicate experiments.

Fig. 2. Time course of net glycogen accumulation in primary cultures of hepatocytes isolated from fasted rats

Hepatocytes were isolated from overnight-fasted rats and then cultured without hormones for 18 h in DMEM containing 5 mM-glucose and 10 mM-lactate. After a medium change, the cells were then cultured in the same medium but with glucose increased to 10 mM and no additions (○), 10 nM-insulin (●), 100 nM-DEX (□) and DEX plus insulin (■). Where present, DEX was added 4 h before the increase in glucose. Net glycogen accumulation was then assessed over the times indicated. The results are from a representative experiment.

Fig. 3. Effect of glucose concentration and hormones on glycogen content in primary cultures of hepatocytes isolated from fasted rats

Hepatocytes from overnight-fasted rats were cultured as described in the legend to Fig. 2, except that net glycogen accumulation was assessed over 2 h in the presence of various concentrations of glucose and no additions (○), 10 nM-insulin (●), 10 nM-DEX (□) and DEX plus insulin (■). (a) Absolute glycogen content. (b) Change in glycogen content owing to insulin alone (●) calculated after subtracting the glycogen content in the absence of insulin, and the glycogen content owing to insulin in the presence of DEX (■) calculated after subtracting the glycogen content in the presence of DEX alone. The results are from a representative experiment.

resulted in a maximum effect and that DEX-dependent increases in glycogen accumulation are dose-dependent (1–100 nM), with maximum effects found at 100 nM (results not shown). The DEX concentration of 100 nM is approx. 4 times that (24 nM) equal in potency to 22 μ g of cortisol/ml, which is the upper limit of normal fasting levels in man. This protocol *in vitro* was designed to mimic the temporal changes found *in vivo*, that is, initial exposure to fasting glucose levels (5 mM) followed by exposure to DEX to mimic the early-morning rise in cortisol levels and finally exposure to increased glucose (10 mM) and insulin in order to mimic a morning meal. As shown in Fig. 2, in the absence of hormones, a linear increase in glycogen content is observed only over a 2 h period. In the presence of hormones, glycogen content continues to increase beyond 2 h, with the greatest effect seen in the presence of both DEX and insulin. In all subsequent studies, glycogen accumulation over 2 h was assessed. The effects of glucose and hormones on glycogen accumulation are shown in Figs. 3(a) and 3(b). In the presence of insulin and DEX, glycogen accumulation

Fig. 4. Dose/response curves for the effect of insulin on net glycogen accumulation in primary cultures of hepatocytes isolated from fasted rats

Hepatocytes were cultured as described in the legend to Fig. 2, except that net glycogen accumulation was assessed over 2 h in the presence of 20 mM-glucose and in the presence of various concentrations of insulin (0.01–100 nM). The ED_{50} values (arrows) were 0.45 and 0.35 nM in the absence (○) and presence (●) of DEX respectively. The results represent mean values $\pm$ S.E.M. from four replicate experiments.

relates directly to the glucose concentration at post-prandial (5–10 mM) and supraphysiological values (Fig. 3a). In cultures exposed to DEX, the enhancement of cellular glycogen content by insulin is increased at all glucose concentrations (Fig. 3b). The insulin effect ranged from 68 to 168% in the presence of DEX and was maximal at 7.5 mM-glucose, which is within the normal range of post-prandial plasma glucose levels. The effect of varying insulin concentrations on glycogen accumulation was then assessed. To maximize glycogen accumulation in order to evaluate insulin effects at low concentrations of insulin, the glucose concentration was increased to 20 mM after the second medium change. Dose/response curves are shown in Fig. 4. The concentration of insulin eliciting a half-maximal effect in the absence and presence of DEX is 0.45 and 0.35 nM respectively.

Effect of lactate and glutamine on glycogen accumulation

To assess the effects of lactate and glutamine on glycogen accumulation, glycogen content was measured in HBSS, to which these substrates could be selectively added. Hepatocytes were initially cultured for 18 h in Medium 199 containing 5 mM-glucose and 10 mM-lactate. The subsequent 2 h incubation was in HBSS containing 10 mM-glucose. As shown in Table 1, lactate (10 mM), when added in combination with glutamine (5 mM), enhanced the absolute insulin response under all conditions in both the absence and the presence of DEX. Lactate alone significantly increased the effect of insulin only in the presence of DEX. In contrast, glutamine alone did not significantly enhance the insulin effect either in the absence or the presence of DEX.

Table 1. Effects of substrates and hormones on net glycogen accumulation in primary cultures of hepatocytes isolated from fasted rats

Hepatocytes from overnight-fasted rats were cultured for 18 h in Medium 199 containing 5 mM-glucose and 10 mM-lactate. The cells were then washed and incubated in HBSS containing 10 mM-glucose and, where indicated, glutamine (5 mM), lactate (10 mM), insulin (10 nM) and DEX (100 nM). Where present, DEX was added 4 h before the switch to HBSS. Net glycogen accumulation over 2 h was then assessed. The results represent means $\pm$ S.E.M. from nine replicate experiments (* $P < 0.05$; ** $P < 0.005$, *** $P < 0.001$; † $P < 0.1$). Statistical significance was determined by paired t tests. The insulin responses in hepatocytes incubated with substrates were compared with those of hepatocytes incubated with glucose alone.

Substrate	Hormones	Glycogen content (nmol/mg of protein)	Insulin response (Δ)
Glucose	None	18 $\pm$ 3	–
	Insulin	29 $\pm$ 5	11 $\pm$ 3
	DEX	36 $\pm$ 6	–
	DEX + insulin	50 $\pm$ 11	14 $\pm$ 7
+ Lactate	None	29 $\pm$ 3	–
	Insulin	47 $\pm$ 7	18 $\pm$ 5†
	DEX	55 $\pm$ 7	–
	DEX + insulin	84 $\pm$ 13	29 $\pm$ 8*
+ Glutamine	None	33 $\pm$ 3	–
	Insulin	44 $\pm$ 5	11 $\pm$ 4
	DEX	56 $\pm$ 6	–
	DEX + insulin	74 $\pm$ 12	18 $\pm$ 7
+ Lactate/ glutamine	None	41 $\pm$ 4	–
	Insulin	65 $\pm$ 8	24 $\pm$ 5**
	DEX	66 $\pm$ 10	–
	DEX + insulin	111 $\pm$ 18	45 $\pm$ 11***

Labelling with [3-³H]glucose and [U-¹⁴C]glucose

To evaluate the metabolic pathways by which glucose is incorporated into glycogen, we used [3-³H]glucose and [U-¹⁴C]glucose as tracers when the glucose concentration in HBSS was increased to 10 mM. The data from these studies are presented in Table 2. In the presence of glucose alone, all of the accumulated glycogen (21 nmol/mg of protein) is synthesized from glucose, with 62% via the direct and 38% via indirect pathways. The addition of insulin or DEX alone results in enhanced glycogen accumulation owing to stimulation of both pathways of glycogen formation. The direct pathway is enhanced 31–69% and the indirect pathway is stimulated 100–111% in the presence of insulin or DEX. Under these conditions, nearly equal contributions of glycogen accumulation occur via the direct (49–54%) and indirect (46–51%) pathways. In the presence of DEX alone, or in combination with insulin, non-glucose precursors partially contribute to the observed increase in glycogen content.

In the presence of glucose plus lactate or glutamine, glycogen content is enhanced; however, a major difference is observed in the mode of action. In the presence of glucose and lactate and in the absence of hormones, the incremental change of 8 nmol of

Table 2. Effects of substrates and hormones on net glycogen accumulation from glucose and non-glucose precursors in primary cultures of hepatocytes isolated from fasted rats

Hepatocytes from overnight-fasted rats were cultured in Medium 199 and then incubated in HBSS as described in the legend to Table 1, except that [$3\text{-}^3\text{H}$]- or [$\text{U-}^{14}\text{C}$]-glucose was added to a final concentration of $0.75\text{ }\mu\text{Ci/ml}$. Net glycogen accumulation and synthesis over 2 h were then assessed. The results represent mean values from three to five experiments.

Hormones	Substrate	Glycogen content (nmol/mg of protein)	Δ	Glycogen synthesis from glucose					Non- glucose‡	Δ
				Direct and indirect*	Direct†	Δ	Indirect	Δ		
None	Glucose	21	—	22	13	—	9	—	—1	—
	+ Lactate	29	8	24	16	3	8	—1	5	6
	+ Glutamine	31	10	30	21	8	9	0	1	2
	+ Lactate/ glutamine	39	18	32	23	10	9	0	7	8
Insulin	Glucose	36	—	35	17	—	18	—	1	—
	+ Lactate	46	10	38	26	9	12	—6	8	7
	+ Glutamine	44	8	43	25	8	18	0	1	0
	+ Lactate/ glutamine	61	25	57	35	18	22	4	4	3
DEX	Glucose	45	—	41	22	—	19	—	4	—
	+ Lactate	59	14	38	26	4	12	—7	21	17
	+ Glutamine	63	18	62	35	13	27	8	1	—3
	+ Lactate/ glutamine	62	17	47	36	14	11	—8	15	11
DEX + insulin	Glucose	64	—	54	32	—	22	—	10	—
	+ Lactate	102	38	60	48	16	12	—10	42	32
	+ Glutamine	110	46	86	55	23	31	9	24	14
	+ Lactate/ glutamine	122	58	76	64	32	12	—10	46	36

* Calculated from incubations with [$\text{U-}^{14}\text{C}$]glucose.

† Calculated from incubations with [$3\text{-}^3\text{H}$]glucose.

‡ Glycogen content minus direct and indirect synthesis from glucose.

glycogen/mg of protein results from a major contribution (6 nmol/mg) from non-glucose precursors. The contribution of non-glucose precursors is also observed in hepatocytes treated with insulin and lactate (7 nmol/mg), and is most pronounced in the presence of DEX and lactate (17 nmol/mg) or DEX plus insulin and lactate (32 nmol/mg). In contrast, the relative contribution of the indirect pathway of glycogen accumulation from glucose is decreased in hepatocytes incubated with lactate in the presence of DEX alone or with DEX plus insulin. These increases in the relative contribution of non-glucose precursors, especially in the presence of DEX, are consistent with the observed decreases in the relative specific radioactivities of [^{14}C]glycogen glucose in hepatocytes exposed to lactate (Table 3). The relative specific radioactivity of [^{14}C]glycogen glucose in the presence of glucose alone is 1.0. This indicates that all of the glycogen is formed from glucose. The primary effect of lactate, therefore, is to serve as a gluconeogenic substrate for glycogen synthesis in hepatocytes, a function which is enhanced with DEX pretreatment.

The increased glycogen synthesis observed in glutamine-treated hepatocytes occurs through mechanisms different from lactate. In the absence of hormones, the increased glycogen content (from 21 to 31 nmol) is nearly all derived from glucose (Table 1). The addition of insulin or DEX alone, or in combination, results in enhanced glycogen accumulation from glucose with no contribution from non-glucose precursors found. Only

in the presence of insulin plus DEX is a measurable increase (14 nmol) in glycogen accumulation from non-glucose precursors found. In the presence of both lactate and glutamine, the increased glycogen content appears to be the result of a composite of lactate-dependent enhancement of gluconeogenesis and glutamine-enhancement of glycogen accumulation from glucose (Table 2). As also seen in glucose-treated hepatocytes supplemented with lactate alone, the non-glucose contribution to the overall glycogen accumulation in hepatocytes supplemented with lactate and glutamine is substantially greater when insulin and DEX are both present. The changes in the relative specific radioactivities of [^3H]- and [^{14}C]-glycogen glucose are intermediate between those of lactate alone and glutamine alone (Table 3).

DISCUSSION

Hormonal and substrate regulation of glycogen accumulation

To address questions related to the regulation of glycogen deposition by hormones and substrates, we first established a model *in vitro* in which we attempt to mimic closely the temporal relationship between glucocorticoids, post-prandial glucose and insulin found *in vivo*. By using this approach, net glycogen accumulation can be directly related to glucose concentration at post-prandial glucose concentrations (5–10 mM). More-

Table 3. Effects of substrates and hormones on the relative specific radioactivities of [³H]- and [¹⁴C]-glycogen glucose in primary cultures of hepatocytes isolated from fasted rats

Relative specific radioactivity = (c.p.m. of glycogen glucose/nmol of glycogen glucose)/(c.p.m. of medium glucose/nmol of medium glucose). The values are calculated from the data in Table 2.

Hormone	Substrate	Relative specific radioactivity	
		[³ H]Glycogen glucose	[¹⁴ C]Glycogen glucose
None	Glucose	0.62	1.05
	+ Lactate	0.53	0.82
	+ Glutamine	0.67	0.97
	+ Lactate/ glutamine	0.59	0.83
Insulin	Glucose	0.46	0.96
	+ Lactate	0.56	0.82
	+ Glutamine	0.56	0.99
	+ Lactate/ glutamine	0.57	0.94
DEX	Glucose	0.49	0.90
	+ Lactate	0.44	0.65
	+ Glutamine	0.57	0.99
	+ Lactate/ glutamine	0.58	0.76
DEX + insulin	Glucose	0.50	0.84
	+ Lactate	0.47	0.59
	+ Glutamine	0.55	0.86
	+ Lactate/ glutamine	0.53	0.62

over, the ability of insulin and DEX to enhance net glycogen accumulation is demonstrable with acute exposure periods (2–4 h) that reflect the state *in vivo*. Heretofore, studies investigating the hormonal regulation of glycogen synthesis in cultured rat hepatocytes have employed chronic treatments (6–48 h) with insulin, glucocorticoids or both (Fleig *et al.*, 1984, 1985, 1987; Lopez *et al.*, 1984; Schudt, 1979; Spence & Koudelka, 1985; Vanstapel *et al.*, 1982; Weber & Kletzien, 1982). The enhanced effect of insulin that we find in the presence of DEX is consistent with data *in vivo* demonstrating decreased glycogen synthesis in the liver of starved (48 h) adrenalectomized animals (Cardell, 1977; Margolis & Curnow, 1983, 1984; Stalmans *et al.*, 1987) and the study by Miller *et al.* (1984) showing a glucocorticoid requirement in the restoration of insulin-sensitive glycogen metabolism in primary cultures of hepatocytes from diabetic rats.

In the absence of insulin, as found in hypoinsulinaemic diabetes mellitus, glycogen accumulation also occurs, but at approximately one-half the rate found in the presence of insulin at all glucose concentrations. For example, it requires between 10 and 20 mM-glucose in the presence of DEX alone to achieve the same glycogen content as in hepatocytes incubated with 5–10 mM-glucose in the presence of DEX plus insulin (Fig. 3a). Thus, although the efficiency of glycogen synthesis is decreased in the absence of insulin, glycogen synthesis still proceeds at high substrate (glucose) concentrations,

perhaps explaining the abundance of glycogen in the livers of some diabetic people and animals in the presence of a relative or absolute deficiency of insulin.

Insulin dose/response curves indicate that the concentration of insulin eliciting a half-maximal response is nearly identical in hepatocytes incubated in the absence and presence of DEX. Schudt (1979) reported that chronic (48 h) exposure of cultured hepatocytes to the glucocorticoid triamcinolone (1 μ M) resulted in an increased glycogen accumulation from glucose, with no significant change in insulin sensitivity. In contrast with DEX-sensitive glycogen accumulation, we have previously reported (Amatruda *et al.*, 1983) that treatment of hepatocytes with DEX (20 h) leads to a greater than 3-fold decrease in insulin sensitivity (from 0.66 to 2.1 nM) with regard to lipogenesis, concomitant with an enhancement of maximum responsiveness. The ED₅₀ for insulin-sensitive glycogenesis (0.35 nM) in DEX-treated hepatocytes is close to portal-vein insulin concentrations found *in vivo* in rats (Misbin *et al.*, 1976), and is more sensitive than other biological parameters which we have evaluated, such as lipogenesis (Amatruda *et al.*, 1982, 1983; Caro & Amatruda, 1982a; Salhanick *et al.*, 1983) and aminoisobutyric acid uptake (Amatruda *et al.*, 1982; Caro & Amatruda, 1982b; Cech *et al.*, 1980). This level of insulin is compatible with the regulation of plasma glucose concentration which occurs *in vivo*, although the mechanism of differential sensitivity to insulin is at present unknown.

Contributions of the direct and indirect pathways of glycogen accumulation from glucose

Spence & Koudelka (1985) reported that the specific radioactivity of [³-³H]- or [¹⁴C]-glycogen glucose was decreased by approx. 60 % in hepatocytes prepared from 1-day-fasted rats, indicating that non-glucose carbon was entering glycogen synthesis. Similarly, Parniak & Kalant (1985) demonstrated an indirect pathway of glycogen formation from glucose (approx. 25 %) in hepatocytes from fed rats. These findings would suggest that glycolysis and gluconeogenesis are occurring simultaneously in the liver. However, since in those studies (Spence & Kodelka, 1985; Parniak & Kalant, 1985) hepatocytes were maintained in a complete medium containing amino acids as well as pyruvic acid, it is difficult to assess the relative contributions of the substrates in the medium to observed changes in glycogen content. The results from the present study evaluating the contributions of the direct and indirect pathways of glycogen synthesis from glucose confirm and extend the observations made by Spencer & Koudelka (1985) and Parniak & Kalant (1985). In the presence of glucose alone and in the absence and presence of hormones, the contributions of the direct and indirect pathways of glycogen formation are comparable. In the presence of glucose plus lactate, the relative contribution of non-glucose precursors to glycogen accumulation increases substantially. Decreases in the relative specific radioactivity of [¹⁴C]glycogen glucose suggest a dilution of the lactate pool from glucose. Because of this possible dilution of pool sizes, we are unable at present to estimate the extent to which the incorporation of lactate derived from glucose metabolism is also enhanced. In the presence of lactate, however, most of the new glycogen formed is derived from the added lactic acid and not from glucose. Although we did not measure glucose

formation from exogenous lactic acid, Newgard *et al.* (1984a) reported that glucose concentrations lower (7–8 mM) than that used in the present study (10 mM) suppress hepatic glucose-6-phosphatase. Therefore we suspect that the proportion of lactate being channelled to glucose in our cultured cells would not be released from the cell as glucose but would be incorporated into glycogen. In the presence of DEX alone, or in combination with insulin, the gluconeogenic contribution of exogenous lactate as well as from endogenous precursors was most pronounced. Although not measured in the present study, others (Fleig *et al.*, 1984; Hoppner *et al.*, 1986; Katz *et al.*, 1979; Salavert & Iynedjian, 1982) have shown that, in the presence of glucocorticoids, the activities of a number of gluconeogenic enzymes are increased in isolated hepatocytes.

In the presence of glucose plus glutamine, increased accumulation of glycogen is observed through a stimulation of both the direct and indirect pathway of glycogen synthesis from glucose. Unlike lactate, however, glutamine does not serve as a C₃ source for glycogen synthesis. Our results are consistent with the observations by others (Boyd *et al.*, 1981; Chen & Lardy, 1985; Katz *et al.*, 1976; Rognstad, 1985) that glutamine-dependent stimulation of glycogen formation is through an activation of glycogen synthase. In this regard, Lavoinne *et al.* (1987) have shown that, in rat hepatocytes isolated from fasted rats and incubated in the presence of 10 mM-glutamine, a correlation is found between the rate of glycogen synthesis and glycogen synthase activity. It was suggested that this activation is at the level of a stimulation of glycogen synthase phosphatase.

In the present study, we describe the ability of glucose, insulin or DEX to enhance hepatocyte glycogen content. Using labelled glucose, we were able to find enhanced activity of both the direct and indirect pathways of glycogen formation from glucose in response to addition of either insulin or DEX. The mechanisms by which glucose, insulin or glucocorticoids each enhances glycogen synthesis, however, remain a matter of controversy (reviewed by Stalmans *et al.*, 1987). For example, Fleig *et al.* (1985, 1987) reported stimulation of glycogen synthase activity in insulin-treated or DEX-treated cultured hepatocytes, and Ciudad *et al.* (1988) described the activation of glycogen synthase in isolated rat hepatocytes by mechanisms involving glucose-dependent alterations in the intracellular concentrations of glucose 6-phosphate and adenine nucleotides. Further studies will be needed to address these issues.

Landau and co-workers (Landau & Wahren, 1988; Scofield *et al.*, 1985) have presented evidence that questions the reliability of employing only 3-³H-labelled and ¹⁴C-labelled glucose to estimate the direct and indirect pathways of hepatic glycogen. Unreliable estimations could occur owing to loss of ¹⁴C in the C-1 position as ¹⁴CO₂ at the 6-phosphogluconate dehydrogenase step of the pentose shunt. Given these concerns, Spence & Koudelka (1985) compared their data obtained from cultured hepatocytes incubated with [3-³H,U-¹⁴C]-glucose with studies using [6-³H,6-¹⁴C]glucose or [3-³H,1-¹⁴C]glucose and found identical results. Similarly, Newgard *et al.* (1984b) observed that the dilution of the specific radioactivity of hepatic glycogen glucose was identical whether [1-¹⁴C]- or [6-¹⁴C]-glucose was administered to fasted rats. In other studies using intact livers from fasted rats, Scofield *et al.* (1985) reported

pentose shunt activity to be low (4–9%), and Shulman *et al.* (1985, 1987) estimated a minimal ($\leq 3\%$) glucose recycling at the triose-phosphate isomerase step to glycogen synthesis. Although in our present study using primary cultures of hepatocytes pentose-shunt activity and glucose cycling through triose phosphates were not assessed, we would expect minimal losses of label through these alternative pathways.

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Bartels *et al.* (1987) have presented evidence in studies using perfused rat livers for the predominance of glycolysis and gluconeogenesis in the perivenous and periportal areas of the liver respectively. In the present study, we did not attempt to isolate and culture perivenous- and periportal-derived hepatocytes, and future studies with isolated hepatocytes will be needed to substantiate the existence of metabolic zonations for the direct and indirect pathways of glycogen formation from glucose and the effects of hormones on these subpopulations of hepatocytes.

Conclusion

We have defined favourable incubation conditions under which hepatocytes demonstrate net glycogen production, and evaluated the effects of hormones and substrates as well as the mechanisms by which glucose is incorporated into glycogen. Further studies will be necessary to define more precisely the mechanisms by which gluconeogenic substrates, amino acids and hormones regulate hepatic glycogen content in normal and altered metabolic states.

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