Role of Glucose, Insulin, and Glucagon in Glycogen Mobilization in Human Hepatocytes

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This study investigated the role of glucose, insulin, and glucagon in the activation of glycogen catabolism in cultured human hepatocytes. Basal glycogenolysis in unstimulated human hepatocytes was low (only 19% of initial glycogen content was degraded in a 4-h incubation) and insensitive to changes in external glucose concentration (from 10 to 0 mM). Both glycogenolysis and glucose output could be significantly stimulated by 35 ng/L glucagon or 10^5 pM dibutyryl cAMP; half-maximal effect was found with 28×10^2 ng/L glucagon and 4×10^5 pM dibutyryl cAMP. After a 3-h exposure to 35×10^3 ng/L glucagon, >90% of glycogen content of human hepatocytes was mobilized. This caused a 4.6-fold increase in the rate of glucose output to the medium compared with nonstimulated cells. About 85% of degraded glycogen rendered glucose to the medium. Insulin (10^4 pM) was able to totally supress basal glycogenolysis; insulin was also essential to reverse the action of glucagon in hepatocytes incubated with glucagon, whereas glucose alone, even at postprandial concentration, was unable to reverse glucagon action. In summary, these experiments show that the mobilization of glycogen stores of human hepatocytes, as it occurs during the postabsorptive periods in humans, is largely dependent on the presence of glucagon and is not simply due to a decrease of external glucose. Insulin, on the other hand, was essential to suppress both basal and glucagon-activated glycogenolysis. *Diabetes* 40:263–68, 1991

A major function of the liver in hemostasis in the body is preventing blood glucose from decreasing below a certain level during the postabsorptive state and fasting. This involves activation of two main processes: glycogenolysis and gluconeogenesis. During the postabsorptive state as blood glucose concentration falls, insulin secretion decreases, and glucagon secretion increases (1,2). In these conditions, glucose is produced by the liver mainly from glycogenolysis (3–6). Most of our knowledge about the control of glycogen degradation has been acquired from animal studies, particularly with rats (7–9). In humans, the fall in plasma insulin from postprandial to basal concentrations is crucial to the stimulation of hepatic glycogenolysis, but the precise role of glucagon and glucose concentrations in this circumstance is less clear (2).

Glycogen metabolism in humans has been studied on the basis of rather complicated in vivo turnover studies with isotopes (4), measurement of splanchnic substrate balance (5,6), or determination of the glycogen content on serial liver biopsies (3). These approaches have serious experimental drawbacks, and the direct evidence of the role of hormones and glucose on human liver is therefore limited. The use of biochemically well-characterized human hepatocytes in primary culture provides a unique in vitro experimental system in which hormonal and nutritional modulators of human liver functions can be tested separately (10,11). The aim of this study was to investigate the role that glucose, insulin, and glucagon concentrations play in the control of glycogen breakdown in human hepatocytes during the postabsorptive state.

Our results show that the mobilization of glycogen in human hepatocytes is essentially dependent on elevation of glucagon concentration. Low levels of glucose alone did not increase the glycogenolytic rate per se.

RESEARCH DESIGN AND METHODS

Surgical liver biopsies (1–3 g) were obtained from patients undergoing cholecystectomy after informed consent was obtained. None of the patients were diabetic or had any other endocrine or hepatic pathology. Patients did not receive

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medication during the week before surgery and were subjected to an overnight fast. Analytical data of serum from liver donors showed no abnormalities in liver function. Total bilirubin concentration ranged from 5 to 15 mg/L, and conjugated bilirubin was <3 mg/L. Glutamic-oxalacetic transaminase and glutamic-pyruvic transaminase never exceeded 50 U/L, and γ-glutamyl transpeptidase was <70 U/L. Morphological analysis did not reveal pathological alterations.

Hepatocyte isolation was performed within 1 hr after resection by microperfusion of the biopsies (10,11). Briefly, the tissue was first perfused with 250 ml of calcium-free buffer (137 mM NaCl, 2.68 mM KCl, 0.7 mM Na_2HPO_4, 10 mM HEPES, 10 mM glucose, and 0.5 mM EGTA, pH 7.5) at 37°C, with a 10-ml/min per catheter flow rate. The washing perfusate was not recirculated. The second step was a recirculating perfusion with the same buffer without EGTA, containing 5 mM $CaCl_2$ and 0.5 mg/ml of collagenase (Boehringer Mannheim, Mannheim, FRG; enzyme activity 0.37 U/mg), at the same flow rate and temperature. Perfusion was continued for ~30 min until sufficient softening of the tissue. The perfusion buffers were continuously gassed with 5% CO_2/95% O_2. The cellular suspension was washed in ice-cold buffer and centrifuged for 2 min at 50 × *g*. The washing procedure was repeated with ice-cold Ham's F-12 medium supplemented with 0.2% bovine serum albumin (BSA) and 10^4 pM insulin.

Cell viability, assessed by the trypan blue dye-exclusion test, oscillated between 85 and 90%. Hepatocytes were then suspended in warm (37°C) Ham's F-12 medium supplemented with 0.2% BSA, 2% newborn calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10^4 pM insulin, at a final density of 0.5 × 10^6 viable cells/ml. Cells were seeded on fibronectin-coated (12) plastic culture dishes (3.5 cm diam) at a final density of 75,000 viable cells/cm² in appropriate volume of medium and incubated at 37°C in a humified 5% CO_2/20% O_2 gas atmosphere. To remove nonattached cells, the culture medium was changed 1 h after seeding. Twenty-four hours later, after three washes, cells were shifted to serum-free medium. Ham's F-12 medium is a complex mixture of inorganic salts, vitamins, amino acids, and other nutrients (13). It contains 0.001 M pyruvate, 10^3 μM glutamine, and 100 μM alanine as major gluconeogenic substrates. Experiments were initiated by incubating cells in fresh medium containing the hormone and glucose concentrations indicated.

Glycogenolysis was determined as net variation in glycogen content during the incubation period. At indicated times, monolayers were carefully rinsed with cold saline and quickly frozen in liquid N_2. Hepatocytes were harvested in 1 ml of cold saline, an aliquot was taken to determine protein content, and the remainder was processed in the same tube for glycogen extraction and assay as described previously (14). Glycogen was extracted by digestion with 30% KOH and subsequent precipitation and reprecipitation with ethanol (66% final concn) and was later assayed with the anthrone reagent (15). Protein content of monolayers was determined with BSA as standard (16). Glycogen content was finally expressed as nanomoles of glucose equivalents per milligram of cellular protein. Glycogen standards containing albumin as exogenous protein were processed in the same way as the samples. The recovery of glycogen during the whole procedure was 93 ± 4% for standards containing 15–150 nmol glucose. Glucose output to the incubation medium was assayed by the glucose oxidase method in neutralized perchloric acid supernatants from aliquots taken at regular intervals (17).

Results in this study correspond to experiments performed in 13 independent hepatocyte cultures obtained from 13 different liver biopsies. Data are expressed as means ± SD for cultures as indicated in figure legends. Statistical significance of differences was performed with analysis of variance and subsequent Newman-Keuls' test for multiple comparison where appropriate. Other comparisons were made by the Student's *t* test. $P < 0.05$ was considered significant.

RESULTS

Human hepatocytes after 24 h in culture had an average mean ± SD glycogen content of 1324.6 ± 458.4 nmol glucose/mg as determined in 13 different cell preparations. In the absence of insulin, basal glycogenolysis accounted for a 19% reduction of the initial glycogen content after a 4-h incubation (Table 1). Adding 10^4 pM insulin to culture me-

TABLE 1
Basal glycogenolysis in human hepatocytes

Biopsy	Initial glycogen content at time 0 (nmol glucose/mg)	Final glycogen content (nmol glucose/mg)		Net glycogen degradation in cells without insulin	
		+ Insulin	− Insulin	nmol glucose · mg^{-1} · 4 h^{-1}	% of initial
1	1383.9 ± 98.8	1375.6 ± 155.0	1019.5 ± 43.6	364.4 ± 43.6	26.3
2	640.6 ± 25.6	617.5 ± 6.4	461.6 ± 19.6	179.0 ± 19.6	27.9
3	491.8 ± 14.2	474.0 ± 4.2	445.6 ± 12.5	46.2 ± 12.5	9.4
4	1316.8 ± 56.5	1290.5 ± 118.5	1113.2 ± 106.0	203.6 ± 106.0	14.9
5	924.6 ± 167.4	941.2 ± 129.0	789.4 ± 60.6	135.1 ± 60.0	14.6
6	1443.7 ± 150.2	1469.7 ± 161.5	1144.9 ± 122.6	298.8 ± 122.6	20.7
Mean ± SD	1033.6 ± 407.7	1028.1 ± 416.6	829.0 ± 316.3	204.5 ± 114.0	19.0 ± 7.3

Cultured (24 h) hepatocytes from 6 different biopsies were incubated in fresh Ham's F-12 medium containing 10 mM glucose with or without 10^4 pM insulin. Table shows the mean ± SD glycogen content for 3 or 4 dishes of cells from separate cultures, determined at time 0 and after a 4-h incubation period. Glycogen content is expressed as nmol glucose equivalent/mg cellular protein. Net glycogen degradation was calculated as the mean of the values obtained by subtraction of the mean of the initial content from the final content in each dish of cells after incubation in the absence of insulin.

dium abolished this degradation, and 24 h later, mean ± SD glycogen content of hepatocytes was similar to initial values (1144.8 ± 586.4 nmol glucose/mg, n = 6 cultures; Table 1).

To investigate whether glycogenolysis could be activated by low glucose concentration, we incubated human hepatocytes in Ham's F-12 medium containing decreasing amounts of glucose. As shown in Fig. 1, this is not the case, and lowering glucose concentration in external medium did not lead to a significant increase of net glycogen degradation on human hepatocytes. Basal glycogenolysis, even in the total absence of glucose, was similar to that determined in the absence of insulin (Table 1), and at the end of the 4-h incubation period, hepatocytes still preserved 70–80% of their initial glycogen content. In these conditions, the glucose released from hepatocytes to the medium accounted for ~85 ± 8% of the hydrolyzed glycogen.

Glucagon and dibutyryl cAMP activated glycogenolysis in a dose-dependent manner (Fig. 2). Hepatocytes were sensitive to glucagon concentrations as low as 35 ng/L. Half-maximal effect was elicited by 28 × 10^2 ng/L, and maximal effects were observed with 35 × 10^3 ng/L glucagon. Dibutyryl cAMP stimulated glycogenolysis with a half-maximal response at 4 × 10^5 pM. Maximal effect was exerted by 10^8 pM dibutyryl cAMP. Maximal glycogenolytic rates were similar for both agents (~3.4-fold basal glycogenolysis), and ~90% of the glycogen stores were hydrolyzed within a 3-h incubation. The maximal glycogen degradation caused by 35 × 10^4 ng/L glucagon and 10^9 pM dibutyryl cAMP (1206.0 ± 20.4 and 1295.0 ± 36.1 nmol glucose/mg, respectively) in the absence of glucose was equivalent to that found when hepatocytes were incubated with the same hormone concentration but in the presence of 10 mM glucose (1155.9 ± 36.4 and 1197.2 ± 33.5 nmol glucose/mg, respectively).

The output of glucose after stimulating hepatocytes with 35 × 10^3 ng/L glucagon or 10^8 pM dibutyryl cAMP in glucose-free medium proceeded linearly for at least 75 min, decreasing slowly thereafter (data not shown). During this initial period, 35 × 10^3 ng/L glucagon stimulated mean ± SD glucose output from 1.6 ± 0.2 to 7.4 ± 1.4 nmol glucose released · mg^{-1} · min^{-1} (n = 3 different cultures), a 4.6-fold increase compared with control values. Similarly, 10^8 pM dibutyryl cAMP increased glucose output to 7.7 ± 0.3 nmol glucose released · mg^{-1} · min^{-1}. Glycogen measurement at the end of the incubation (3 h) showed that glucose released to the medium accounted for 81 ± 14% of the depleted glycogen (n = 3 different biopsies). Glucose output in the absence of hormonal stimulus was constant over the complete period studied (1.6 ± 0.2 nmol glucose released · mg^{-1} · min^{-1}).

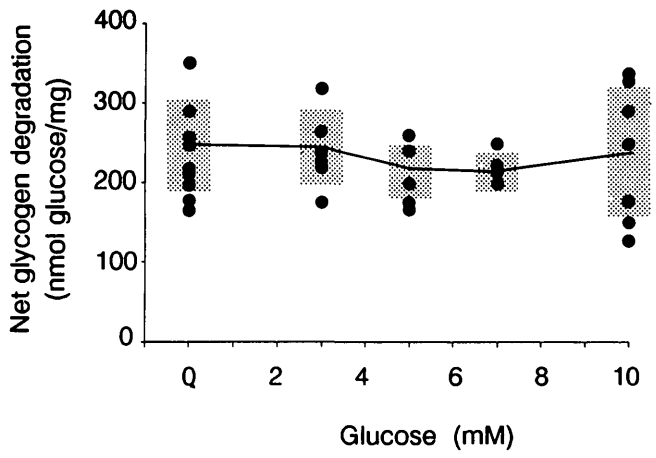

FIG. 1. Effect of glucose concentration on basal glycogenolysis. Cultured (24 h) human hepatocytes were incubated in hormone-free Ham's F-12 culture medium containing increasing amounts of glucose. Glycogen content of monolayers was assayed at beginning and end of 4-h incubation. Individual data points from 3 independent hepatocyte cultures (*shaded area*, mean ± SD) are shown and are expressed as net glycogen degraded in 4 h. Initial glycogen content of each culture was 640.0 ± 25.6, 1316.8 ± 56.5, and 1522.2 ± 106.5 nmol glucose/mg, respectively.

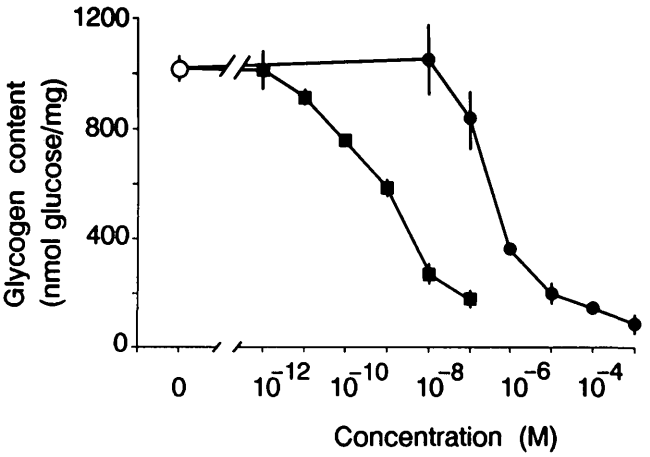

Fig. 2. Dose-response activation of glycogenolysis by glucagon and dibutyryl cAMP. Cultured (24 h) human hepatocytes were incubated in glucose-free Ham's F-12 medium containing increasing concentrations of glucagon (■) or dibutyryl cAMP (●). Glycogen content was assayed at beginning and end of 3-h incubation. Values are means ± SD of 4 or 5 different plates from representative experiment and correspond to glycogen content found at end of incubation. Initial glycogen content was 1383.9 ± 98.8 nmol glucose/mg. Data points with no SD bars drawn had SD less than area of symbol. Values versus control are significant ($P < 0.05$ by Student's t test) at 35 ng/L (10 pM) glucagon and 10^5 pM dibutyryl cAMP. x-Axis, glucagon concentrations.

To evaluate the ability of insulin and/or glucose to reverse glucagon-activated glycogenolysis, net glycogen accumulation was determined in glycogen-depleted cells incubated with increasing glucose concentrations in the presence or absence of 10^4 pM insulin and with or without the simultaneous presence of 35 × 10^2 ng/L glucagon.

When glucagon was absent from the culture medium, glucose alone promoted dose-dependent glycogen accumulation, which was significant at concentrations ≥5 mM (Fig. 3*A*). If 10^4 pM insulin was also present, net increase in glycogen content was significantly higher than controls for all glucose concentrations tested. In these conditions, insulin-induced glycogen accumulation as a function of external glucose concentration was significantly higher ($P < 0.01$) for all glucose concentrations and was additive to that caused by glucose alone (Fig. 3*A*).

On the other hand, glucose at physiological concentrations was unable to cause net glycogen accumulation if glucagon was present in the incubation mixture (Fig. 3*B*). In these conditions, the presence of insulin was essential to counteract glucagon action and to permit a significant ($P <$

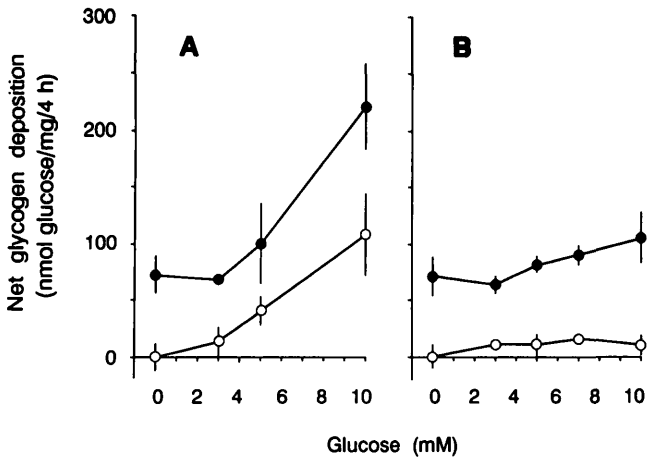

Fig. 3. Reversal of basal and glucagon-activated glycogenolysis by glucose and insulin. Cultured (24 h) hepatocytes were glycogen depleted by preincubation with 35 × 10^3 ng/L glucagon for 3 h. After extensive washing, glycogen content was determined, and experiment was initiated with addition of fresh Ham's F-12 medium containing indicated amounts of glucose, with (●) or without (○) 10^4 pM insulin, in the absence (*A*) or presence (*B*) of 35 × 10^2 ng/L glucagon. Glycogen content was determined after 4-h incubation. Results are expressed as net increases in glycogen content. Data represent mean ± SD value of 3 independent cultures (3–5 plates/culture and variable). Initial glycogen content of each culture was 38.3 ± 10.2, 127.7 ± 8.5, and 149.7 ± 23.2 nmol glucose/mg, respectively.

0.01) increase in glycogen content for any condition tested. In fact, addition of insulin (10^4 pM) caused a significant ($P < 0.01$) net increase in glycogen content even in the absence of glucose (71.5 ± 16.4 nmol glucose/mg) that was equivalent to that caused by the hormone in the same incubation conditions but in the absence of glucagon (72.7 ± 16.5 nmol glucose/mg). Insulin-induced net glycogen deposition in glucagon-exposed cells increased significantly as a function of external glucose concentration (Fig. 3*B*).

DISCUSSION

Different experimental approaches in vivo (3–6) have shown that, during the postprandial state, glycogenolysis accounts for ~75% of overall hepatic glucose output in humans. Major signals for the activation of this process are thought to be decreased blood glucose levels associated with decreased concentration of insulin and enhanced concentration of glucagon in the portal vein (1,2). This study was undertaken to examine the relevance of these factors on the activation of glycogen breakdown in adult human hepatocytes. The metabolic competence, hormone responsiveness, and suitability of the human hepatocyte culture system used in this study have been reported previously (10,11,18,19).

Human hepatocytes in culture retain high levels of glycogen (~4.5% of hepatocyte wet wt) similar to those of the liver in fed healthy individuals (1). In the absence of insulin, basal glycogenolysis represents an average value of 204.5 ± 104.1 nmol glucose · mg^{-1} · 4 h^{-1} (mean ± SD; n = 6 biopsies). Our results have shown that this basal glycogenolysis is insensitive to changes in external glucose (Fig. 1) but results in dose-dependent stimulation by glucagon and dibutyryl cAMP (Fig. 2). Maximal effect (a 3.4-fold increase) was observed with 35 × 10^3 ng/L glucagon, and, after a 3-h incubation, 90% of the glycogen stores had been mobilized. Glucose output to the culture medium accounted for 80–90% of mobilized glycogen, a situation similar to that found in humans in vivo after administration of pharmacological doses of glucagon (20) or during the postabsorptive state (4).

Blood glucose in a healthy person oscillates from a basal level of ~4–6 mM to postprandial levels that rarely exceed 8 mM. In humans soon after cessation of absorption, glycogenolysis gradually increases, and most of the glycogen stores are depleted in ~4 h (21). From in vivo studies, it was assumed that the reduction in insulin levels associated to a fall in blood glucose was the main factor involved in the activation of glycogenolysis in the postabsorptive state, the role of glucagon being less clear (2). The findings that, in human hepatocytes, a decrease of external glucose concentration did not lead to an increase on the glycogenolytic rate on one side (Fig. 1) and that basal glycogenolysis was suppressed by 10^4 pM insulin (Table 1) suggest that hepatocyte basal glycogenolysis is due to a lack of insulin in the incubation medium. However, net glycogen degraded in these conditions (low glucose and in the absence of insulin) was only 20% of initial content after 4 h of incubation, far from that observed in vivo (21). Results in cultured human hepatocytes showing that the presence of glucagon is a necessary component in the rapid activation of glycogenolysis (90% of glycogen stores mobilized within 3 h) suggest that the hormone could be playing the same role during the postabsorptive state in vivo. In agreement with this is the fact that in vivo there is a significant hypoglycemia (25% fall in blood glucose levels) when glucagon secretion is inhibited during the postabsorptive state both in healthy (22,23) and diabetic (24) subjects.

Glucagon effects were observed in vitro at concentrations that can be considered physiologically relevant. Peripheral concentration of glucagon in humans can increase up to three- to fourfold its basal level, with values in the range 50–300 ng/L (1,25). Glucagon and insulin are secreted into the mesenteric vein and first reach the liver via the portal vein. Because a major proportion of the hormones (50% of insulin and up to 81% of glucagon) are degraded through the first liver passage, its portal concentration must be higher than the peripheral one (1). In fact, it has been shown that liver is exposed to insulin concentrations 3–10 times greater than those determined in the systemic circulation (26). A similar situation must occur for glucagon, and the concentrations of glucagon in this study found to be active on human hepatocytes can be regarded as very close to the concentrations reaching the liver in vivo.

The role of insulin and glucose in the reversion of glycogenolysis was also investigated. Glycogen-depleted hepatocytes were used to simulate the fasting-refeeding transition in humans. As shown in RESULTS, human hepatocytes are sensitive to glucose, which caused a dose-dependent increase of glycogen content in the absence of insulin. Addition of insulin to glucose caused a further increase of glycogen content, but the effect of the hormone was crucial in cells incubated in the presence of 35 × 10^2 ng/L glucagon, where physiological concentrations of glucose were unable to reverse glucagon action. These observations reflect the antagonistic action of insulin on glucagon-activated

glycogenolysis, which was also found in rat hepatocytes (27).

In humans, small increments in plasma insulin caused by discrete glucose infusion are thought to be responsible even more than glucose itself for the drastic decrease in liver glucose output (2). Under these circumstances, plasma glucagon levels do not decrease, and insulin acts mainly through inhibition of glycogenolysis (2). In agreement with this, our results in cultured human hepatocytes have shown that insulin, rather than glucose, is able to suppress the action of physiological concentrations of glucagon.

Also noticeable is the effect caused by insulin itself when cells are incubated in the absence of glucose. The increase in glycogen content in these conditions was equivalent whether glucagon was present and has to be the result of hepatocyte utilization of gluconeogenic substrates (such as glutamine, pyruvate, or alanine, which are present in the culture medium) as alternative sources for glucose that, because of an insulin action stimulating glycogen synthesis and blocking subsequent glycogen degradation, may result in net glycogen accumulation. Evidence for the operativeness of this indirect pathway for glycogen synthesis first demonstrated in rat hepatocytes (28) has been shown in human liver in vivo (29–31).

The extrapolation of in vitro results to the complex in vivo organism is always difficult; however, the information obtained from primary cultured human hepatocytes can be valuable and is significant to get better knowledge of the regulation of hepatic metabolism in human liver. In fact, results in this study might have significant implications concerning the use of rat models to study hepatic carbohydrate metabolism in humans. As far as the behavior of cultured cells may reflect the functional activity of hepatocytes in the intact organism, our results support the idea that human hepatocytes strictly depend on glucagon to produce rapid mobilization of glycogen stores, whereas insulin is essential to reverse both basal and glucagon-activated glycogenolysis. This greatly contrasts with what we and others had observed in hepatocytes from fed rats incubated with 5–10 mM glucose (conditions equivalent to those used in this study for human hepatocytes) (14,27,32–35). In rat hepatocytes, basal glycogenolysis (in the absence of glucagon) was extremely high (6-fold that determined herein for human hepatocytes) despite the presence of glucose and insulin at postprandial levels (32). In addition, glycogen-depleted rat liver cells do not accumulate glycogen in response to physiological glucose alone (up to 10 mM), and insulin stimulus is low and transient, followed by a quick degradation of the glycogen accumulated (14,32).

This suggests that glycogenolysis may be a prevalent process in rat hepatocytes, whereas in human hepatocytes, basal glycogenolysis is low, and glucagon is required for a rapid mobilization of glycogen. The existence of interspecies differences in glycogen metabolism between human and rat hepatocytes also anticipates quantitative differences in the regulation of the enzymes involved.

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