INSULIN REGULATION OF GLYCOGEN SYNTHASE IN THE ISOLATED RAT HEPATOCYTE

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SUMMARY

Isolated rat hepatocytes have been prepared by a modified technique of collagenase digestion. This method yields cells which, on exposure to insulin, exhibit the rapid activation of glycogen synthase characteristic of the response observed when the liver is studied in situ. Activation of glycogen synthase I by insulin and added glucose is observed in a complex medium containing amino acids and vitamins. The conditions of cell isolation are critical to the preservation of insulin responsiveness in the isolated rat hepatocyte.

The influence of insulin on hepatic metabolism has been well studied in the intact organism, in the isolated perfused liver, and in the liver slice. While these systems have provided extensive and complementary information, each possesses well-known limitations. Preparations of isolated hepatocytes offer a unique combination of advantages for the study of the hormonal control of hepatic metabolism. Not only does each hepatocyte have uniform and ready access to a defined bathing medium, but the effects of several incubations on both intracellular and extracellular components may be measured simultaneously. Nevertheless, few studies have documented the influence of insulin, as the sole hormone, on the isolated hepatocyte. Herein, we report the preparation of isolated rat hepatocytes which exhibit, on exposure to insulin, alterations in glycogen synthase activity characteristic of the liver in situ; the conditions of cell isolation which appear critical to the preservation of this response are detailed.

METHODS AND MATERIALS

Male Sprague-Dawley rats (Charles River Laboratories) weighing 200-250 grams and maintained on standard rat chow (Purina) were fasted for 48 hours prior to hepatocyte isolation. Hepatocytes were isolated by a modification of the method of Seglen (1). The rat was anesthetized with intraperitoneal amobarbital

(0.2 mg/g weight). Following cannulation of the portal vein and inferior vena cava above the diaphragm, liver perfusion was initiated with Ca^{++}-free Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4; 37°C) containing 5.5 mM glucose at 40 ml/min. After perfusion with 300 ml of buffer, the liver was removed and placed in a recirculating perfusion system contained in a jacketed temperature controlled hood. Oxygenation of the perfusate with 95% O_2/5% CO_2 was accomplished by a simple membrane oxygenator (2). The perfusate was then switched to KRB (pH 7.4) containing 5 mM Ca^{++}, 5.5 mM glucose and collagenase 0.5 mg/ml (Worthington Biochemical Co., Type I, Lot #CLS 45E059) for 10 minutes. The liver was then removed and gently teased apart into ice-cold KRB (pH 7.4) containing 5.5 mM glucose. The digest was poured through a double layer of 100-mesh silk cloth and the filtrate transferred to plastic conical tubes. Cells were then harvested by centrifugation (50 x g x 2 min); the supernatant was discarded and the cells were gently resuspended in the same buffer. This washing procedure was repeated twice. The cell pellet was then suspended in Minimal Essential Medium (MEM) (Grand Island Biological Company) containing 25 mM HEPES buffer (pH 7.4) and 10 mM glucose equilibrated with 95% O_2/5% CO_2. The suspension was distributed into polyethylene vessels such that the depth of the medium did not exceed 1 cm. The vessels were gassed with 95% O_2/5% CO_2 and "preincubated" for 30 minutes at 37°C with shaking at 90 oscillations per minute. The suspension was then refiltered; the cells were harvested by centrifugation as above and resuspended in fresh MEM for metabolic studies.

In studies undertaken to characterize the influence of insulin and glucose on glycogen synthase activity, aliquots of cell suspensions were removed from incubation vessels at various time points following exposure to insulin or added glucose and the cells were sedimented by centrifugation (1000 x g x 30 seconds). The medium was removed and the cell pellet immediately frozen and stored at -70°C. To assay for glycogen synthase, the cell pellet was homogenized in an ice-cold medium containing Tris chloride (0.01 M; pH 7.8), sucrose (250 mM), potassium fluoride (50 mM) and EDTA (5 mM). Glycogen synthase activity was measured by the method of Villar-Palasi et al (3). Synthase I activity was determined in the presence of Na_2SO_4 (10 mM) and total activity (D + I) in the presence of glucose-6-phosphate. Results are expressed as %I activity (I/D + I x 100). Cell protein was determined by the method of Lowry et al (4).

RESULTS

Hepatocytes incubated at 37°C in MEM containing 25 mM HEPES (pH 7.4) and 10% rat serum maintained their viability for at least two hours. The total glycogen synthase activity (D + I) per mg of cell protein remained constant throughout this period. As indicated in Figure 1, in 10 mM glucose 8-10% of this activity was measurable in the I form. If the extracellular glucose was raised to 50 mM, there was a prompt increase in the I form detectable within the first two minutes, reaching a peak at 15 minutes and declining to control levels by two hours. Similar activation was observed if 10 mM glucose was added to a medium free of glucose. The total glycogen synthase activity remained constant over this interval.

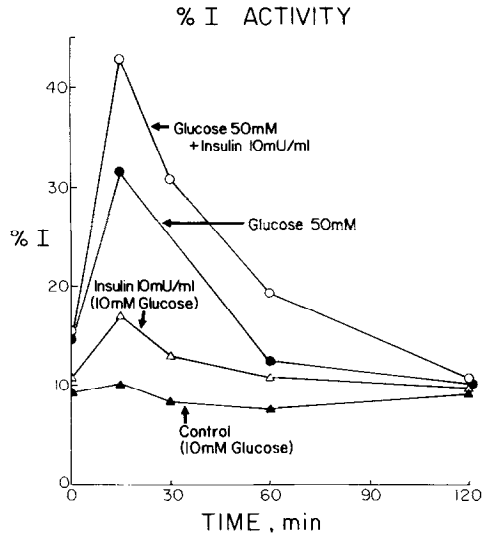

Figure 1. Hepatocytes, suspended in MEM containing 25 mM HEPES, 10 mM glucose and 10% rat serum at a concentration of 3-4 x 10^6 cells/ml, were incubated at 37°C in a metabolic shaker at 90 oscillations per minute. Additions were made as indicated. The apparent zero time increase in I activity in 50 mM glucose is due to glucose addition prior to zero time (-2 minutes). One ml aliquots are taken at the indicated time points and processed as detailed below.

When insulin was added to the hepatocyte suspension, a prompt increase in the I form of glycogen synthase was easily detected, even in the presence of the simultaneous stimulation by 50 mM glucose (Figure 1). As with glucose, the total synthase activity in the presence of insulin did not change (data not shown). The peak stimulation due to insulin was observed within 15 minutes; the magnitude of the peak stimulation in glycogen synthase I activity due to insulin in 13 experiments, at a variety of ambient glucose concentrations was 40.7 $\pm$ 4.7% (SEM). The period of declining glycogen synthase I activity was not associated with alterations in cell viability as judged by trypan blue exclusion. Furthermore, the addition of glucose or insulin to control cells after 60 minutes of incubation resulted in a prompt increase in glycogen synthase I activity.

Figure 2 illustrates that hepatocytes incubated in MEM/HEPES did not require rat serum for the insulin or glucose stimulation of glycogen synthase

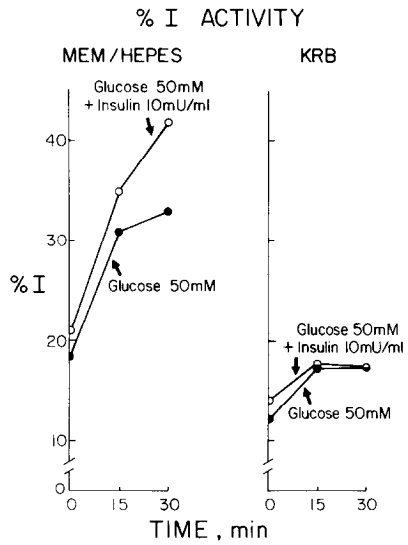

Figure 2. Hepatocytes from single preparations were suspended in either MEM/HEPES without rat serum (left) or KRB (right), with the additions indicated.

I activity. However, in KRB, the ability of glucose to activate hepatocyte glycogen synthase I was markedly attenuated and the stimulation by insulin was abolished. Supplementation of KRB buffer with HEPES buffer (Figure 3) did not restore the responsiveness of glycogen synthase to either glucose or insulin. Despite the lack of insulin stimulation of glycogen synthase in cells incubated in KRB, cell viability (by trypan blue exclusion) was maintained identically in KRB and MEM.

DISCUSSION

Hepatocytes prepared by the present method exhibit, on exposure to insulin, rapid activation of glycogen synthase I activity. It is of interest to compare the present results to those of Miller and Larner (5) obtained with the perfused rat liver. They observed enhanced glycogen synthase I activity due to insulin at 6 and 15 minutes after hormone exposure with a return to a glucose-stimulated baseline thereafter. The preservation of a similar temporal profile in the isolated hepatocyte indicates that this response reflects regulatory mechanisms intrinsic to the hepatocyte. However, the nature of the mechanisms responsible

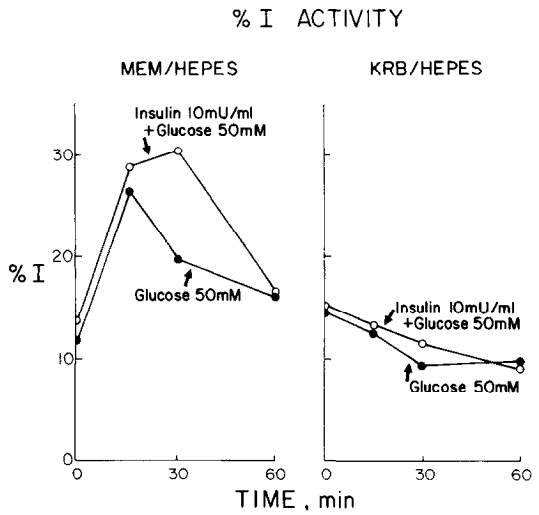

Figure 3. Hepatocytes from single preparations were suspended in MEM/HEPES (25 mM) or KRB/HEPES (25 mM). The NaCl concentration in the latter buffer was adjusted to maintain ionic strength. Additions were made as indicated.

for both the initial activation and subsequent decline in glycogen synthase I we observe in the presence of insulin and glucose are at present unclear.

Only a few reports have documented the ability of insulin, as the sole hormone, to alter the metabolism of isolated hepatocytes (6-9). We have attempted several other methods of hepatocyte isolation (10-12). In our hands, although these techniques yield viable hepatocytes that exhibit an increase in glycogen synthase I on addition of extracellular glucose, no response in glycogen synthase I to insulin can be elicited. While the critical modifications in the present technique of cell isolation responsible for the preservation of insulin sensitivity cannot be precisely determined, it appears that the conditions of collagenase treatment are of preeminent importance. In screening lots of collagenase for hepatocyte isolation, we have relied on our ability to prepare isolated adipocytes (13) with high insulin sensitivity, as measured by at least a three-fold stimulation of $^{14}CO_2$ production from U-[^{14}C]glucose on insulin exposure at 100 uU/ml. Lots of collagenase which yield adipocytes poorly responsive to insulin have yielded insulin-unresponsive hepatocytes. Furthermore, even employing an acceptable lot of collagenase, if the exposure

to the enzyme is prolonged from 10 to 30 minutes, the hepatocytes obtained, although unimpaired in their ability to exclude trypan blue and possessed of glycogen synthase responsive to extracellular glucose, do not exhibit insulin stimulation of glycogen synthase. We expect that the conditions of enzyme exposure will require variation with individual preparations of crude collagenase.

The activation of glycogen synthase by insulin and glucose in the isolated hepatocyte appears to require a complex medium with amino acids and vitamins; rat serum is not an essential constituent. Wagle (9) has previously reported an increase in insulin sensitivity with the addition of 5 mM lactate and 5 mM of amino acids to incubated hepatocytes. The role of the amino acids in modulating the insulin response is unknown at present.

In summary, we have demonstrated a technique for the isolation of viable rat hepatocytes that maintain *in vitro* the insulin responsiveness characteristic of the liver *in situ* as measured by the rapid activation of glycogen synthase I. This system should permit detailed studies of the effects of insulin on several aspects of hepatic metabolism.

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