STUDIES ON THE IN VITRO EFFECTS OF INSULIN ON GLYCOGEN SYNTHESIS AND ULTRA-STRUCTURE IN ISOLATED RAT LIVER HEPATOCYTES*

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SUMMARY: Rat liver hepatocytes were isolated by collagenase in vitro perfusion technique and effect of insulin on glycogen synthesis and ultra-structure was studied. Addition of insulin stimulated glycogen synthesis and maintained better cellular structure. Synthesis of glycogen was linear in isolated hepatocytes when incubated with various concentrations of glucose (0-800 mg%) reaching initial levels. Concanavaline A inhibited epinephrine stimulated glycogenolysis but had no effect on glucagon stimulated glycogenolysis. These studies indicate that insulin is required for glycogen synthesis and for maintaining hepatocytes ultrastructure. Furthermore, isolated hepatocytes retain various receptors and that different hormones utilize different receptor sites.

Insulin has long been known to play an important role in the regulation of carbohydrate and protein metabolism in liver. However, the specific nature of its action at the cellular level is poorly understood. Control of glycogen synthesis and maintenance of intracellular structure are closely interrelated and may be regulated by insulin. The question of whether or not insulin actually enhanced hepatic glycogenesis has remained controversial due to an absence of direct evidence obtained in vitro. In the present study, we report direct effects of insulin on glycogen synthesis and in maintenance of intracellular structures in isolated rat hepatocytes.

MATERIALS AND METHODS

Male fed Cox rats (180-200 g) were used for all these studies. Rat liver parenchymal cells were isolated by collagenase in vitro perfusion technique (1). Approximately 70 mg of wet cells (140,000/mg) were incubated in 3 ml of Umbreit Ringer 25 mM $NaHCO_3$ buffer (with no albumin) with various concentrations of

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TABLE I

Effect of Insulin on Glycogen Synthesis and Concanavalin A on Glycogenolysis in Isolated Rat Liver Hepatocytes

Conditions of Incubation	Glycogen Synthesis uMoles glucose/g*
Isolated hepatocytes unincubated	125 ± 15
Isolated hepatocytes incubated with no substrate	15.2 ± 2.2
Isolated hepatocytes incubated with no substrate + 100 u Units insulin	22.0 ± 4
Isolated hepatocytes incubated with 5 mM glucose, 5 mM lactate, 5 mM amino acids mixture	40.5 ± 8
Isolated hepatocytes incubated with 5 mM glucose, 5 mM lactate, 5 mM amino acid mixture & 100 u Units insulin	65.8 ± 12

*Approximately 70 mg of isolated hepatocytes were incubated in 3 ml of Ringer bicarbonate medium for 2 hrs at 37°C. Values are mean ± SEM of six observations.

Hormone Addition	Glycogenolysis uMoles glucose release in medium/g/hr**
None	44.8 ± 4.6
Epinephrine (10^{-6})	65.2 ± 7
Epinephrine (10^{-6}M) + Concanavalin A (100γ)	42.6 ± 5
Glucagon (10^{-6}M)	78.6 ± 8.0
Glucagon (10^{-6}M) + Concanavalin A (100γ)	76.5 ± 9

**Approximately 70 mg of isolated hepatocytes were incubated in 3 ml of Ringer bicarbonate medium for 1 hr at 37°C. Values are mean ± SEM of six observations.

hormones at 37°C and at 90 oscillations per min as described previously (2). At the end of the incubation period the vials contents were placed in conical centrifuge tubes and were then centrifuged at 2000 rpm in an International centrifuge for 10 min. The supernatent was assayed for glucose by glucose oxidase method (3). Cellular glycogen was precipitated by the method of Good et al. (4), washed, hydrolyzed and assayed as glucose (3). The ultrastructure studies were carried out by thin section electron microscopy on the Siemans IA polarizing electron microscope. The tissue was fixed with 6% glutaraldehyde buffered with 0.1 m sodium cacodylate as the initial fixative and 1% osmium tetraoxide with 0.1 m sodium cacodylate buffer as a post fixative. A standard graded alcohol dehydration and embedding schedule in Epon 812 was followed to obtain thin sections for electron microscopy.

RESULTS AND DISCUSSION

The effect of insulin on glycogen synthesis and concanavalin A on glycogenolysis is summarized in Table I. Glycogen content was significantly lowered when the cells were incubated without any substrate. However, when glucose, lactate and amino acids mixture were added, significantly higher levels of glycogen were maintained by the isolated hepatocytes. Under all conditions studied, added insulin showed higher levels of glycogen. This is also further confirmed by electron micrograph (Figure 1A, B) of an hepatocyte taken immediately after incubation. Higher intracellular glycogen can be seen in cells incubated with insulin (Figure 1B) as compared to control without insulin (Figure 1A). In addition, it can also be seen that better mitochondrial and endoplasmic reticulum structure is maintained in the presence of insulin. Cells incubated with no insulin (Figure 1A) show two types of mitochondria that are less dense in metrix and are granulated. More smooth endoplasmic reticulum as tubular profiles is also present in these cells. In cells incubated with insulin (Figure 1B), there are more single strands of rough endoplasmic reticulum, more glycogen granules and only one type of dense mitochondria. Furthermore, it was also observed that radio-

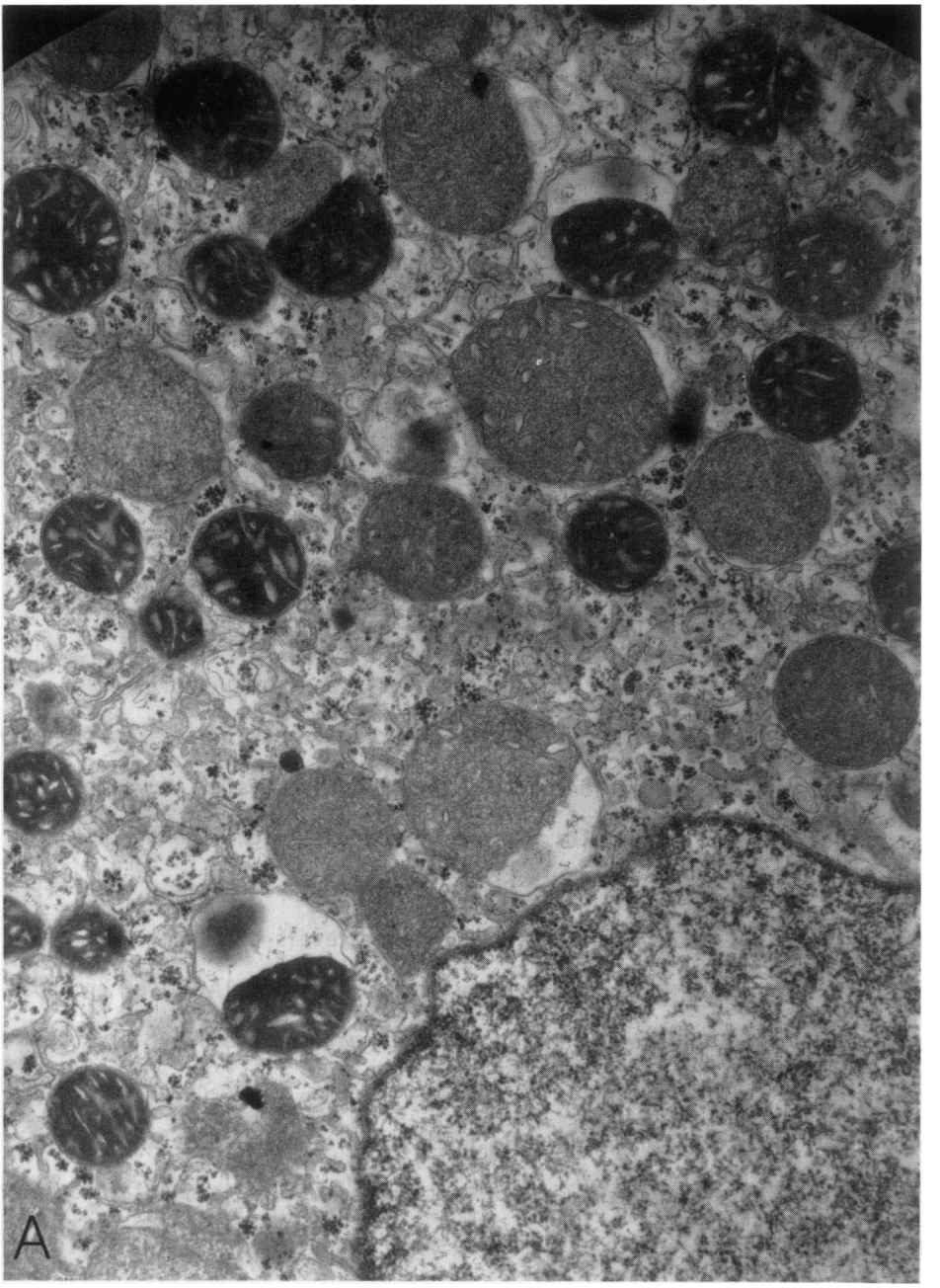
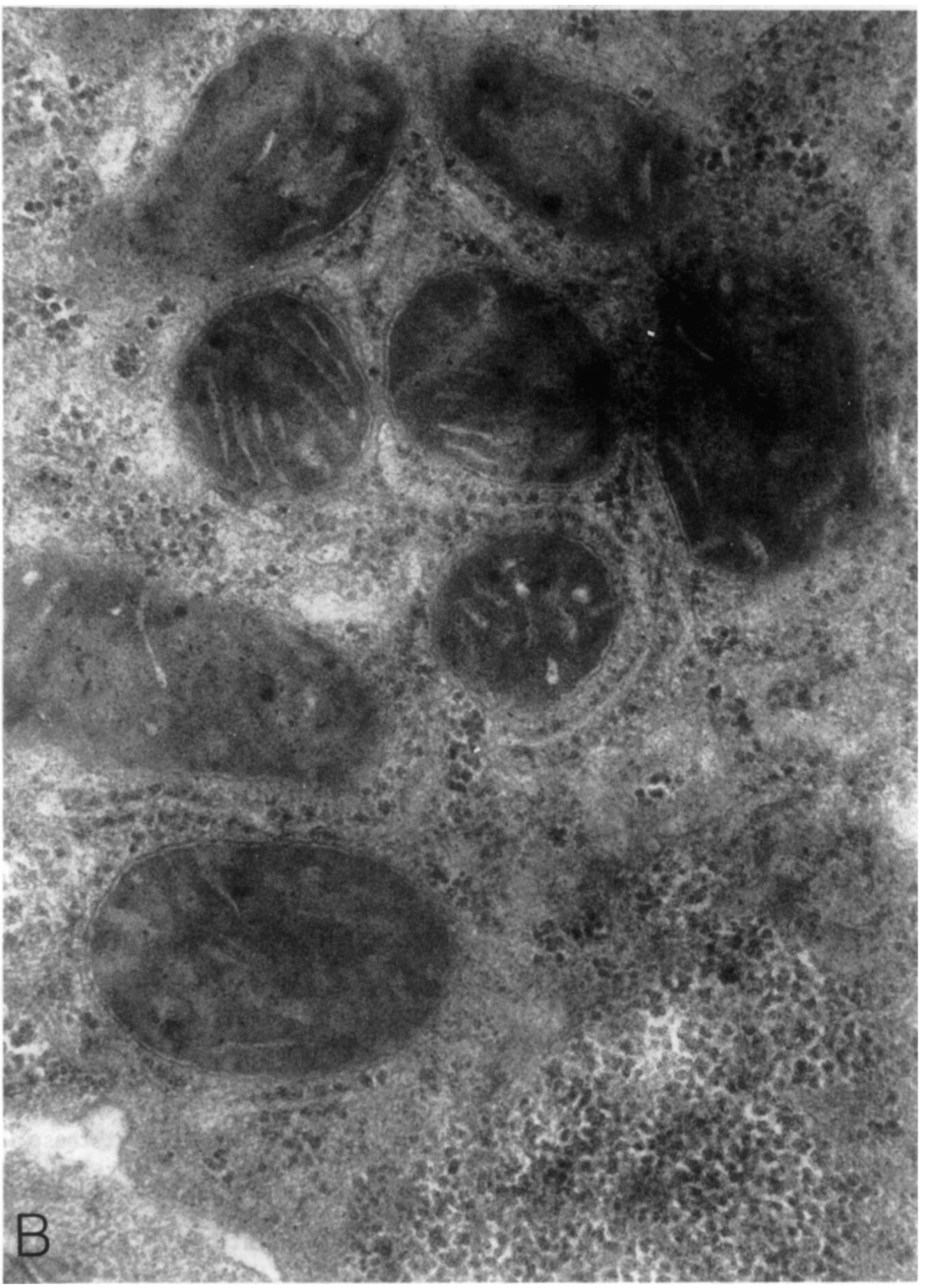


Figure 1: Electron micrograph (A: 24,000 x mag; B. 53,000 x mag) of isolated hepatocyte taken after 6 hours of incubation. Cells were incubated in 3 ml of Ringer bicarbonate medium in the presence of 10 mM glucose and 5 mM isoleucine and with (10 mUnits; Figure B) and without insulin (Figure A) at 37° C.



activity from added U-C^{14} glucose was incorporated into glycogen indicating new glycogen was synthesized.

Addition of 100γ of concanavalin A (Table I) completely abolished epinephrine-stimulated glycogenolysis but had no effect on glucagon stimulated

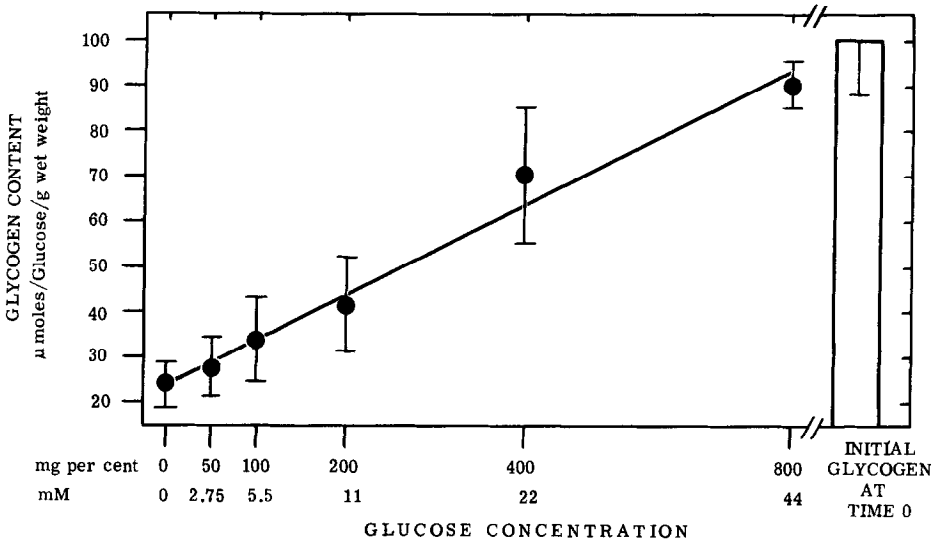


Figure 2: Synthesis of glycogen by isolated hepatocytes incubated with various concentrations of glucose. Approximately 70 mg of isolated cells were incubated in 3 ml of Ringer bicarbonate medium for 2 hrs at 37°C. Values are mean ± SEM of six observations.

glycogenolysis, indicating that these hormones utilize different receptor sites. Recently Cuatrecasas et al. (5,6) have shown that concanavalin A is as effective as insulin in enhancing glucose transport and in inhibiting epinephrine stimulated lipolysis in isolated adipocytes. They have suggested that this response may be brought about by an interaction with insulin receptor structures which may be glycoproteins. As concanavalin A had no effect on glucagon stimulated glycogenolysis, it would indicate that these hormones utilize different receptors or receptor sites for their action.

Studies on glycogen synthesis with various concentrations of glucose are summarized in Figure 2. It can be seen that glycogen synthesis is linear in the presence of various concentrations of glucose and initial glycogen content could be maintained with 800 mg% glucose as the substrate. In addition, it was also observed that radioactivity from added U-C^{14}-glucose was incorporated into glycogen, indicating new glycogen was synthesized. A

small amount of (12-15 umoles/g/hr) net glycogen synthesis was also observed in fasted rats when isolated cells were incubated with glucose (5 mM), lactate (5 mM) and amino acids mixture (5 mM).

Control of glycogen synthesis by insulin (7-9) has remained controversial due to the lack of direct evidence obtained *in vitro* and due to several negative reports *in vivo* (10) and *in vitro* (11). Recently Miller and Larner (7) using perfused liver have shown that insulin stimulated glycogen synthesis and activated glycogen synthase. Furthermore, it also activated glycogen synthase previously inactivated by glucagon in isolated perfused livers from normal rats. This change in synthase activity was associated with a decrease in synthase (protein) kinase activity and tissue cyclic AMP levels when insulin was used to antagonize the effect of glucagon. We have reported previously (2) that insulin antagonized the stimulatory effect of glucagon or epinephrine on glycogenolysis in isolated hepatocytes. Mortimore *et al.* (12,13) have shown that insulin and amino acids reverse the enlargement of hepatic lysosomes in the perfused liver and also inhibit proteolysis. Thus, the results presented here clearly demonstrate for the first time that glycogen synthesis is directly controlled by insulin both *in vivo* and *in vitro* and insulin also functions in maintaining the intracellular structure of hepatocytes.

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