Effect of Insulin on Glycogen and Protein Synthesis in Monolayer Cultures of Hepatocytes from Normal and Alloxan Diabetic Rats

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Summary. The effects of insulin on net glycogen synthesis and amino acid incorporation into protein were studied in cultured hepatocytes from adult normal and alloxan diabetic rats. Insulin stimulated glycogen synthesis in monolayer cells throughout a four day culture period and enhanced leucine incorporation into protein more effectively in normal cells with high glycogen levels than in cultured diabetic cells. These differences correlate well with the observed cellular ultrastructures which were maintained much better in the presence of insulin. Restoration of the morphological changes of alloxan diabetic hepatocytes to normal liver cell structures can be observed at any time during the culture period by giving insulin continuously.

Key words: Insulin, glycogen, amino acid, alloxan diabetes, cultured hepatocytes.

It has been generally accepted that insulin plays an essential role in the regulation of carbohydrate and protein metabolism in liver tissue. The specific nature of its action at cellular level has been previously investigated in perfused liver [1, 2] and in hepatocyte suspensions [3, 4], but various attempts to obtain hepatocytes capable of efficient glycogen synthesis during a several day culture period have not been successful. Cultured hepatocytes have, however, become increasingly popular as a valuable system for investigating many liver biochemical func-

tions in vitro [5, 6]. We set out to develop a hepatocyte preparation capable of glycogen synthesis from normal gluconeogenic substrates. It was, therefore, of considerable interest to see if these cultured hepatocytes, especially from diabetic animals could synthesize cellular glycogen in the presence of insulin, and if amino acid incorporation into protein could be stimulated by the hormone even after several days in culture. In the present study we report the stimulatory effect of insulin on hepatic glycogen and protein synthesis and interrelate it with ultrastructural alterations in monolayer cultures of normal and diabetic rat hepatocytes.

Materials and Methods

Isolation and Cultivation of Hepatocytes

Male Sprague-Dawley rats weighing 100–150 g were used throughout. All rats were kept on a standard laboratory diet (Altromin®) and tap water ad libitum. The animals had easy access to food from a dish placed on the floor. This procedure was recently reported to enhance significantly liver glycogen content [7]. Hepatocytes were isolated by a modified method of collagenase/hyaluronidase (50 and 100 mg, respectively, per 100 ml Hank's solution) digestion of liver slices, which is especially suitable for harvesting a large number of viable cells from small liver pieces, e.g. biopsy samples [8]. The total cell yield and the viability index (percentage of viable cells) were calculated in duplicate with a haemocytometer; viability was based on the ability of cells to exclude the dye, trypan blue [9]. The isolation procedure for tissue culture was performed with sterile solutions and strict aseptic techniques were used throughout. Gentamycin (75 µg/ml) was

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added to (Dulbecco's) phosphate buffered saline (PBS) [9] for cell washing and the enzyme solution was always prepared freshly and sterilized by passage through a washed Millipore filter (0.2 μ m pore size). MEM (Eagle) containing 20% fetal bovine serum, 10% tryptose phosphate broth, 2 mmol/l glutamine, antibiotics and 4 mmol/l leucine was used as the culture medium. After diluting the cell suspension with culture medium to 10^6 cells/ml, 3 ml sterile cell suspension were placed into tissue culture flasks under strict aseptic conditions in a laminar air flow cabinet. From each liver more than 80 monolayers were obtained. The suspended cells were carefully dispersed on the bottom and the flasks were placed in an incubator at 35°C. Visual examination with an inverted microscope revealed that the cells were in contact in a single layer in the flasks. A complete change of the medium was carried out after the first 4 hours of incubation and subsequently every 12 hours. The average time needed for the whole procedure from sacrificing the rats to starting the final culture was not more than 3 hours. Insulin (200 μ U/ml) was given directly into the culture medium when indicated.

Analytical Procedures

At the end of the culture period the plastic tissue culture flasks were cut open with a heated scalpel. Cells were incubated in 2 ml tissue culture medium containing 0.1 μ Ci [U - 14 C]-leucine and the incorporation of labelled amino acid into protein was measured after 60, 120, 180, and 210 min [10]. For glycogen determination cells were washed twice with glucose free PBS [9], scraped out of the culture flasks by a rubber policeman with 1 ml 0.6 mol/l HC10₄, and homogenized on ice. Cellular glycogen was then assayed by a direct enzymatic procedure [11] and protein content was estimated according to Lowry et al. [12].

Preparation of Electron Micrographs

The ultrastructural studies were carried out by thin section electron microscopy on a Siemens polarizing electron microscope (Elmiskop 102). Cells, immediately after the isolation or after the culture period, were fixed with 2% glutaraldehyde in 0.1 mol/l sodium cacodylate and 0.2 mol/l saccharose (pH 7.4) for 2 h at room temperature. They were rinsed with 0.1 mol/l sodium phosphate buffer and postfixed for 30 min in a final solution of 20 g/l osmium tetroxide in the above mentioned sodium cacodylate-saccharose-buffer. The cells were then dehydrated with increasing concentrations of

ethanol up to 100%, infiltrated with propylene oxide and finally embedded in a mixture of propylene oxide and Araldit®. After polymerization at 90° C resin blocks were detached from the plastic tissue culture flasks by an immediate deep-freezing in liquid nitrogen. Cells were then covered again with the propylene oxide/Araldit® mixture and the solid blocks finally were thin sectioned (500 Å) with a LKB ultratome III. Finally sections were stained with uranylacetate (20 g/l) in absolute ethanol and post-stained with lead citrate.

Alloxan Diabetes

Alloxan diabetes was produced by subcutaneous injection of alloxan monohydrate (180 mg/kg) solution which was freshly prepared in 50 mmol/l citrate buffer (pH 4.0) and injected immediately. After five days the animals used in these experiments had a blood glucose level of more than 350 mg/100 ml.

Materials

Fetal bovine serum (FBS), tryptose phosphate broth, antibiotics (penicillin/streptomycin) and minimum essential medium (MEM) (Eagle) containing 100 mg/100 ml glucose were purchased from Flow Laboratories, Bonn. The same batch of FBS was used for all experiments. Insulin content of the serum was approximately 2 μ U/ml, whereas the presence of insulin was not detectable in the culture medium. Therefore, the concentration of insulin contributed by the addition of serum to the culture medium is negligible. Collagenase, type I (approximately 125–200 U per mg solid) and hyaluronidase, type I (approximately 300 U/mg) were from Sigma, München, and all biochemical compounds for the enzymatic assays were from Boehringer, Mannheim. [U - 14 C]-leucine was from the Radiochemical Centre Amersham (England), insulin (Depot beef insulin 40 IU/ml) was from Hoechst, Frankfurt and tissue culture flasks were obtained from Nunc Products, Roskilde (Denmark).

Results

Isolated hepatocytes became adherent to the collagen-coated bottom of the tissue culture flasks within the first 6 hours. Efficiency of attachment was 65% in normal and 50% in diabetic cells. Diabetic cells were found to be more fragile than normal cells. After 24–30 h, adaptation of the cells to the in vitro conditions appeared to be complete and the typical morphology of confluent hepatic

Fig. 1. Hepatocytes from alloxan diabetic rats in nonproliferating monolayer culture. Light micrograph (240 $\times$) of cells stained with haematoxylin and eosin 24 hours after complete enzymatic disaggregation of liver tissue. The adaptation of the parenchymal cells to the in vitro conditions appeared to be complete. Cells were attached at the bottom of the tissue culture flasks, they were flattened out and the initially round cells assumed a cuboidal shape

parenchymal cells in nonproliferating monolayer culture was seen with the inverted light microscope (Fig. 1). Cells retained their epithelioid shape even when they were not in contact with others and approximately 20% were recognized as binucleated cells. The addition of insulin to the culture medium accelerated the time course of plating and cell aggregation in normal [13] and diabetic hepatocytes.

The effects of insulin on net glycogen synthesis in nonproliferating monolayer cultures from normal and diabetic animals are summarized in Figure 2. Immediately after isolation of hepatocytes the cellular glycogen content was only 33 $\mu\text{g}/\text{mg}$ protein in normal and 9 $\mu\text{g}/\text{mg}$ in diabetic cells. However, after cell attachment, new glycogen synthesis was found to occur. At the end of the four day culture period, the glycogen content of hepatocytes cultured from normal and alloxan diabetic rats amounted to 123 $\mu\text{g}/\text{mg}$ and 76 $\mu\text{g}/\text{mg}$, respectively. The presence of insulin (200 $\mu\text{U}/\text{ml}$) further enhanced glycogen formation during the same culture time up to 163 $\mu\text{g}/\text{mg}$ in diabetic and 189 $\mu\text{g}/\text{mg}$ in control cells.

The incorporation of [^{14}C]-leucine into pro-

Fig. 2. Effect of insulin on glycogen content of cultured hepatic parenchymal cells from normal and alloxan diabetic rats. Hepatocytes were maintained in nonproliferating monolayers for 96 hours using standard culture medium containing 100 mg/100 ml glucose and 2 mmol/l glutamine. Glycogen was assayed immediately after isolation (zero time) and after a four day culture period in the presence and absence of insulin (200 $\mu\text{U}/\text{ml}$). Values ($\mu\text{g}/\text{mg}$ protein) are means $\pm$ SEM of five observations

Fig. 3. Electron micrograph (16,200 $\times$) of three hepatocytes in nonproliferating monolayer culture four days after plating. Normal nucleus, endoplasmic reticulum, mitochondria, lysosomes (L) and Golgi complex (GC) can be seen. Intercellular spaces frequently show structures similar to bile canaliculi (BC), microvilli (MV) which regenerate during the culture period, zonulae adherens (A) and desmosome like junctions (D)

tein in four day cultured hepatocytes from normal and alloxan diabetic animals is summarized in Table 1. The incorporation of labelled leucine, which started at similar levels in cells from normal and diabetic animals, increased linearly with the progressive incubation time. The presence of insulin (200 μ U/ml) resulted in enhanced amino acid incorporation during all the incubation periods studied. This increase was significant for the control cells which had high glycogen level at the start of the experiment. The effect of insulin on alloxan diabetic

cells which had a lower glycogen level was less pronounced and not statistically significant.

Studies on the ultrastructure of freshly isolated cells demonstrated distinct cell membranes, normal mitochondria with cristae and many parallel strands of rough endoplasmic reticulum. Isolated cells from diabetic rats showed — in contrast to the control cell preparation — very low cellular glycogen, less parallel strands of polyribosomes, more smooth endoplasmic reticulum and an increase in lipid and large vacuoles as observed recently [14]. The amount of

Table 1. Effect of insulin on the incorporation of leucine into protein in cultured hepatocytes from normal and alloxan diabetic rats

Time of incubation (min)		60	120	180	210
	Glycogen levels ($\mu\text{g}/\text{mg}$ protein)				
Control		908 $\pm$ 111	1853 $\pm$ 258	2431 $\pm$ 186	2921 $\pm$ 266
	123 $\pm$ 17				
Control + Insulin (200 $\mu\text{U}/\text{ml}$)		1282 $\pm$ 149*	2197 $\pm$ 181*	2969 $\pm$ 204*	3835 $\pm$ 241*
Alloxan diabetes		880 $\pm$ 181	1520 $\pm$ 99	2299 $\pm$ 376	2689 $\pm$ 406
	76 $\pm$ 10				
Alloxan diabetes + insulin (200 $\mu\text{U}/\text{ml}$)		1038 $\pm$ 70	1704 $\pm$ 39	2502 $\pm$ 249	2734 $\pm$ 262

Hepatocytes from adult normal and alloxan diabetic animals were isolated for tissue culture by a modified enzymatic procedure using liver slices [7]. After four days tissue culture flasks were cut open and hepatic parenchymal cells were incubated with 2 ml culture medium containing 0.1 μCi [^{14}C]-leucine with or without insulin. Values (dpm/mg protein) are means $\pm$ SEM of four observations. Significant differences ($P < 0.05$) between control and insulin treated cells (Student's 't' test) are marked with asterisks

Fig. 4. Electron micrograph (27,200 $\times$) of a hepatocyte from an alloxan diabetic rat maintained for 96 h in nonproliferating monolayer culture without addition of insulin. Normal nuclear and mitochondrial structures are seen. However, there is a significant reduction in polysome strands, a decrease in glycogen content, an increase in smooth endoplasmic reticulum (SER) and large lipid droplets (LD)

microvilli on the surface of the cell membrane was found to be reduced in both series of experiments.

Hepatocytes *in vitro* after four days in monolayer culture showed a well-developed lamellar rough endoplasmic reticulum. Mitochondria were normal with regard to membrane integrity, size and form of cristae [15]. Intercellular boundaries of the close contacts of the cell membranes were frequently interrupted by structures similar to bile canaliculi (Fig. 3). Intercellular space of neighbouring cells was filled with microvilli formed by plasma membrane evaginations of both apposing cells. These microvilli, newly grown during the culture time, have been suggested as essential for hormonal responses at physiological concentrations [16]. Along the intercellular boundaries structures were characterized as zonulae adherens, zonulae occludens and desmosomes. Cultured alloxan hepatocytes without insulin showed large inclusions of lipid droplets, an

increase in free ribosomes, more smooth endoplasmic reticulum and a decrease in polysome strands (Fig. 4). When insulin was present in the culture medium, restoration of the morphological changes of alloxan diabetic hepatocytes to normal liver cell structures was observed (Fig. 5 A). The increased intracellular glycogen (Fig. 5 B) was mainly of a size similar to ribosomes [17].

Discussion

Freshly isolated hepatocytes from normal and alloxan diabetic rats were observed to have low glycogen levels. This may have been due to a deficient supply of nutritive substances and to the altered metabolic conditions during the isolation procedure [18]. Re-synthesis of glycogen occurs during the culturing of the hepatocytes even in the absence of insulin. This

Fig. 5. Electron micrograph of hepatocytes from alloxan diabetic rats cultured 96 h in the presence of insulin. **A** (opposite page) Electron micrograph (20,400 $\times$) showing the recovery of hepatocytes from alloxan diabetic rats to normal hepatic cell structure as described in Figure 3. **B** Electron microscopic section (39,100 $\times$) showing cellular glycogen (GL) and rough endoplasmic reticulum (RER) along with occasional RER which encircle adjacent mitochondria in single profiles (arrows). These structures are similar to those observed in freshly isolated hepatocytes from normal adult rats.

glycogen formation can be attributed to the presence of certain gluconeogenic amino acids, such as glutamine, in the standard culture medium. Hems et al. [1] demonstrated that the presence of gluconeogenic precursors stimulate glycogen synthesis in the perfused liver of the starved rat. More recently, Katz et al. [19] reported an effect of amino acids on glycogen synthesis in isolated hepatocytes. However, addition of insulin resulted in an increased glycogen synthesis in cultured parenchymal cells from both normal and diabetic rats.

Stimulation of glycogen synthesis and activation of glycogen synthetase by insulin have previously been shown in perfused livers [1, 2], but these effects have remained controversial due to several negative reports [20, 21]. Wagle et al. [3], however, using isolated hepatocytes reported recently that insulin directly controls glycogen synthesis *in vivo* and *in vitro* which was corroborated by an insulin stimulated enhancement of [U- 14 C]-glucose incorporation into glycogen [4]. Our results with cultured hepatocytes are consistent with these observations.

Insulin is also generally accepted as a protein-anabolic hormone and Crane and Miller [22]

showed that insulin and cortisol succinate enhanced plasma protein synthesis. The studies presented here demonstrate that cultured hepatocytes are capable of incorporating 14 C-amino acid into protein linearly even after several days in culture. The stimulatory effect of insulin on protein synthesis was more apparent in normal cultures which had high levels of glycogen. The failure to observe a greater effect of insulin with alloxan diabetic cells may be related to their lower glycogen level. It has been reported that with freshly isolated hepatocyte suspensions, high glycogen levels are required to observe an insulin effect on leucine incorporation [23]. Insulin was also recently found to induce transport of α -aminoisobutyric acid in primary cultures of adult rat liver parenchymal cells [24].

The interrelationship between glycogen levels and protein synthesis in the presence of the hormone correlates with the cellular ultrastructure. It was found that the addition of insulin to diabetic cell culture resulted in restoration of normal liver cell structure. This effect could be observed at any time during the entire culture period. The hormone also seems to act as a stabilizing factor in monolayer cul-

tures to maintain rough endoplasmic reticulum in intact liver cells. These changes are similar to those previously reported for the effect of insulin administration on liver morphology of alloxan diabetic rats in vivo [25]. Additionally, cultured hepatocytes preserve their sensitivity to hormones even after several days. This may be due to the new microvilli formed during the culture period.

The results presented here demonstrate a system which offers the possibility to observe continuously the recovery of chemically injured hepatocytes. The data indicate that cultured hepatocytes are sensitive to hormones and are able to perform complex metabolic functions for several days. They behave in many respects [26, 27] like normal adult rat liver. The monolayer culture system may have considerable potential for future work on the mechanism of action of insulin and it should prove to be suitable for various in vivo/in vitro comparative studies of liver biochemical function.

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