Insulin controls key steps of carbohydrate metabolism in cultured HT29 colon cancer cells

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Effects of insulin on key steps of carbohydrate metabolism were investigated in cultured HT29 colon cancer cells by two different approaches, i.e. incubation of the cells either in the absence or in the presence of glucose in the medium. In glucose-deprived cells, insulin decreased glycogen breakdown, but did not affect polysaccharide levels when glucose was present. Glycogen synthase became activated after insulin treatment in both conditions, even though the activation was more evident when glucose was omitted. No effect on glycogen phosphorylase activity was evident under our experimental conditions. In cells incubated with glucose, the hormone stimulated in a dose-dependent manner the rates of glucose uptake and lactate release. Concomitantly with the increase in glycolytic rate, insulin caused a strong increase in fructose 2,6-bisphosphate. This effect was not observed in the absence of glucose. It is concluded that the carbohydrate metabolism of cultured HT29 cells responds to insulin, making this biological model suitable for investigations *in vitro* on the mechanism of insulin action.

INTRODUCTION

Studies on tumour cells *in vivo* and studies on cultured cancer cells have focused attention on the alterations of carbohydrate metabolism which are associated with the neoplastic state. These include exaggerated glycolysis, which converts glucose into lactate even in aerobic conditions (Weinhouse, 1972; Eigenbrodt & Glossmann, 1980; Denis-Pouxviel *et al.*, 1987), and glycogen accumulation in cancer cells derived from epithelial tissues which normally do not store glycogen, such as colon mucosa (Rousset *et al.*, 1979, 1981). In addition, impaired response of metabolism to hormones was considered common in cancer cells (Weber, 1963).

Over the last few years, the carbohydrate metabolism of the HT29 cell line derived from a human colon adenocarcinoma has been extensively studied (Paris *et al.*, 1983; Rousset *et al.*, 1984; Denis *et al.*, 1984, 1985*b*, 1986*a,b*). By studying the binding of specific radioactive ligands, it was shown that HT29 cells possess receptors to vasoactive intestinal peptide (Laburthe *et al.*, 1978), receptors to insulin (Cézard *et al.*, 1981; Mountjoy *et al.*, 1983) and α_2 -adrenoceptors (Carpéné *et al.*, 1983; Bouscarel *et al.*, 1985). Further investigations have also indicated that stimulation of HT29 cells by vasoactive intestinal peptide and/or α_2 -agonists led to biological responses, including changes in concentrations of cyclic AMP and Fru-2,6- P_2 (Bouscarel *et al.*, 1985; Denis *et al.*, 1986*a*). Insulin has been mainly considered as a growth factor in cell culture (Barnes & Sato, 1980), and previous experiments with insulin on HT29 cells were limited to its effect on nucleic acid synthesis and mitosis (Cézard *et al.*, 1981) or on receptor regulation (Mountjoy *et al.*, 1983).

Therefore, we decided to study in these cultured cells, which possess insulin receptors, the regulatory effect of insulin on some key indices of carbohydrate metabolism.

The chosen parameters attending glycogen metabolism were glycogen levels and activities of glycogen synthase and glycogen phosphorylase. Glycolysis was estimated through the rates of glucose uptake and lactate release. We also measured the levels of Fru-2,6- P_2 , a powerful regulator of the glycolytic pathway in HT29 cells (Denis *et al.*, 1985*a*, 1986*a*).

In the present work, we describe the important role of insulin in modulating carbohydrate metabolism in HT29 cells and the dependence of these effects on the presence of glucose in the incubation medium.

MATERIALS AND METHODS

Drugs and chemicals

Insulin from pig pancreas (24 i.u./mg of protein; I 3505) was from Sigma (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle medium (DMEM) was from Eurobio (Paris, France), and fetal-calf serum, previously tested to be suitable for HT29-cell culture, was from Seromed (Berlin, Germany). All other chemicals were purchased from Sigma or from Serva (Heidelberg, Germany), and were of the highest purity grade.

Cell culture

The HT29 cell line has been established in permanent culture from a human colon carcinoma by Dr. J. Fogh (Sloan Kettering Institute for Cancer Research, Rye, NY, U.S.A.) (Fogh *et al.*, 1977). The cells were routinely seeded at a density of 2×10^4 cells/cm² in 35 mm-diameter plastic Petri dishes (Falcon; Becton Dickinson, Grenoble, France) and grown at 37 °C, under a 5% CO₂/air atmosphere, in DMEM containing 25 mM-glucose, supplemented with 10% (v/v) fetal-calf serum.

Abbreviations used: Fru-2,6- P_2 , fructose 2,6-bisphosphate; DMEM, Dulbecco's modified Eagle's medium.

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During the exponential phase of growth, the culture medium was changed every 48 h. Under these conditions, cell confluency was reached after 10 days of culture. During stationary phase, medium was changed every 24 h, to avoid any nutrient exhaustion.

Experimental protocol

All experiments were performed with attached post-confluent cells. At least 20 h before starting the experiments, the standard culture medium was removed and replaced by 2 ml of serum-free medium. In this way, possible interference from serum hormones or growth factors was avoided. Experiments were started by adding insulin at the appropriate concentration (usually 150 nM) in less than 20 μ l. For each experimental point, three dishes were run in parallel. At given time, 50 μ l medium samples were taken for glucose and lactate determination, medium was removed and the cell layer was rapidly frozen.

Analytical procedures

Fru-2,6- P_2 concentration was determined as follows. Frozen cell layers were scraped into ice-cold 0.1 M-NaOH (1 ml) and sonicated for 10 s. A portion of the homogenate was used for protein determination (Bradford, 1976). The rest of the homogenate was heated at 80 °C for 5 min and used to assay Fru-2,6- P_2 by the method of Van Schaftingen *et al.* (1982) as modified by Van Schaftingen & Hers (1984).

Glycogen was measured enzymically with amyloglucosidase (Keppler & Decker, 1984).

Glycogen synthase and glycogen phosphorylase were assayed after scraping frozen cell layers into 0.2 ml of ice-cold 10 mM-Tris/HCl buffer (pH 7.4) containing 150 mM-EDTA, 0.6 M-sucrose, 1 mM-phenylmethanesulphonyl fluoride, 1 mM-benzamidine, 25 μ g of leupeptin/ml and 50 mM-2-mercaptoethanol. The resulting suspension was sonicated for 10 s, and centrifuged at 13000 g for 10 min at 4 °C. The supernatant was tested for both enzymatic activities. Glycogen synthase activity ratio was measured by the low-glucose 6-phosphate/high-glucose 6-phosphate method as described by Guinovart *et al.* (1979). Glycogen phosphorylase activity was measured by the method of Wang & Esmann (1972). The assay mixture contained 200 mM-KF, 100 mM-glucose 1-phosphate, 1% rabbit liver glycogen, 1 mM-caffeine, 100 mM- $[^{14}C]$ glucose 1-phosphate (0.02 μ Ci/ μ mol), with or without 1 mM-AMP.

Glucose concentrations were measured by using glucose oxidase in a Beckman Glucose Analyzer. Concentrations of L-lactate were measured by using lactate dehydrogenase and NAD⁺ (Hohorst & Bergmeyer, 1974).

All reported values refer to means $\pm$ S.E.M. of three separate experiments, and Student's *t* test was used for comparing the values, significance being admitted for $P < 0.05$.

RESULTS

Effect of insulin on glycogen levels

The ability of insulin to modify glycogen levels was studied in HT29 cells kept in normal or in glucose-deprived medium, in the absence of fetal-calf serum.

Fig. 1. Effect of 150 nM-insulin (■) on glycogen content in cultured post-confluent HT29 cells: comparison with controls (□)

At zero time, cells were given fresh serum-free medium (DMEM) containing 25 mM-glucose (a) or no glucose (b). Reported values are means $\pm$ S.E.M. for three parallel experiments: *significant difference ($P < 0.05$).

As shown in Fig. 1, cells maintained a high glycogen level during the incubation period, but no significant effect of insulin was observed when cells were incubated in glucose-containing medium (Fig. 1a). Incubation in the absence of glucose (Fig. 1b) induced by itself a breakdown of glycogen after a 20 min lag period. After 1 h, the level was only about one-third the original value. In glucose-deprived cells incubated without insulin, the glycogen level continued to decline, and glycogen was essentially absent after 6 h. On the contrary, when insulin was present in the medium, cells did not become completely deprived of glycogen, and they maintained, even after 10 h, a glycogen content which was approx. 30% of that determined in cells incubated with glucose and insulin. Therefore, the main effect of insulin, as regards glycogen level, is to prevent cells completely degrading their glycogen content, as occurs in the absence of insulin.

Effect of insulin on glycogen synthase and phosphorylase

We next studied the effects of insulin on the enzyme responsible for the synthesis of glycogen, glycogen synthase, in the absence or in the presence of glucose in the medium.

In cells incubated in the presence of glucose (Fig. 2a), insulin produced a significant activation (1.4-fold) of glycogen synthase, which was maintained for the whole period of incubation. It appears that cells incubated in the presence of insulin always showed a significantly

Fig. 2. Effect of 150 nM-insulin (■) on glycogen synthase activity ratio (assayed with low/high glucose 6-phosphate concentration) in cultured post-confluent HT29 cells: comparison with controls (□)

At zero time, cells were given fresh serum-free medium (DMEM) containing 25 mM-glucose (a) or no glucose (b). Reported values are means $\pm$ S.E.M. for three parallel experiments: *significant difference ($P < 0.05$).

higher glycogen synthase activity ratio than cells incubated in the absence of hormone.

Glycogen synthase responded in a biphasic manner to glucose deprivation (Fig. 2b). For the first 20–30 min the low-glucose 6-phosphate/high-glucose 6-phosphate activity ratio decreased. Afterwards, glycogen synthase activity ratio steadily increased and, in cells incubated in the absence of insulin, it reached values close to 1, revealing complete activation after 4–6 h. In cells incubated with insulin this biphasic behaviour was also observed. However, a significant effect of the hormone was observed in the second phase: glycogen synthase activity ratio, after reaching a minimum after 20–30 min, showed a dramatic increase from 0.2 to 0.85 within 30 min, total activation being attained after 2 h.

Again, the effects of insulin on glycogen synthase are much more evident in the absence of glucose than in its presence.

The activity ratio of glycogen phosphorylase slowly increased after incubation of the cells for 2 h in the presence of glucose (Fig. 3a). However, in these conditions, the activity of the enzyme was not modified by treatment of the cells with insulin.

Glycogen phosphorylase activity ratio showed a biphasic response to glucose deprivation (Fig. 3b). The activity ratio of the enzyme increased for the first minutes, reaching its maximum after 5 min of incubation. When

Fig. 3. Effect of 150 nM-insulin (■) on glycogen phosphorylase activity ratio (assayed without/with AMP) in cultured post-confluent HT29 cells: comparison with controls (□)

At zero time, cells were given fresh serum-free medium (DMEM) containing 25 mM-glucose (a) or no glucose (b). Reported values are means $\pm$ S.E.M. for three parallel experiments. There were no significant differences at $P < 0.05$.

glucose-deprived HT29 cells were incubated in the presence of insulin, no effect of the hormone was observed on glycogen phosphorylase – AMP/ + AMP activity ratio.

Rates of glucose uptake and lactate release

As shown in Fig. 4, glucose uptake was significantly stimulated by insulin at doses in the range 15–150 nM. A dose-response effect was evident, since glucose consumption, expressed as nmol/h per mg of protein, is calculated to be 420 in controls and 580, 610 and 640 with 15 nM-, 75 nM- and 150 nM-insulin respectively. Lower doses of insulin (1.5 nM and 7.5 nM) were also found to be effective as compared with controls (results not shown). For lactate production, a time- and dose-dependent effect of insulin was also observed.

Fru-2,6- P_2 concentrations

Insulin produced a dramatic increase in Fru-2,6- P_2 in cells incubated in the presence of glucose (Fig. 5a). After 2 h, the value was twice the control. Afterwards, a slow decrease was noticed. However, in cells incubated in the absence of glucose, Fru-2,6- P_2 concentrations showed a biphasic shift (Fig. 5b). They increased for the first 20 min, then decreased very rapidly, reaching very low values at 60 min and later. In the presence of insulin, this evolution pattern was not modified.

Fig. 4. Effect of insulin on rates of glucose uptake and lactate release in cultured post-confluent HT29 cells: comparison with controls

Cells were cultured in a serum-free medium for 20 h before the start of experiments. At zero time, medium was replaced by fresh serum-free medium (DMEM) containing 25 mM-glucose and (■) 15 nM-, (▲) 75 nM- or (◆) 150 nM-insulin. No insulin was given to controls (□). The Figure is representative of five identical experiments.

DISCUSSION

It has been shown previously that HT29 cells possess insulin receptors, leading to the suggestion that this hormone could regulate the metabolism of these cultured cancer cells besides acting as a growth factor.

The experiments reported here clearly indicate that insulin stimulates glucose uptake and lactate production in post-confluent HT29 cells. This effect on glycolytic rate appears to be dose-dependent. The insulin concentrations that we used in our experiments are of the same order of magnitude as those used by other authors working on isolated cells (Khoo *et al.*, 1973; Lawrence *et al.*, 1977; Lawrence & Larner, 1978). Ciudad *et al.* (1981) used 10 nM-insulin in experiments on glycogen synthase activation in isolated hepatocytes. Parkes & Grieneringer (1985) used insulin concentrations ranging from 1 to 100 nM on cultured chicken hepatocytes and considered the lowest concentrations as 'physiological' ones, but used 35 nM for testing the ability of insulin to control glycogen metabolism in their model.

Under our experimental conditions, insulin is found to modify the evolution pattern of glycogen synthase activation ratio in the direction of a higher activity of the enzyme as compared with controls. This effect is more

Fig. 5. Effect of 150 nM-insulin (■) on Fru-2,6-P₂ concentration in cultured post-confluent HT29 cells: comparison with controls (□)

At zero time cells were given fresh serum-free medium (DMEM) containing 25 mM-glucose (a) or no glucose (b). Reported values are means $\pm$ S.E.M. for three parallel experiments: *significant difference ($P < 0.05$).

marked for glucose-deprived cells. By contrast, no clear effect of the hormone was evident in any case when the glycogen phosphorylase activity ratio is considered. These results are in accordance with the observations by Khoo *et al.* (1973), Lawrence *et al.* (1977) and Lawrence & Larner (1978, 1979) on rat adipocytes and those by Ciudad *et al.* (1981) on rat hepatocytes. According to most authors, the effects of insulin on glycogen metabolism are unrelated to changes in cyclic AMP concentrations, which explains the lack of effect on glycogen phosphorylase. They rather involve increases in glucose transport and phosphorylation and specific activation of glycogen synthase phosphatase.

From the present experiments, and as previously reported (Paris *et al.*, 1983), it appears that glucose deprivation by itself induces strong and phasic modifications of both glycogen synthase and glycogen phosphorylase activities. The short-term effect (0–20 min) of glucose deprivation involves inactivation of the glycogen synthase and clear activation of glycogen phosphorylase. After 1 h, glycogen phosphorylase activity returns to basal values (close to 70% activation), whereas the glycogen synthase activation state increases to reach a stable value (85% activation) within 4 h.

As previously reported (Paris *et al.*, 1983; Rousset *et al.*, 1984), such variations of the activation state of the enzymes do not correlate with the evolution pattern of glycogen level, which, after a lag period of 30 min, decreases gradually down to a trace level as a result of

absence of glucose. Again in this case, the effect of insulin is to boost the activation of glycogen synthase, underscoring that at least a part of the effect of the hormone on the enzyme is not dependent on the presence of glucose. It also appears that insulin prevents the total degradation of glycogen in glucose-deprived cells, as also observed in cultured liver cells by Parkes & Grieninger (1985).

Insulin increases Fru-2,6- P_2 in HT29 cells cultured in the presence of glucose. This effect closely resembles that observed in other tissues, such as skeletal muscle (Bosca *et al.*, 1985a), heart (Rider & Hue, 1984) or fibroblasts (Bosca *et al.*, 1985b). The early and transitory increase in Fru-2,6- P_2 which occurs in control cells is a consequence of the change of medium, as reported in cultured neoplastic liver cells (Loiseau *et al.*, 1985) and in HT29 cells (Denis *et al.*, 1986b). The strong accumulation of Fru-2,6- P_2 which is induced by insulin in cells cultured with glucose is concomitant with the increase in glycolysis, and could be due to the increased glucose uptake leading to increased availability of phosphorylated hexoses which are precursors for Fru-2,6- P_2 . No such effect of insulin is seen when glucose is lacking, suggesting that the action of insulin on Fru-2,6- P_2 is indeed concentration mediated by the increase in substrate supply.

From all the data reported here, we can conclude that, in HT29 cells, an effect of insulin is observed on all investigated indices of carbohydrate metabolism. The presence or absence of glucose is sometimes necessary to obtain evidence of the effect. Cultured HT29 cells appear to be a suitable model for investigations *in vitro* on the mechanism of insulin action.

This work was made possible thanks to the 'Action Intégrée Franco-Espagnole' no. 103/131. It was also supported by grant FIS 88/0853.

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Received 12 December 1988/13 February 1989; accepted 27 February 1989