Inhibition of Glycogenolysis and Glycogen Phosphorylase by Insulin and Proinsulin in Rat Hepatocyte Cultures

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SUMMARY

The inhibitory action of insulin and proinsulin on basal and glucagon-activated glycogenolysis was studied in cultured rat hepatocytes containing [^{14}C]glycogen. Insulin or proinsulin given as sole hormones in the presence of 5 mM glucose decreased basal release of [^{14}C]glucose from [^{14}C]glycogen to 20%. Half-maximal effective concentration of insulin was ~0.15 nM and of proinsulin was ~5 nM. Inhibition of [^{14}C]lactate release from [^{14}C]glycogen required slightly higher hormone concentrations with a similar difference in potency for insulin and proinsulin.

The glucagon-stimulated release of [^{14}C]glucose was completely blocked by insulin or proinsulin with half-maximal effective concentrations of ~0.2 and ~8 nM, respectively. In contrast, release of [^{14}C]lactate in the presence of glucagon was increased slightly by insulin and proinsulin.

Basal and glucagon-activated phosphorylase activity was inhibited by ~50% in a dose-dependent manner by both hormones, with differences in potency similar to those for the inhibition of glycogenolysis.

These data point to a direct regulatory role of insulin in the control of hepatic glycogen breakdown even when acting as sole hormone. The results do not support the notion of a preferential inhibitory potency of proinsulin on hepatic glycogenolysis. *Diabetes* 36:551–55, 1987

The contribution of insulin to the regulation of hepatic glycogen metabolism has been shown by several in vivo and in vitro studies (1,2). However, considerable debate persists about its relative importance compared with other regulatory factors such as glucose; "catabolic" hormones, e.g., glucagon and epinephrine/norepinephrine; and the autonomic hepatic nerves, which are known to affect glycogenesis and glycogenolysis (3–5). The contradictory data originate from different experimental conditions; in particular, in vitro studies did not show consistent effects of insulin when acting as sole hormone (3). It has been shown in in vivo studies that insulin suppresses hepatic glycogenolysis and glycogen phosphorylase activity (6,7); however, when isolated hepatocytes from fed rats were used, an augmentation of glucose-induced inactivation of phosphorylase was reported for insulin (8).

Proinsulin—the single-chain molecular precursor of insulin—has received recent interest because of its potentially preferential action in the inhibition of hepatic glucose production, compared with its stimulative action on glucose-consuming tissues like myocytes and adipocytes (9,10). Experiments with perfused livers from fed rats demonstrated a high potency of biosynthetic human proinsulin (similar to that of insulin) in suppressing glucagon-activated glucose output (11). In our work, cultured rat hepatocytes containing labeled glycogen were used to further characterize the inhibitory actions of insulin and proinsulin on basal and glucagon-activated glycogenolysis. In addition, the suppressive action of both hormones on basal and glucagon-augmented glycogen phosphorylase activity was studied.

MATERIALS AND METHODS

Materials. Chemicals were reagent grade and from commercial sources. Enzymes were obtained from Boehringer Mannheim (Mannheim, FRG); bovine serum albumin, glucagon, and bovine insulin were from Serva (Heidelberg, FRG); and biosynthetic human proinsulin was a gift from Eli Lilly (Indianapolis, IN; Lot L 31-102-165). Collagenase was from Worthington (Biochrom, Berlin, FRG), and D-[U-^{14}C]glucose was from New England Nuclear (Dreieich, FRG).

Animals. Male Wistar rats (180–230 g) were kept on a 12-h day/night rhythm (dark period from 2000 to 0800 h) and were allowed free access to the standard diet ssniff R 15 (ssniff, Soest, FRG).

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Received for publication 10 July 1986 and accepted in revised form 6 October 1986.

Cell culture. Hepatocytes were isolated from fed rats by a recirculating collagenase perfusion in situ, as previously reported (12). Cell preparations yielded >90% viable cells. Cells were suspended in medium 199 containing 5 mM glucose and were cultured on 60-mm Falcon plastic dishes (12). For the first 2 h, medium was supplemented with 4% newborn calf serum, 1 nM insulin, and 0.1 μM dexamethasone. After the first medium change (3 h, 2.5 ml/dish), serum was omitted, the initial insulin concentration was kept at 0.5 nM, and the dexamethasone concentration was held at 0.1 μM. The gas atmosphere contained 5% (vol/vol) CO_2, 16% (vol/vol) O_2, mimicking arterial tensions, and 79% (vol/vol) N_2.

After 22 h, the medium was changed again to contain 20 mM glucose, 10 nM insulin, and 0.1 μM dexamethasone to favor synthesis of glycogen. For the determination of the glycogenolytic rate, glycogen was labeled with D-[U-^{14}C]-glucose (0.08 μCi/μmol glucose) from 22 to 46 h. The labeled precursor was removed after 46 h by three successive medium changes. The final medium for the experiments (2 ml/dish) contained 5 mM glucose, 2 mM lactate, 0.1 μM dexamethasone, and hormones as indicated in the figures. Dishes were then allowed 45 min to preincubate before the time 0 samples (130 μl medium) were taken. Release of

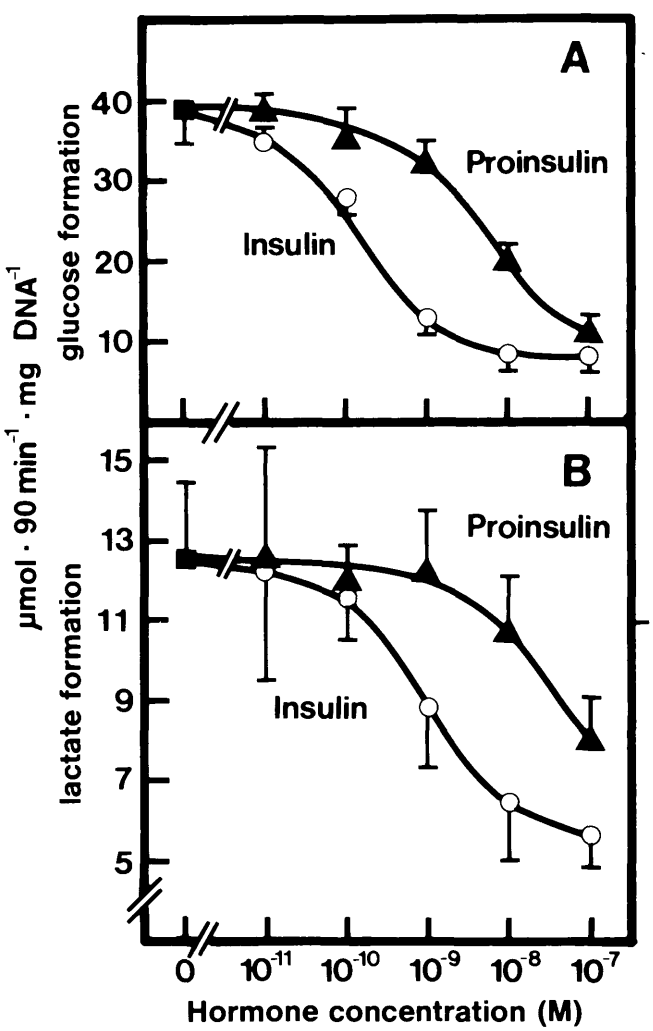

FIG. 1. Decrease of basal glycogenolysis by insulin and proinsulin. Glycogen was prelabeled by incubation of cultures with 20 mM D-[U-^{14}C]glucose and 10 nM insulin from 24 to 48 h. Cells were washed *free from radioactive precursor and provided with fresh medium* 199 containing 5 mM glucose, 2 mM lactate, 0.1 μM dexamethasone, and initial hormone concentrations as shown. [^{14}C]glycogen breakdown to [^{14}C]glucose (*A*) and [^{14}C]lactate (*B*) was determined for 90 min. Values are means ± SE for average values of 3 different cell preparations with 3 culture dishes for individual determinations (at hormone concentrations of 1 nM, $P < .01$ by Student's *t* test for paired data).

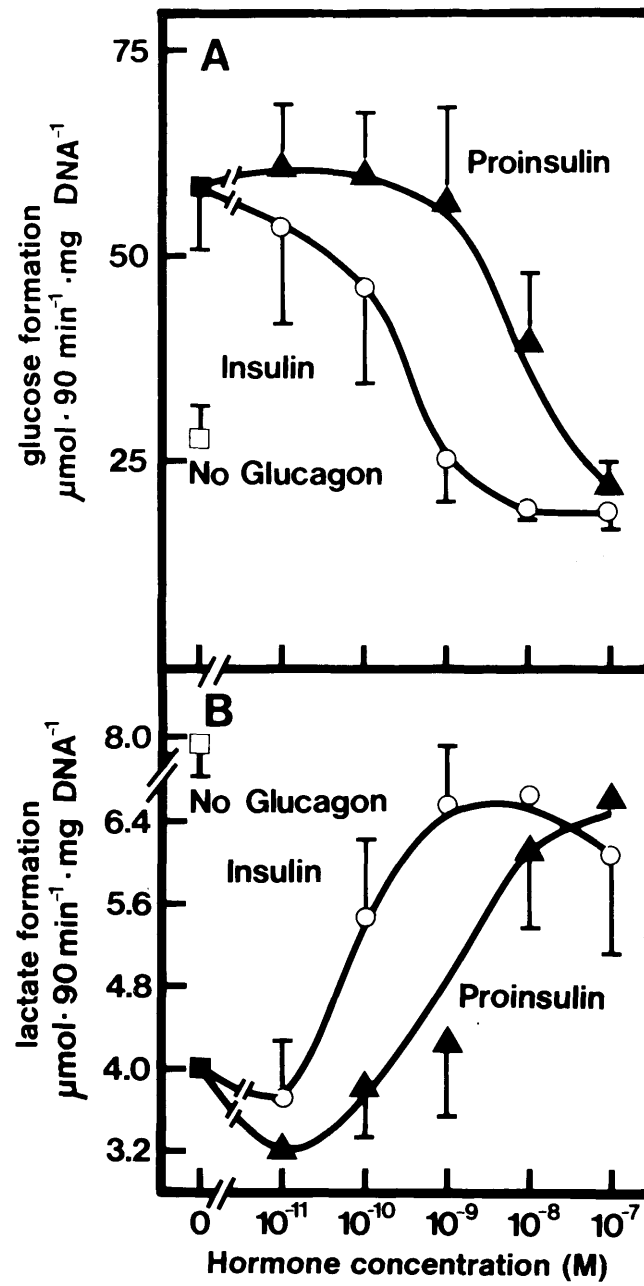

FIG. 2. Inhibition of glucagon-activated glycogenolysis by insulin and proinsulin. Glycogen was prelabeled, and cells were then incubated for 90 min in medium 199 with additions as described in Fig. 1, except that besides proinsulin or insulin, medium contained 0.1 nM glucagon. [^{14}C]glycogen breakdown to [^{14}C]glucose (*A*) and [^{14}C]lactate (*B*) was determined. Control without insulin, proinsulin, and glucagon is given by *open square*. Values are means ± SE for the average values from 3 different cell preparations with 3 culture dishes for individual determinations (at hormone concentrations of 1 nM, $P < .025$ by Student's *t* test for paired data).

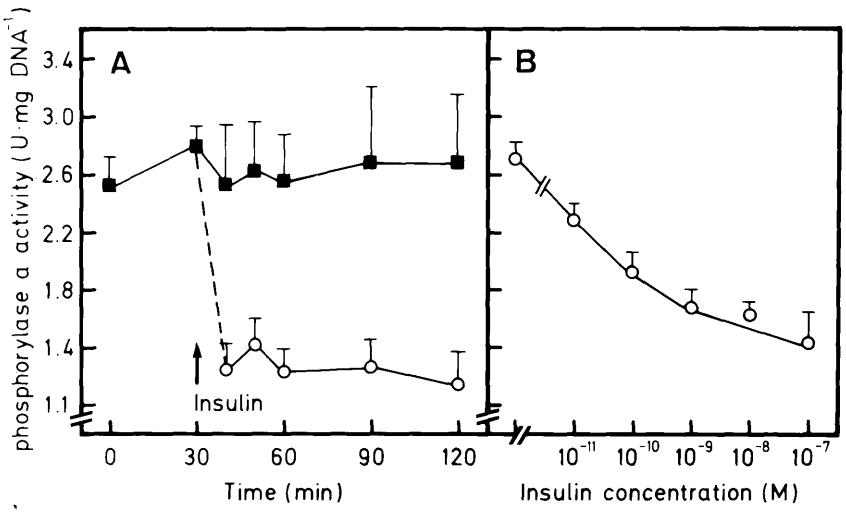

FIG. 3. Inhibition of basal glycogen phosphorylase activity by insulin. Cells were cultured as described for measurement of metabolic activities (Fig. 1) except that glycogen was synthesized from nonradioactive 20 mM glucose during preculture from 24 to 48 h. For experiments, cells were supplied with fresh medium 199 containing 5 mM glucose, 2 mM lactate, and 0.1 μM dexamethasone. *A*: time course. Insulin (0.1 μM) was added to dishes after 1-h preincubation period. *Points* represent means ± SE for average values from 3 cell preparations with 2 dishes for individual determinations (at 50-min time point, $P < .01$ by Student's *t* test for paired data). U corresponds to μmol · min^{-1}. *B*: dose-response curve. Insulin at initial concentrations indicated was added together with fresh medium at 48 h, and cells were then incubated for another 45 min. Values are means ± SE for average values of 3 cell preparations with 2 dishes for individual determinations.

[^{14}C]glucose and [^{14}C]lactate was linear for 120 min under experimental conditions. When glucagon was added, a small increase of glucose concentration in the medium was observed (maximum of <1 mM after 90 min in glucagon-treated cells). When glycogen phosphorylase activity was measured, medium was rapidly suctioned off, and the dishes were immersed in liquid N_2.

Assays. [^{14}C]glucose and [^{14}C]lactate were separated by ion-exchange chromatography as described (13). Incorporation of label into glycogen was determined after treatment of cell homogenates with amyloglucosidase (14) and estimation of the liberated glucose by the glucose dehydrogenase method (15).

Cells were processed for the measurement of glycogen phosphorylase activity according to the method of Stalmans and Hers (16) in 50 mM glycylglycine (pH 7.4), 100 mM NaF, 20 mM EDTA, and 0.5% glycogen (0.5 ml/dish) and sonicated for 5 s at low intensity. One hundred microliters of this homogenate were incubated for 20 min with 100 μl of a solution containing 100 mM glucose-1-phosphate, 2% glycogen, 300 mM NaF, and 1mM caffeine at pH 6.4. Protein was precipitated with 100 μl 20% trichloroacetic acid, and the supernatant was used for determination of inorganic phosphate. DNA was estimated according to the method of Oliver et al. (17).

RESULTS

Decrease of basal glycogenolysis by insulin and proinsulin. Insulin and proinsulin caused an identical maximal decrease of [^{14}C]glucose release from [^{14}C]glycogen to ~20% (Fig. 1*A*). The half-maximal effective hormone concentrations were ~0.15 nM for insulin and ~5 nM for proinsulin.

Inhibition of [^{14}C]lactate release required slightly higher hormone concentrations (Fig. 1*B*); half-maximal effective concentrations revealed a similar difference in potency between insulin and proinsulin (1 nM for insulin and 50 nM for proinsulin).

Inhibition of glucagon-activated glycogenolysis by insulin and proinsulin. When glycogenolysis was activated by glucagon, [^{14}C]glucose release from [^{14}C]glycogen was increased approximately twofold compared with basal release rates. Insulin and proinsulin suppressed the glucagon-dependent increase completely and further reduced glucose release compared with non-glucagon-activated cells to <30%. Half-maximal effective hormone concentrations demonstrated a difference in potency (~0.3 nM for insulin and ~8 nM for proinsulin) (Fig. 2*A*).

The release of [^{14}C]lactate was reduced by glucagon from ~8 to ~4 μmol · 90 min^{-1} · mg DNA^{-1}, in line with an inhibition of glycolysis and an activation of gluconeogenesis. Insulin and proinsulin increased the glucagon-depressed [^{14}C]lactate release again (Fig. 2*B*).

Inhibition of basal glycogen phosphorylase activity by insulin. Basal phosphorylase activity was reduced by insulin to ~50% at 10 min after hormone addition (Fig. 3*A*), suggesting an interconversion of the enzyme by chemical modification as the molecular mechanism of inactivation. The dose-response curve demonstrates a half-maximal effective insulin concentration of ~0.05 nM (Fig. 3*B*).

Inhibition of glucagon-dependent activation of glycogen phosphorylase by insulin and proinsulin. Glucagon increased phosphorylase activity approximately twofold compared with nonstimulated cells. This activation was antagonized by insulin and proinsulin in a dose-dependent manner (Fig. 4) with a difference in potency similar to that observed for glycogenolysis.

DISCUSSION

We have shown that insulin and proinsulin, when acting as sole hormones, inhibit hepatic glycogenolysis and decrease hepatic glycogen phosphorylase activity. The difference observed in the potency of insulin and proinsulin was similar to that reported for the modulation of other metabolic processes in other target organs.

The role of insulin in the regulation of hepatic glycogenolysis has been the subject of in vivo and in vitro studies previously. Although in vivo studies demonstrated a suppression of hepatic glycogenolysis (6) and phosphorylase activity (7,18) by insulin, the problem of whether insulin alone can exert a direct antiglycogenolytic effect remained unresolved because in vitro studies did not show consistent ef-

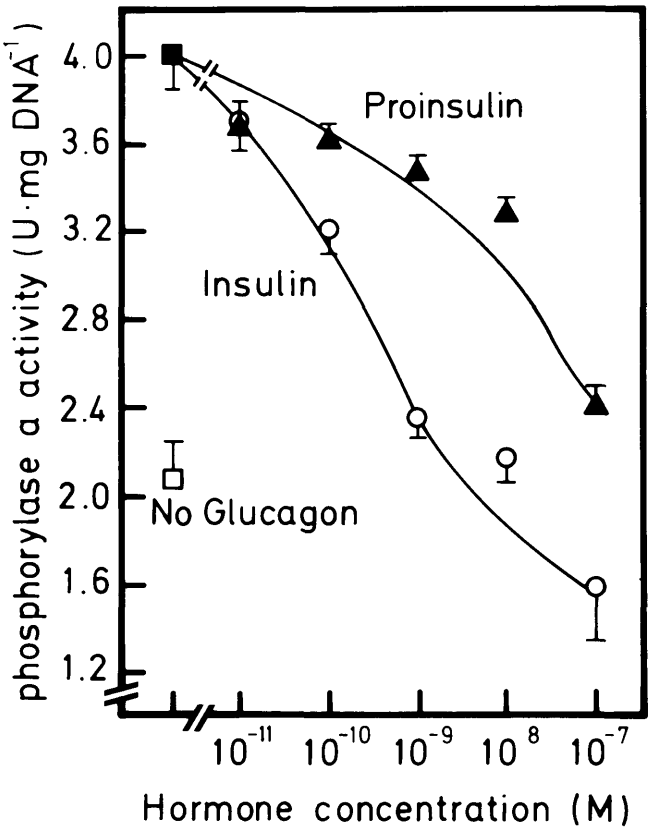

FIG. 4. Inhibition of glucagon-dependent activation of glycogen phosphorylase by insulin and proinsulin. Cells were cultured as described in Fig. 3. With medium change at 48 h, insulin or proinsulin at concentrations indicated were added together with 0.1 nM glucagon; cells were then incubated for another 45 min. Control without insulin, proinsulin, and glucagon is given by *open square*. Values are means ± SE for average values of 2 cell preparations with 3 dishes for individual determinations (at hormone concentrations of 1 nM, $P < .01$).

fects of the hormone (3). Previously, it was shown in isolated hepatocytes that insulin, when added as the sole hormone, decreased the disappearance of radioactivity from glycogen prelabeled with [^{14}C]glucose (19). However, glycogenolysis was not directly measured in these experiments.

In our work, we clearly demonstrated that in hepatocyte cultures with serum-free media, insulin and proinsulin decreased the release of [^{14}C]glucose and [^{14}C]lactate from [^{14}C]glycogen when acting as sole hormones in the presence of 5 mM glucose. Dose-response curves revealed considerable effects at physiologic concentrations of insulin, supporting the biological significance of this observation. This decrease of glycogenolysis was accompanied by an insulin-dependent suppression of glycogen phosphorylase activity, which can be regarded as the major regulatory mechanism of insulin action. However, the possibility should be considered that insulin might have also acted at other sites, e.g., with respect to [^{14}C]glucose release at glucose-6-phosphatase (20,21) and [^{14}C]lactate output at phosphofructokinase and pyruvate kinase (22,23). The difference in half-maximal effective concentrations for insulin between the release of [^{14}C]glucose and [^{14}C]lactate from [^{14}C]glycogen could indicate that, besides glycogen phosphorylase, different regulatory control sites must be involved. Because insulin inhibited glycogen phosphorylase and at the same time activated pyruvate kinase (23), it must be expected that higher insulin concentrations are required to inhibit [^{14}C]lactate formation from [^{14}C]glycogen than [^{14}C]glucose release, as was observed (Fig. 1). A slight inhibition of glycogen phosphorylase by insulin has also been observed previously in hepatocyte suspensions, yet only in the presence of high (10 mM) glucose concentrations (8).

In addition, the known antagonistic action of insulin on glucagon-activated glycogenolysis was also observed in this work with respect to [^{14}C]glucose but not [^{14}C]lactate release. The glucagon-stimulated [^{14}C]glucose release was decreased by insulin; this was associated with an appropriate reduction of phosphorylase activity, confirming the role of this enzyme as the primary regulatory site for glucose release from glycogen. In the presence of glucagon, the release of [^{14}C]lactate from glycogen was reduced rather than increased, reflecting the antiglycolytic/gluconeogenic action of glucagon. The glucagon-decreased [^{14}C]lactate output was enhanced again by insulin and proinsulin, in line with partially relieving the antiglycolytic/gluconeogenic action of glucagon.

Proinsulin has received interest as a possible therapeutic agent in patients with diabetes mellitus because in vivo and in vitro work suggested a preferential inhibitory action on hepatic glucose production (9,11). Studies in the perfused rat liver demonstrated an almost identical potency of proinsulin and insulin in decreasing glucagon-activated glucose release, in contrast to the severalfold difference in potency between both hormones in the modulation of other hepatic and nonhepatic metabolic pathways observed previously and in this in vitro work (24,25). Although reliable estimates for half-maximal effective proinsulin concentrations were not possible under all experimental conditions (e.g., inhibition of glucagon-stimulated phosphorylase activity), it is evident that severalfold higher proinsulin concentrations are required to achieve a response identical to that observed for insulin at submaximal concentrations.

Currently, a simple explanation for the discrepancy between the proinsulin sensitivity of glycogenolysis in the perfused liver and in cell culture cannot be given. The perfused liver might be regarded as an experimental in vitro condition reflecting physiological events more closely than cultured hepatocytes; however, because our findings agree with reports on differences in receptor-binding affinity of insulin and proinsulin in liver membrane preparations (26) and because changes in metabolism were paralleled by changes in enzyme activity at physiologic hormone concentrations (as observed in our work), it must be assumed that the available direct experimental evidence for a preferential inhibitory action of proinsulin on hepatic glucose production is sparse.

ACKNOWLEDGMENTS

The excellent technical assistance of S. Zachmann and K. Unthan is gratefully acknowledged.

This work was supported by Grant Cr 20/19-4 from the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, FRG.

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