Insulin-like Action of Proinsulin on Rat Liver Carbohydrate Metabolism In Vitro

IRMELIN PROBST, HEINZ HARTMANN, KURT JUNGERMANN, AND WERNER CREUTZFELDT

SUMMARY

Short-term effects of human proinsulin on metabolic rates and its long-term action on enzyme induction were studied in primary cultures of rat hepatocytes and in the perfused rat liver, and compared with the effects of bovine insulin. In the perfused rat liver, proinsulin decreased the glucagon-dependent increase of glycogenolysis. The action of 0.5 nM glucagon was almost completely suppressed by 100 nM proinsulin. Proinsulin and insulin showed similar potency.

In cultured rat hepatocytes, proinsulin stimulated glycolysis up to fivefold with a half-maximal effective dose of 30 nM. Proinsulin induced the key glycolytic enzymes glucokinase and pyruvate kinase by twofold and antagonized the glucagon-dependent induction of phosphoenolpyruvate carboxykinase with a half-maximal effective dose at 3 nM. For the effects in cultured hepatocytes, about 100-fold higher concentrations of proinsulin than of insulin were required.

DIABETES 1985; 34:415–19.

Hepatic carbohydrate metabolism is regulated by a variety of circulating hormones[1,2] and by the autonomic nervous system.[3,4] Among the hormonal signals, insulin is of major importance in controlling short-[5,6] and long-term[7,8] regulatory events. Proinsulin—the single chain molecular precursor of insulin—has been detected in peripheral blood of different species and may account for up to 40% of the "insulin" measured in the fasting state.[10] Exceedingly high concentrations of proinsulin have been demonstrated in patients with insulinomas.[11–13]

Short-term biologic effects of proinsulin have been investigated in a few studies under in vivo and in vitro conditions; long-term actions of proinsulin have not been studied so far. In vivo proinsulin has been shown to produce hypoglycemia in a variety of species including man.[14–16] In vitro, it has been demonstrated that proinsulin exerts insulin-like actions in isolated fat cells[17,18] and rat diaphragm.[19] Proinsulin binding to isolated plasma membranes of rat liver has been documented.[20] On the basis of dose-response curves, it was concluded from these studies that proinsulin exerts approximately 3–10% of the biologic activity of insulin.

Early work, using the eviscerated, hepatectomized rat model,[21] and recent results obtained during euglycemic clamp studies in man suggested a preferential action of proinsulin on hepatic carbohydrate metabolism.[22,23]

The aim of the present study was to investigate proinsulin action on short- and long-term regulatory events using the in situ perfused rat liver and primary cultures of adult rat hepatocytes. It was found that proinsulin in the isolated systems (1) controls flux through glycolysis and glycogenolysis and (2) induces key enzymes of hepatic carbohydrate metabolism. Dose-response curves show that proinsulin has a similar potency as insulin in suppressing hepatic glucose output, while for the effects on glycolysis and on enzyme induction 100-fold higher concentrations of proinsulin are required.

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 from Serva (Heidelberg, FRG); and biosynthetic human proinsulin was a gift from Eli Lilly and Company (Indianapolis, Indiana). Collagenase was from Worthington (Freehold, New Jersey) and D-[U-^{14}C]-glucose from Amersham-Buchler (Braunschweig, FRG).

Animals. Male Wistar rats 150–200 g were kept on a 12-h day/night rhythm (dark period from 8:00 p.m. to 8:00 a.m.) and were allowed free access to the standard diet Altromin (Altromin, Lage, FRG).

Liver perfusion. All experiments were started at 9:30 a.m. when the animals were just at the beginning of the postab-

From the Department of Biochemistry (I.P., K.J.) and Department of Medicine (H.H., W.C.), Division of Gastroenterology and Metabolism, University of Göttingen, FRG.
Address reprint requests to Prof. Dr. med. W. Creutzfeldt, Medizinische Universitätsklinik, Robert-Koch-Strasse 40, D-3400 Göttingen, FRG.
Received for publication 18 April 1984 and in revised form 10 September 1984.

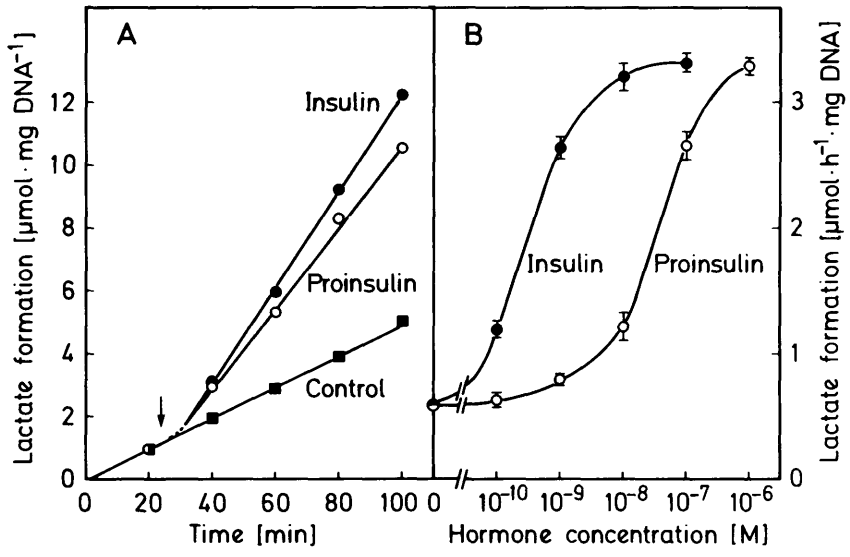

FIGURE 1. Stimulation of glycolysis by proinsulin and insulin. (A) Time course. Cells were cultured with 10 nM insulin for 46 h. The arrow marks the addition of 100 nM proinsulin or insulin to the plate. (B) Concentration response curves. Cells were cultured for 46 h with 0.5 nM insulin. Glycolysis was measured for 3 h. Insulin degradation amounted to <10% during this time. The difference between A and B in the basal glycolytic rate and the degree of stimulation by hormone is due to the different insulin concentrations during the 46-h cell culture. Values are means ± SEM of nine dishes from three different cell preparations.

sorptive phase with an average hepatic glycogen content of 400 µmol glycosyl units/g liver^{-1}.[24] After anesthesia by intraperitoneal injection of pentobarbital (60 mg/kg body wt), the experiments were performed in a 37°C perfusion chamber (Krannich, Göttingen, FRG). Livers were perfused without recirculation via the portal vein; hepatic venous samples were collected every minute.

Perfusion media consisted of 30% (vol/vol) washed bovine red cells, suspended in Krebs-Ringer bicarbonate buffer containing 2% (wt/vol) of bovine serum albumin. Medium glucose and lactate concentrations were adjusted to 5 mmol/L and 2 mmol/L, respectively. The flow rate was kept at a rate of 1.8–2.0 ml/g liver, resembling physiologic flow rates.[25,26] After an equilibration period of 45 min, samples were collected and hormones infused into the portal vein for the indicated time periods. Glucose and lactate concentrations were measured photometrically with standard enzymatic methods using hexokinase plus glucose-6-phosphate dehydrogenase and lactate dehydrogenase.

Cell culture. Hepatocytes were isolated from fed rats by a recirculating collagenase perfusion in situ.[7] Cell preparations yielded >90% viable cells. Cells were suspended in medium 199 containing 5 mM glucose and no lactate and cultured in Falcon plastic dishes. For the first 2 h, medium was supplemented with 4% fetal calf serum and 1 nM insulin. After the first medium change (2 h, 2.5 ml/dish), serum was omitted, the initial insulin concentration was kept at 0.5 nM or 10 nM (Figure 1A), and dexamethasone concentration was 100 nM. The gas atmosphere contained 5% (vol/vol) CO_2, 16% (vol/vol) O_2, and 79% (vol/vol) N_2. Medium was changed again at 6 h, 20 h, and 46 h.

Determination of the glycolytic rate. Culture plates were washed once and incubated in hormone-free medium for 1–2 h. Medium was changed again twice (2 ml/dish), then supplemented with 0.1 µM dexamethasone, 2 mM lactate, and the initial insulin and proinsulin concentrations as indicated. After 20-min preincubation ^{14}C-glucose (20 µl, 0.3–0.6 µCi/dish) was added and the zero time samples taken after another 20 min. The product ^{14}C-lactate was separated by ion-exchange chromatography as described previously.[5] Lactate formation was linear with time for all conditions tested. Triplicates were taken for each assay point.

Assays. Cells were processed for glucokinase and pyruvate kinase assays using an Ultra-Turrax (Janke and Kunkel KG, Staufen, FRG) in 25 mmol/L glycylglycine, 35 mmol/L KCl, 6 mmol/L $MgSO_4$, 5 mmol/L EDTA, and 10 mmol/L 2-mercaptoethanol at pH 7.5. Enzyme activities were determined as described,[22] with the extra addition of 0.1 mM fructose-1,6-bisphosphate in the pyruvate kinase assay. For the phosphoenolpyruvate carboxykinase assay, cells were homog-

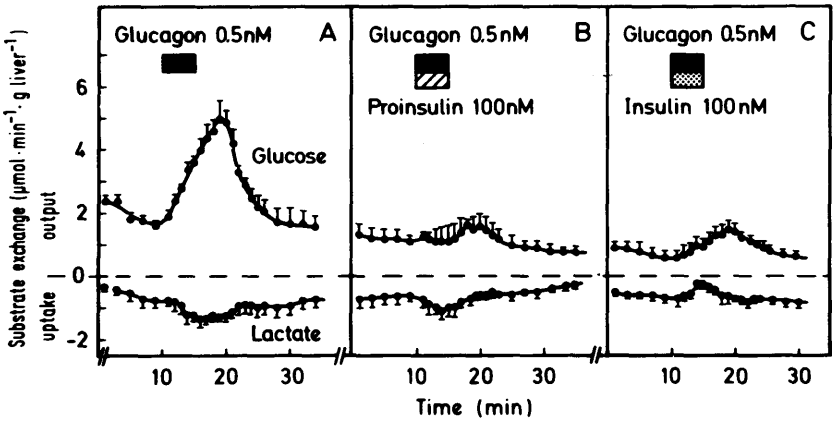

FIGURE 2. Suppression of glucagon-dependent stimulation of glycogenolysis in the in situ, perfused rat liver by proinsulin. In (A), only glucagon (0.5 nM), and in (B) and (C) proinsulin (100 nM) and insulin (100 nM) were infused simultaneously with glucagon into the portal vein. The bars indicate infusion periods of 5 min (11–15 min). Values are means ± SEM of three experiments.

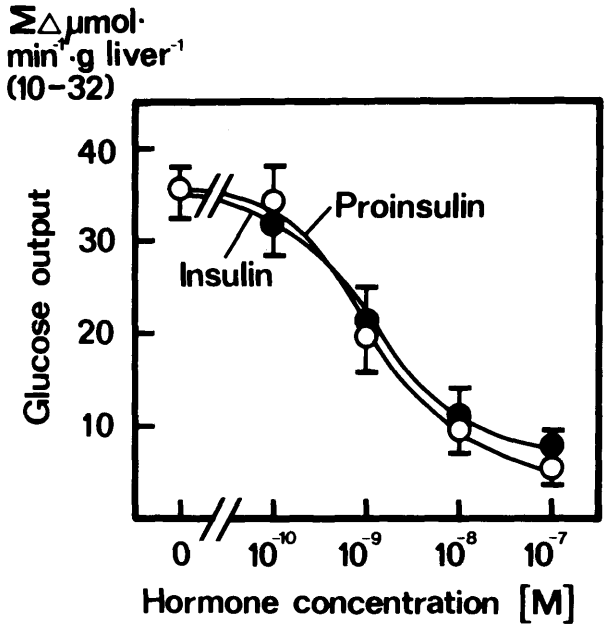

FIGURE 3. Dose-response curve for the suppressive action of proinsulin and insulin on glucagon-dependent glycogenolysis in the perfused rat liver. Hormones were infused as indicated in Figure 2. Glucose output was calculated by integrating the area under the curve for glucose output from the beginning of hormone infusion to minute 32 and subtracting the preinfusion values. Values are means ± SEM of at least four experiments.

enized in 50 mM Tris/HCl, pH 8.1, containing 0.25 mM $MnCl_2$ and 1 mM dithioerythritol; enzyme activity was determined according to Seubert and Huth[27] and DNA according to Oliver et al.[28]

RESULTS

Short-term stimulation of glycolysis by proinsulin. In liver cells maintained in primary culture for 48 h, addition of proinsulin or insulin at a final concentration of 100 nM resulted in an up to threefold increase of glycolytic flux (Figure 1A). Maximal stimulation was obtained within 5 min and lasted for at least 4 h. Obviously, the cultured cells did not become desensitized toward the hormones during this time period. Better time resolution was not technically feasible with this dish culture system.

Proinsulin concentrations of 10 nM stimulated glycolysis significantly. The half-maximal effective dose for proinsulin was 30 nM. In comparison, insulin was almost 100-fold more effective (Figure 1B).

Suppression of the glucagon-dependent stimulation of glycogenolysis by proinsulin. Infusion of glucagon into the portal vein at a final concentration of 0.5 nM resulted in a threefold increase of hepatic glucose output and in a slight increase of hepatic lactate uptake (Figure 2A). On cessation of hormone infusion, hepatic glucose output and lactate uptake returned to preinfusion levels. When proinsulin at a final concentration of 100 nM was infused simultaneously with glucagon, the observed glucagon-dependent effects were almost completely abolished (Figure 2B); insulin was equally effective (Figure 2C). In a dose range from 10^{-10} to 10^{-7} M, proinsulin and insulin had identical potency (Figure 3).

Induction of glucokinase and pyruvate kinase by proinsulin. Treatment of cultured hepatocytes for 48 h with 100 nM proinsulin or 10 nM insulin led to an increase of glucokinase and pyruvate kinase activity by twofold when compared with cells maintained under basal conditions with 0.1 nM insulin (Table 1). The time course and previous work using specific antibodies under similar conditions[8,29] show that increase in enzyme activity is due to an increase in enzyme protein. Whether this is due to an altered enzyme synthesis or degradation can obviously not be deduced from these experiments.

Antagonism of proinsulin to the glucagon-dependent induction of phosphoenolpyruvate carboxykinase. Glucagon, at a concentration of 0.1 nM, induced phosphoenolpyruvate carboxykinase by 2.7-fold when the hormone was present in the culture medium for 4 h. Previous work using a specific antibody demonstrated that the observed increase in enzyme activity was due to an increased amount of the

TABLE 1
Long-term inducing effect of insulin and proinsulin on glucokinase and pyruvate kinase in cultured rat hepatocytes

	Enzyme activity (µmol/min · mg DNA)	
Culture	Glucokinase	Pyruvate kinase
Control	0.43 ± 0.027	6.6 ± 0.13
Proinsulin	0.84 ± 0.03	10.5 ± 0.08
Insulin	0.89 ± 0.03	11.6 ± 0.06

After the 2-h attachment phase, cells were cultured for 46 h with 100 nM dexamethasone and the initial concentrations of either 0.5 nM insulin (control), 10 nM insulin, or 100 nM proinsulin. Values are means ± SEM from six dishes of two different preparations.

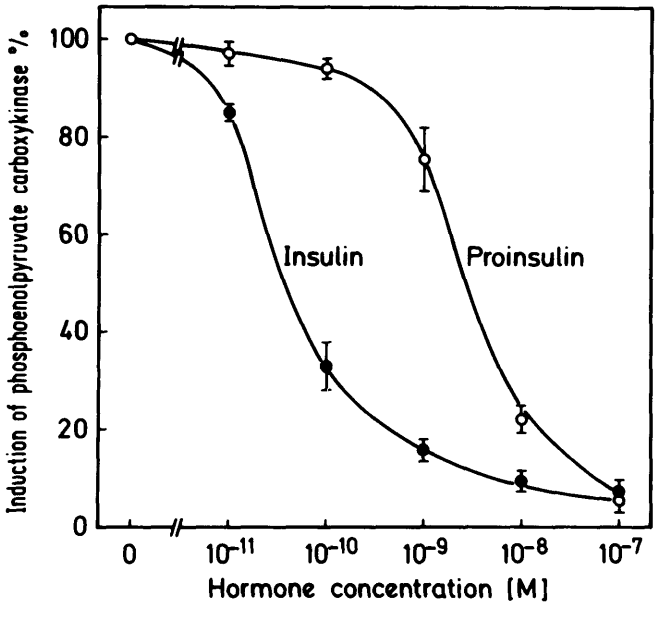

FIGURE 4. The antagonistic action of proinsulin and insulin on the induction of phosphoenolpyruvate carboxykinase by glucagon. Cells were cultured for 24 h. The induction experiment was performed with 0.1 nM glucagon for 4 h in the presence of the initial proinsulin and insulin concentrations as indicated. Enzyme induction from 0% to 100% corresponds to uninduced and fully induced enzyme activity (0.5–1.35 U/mg DNA). Values are means ± SEM of six dishes from two different cell preparations.

enzyme.[30] Proinsulin antagonized this induction with a half-maximal effective dose at 3 nM; in comparison, insulin exerted the same effect at considerably lower concentrations (Figure 4).

DISCUSSION

In the present study, it has been shown in vitro that human proinsulin exerts insulin-like, short-term effects on hepatic glycolysis and glycogenolysis, and induces key enzymes of hepatic carbohydrate metabolism. In comparison to insulin, proinsulin is considerably less effective in controlling glycolysis and enzyme induction; however, it is of equal potency in suppressing hepatic glucose output of the perfused liver.

When glycolysis was studied in cultured hepatocytes, the identical time course of stimulation by insulin and proinsulin observed suggests that proinsulin acted as such rather than being cleaved to insulin by the hepatocytes before stimulation of glycolysis. This interpretation is in accord with earlier work, demonstrating that proinsulin is neither degraded by the liver[31,32] nor split to insulin and C-peptide at the receptor site.[33]

In stimulating glycolysis, insulin was almost 100-fold more effective than proinsulin. This may partially be explained by the observation of a decreased affinity of proinsulin to the insulin receptor as shown in liver membrane preparations.[20] At present, no explanation can be given why proinsulin and insulin suppressed the glucagon-dependent stimulation of glycogenolysis in the perfused liver with almost identical potency. Considering the observation of decreased proinsulin binding to membrane receptors, the underlying molecular events may involve specific postreceptor mechanisms.

Under physiologic conditions, proinsulin concentrations in the peripheral venous blood do not exceed 0.3–0.4 nmol/L.[10] The proinsulin-to-insulin ratio in the peripheral circulation has been shown to be about 10–20% on a molar basis. In portal blood, this ratio can be expected to be even smaller, because insulin—in contrast to proinsulin—is degraded by the liver.[34,35] In fasting subjects, proinsulin may account for up to 40% of the measured "insulin" in the peripheral venous blood. This high proinsulin percentage in the fasting state is primarily due to the 4–5-fold increased plasma half-life of proinsulin as compared with insulin.[36] The results obtained in this study demonstrate that proinsulin has only about 1% of the activity of insulin in regulating glycolysis and enzyme inductions; since it accounts for only 10–40% of the total IRI, its physiologic importance should be negligible. In insulinoma patients, proinsulin concentrations up to 12 nM have been measured,[10] which is about 30–120-fold higher than the normal insulin level. Proinsulin may account for up to 90% of the measured "insulin".[11–13] In this situation, especially considering the very low proportion of insulin simultaneously present, proinsulin effects on hepatic glycolysis and enzyme inductions may become important.

Previous results obtained in the eviscerated rat model[21] and recent data derived from euglycemic clamp studies[22,23] suggest a preferential action of proinsulin on the liver while leaving peripheral tissues, e.g., adipocytes and myocytes, less affected. This preferential action may be due to the observed relatively higher potency of proinsulin in suppressing glycogenolysis. Proinsulin was effective at nearly physiologic concentrations and its potency in the perfused rat liver was almost identical to that of insulin.

ACKNOWLEDGMENTS

We thank K. Unthan and S. Zachmann for their excellent technical assistance.

This work was supported by the Deutsche Forschungsgemeinschaft, D-5300 Bonn-Bad Godesberg, FRG.

REFERENCES

[1] Hers, H. G., and Hue, L.: Gluconeogenesis and related aspects of glycolysis. Annu. Rev. Biochem. 1983; 52:617–53.

[2] Hue, L., and Van de Werve, G., Eds. Short-term Regulation of Liver Metabolism. Amsterdam-New York, Elsevier/North Holland Biomedical Press, 1981.

[3] Lautt, W. W.: Hepatic nerves: a review of their functions and effects. Can. J. Physiol. Pharmacol. 1980; 58:105–23.

[4] Hartmann, H., Beckh, K., and Jungermann, K.: Direct control of glycogen metabolism in the perfused rat liver by the sympathetic innervation. Eur. J. Biochem. 1982; 123:521–26.

[5] Probst, I., and Jungermann, K.: Short-term regulation of glycolysis by insulin and dexamethasone in cultured rat hepatocytes. Eur. J. Biochem. 1983; 135:151–56.

[6] Nyfeler, F., Fasel, P., and Walter, P.: Short-term stimulation of net glycogen production by insulin in rat hepatocytes. Biochim. Biophys. Acta 1981; 675:17–23.

[7] Katz, N. R., Nauck, M. A., and Wilson, P. T.: Induction of glucokinase by insulin under the permissive action of dexamethasone in primary rat hepatocyte cultures. Biochem. Biophys. Res. Commun. 1979; 88:23–29.

[8] Spence, J. T., Merrill, M. J., and Pitot, H. C.: Role of insulin, glucose, and cyclic GMP in the regulation of glucokinase in cultured hepatocytes. J. Biol. Chem. 1981; 256:1598–1603.

[9] Steiner, D. F., Cunningham, D. D., Spigelman, L., and Aten, B.: Insulin biosynthesis: evidence for a precursor. Science 1967; 160:697–700.

[10] Heding, L. G., Faber, O., Kasperska-Czyzykowa, T., Sestoft, L., and Turner, R.: Radioimmunoassay of proinsulin and hyperproinsulinemic states. *In* Proinsulin, Insulin, and C-Peptide. Baba, S., Kaneko, T., Yanaihara, N., Eds. Amsterdam-Oxford, Excerpta Medica, 1979:254–61.

[11] Melani, F., Ryan, W. G., Rubenstein, A. H., and Steiner, D. F.: Proinsulin secretion by a pancreatic beta cell adenoma. N. Engl. J. Med. 1970; 283:713–19.

[12] Gorden, P., Sherman, B., and Roth, J.: Proinsulin-like component of circulating insulin in the basal state and in patients and hamsters with islet cell tumors. J. Clin. Invest. 1971; 50:2113–22.

[13] Creutzfeldt, W., Arnold, R., Creutzfeldt, C., Deuticke, U., Frerichs, H., and Track, N. S.: Biochemical and morphological investigations of 30 human insulinomas. Diabetologia 1973; 9:217–31.

[14] Chance, R. E., Ellis, R. M., and Bromer, W. W.: Porcine proinsulin: characterization and amino acid sequence. Science 1968; 161:165–67.

[15] Lazarus, N. R., Penhos, J. C., Tanese, T., Michaels, L., Gutman, R., and Recant, L.: Studies on the biological activity of porcine proinsulin. J. Clin. Invest. 1970; 49:487–96.

[16] Galloway, J. A., Root, M. A., Chance, R. E., Rathmacher, R. P., Challoner, D. R., and Shaw, W. N.: In vivo studies of the hypoglycemic activity of porcine proinsulin. Abstract. Diabetes 1969; 18 (Suppl. 1):341A.

[17] Gliemann, J., and Sørensen, H. H.: Assay of insulin-like activity by the isolated fat cell method. IV. The biologic activity of proinsulin. Diabetologia 1970; 6:499–504.

[18] Podlecki, D. A., Frank, B. H., and Olefsky, J. M.: In vitro characterization of biosynthetic human proinsulin. Diabetes 1984; 33:111–18.

[19] Shaw, W. N., and Chance, R. E.: Effect of porcine proinsulin in vitro on adipose tissue and diaphragm of the normal rat. Diabetes 1968; 17:737–42.

[20] Freychet, P., Roth, J., and Neville, D. M.: Insulin receptors in the liver: specific binding of [^{125}I]-insulin to the plasma membrane and its relation to insulin bioactivity. Proc. Natl. Acad. Sci. USA 1971; 68:1833–37.

[21] Willms, B., Appels, A., Söling, H. D., and Creutzfeldt, W.: Lack of hypoglycemic effect of bovine proinsulin in eviscerated, hepatectomized rats. Horm. Metab. Res. 1969; 1:199–200.

[22] Revers, R., Olefsky, J., Schmeiser, L., Kolterman, O., Frank, B., Galloway, J., Bergenstal, R., and Blix, P.: The effects of biosynthetic human proinsulin on carbohydrate metabolism. Abstract. Diabetes 1983; 32:54A.

[23] Bergenstal, R. M., Cohen, R. M., Lever, E., Polonsky, K., Jaspan, J., Blix, P. M., Revers, R., Olefsky, J. M., Kolterman, O., Steiner, K., Cherrington, A., Frank, B., Galloway, J., and Rubenstein, A. H.: The metabolic effects of biosynthetic human proinsulin in individuals with type I diabetes. J. Clin. Endocrinol. Metab. 1984; 58:973–79.

[24] Jungermann, K., Heilbronn, R., Katz, N., and Sasse, D.: The glucose/

glucose-6-phosphate cycle in the periportal and perivenous zone of rat liver. Eur. J. Biochem. 1982; 123:429–36.

[25] Dhumeaux, D., and Berthelot, P.: Measurement of hepatic blood flow in the rat. Transhepatic catheterization of the hepatic veins. Biol. Gastroenterol. 1973; 6:49–54.

[26] Ossenburg, E. W., Danis, P., and Benhamou, J. P.: Hepatic blood flow in the rat: effect of portocaval shunt. J. Appl. Physiol. 1974; 37:806–808.

[27] Seubert, W., and Huth, W.: On the mechanism of gluconeogenesis and its regulation. II. The mechanism of gluconeogenesis from pyruvate and fumarate. Biochem. Z. 1965; 343:176–91.

[28] Oliver, I. T., Edwards, A. M., and Pitot, H. C.: Hormonal regulation of phosphoenolpyruvate carboxykinase in primary cultures of adult-rat liver parenchymal cells. Eur. J. Biochem. 1978; 87:221–27.

[29] Noguchi, T., Inoue, H., and Tanaka, T.: Regulation of rat liver L-type pyruvate kinase mRNA by insulin and by fructose. Eur. J. Biochem. 1982; 128:583–88.

[30] Nauck, M., Wölfle, D., Katz, N., and Jungermann, K.: Modulation of the glucagon-dependent induction of phosphoenolpyruvate carboxykinase and tyrosine aminotransferase by arterial and venous oxygen concentrations in hepatocyte cultures. Eur. J. Biochem. 1981; 119:657–61.

[31] Rubenstein, A. H., Pottenger, L. A., Mako, M., Getz, G. S., and Steiner, D. F.: The metabolism of proinsulin and insulin by the liver. J. Clin. Invest. 1972; 51:912–21.

[32] Katz, A. I., and Rubenstein, A. H.: Metabolism of proinsulin, insulin and C-peptide in the rat. J. Clin. Invest. 1973; 52:1113–21.

[33] Freychet, P.: The interactions of proinsulin with insulin receptors on the plasma membrane of the liver. J. Clin. Invest. 1974; 54:1020–31.

[34] Horwitz, D. L., Starr, J. I., Mako, M. E., Blackard, W. G., and Rubenstein, A. H.: Proinsulin, insulin and C-peptide concentrations in human portal and peripheral blood. J. Clin. Invest. 1975; 55:1278–83.

[35] Ishida, T., Lewis, R. M., Hartley, C. J., Entman, M. L., and Field, J. B.: Comparison of hepatic extraction of insulin and glucagon in conscious and anesthetized dogs. Endocrinology 1983; 112:1098–1109.

[36] Sonksen, P. H., Tompkins, C. V., Srivastava, M. C., and Nabarro, J. D. N.: A comparative study of the metabolism of human insulin and porcine proinsulin in man. Clin. Sci. Mol. Med. 1973; 45:633–54.