The Effect of Insulin on the Glycogenolytic Cascade and on the Activity of Glycogen Synthase in the Liver of Anaesthetized Rabbits

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1. The administration of insulin to anaesthetized rabbits caused the inactivation of liver phosphorylase and phosphorylase kinase, but did not change either the hepatic concentration of cyclic AMP or the activity of cyclic AMP-dependent histone kinase. All measured parameters were increased by the subsequent administration of glucagon. 2. Activation of glycogen synthase by insulin was only observed when phosphorylase had been strongly inactivated.

It has become widely accepted that insulin promotes the arrest of glycogenolysis and the deposition of glycogen in the liver (for a review see Stalmans, 1976). Few studies, however, have dealt with the effect of the hormone *in vivo* on the enzymic changes that underly this metabolic switch. A partial inactivation of phosphorylase has been observed inconsistently in anaesthetized rats (Stalmans *et al.*, 1974) and much more systematically in rhesus monkeys (Curnow *et al.*, 1975). In the latter case, the conversion of phosphorylase *a* into phosphorylase *b* was almost complete, and was followed by the activation of glycogen synthase.

In the preceding paper (van de Werve *et al.*, 1977) it has been shown that the activation of phosphorylase in the liver cannot always be related to a change in the state of activation of phosphorylase kinase. It was therefore of considerable interest to examine by which mechanism insulin causes the inactivation of phosphorylase in the liver *in vivo*. Rabbits were chosen for the present work, since they have been reported to show a good hepatic response to insulin (Berthet *et al.*, 1956). We show that the hormone caused a rapid inactivation of both phosphorylase and phosphorylase kinase with no change in the concentration of cyclic AMP and the state of activation of cyclic AMP-dependent protein kinase.

Materials and Methods

Handling of animals

For the experiments, 11 male rabbits from various breeds, weighing 2–4 kg and fed *ad libitum*, were anaesthetized with sodium pentobarbital and ether as described by Shimazu & Amakawa (1975). Laparotomy was started 10–15 min after the injection of pentobarbital. While the animals were kept anaes-

thetized by further intermittent application of ether, hormones were injected and liver samples weighing about 0.5 g were removed, according to various time-schedules. The liver specimens were immediately quick-frozen between aluminium blocks precooled in liquid N₂ and stored at –20°C until further processed. When the fragment was taken from a small lobe, bleeding was controlled by a ligature as described in the preceding paper (van de Werve *et al.*, 1977); when the sample was cut from the margin of a major lobe, the wound was covered with several layers of a haemostatic compress wrapped in a sheet of Parafilm (American Can Co., Greenfield, CT, U.S.A.), which was securely fastened with a clothes-peg.

The data shown in Fig. 1 were obtained with six white New Zealand rabbits that were handled on the same day according to a standardized scheme. The first biopsy was taken 40 min after the injection of pentobarbital; 6 min later a second sample was cut off, 2 min before the administration of either insulin (2 i.u./kg) or an equivalent volume (0.4 ml/kg) of 0.9% NaCl through an ear vein. Four additional biopsies were taken 3, 8, 13 and 18 min after this injection. Then 2 min after the last biopsy, glucagon (0.5 mg dissolved in 1 ml of 0.9% NaCl) was injected into the inferior vena cava. One or two more samples were then obtained, 2 and/or 5 min after the latter injection.

Assay of cyclic AMP-dependent histone kinase

Frozen liver biopsies were cooled by immersion in liquid N₂, weighed, and homogenized in a Potter-Elvehjem homogenizer in 10 vol. of an ice-cold solution containing 0.25 M-sucrose, 4 mM-EDTA, 10 mM-theophylline and 10 mM-sodium phosphate, pH 7.4; immediately after the preparation of each homo-

genate, a 50 μ l portion was transferred to a tube immersed in a cooling mixture (solid CO₂ in acetone), and containing 0.45 ml of the same solution. The tubes were kept in the cooling mixture until further processing (about 1 h). At 2–5 min before its contents were assayed, each tube was swirled in a water bath at 30°C until thawed. It has been checked that this procedure yields enzyme activities identical with those determined on unfrozen homogenates assayed 5 min after the preparation of the latter.

The assay was performed as described in the preceding paper (van de Werve *et al.*, 1977), except that, in addition, the activity of each sample was also measured in the presence of 58 μ g of the heat-stable protein inhibitor of protein kinase (Walsh *et al.*, 1971); it has been verified that this amount of inhibitor completely abolishes the activity of cyclic AMP-dependent histone kinase under our assay conditions. To render the assay specific for the cyclic AMP-dependent histone kinase, the activity measured in the presence of the protein inhibitor was subtracted from the activities measured in the absence of the inhibitor. The values determined in the presence of cyclic AMP and those determined in the presence of the inhibitor were constant in the different biopsies obtained from a single rabbit.

Other assays and materials

Phosphorylase *a* and glycogen synthase *a* were assayed in a single homogenate as previously described (Stalmans *et al.*, 1974), except that the concentration of glycylglycine in the homogenization medium was decreased to 10 mM. Phosphorylase kinase was determined as reported in the preceding paper (van de Werve *et al.*, 1977). Cyclic AMP was measured as described by van de Werve *et al.* (1974). For the determination of glucose in frozen liver samples, the procedure of De Wulf & Hers (1967) was followed. Glucose oxidase free of glucoamylase (type V) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The sources of other chemicals are specified either in the preceding paper (van de Werve *et al.*, 1977) or by van de Werve *et al.* (1974).

Results

Inactivation of phosphorylase

Fig. 1 shows that the administration of insulin to rabbits caused a rapid inactivation of phosphorylase which, within 3 min, lost about 60% of its activity. This low activity of phosphorylase *a* persisted for the next 15 min. Subsequently the enzyme was rapidly re-activated by the administration of glucagon. Phosphorylase kinase was concomitantly inactivated and was also re-activated by the administration of glucagon. In contrast, insulin did not change the hepatic concentration of cyclic AMP or the activity of cyclic AMP-dependent histone kinase; these two

Fig. 1. Effect of the successive administration of insulin and glucagon on the concentration of cyclic AMP and on the activities of cyclic AMP-dependent histone kinase, of phosphorylase kinase, and of phosphorylase in the liver.

At zero time, three rabbits received insulin (●) and three others received 0.9% NaCl (○). Glucagon was injected into all animals at 20 min. Values shown are means $\pm$ S.E.M.; those without vertical bars are means of determinations on two biopsies. The concentration of glucose in the biopsies, which was initially 1.92 ± 0.29 mg/g of liver (mean $\pm$ S.E.M.; $n = 6$), fell to 1.43 ± 0.31 at 18 min after the administration of insulin, and remained constant in the control animals.

parameters were increased by the administration of glucagon.

Activation of glycogen synthase

Before treatment and after the injection of 0.9% NaCl, the liver contained a very small amount of synthase *a* (mean $\pm$ S.E.M. = 13 ± 0.7 units/g of liver; $n = 38$). After the administration of insulin a significant conversion of synthase *b* into *a* was observed in only one of the three rabbits shown in Fig. 1, and in two other animals out of four in another series of rabbits subjected to different time-schedules. Fig. 2 shows the whole of our data on the activity of phosphorylase *a* and of synthase *a* in 63 biopsies obtained from four NaCl-treated and seven insulin-treated

Fig. 2. Correlation between the activities of phosphorylase *a* and glycogen synthase *a* in rabbit liver biopsies

○, Liver samples from rabbits before treatment or after the injection of 0.9% NaCl; ●, biopsies taken after administration of insulin. The vertical broken line indicates the activity of phosphorylase *a* at or below which a significant activation of glycogen synthase was observed.

rabbits. It is apparent, as also shown in Fig. 1, that a first action of insulin has been to decrease the activity of phosphorylase. Further, in the few liver samples that contained a particularly low amount of phosphorylase *a* (≤ 0.8 unit/g of liver), a second effect of insulin has been to provoke conversion of synthase *b* into *a*. Whenever a significant amount of either phosphorylase or glycogen synthase was present in the active form, the other enzyme was almost completely in the inactive form.

Discussion

Inactivation of phosphorylase

Since insulin provoked an important and synchronous inactivation of phosphorylase and of phosphorylase kinase, it is difficult to escape the conclusion that the latter change is responsible for the former. The question how insulin caused the inactivation of phosphorylase kinase cannot be readily answered. We do not know if the presence of glucagon or other effectors in the portal blood is required for this insulin effect, as it is in the case of the isolated hepatocyte (van de Werve *et al.*, 1977). However, it appears that current concepts of insulin action are unable to explain the effects observed *in vivo*. Insulin could act by lowering the hepatic concentration of cyclic AMP; such an effect can be readily demonstrated with isolated liver preparations when the concentration of the nucleotide has been previously increased by glucagon or adrenaline

(Exton *et al.*, 1971). However, neither in the present work nor in the rhesus monkeys studied by Curnow *et al.* (1975) did insulin measurably affect the hepatic concentration of cyclic AMP. In this respect, the objection is often raised that a large part of the total amount of cyclic AMP in basal conditions might be bound and thus metabolically inactive; therefore a hardly detectable decrease in the concentration of the nucleotide could produce an important effect. This objection does not apply to the present results, since insulin did not change the activity of the cyclic AMP-dependent protein kinase.

Since neither the concentration of cyclic AMP nor the activity of protein kinase were affected by insulin, it appears likely that the hormone acted through a messenger different from cyclic AMP, possibly a cation; an ionic change could influence the activity of phosphorylase kinase (cyclic AMP-dependent protein kinase or maybe a different kinase) or phosphorylase kinase phosphatase.

Activation of glycogen synthase

Whereas the inactivation of phosphorylase was a constant feature after the administration of insulin, the activation of glycogen synthase occurred only occasionally. The data presented in Fig. 2 show that the activation of the synthase was only observed when phosphorylase *a* activity had reached a very low value. The same pattern has previously been noted in a study with anaesthetized mice treated with glucose (Stalmans *et al.*, 1974). It indicates that, in the intact animal, glycogenolysis is arrested before glycogen synthesis starts. A sequential inactivation of phosphorylase and activation of glycogen synthase has indeed been observed in anaesthetized rats treated with glucose (Stalmans *et al.*, 1974) and in monkeys treated with glucose or with insulin (Curnow *et al.*, 1975); it also occurred in isolated hepatocytes, whether the inactivation of phosphorylase was provoked by a rise in the concentration of glucose or of K^+ in the incubation medium (Hue *et al.*, 1975). The sequential change in the activities of the two enzymes has been explained by the observation that phosphorylase *a* strongly inhibits glycogen synthase phosphatase, as discussed in detail elsewhere (Hers, 1976; Stalmans, 1976). Stalmans & Hers (1973) have called attention to the sequential inactivation of phosphorylase and activation of glycogen synthase that occurred after insulin administration to a diabetic dog, studied in detail by Bishop *et al.* (1971). It appears therefore that the profound inhibition of glycogen synthase phosphatase by phosphorylase *a* is a general control mechanism of glycogen metabolism in the liver.

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