Effects of diabetes on the expressed and total activities of 3-hydroxy-3-methylglutaryl-CoA reductase in rat liver *in vivo*

Reversal by insulin treatment

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1. The expressed and total activities of HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase (EC 1.1.1.34) were measured in microsomal fractions prepared from cold-clamped liver samples [Easom & Zammit (1984) *Biochem. J.* **220**, 733–738] from control or insulin-treated diabetic animals. 2. Streptozotocin-induced diabetes resulted in a marked decrease in total activity of HMG-CoA reductase and in the fraction of the enzyme in the active form, but appreciable effects were only observed in the liver of animals in which the blood glucose was above 20 mM. 3. Intravenous infusion of insulin into diabetic rats resulted in a rapid (<20 min) and total dephosphorylation of the enzyme *in vivo* without any change in total activity. 4. Longer-term (4 h) treatment with insulin (injected intraperitoneally) produced a rapid increase in expressed/total HMG-CoA reductase activity ratio to about 90%, followed, after a lag of 2–3 h, by a 5–6-fold increase in total activity. 5. These observations are discussed with respect to the possible role of insulin in generating and maintaining the respective diurnal rhythms in total and in expressed/total HMG-CoA reductase activity ratio observed for normal animals *in vivo* [Easom & Zammit (1984) *Biochem. J.* **220**, 739–745].

An important role for insulin in the regulation of the activity of HMG-CoA reductase (EC 1.1.1.34) in the liver has been evident for a considerable time. Thus Lakshmanan *et al.* (1973) demonstrated that administration of insulin to normal or diabetic rats resulted in an increase in hepatic reductase activity and rate of cholesterogenesis even if the treatment was performed in the middle of the light period and in animals that did not have access to food. Similarly it has been established that streptozotocin-induced diabetes in rats results in a marked decrease in the total activity of HMG-CoA reductase in the liver (Huber *et al.*, 1973; Lakshmanan *et al.*, 1973; Uchida *et al.*, 1979; Young *et al.*, 1982). However, relatively few studies have attempted to study the possible role of insulin in modulating the fraction of HMG-CoA reductase in the active form, i.e. in altering the phosphorylation state of the enzyme. Ingebritsen *et al.* (1979) showed that in isolated hepatocytes the expressed/total HMG-CoA reductase activity ratio was

increased by inclusion of insulin in the incubation medium. On the contrary, studies on the enzyme in the liver of diabetic rats *in vivo* have suggested that insulin-deficiency either does not affect this ratio (Goodman *et al.*, 1982) or actually increases it (Young *et al.*, 1982). These data, coupled with others that have indicated that the rate of hepatic cholesterogenesis is unaffected on a unit-weight basis in the diabetic rat (Feingold *et al.*, 1982), would not be reconcilable with a cholesterogenic role for insulin *in vivo*.

We have previously described a technique that enables the rapid sampling of rat liver for the preparation of liver microsomal fractions in which the phosphorylation state of HMG-CoA reductase that occurs in the intact animal *in vivo* is preserved *in vitro* (Easom & Zammit, 1984a). Using this cold-clamping technique, we have demonstrated that marked diurnal modulation of the phosphorylation state of the enzyme occurs *in vivo* in the normal animal (Easom & Zammit, 1984b). One of the differences between the characteristics of this diurnal cycle and that observed for the total activity of HMG-CoA reductase is a 2–3 h phase

Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

separation between the respective peaks and nadirs of the two cycles (Easom & Zammit, 1984b). These observations suggest that the two rhythms could be generated by related hormonal effects, but through different mechanisms.

Since circulating insulin concentrations *in vivo* also undergo a diurnal cycle (Peret *et al.*, 1973; Bruckdorfer *et al.*, 1974; Knox *et al.*, 1979), the peak of which coincides with that of the diurnal rhythm of expressed/total HMG-CoA reductase activity described by us (Easom & Zammit, 1984b), we decided to investigate further the effects of insulin deficiency and supplementation of the hormone on this ratio *in vivo*. In contrast with previous studies (see above), we find that diabetes is accompanied by a marked decrease in the fraction of HMG-CoA reductase in the active form *in vivo* and that this decrease can be rapidly reversed by insulin treatment of diabetic animals. In addition, it is shown that a 2–3 h difference exists between the effects of insulin on the expressed/total activity ratio and those on total activity, thus supporting the suggestion that insulin may play an important role in the generation and maintenance of both the respective diurnal cycles involved in the regulation of hepatic HMG-CoA reductase activity *in vivo* (Easom & Zammit, 1984b).

Materials and methods

Animals

Male Wistar rats were housed from birth in a light-controlled room (dark phase 03:30–15:30 h) and maintained as described previously (Zammit, 1980, 1981) on Oxoid rat breeding diet (Oxoid, Basingstoke, Hants., U.K.) given *ad libitum*. The animals were used within a weight range of 160–200 g (i.e. 5–6 weeks old). They were handled for several days before being killed in order to train them to the experimental protocol and to minimize stress on the day of the experiment. Rats were made diabetic by intraperitoneal injection of streptozotocin (150 mg/kg body wt.) as described by McNeillie & Zammit (1982) and were used 7–8 days afterwards. For the first 2 days they were given a 1% glucose solution to drink.

Chemicals and enzymes

The sources of these were as described by Easom & Zammit (1984a). In addition, streptozotocin was generously given by the Upjohn Co. (Kalamazoo, MI, U.S.A.). Crystalline insulin was from Sigma (Poole, Dorset, U.K.).

Sampling of liver, preparation of liver homogenates and separation of microsomal fractions

These were performed essentially as described

previously (Easom & Zammit, 1984b). In brief, liver samples (approx. 1 g) were obtained by cold-clamping of the left lateral lobe of the liver in rats anaesthetized 25 min earlier by an intraperitoneal injection of pentobarbitone (60 mg/kg body wt.). The liver samples were homogenized in a medium containing 50 mM-sucrose, 10 mM-Tris/HCl (pH 7.4), 100 mM-KF, 10 mM-EDTA, 1 mM-dithiothreitol and 0.5 mM-phenylmethanesulphonyl fluoride. Microsomal fractions were prepared and washed either in fluoride-containing medium or in medium in which fluoride was replaced by an equivalent concentration of KCl (see Easom & Zammit, 1984b).

Activation and assay of HMG-CoA reductase

HMG-CoA reductase activity was measured either directly, in fluoride-washed microsomal fractions or after dephosphorylation of the enzyme by incubation with exogenous phosphoprotein phosphatase (Easom & Zammit, 1984b). The enzyme assay used was that of Shapiro *et al.*, (1974). Microsomal protein concentration was measured as described by Lowry *et al.* (1951).

Determination of blood glucose

After sampling of the liver, a sample of blood was withdrawn with a hypodermic syringe from the aorta and mixed with an equal volume of ice-cold 3% (v/v) HClO₄. The concentration of glucose in the neutralized supernatant obtained after sedimentation of the protein was measured as described by Bergmeyer *et al.* (1974).

Results

Effect of diabetes on expressed and total HMG-CoA reductase activities in rat liver

The induction of diabetes by the dose of streptozotocin used (150 mg/kg body wt., injected intraperitoneally) was effective to variable extents in individual animals, judging by the wide range of hyperglycaemia resulting 7 days after the treatment. Thus the blood glucose concentrations in streptozotocin-treated animals ranged from 10 to 38 mM. Using the degree of hyperglycaemia as an indirect index of the degree of insulin deficiency induced by streptozotocin treatment, we were able to assess the effects of increasing severity of insulin deficiency on the total and the expressed/total HMG-CoA reductase activity ratio *in vivo*. The results are presented in Fig. 1, showing that, for both parameters, moderate insulin deficiency, resulting in a hyperglycaemia equivalent to 20 mM-glucose or less, did not have any significant effect. However, for animals that developed a glycaemia in excess of 20 mM-glucose there was a distinct inverse relationship between blood glucose con-

Fig. 1. Effects of different degrees of insulin deficiency on total HMG-CoA reductase activity (a) and on the expressed/total activity ratio of the enzyme (b) in hepatic microsomal fractions from diabetic rats

The values given by the solid bars ($\pm$ S.E.M. $n = 4$) relate to the parameters in normal animals at 2 h into the dark period (D2). The straight lines were obtained by performing linear-regression analyses of the data obtained for rats which had blood glucose ≥ 20 mm. The correlation coefficients were -0.38 in (a) and -0.82 in (b).

Easom & Zammit, 1984b), the correlation between blood glucose concentration and total activity appeared to be less strong (at least on an empirical, linear, basis) than that obtained for the expressed/total activity ratio (which is independent of the absolute specific activity of the enzyme and its variability between animals). However, the sensitivity of the two parameters for HMG-CoA reductase to insulin deficiency was similar. In this respect the total activity and the expressed/total activity attained at the degree of hypoinsulinaemia that resulted in blood glucose concentrations above 35 mm were similar to the lowest values observed physiologically in normal animals at the respective nadirs of diurnal rhythms of the two parameters (Easom & Zammit, 1984b).

Effect of insulin administration

For these experiments only diabetic rats that had blood glucose concentrations in excess of 30 mm were used. Two series of experiments were performed: (1) insulin was administered acutely by intravenous infusion (femoral vein) into anaesthetized rats 4 h into the dark period (D4), and the liver was sampled 15–20 min later; and (2) medium-term insulin supplementation was effected by intraperitoneal injection into conscious rats every 60 min for 4 h, commencing 2 h into the dark period (D2). The rats were anaesthetized 20 min before sampling of the liver. This latter procedure was adopted to produce a more gradual increase in insulin, such as would be expected to occur *in vivo*.

In the acute (intravenous infusion) experiments (6 units of insulin infused), the blood glucose concentration was decreased from 31.2 ± 2.7 to 21.5 ± 1.7 mm (means $\pm$ S.E.M., $n = 4$) after 15–20 min. This treatment did not affect total activities of HMG-CoA reductase in liver microsomal fractions, but it increased the expressed/total activity ratio from $48.1 \pm 2.5\%$ ($n = 11$) to $99.6 \pm 5.6\%$ ($n = 4$), i.e. it resulted in the total dephosphorylation and activation of HMG-CoA reductase in the liver of the intact animals. In control animals infused with 0.9% NaCl, there was no change in the fraction of HMG-CoA reductase in the active form.

In the medium-term experiments (intraperitoneal injection of insulin) a very gradual decrease in blood glucose concentration was achieved, resulting in near-normoglycaemia after 3–4 h (Fig. 2) of insulin treatment. Both the expressed/total activity ratio and total HMG-CoA reductase activity were increased by this treatment, such that for both parameters the values increased from those characteristic of diabetic animals to those observed at 2–6 h into the dark period in normal animals (Easom & Zammit, 1984b). In these experiments the

centration and the values of the two parameters of HMG-CoA reductase activity. Because of the variability routinely encountered in the absolute values of HMG-CoA reductase activity (see also

Fig. 2. Decrease in blood glucose concentration in diabetic rats injected hourly intraperitoneally with insulin. Values are means ($\pm$ S.E.M.) for three to five determinations.

increase in expressed/total ratio was not as rapid or as extensive as that observed with intravenous infusion of insulin, but it was evident within the first 20 min after intraperitoneal injection of insulin and was maximal by 60 min, at which time 80% of the enzyme was in the active state (Fig. 3a). Control diabetic animals injected with 0.9% NaCl showed a continuous small increase in expressed/total activity ratio over this 4 h period. This is presumed to have been due to residual insulin secretion in these animals, and suggests that even in relatively severe diabetes (30–40 mM glucose in whole blood) diurnal changes in the fraction of HMG-CoA reductase in the active form may occur; however, the peak of this rhythm may be considerably later in diabetic animals than in normal rats. The effect was not studied further.

After intraperitoneal injection of insulin, the stimulatory effect of insulin on total HMG-CoA reductase activity occurred 2–3 h later than that observed for the fraction of the enzyme in the active form (Fig. 3b). Thus, even after 2 h of the commencement of insulin treatment there was no detectable increase in total HMG-CoA reductase activity, whereas at 3 h the maximal effect had occurred (Fig. 3b). At its height, this effect resulted in total reductase activities similar to those observed in normal animals 6 h into the dark period (Easom & Zammit, 1984b).

Fig. 3. Effect of intraperitoneal infusion of insulin on (a) the expressed/total HMG-CoA reductase activity ratio and (b) the total activity of the enzyme in liver microsomal fractions of diabetic rats *in vivo*.

Rats with blood glucose concentrations > 30 mM were treated with insulin (▲). Control animals (○) were infused with 0.9% NaCl. Values are means ($\pm$ S.E.M.) for three to five determinations.

Discussion

The role of insulin in promoting the synthesis of HMG-CoA reductase in the liver has long been appreciated (see the introduction), although other work (Nakayama & Nakagawa, 1977; Young *et al.*, 1982) has indicated that increases in enzyme synthesis in diabetic rats do not have an obligatory requirement for insulin and can be induced by cholestyramine feeding or by the correction of diabetic hyperphagia. The involvement of insulin in modulating the fraction of HMG-CoA reductase in the active form *in vivo* has also been equivocal. Thus Goodman *et al.* (1982) reported that this fraction was the same in normal and diabetic rats, and the results of Young *et al.* (1982) suggested that it is increased in diabetes.

In the present study we have used the cold-clamping technique described previously (Easom & Zammit, 1984a) to obtain values for the expressed/total HMG-CoA reductase activity ratio in rat liver *in vivo* under conditions of insulin deficiency and supplementation. The results show that in diabetes the fraction of the enzyme in the active form is markedly depressed at the time in the dark phase that coincides with the peak of the diurnal rhythm of this ratio in normal animals. However, there was evidence that a diurnal cycle in this ratio was not entirely eliminated by the severity of diabetes induced in these studies, although it appears that the peak in this attenuated cycle may occur later than in normal animals. This may result from diurnal variations in circulating insulin concentrations at much lower absolute concentrations of the hormone. Intravenous infusion of insulin into diabetic animals resulted in very rapid and full activation (dephosphorylation) of the existing enzyme protein in the liver of diabetic animals. When insulin was delivered less acutely, by intraperitoneal injection, the expressed/total HMG-CoA reductase activity ratio was restored to values observed for normal animals at the peak of its diurnal cycle (i.e. approx. 80% active; Easom & Zammit, 1984b). These observations suggest that acute hyperinsulinaemia (intravenous infusion) overcomes any counter-regulatory effects resulting from the ensuing decrease in blood glucose. The mode of action of insulin in promoting HMG-CoA reductase dephosphorylation is not known. Insulin may act primarily by activating protein phosphatase-1 through its putative effects on glycogen synthase kinase-3 and inhibitor-1 (see Cohen, 1980) if the effects of the hormone in the liver are similar to those described for adipose tissue (Nemenoff *et al.*, 1983). The particular phosphoprotein phosphatases involved in the bicyclic phosphorylation-dephosphorylation mechanism through which the activity of HMG-CoA reductase is regulated may be phosphatase-2C

(which has the highest specific activity against reductase; Ingebritsen *et al.*, 1983a) and phosphatase-1, for which reductase is also a substrate and which is the only phosphatase with a microsomal localization (Ingebritsen *et al.*, 1983b). If (indirect) activation of phosphatase-1 by insulin is involved in the acute increase in the expressed/total HMG-CoA activity ratio *in vivo* in diabetic rats, it would have to occur against the background of a depressed (50%) activity of phosphatase-1 in the liver of diabetic animals (Foulkes & Jefferson, 1984).

The experiment in which insulin treatment was performed over a 4 h period also documented the temporal relationship that exists between the insulin-mediated dephosphorylation of HMG-CoA reductase *in vivo* and the onset of the increase in enzyme synthesis and total enzyme activity. The lag period between the two effects was 2–3 h. It is noteworthy that (i) the magnitude of this lag period is similar to the phase separation observed between the respective diurnal rhythms of the expressed/total HMG-CoA reductase activity ratio and of the total activity *in vivo* (Easom & Zammit, 1984b), and (ii) that the peak in the diurnal cycle of insulin concentrations *in vivo* also occurs at about 2 h into the dark period (Peret *et al.*, 1973; Knox *et al.*, 1979), i.e. when the fraction of the enzyme in the active form is almost at its peak in normal animals *in vivo* (Easom & Zammit, 1984b). Therefore it is possible that both the diurnal rhythms involved in the modulation of HMG-CoA reductase activity are generated by the diurnal changes in circulating insulin concentrations, through the different effects of the hormone on enzyme synthesis and dephosphorylation.

The data presented in Fig. 1 suggest that both parameters are sensitive to the same range of insulin concentrations *in vivo*. In both instances, moderate hypoinsulinaemia, which results in an increase in blood glucose to approx. 20 mM, had no discernable effect. In the more severely diabetic animals (blood glucose 20–38 mM) there was a negative correlation between the severity of hyperglycaemia and both total activity of HMG-CoA reductase and the fraction of the enzyme in the active form. These observations suggest one reason for the rather variable effects of streptozotocin-induced diabetes on the rate of hepatic cholesterologenesis reported in the literature (see above): only animals in which the extent of hypoinsulinaemia induced experimentally is sufficiently severe to increase blood glucose above 20 mM would exhibit any significant depression of expressed HMG-CoA reductase activity and cholesterologenesis *in vivo*.

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