Acetazolamide-insensitive carbonic anhydrase activities in liver and tonic skeletal muscle of adult male rats with streptozotocin-induced diabetes mellitus

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One of the four discrete isoenzymes of carbonic anhydrase hitherto characterized, CA III, has the lowest turnover rate and the greatest resistance to inhibition by sulphonamides. Streptozotocin-induced diabetes mellitus resulted in a reduction in acetazolamide-resistant activity of carbonic anhydrase in the liver, but not in tonic skeletal muscle, of adult male rats. The hepatic activity declined with apparent first-order kinetics [calculated rate constant (k) 0.089 day^{-1}] to a minimum of approx. 6% of control values; the reduction in activity was moderated by administration of insulin.

INTRODUCTION

At least four genetically discrete mammalian isoenzymes of carbonic anhydrase (CA; carbonate dehydratase, EC 4.2.1.1) have now been fully or partially characterized (Tashian, 1989). One, known as CA III, is exceptionally resistant to inhibition by micromolar concentrations of acetazolamide and has a lower turnover rate than the other principal isoenzymes (Tashian *et al.*, 1980). Immunocytochemical techniques have been used to demonstrate the presence of CA III in epithelia in salivary-gland ducts, bronchioles, caecum, ascending colon and vas deferens, as well as in adipocytes in mice (Spicer *et al.*, 1990), but the greatest activities in the rat are found in the cytosol of skeletal muscle and hepatocytes (Shiels *et al.*, 1984).

The quantity of CA III expressed in rat skeletal muscles varies with fibre type, being maximal in the type I (slow-twitch oxidative) fibres predominating in muscles of tonic function (Riley *et al.*, 1982), wherein it represents up to 54% of the soluble sarcoplasmic protein and approx. 4% of total protein (Shiels *et al.*, 1984). Expression of the CA III gene in skeletal muscle may also be influenced by neuronal activity, for increased quantities of the isoenzyme are found in fast-twitch rat muscles after denervation, although the same procedure has little effect on CA III in the slow-twitch soleus muscles (Carter *et al.*, 1988), and by thyroid hormone, as thyroidectomy has been shown to increase the concentration of CA III in soleus, with no accompanying change in fibre type or distribution (Jeffery *et al.*, 1987). Some variations also occur with age; CA III concentrations in soleus rise to a maximum at about 100 days, with no subsequent decline occurring until after 600 days, no difference in activity (or in variation of activity with age) being evident between the sexes (Jeffery *et al.*, 1988).

In the liver of the male rat the concentration of CA III may be up to 20-fold greater than in the age-matched female (Jeffery *et al.*, 1986), reaching a maximum of more than 9% of the total soluble cytosolic protein at 100 days of age (Carter *et al.*, 1984) and then declining after 400 days (Jeffery *et al.*, 1988). An immunofluorescence study has demonstrated that much of the CA III in the male rat liver lobule is located in cells close to the central vein (Carter *et al.*, 1987). CA III concentrations in rat liver have been shown to be increased by administration of testosterone (Shiels *et al.*, 1983), but it now appears that this effect is indirect, being mediated by alteration of the pattern of

release of pituitary growth hormone (GH) into the circulation (Jeffery *et al.*, 1990). Further potential evidence of endocrine control of the hepatic expression of CA III was provided in a recent paper by Dodgson & Watford (1990), in which they reported that the activity of the isoenzyme in hepatocytes of male rats is reduced in acute diabetes mellitus induced by injection of streptozotocin. We now report an investigation of the effects of chronic, rather than acute, streptozotocin-induced diabetes mellitus and also concurrent administration of insulin upon the activities of CA III found in skeletal muscle as well as in the liver of adult male rats.

MATERIALS AND METHODS

In the first of two experiments, diabetes mellitus was induced in 24 adult male Sprague–Dawley rats (initial body mass 180–280 g, fed a stock diet *ad libitum*) by intraperitoneal injection of streptozotocin (45 mg/kg body mass, in 0.3 ml of 0.02 M-sodium citrate buffer, pH 4.5) on 2 successive days. In addition, 12 of those rats subsequently received subcutaneous injections of a zinc-insulin suspension (Human Monotard; Novo Industri, Bagsvaerd, Denmark; 25 i.u./kg body mass) on alternate days for the duration of the experiment, whereas the others remained untreated. Both groups were killed by decapitation in the period 83–146 days after the first injection of streptozotocin. This experiment also involved two groups of citrate-buffer-injected control animals: 12 were killed at the same age as the two groups of diabetic rats, and a further 12 as they attained body-mass values similar to the range found in the untreated diabetic group at the time of their death.

In the second experiment, diabetes mellitus was similarly induced in 62 adult male Sprague–Dawley rats (initial body mass 180–275 g) that were subsequently killed by decapitation at intervals ranging from 2 to 19 days after the first injection of streptozotocin.

Blood plasma samples were analysed for glucose by a glucose oxidase–peroxidase test combination (Glucose Liquicolor; Human G.m.b.H., Taunusstein-Neuhof, Germany), and samples of whole blood were haemolysed by addition to 15 vol. of 0.14 M- NH_4Cl in 0.02 M-Tris/HCl buffer, pH 7.2. Peripheral segments of three separate lobes of each liver were pooled and disrupted with a Potter–Elvehjem homogenizer in 5 vol. of 0.002 M-sodium Hepes buffer, pH 7.4, containing 0.22 M-D-mannitol, 0.07 M-

Abbreviations used: CA III, carbonic anhydrase isoenzyme III; MU, operational unit of carbonic anhydrase activity (Maren, 1967); G6PDH, glucose-6-phosphate dehydrogenase; GH, growth hormone.

sucrose and BSA (0.5 g/l) before centrifugation for 60 min at 4 °C and 100000 *g* (r_{av} 5.5 cm). After excision, weighing and coarse dissection, the soleus muscles of each rat were combined and incubated for 2 h at 37 °C in 10 vol. of 0.01 M-sodium 5,5'-diethylbarbiturate buffer, pH 7.4, containing 1 mM-dithiothreitol, 1 mM-CaCl₂, Triton X-100 (10 ml/l) and collagenase (1 mg/ml; type IV, Sigma). EDTA was then added to obtain a concentration of 5 mM, and the muscles were homogenized, stored at -20 °C for 16–24 h and thawed before centrifugation for 30 min at 4 °C and 10000 *g* (r_{av} 5.5 cm). The pellet was resuspended in 10 vol. of the muscle incubation buffer (but with 5 mM-EDTA substituted for CaCl₂), re-centrifuged as described above, and the muscle supernatant fluids combined.

Carbonic anhydrase activity was determined in the diluted haemolysates and supernatant fluids at 25 °C by the method of Livesey (1977) in a stopped-flow rapid-kinetics cell (Hi-Tech Scientific, Salisbury, Wilts., U.K.), the operational unit of enzyme activity (MU) being as defined by Maren (1967). CA III activity in the supernatants of the liver and muscle homogenates was differentiated from that of other isoenzymes of carbonic anhydrase that were also present by the inclusion in the assay medium of 3 μ M-acetazolamide (Carter *et al.*, 1981; Frémont *et al.*, 1989). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (G6PDH) was assayed in the liver supernatants and haemolysates by the method of Kornberg & Horecker (1955), one unit of activity being defined as the quantity of enzyme that catalysed the transformation of 1 μ mol of substrate/min at 25 °C. The liver activities were corrected for the contribution by blood trapped within the tissue at the time of excision as determined by the principle described by Danielli (1941), using the techniques of Gay *et al.* (1981). Soluble protein was determined by the method of Markwell *et al.* (1978), with lysozyme (BDH) as the standard.

In the first experiment the results from the four different treatment groups were tested by Fisher's Analysis of Variance. If the variance ratio, *F*, for a variable was statistically significant ($P < 0.05$), multiple comparisons among the means were made by the non-parametric Kruskal and Wallis test.

RESULTS

The data obtained in the first experiment (Table 1) show that administration of streptozotocin resulted in statistically sig-

nificant sustained hyperglycaemia and retardation of growth, both effects being moderated by the administration of insulin on alternate days. In contrast with an absence of significant variation between treatment groups with respect to the total activity of

Fig. 1. Activities at 25 °C of CA III and G6PDH in male rat liver cytosol

The activities are shown as functions of time elapsed since the administration of streptozotocin.

Table 1. Effects of streptozotocin-induced chronic diabetes mellitus on activities at 25 °C of carbonic anhydrase in rat tissues

The values represent the means $\pm$ S.E.M. for 12 rats in each treatment group. Statistically significant ($P < 0.05$) differences exist between means on the same line that do not bear a common superscript.

Parameter	Treatment ...	Age-matched controls	Body-mass-matched controls	Chronic diabetes	Chronic diabetes with exogenous insulin
Body mass (g) at end of experiment		518 ^a $\pm$ 10	283 ^b $\pm$ 24	255 ^b $\pm$ 20	408 ^c $\pm$ 13
Concentration of glucose in plasma (mg/100 ml)		134 ^a $\pm$ 7	137 ^a $\pm$ 5	517 ^b $\pm$ 9	282 ^a $\pm$ 44
Total carbonic anhydrase activity in blood (MU/ μ l)		62.7 $\pm$ 2.2	56.5 $\pm$ 2.6	62.9 $\pm$ 1.6	55.1 $\pm$ 3.9
CA III activity in:					
Liver					
MU/g of tissue		1040 ^a $\pm$ 48	1000 ^a $\pm$ 52	64 ^b $\pm$ 12	555 ^c $\pm$ 44
MU/mg of protein		21.7 ^a $\pm$ 1.6	20.4 ^a $\pm$ 1.2	1.3 ^b $\pm$ 0.2	11.4 ^c $\pm$ 0.8
Soleus					
MU/mg of tissue		3.51 $\pm$ 0.17	3.51 $\pm$ 0.11	4.32 $\pm$ 0.51	3.05 $\pm$ 0.32
MU/mg of protein		89.4 $\pm$ 5.6	81.8 $\pm$ 3.1	95.2 $\pm$ 9.9	71.6 $\pm$ 7.4

Fig. 2. Correlation of activities at 25 °C of CA III and G6PDH in male rat liver cytosol

carbonic anhydrase in whole blood, chronic diabetes mellitus resulted in a profound reduction in the activity of CA III in the liver, whether that activity was expressed on the basis of fresh tissue mass or of the mass of extracted soluble protein. The group with chronic diabetes treated with insulin had hepatic CA III activities intermediate between the control and the untreated diabetic values. Comparatively little effect of the various treatments on CA III was observed in the soleus muscles, with small (but statistically non-significant) elevations in activity in chronic diabetes mellitus altered by exogenous insulin to reductions below the control values. No statistically significant differences in CA III activities in either liver or soleus were found between the age-matched and body-mass-matched control groups, indicating that the ages of the animals lay in the range within which hepatic and muscle CA III in the rat exhibit little variation with growth (Jeffery *et al.*, 1988).

Fig. 1 shows the rate of change of the activities of both CA III and G6PDH in the liver during their initial decline after the administration of streptozotocin in the second experiment. Least-squares estimates of the regressions of the data on semi-logarithmic plots against time indicated that decay of the activities exhibited apparent first-order kinetic characteristics, the specific rate constant estimated from the regression of hepatic CA III on time being 0.0885 ± 0.0036 (estimate $\pm$ S.E.M.) day^{-1} , and hence the half-time for the decay in activity was 7.83 ± 0.32 days. A remarkably similar result was obtained for the decline in hepatic activity of G6PDH, with a specific rate constant of 0.0850 ± 0.0145 (estimate $\pm$ S.E.M.) day^{-1} and a half-time of 8.16 ± 1.39 days. Fig. 2 reveals that there was also a strong correlation between the activities of these two enzymes measured in individual rats during the same period (Pearson product-moment correlation coefficient, $r_s = 0.59$; $P < 0.0001$), although the parallel slopes of their declines in acute diabetes mellitus may not necessarily be sustained over a longer period of observation.

DISCUSSION

The primary aim of the first experiment in this investigation was to compare the effect of chronic streptozotocin-induced diabetes mellitus on the activity of CA III in adult male rat liver with the effect of acute diabetes. A 60% reduction in activity relative to control values only 6 days after the administration of

streptozotocin has been reported by Dodgson & Watford (1990). However, we observed a greater decline, to a mean of 6% of control values, in a group studied over the range of 83–146 days after receiving the first injection of streptozotocin. This probably approaches the minimum activity of CA III expressed in the male rat liver in chronic diabetes, as we observed a reduction to $11 \pm 2\%$ of control values (mean $\pm$ S.E.M.; $n = 8$) in a separate experiment (J. B. Moynihan & S. Ennis, unpublished work) in which the duration of diabetes was only 36–44 days. With the finding that the activities in the body-mass-matched control rats were similar to those found in the age-matched control animals, there is no apparent association between the reduced activity of CA III in the liver and the simultaneous inhibition of growth that occurred in the diabetic group.

The fall in hepatic CA III activity after induction of diabetes was attenuated by the administration of insulin at a rate that had been selected to produce hypoglycaemia and growth promotion insufficient to restore fully the concentration of glucose in the plasma and the rate of increase of body mass to the values observed in the age-matched control group. The effect of administration of streptozotocin on hepatic CA III activity therefore appears to be a consequence of the destruction of the insulin-secreting β -cells of the islets of Langerhans rather than a direct effect of the drug upon hepatocytes. Further studies will be necessary to determine whether higher rates of administration of insulin may restore normal, or even achieve supranormal, activity of CA III in the liver of the diabetic male rat. At present it is not certain whether the reduction in CA III activity in the diabetic male rat liver reported here and by Dodgson & Watford (1990) represents a hitherto-unknown endocrine or metabolic control mechanism for this isoenzyme, or whether it is the result of an altered pattern of release of GH from the pituitary. The latter possibility is consistent with the more frequent release and higher serum concentrations of GH in young diabetic patients (juvenile-onset, insulin-dependent diabetes) than in controls (Johansen & Hansen, 1969). This resembles the greater frequency of release of GH in female rats in comparison with males, resulting in the expression of CA III in the female rat liver at mean concentrations of only 7.2% (Jeffery *et al.*, 1986) or 5.9% (Jeffery *et al.*, 1990) of those found in the male. Thus the magnitude of the maximum decline in activity of CA III in the liver of the male rat with chronic diabetes observed in the present study corresponds to the effect expected with a transition from the male pattern of endogenous GH secretion to that of the female (Jeffery *et al.*, 1990).

The data presented in Fig. 2 support involvement of the cytosolic isoenzyme CA III in hepatic lipogenesis in the male rat, showing a statistically significant correlation of the activities of CA III with those of G6PDH in the liver. Inhibitors of carbonic anhydrase also inhibit fatty acid synthesis in mouse liver (Rous & Favarger, 1963) and in reptilian liver (Herbert & Coulson, 1984). Furthermore, Cao & Rous (1978) demonstrated that acetazolamide inhibited acetyl-CoA carboxylase (EC 6.4.1.2) (the regulatory step in fatty acid synthesis) in a cytosolic fraction from mouse liver, but only at concentrations of acetazolamide sufficient to inhibit the CA III isoenzyme of carbonic anhydrase. Such concentrations had no inhibitory effect on fatty acid synthetase activity. Hepatic CA III may therefore be involved in fatty acid synthesis by providing bicarbonate ions (HCO_3^-) to acetyl-CoA carboxylase, which has a comparatively high $K_m(\text{HCO}_3^-)$ of 3 mM (Numa *et al.*, 1965), just as mitochondrial carbonic anhydrase (CA V) has been shown (Dodgson & Forster, 1986) to function in the synthesis of urea by the provision of HCO_3^- to carbamoyl phosphate synthetase (ammonia) (EC 6.3.4.16), with a $K_m(\text{HCO}_3^-)$ of 5.3 mM (Guthöhrlein & Knappe, 1969), and in gluconeogenesis by supplying HCO_3^- to

pyruvate carboxylase (EC 6.4.1.1), which has a $K_m(\text{HCO}_3^-)$ of 2 mM (Utter *et al.*, 1975). Other putative roles for the cytosolic CA III in hepatic lipogenesis include facilitation of diffusion of CO_2 away from the active sites of malate dehydrogenase (oxaloacetate-decarboxylating) (NADP^+) (EC 1.1.1.40) and phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44) in order to reduce product inhibition of those enzymes.

As substantial quantities of CA III are also found in tonic skeletal muscle, a further aim of our study was to determine whether the effect of diabetes mellitus upon the activity of this isoenzyme in the liver may be reproduced in the soleus, selected as an example of a skeletal muscle with an exceptionally high content of type I fibres (Sréter & Woo, 1963) and hence containing a relatively high concentration of CA III in control animals (Shiels *et al.*, 1984). The results show a marked difference between liver and muscle in the effects of chronic diabetes on CA III, perhaps reflecting differences in physiological roles of the isoenzyme in those two tissues. It has been suggested that the function of CA III in sarcoplasm is to facilitate diffusion of CO_2 within the muscle fibre (Gros *et al.*, 1984), but Moynihan (1990) found that chronic expansion of the vascular bed and hence shortening of the overall diffusion path of CO_2 in soleus did not influence the activity of CA III in the tissue, and there is a poor correlation between CA III concentration and the capacity of the muscle fibres for aerobic energy metabolism (Frémont *et al.*, 1988). Facilitation of CO_2 movement in skeletal muscle by CA III is perhaps more important during sustained contractions, for Barclay (1987) reported that inhibition of the isoenzyme in mouse soleus would only impair the development of tetanic tension in the muscle in hypercapnia, with little effect at normal systemic concentrations of CO_2 . An alternative proposal for the function of CA III in muscle is that it is involved in control of utilization of glycogen in type I fibres (Côté *et al.*, 1989), but this is not supported by our finding that the activity of the isoenzyme in soleus shows relatively little change with the profound metabolic disturbances, including impaired glycogen synthesis, of chronic diabetes. As thyroidectomy results in increased concentrations of CA III without apparent alteration in fibre type or distribution in rat soleus (Jeffery *et al.*, 1987) the statistically non-significant increase in activity of the isoenzyme in that muscle with chronic diabetes may be a consequence of the reduction in circulating concentrations of thyroid hormones that occurs in streptozotocin-diabetic rats (Xiang *et al.*, 1988) rather than a direct effect upon the tissue of hypoinsulinaemia or hyperglycaemia. Histochemical investigations should reveal whether changes in whole-muscle CA III activity are associated with alterations in the proportion of type I fibres in the soleus in diabetes mellitus.

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