The effect of streptozotocin-induced diabetes on phenylalanine hydroxylase expression in rat liver

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The impact of experimentally induced diabetes on the expression of rat liver phenylalanine hydroxylase has been investigated. A significant elevation in maximal enzymic activity was observed in diabetes. This was associated with significant increases in the amount of enzyme, the phenylalanine hydroxylase-specific translational activity of hepatic RNA and the abundance of phenylalanine hydroxylase-specific mRNA. These changes in phenylalanine hydroxylase expression were not observed when diabetes was controlled by daily injections of insulin. These results are discussed in relation to the hormonal control of phenylalanine hydroxylase gene expression.

INTRODUCTION

Phenylalanine hydroxylase [L-phenylalanine, tetrahydrobiopterin: oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1] catalyses the initial step of phenylalanine degradation in the liver:

The activity of the enzyme is subject to precise, yet flexible, regulation, so that phenylalanine and tyrosine homeostasis can be achieved despite fluctuations in either dietary protein intake or protein turnover. The enzyme is controlled, in the short term, by reversible phosphorylation (Donlon & Kaufman, 1978; Fisher & Pogson, 1984) and in the longer term by alterations in enzyme concentration.

The regulation of phenylalanine hydroxylase protein turnover has been subject to extensive investigation (Haggerty *et al.*, 1973; Baker & Shiman, 1979; Sorimachi *et al.*, 1981). These studies have established a role for corticosteroids in the maintenance of enzyme concentration in cultured hepatoma cells. Corticosteroids appear to exert their effect on phenylalanine hydroxylase expression at a transcriptional level (Chiappelli *et al.*, 1981; Dahl & Mercer, 1986). These hormones may also influence phenylalanine hydroxylase expression *in vivo* (Haggerty *et al.*, 1983) and in isolated liver cells (Stanley *et al.*, 1985).

Experimentally induced diabetes is associated with a significant increase in phenylalanine hydroxylation in isolated liver cells (Carr & Pogson, 1981), which reflects an increase in maximal enzymic activity (Donlon & Beirne, 1982). Changes in the phosphorylation status of the enzyme undoubtedly occur after the onset of diabetes (Donlon & Beirne, 1982). More recent work, however, has indicated that diabetes is associated with a significant elevation in the amount of total phenylalanine hydroxylase protein present in liver cell extracts (Howard & Donlon, 1985; Santana *et al.*, 1985). This may reflect

an alteration in the rate of phenylalanine hydroxylase synthesis relative to the rate of total protein synthesis (Santana *et al.*, 1985).

The present paper seeks to clarify the mechanisms underlying the changes in phenylalanine hydroxylase expression which occur after the onset of streptozotocin-induced diabetes in rats. Portions of this work have appeared in preliminary form (Taylor *et al.*, 1988).

MATERIALS AND METHODS

Chemicals

Guanidine hydrochloride, 6-methyltetrahydropteridine, oligo(dT)-cellulose, L-methionine, L-phenylalanine, phenylmethanesulphonyl fluoride, rabbit anti-sheep serum and streptozotocin were from Sigma. Peroxidase-labelled rabbit anti-sheep IgG was from Nordic Immunological Laboratories, Maidenhead, Berks., U.K. Chymostatin, leupeptin and pepstatin were from the Peptide Institute Inc., Osaka, Japan. Guanidinium thiocyanate (purum grade) was from Fluka. Tetra-aminobiphenyl hydrochloride was from BDH. L-[4,5-³H]Leucine (120 Ci/mmol), L-[³⁵S]methionine (800 Ci/mmol), [α -³²P]dCTP (3000 Ci/mmol), PCS scintillator cocktail, NCS solubilizer and rabbit reticulocyte lysates (N.90) were from Amersham International, Amersham, Bucks., U.K. Plasmid pPCK10 was a gift from Professor R. W. Hanson, Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, OH, U.S.A.

All other chemicals were of the purest grade available from standard suppliers.

Animals

Male Wistar rats (University of Liverpool breeding colony) weighing 180–220 g were used throughout. Animals were fed *ad libitum* [Labsure Animal Diet (CRM); C. Hill Group, Poole, Dorset, U.K.].

Diabetes was induced by intravenous injection of

Abbreviations used: poly(A)⁺ RNA, polyadenylated RNA; PEPCK, phosphoenolpyruvate carboxykinase.

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streptozotocin (50 mg/kg body wt.) in 0.9% (w/v) NaCl. Glucosuria was checked with Clinistix (Ames Co., Stoke Poges, Slough, Bucks., U.K.). Animals were used 10 days after injection. Where indicated, diabetes was controlled by subcutaneous injection of 10–20 units of insulin–zinc suspension (Novo Ultratard: Farillon Ltd., Romford, Essex, U.K.)/day. Diabetes, and its control, was confirmed by serum glucose analysis [normal, 6.0 ± 0.9 (6); uncontrolled diabetes, 26.8 ± 2.3 (10); insulin-controlled diabetes, 3.5 ± 0.7 (10) mM-glucose: means $\pm$ S.E.M. for the numbers of different animals shown in parentheses].

Extraction and assay of phenylalanine hydroxylase

Immediately after rats were killed by cervical dislocation, pieces of liver were minced and then homogenized (4 ml of extraction buffer/g fresh wt. of liver) at 4 °C by using a Ystral disperser. Extraction buffer containing 250 mM-sucrose, 20 mM-Tris/HCl, 2 mM-sodium phosphate, 5 mM-EDTA, 100 mM-NaF, 0.5 mM-phenylmethanesulphonyl fluoride, leupeptin (20 μ g/ml), pepstatin (2 μ g/ml) and chymostatin (2 μ g/ml), pH 7.4, was used. After centrifugation at 40 000 *g* for 30 min at 4 °C, the supernatant was assayed for phenylalanine hydroxylase activity at 30 °C as described by Shiman *et al.* (1979). The protein concentration of each extract, as determined by the method of Lowry *et al.* (1951), was not affected by the induction of diabetes (normal, 33.5 ± 3.7 ; diabetes, 35.0 ± 2.8 ; insulin-controlled diabetes, 28.9 ± 1.1 mg of protein/ml of extract: means $\pm$ S.E.M. for at least three different animals in each group). Hence, although diabetes was associated with a slight decrease in soluble protein when expressed on the basis of DNA (results not shown), which is a reflection of protein content per cell, soluble protein content was unchanged on the basis of tissue weight.

Immunoblotting of phenylalanine hydroxylase

Liver extracts, prepared as described above, were subjected to SDS/polyacrylamide gel electrophoresis essentially as described by Laemmli (1970) and then transferred to nitrocellulose filters (Towbin *et al.*, 1979). Immunoblotting was performed with a sheep antiserum to rat liver phenylalanine hydroxylase (Fisher & Pogson, 1984). Antibody binding was revealed by incubation with peroxidase-labelled rabbit anti-sheep IgG with subsequent addition of tetra-aminobiphenyl hydrochloride and H₂O₂. Immunoblots were quantified by densitometric scanning at 500 nm with a Zeiss chromatographic scanner. A linear relationship between protein load and absorption was verified for each blot.

Isolation of RNA from liver

RNA was isolated from liver essentially as described by Chirgwin *et al.* (1979). Briefly, freshly excised liver was homogenized in a guanidinium thiocyanate-based solution (20 ml/g fresh wt. of liver) by using a Ystral tissue disperser. After centrifugation at 10 000 *g* for 10 min at 10 °C, RNA was precipitated with ethanol from the resulting supernatant. RNA was subsequently purified by suspension in a guanidine hydrochloride solution and re-precipitated with ethanol twice. Purified RNA precipitates were dissolved in water and stored at –70 °C. The amount of RNA obtained in this way was similar for each group of animals (normal, 5.7 ± 0.2 ; diabetes, 6.5 ± 0.2 ; insulin-controlled diabetes, $6.8 \pm$

0.5 mg of RNA/g fresh wt. of liver: means $\pm$ S.E.M. for at least three different animals in each group). Although RNA was, like soluble protein, lost on the basis of cellular DNA content after the induction of diabetes, it was essentially unchanged when expressed on the basis of fresh tissue weight.

Poly(A)⁺ RNA was purified from total RNA by affinity chromatography on oligo(dT)–cellulose as described by Clemens (1984).

Translational capacity of poly(A)⁺ RNA

Purified poly(A)⁺ RNA was used for translation experiments *in vitro*. Total protein synthesis was estimated from the incorporation of [4,5-³H]leucine into trichloroacetic acid-insoluble material after deacylation. Briefly, poly(A)⁺ RNA (0.25 μ g) was incubated at 30 °C with 0.02 ml of a rabbit reticulocyte lysate and 3.5 μ Ci of [4,5-³H]leucine. Translation was terminated by treating 0.002 ml portions of the mixture with 0.50 ml of 1 M-NaOH containing 1.5% (w/v) H₂O₂. After incubation at 37 °C for 10 min, 1.0 ml of ice-cold 25% (w/v) trichloroacetic acid containing 2% (w/v) casein hydrolysate was added, and the resulting mixture was incubated at 4 °C for 1 h. After centrifugation at 12 000 *g* at 4 °C for 5 min, the trichloroacetic acid-insoluble material was washed twice with 10% trichloroacetic acid and then solubilized by addition of 0.1 ml of NCS tissue solubilizer. The resulting solutions were counted for radioactivity in the presence of 1.0 ml of PCS scintillation fluid. Protein-synthesis rates were calculated from the radioactivity of these samples over the linear portion of the time course of the assay.

Phenylalanine hydroxylase synthesis was estimated from the incorporation of [³⁵S]methionine into phenylalanine hydroxylase-specific immunoprecipitable material. Briefly, poly(A)⁺ RNA (0.5 μ g) was incubated at 30 °C with 0.04 ml of lysate and 15.0 μ Ci of [³⁵S]methionine. Incorporation of [³⁵S]methionine was terminated by addition of 0.01 ml of 10 mM-methionine. Then 0.02 ml of phenylalanine hydroxylase-specific antiserum was added to the translation mixture, followed by 0.02 ml of anti-sheep serum. The mixture was allowed to precipitate overnight at 4 °C, and immunoprecipitates were collected by centrifugation at 12 000 *g* for 5 min at 4 °C. Precipitates were purified by the two-step sucrose-gradient procedure of Wicks & Su (1978) and then analysed by SDS/polyacrylamide-gel electrophoresis.

The distribution of radioactivity within gels was assessed by solubilization of 1.0 mm slices in 0.20 ml of 30% (w/v) H₂O₂ at 70 °C for 5 h. The resulting samples were counted for radioactivity in the presence of 2.0 ml of PCS scintillation fluid. The major source of radioactivity in the gels was the phenylalanine hydroxylase polypeptide (50 kDa; Donlon & Kaufman, 1980). Enzyme synthesis was calculated from the change in radioactivity of this polypeptide over the linear portion of the time course of the assay. Translational activity was linear over the range of RNA concentrations used in all assays (results not shown).

Northern-blot analysis of RNA

Total RNA (10 μ g) was electrophoresed under denaturing conditions in 1% (w/v) formaldehyde/agarose gels essentially as described by Maniatis *et al.* (1982). After electrophoresis, gels were stained with ethidium bromide and viewed under u.v. light to locate

the 28 S and 18 S rRNA species. The resolved RNA was then transferred from the gel to nitrocellulose filters by capillary blotting. Filters were probed with the 2020 bp cDNA insert of the phenylalanine hydroxylase plasmid (pRLPH 3: cloned as described by Dahl & Mercer, 1986) or a 1500 bp cDNA insert of the cytosolic phosphoenolpyruvate carboxykinase [PEPCK; GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32] plasmid (pPCK10: cloned as described by Yoo-Warren *et al.*, 1983). The cDNAs were labelled with [α - 32 P]dCTP by the random-priming method (Feinberg & Vogelstein, 1983, 1984). The probes each had a specific radioactivity of at least 10^8 c.p.m./ μ g of DNA. Pre-hybridization (12 h) and hybridization (36 h) were carried out at 60 °C in the presence of $6 \times$ SSC ($1 \times$ SSC is 150 mM-NaCl, 15 mM-sodiumcitrate, pH 7.0)/ $10 \times$ Denhardt's solution/0.1% (w/v) SDS/0.1% (w/v) salmon sperm DNA and $6 \times$ SSC/ $2 \times$ Denhardt's solution/0.1% SDS/0.1% salmon sperm DNA respectively. After hybridization, the filters were washed in the presence of $1 \times$ SSC and 1% SDS, then with $0.5 \times$ SSC and 1% SDS. The filters were autoradiographed by using Fuji (RX) X-ray film and intensifying screens. mRNA was quantified by densitometric scanning of the autoradiographs at 600 nm. A linear relationship between RNA load and absorbance peak area was verified for each blot.

RESULTS AND DISCUSSION

Alterations in phenylalanine hydroxylase in diabetes

Uncontrolled diabetes was associated with an approx. 2-fold elevation in hepatic phenylalanine hydroxylase activity when measured with synthetic cofactor (which is insensitive to differences in enzyme phosphorylation state: Abita *et al.*, 1976). Control of diabetes by daily injection of insulin abolished this change in maximal enzymic activity [normal, 6.8 ± 0.3 ; uncontrolled diabetic, 12.6 ± 0.6 ($P < 0.05$); controlled diabetes, 4.7 ± 0.8 nmol of tyrosine formed/min per mg of protein: means $\pm$ S.E.M. for at least three different animals in each group]. This alteration of enzymic activity was confined to the liver. Analysis of the phenylalanine hydroxylase activity of the kidney cortex, the only other significant source of the enzyme in mammals (Berry *et al.*, 1972), failed to reveal any significant changes after the induction of diabetes (results not shown).

Immunoblot analysis of liver extracts (see Fig. 1) confirmed that diabetes-related alterations in enzymic activity closely reflected changes in the abundance of phenylalanine hydroxylase protein. Quantification of immunoblots revealed an approximate doubling in the amount of phenylalanine hydroxylase protein in liver extracts from diabetic animals [uncontrolled diabetic 199 ± 7 ($P < 0.05$), controlled diabetic $82 \pm 16\%$ of normal: means $\pm$ S.E.M. for at least three different animals in each group]. These observations are consistent with the limited immunotitration data provided by ourselves (Santana *et al.*, 1985) and other workers (Howard & Donlon, 1985).

It is possible to make an estimate of the relative contributions made by alterations in both enzyme amount and enzyme phosphorylation status to the overall change in enzyme activity which occurs in diabetes. This is most simply done by comparison of phenylalanine hydroxylation flux in isolated liver cells, supported by endogenous tetrahydrobiopterin and hence sensitive to

Fig. 1. Immunoblot analysis of phenylalanine hydroxylase

Immunoblots were obtained from liver extracts subjected to SDS/polyacrylamide-gel electrophoresis, transferred to nitrocellulose and immunostained with a phenylalanine hydroxylase-specific antiserum. Lane 1, no treatment; lane 2, uncontrolled diabetes; lane 3, insulin-controlled diabetes. The relative mobilities of a series of molecular-mass markers, after SDS/polyacrylamide-gel electrophoresis, are indicated on the left: A, bovine serum albumin (66 kDa); B, egg albumin (45 kDa); C, glyceraldehyde-3-phosphate dehydrogenase (36 kDa); D, carbonic anhydrase (29 kDa); E, trypsinogen (24 kDa).

changes in enzyme phosphorylation state, with maximal enzyme activity of liver extracts measured in the presence of an artificial cofactor. This type of analysis, using data presented here and previously (Santana *et al.*, 1985), indicates a modest (approx. 20%) increase in the ratio of hydroxylation flux to maximal enzyme activity after the induction of diabetes. This is indicative of an elevation in enzyme phosphorylation state in diabetes, as reported by Donlon & Beirne (1982). It therefore appears the alteration in the amount of enzyme is the predominant factor in the diabetes-induced increase in phenylalanine hydroxylase activity.

Influence of diabetes on translational activity of RNA

We have previously shown that there is an approx. 3-fold increase in the ratio of phenylalanine hydroxylase synthesis to total protein synthesis in liver cells from diabetic rats (Santana *et al.*, 1985). In order to establish the biochemical mechanism underlying this observation, we assessed the impact of diabetes on the functional

Fig. 2. Effect of diabetes on the translational activity of hepatic poly(A)⁺ RNA

Poly(A)⁺RNA was purified from liver and translational activity was assessed in a reticulocyte translation system as described in the Materials and methods section. □, No treatment; ●, uncontrolled diabetes; ■, insulin-controlled diabetes. Results are means $\pm$ S.E.M. for three different RNA preparations from each group of animals.

activity of hepatic poly(A)⁺ RNA. Fig. 2 shows typical time courses for poly(A)⁺-RNA-directed protein synthesis in a reticulocyte-lysate translation system. Synthesis was essentially linear, after an initial lag period of approx. 10 min, for up to 1 h.

Diabetes decreased the total translational activity of hepatic poly(A)⁺ RNA by approx. 49%. Control of diabetes by daily insulin treatment largely prevented this fall in translational activity. However, it is not clear why insulin treatment failed to restore total mRNA activity fully to control values throughout the time course of translation. These observations are consistent with the observation that hepatic protein synthesis is decreased in diabetic animals both *in vivo* (McNurlan & Garlick, 1979; Albertse *et al.*, 1980) and in isolated liver cells (Wagle *et al.*, 1975; Bellemann *et al.*, 1977). These findings may reflect a role for insulin in mediating a general enhancement of total mRNA activity in the liver (Hill *et al.*, 1981).

In contrast with total protein synthesis, phenylalanine hydroxylase-specific template activity was significantly elevated in poly(A)⁺ RNA isolated from livers of diabetic animals [normal, 0.13 ± 0.02 (3); uncontrolled diabetes, 0.29 ± 0.03 (5) ($P < 0.05$); controlled diabetes, 0.06 ± 0.01 (3) ($P < 0.05$) phenylalanine hydroxylase synthesis as % of total protein synthesis: means $\pm$ S.E.M. for the numbers of different animals shown in parentheses]. These estimates of phenylalanine hydroxylase-specific synthetic capacity are consistent with the relatively low abundance of phenylalanine hydroxylase-specific mRNA in liver (Robson *et al.*, 1982).

It is clear from the foregoing data that the effects of diabetes on phenylalanine hydroxylase-specific

translational capacity closely parallel the effects on enzyme synthesis and enzyme abundance in liver cells.

Quantification of phenylalanine hydroxylase-specific mRNA

In order to determine if the changes in poly(A)⁺ RNA template activity reflected changes in the relative abundance of the phenylalanine hydroxylase-specific mRNA, total RNA was subjected to Northern-blot analysis using a cloned phenylalanine hydroxylase cDNA probe. In addition, the RNA was probed with a cloned PEPCK cDNA probe. The impact of diabetes on PEPCK expression is well characterized (Cimbala *et al.*, 1982); therefore, by probing for PEPCK mRNA we were able to confirm that each of our RNA samples contained similar amounts of undegraded poly(A)⁺ RNA.

The relative abundance of the 2800-nucleotide PEPCK mRNA was substantially elevated in diabetes, unless the diabetes was controlled by insulin treatment, as shown by densitometric analysis of Northern blots [uncontrolled diabetes, 360 ± 61 ($P < 0.05$); insulin-controlled diabetes, 72 ± 37 % of normal: means $\pm$ S.E.M. for at least three different RNA preparations in each group]. Our results

Fig. 3. Northern-blot analysis of hepatic RNA

Total RNA was subjected to Northern-blot analysis using a cloned phenylalanine hydroxylase probe as described in the Materials and methods section. Lane 1, no treatment; lane 2, uncontrolled diabetes; lane 3, insulin-controlled diabetes. The relative mobilities of a series of size markers, after formaldehyde/agarose-gel electrophoresis, are indicated on the left: 28 S RNA (4.7 kb), PEPCK mRNA (2.8 kb), serum albumin mRNA (2.4 kb) and 18 S RNA (1.9 kb).

are consistent with those of other workers, who have shown a substantial increase in PEPCK mRNA abundance in diabetes (Cimbala *et al.*, 1982). This accounts for the enhancement of PEPCK activity in diabetes (Wimhurst & Manchester, 1970; Chang & Schneider, 1971).

Fig. 3 shows that there are two, partially resolved, species of mRNA which hybridize to this phenylalanine hydroxylase-specific cDNA probe. Dahl & Mercer (1986) have shown that these are derived from alternative polyadenylation signals, and they represent mRNA species of approx. 2000 and 2400 nucleotides. Fig. 3 shows that an increase in the abundance of phenylalanine hydroxylase-specific mRNA occurred in diabetes. The elevation in phenylalanine hydroxylase-specific mRNA was significantly diminished when diabetes was controlled by daily injection of insulin. The alterations in phenylalanine hydroxylase mRNA were quantified by densitometric analysis of the Northern blots [uncontrolled diabetes, 295 ± 22 ($P < 0.05$); insulin-controlled diabetes 148 ± 13 % of normal: means $\pm$ S.E.M. for at least three different RNA preparations in each group]. The change in phenylalanine hydroxylase mRNA abundance in diabetes closely paralleled the increase in enzymic activity, amount of enzyme and phenylalanine hydroxylase-specific translational activity of RNA, seen after the onset of diabetes (see above).

General discussion

In this paper we have shown that the increased amount of phenylalanine hydroxylase protein and the elevated functional activity of phenylalanine hydroxylase-specific mRNA which are characteristic of the diabetic state are largely a result of an increase in the relative abundance of the phenylalanine hydroxylase-specific mRNA. There is therefore control of phenylalanine hydroxylase expression at a pre-translational level. This control may reflect alterations in transcription, mRNA maturation or, alternatively, mRNA stability (Raghow, 1987). Analysis of the effect of diabetes on the synthesis of mRNA *de novo* will be required to elucidate fully the processes underlying the observed alterations in phenylalanine hydroxylase mRNA abundance.

It should be noted that, in addition to an altered [insulin]/[glucagon] ratio, streptozotocin-induced diabetes is associated with alterations in the circulating levels of a variety of other hormones which may play key roles in the regulation of phenylalanine hydroxylase expression. Circulating levels of corticosteroids are elevated within a few days of the induction of diabetes (De Nicola *et al.*, 1976; Rhees *et al.*, 1983). It would appear, however, that alterations in corticosteroid concentrations are unlikely to account for the present observations. Previous work has failed to show major changes in maximal phenylalanine hydroxylase activity in liver cell extracts prepared from adrenalectomized or dexamethasone-treated adrenalectomized rats (Stanley *et al.*, 1985). It is only through analysis of the effect of different combinations of hormones, on enzyme expression in long-term liver cell cultures, that an indication of which hormones influence the expression of phenylalanine hydroxylase in diabetes will be obtained. Limited experiments of this type have already suggested a role for cyclic AMP in the development of phenylalanine hydroxylase activity and accumulation of phenylalanine hydroxylase-specific mRNA in foetal-rat liver cells (Yeoh *et al.*, 1988).

The impact of diabetes on phenylalanine hydroxylase expression bears some resemblance to its effect on PEPCK expression. In the latter case the molecular mechanisms underlying the effect of diabetes, specifically the role of cyclic AMP and insulin in the control of transcription, are well known (Cimbala *et al.*, 1982; Schubart, 1986; Magnuson *et al.*, 1987; Sasaki & Granner, 1988). Little is known about the molecular basis of the hormonal control of phenylalanine hydroxylase gene transcription. In this respect it is, perhaps, of some interest that the gene for tyrosine hydroxylase is under the control of cyclic AMP. A cyclic AMP regulatory element, analogous to that found in the promoter of the PEPCK gene, has been identified in the tyrosine hydroxylase promoter (Lewis *et al.*, 1987). The possible involvement of a similar regulatory element in the control of the expression of the evolutionarily related phenylalanine hydroxylase gene remains to be established.

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