The effect of experimental diabetes on phenylalanine metabolism in isolated liver cells

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1. Chronic (10-day) diabetes was associated with increased metabolic flux through phenylalanine hydroxylase in isolated liver cells. This flux was stimulated by $0.1\ \mu\text{M}$ -glucagon, but not by $10\ \mu\text{M}$ -noradrenaline; $0.1\ \mu\text{M}$ -insulin affected neither basal nor glucagon-stimulated flux. 2. The increased rate of phenylalanine hydroxylation in diabetes was accompanied by parallel increases in enzyme activity (as measured with artificial cofactor) and immunoreactive-enzyme-protein content. 3. In contrast with total protein synthesis, which decreased, phenylalanine hydroxylase synthesis persisted at the control rate in cells from diabetic animals. 4. These findings are discussed in relation to the hormonal regulation of the hydroxylase and the known metabolic consequences of chronic diabetes.

The first step of phenylalanine degradation in the liver involves hydroxylation catalysed by phenylalanine hydroxylase [L-phenylalanine,tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1]:

The activity of the hydroxylase is controlled, in the short term, by a phosphorylation–dephosphorylation process (Donlon & Kaufman, 1978; Fisher & Pogson, 1984), and, in the long term, by changes in enzyme concentration.

Regulation of enzyme turnover has been well investigated in hepatoma cell lines (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 cells, control being exerted at a transcriptional site (Chiappelli *et al.*, 1981). There is conflicting evidence for a role of insulin in the control of phenylalanine hydroxylase activity in hepatoma cells: Tourian (1975) reported that insulin enhances enzyme activity by a cycloheximide-sensitive mechanism, but Sorimachi *et al.* (1981) failed to demonstrate any such inductive effect of insulin.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Carr & Pogson (1981) found that experimentally induced diabetes in rats is associated with a significant increase in metabolic flux through the phenylalanine catabolic pathway in isolated hepatocytes. Donlon & Beirne (1982) have also observed an increase in enzyme activity of liver extracts from diabetic rats. It was suggested that changes in the phosphorylation state accounted for altered enzyme activity over the first 3 days of induced diabetes. Chronic (7-day) diabetes, however, was associated with an increase in artificial-cofactor-dependent activity, indicative of increased enzyme protein concentration [assays with artificial cofactors are insensitive to changes in enzyme phosphorylation state (Abita *et al.*, 1976; Milstien *et al.*, 1976)]. These observations contrast with the finding that total protein synthesis is decreased in liver cells from diabetic animals both when freshly isolated (Wagle *et al.*, 1975) and in primary culture (Bellemann *et al.*, 1977). In addition, insulin stimulates general protein synthesis in the liver and in liver cells from normal animals; this may involve enhancement of translation efficiency (Hill *et al.*, 1981) or polyribosome stabilization (Wagle & Sampson, 1975). Inhibition of proteolytic activity is another feature of insulin action (Aronson, 1980; Draznin & Trowbridge, 1982).

The present paper seeks to clarify the mechanisms involved in the changes in phenylalanine metabolism in experimental diabetes.

Materials and methods

Animals

Male Sprague-Dawley rats (University of Manchester 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 the intravenous injection of streptozotocin (60 mg/kg body wt.) in 0.9% (w/v) NaCl. Glucosuria was checked with Clinistix (Ames Co., Stoke Poges, Slough, Bucks., U.K.). Diabetic animals were used 10 days after treatment.

Chemicals

Reagents and radiochemicals were obtained from the sources given previously (Fisher & Pogson, 1984) with the following additions. Waymouth's MB 752/1 medium was from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.). Foetal-calf serum was from Gibco Bio-Cult. (Paisley, Scotland, U.K.). L-[4,5-³H]Leucine (sp. radioactivity 131 Ci/mmol) was from Amersham International (Amersham, Bucks., U.K.). Noradrenaline [(–)-arterenol bitartrate] was from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). Streptozotocin was a gift from Dr. D. C. N. Earl (ICI Pharmaceuticals Division, Macclesfield, Cheshire, U.K.), and bovine monocomponent insulin was a gift from the Wellcome Foundation (Dartford, Kent, U.K.).

Preparation and incubation of hepatocytes

Cells were prepared as described previously (Elliott *et al.*, 1976). In all experiments, metabolic integrity was assessed by measurement of ATP content (Dickson & Pogson, 1977). Liver cells from normal fed rats contained > 7.50 nmol of ATP/mg dry wt.; those from diabetic rats contained > 5.50 nmol of ATP/mg dry wt. This is consistent with other reports (Siess & Wieland, 1976).

For flux experiments, cells were incubated in a simple salts medium (Krebs & Henseleit, 1932) supplemented with 2% (w/v) bovine serum albumin (freed of fatty acids and other materials as described by Chen, 1967) and containing 2.5 mM-CaCl₂. Cells were preincubated for 30 min with lactate/pyruvate (9:1; final total concn. 10 mM) and glucose (10 mM). Incubations (volume 2 ml) were as previously described (Smith & Pogson, 1980).

For experiments involving protein synthesis, cells were incubated in an orbital shaking water bath as described by Dickson & Pogson (1977). Waymouth's MB 752/1 medium, supplemented with 5% (v/v) foetal-calf serum (freed of low-M_r

components by treatment with charcoal and subsequent dialysis) was used throughout.

Measurement of metabolic flux

L-Phenylalanine was added to a final concentration of 0.05 mM (final sp. radioactivity 1.5 Ci/mol). Effectors were added 2 min before phenylalanine. All such effectors were prepared immediately before use in a salts solution (Krebs & Henseleit, 1932); with glucagon and insulin, the medium was supplemented with 2% (w/v) bovine serum albumin. The inclusion of ascorbate and catechol (final concns. 1 mM and 3 mM respectively), to prevent oxidation of noradrenaline, had no significant effect on the magnitude of hormone-induced flux changes. All incubations were terminated with 0.2 ml of 2 M-HClO₄. Flux through phenylalanine hydroxylase was assessed by the ³H-release assay (Fisher & Pogson, 1984). All flux data presented here are based on initial rates over a 30 min period.

Measurement of enzyme activity in cell extracts

Phenylalanine hydroxylase was extracted and assayed at 25°C as described by Shiman *et al.* (1979), except that cells were disrupted by three cycles of freezing (liquid N₂) and thawing (25°C). One unit of enzyme activity is defined as the amount that catalyses the formation of 1 μmol of tyrosine/min.

Cyclic AMP assay

The cyclic AMP content of deproteinized neutral cell extracts was assayed by the protein-binding method of Tovey *et al.* (1974).

Protein content

This was measured by the method of Lowry *et al.* (1951).

Immunotitration of phenylalanine hydroxylase

Liver cells were incubated for 30 min before disruption and extraction as described above. Soluble extracts (0.10 ml) were incubated in 0.1 M-potassium phosphate, pH 6.8, with various amounts (up to 0.15 ml) of an anti-(phenylalanine hydroxylase) antibody raised in sheep (Fisher & Pogson, 1984). The mixtures (final volume 0.50 ml) were incubated at 25°C for 30 min and then at 4°C for 2 h. Immunoprecipitates were collected by centrifugation at 12000g for 10 min. The resulting supernatants were assayed for enzyme activity. Hydroxylase activity was not removed from cell extracts by control pre-immune serum (results not shown).

Protein synthesis in isolated liver cells

Liver cells were incubated for up to 6 h in the presence of 400 μM-L-[4,5-³H]leucine (final sp.

radioactivity 25 Ci/mol). The rate of total protein synthesis was determined as follows. At 1 h intervals, portions of cell suspensions (0.10 ml) were acidified by addition of 0.10 ml of ice-cold 10% (w/v) trichloroacetic acid. Precipitated protein was collected by centrifugation at 12000g for 5 min at 4°C. Precipitates were washed twice with 5% (w/v) trichloroacetic acid before solubilization in 0.03 ml of NCS, addition of 2.0 ml of PCS scintillator cocktail and measurement of radioactivity. No significant contamination with lipid material was detected in these fractions as judged by washing precipitates with ethanol (results not shown).

The intracellular concentration and specific radioactivity of leucine were determined by amino acid analysis (LKB 4400 analyser; Na⁺ form cation exchanger) of liver cell extracts. Through the incubation period no significant difference was found in concentration (normal, 1.88 ± 0.22 mM; diabetic, 1.40 ± 0.09 mM; means $\pm$ S.E.M. for three independent cell preparations after 4 h incubation) or specific radioactivity (normal, 11877 ± 471 d.p.m./nmol, diabetic 13387 ± 2375 d.p.m./nmol; means $\pm$ S.E.M. for three independent cell preparations after 4 h incubation). These observations are consistent with those of other workers, who found that intracellular leucine pools were of similar sizes in liver cells from normal and diabetic animals (Berry *et al.*, 1980).

Phenylalanine hydroxylase synthesis was measured as follows. Portions (1.0 ml) of cell incubation mixtures (as used for assessment of total protein synthesis: see above) were removed and cells were sedimented by centrifugation for 5 s in an Eppendorf Microfuge. Cells were washed twice with ice-cold incubation medium (containing 400 μ M-leucine, but without radioactive leucine) to stop incorporation of radioactivity into proteins. The incorporation of radioactivity into phenylalanine hydroxylase was determined after immunoprecipitation as described previously (Fisher & Pogson, 1984). A 1.5-fold excess of antibody was used to ensure precipitation of all antigen.

The specificity of the antibody preparation was assessed by SDS/polyacrylamide-gel-electrophoretic analysis (Laemmli, 1970) of immunoprecipitates formed between [³H]leucine-labelled cell extracts and the antibody. This antibody preparation was monospecific, the major source of radioactivity being the hydroxylase monomer (*M*, 50000; Donlon & Kaufman, 1980). The contaminating sources of radioactivity amounted to less than 15% of total radioactivity and did not change with cell incubation time. Similar results were obtained with extracts obtained from liver cells of diabetic animals. It was therefore unnecessary to

analyse all samples by SDS/polyacrylamide-gel electrophoresis.

Rates of synthesis were calculated from the slope of the time course of incorporation of radioactivity into immunoprecipitates or trichloroacetic acid-insoluble protein over the first 4 h of incubation.

Expression of results

Chronic diabetes was associated with a fall of approx. 13% in liver-cell dry weight, as determined by assay of DNA (results not shown). This change did not affect the outcome of statistical analyses; data are expressed on a tissue-dry-weight basis throughout.

Results and discussion

Flux through phenylalanine hydroxylase

Table 1 shows the effect of various hormones on phenylalanine hydroxylation by liver cells isolated from normal adult rats and chronic diabetic animals. A physiological substrate concentration of 0.05 mM was used throughout; the resulting metabolic flux is known to be highly sensitive to hormonal influences on the enzyme (Carr & Pogson, 1981). This may reflect the effect of phosphorylation on the affinity of the enzyme for phenylalanine as an activator (Shiman *et al.*, 1982).

In agreement with our previous observations (Carr & Pogson, 1981), experimental diabetes was associated with an approximate doubling of flux through the hydroxylase. The increased basal flux was further enhanced by exposure of cells to glucagon. This is consistent with there being an increased pool of enzyme which remains susceptible to covalent modification as in the normal animal. This was confirmed by the observation that the maximal enzyme activity of cell extracts, measured by an assay with artificial cofactor, was substantially increased in the diabetic state (see Table 1). Exposure of cells to insulin (0.1 μ M) had no effect on basal or glucagon (0.1 μ M)-stimulated flux rates. Previous work has indicated that phenylalanine hydroxylase is sensitive to α -adrenergic effectors (Garrison & Wagner, 1982; Fisher *et al.*, 1984). These agents stimulate enzyme phosphorylation, with a concomitant increase in hydroxylation in cells (Fisher *et al.*, 1984). This was again confirmed, but, in contrast, no effect was seen in liver cells from diabetic animals (see Table 1).

It has been suggested that α_1 -receptor-dependent changes in intracellular cyclic AMP content may be associated with α -agonist action (Morgan *et al.*, 1983a,b). We were, however, unable to detect significant changes in cyclic AMP content of cells from normal rats (males, body wt. 180–220 g) when these were exposed to noradrenaline (10 μ M)

Table 1. *Hormonal responsiveness of phenylalanine hydroxylation in liver cells from normal and diabetic rats*
 Rates were measured as described in the Materials and methods section, and expressed as nmol/h per mg dry wt. (based on initial rates over 30 min); means $\pm$ S.E.M. are given for six independent cell preparations in each case. Differences between experimental and control cells were tested by Student's *t* test: **P* (versus control) <0.05 ; other differences not significant. Differences between cells with and without insulin in paired experiments were similarly tested: †*P* (versus cells without insulin) <0.05 ; other differences not significant. Maximal enzyme activity was determined as described in the Materials and methods section and is expressed as nmol/h per mg dry wt. Means $\pm$ S.E.M. are given for six independent cell extracts in each case. The difference between normal and diabetic rats was tested by Student's *t* test: ‡*P* (versus normal) <0.01 .

	Phenylalanine hydroxylation (nmol/h per mg dry wt.)	
	Normal rats	Diabetic rats
Control	4.55 $\pm$ 0.16	10.13 $\pm$ 0.82
0.1 μ M-Insulin	4.63 $\pm$ 0.17	9.64 $\pm$ 0.63
10 μ M-Noradrenaline	6.32 $\pm$ 0.14*	9.96 $\pm$ 1.07
0.1 μ M-Glucagon	8.43 $\pm$ 0.33*	16.45 $\pm$ 1.55*
0.1 μ M-Insulin + 0.1 μ M-glucagon	8.23 $\pm$ 0.43*	16.90 $\pm$ 1.54*
0.1 μ M-Insulin + 10 μ M-noradrenaline	5.18 $\pm$ 0.26†	10.63 $\pm$ 0.77
0.1 μ M-Glucagon + 10 μ M-noradrenaline	7.97 $\pm$ 0.34*	13.54 $\pm$ 0.88*
0.1 μ M-Glucagon + 0.1 μ M-insulin + 10 μ M-noradrenaline	6.92 $\pm$ 0.22*†	13.51 $\pm$ 0.70*
Maximal phenylalanine hydroxylase activity in cell extracts	227 $\pm$ 15‡	410 $\pm$ 34‡

(results not shown). This finding is similar to that of other workers (Pushpendran *et al.*, 1984). It appears, therefore, that α -adrenergic control of phenylalanine hydroxylase in our normal rats is exerted through a cyclic-AMP-independent mechanism. We have previously shown that the calcium ionophore A23187 mimics the effects of α -adrenergic agents on phenylalanine hydroxylase (Fisher *et al.*, 1984). This indicates a possible role for Ca^{2+} in the α -adrenergic control of the hydroxylase. Interestingly, Schworer & Soderling (1983) have shown that phenylalanine hydroxylase is a substrate for a Ca^{2+} -calmodulin-dependent protein kinase.

The lack of α -adrenergic stimulation of phenylalanine hydroxylase in cells from diabetic animals might be related to a decrease in the number of α -adrenergic membrane receptors in the liver in chronic diabetes (Dighe *et al.*, 1984). Alternatively, there may be a lesion in the Ca^{2+} -dependent signal-transduction process.

The inhibitory effect of insulin on the adrenergic response in normal rats (see Table 1) has been previously noted (Blackmore *et al.*, 1979; Dehaye *et al.*, 1981). This has been associated with Ca^{2+} redistribution brought about by high concentrations of insulin (Blackmore *et al.*, 1979).

The combination of glucagon and noradrenaline caused an enhancement of flux not significantly different from that caused by glucagon alone. This response was significantly diminished by insulin only in cells from normal rats. The lack of additivity of the glucagon and adrenergic re-

sponses is consistent with there being only one major phosphorylation site on the phenylalanine hydroxylase subunit (Wretborn *et al.*, 1980).

Immunotitration of phenylalanine hydroxylase

Cell extracts from normal and diabetic animals were immunotitrated to determine the antibody equivalence points for the enzyme in the two physiological states. As shown in Fig. 1, the immunotitration curves were parallel. The equivalence point of normal cell extracts ($2.06 \pm 0.12 \mu\text{l}$ of antibody/munit of activity; mean $\pm$ S.E.M. for three independent cell preparations) was not significantly different from that of those from diabetic animals ($2.39 \pm 0.30 \mu\text{l}$ of antibody/munit of enzyme activity; mean $\pm$ S.E.M. for three independent cell preparations). This is consistent with an increased amount of phenylalanine hydroxylase protein in the liver of diabetic animals (Donlon & Beirne, 1982), rather than with modification of existing enzyme molecules.

Protein synthesis in isolated liver cells

Protein-synthesis rates (both total protein and phenylalanine hydroxylase synthesis) in liver cells isolated from normal and chronic-diabetic rats remained linear for at least 4 h (Fig. 2). Consistent with the observations of other workers (Wagle *et al.*, 1975; Bellemann *et al.*, 1977; McNurlan & Garlick, 1981), we observed a significant decrease in total protein synthesis in cells from diabetic animals [normal, $234 (\pm 15) \times 10^3$, diabetic, $81 (\pm 12) \times 10^3$ d.p.m. incorporated/per mg dry wt.;

Fig. 1. *Immunotitration of phenylalanine hydroxylase* Immunotitration was carried out as described in the Materials and methods section, with cell extracts from normal (■) and diabetic (●) rats. Each point represents the mean $\pm$ S.E.M. for three, or the mean of two, independent experiments.

means $\pm$ S.E.M. for three independent observations; $P < 0.01$). This decrease has been attributed to altered polyribosome morphology (Bellemann *et al.*, 1977), decreased initiation of protein synthesis (Stanchfield & Yager, 1979) or decreased translational efficiency (Hill *et al.*, 1981). The addition of insulin to the incubation medium did not improve the total protein-synthesis rate of cells from diabetic animals (results not shown).

In contrast with total protein synthesis, the rate of synthesis of phenylalanine hydroxylase was unchanged in cells isolated from diabetic animals when compared with cells from normal animals (normal, 299 ± 27 , diabetic, 295 ± 22 d.p.m. incorporated/h per mg dry wt.; means $\pm$ S.E.M. for three independent observations). As a result, the ratio of the rate of synthesis of the hydroxylase to that of total protein synthesis was increased approx. 3-fold in the diabetic state. The mechanism responsible for this maintained rate of synthesis is unclear, although it may be related to changes in plasma corticosterone concentrations which have been reported to occur in streptozotocin-induced chronic-diabetic rats (Rhees *et al.*, 1983). Donlon & Beirne (1982) have reported that in liver extracts from controlled diabetic (i.e. insulin-treated) animals enzyme activity was not significantly differ-

Fig. 2. *Protein synthesis in isolated liver cells* The Figure represents a typical total protein (a) and phenylalanine hydroxylase (b) synthesis experiment. Cells from normal (■) and 10-day-diabetic (●) rats were incubated and treated as described in the text. Each point represents the mean $\pm$ S.D. for triplicate samples. Where not shown, error bars lie within the point.

ent from that of the uncontrolled diabetic. Hence insulin insufficiency alone seems unlikely to account for the maintained rate of phenylalanine hydroxylase synthesis.

In addition to changes in protein synthesis, protein-degradation rates also change during diabetes. During the onset of experimental diabetes, an increase in overall rates of protein degradation

has been reported (Garlick, 1980); this may be a direct result of insulin deficiency (Mortimore & Schworer, 1980; Hopgood & Ballard, 1980). This enhancement of liver proteolysis does not persist; after 4 days of diabetes, proteolytic capacity declines below normal (Garlick, 1980). This phenomenon probably reflects a re-adjustment to the low protein-synthesis rate associated with the diabetic state (Pain *et al.*, 1983). If hydroxylase degradation was decreased in this way, a maintained synthesis rate could account for the increased amount of antigen seen.

General discussion

In this paper we have shown that diabetes is associated with enhanced flux through phenylalanine hydroxylase. This appears to be largely attributable to alteration in the amount of hydroxylase protein rather than to modification (for example, through phosphorylation) of pre-existing molecules. Short-term regulation of phenylalanine hydroxylase is also changed during streptozotocin-induced diabetes. Sensitivity to glucagon is retained, whereas that to adrenergic agents is lost. Although the latter finding may be related to the various secondary effects of streptozotocin treatment (Srivastava *et al.*, 1982), the possibility remains that this may be an important feature of the chronic diabetic state. Evidence exists for abnormal adrenergic control in untreated human diabetes mellitus (Arner & Östman, 1976).

The question as to how the synthesis of phenylalanine hydroxylase is maintained preferentially in chronically diabetic animals remains to be answered. In this context, it is noteworthy that the change in the hydroxylase activity is similar to those observed for other enzymes of amino acid metabolism and gluconeogenesis.

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