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

M. Angélica SANTANA, Michael J. FISHER, Alison J. BATE and Christopher I. POGSON*
Department of Biochemistry, University of Manchester, Oxford Road, Manchester M13 9PL, U.K.

(Received 17 September 1984/Accepted 3 December 1984)

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 \( \mu M \) glucagon, but not by 10 \( \mu M \) noradrenaline; 0.1 \( \mu M \) 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]:

\[
\text{L-Phenylalanine + tetrahydropteridine + O}_2 \rightarrow \text{L-tyrosine + dihydropteridine + H}_2\text{O}
\]

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 (Haggert \textit{et al.}, 1973; Baker & Shiman, 1979; Sorimachi \textit{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 \textit{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 \textit{et al.} (1981) failed to demonstrate any such inductive effect of insulin.

Abbreviation used: SDS, sodium dodecyl sulphate.

* Present address: Biochemistry Department, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, U.K.

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 \textit{et al.}, 1976; MILSTIEN \textit{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 (Wagge \textit{et al.}, 1975) and in primary culture (Bellemann \textit{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 \textit{et al.}, 1981) or polyribosome stabilization (Wagge & Sampson, 1975). Inhibition of proteolitic 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 Clinitest (Ammes 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 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.
Effect of diabetes on phenylalanine metabolism

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 ± 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 ± 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 μm-leucine, but without radioactive leu- cine) 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-electrophore- tic 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 μM) had no effect on basal or glucagon (0.1 μM)-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 α5-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 μM)
(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 Ca2+ in the α-adrenergic control of the hydroxylase. Interestingly, Schworer & Soderling (1983) have shown that phenylalanine hydroxylase is a substrate for a Ca2+-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 Ca2+-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 Ca2+ 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 responses 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 ± 0.12 µl of antibody/munit of activity; mean ± S.E.M. for three independent cell preparations) was not significantly different from that of those from diabetic animals (2.93 ± 0.30 µl of antibody/munit of enzyme activity; mean ± 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 (± 15) × 103, diabetic, 81 (± 12) × 103 d.p.m. incorporated/per mg dry wt.;

---

**Table 1. Hormonal responsiveness of phenylalanine hydroxylation in liver cells from normal and diabetic rats**

<table>
<thead>
<tr>
<th></th>
<th>Normal rats</th>
<th>Diabetic rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.55 ± 0.16</td>
<td>10.13 ± 0.82</td>
</tr>
<tr>
<td>0.1 µM-Insulin</td>
<td>4.63 ± 0.17</td>
<td>9.64 ± 0.63</td>
</tr>
<tr>
<td>10 µM-Noradrenaline</td>
<td>6.32 ± 0.14*</td>
<td>9.96 ± 1.07</td>
</tr>
<tr>
<td>0.1 µM-Glucagon</td>
<td>8.43 ± 0.33*</td>
<td>16.45 ± 1.55*</td>
</tr>
<tr>
<td>0.1 µM-Insulin + 0.1 µM-glucagon</td>
<td>8.23 ± 0.43*</td>
<td>16.90 ± 1.54*</td>
</tr>
<tr>
<td>0.1 µM-Insulin + 10 µM-noradrenaline</td>
<td>5.18 ± 0.26†</td>
<td>10.63 ± 0.77</td>
</tr>
<tr>
<td>0.1 µM-Glucagon + 10 µM-noradrenaline</td>
<td>7.97 ± 0.34*</td>
<td>13.54 ± 0.88*</td>
</tr>
<tr>
<td>0.1 µM-Glucagon + 0.1 µM-insulin + 10 µM-noradrenaline</td>
<td>6.92 ± 0.22†</td>
<td>13.51 ± 0.70*</td>
</tr>
<tr>
<td>Maximal phenylalanine hydroxylase activity</td>
<td>227 ± 15†</td>
<td>410 ± 34†</td>
</tr>
</tbody>
</table>

*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 ± 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.
Effect of diabetes on phenylalanine metabolism

Fig. 1. **Immunotitration of phenylalanine hyroxylase**
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 ± S.E.M. for three, or the mean of two, independent experiments.

![Graph showing enzyme activity against antibody concentration](image)

Means ± 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 hyroxylase 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 ± S.E.M. for three independent observations). As a result, the ratio of the rate of synthesis of the hyroxylase 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 different from that of the uncontrolled diabetic. Hence insulin insufficiency alone seems unlikely to account for the maintained rate of phenylalanine hyroxylase 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

![Graph showing protein synthesis against time](image)

**Fig. 2. Protein synthesis in isolated liver cells**
The Figure represents a typical total protein (a) and phenylalanine hyroxylase (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 ± S.D. for triplicate samples. Where not shown, error bars lie within the point.
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
decreases 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 main-
tained synthesis rate could account for the in-
creased 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 hydroxy-
lase 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 re-
tained, whereas that to adrenergic agents is lost.
Although the latter finding may be related to the
various secondary effects of streptozotocin treat-
ment (Srivastava et al., 1982), the possibility
remains that this may be an important feature of
the chronic diabetic state. Evidence exists for an-
normal adrenergic control in untreated human

The question as to how the synthesis of
phenylalanine hydroxylase is maintained preferen-
tially 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.

We thank the Medical Research Council for financial
support.

References

Abita, J.-P., Milstien, S., Chang, N. & Kaufman, S.
(1976) J. Biol. Chem. 251, 5310–5314
Baker, R. E. & Shimam, R. (1979) J. Biol. Chem. 254,
9633–9639
Bellemann, P., Fry, J. R., Bridges, J. W., Scholte, W. &
19, 535–540
Blackmore, B. F., Assimacopoulos-Jeannet, F., Chan,
2834
655–660
Dehaye, J.-P., Hughes, B. P., Blackmore, P. E. & Exton,
Dickson, A. J. & Pogson, C. I. (1977) FEBS Lett. 83,
27–32
Dighe, R. R., Rojas, F. J., Birnbaumer, L. & Garber,
Commun. 108, 746–751
6657–6659
2146–2152
11988–11993
Elliott, K. R. F., Ash, R., Pogson, C. I., Smith, S. A. &
Crisp, D. M. (1976) in Use of Isolated Liver Cells and
Kidney Tubules in Metabolic Studies (Tager, J. M.,
Söling, H.-D. & Williamson, J. R., eds.), pp. 139–143,
North-Holland, Amsterdam
79–85
Biochem. J. 219, 87–90
Garlick, P. J. (1980) Protein Degradation in Health and
Disease; Ciba Found. Symp. 75, 348–350
13135–13143
Haggerty, D. F., Young, P. L., Popjak, G. & Carnes,
Chem. 256, 1510–1513
Degradation in Health and Disease; Ciba Found. Symp.
75, 205–218
Krebgs, H. A. & Henseleit, K. (1932) Hoppe-Seyler's
Z. Physiol. Chem. 210, 33–66
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall,
24, E238–E245
Milstien, S., Abita, J.-P., Chang, N. & Kaufman, S.
J. Biol. Chem. 258, 5103–5109
Eur. J. Pharmacol. 96, 1–10
Degradation in Health and Disease; Ciba Found. Symp.
75, 281–305
Am. J. Physiol. 245, E604–E610
Pushpandran, C. K., Corvera, S. & Garcia-Sainz, J. A.
Horm. Metab. Res. 15, 353–354
Chem. 257, 839–844
Shiman, R., Mortimore, G. E., Schworer, C. M. & Gray,

1985
Effect of diabetes on phenylalanine metabolism
