Vol. 108, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1982 Pages 746-751

MODULATIONS OF RAT HEPATIC PHENYLALANINE HYDROXYLASE DUE TO INDUCED DIABETES OR HIGH-PROTEIN DIET

John Donlon and Edel Beirne,

Department of Biochemistry,
University College, Galway,
Ireland.

Received July 9, 1982

SUMMARY: The tetrahydrobiopterin-dependent activity and apparent state of phosphorylation of rat hepatic phenylalanine hydroxylase is stimulated three days after induction of diabetes by the administration of streptozotocin. This response can be inhibited by concurrent treatment with insulin. Animals that are subjected to the diabetic state for five to seven days show an apparent increase in the amount of hydroxylase present. In addition, rats fed on a high-protein diet for one week have elevated tetrahydrobiopterin-dependent hydroxylase activity and an altered distribution of the multiple forms of this enzyme. These observations support the conclusion that rat hepatic phenylalanine hydroxylase can be modulated *in vivo* both by transient and sustained mechanisms that are responsive to an altered physiological status.

INTRODUCTION: Phenylalanine hydroxylase (EC 1.14.16.1) from extracts of rat liver can be resolved into three forms by chromatography on calcium phosphate-cellulose (1). The two major forms and a further more phosphorylated form can be separated on the basis of their phosphate content (2). The activity of rat liver phenylalanine hydroxylase can be stimulated, through cyclic AMP-dependent phosphorylation *in vivo*, in response to a pharmacological dose of glucagon (3). This response is observed when hydroxylase activity is assayed using the natural cofactor, tetrahydrobiopterin (4), and it does not affect the amount of enzyme present as measured with a synthetic cofactor, such as dimethyltetrahydropteridine. Stimulation of activity has also been correlated with a conversion of the multiple forms to a single fully phosphorylated form (3). The addition of glucagon, within the physiological range of concentrations, to isolated rat hepatocytes also increases tetrahydrobiopterin-dependent phenylalanine hydroxylase activity (5).

0006-291X/82/180746-06$01.00/0

In order to further explore the regulation of rat liver phenylalanine hydroxylase *in vivo*, under conditions where physiological variations in glucagon and/or insulin levels occur, we have studied some physiological models:- (i) animals that were subjected to streptozotocin-induced diabetes (6); (ii) diabetic animals being treated with insulin; and (c) rats that were fed on a high-protein, low-carbohydrate diet (7). We show here that rat hepatic phenylalanine hydroxylase activities, as assayed with tetrahydrobiopterin and also 6,7-dimethyltetrahydropteridine, and the constitution of the multiple forms of this enzyme can be altered in response to diabetes or a high-protein diet.

MATERIALS AND METHODS: Heparin, aprotinin, streptozotocin, ammonia diagnostic kit, vitamin-free casein, dithiothreitol, bovine serum albumin and all the vitamin components of the special diets were purchased from Sigma Chemical Co., Poole, England. Corn oil and cod liver oil were obtained locally. Biopterin and 6-methyltetrahydropteridine were from CP Laboratories, Bishops Stortford, England. Tetrahydrobiopterin was obtained by catalytic hydrogenation of biopterin over PtO_2 (8). The insulin zinc suspension (Ultralente) was a Wellcome product. Catalase, gluketur test strips and the glucose test combination were supplied by The Boehringer Corporation Ltd., Blackrock, Dublin, Ireland.

Male Sprague-Dawley rats (approx. 150 g), obtained from Northwestern Laboratories, Ballina, Ireland, were allowed free access to food and water. The special diets were formulated according to Pitot *et al* (9) except that 55 ml of cod liver oil was substituted for Oleo percomorphum. The salt mix (per kg diet) was prepared from:- 30 g $CaCl_2$; 100 mg $CuSO_4$; 500 mg ferric citrate; 500 mg $MgCl_2$; 200 mg $MnSO_4$; 7.5 g Na_2HPO_4; 2.5 g K_2HPO_4; 7.5 g NaH_2PO_4; 2.5 g KH_2PO_4 and 5 mg zinc acetate. The glucose was replaced by varying the amounts of vitamin-free casein to give diets that were 16% protein (i.e. equivalent to standard chow used) and 91% protein, respectively.

Streptozotocin (freshly prepared in 50 mM sodium citrate, pH 4.6) was administered intravenously, through a tail vein, in a single dose of 70 mg/kg body weight. Where stated, approx. two units of insulin were given intraperitoneally, as a daily dose, commencing 24 hours after streptozotocin. At selected times the animals were killed by decapitation. The livers were rapidly excised and placed in cold 0.15 M KCl. Blood samples were collected into small beakers containing 1 mg heparin, 2 mg Na_2EDTA and between 1 and 2 (trypsin inhibitor) units of aprotinin. Liver extracts were prepared by homogenization with 3 volumes of cold 0.15 M KCl using a Teflon homogeniser. Supernatants obtained after centrifugation at 30,000 x g for 90 min. at 4°C were stored at -20°C for subsequent assays and protein determinations (3). Four rats were used in each group and the means of specific activities $\pm$ S.D. were employed to calculate p values by Student's t-test. Analytical scale calcium phosphate-cellulose chromatography was performed essentially as described previously (2). Elution was performed with a linear gradient of potassium phosphate, 25 to 150 mM, pH 6.8, containing 0.15 M KCl, total volume 25 ml. The recovery of 6-methyltetrahydropterin-dependent hydroxylase activity from these columns was 70-75%.

RESULTS: Hyperglycemia and glycosuria were present 24 hours after streptozotocin administration. A typical time course for the alterations

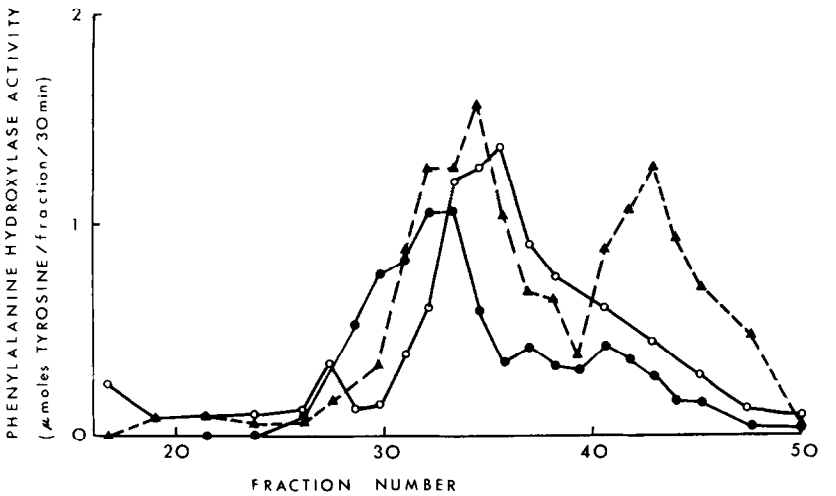

Fig. 1. Calcium phosphate-cellulose chromatography of phenylalanine hydroxylase from 1 ml aliquots of liver extracts from: (a) control rats (o——o); (b) animals that had been exposed to streptozotocin-induced diabetes for three days (▲--▲); (c) diabetic animals treated with insulin (●——●). Beef liver catalase, included as a marker, eluted just ahead of the principal phenylalanine hydroxylase peak.

to tetrahydrobiopterin-dependent and dimethyltetrahydropterin-dependent phenylalanine hydroxylase activities, together with plasma glucose levels, is detailed in Table 1. Three days following administration of the drug, there was an approx. 2-fold elevation of tetrahydrobiopterin-dependent activity with a slight stimulation (32%) of activity with the synthetic cofactor. The distribution of the multiple forms of the enzyme in this group (Fig. 1) shows an increased proportion of the more phosphorylated

Table 1. Phenylalanine hydroxylase activities and plasma glucose levels of normal and diabetic rats.

Exposure time	Group	Phenylalanine hydroxylase activity (μmoles Tyrosine/30 min/mg) Tetrahydrobiopterin	Dimethyltetrahydropterin	Plasma glucose (mg %)
One day	Controls	0.025 ± 0.002	0.375 ± 0.015	121 ± 8
	Streptozotocin	0.032 ± 0.002 (P <0.01)	0.351 ± 0.02 (P <0.05)	413 ± 21
3 days	Controls	0.023 ± 0.005	0.403 ± 0.078	124 ± 5
	Streptozotocin	0.058 ± 0.01 (P <0.001)	0.576 ± 0.076 (P <0.02)	428 ± 36
5 days	Controls	0.029 ± 0.003	0.509 ± 0.073	126 ± 8
	Streptozotocin	0.047 ± 0.003 (P <0.001)	0.697 ± 0.022 (P <0.01)	525 ± 72
7 days	Controls	0.025 ± 0.003	0.486 ± 0.063	130 ± 6
	Streptozotocin	0.042 ± 0.004 (P <0.001)	0.665 ± 0.028 (P <0.01)	524 ± 41

Table 2. Dietary protein content and phenylalanine hydroxylase activity.

Regimen	Phenylalanine hydroxylase activity (μmoles Tyrosine/30 min/mg)	
	Tetrahydrobiopterin	Dimethyltetrahydropterin
Standard chow	0.025 ± 0.003	0.395 ± 0.127
16% Protein	0.025 ± 0.004	0.37 ± 0.03
91% Protein	0.073 ± 0.003	0.493 ± 0.049

form III. The stimulation of tetrahydrobiopterin-dependent hydroxylase activity and the alteration to the chromatographic pattern was reversed or prevented by the administration of insulin, even in the presence of continued hyperglycemia (see Fig. 1).

Following further exposure to the diabetic state (5-7 days) there was a sustained highly significant elevation of the tetrahydrobiopterin-dependent activity. Additionally, under these conditions, there was evidence of a significant increase in the amount of hydroxylase present as determined by assay with 6,7-dimethyltetrahydropterin, consistent with a previous report (10). Continued concurrent treatment with insulin up to such time points had only small effects on both activities. Calcium phosphate-cellulose chromatography of extracts from livers of animals exposed to the diabetic state for seven days resulted in a diffuse pattern with elution of activity occurring throughout the gradient (data not shown).

Rats that were fed on the high-protein, low-carbohydrate diet for one week had 3-fold elevation of their tetrahydrobiopterin-dependent phenylalanine hydroxylase activity without any significant alteration in plasma glucose levels or in the amount of enzyme present, as assayed with synthetic cofactor (Table 2). The special diet having 16% protein did not produce any differences when compared to the standard chow. Calcium phosphate-cellulose chromatography of extracts having stimulated activity revealed the presence of increased proportions of the more phosphorylated forms (Fig. 2). One complication introduced by the high-protein diet was a significant elevation of plasma ammonia concentration.

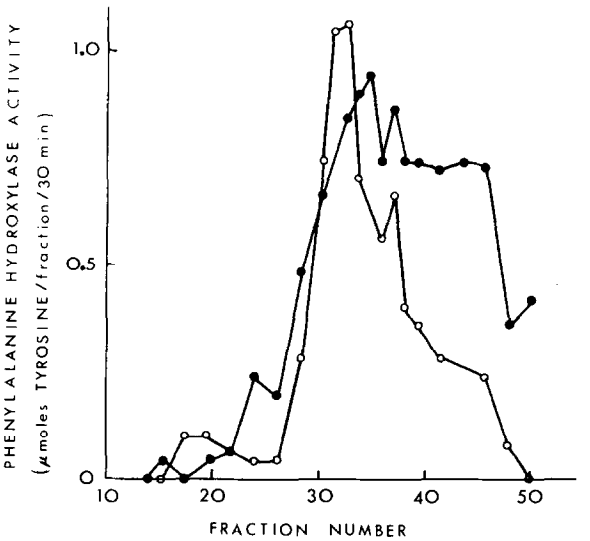

Fig. 2. Calcium phosphate-cellulose chromatography of 1 ml aliquots of liver extracts from control rats (o——o) and from animals fed on a 91% protein diet for one week (●——●).

DISCUSSION: These studies have provided evidence of more sustained mechanisms for the regulation of rat liver phenylalanine hydroxylase *in vivo* than had been previously observed (2,5,10). In the case of the animals subjected to streptozotocin-induced diabetes, this enzyme would appear to be subject to a biphasic modulation i.e. short-term regulation (most striking after three days) and long-term apparent amplification of the amount of enzyme present (from five days onwards). The effects at the earlier stages of diabetes and with the high-protein diet can be explained on the basis of the maintenance of increased proportion of the more phosphorylated forms, having greater activity with tetrahydrobiopterin (2-3). Consistent with this view, samples which had been activated above basal levels *in vivo* were less responsive to further stimulation by phosphorylation *in vitro* (11). The observed changes, mediated through cyclic AMP, could result from either hypoinsulinemia and/or hyperglucagonemia, absolute or relative, which occur under these conditions (12,7). The fact that insulin reversed or prevented these effects suggests that insulin also plays a role in controlling the basal activity and constitution of the multiple forms of phenylalanine hydroxylase.

Streptozotocin was selected as an inducer of diabetes in preference to alloxan because of the latter's complicating effects on glucagon secretion (13). Nevertheless, it is clear that streptozotocin has many varied secondary effects on metabolism in rodents (14). Hence it is not clear what factor(s) may be responsible for the apparent increased amount of phenylalanine hydroxylase from five days following induction of diabetes.

The response of hepatic phenylalanine hydroxylase to a high-protein diet presents a good model for further studies on (a) the long-term regulation of its activity *in vivo* and (b) the metabolic significance of such modulation. Other studies have pointed to alternative and/or complementary mechanisms through which the activity of this enzyme could be amplified *in vivo* (15-16).

ACKNOWLEDGEMENT: We thank the Department of Health and Human Services, U.S.A. (Grant No. RO3 MH 35534) and the Medical Research Council of Ireland for grants in support of these studies. We are grateful to Mr. Daniel Collins for assistance in the care and handling of animals.

REFERENCES

1. Barranger, J.A., Geiger, P.J., Huzino, A., and Bessman, S.P. (1972) Science 175, 903-905.
2. Donlon, J., and Kaufman, S. (1980) J. Biol. Chem. 255, 2146-2152.
3. Donlon, J., and Kaufman, S. (1978) J. Biol. Chem. 253, 6657-6659.
4. Kaufman, S. (1963) Proc. Natl. Acad. Sci. U.S.A. 50, 1085-1093.
5. Abita, J.-P., Chamras, H., Rosselin, G., and Rey, F. (1980) Biochem. Biophys. Res. Commun. 92, 912-918.
6. Rakieten, N., Rakieten, N.C., and Nadkarni, M.W. (1963) Cancer Chemother. Rep. 21, 91-98.
7. Eisenstein, A.B., Strack, I., and Steiner, A. (1974) Metabolism 23, 15-23.
8. Pohland, A., Flynn, E.H., Jones, R.G., and Shive, W. (1951) J. Amer. Chem. Soc. 73, 3247-3252.
9. Pitot, H.C., Potter, V.R., and Morris, H.P. (1961) Cancer Res. 21, 1001-1008.
10. Carr, F.P.A., and Pogson, C.I. (1981) Biochem. J. 198, 655-660.
11. Abita, J.-P., Milstien, S., Chang, N., and Kaufman, S. (1976) J. Biol. Chem. 251, 5310-5314.
12. Stearns, S.B., and Benzo, C.A. (1981) Streptozotocin: Fundamentals and Therapy, pp. 185-212, Elsevier/North-Holland Biomedical Press, Amsterdam.
13. Östenson, C.-G. (1980) Biochem. J. 188, 201-206.
14. Chang, A.Y. (1981) Streptozotocin: Fundamentals and Therapy, pp. 109-126, Elsevier/North-Holland Biomedical Press, Amsterdam.
15. Shiman, R., and Jefferson, L.S. (1982) J. Biol. Chem. 257, 839-844.
16. Hasegawa, H., and Kaufman, S. (1982) J. Biol. Chem. 257, 3084-3089.