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Dopamine-β-Hydroxylase Activity in Plasma, Spleen and Adrenal Gland of Streptozotocin-Diabetic Rats: Correlation with Cataracts

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In streptozotocin-diabetic rats a large increase in plasma dopamine-β-hydroxylase activity was observed. This increase returned to control values with sufficient insulin doses (6 I.U/day); lower insulin doses did not allow normal level to be reached, a dose-dependent decrease being observed. Although the glucemia levels in the diabetic state are responsible for the plasma dopamine-β-hydroxylase, there is no exact ratio between these two parameters when diabetic animals are treated with different insulin doses which suggests not only the clearance of plasmatic dopamine-β-hydroxylase, but a contribution from exocytotic tissues as well. In the experimental conditions, before cataracts appeared, the animals about to develop opaque lenses showed a greater dopamine-β-hydroxylase activity than those which were to remain without this complication. After three months in diabetic state, the severity of disease was evident in the animals with cataracts since they showed a significantly higher function of the sympathoadrenal axis, expressed in spleen and adrenal dopamine-β-hydroxylase activity. Plasma dopamine-β-hydroxylase, as a minority glycoprotein can be considered a useful parameter of other mannose-terminal glycoproteins without having a well-known function, and also as a high risk protein, the accumulation of which in several places contributes to the complex pathogenic mechanism of diabetes complications.

Key words: Dopamine-β-hydroxylase, Glycoproteins, Sympathoadrenal system.

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The role of the sympathoadrenal system in the evolution of the diabetic state is unknown. In diabetes mellitus, hyperactivity of the peripheral (5, 6, 12) and central sympathetic systems (22) as well as adrenomedullary stimulation (8), has been reported.

Dopamine-β-hydroxylase (DBH) is a copper-containing glycoprotein, which catalizes the formation of norepinephrine from dopamine (9). DBH is localized inside storage granules, together with catecholamines and other substances, in chromaffin cells and sympathetic nerve endings (21). When tissues are properly stimulated, the storage granule content is released via a process called exocytosis (20). As existence of DBH in plasma has been reported (30, 31) the determination of plasma DBH activity could serve as an index of sympathoadrenal function (2, 7, 10, 23). However, in a previous work, it was reported (18) that plasma glucose levels can modulate the plasma DBH activity since its clearance from plasma is carried out via a lectin receptor which is specific for mannose/glucose/N-acetyl-glucosamine; thus, glucose can compete with the mannose terminal sugar of DBH. In this way, in several situations generally accepted as sympathoadrenal stimulants, both plasma and tissue DBH activity have been studied together with glucose levels. Plasma DBH activity could serve as a parameter of sympathetic and adrenal function if no simultaneous modification occurs in the glucemia (17).

The aim of the present study was to determine if the increase in plasma DBH activity in streptozotocin-diabetic rats is due only to a diminished catabolic clearance or if an increase in synthesis and release from the sympathetically innervated tissues and adrenal gland can be considered. On the other hand, an interesting aspect of the experimental diabetic state is the development of cataracts; however, there is little information regarding the substances that can precipitate in the crystalline, thereby originating its opacity (28, 29), but this complication correlates well with the severity of the disease, and no studies were undertaken to find a correlation between the appearance of cataracts and plasma DBH values, and tissue levels in adrenal gland and spleen.

Materials and Methods

Male Wistar rats, weighing 150±15 g, usually 7 weeks old, were housed in a room with controlled temperature (20 °C), and had water and food *ad libitum*. The animals were anesthetized with ether before (basal determination) and after treatment, and 1 ml of heparinized blood was obtained by cardiac puncture.

Diabetes mellitus induction was carried out by injecting streptozotocin (STZ, Sigma) (65 mg/kg body weight, dissolved in 0.5 ml buffer citrate, pH 4.5) by cardiac puncture. Control animals were used by injecting the corresponding buffer at an equal volume by the same route.

Slow insulin (Novo-lente MC, Novo Industries) in doses of 6, 4 and 2 I.U. was subcutaneously administered daily during 8 weeks, after 2 weeks of the diabetes induction.

The eyes of all animals were examined once weekly with the aid of an intense, focused light source in an otherwise darkened room.

A basal blood sample (1 ml) was obtained by cardiac puncture in a heparinized syringe; blood was immediately transferred to chilled tubes and then centrifuged in the cold, at 10,000 × *g* for 10 min; plasma was frozen at —20 °C until DBH assay.

After deproteinization, 0.1 ml of the blood obtained by cardiac puncture was used for plasma glucose assay by means of a glucose oxidase technique (32).

Adrenal glands and spleen were rapidly dissected, weighed and homogeneized with an ice-cold 5 mM Tris-HCl, pH 7.3, containing 0.2 % Triton-X-100 (Merck). The homogenates were centrifuged, in the cold, at 27,000 × *g* for 20 minutes and the supernatants assayed immediately for DBH activity.

DBH activity of plasma and tissue was assayed by a sensitive procedure using tyramine as substrate (11). Briefly, tyramine is converted into octopamine by the DBH in the sample, in presence of copper sulphate as inhibitor of endogenous inactivators of the enzyme. After incubation, octopamine is methylated by addition of ^{14}C-S-adenosyl-methionine (59 μCi/μmol, Amersham, U.K.) and phenylethanolamine-N-methyl transferase (PNMT). The resulting ^{14}C-octopamine is extracted and the radioactivity measured in a scintillation counter.

The phenylethanolamine - N - methyl transferase (PNMT) necessary for the second step of the reaction was partially purified in our laboratory using the method of AXELROD (3), modified by MOLINOFF *et al.* (16). Its specific activity was 0.46 nmoles synephrine/mg/h.

In order to overcome the effects of endogenous inhibitors of DBH, the appropriate copper concentration was selected: 16.6 and 33.3 μmol/tube for adrenal and spleen homogenates, respectively, and 47.6 μmol/tube for plasma. The optimal sample volume was also previously tested: 5 and 10 μl for adrenal and spleen homogenates, respectively, and 25 μl for plasma. The optimal tyramine concentration was 0.645 mM. Internal octopamine standards containing 0.2 nmol/tube were used, as well as blanks (with the sample heated at 95 °C for 4 min). All samples, standards and blanks were assayed in duplicate and in the cold (+4 °C). The variability between replicates was 5.6 % for adrenal, 4.9 % for spleen and 4.1 % for plasma. Adequate inactivation of enzyme inhibitors was further tested by adding a predetermined amount of a partially purified bovine adrenal DBH to a duplicate of each sample. Using these aliquots of tissue homogenates and plasma, the recoveries were always greater than 90 %, so that data were not corrected for recoveries. The first step of the reaction was run for 30 and 60 min for tissue and plasma assay, respectively; the second step for 30 min in all cases. One unit DBH activity was defined as the formation of 1 nmol octopamine, measured as ^{14}C-synephrine/h incubation at 37 °C.

The contrast between means was carried out using a double analysis of variance. The variance of random error was isolated and determined by Student's t test, thereby allowing the uniformity of two means to be contrasted. Values are expressed as the mean ± standard errors of the means. A p value < 0.05 was considered to indicate a significant difference.

Results

Plasma dopamine-β-hydroxylase activity in streptozotocin-diabetic rats; correlation with cataract appearance. Plasma DBH activity and glucemia are significantly increased in STZ-diabetic rats with respect to their own basal value before diabetic induction (fig. 1). Experimental animals were classified in three groups according to the development of the disease: a) animals which died between 2 and 10 weeks (27 %), b) animals which developed cataracts after 6 weeks (41%), c) animals without cataracts during the three months of experimental studies (32 %).

After two weeks of diabetes induction the highest increase in plasma DBH, but not in glucemia values, corresponded to group a), which was significant with regard to control animals ($p < 0.001$) and also with regard to group c) ($p < 0.001$) (fig. 1). Plasma DBH activity was significantly greater in animals that would develop cataracts (group b) compared to group c, this increase reaching maximum value two weeks after diabetes induction ($p < 0.001$), taking into account that the diabetic cataracts only appeared 6 weeks after STZ injection. Glucose levels were

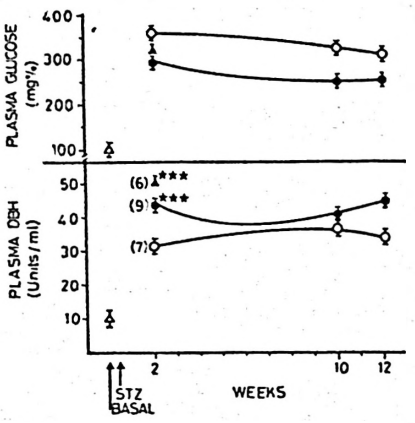

Fig. 1. *Plasma dopamine-beta-hydroxylase (DBH) and glycemia values in streptozotocin (STZ)-diabetic rats.*
Wistar rats injected with STZ, in the 7th week of life, which developed cataracts (●) or not (○), either died (▲) during the evolution period, and basal values (Δ). Each value represents the mean ± SEM with the number of animals in brackets. * * * $p < 0.001$ between diabetic rats without cataracts and the other groups. One unit of DBH is expressed as the formation of 1 nmol synephrine/hour.

not significantly different between the two groups, only having a slow increase in group c).

Tissue dopamine-β-hydroxylase activity in streptozotocin-diabetic rats. Three months after STZ-administration, the diabetic animals showed an increase in adrenal DBH activity, being significant ($p < 0.05$) for diabetic rats (group c) and for diabetic rats developing cataracts ($p < 0.01$, group b) with respect to controls (fig. 2). When a comparison was made between groups b) and c), a significant difference was also obtained ($p < 0.05$). The weight of the adrenal glands was greater ($p < 0.001$) in the diabetic animals than in control ones. When adrenal DBH activity was expressed in mg of this tissue, the only significant increase observed was between control and diabetic rats with cataracts, being 2.2 ± 0.3 and 4.09 ± 0.02 U/mg tissue ($p < 0.001$), respectively. As spleen is one of the most sympathetically innervated tissues, it was chosen for studies of DBH levels in STZ-diabetic rats. As it happened with the adrenal glands, spleen DBH activity increases in both diabetic groups, although it is more significant in animals with cataracts ($p < 0.001$) (fig. 2).

Insulin effect on plasma DBH activity and glucemia values. The mean DBH activities in plasma from several groups are shown in table I and the corresponding glucemia values for the same groups in table I. All animals in the second week of experimental diabetes, just before insulin treatment, showed a significant increase of DBH (42.7±3.1 U/ml, $p < 0.001$) and glucemia (311.7 ± 48.5 mg/100 ml, $p < 0.001$) compared to the control group.

When diabetic animals were treated with insulin, plasma DBH and glucose

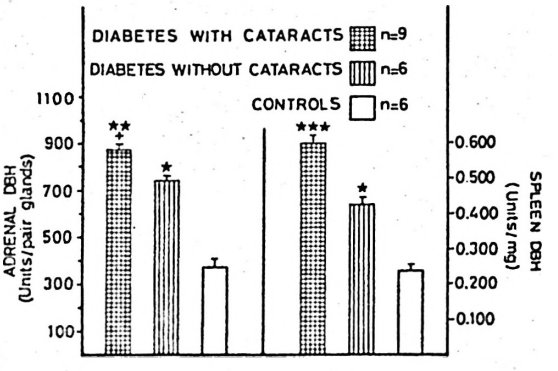

Fig. 2. *Tissue dopamine-beta-hydroxylase (DBH) activity.*
Adrenal and spleen DBH activity in diabetic and control rats after 12 weeks of STZ or vehicle injection, respectively. Each bar represents the mean ± SEM * $p < 0.05$, * * $p < 0.01$, * * * $p < 0.001$ between control and diabetic animals; + $p < 0.05$ between diabetic rats.

Table I. *Plasma dopamine-β-hydroxylase activity and glucemia values in control group and diabetic rats treated with different doses of insulin.*

Wistar rats injected with buffer (control) or STZ in the 7th week of life were treated with different insulin doses. Plasma DBH and glycemia were determined in the 13th and 17th week, 4 and 8 weeks after insulin respectively, except control animals (no insulin). Each value represents the mean ± S.E.M. (U/ml plasma DBH and mg % plasma glycemia) of six animals per group. P was determined by Student's t-test between control and 4th week (p1) and 8th week post-insulin (p2).

Insulin (U.I.)	Plasma DBH (U/ml)				Glucemia values (mg/100 ml)			
	4th week	p1	8th week	p2	4th week	p1	8th week	p2
—	9.6 ± 1.3		8.2 ± 0.9		101.4 ± 2.2		95.7 ± 2.4	
6	10.1 ± 0.5	N.S.	9.8 ± 0.3	N.S.	119.8 ± 35.5	N.S.	115.0 ± 18.7	N.S.
4	14.2 ± 1.1	< 0.02	17.0 ± 1.2	< 0.001	199.3 ± 18.4	< 0.01	167.9 ± 23.2	< 0.01
2	17.1 ± 1.7	< 0.001	18.8 ± 2.3	< 0.001	245.8 ± 34.0	< 0.001	185.9 ± 27.6	< 0.001

diminished in a both similar and dose-dependent way. With 6 I.U/day of insulin, plasma DBH and glucose levels were equivalent to those of control animals, 4 and 8 weeks after treatment. However, using 2 or 4 I.U/day of insulin the high glucose levels were not accompanied by a proportional rise in DBH.

Discussion

The large increase in plasma DBH activity for diabetic animals has been previously reported (4, 24). When these animals are treated with sufficient insulin doses, the plasma DBH levels return to control values showing, with time, an evolutionary pattern similar to that of the control rats (18). When lower insulin doses are administered, plasma DBH activity does not return to control values, a dose-dependent decrease being observed.

A certain parallel exists between DBH and glucose in plasma, since glucose and other non-metabolisable sugar analogues compete with the mannose terminal residue of DBH for clearance at their catabolic receptor, resulting in an increase in the turnover time of this enzyme in plasma (18). Similarly, glucemia levels in the diabetic state are responsible for plasma DBH activity because animals treated with low doses of insulin show a higher DBH activity only if glucemia is also increased.

Nevertheless, when plasma DBH and glucemia were studied at 4 and 8 weeks after insulin treatment (4 and 2 I.U/day) there was no exact ratio between these two parameters, and the higher glucemia values (4th week) did not correspond to the higher plasma DBH activity (8th week). This suggests that during the evolution of the diabetic state, plasma DBH levels are not only affected by clearance but, possibly also by the exocytotic levels of neural tissues (5, 6, 8, 12).

Since plasma DBH is a glycoprotein (15) there could well be a possible link between its levels and the evolutionary pattern of the diabetic state, with complications such as cataracts (13), micro-angiopathies (14, 26), blood coagulation disorders (19), nephropathies (1), etc. In the present paper, the presence of cataracts has been chosen as a parameter of these complications since it is easy to observe and is blood-less. In the experimental conditions, rats usually developed cataracts from the 6th week after STZ-administration. Before the appearance of cataracts a significant increase in plasma DBH activity existed between diabetic rats with cataracts compared to those without. No significant difference could be observed in glucemia values. Thus, plasma DBH values must be influenced not only by glucemia but also by some other factor.

DBH levels in adrenal glands and spleen were therefore studied to identify this influence and to see if any changes take place as already pointed out by some authors (5, 6, 8, 12). Recently, SOCHOR *et al.* (25) have shown an increase in glucose metabolism with adrenal gland hypertrophy in response to the chronic stress resulting from the diabetic state. In the present results, adrenal glands from diabetic animals show a significant increase in their weight and DBH content, the highest increase being for animals with cataracts. Similar behaviour is shown by spleen, taken as representative of sympathetically innervated tissues. The diabetic state always implies a higher activity of the sympathoadrenal axis. On the other hand, if the presence of cataracts is considered as an index of the disease's severity, plasma DBH from these animals showed a greater increase prior to cataracts appearance; the higher adrenal and spleen levels lead to conclude that this increase is due not only to a diminished clearance but also to a pos-

sible increase in the release from tissue source.

Nevertheless, it is necessary to bear in mind that although plasma DBH is a glycoprotein found in smaller quantities, it can serve as a useful parameter, indicative of the presence of other glycoproteins with the same terminal sugar (18, 27). If these proteins accumulate in plasma, they could denaturalize and precipitate in several places, thereby contributing to the pathogenic mechanism in diabetes complications. This could explain the accumulation of positive periodic acid-schiff staining material in the basal membrane of vessels (26) and in diabetic nephropathy (1). Thus, DBH and other glycoproteins with unknown function in plasma can be considered as high-risk proteins and their levels constituting an indicative parameter for further diabetes complications, at least in rats.

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Resumen

La inducción de diabetes mellitus en ratas, por estreptozotocina, produce un gran incremesto de la actividad plasmática de dopamina-beta-hidroxilasa (DBH-p), que se normaliza con dosis suficientes de insulina (6 I.U/día); menores dosis de insulina no la normalizan aunque se observa un descenso dosis dependiente. Aunque la hiperglucemia diabética es responsable de la DBH-p, no hay una relación exacta entre ambos parámetros cuando los animales diabéticos son tratados con distintas dosis de insulina; esto sugiere no sólo la participación del aclaramiento plasmático de la DBH sino también la de los tejidos exocíticos. Antes de la aparición de cataratas, los animales que más tarde las desarrollan presentan una mayor DBH-p que los que permanecen sin esta complicación. Tras 3 meses de evolución diabética, la severidad de la esfermedad es evidente en los animales con cataratas, ya que muestran un aumento importante de la actividad simpatoadrenal, medida como actividad de DBH adrenal y esplénica. La DBH, como glicoproteína minoritaria, puede ser considerada como un parámetro útil de otras glicoproteínas con manosa terminal sin función específica conocida y además como proteína de alto riesgo, de tal manera que su acumulación en diversos tejidos contribuye a la patogenia de las complicacioses diabéticas.

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