The Functional State of Sympathetic Nerves in Spontaneously Diabetic Mice

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SUMMARY

I investigated biochemical parameters of sympathetic nerve function in spontaneously diabetic mice (C57 BL/KsJ *db/db*) and in their lean littermates. The concentration of norepinephrine (NE) in organs innervated by sympathetic nerves was significantly reduced in the heart, kidney, and salivary glands of mice (24 weeks old) with severe diabetic-like symptoms (blood glucose > 300 mg./100 ml.). In the spleen, vas deferens, and adrenal glands of the same animals the NE levels were not changed in relation to control. Other measurements of NE in young (six weeks old) diabetic mice revealed no differences between diabetic and nondiabetic controls.

The turnover of NE, a measure of the functional state of sympathetic nerves, decreased significantly in the heart and salivary glands of 24-week-old mice but remained unchanged in the kidney and spleen. In young, diabetic mice the rates of NE turnover in several organs were similar to those found in age-matched controls. The hearts of 24-week-old diabetic mice contained significantly less dopamine-β-hydroxylase (DBH), an intraneuronal enzyme active in the terminal step of NE biosynthesis. The kidney of the same animals was hypertrophic and showed a massive elevation of monoamine oxidase (MAO), an enzyme that degrades NE to inactive products.

Other experiments showed that the regeneration of sympathetic neurons that follows the reversible chemical denervation with 6-hydroxydopamine was comparable in diabetic and nondiabetic animals. It appears that mice with spontaneous diabetes show changes of sympathetic nerve function similar to those noted in diabetic patients with autonomic neuropathy. DIABETES *27*:969-74, October, 1978.

Neuropathies of the autonomic nervous system, which are common in long-term diabetic patients, cause disturbances of vascular reflexes and cardiac rhythm and frequently produce impairment of reproductive functions.[1,2] Although symptoms of diabetic neuropathy are well defined, the syndrome is still characterized in terms of the functions affected. Nothing is known concerning the nature of the nerve defect or its pathogenesis.

In view of the relevance of neurologic factors to the complications of diabetes, I have investigated the function of the sympathetic nervous system in experimental diabetes. The principal objective of the present investigation was to determine whether nerve lesions similar to those found in diabetic patients with autonomic neuropathy could be reproduced in animals with spontaneous diabetes. In this report, I describe several biochemical parameters of sympathetic nerve function in diabetic mice (*db/db*). Sympathetic nerve activity is partially suppressed in some organs of diabetic mutants; this reduction is related to the severity of the diabetic condition.

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MATERIALS AND METHODS

Male and female C57 BL/KsJ *db/db* (diabetic) and C57 Bl/KsJ (nondiabetic) mice were purchased from Jackson Laboratories. Some mice were used when they were six weeks old, and others were maintained, with food and water ad libitum, up to 24 weeks. Weight gain was recorded in a group of male mice at weekly intervals. Blood glucose was determined on jugular blood by the enzymatic method (Glucostat, Worthington). Norepinephrine (NE) of mouse tissues was estimated by either the spectrophotofluorimetric method[3] or the sensitive radioenzymatic procedure,[4] in which methylation of NE was done with phenylethanolamine-N-transferase (PNMT) and a radiolabeled methyl donor ($[^3H]CH_3$-S-adenosyl-L-methionine). Studies on NE turnover were performed in male mice by measuring the rate of decline of endogenous NE after blocking its synthesis at the level

of hydroxylation of tyrosine, which is the rate-limiting step.[5] The synthesis was inhibited by administering α-methyl-p-tyrosine methyl ester (250 mg. per kilogram intraperitoneally every three hours).[6] Groups of mice (four to five) were killed at two, four, and eight hours after drug administration; a group of untreated mice served as the zero-time group. The turnover rates of NE were calculated by multiplying the rate constants of NE decline (K) by the steady-state amine levels. The intraneuronal enzymes dopamine-β-hydroxylase (DBH), a catalyst for the hydroxylation of dopamine to NE,[7] and monamine oxidase (MAO), which deaminates NE,[8,9] were assayed in heart and kidney homogenates (1:20 in 5 mM Tris buffer, pH 7.5, containing Triton, 0.1 per cent). DBH was determined by a radioenzymatic procedure[10] using tyramine (0.15 mM) as substrate and octopamine (55 pmol) as standard. MAO activity was measured with kynuramine as substrate, and the results were expressed in terms of amount of 4-hydroxyquinoline formed.[11] Both enzymatic activities were measured at 37°C. in air with 30-minute incubation for DBH and 10-minute for MAO. Statistical analysis was performed by Student's *t*-test with paired or unpaired comparisons. Adenosyl-1-methionine S-[methyl-^{3}H] was obtained from New England Nuclear (Boston, Mass.). All other chemicals were obtained from Sigma, St. Louis, Mo. Phenylethanol-amino-N-methyltransferase was isolated from ox adrenal gland.[10]

RESULTS

Obesity and hyperglycemia are features of young (six weeks) diabetic mice (C57 BL/KsJ *db/db*) (table 1). With age, the body weight doubles as compared with control mice, and hyperglycemia becomes more marked (table 1). These data document the inexorable progression of the diabetic condition in these mutants.

Concentrations of norepinephrine (NE) measured in adrenergically innervated tissues of six-week-old diabetic mice were quite similar to those found in nondiabetic controls (table 2). In older (24 weeks) diabetic mice, however, we noted a significant depletion of NE in several tissues (table 2). Highest reductions were observed in salivary glands (45 per cent) and kidney (43 per cent). In other organs of 24-week-old mice, namely spleen, vas deferens, and adrenal glands, there were no significant differences in NE concentration between diabetic and nondiabetic mice.

Since the rate of utilization of transmitter is a function of the nervous activity, the measurements of NE disappearance, after blockade of its synthesis (turnover rates), are an indication of the functional state of sympathetic nerves. Figures 1 and 2 represent the time course of NE decline that follows the administration of α-methyl-p-tyrosine observed in heart and kidney of mildly diabetic (six weeks) and severely diabetic (24 weeks) mice. As shown in figure 1, the rate of decline

TABLE 1

Body weight and blood glucose concentration in male *db/db* mice at different ages

Age (wk.)	Weight (gm.)		Blood glucose (mg./100 ml.)	
	Control	*db/db*	Control	*db/db*
6	16.5 ± 0.68	28.8 ± 1.10	112 ± 10.5	258 ± 6.4
12	26.5 ± 0.40	54.3 ± 0.60	103 ± 7.27	289 ± 12.26
24	31.5 ± 0.79	66.9 ± 1.0	110 ± 8.5	389 ± 6.36

TABLE 2

Norepinephrine concentrations in organs of *db/db* mice at six and 24 weeks of age

Organ	Norepinephrine (ng./gm. ± S.E.M.) 6 Weeks		24 Weeks	
	Control	*db/db*	Control	*db/db*
Heart	453 ± 44 (8)	468 ± 32 (16)	542 ± 16.43 (4)	431 ± 30.62 (4)
Kidney	321 ± 35.5 (8)	308 ± 3.8 (8)	171 ± 0.66 (4)	98.5 ± 11.9 (4)
Salivary gland	2,970 ± 173 (14)	2,310 ± 111 (12)	4,450 ± 180 (4)	2,470 ± 150 (4)
Spleen	675 ± 64 (16)	699 ± 86 (16)	647 ± 105 (4)	719 ± 58 (4)
Vas deferens	—	—	9,814 ± 1,275 (4)	11,547 ± 717 (4)
Adrenal gland*	—	—	5,030 ± 390 (4)	6,460 ± 1,030 (3)

Number of observations is in parentheses. *Values for adrenal gland are expressed in nanograms per pair.

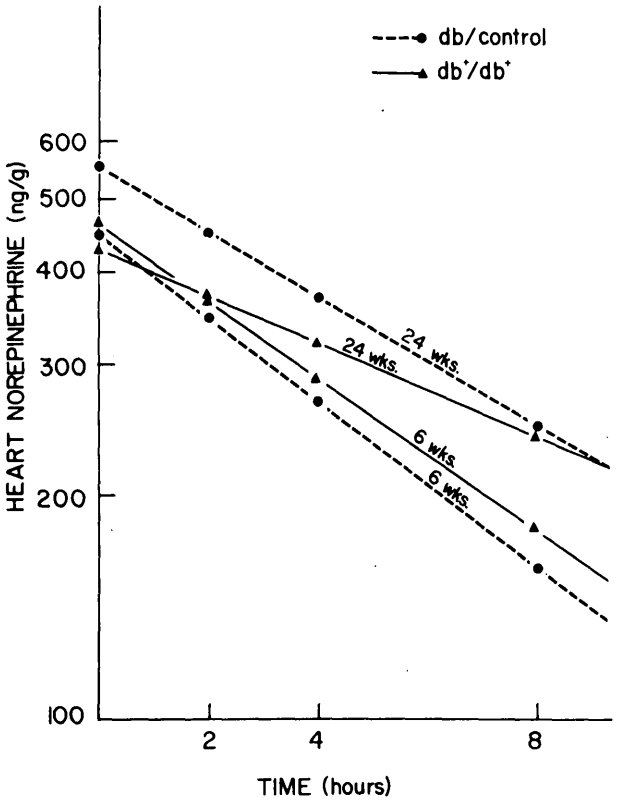

FIG. 1. Turnover of norepinephrine in the hearts of diabetic (C57 BL/KsJ *db/db*) and nondiabetic (C57 BL/KsJ) mice. The lines represent the decline of endogenous norepinephrine after administration of α-methyl-p-tyrosine methyl ester (250 mg. per kilogram intraperitoneally at zero time followed by 125 mg. per kilogram every three hours). Each point is the average of four values. Regression lines were fitted by the method of least square. These slope values (± S.E.) were obtained: 0.13 ± 0.01 (six weeks, control); 0.12 ± 0.02 (six weeks, *db/db*); 0.10 ± 0.02 (24 weeks, control); 0.07 ± 0.01 (24 weeks, *db/db*). Correlation coefficients were: 0.92 and 0.78, respectively, for six-week-old control and diabetic mice; 0.86 and 0.81 for 24-week-old control and diabetic mice.

of cardiac NE is identical in six-week-old diabetic and nondiabetic mice but visibly different in 24-week-old diabetic mice. The values of the slopes were, in fact, similar in the six-week-old animals (0.12 versus 0.13) and 24-week-old controls (0.12) but considerably smaller in the older diabetic (0.07). The values of NE turnover for several organs of diabetic and control mice are summarized in table 3. It is evident that the sympathetic nerves to the heart of old (24 weeks) diabetic mutants had a much slower NE turnover (31.2 ng. per gram per hour) than those of age-matched controls (55.1 ng. per gram per hour).

In the adrenergic nerve terminals of the kidney, there was no difference in the rate of NE disappearance between six-week-old *db/db* mice and controls (figure 2); however, in older mice, I observed a reduction of NE concentration at the steady state in control and diabetic animals (table 3). A closer analysis of the data concerning the pool of NE in the kidneys of diabetic mice (24 weeks old) reveals a marked hypertrophy of the kidney (140 per cent) (table 4) not accompanied by a proportional increase in total NE content; thus, the concentration of amine (nanograms per gram) is decreased (43 per cent, $p < 0.001$) (table 4).

The adrenergic fibers innervating the salivary glands of *db/db* mice showed (figure 3) a considerably slower decline of NE than controls (K values: 0.12 versus 0.24); the steady-state NE pool and the values of turnover were also markedly reduced. In the adrenergic nerves to the spleen of diabetic mice, the rate of transmitter turnover was similar to that observed for controls (table 3).

DBH and MAO Activity in Peripheral Sympathetic Nerves of Diabetic Mice

Since the level of enzymes necessary for the metabolism of NE often reflects neuronal activity, I thought that measurements of activity of DBH, the last catalyst in the synthesis of NE, and of MAO, which inactivates NE mainly intraneuronally, would provide additional information on the functional state of sympathetic nerves. The results of these experi-

TABLE 3
Turnover of norepinephrine (NE) in organs of *db/db* mice at six and 24 weeks of age

		6 Weeks		24 Weeks	
Organ		Conc. of NE at steady state (ng./gm. ± S.E.M.)	NE turnover rate (ng./gm./hr.)	Conc. of NE at steady state (ng./gm. ± S.E.M.)	NE turnover rate (ng./gm./hr.)
Heart	Control	448 ± 27.4	58.5	549.6 ± 16.3	55.1
	db/db	456 ± 34.2	54.0	424.1 ± 30.5	31.2
Kidney	Control	321 ± 35.5	24.1	186.4 ± 0.70	18.7
	db/db	308 ± 3.08	25.5	103.4 ± 1.94	16.1
Spleen	Control	—	—	613.0 ± 105	93.2
	db/db	—	—	731.0 ± 58.5	96.4
Salivary gland	Control	—	—	3,550 ± 183.5	885.4
	db/db	—	—	2,080 ± 158.9	258.3

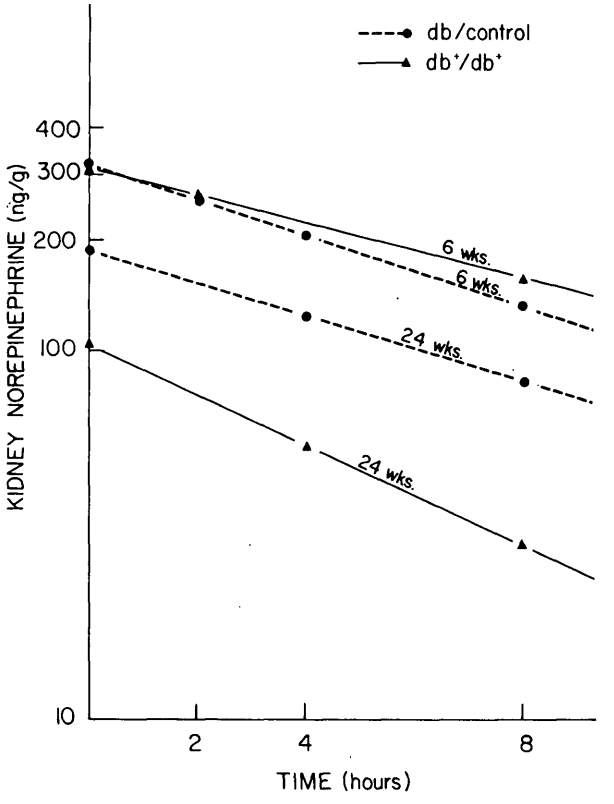

FIG. 2. Turnover of norepinephrine in the kidneys of diabetic (C57 BL/KsJ *db/db*) and nondiabetic (C57 BL/KsJ) mice. Details of treatment are reported in the legend to figure 1. These slope values (± S.E.) were obtained: 0.11 ± 0.01 and 0.08 ± 0.01, respectively, for six-week-old control and diabetic mice; 0.10 ± 0.02 and 0.15 ± 0.02 for 24-week-old control and diabetic mice. Correlation coefficients were 0.86 for six-week-old control and diabetic mice, 0.77 for 24-week-old control, and 0.91 for 24-week-old diabetic mice.

ments, summarized in table 5, show a marked decrease (34 per cent, $p < 0.005$) of DBH in the cardiac nerves of severely diabetic animals and a massive increase (+ 340 per cent, $p < 0.001$) of MAO in the kidneys of the same animals.

Effect of 6-OH-Dopamine on Peripheral Adrenergic Neurons of Diabetic Mice

Administration of the neurotoxic substance 6-OH-dopamine causes a reversible degeneration of

TABLE 4

Kidney weight and norepinephrine (NE) content in 24-week diabetic (*db/db*) mice

Mice	Wet weight (gm.)	NE ng./kidney	NE ng./gm.
Control	0.22 ± 0.01	37.5 ± 1.95	171.0 ± 0.66
db/db	0.53 ± 0.04*	41.5 ± 3.12	98.5 ± 11.9*

Each value is the mean ± S.E. of six observations.
*P < 0.001.

TABLE 5

Dopamine-β-hydroxylase (DBH) and monoamine oxidase (MAO) activities in organs of *db/db* mice

Organ		DBH synephrine formed (nmol/gm./hr.)	MAO 4-hydroxyquinoline formed (nmol/gm./hr,)
Heart	Control	89.8 ± 7.2	696.3 ± 44
	db/db	59.4 ± 2.5*	652.0 ± 60
Kidney	Control	55.5 ± 2.4	118.1 ± 13.1
	db/db	48.5 ± 3.0	406 ± 40†

Values are means ± S.E. of five observations.
Significantly different from controls: *P < 0.005, †P < 0.001.

adrenergic nerves characterized by the disappearance of NE from nerve terminals.[12] The reappearance of NE in nerve endings measures the regenerative properties of the neurons. The levels of NE in organs of diabetic and nondiabetic mice two weeks after the administration of 6-OH-dopamine were compared and expressed as per cent of those found in the respective controls (diabetic and nondiabetic) not treated with the neurotoxin. As shown in figure 4, the resto-

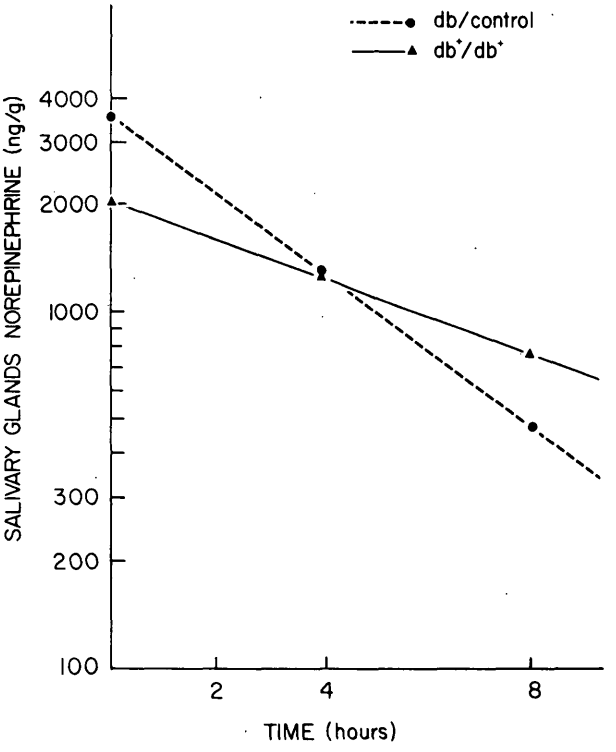

FIG. 3. Turnover of norepinephrine in the salivary glands of diabetic (C57 BL/KsJ *db/db*) and control (C57 BL/KsJ) mice. Treatments are those reported in legend to figure 1. In control animals the slope of NE decline was 0.25 ± 0.04 and the correlation coefficient was 0.88. In diabetic mice the values were slope: 0.12 ± 0.02, and correlation coefficient: 0.73.

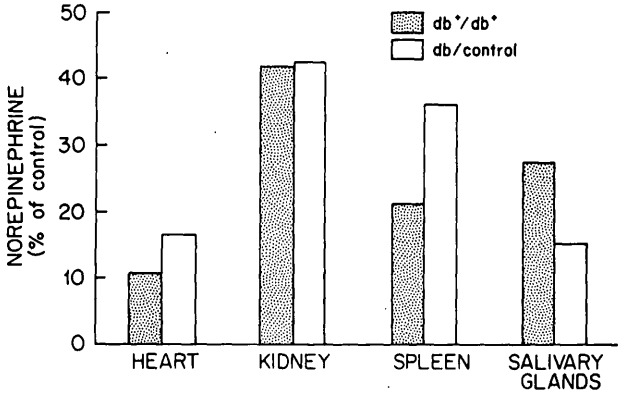

FIG. 4. Restoration of norepinephrine after 6-hydroxy-dopamine in diabetic (C57 BL/KsJ *db/db*) and control (C57 BL/KsJ) mice. 6-OH-Dopamine HBr (250 mg. per kilogram) was given intraperitoneally 15 days before being killed to diabetic and control (24-week-old) mice. Bars refer to five observations for each tissue. Control levels (nontreated mice) or norepinephrine are those reported in table 2.

ration of NE is lowest in the heart (11 per cent for *db/db* and 16 per cent for control) and highest in kidney (42 per cent for both groups). In general, no differences in regenerative properties were noted between the neurons of diabetic mice and their controls; however, the spleen of control animals seemed to recover the NE at a faster rate while the opposite was observed for salivary glands.

DISCUSSION

The results of this investigation indicate that the spontaneous diabetes occurring in mice is associated with changes of peripheral adrenergic nerve function. I find profound depletion of NE in some organs of diabetic mice accompanied by an alteration of the rate of NE turnover in some nerve fibers. These changes of nerve function seem to correlate with the severity of the experimental diabetic syndrome, since they are only evident in animals with marked hyperglycemia and they coincide with the decline of plasma and pancreatic insulin observed in old diabetic mice.[13]

The data on NE turnover suggest an inhibition of sympathetic nerve activity in the heart and salivary glands of diabetic mutants. The decreased levels of DBH (table 5), the enzyme active in NE synthesis, and the slower decline of NE after blockade of its synthesis are clear indications that cardiac adrenergic nerves of diabetic mice synthesize less transmitter and utilize it at a slower rate (table 3).

Recently, Neubauer et al.[14] found a conspicuous decrease of NE concentration in the heart and blood vessels of diabetic patients with autonomic neuropathy. Thus, it appears that diabetes of long standing causes similar changes in the cardiac adrenergic nerves of man and mice.

The adrenergic innervation of the kidney of spontaneously diabetic animals is affected differently than in other tissues. The concentration of NE in this organ is severely reduced compared with controls. However, the loss of transmitter is entirely related to the massive elevation of MAO (table 5), the enzyme that degrades NE to inactive products, and not to a reduced synthesis of NE in the renal nerves, since the turnover of transmitter is comparable to that of control animals (table 3). It is of interest that hypertrophy of the kidneys is common to several species with diabetes, including man.[15,16]

Among several mechanisms that could reduce sympathetic nerve activity in the heart and salivary glands, the possibility that a lack of regenerative properties in the nerve was caused by the diabetic condition was examined. I studied the regeneration of adrenergic nerves observed in animals treated with the neurotoxin 6-OH-dopamine because it has been well characterized.[17] My experiments, however, do not show any difference between diabetic and nondiabetic as far as the restoration of NE tissue levels is concerned and, therefore, I conclude that experimental diabetes does not influence the regeneration of nerves damaged by toxic substances.

In my view, the altered glucose metabolism and the declining plasma levels of insulin, characteristic of diabetic mice, do not influence directly the sympathetic nerve function. If these were causative factors, a diffused change of sympathetic nervous activity would have been observed. In contrast my data show a suppression in selected nerve tracts reaching the heart and salivary glands. The origin of the abnormalities of nerves in diabetes has been the subject of controversy. The prevailing hypothesis favors ischemia, a consequence of vascular disease, as the primary cause of nerve defect. The localized functional lesions of sympathetic nerves observed in this investigation are inconsistent with the ischemic hypothesis, and their presence suggests that the defect of adrenergic nerves is unrelated to the progression of pervasive vascular disease.

It is useful to consider that hyperglycemia might influence cardiac sympathetic activity indirectly through an effect on the central nervous system. Since glucose concentration affects the activity of neurons in the hypothalamus,[18] a brain area that also exercises a powerful control upon the cardiac sympathetic output,[19] it is conceivable that prolonged elevation of glucose might cause the changes of nerve function

noted in diabetic mice. In keeping with this notion of disturbances of central autonomic neurons, I find (unpublished) greatly elevated levels of NE (twice the control) in the hypothalamus of severely diabetic mice (24 weeks old).

Whatever mechanism might be at work, the present observations demonstrate that genetically determined diabetes is accompanied by a gradual deterioration of selected fibers of the peripheral adrenergic system.

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