Changes in the immunoreactivity of protein gene product (PGP) 9.5 in the cochlea of spontaneously diabetic WBN/Kob rats

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Summary The mechanism of hearing impairment due to diabetes mellitus was examined in relation to changes in the level of the immunoreaction for the protein gene product 9.5 in the cochlea of spontaneously diabetic WBN/Kob rats. At 7 months (33 weeks) of age, when half of the males of this strain manifest diabetes, male WBN/Kob rats were divided into two groups as follows: one group consisted of prediabetic animals showing slightly decreased tolerance to glucose with a normal plasma concentration of glucose, normal urinary excretion of glucose, and functional hearing impairment (assessed in terms of elevation of hearing threshold). The second group consisted of diabetic animals with glucose intolerance, high plasma glucose level, polyuria, urinary glucose excretion, and more apparent elevation of hearing threshold. According to morphometric analysis of the spiral

ganglion, the number of ganglion cells was significantly smaller in both the prediabetic and the diabetic animals than in the age-matched control Wistar rats. The staining intensity for protein gene product 9.5 was increased in some spiral ganglion cells of diabetic animals, but decreased in others according to quantitative immunohistochemical analysis. On the other hand, the immunoreactivity for protein gene product 9.5 was similar in the prediabetic animals to that in the control Wistar rats. These results suggest that numerical and immunohistochemical changes in the spiral ganglion cells reflect the onset and degree of the diabetic hearing impairment. [Diabetologia (1997) 40: 173–178]

Keywords PGP 9.5, hearing impairment, diabetes mellitus, WBN/Kob rats, cochlea.

In our previous study, hearing impairment of spontaneously diabetic WBN/Kob rats was first detected as an elevation of the hearing threshold in the auditory brainstem response in animals expressing glucose intolerance, then as a decrease in the number of spiral ganglion cells in the cochlea, and finally as oedematous changes in the stria vascularis [1]. Atrophy of the spiral ganglion was also reported as the most common pathological change in the cochlea of diabetic patients [2]. On the other hand, functional

changes in the spiral ganglion cells caused by diabetes mellitus remain to be examined.

Protein gene product (PGP) 9.5 is a ubiquitin carboxyl-terminal hydrolase [3] prevalent in cells of neural origin [4–6]. Although the functional significance of PGP 9.5 is still unclear, this protein may play an essential role in the ubiquitin-related metabolism in nervous tissues. Recently, PGP 9.5 has been increasingly used as a convenient neural marker in neurophysiological studies [7–12]. In the present study, therefore, we examined the immunoreactivity for PGP 9.5 in the spiral ganglion cells of the cochlea in prediabetic and diabetic WBN/Kob rats by an immunohistochemical method with computer-assisted image analysis and compared the results with those obtained from normal age-matched Wistar rats in order to reveal the functional changes in the spiral ganglion cells from a quantitative point of view.

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Abbreviations: PBS, Phosphate buffered saline; PGP, protein gene product.

Materials and methods

Animals. Fourteen WBN/Kob and seven Wistar male rats were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan) and kept under observation from 1 to 7 months of age. The animals were individually housed in stainless steel cages (310 × 270 × 175 mm) and fed standard rat chow (MB-3; Funabashi Farm, Chiba, Japan) and tapwater ad libitum in an animal room with a ventilation capacity of 15 exchanges of room air per hour under controlled temperature ($23 \pm 2^\circ\text{C}$), controlled relative humidity ($55 \pm 10\%$), and a 12-h light/12-h dark cycle (lights on 07.00 to 19.00 hours).

Antiserum. Rabbit anti-human PGP 9.5 polyclonal serum used in this study was purchased from UltraClone (Wellow, Isle of Wight, UK). The location of the antigen-antibody reaction was visualized by the use of a streptavidin-biotin staining kit (Histofine SAB-PO; Nichirei, Tokyo, Japan).

Clinical examinations. Urine samples were checked for their glucose level with a Multistix (Bayer-Sankyo, Tokyo, Japan) every 2 weeks throughout the experimental period to determine the time of onset of diabetes. At 7 months (33 weeks) of age, when half of the animals examined were diabetic, body weight, urinary volume, and plasma and urinary glucose levels (hexokinase method, autoanalyzer; Cobas Fara, Roche, Basel, Switzerland) were measured in all of the rats. In addition, the animals were treated orally with glucose (2 g/kg), and their plasma glucose levels were determined at 30, 60, and 120 min after glucose administration to ascertain glucose tolerance status.

Auditory brainstem response. A few days after the glucose tolerance test, auditory brainstem response was recorded to estimate the hearing threshold of each rat as described in our previous study [1].

Tissue preparation. The day after the recording of auditory brainstem response, the animals were anaesthetized with pentobarbital sodium (50 mg/kg body weight, i.p.) and perfused transcardially with physiological saline followed by Bouin's fixative without acetic acid for light microscopic immunohistochemistry. After decapitation, the temporal bones including the inner ear were dissected out and immersed in the same fixative for 2–4 h. The specimens were decalcified in Plank-Rychlo's solution [13] overnight and embedded in paraffin by routine procedures. Paraffin sections were cut serially at 4 μm , deparaffinized with xylene, and stained with haematoxylin and eosin (H. E.) or processed for immunohistochemistry.

Immunohistochemical examinations. Deparaffinized sections were treated with 3% H_2O_2 in methanol for 10 min to inactivate endogenous peroxidase, rinsed in phosphate-buffered saline (PBS) for 10 min, and then incubated with blocking serum for 20 min at room temperature. Next, the sections were treated with anti-PGP 9.5 serum (diluted 1:2000) overnight at 4°C . After rinsing 6 times in PBS, 5 min each time, sections were incubated with a biotinylated secondary antibody (goat anti-rabbit IgG) for 30 min at room temperature. After being rinsed 4 times in PBS, 5 min each time, the sections were incubated with streptavidin-peroxidase and washed with PBS under the same conditions as the secondary antibody treatment. Finally, they were treated with 3,3'-diaminobenzidine- H_2O_2 substrate medium, rinsed in tapwater, and counterstained with haematoxylin [14]. The characterization of the antiserum was described elsewhere [5]. Control immunohistochemical stainings were performed with normal rabbit serum or PBS to

Fig. 1. Average body weight of male prediabetic and diabetic WBN/Kob rats and of male Wistar rats at 7 months of age. Each column represents the mean $\pm$ SD ($n = 7$)

replace the antiserum for the confirmation of its specificity. No specific reaction products were observed after the control staining.

Morphometric analysis. Sections stained with H. E. were examined morphometrically with an image analyser (Luzex 3; Nireco, Tokyo, Japan) to measure the area of Rosenthal's canal of the cochlea occupied by spiral ganglion cells for the calculation of the ratio of cell area to the canal area. Immunohistochemically stained sections were also examined to count the number of spiral ganglion cells immunoreactive to the anti-PGP 9.5 serum. The intensity of immunoreaction for PGP 9.5 was graded with the image analyser to determine the number of cells immunopositive for the antiserum as follows: i.e., negative, slight, moderate, or intense. Twenty fields were sampled at random in all turns from the base to the apex of the cochlea and were analysed for each animal.

Statistical analysis

All data are expressed as the mean $\pm$ SD, and analysed statistically by Student's *t*-test. A difference from the respective control was regarded as statistically significant at the $p < 0.05$ level.

Results

Clinical findings. The onset of diabetes in the WBN/Kob rats was observed around the age of 6 months, and the cumulative incidence of diabetes reached 50% (7 of 14) at the age of 7 months. The average body weight of the diabetic WBN/Kob rats was slightly, though not significantly, lower than that of the prediabetic WBN/Kob or the control Wistar rats. There was no difference in the average body weight between the prediabetic WBN/Kob rats and the control Wistar rats (Fig. 1).

The plasma concentration of fasting glucose was significantly higher in the diabetic WBN/Kob rats than in the prediabetic WBN/Kob or the control Wistar rats. In the glucose tolerance test, the plasma

Fig. 2. Blood glucose levels after glucose administration in male prediabetic (▨) and diabetic (■) WBN/Kob rats and in male Wistar rats (□) at 7 months of age. Each column represents the mean $\pm$ SD ($n = 7$). * $p < 0.05$; ** $p < 0.01$

glucose levels were significantly higher in the prediabetic and diabetic WBN/Kob rats than in the control Wistar rats at 30, 60, and 120 min after the oral administration of glucose (2 g/kg), and two to three times higher in the diabetic WBN/Kob rats than in the prediabetic rats. In the prediabetic and diabetic WBN/Kob rats, the plasma glucose levels showed no difference between 30 and 120 min after glucose administration because of the retardation of its disappearance from the plasma. These results are summarized in Figure 2.

The urinary volume and glucose excretion showed a significant and marked increase in the diabetic WBN/Kob rats in comparison with those in the prediabetic WBN/Kob rats or the control Wistar rats (Fig. 3).

Hearing threshold in ABR. The average hearing thresholds of the right ear of the prediabetic and diabetic WBN/Kob rats and of the control Wistar rats were 54 ± 4 , 63 ± 3 , and 39 ± 3 dB hearing level, respectively, and were similar to those of the left ear. The hearing thresholds of the prediabetic and diabetic WBN/Kob rats were significantly higher than the threshold in the control Wistar rats. The hearing threshold was significantly higher in the diabetic WBN/Kob rats than in the prediabetic rats (Fig. 4).

Immunohistochemical findings. In the cochlea of the prediabetic WBN/Kob and control Wistar rats, almost all of the neurons in the spiral ganglion showed moderate immunoreactivity for PGP 9.5 in their cytoplasm and nucleus, and only a few were strongly immunopositive. The cochlear nerve fibers in the spiral ganglion were slight to moderate in immunoreactivity. Immunopositive nerve fibers were also observed within the organ of Corti. Satellite cells in the spiral ganglion were immunonegative for PGP 9.5 (Fig. 5,

Fig. 3. Average urinary volume and glucose excretion in male prediabetic and diabetic WBN/Kob rats and in male Wistar rats at 7 months of age. Each column represents the mean $\pm$ SD ($n = 7$). Significant difference from the prediabetic WBN/Kob rats or the control Wistar rats ** $p < 0.01$

Fig. 4. Average hearing thresholds (right ear) in male prediabetic and diabetic WBN/Kob rats and in male Wistar rats at 7 months of age. Each column represents the mean $\pm$ SD ($n = 7$). Significant differences are as indicated ** $p < 0.01$

Fig. 6A). In the diabetic WBN/Kob rats, the immunoreactivity for PGP 9.5 was almost negative in approximately 30% of the neurons in the spiral ganglion, though it was increased in some of the remaining neurons and was stronger than that in the

Fig 5A,B. Serial sections (A, B) of the cochlea in a control male Wistar rat at 7 months of age. A: H. E., B: PGP 9.5 immunostaining $\times 210$

prediabetic WBN/Kob and the control Wistar rats. There was no difference in the immunoreactivity of the nerve fibers between the prediabetic and diabetic WBN/Kob rats (Figs. 6 A and B).

Morphometric findings. In the cochlea of the prediabetic and diabetic WBN/Kob rats, the spiral ganglion cells were evidently decreased in number. The area of the spiral ganglion cells was significantly decreased in the prediabetic and diabetic WBN/Kob rats in comparison with that in the control Wistar rats (Table 1). The number of spiral ganglion cells negative or slightly immunopositive for PGP 9.5 was significantly larger in the diabetic WBN/Kob rats than in the prediabetic WBN/Kob rats and the control Wistar rats. Additionally, in the diabetic WBN/Kob rats, the number of intensely immunopositive spiral ganglion cells was significantly larger than in the prediabetic and the control animals (Table 2). One out of five prediabetic WBN/Kob rats showed an apparent increase in the number of intensely immunopositive spiral ganglion cells (239 cells) with a very slight decrease in the total number of cells (1352 cells) in comparison with the average numbers, 95 ± 14 cells and 1572 ± 139 cells, respectively, in the control animals.

Fig 6A,B. Spiral ganglion in a male prediabetic (A) and a male diabetic WBN/Kob rat (B) at 7 months of age. In B the number of immunopositive spiral ganglion cells is reduced, and some of the remaining cells show an increased staining intensity for PGP 9.5. PGP 9.5 immunostaining $\times 330$

Discussion

The spontaneous diabetic syndrome has been reported in aged males of the WBN/Kob rat strain [15–22]. These animals show reduced glucose tolerance at 5 months of age and develop the spontaneous diabetic syndrome around 9 months of age [18]. With respect to clinical examinations, half of the WBN/Kob rats used in the present study were normal at 7 months of age in terms of their fasting plasma glucose levels and negative in urinary glucose excretion, but were slightly intolerant for glucose, and thus were regarded as being in the prediabetic state. On the other hand, the remaining animals were judged to be diabetic by their slight loss of body weight, hyperglycaemia, glucose intolerance, polyuria, and glycosuria. These findings suggest that the prediabetic condition rapidly becomes diabetic in the WBN/Kob rats at 7 months of age.

The hearing impairment found in diabetic patients has been reported to be possibly caused by severe atrophy of the spiral ganglion in the cochlea [2]. In the WBN/Kob rats, hearing impairment induced by diabetes is first detected as an elevation of the hearing

Table 1. Average ratio of the area of spiral ganglion cells to the area of Rosenthal's canal in WBN/Kob and Wistar rats

Strain	State	No. of animals	Ratio of cell area to canal area (%) ^a
Wistar	Normal	5	73.6 ± 3.7
WBN/Kob	Prediabetic	5	55.2 ± 9.3
	Diabetic	5	49.5 ± 6.4

^a Twenty fields of Rosenthal's canal per animal were examined. Significant difference ^b $p < 0.01$

threshold along with glucose intolerance, and, secondly, as an extensive decrease in the number of spiral ganglion cells in the cochlea [1]. In the present study, the hearing threshold was significantly higher in both prediabetic and diabetic WBN/Kob rats than in the control Wistar rats, and was also significantly higher in the diabetic than in the prediabetic rats. Morphometric analysis revealed that the population of the spiral ganglion cells was significantly decreased in the prediabetic and diabetic WBN/Kob rats at 7 months of age in comparison with that of the age-matched control Wistar rats.

PGP 9.5 is a novel neuron-specific protein found in all types of mammalian nerves [4, 7–10]. This protein is uniformly distributed in the cytosol and nucleoplasm as a soluble protein in the spiral ganglion cells of the cochlea [11, 12]; therefore, changes in the immunoreactivity for anti-PGP 9.5 serum may reflect structural and functional alterations of these cells. In the present study, quantitative immunohistochemical analysis using an image analyser demonstrated that the number of spiral ganglion cells immunopositive for PGP 9.5 was significantly decreased in the diabetic WBN/Kob rats, whereas that of the prediabetic animals was comparable to that of the age-matched controls. In addition, the immunopositive spiral ganglion cells showed a more intense immunoreaction in the diabetic WBN/Kob rats than in the prediabetic and the control animals. These results may suggest an important relationship between the onset and extent of diabetes and the PGP 9.5-immunoreactivity. Since PGP 9.5-immunoreactivity was increased at the early stage of diabetes, but decreased at the later stage in cutaneous nerves of diabetic patients [10], the strength of PGP 9.5-immunoreactivity may reflect

the amount of PGP 9.5, and the amount of PGP 9.5 may be variable in the spiral ganglion cells according to the diabetic stage. In addition, since one animal in the prediabetic stage showed an apparent increase in the number of intensely immunopositive spiral ganglion cells with a very slight decrease in the total number of cells, the staining intensity for PGP 9.5 may increase in the spiral ganglion cells prior to their death.

In the diabetic WBN/Kob rats, the number of spiral ganglion cells with intense immunoreactivity for PGP 9.5 was significantly increased. The number of spiral ganglion cells with intense immunoreactivity in the control group was comparable to the staining in the prediabetic WBN/Kob rats and less than that in the diabetic WBN/Kob rats. Although intensely immunopositive cells were suggested to be type II ganglion cells [12], their intense immunoreactivity seems to reflect the difference in functional state of ganglion cells regardless of whether they were type I and type II cells.

As PGP 9.5 is a ubiquitin carboxyl-terminal hydrolyase [3], the present results suggest that the degree of hearing impairment induced by diabetes is associated with the ubiquitin-related metabolism in the spiral ganglion cells. The intense immunoreactivity for PGP 9.5 may suggest the increase of waste protein in ganglion cells to be decomposed through ubiquitin. Anomalous accumulation of ubiquitin immunoreactivity was reported in the rat brain during normal aging [23]. On the other hand, the reduced immunoreactivity for PGP 9.5 in the diabetic animals may imply the decrease in the amount of ubiquitin in a subset of spiral ganglion cells, in this population.

In conclusion, the present results suggest that the diabetic hearing impairment is characterized by the decline in the spiral ganglion cell population with an elevation of hearing threshold, and followed by their functional alteration as indicated by the change in their immunoreactivity for PGP 9.5. The present results also suggest that the immunohistochemical analysis of PGP 9.5 is a useful technique with which to demonstrate the development of diabetic neuropathy.

Table 2. Average number of spiral ganglion cells immunoreactive for PGP 9.5 in WBN/Kob and Wistar rats

Strain	State	No. of animals	Total number of cells ^a	PGP 9.5-immunoreactive cells		
				Negative or slight	Moderate	Intense
Wistar	Normal	5	1572 ± 139	48 ± 42	1429 ± 136	95 ± 14
WBN/Kob	Prediabetic	5	1195 ± 126	66 ± 80	1007 ± 70	122 ± 71
	Diabetic	5	1067 ± 148	312 ± 142	431 ± 59	324 ± 77

^a Cells in 20 fields of Rosenthal's canal per animal were counted. ^b $p < 0.01$

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