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Original Article

Superimposition of hypertension on diabetic peripheral neuropathy affects small unmyelinated sensory nerves in the skin and myelinated tibial and sural nerves in rats with alloxan-induced type 1 diabetes

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Abstract: Diabetic peripheral neuropathy (DPN) is a major complication of diabetes mellitus, and hypertension is considered to be a risk factor for DPN in patients with type 1 diabetes (T1DM). However, the morphological effects of hypertension on DPN are unclear. In this study, we investigated the effect of hypertension on DPN by investigating the changes in unmyelinated and myelinated nerve fibers in hypertensive rats with alloxan (AL)-induced T1DM. Thirteen-week-old WBN/Kob rats with AL-induced diabetes were allocated to receive tap water only (AL group), tap water containing 0.5% saline (0.5AN group), or tap water containing 0.75% saline (0.75AN group) for 15 weeks. Hyperglycemia was maintained for 15 weeks, and the animals were euthanized at 28 weeks. By 23 weeks of age, the systolic blood pressure was significantly higher in the 0.75AN and 0.5AN groups than in the AL group and was unchanged in all groups at 28 weeks. The number of intraepidermal sensory unmyelinated nerve fibers was significantly smaller in the 0.75AN and 0.5AN groups than in the AL group. The axonal size in the myelinated tibial and sural nerve fibers was significantly smaller in the 0.75AN group than in the AL group. Furthermore, luminal narrowing and endothelial hypertrophy were observed in the endoneurial tibial nerve vessels in the 0.75AN group. These findings suggest that superimposing hypertension on hyperglycemia may accelerate a reduction in the number of small unmyelinated sensory nerve fibers in the skin and induce mild axonal atrophy in myelinated tibial and sural nerve fibers in rats with AL-induced T1DM. (DOI: 10.1293/tox.2020-0003; J Toxicol Pathol 2020; 33: 161–169)

Key words: diabetes, hypertension, peripheral neuropathy, rat, skin

Introduction

Diabetic peripheral neuropathy (DPN) is one of the major complications of diabetes and the number of patients affected increases with age[1]. Although its exact pathogenesis is not fully understood, the duration of hyperglycemia, poor glycemic control, and impaired insulin action have a strong impact on the development of neuropathy[2]. Poor glycemic control is likely to be the strongest risk factor for worsening neuropathy, but hypertriglyceridemia, elevated body mass index, and smoking also play important roles[3–5]. However, the role of hypertension in DPN remains uncertain[6]. A significant relationship has been found between hypertension and the incidence of neuropathy in patients with type 1 diabetes (T1DM)[3, 7, 8] but not in those with type 2 diabetes (T2DM)[9–15]. The current evidence also supports an association between hypertension and neuropathy in T1DM but not in T2DM[16].

Hypertension has been shown to make a mild additive contribution to DPN in animal models of T1DM (spontaneously hypertensive rats [SHR] with streptozotocin [STZ]-induced diabetes) and T2DM (a genetic hybrid between SHR and Zucker diabetes fatty rats)[17–19]. A previous study by our group also showed slight worsening of myelinated nerve and vascular lesions in rats with the alloxan (AL)-induced T1DM and hypertension[20]. The results of these animal studies indicate that hypertension may have an adverse effect on myelinated sciatic, tibial, and sural nerve fibers. However, there has only been one study on the effect of hypertension on unmyelinated nerve fibers in an animal model of diabetes[17]. That study analyzed intraepidermal sensory unmyelinated nerve fibers (IENFs), which are standard sites for morphologic analysis in DPN studies[17, 19, 21, 22], in SHR rats with STZ-induced T1DM, but failed to show that hypertension affected unmyelinated sensory nerve fibers in T1DM.

Human DPN is characterized by loss of nerve fibers,

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axonal degeneration, and segmental demyelination with a slowing of nerve conduction velocity[2]. Of the many diabetic animal models that have been used to clarify the pathogenesis of neuropathy, AL-induced T1DM model is the most reliable in terms of rapid development of overt hyperglycemia, slowing of nerve conduction velocity, and mild axonal atrophy, but generally does not include overt degenerative neuropathy, demyelination, and loss of peripheral nerve fibers. Diabetic male Wistar Bonn Kobori (WBN/Kob) rats, which develop endocrine insufficiency due to chronic pancreatitis, spontaneously develop endoneurial microangiopathy, diabetes, and severe motor DPN characterized by segmental demyelination and axonal atrophy with slowing of nerve conduction velocity[20, 23–28]. Therefore, it seemed probable that hyperglycemia-related morphologic changes in the peripheral nerves would be found in WBN/Kob rats with diabetes. However, male WBN/Kob rats only show overt hyperglycemia and glucosuria from about 40–60 weeks of age. Therefore, we developed an animal model in which WBN/Kob rats are treated with AL in order to induce T1DM at an early age[27].

The aim of the present study was to investigate the morphologic effect of hypertension created by exposure to low-dose saline on DPN in WBN/Kob rats with AL-induced T1DM.

Materials and Methods

Animals and housing conditions

Male WBN/Kob rats were sourced from Japan SLC, Inc. (Hamamatsu, Japan) and housed in stainless steel cages at a temperature of 20–26°C and relative humidity of 40–70% on a 12/12-h light/dark cycle. The cages were ventilated with fresh filtered air and all rats had free access to tap water and a widely used standard pellet diet for experimental rats (Charles River Formula 1, Oriental Yeast, Tokyo, Japan). During all experimental procedures, the animals were handled in accordance with the principles outlined in the Guide for the Care and Use of Laboratory Animals prepared by our institution (Setsunan University) and the Japanese Association for Laboratory Animal Science. The study was approved by the Committee for Animal Experiments of Setsunan University.

Experimental design

A total of 27 10-week-old male WBN/Kob rats received a single injection of AL (Sigma-Aldrich Japan, Tokyo, Japan) via the tail vein at a dosage of 40 mg/kg. This dosage allows the maximum survival time in rats after the development of signs of diabetes, and induces continuous glycosuria. At 13 weeks of age, the rats were started on tap water only (AL group), tap water containing 0.5% saline (0.5AN group), or tap water containing 0.75% saline (0.75AN group). The saline preparations were sourced from Wako Pure Chemical Industries (Osaka, Japan). The sodium chloride concentrations were selected on the basis of our preliminary findings regarding saline-induced hypertension in rats. A total of 7 rats became moribund or died as a result of necropsy-confirmed ascending urinary tract infection and systemic edema during the study period. The remaining rats were sacrificed at 28 weeks of age for morphological examination.

Monitoring of glycosuria, glycemia, and blood pressure

Urinary glucose levels were measured semiquantitatively in fresh urine using urine test paper (Wako Pure Chemical Industries). Blood glucose levels in the tail vein samples were measured semiquantitatively using the glucose oxidase method (Glutest E; Sanwakagaku, Nagoya, Japan). Urinary and blood glucose levels were measured at monthly intervals. Blood samples from the tail vein and fresh urine samples were collected between 1:00 pm and 4:00 pm to measure the fasting blood glucose level. Blood pressure was measured by the tail cuff method using a non-invasive blood pressure monitor for mice and rats (MODEL MK-2000; Muromachi Kikai Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions as previously reported[20, 27]. The mean of five consecutive measurements was recorded.

Motor and sensory nerve conduction velocity studies

At the end of the experiment, motor and sensory nerve conduction velocities were measured after the rats were anesthetized with ketamine (Ketalar; Sankyo, Tokyo, Japan; 40 mg/kg) and xylazine (Seractal; Bayer, Tokyo, Japan; 2.0 mg/kg) administered intramuscularly (IM). For motor nerve conduction velocity studies, the right sciatic nerve was exposed by making skin incisions at regular intervals between the great trochanter and ankle as previously reported[20, 27]. Bipolar stimulating electrodes were placed on the nerves through the incisions, and bipolar recording electrodes were inserted percutaneously onto either the interossei or lumbrical muscles. Sensory nerve conduction velocity was determined by stimulating the sural nerve distally at the ankle via bipolar electrodes with supramaximal stimulation and recording at the fourth and fifth digits. The conduction velocity was calculated from the onset latency and interelectrode distance values obtained by a Polygraph 360 electromyography system (Nippon-denki-sanei, Tokyo, Japan) with the BioSignal processing program (Nihonsanteku, Osaka, Japan). Hind limb skin temperature was maintained at 37°C.

Histological analysis of peripheral nerves

The animals were euthanized by exsanguination via the abdominal aorta under deep anesthesia with ketamine (Ketalar; 40 mg/kg IM) and xylazine (Seractal; 2.0 mg/kg IM). The right tibial and sural nerves were removed and fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Samples were trimmed, dehydrated in an automated processor, and embedded in paraffin. The left tibial and sural nerves were removed and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). After fixation, the tissue samples were post-fixed in 1.5% osmium tetroxide solution (pH 7.4) for 2 h and processed

into epoxy resin. Semi-thin (1-μm) sections were cut and stained with toluidine blue.

Intraepidermal nerve fiber density

Foot pads were collected from the plantar surface of the hind paw and fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Samples were trimmed, dehydrated in an automated processor, and embedded in paraffin. Sections (80-μm thick) were deparaffinized in xylene and rehydrated with graded ethanol. The slides were rinsed with 0.05 M Tris-buffered saline (pH 7.6), treated with 1% hydrogen peroxide in methanol, and rinsed again with 0.05 M Tris-buffered saline. The slides were incubated with 5% normal goat serum for 5 min and then overnight at 4°C with rabbit polyclonal anti-PGP9.5 antibody (Dako, Santa Clara, CA, USA; diluted 1:200)[29]. The sections were exposed for 60 min to Alexa Fluor 488-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). The slides were then mounted with mounting medium. The number of IENFs crossing the dermal-epidermal junction was quantified according to the guidelines published by the European Federation of Neurological Societies[21]. Five randomly chosen tissue sections from each animal were quantified. Only single IENFs crossing the dermal-epidermal junction were counted, with exclusion of secondary branches and nerve fragments not crossing the dermal-epidermal junction. The data are presented as the number of fibers per millimeter.

Morphometric analysis of tibial and sural nerves

For morphometric analysis, as previously reported[20, 27], semi-thin cross-sections of the distal portions of the tibial and sural nerves were used, with one section of each nerve used per animal. For the tibial nerve samples, a terminal nerve portion approximately 5-mm long from just proximal to the branching of the lateral and medial plantar nerve was used. For the sural nerve samples, a terminal nerve portion approximately 5-mm long from just proximal to the terminal branching was used. Digital images (3,900 × 3,090 pixels; 20× objective lens) were captured using a DC450 camera attached to a DM5500 light microscope (both from Leica Microsystems, Wetzlar, Germany). The sections were analyzed morphometrically by image processing and analysis software (IP Lab version 4.0; BD Biosciences, Rockville, MD, USA). The following morphometric parameters were analyzed: 1) Total fascicular area; 2) number and size (cross-sectional area) of myelinated nerve fibers, myelin, and axons; and 3) mean fiber, axon, and myelin size (cross-sectional area). The fiber occupancy (nerve fiber area/fascicular area) was calculated by dividing the total area of myelinated fibers by the total fascicular area. The fiber density (number of fibers/mm^2) was calculated by dividing the total number of myelinated fibers by the total fascicular area. Histograms for the size frequencies of nerve fibers, axons, and myelin were constructed and the data presented in increments of 10 μm^2.

Statistical analysis

The data are presented as the mean ± standard deviation. A multiple comparison test was performed to detect differences between the three groups. Homogeneity of variance was analyzed by Bartlett's test followed by one-way analysis of variance when the variance was homogeneous. If a significant between-group difference was found, Tukey's test (parametric) was performed to test the differences between mean values. When the variance was heterogeneous, the Kruskal-Wallis *H* (Wilcoxon) test was performed; if a significant difference was found between the groups, the Steel-Dwass test (nonparametric) was performed to test the differences between mean values. All statistical analyses were performed using JMP Academic Suite 13Pro software (SAS Institute Inc., Tokyo, Japan). A P-value < 0.05 was considered statistically significant.

Results

Body weight, glycosuria, and glycemia

There was no significant change in mean body weight between the three groups from 13 weeks of age to the time of scheduled necropsy (0.75AN group, 330.3 ± 24.7 g; 0.5AN group, 327.6 ± 33.0 g; AL group, 322.4 ± 26.3 g). Severe hyperglycemia (>300 mg/dL) and glycosuria (>500 mg/dL, data not shown) continued from the day of AL injection to the time of necropsy (28 weeks) in the three groups (Supplementary Fig. 1). None of the rats had any gait abnormalities up until the time of scheduled necropsy.

Blood pressure

At 23 weeks of age, systolic blood pressure was significantly higher in the 0.75AN and 0.5AN groups (223.7 ± 7.8 mmHg and 178.9 ± 21.9 mmHg, respectively) than in the AL group (118.7 ± 8.5 mmHg); it was also significantly higher in the 0.75AN group than in the 0.5AN group (Fig. 1). The systolic blood pressure in each study group remained almost constant from 23 to 28 weeks.

Motor and sensory nerve conduction velocity

The results of the motor and sensory nerve conduction studies were similar between the three groups (Supplementary Fig. 2).

Intraepidermal nerve fibers

Significant differences in IENFs were observed between the AN and AL groups (Fig. 2). The number of IENFs crossing the dermal-epidermal junction was significantly smaller in the 0.75AN and 0.5AN groups than in the AL group, with no significant difference between the 0.75AN and 0.5AN groups. The epidermal thickness was similar in the three groups, and there were no cases of ulceration or inflammation.

Morphology of the tibial and sural nerves

Myelinated tibial nerve fibers showed slight axonal atrophy in about half of the animals in the 0.75AN group

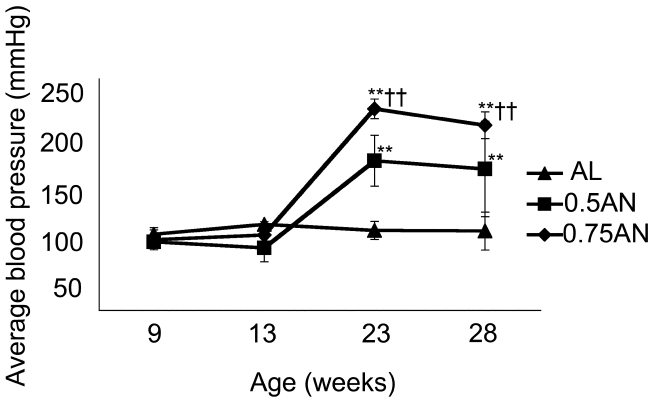

Fig. 1. Changes in systolic blood pressure in diabetic rats with hypertension. The data are expressed as the mean and standard deviation.***P*<0.01 vs. alloxan (AL), ††*P*<0.01 vs. 0.5AN. AL group, rats with alloxan-induced diabetes that received drinking water without saline; 0.5AN group, rats with alloxan-induced diabetes that received 0.5% saline drinking water; 0.75AN group, rats with alloxan-induced diabetes that received 0.75% saline drinking water.

compared to the AL and 0.5AN groups, and the mean axon size tended to be smaller in the 0.75AN group than in the AL group (Fig. 3, Table 1). The frequency histogram for axons in the tibial nerve showed a significant shift to a smaller size in the 0.75AN and 0.5AN groups when compared to the AL group (Fig. 5). Furthermore, the nerve fiber occupancy tended to be lower in the 0.75AN group than in the AL and 0.5AN groups (Table 1). Formation of a myelin ovoid, extension of the endoneurium, luminal narrowing with endothelial hypertrophy, and thickening of the vascular wall were also evident (Fig. 3 and 4). The sural nerves were structurally similar in all three groups; however, the frequency histogram for axons in the sural nerve showed a significant shift to a smaller size in the 0.75AN and 0.5AN groups compared to that in the AL group (Fig. 5).

Discussion

Our present findings show that superimposing hypertension on type 1 DPN accelerates a reduction in the number of small unmyelinated sensory nerve fibers in the skin. The density of IENFs crossing the dermal-epidermal junction is known to be decreased in humans, rats, and mice with T1DM[21, 22, 30–36]. Progressive loss of IENFs is associated with increasingly severe and painful diabetic neuropathy[37]. To the best of our knowledge, there is limited literature on the relationship between hypertension and small unmyelinated sensory nerve fibers in the skin. Edwards *et al.*[38] found a reduction in the number of active sensory nerve fibers in patients with essential hypertension and sensorimotor deficits. In an SHR model, hypertension had no discernable impact on the number of IENFs crossing the dermal-epidermal junction and superimposition of T1DM on hypertension did not change this number[17]. However, the present study, which

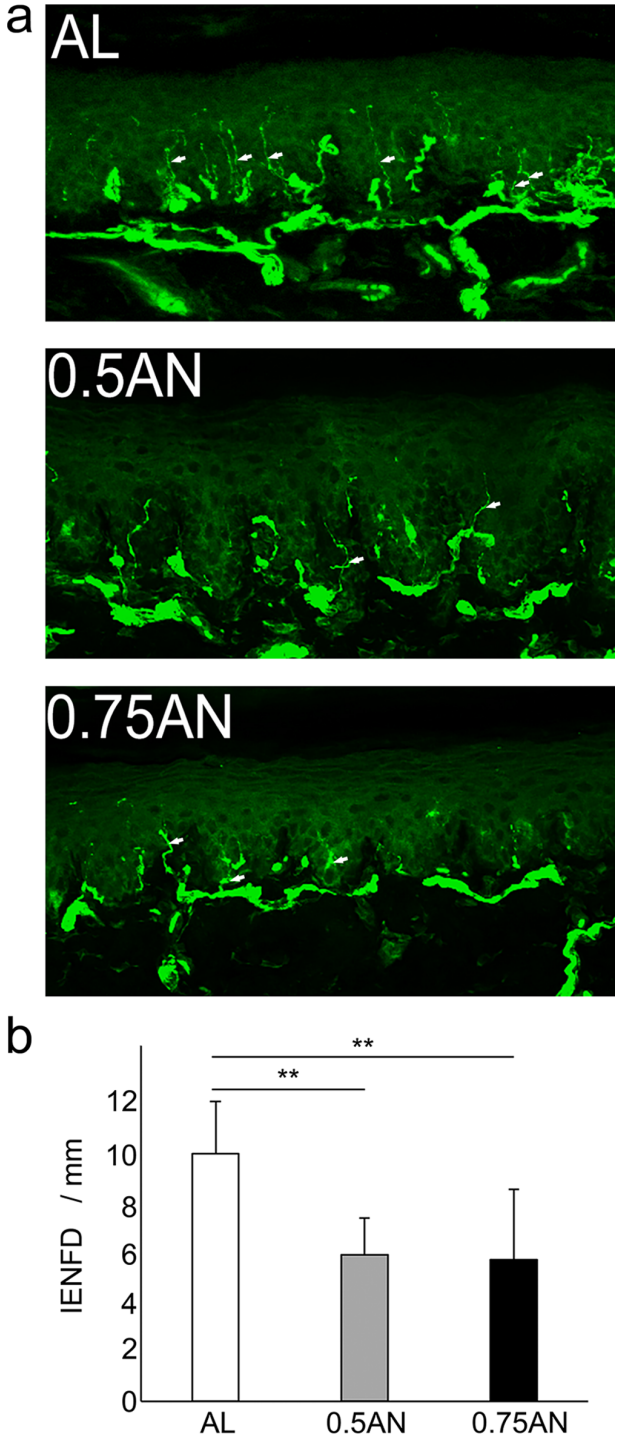

Fig. 2. Images showing a significant decrease in the density of intraepidermal sensory unmyelinated nerve fibers (IENFs) in the hind paw of rats in the 0.5AN and 0.75AN groups. (a) Representative sections of IENFs stained with PGP9.5. (b) The number of IENFs crossing the dermal-epidermal junction is significantly lower in the 0.5AN and 0.75AN groups than in the alloxan (AL) group. The data are expressed as the mean and standard deviation. ***P*<0.01. AL group, rats with alloxan-induced diabetes that received drinking water without saline; 0.5AN group, rats with alloxan-induced diabetes that received 0.5% saline drinking water; 0.75AN group, rats with alloxan-induced diabetes that received 0.75% saline drinking water; IENFs, intraepidermal nerve fibers.

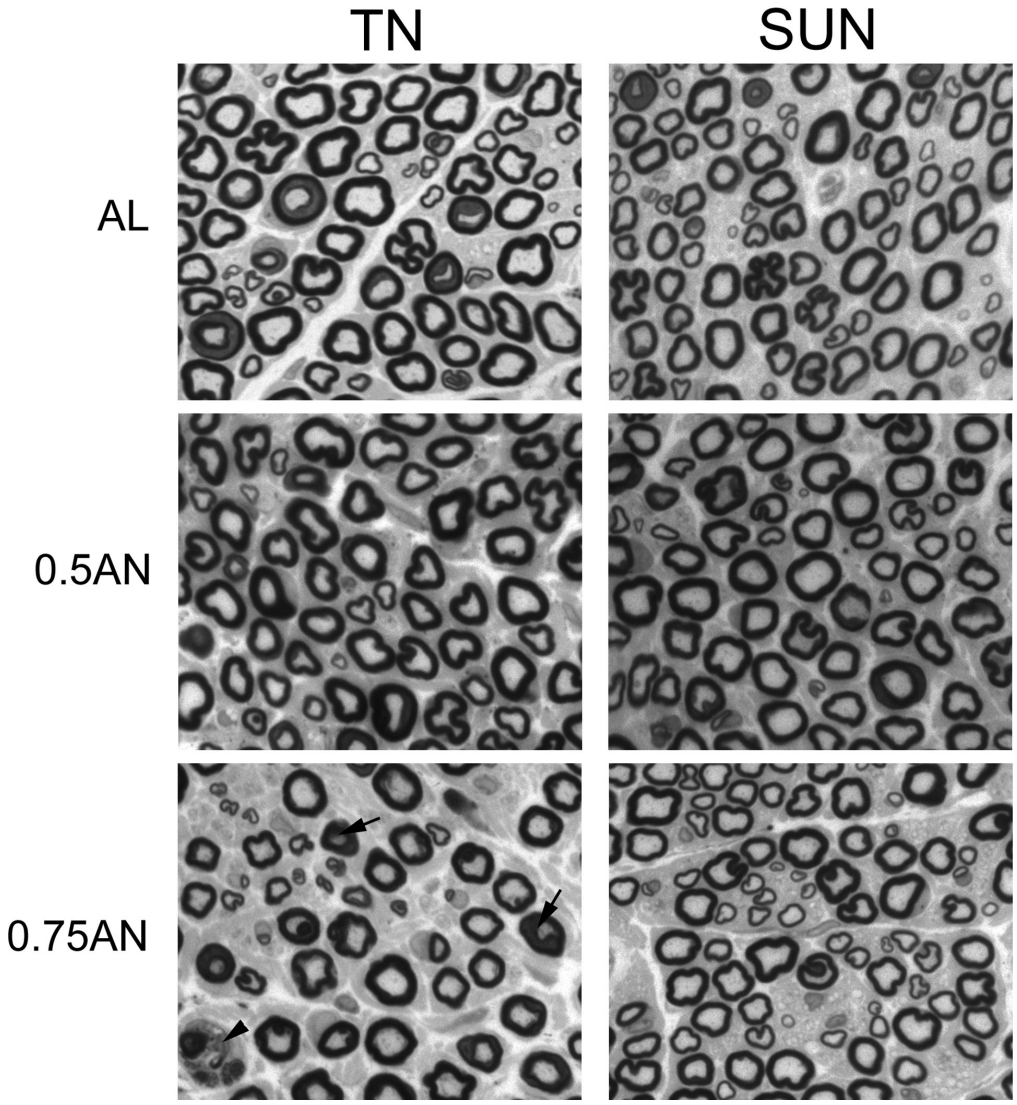

Fig. 3. Representative semi-thin sections of the tibial (TN) and sural (SUN) nerves in the three groups. Myelinated nerve fibers in the 0.75AN group show axonal atrophy (arrows), distension of the myelin sheath, and a myelin ovoid (arrowhead). Alloxan (AL) group, rats with alloxan-induced diabetes that received drinking water without saline; 0.5AN group, rats with alloxan-induced diabetes that received 0.5% saline drinking water; 0.75AN group, rats with alloxan-induced diabetes that received 0.75% saline drinking water.

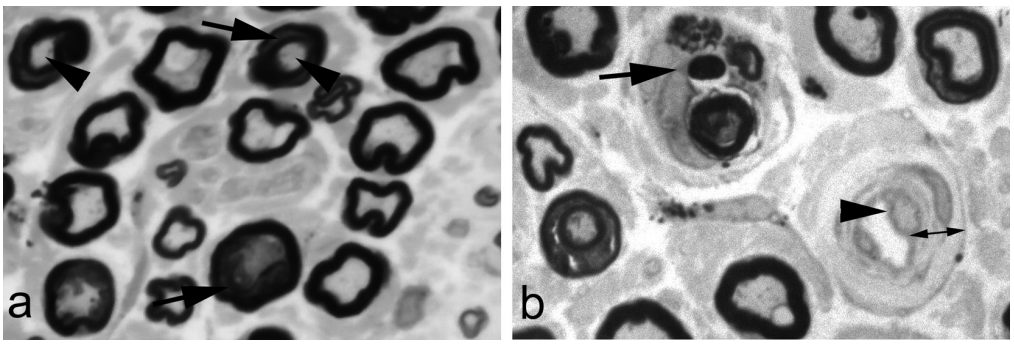

Fig. 4. Representative semi-thin tibial nerve sections in the 0.75AN group. This figure is a magnified view of Fig. 3. (a) Myelinated nerve fibers showing axonal atrophy (arrowheads) and distension of the myelin sheath (arrows). (b) Myelinated nerve fiber shows myelin ovoid (arrow). In endoneurial vessels, luminal narrowing was observed with endothelial hypertrophy (arrowhead) and thickening of the vascular wall (double-headed arrow).

Table 1. Morphometric Analysis of Tibial and Sural Nerves

		Nerve fiber occupancy (%)	Axon/fiber ratio	Mean fiber size (μm²)	Mean myelin size (μm²)	Mean axon size (μm²)
Tibial nerve						
AL group	Mean	52.16	0.34	53.89	36.45	17.44
	SD	5.55	0.04	5.32	3.58	2.67
0.5AN group	Mean	49.62	0.37	52.39	32.95	19.44
	SD	3.32	0.06	7.13	5.27	4.11
0.75AN group	Mean	41.55	0.30	50.26	35.35	14.91
	SD	8.22	0.06	3.07	2.58	2.72
Sural nerve						
AL group	Mean	44.73	0.37	50.01	31.99	18.02
	SD	2.11	0.01	8.81	5.45	3.41
0.5AN group	Mean	48.25	0.36	48.85	31.02	17.83
	SD	4.30	0.06	2.69	3.87	2.73
0.75AN group	Mean	49.14	0.27*†	50.90	36.42	14.49
	SD	2.17	0.03	5.67	3.38	2.69

*P<0.05 vs. alloxan (AL) group; †P<0.05 vs. 0.5AN group (Tukey's test).

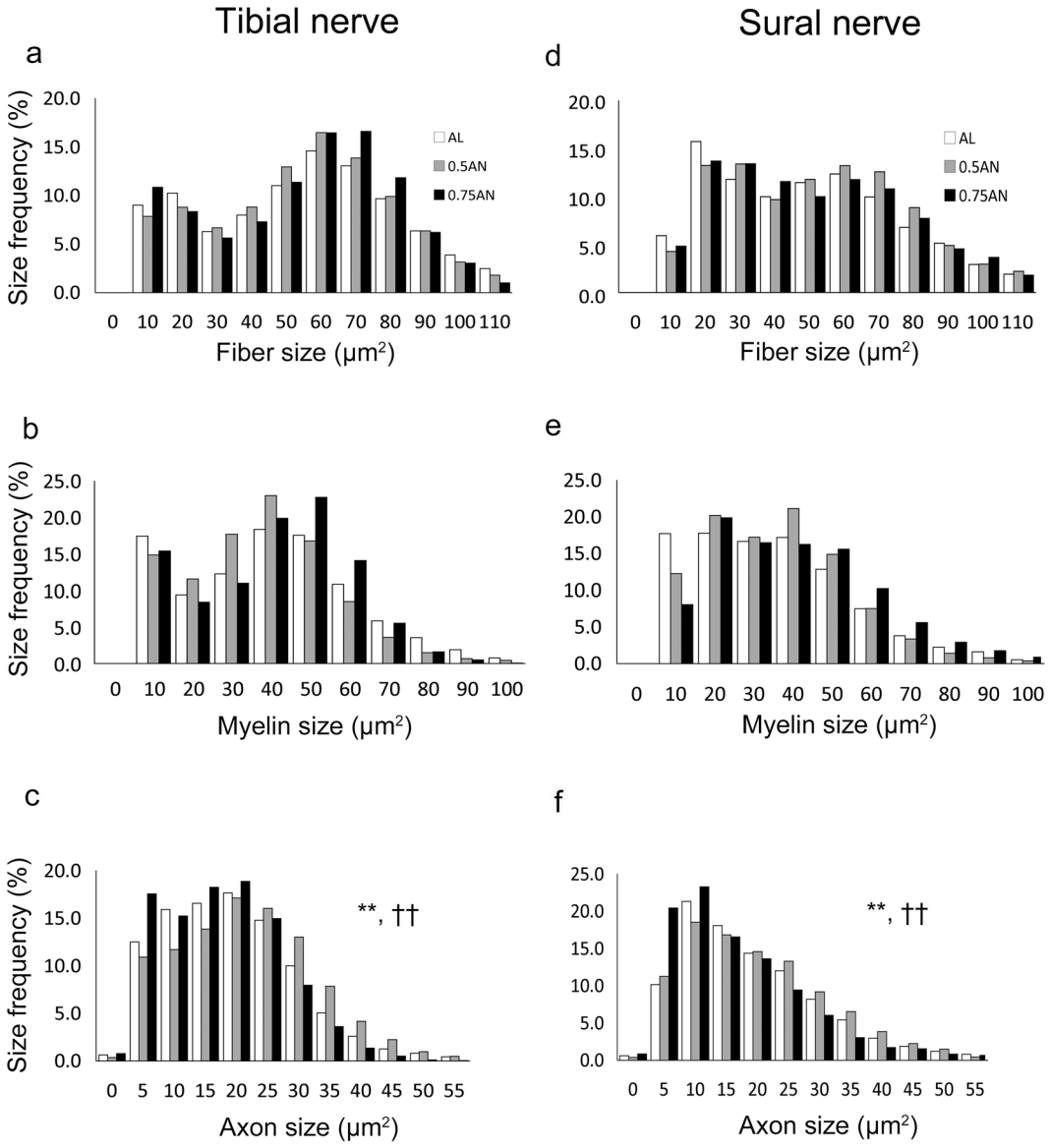

Fig. 5.

was performed in a more suitable animal model, i.e., WBN/Kob rats with T1DM, clearly shows that hypertension can decrease the number of IENFs crossing the dermal-epidermal junction. Our previous study found no significant decrease in unmyelinated small sensory nerve fibers in WBN/Kob rats with T1DM[27]. It was suggested that hypertension could cause worsening of small sensory fiber neuropathy in T1DM.

Moreover, we found that the axons of myelinated tibial and sural nerve fibers were significantly smaller in diabetic rats treated with saline than in those not treated with saline. Axonal atrophy has previously been observed in rats and mice with AL-induced diabetes, and axonal lesions are one of the characteristic changes seen in rodent models of DPN[20, 39–41].Therefore, the axons of the myelinated nerve fibers in all three groups in the present study were considered to be affected by diabetes, as in the previous reports[20, 39–41]. However, hypertension has been reported to affect small myelinated fibers and vessels in the sural nerve in the SHR rat[42, 43]. Sanada *et al.*[42] found that large fibers showed more obvious changes in the myelin sheath, whereas small fibers showed axonal atrophy. Moreover, the vascular supply to the peripheral nerves was found to be impaired by thickening of the arterial wall and luminal narrowing in the interfascicular arteries in the SHR rat[43]. Superimposing severe hypertension on hyperglycemia may accelerate axonal atrophy in small myelinated nerves, hypertrophy of endothelial cells, and luminal narrowing in endoneurial arteries[17, 19, 20]. In the present study, we observed axonal atrophy in myelinated nerve fibers and vascular lesions in hypertensive T1DM rats, which is consistent with the findings of the previous studies. Therefore, hypertension may worsen axonal atrophy in myelinated small nerve fibers in T1DM-related DPN.

The density of IENFs, which are the distal sensory nerve terminals, was significantly decreased by hypertension in the 0.75AN and 0.5AN groups; however, axonal atrophy in the proximal sensory sural nerve was only detected in the 0.75AN group and was not accompanied by a decrease in conduction velocity. These findings suggest that the effect of hypertension on DPN may progress from the distal nerve terminal to the proximal myelinated portion of the nerve, similar to that observed in an STZ-induced animal model of DPN[44, 45].

In conclusion, the present study suggests that superimposing severe hypertension on hyperglycemia may accelerate a reduction in the number of unmyelinated small sensory nerve fibers in the skin and induce mild axonal atrophy of myelinated tibial and sural nerve fibers in rats with AL-induced T1DM. Therefore, the effects of hypertension on myelinated nerve fibers may be different from those on unmyelinated nerve fibers in T1DM.

Disclosure of Potential Conflicts of Interest: Neither of the authors have any conflicts of interest relevant to this study to disclose.

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Fig. 5. Frequency histograms showing the sizes of the myelinated fibers, myelin sheath, and axons in the tibial (a–c) and sural (d–f) nerves. The frequency histogram for the tibial and sural nerves showed a significant shift to a smaller axon size in the 0.75AN and 0.5AN groups when compared to the AL group. ***P*<0.01, 0.75AN vs. AL; ††*P*<0.01, 0.5AN vs. AL. AL group, rats with alloxan-induced diabetes that received drinking water without saline; 0.5AN group, rats with alloxan-induced diabetes that received 0.5% saline drinking water; 0.75AN group, rats with alloxan-induced diabetes that received 0.75% saline drinking water.

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