Neuropathy induced by exogenously administered advanced glycation end-products in rats

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ABSTRACT

Aims/Introduction: Advanced glycation end-products (AGE) have been implicated in the development of diabetic neuropathy. It still remains unknown, however, how AGE cause functional and structural changes of the peripheral nerve in diabetes. To explore the role of AGE in diabetic neuropathy, we examined the peripheral nerve by injecting AGE into normal Wistar rats.

Materials and Methods: Young, normal male Wistar rats were injected intraperitoneally (i.p.) daily for 12 weeks with purified AGE prepared by incubating D-glucose with bovine serum albumin (BSA). A control group received BSA alone. A group of rats given AGE were co-treated with aminoguanidine (50 mg/kg/day, i.p.). Peripheral nerve function and structure, as well as nerve Na^+,K^+-ATPase activity, were examined in these rats. Immunohistochemical expressions of 8-hydroxy-2′-deoxyguanosine (8OHdG) and nuclear factor-κB (NF-κB)p65 were also examined.

Results: Serum AGE levels were increased two to threefold in the AGE-treated group compared with those in the BSA-treated control group. AGE-treated rats showed a marked slowing of motor nerve conduction velocity (MNCV) and decreased nerve Na^+,K^+-ATPase activity compared with those in the BSA-treated group. These changes were accompanied by intensified expressions of 8OHdG and NF-κBp65 in endothelial cells and Schwann cells. Aminoguanidine treatment corrected MNCV delay, Na^+,K^+-ATPase activity, and suppressed the expression of 8OHdG and NF-κB, despite there being no influence on serum AGE levels.

Conclusions: The results suggest that an elevated concentration of blood AGE might be one of the contributing factors to the development of neuropathic changes in diabetes. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2009.00002.x, 2010)

KEY WORDS: Advanced glycation end-products, Neuropathy, Oxidative stress

INTRODUCTION

Non-enzymatic glycation of structural proteins and the formation of advanced glycation end-products (AGE) have been implicated in the pathogenesis of diabetic complications[1,2]. The peripheral nervous system is one of the major targets for AGE-induced tissue damage. Human diabetic nerves exhibit an excessive deposition of AGE that correlates with the severity of pathological changes[3]. In addition to direct deleterious effects of AGE, biological reactions of AGE binding with their receptors for AGE (RAGE) exert tissue injury, resulting in characteristic complications in diabetes[1,4]. Recent studies disclosed that upregulated RAGE expression in diabetes contributes to nerve dysfunction and neuropathic symptoms[5,6]. While RAGE overexpression in diabetic mice accelerated the neuropathic changes[6], deletion of RAGE gene was found to protect the progression of neuropathy in long-term hyperglycemia[7], thus implicating the role of AGE/RAGE interaction in the peripheral nerve injury.

Blood AGE concentrations are known to be elevated in diabetic patients[8,9]. Vlassara *et al.* showed that normal rats, when injected with exogenous AGE, developed pathological lesions of renal glomeruli or aortic walls, reminiscent of diabetic complications[10,11]. In fact, exposure of vessel tissues to AGE causes augmented expression of cell adhesion molecules and vascular endothelial growth factors (VEGF), as well as increased vascular permeability[11,12]. It is yet to be clear, however, if circulatory AGE indeed influence peripheral nerve function and structure, contributing to the establishment of neuropathy. In the present study, we examined if the elevation of circulating AGE in rats given exogenous AGE causes deleterious effects on peripheral nerve tissues and if the induced changes are similar to those described in diabetic neuropathy.

MATERIALS AND METHODS

Preparation of AGE-BSA

The method of AGE production and its purification was carried out according to the description given by Vlassara *et al*[10–12]. In brief, 1.6 g bovine serum albumin (BSA; Fraction V, Sigma, St. Louis, MO, USA) was dissolved together with 3.0 g D-glucose (Wako Chem, Osaka, Japan) in 10 mL of 0.5 M sodium phosphate buffer (pH 7.4). The solution was deoxygenated with nitrogen and sterilized by ultrafiltration (0.45 μm filter) and incubated at 37°C for 90 days under aseptic conditions.

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After unincorporated sugar was removed by dialysis in 20 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl, glucose-modified high molecular weight materials were purified by Heparin-Sepharose CL-4B (Amersham Pharmacia, Uppala, Sweden) column chromatography and used as AGE-BSA. Endotoxin content in all samples was measured by Limulus amoebocyte lysate assay (E-toxate, Sigma) and found to be less than 0.2 ng/mL. The AGE-BSA showed absorption and fluorescent spectra identical to those reported previously (excitation 370 nm; emission 440 nm)[13]. Protein concentration was determined by the Bradford method using BSA as a standard[14].

Animal Studies

Four-week-old, male Wistar rats (160–180 g; Japan Clea, Osaka, Japan) were used. After a 1-week adaptation period, they were divided into four groups (8–10 animals in each group) and AGE was given by intraperitoneal (i.p.) injection for 12 weeks as follows: AGE-low group, 20 mg/kg/day of AGE; AGE-high group, 200 mg/kg/day; AGE + aminoguanidine (AG) group, 20 mg/kg/day of AGE + 50 mg/kg/day of AG i.p. (Wako Chem). For comparison, control rats received BSA alone (BSA group; 20 mg/kg/day BSA). Rats were monitored weekly for bodyweight and biweekly for blood glucose. Blood samples were obtained by tail snipping and fasting blood glucose concentrations were measured by a glucose-oxidase method (Toecho Super II, Kagawa, Japan). All animal experimentation followed the guidelines of Hirosaki University (approval number #M99023).

Tissue Preparation

Animals were killed under deep anesthesia with pentobarbital sodium (50 mg/kg bodyweight i.p., Abbot, Chicago, IL, USA) after 12 weeks of AGE injection by cardiac withdrawal of blood. Blood samples were used to determine AGE concentrations. Thereafter, sciatic nerve tissues were excised and processed for biochemical analysis for Na^+,K^+-ATPase activity, AGE content, and structural analysis of myelinated nerve fibers and endoneurial microvessels.

Measurement of AGE Concentration

For measurement of tissue AGE, peripheral nerve tissues were rinsed with phosphate buffered saline and finely minced with scissors. Lipids were extracted with acetone/chloroform (1:1) by shaking gently overnight at 4°C. Samples were then dried by vacuum centrifugation and resuspended in 0.2 M NaPO4 buffer (pH 7.4). After two rinses with methanol and distilled water, the samples were transferred to HEPES buffer (Gibco, Grand Island, NY, USA) with 0.01 M CaCl2 for 12 h. Then collagenase (type VII, Sigma) was added at a 1:100 (w/w) ratio and the mixture was incubated for 48 h at 37°C under mild shaking. One drop of toluene was added to maintain sterility. Digested samples were then centrifuged at 15 000 *g*, and clear supernatants were examined fluorometrically with a spectrophotometer (model 100-50; Hitachi, Tokyo, Japan) at specific excitation and

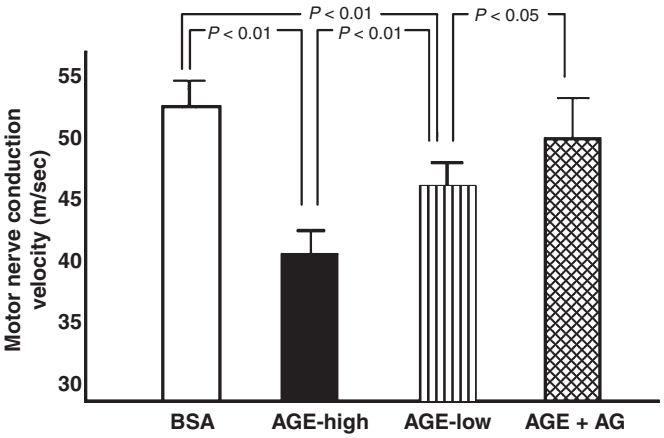

Figure 1 | The results of motor nerve conduction velocity (MNCV) in experimental animals. Compared with the levels of bovine serum albumin (BSA)-treated animals, both the advanced glycation end-products (AGE)-low (20 mg/kg/day) and the AGE-high (200 mg/kg/day) groups showed a significant delay. The aminoguanidine-treated group (AGE-AG) showed a significant improvement of MNCV. Each group consisted of 8–10 animals.

Table 1 | Laboratory data of experimental animals

Group	Number	Bodyweight (g)		Blood glucose (mmol/L)		AGE	
		Initial	End	Initial	End	Serum (AU/mL)	Nerve (AU/prot)
BSA	8	172 ± 54	318 ± 50	4.79 ± 0.29	4.61 ± 0.45	2.06 ± 0.37	0.13 ± 0.09
AGE-high	10	172 ± 63	329 ± 61	4.91 ± 0.39	4.78 ± 0.53	6.15 ± 1.25*,**	0.16 ± 0.12
AGE-low	8	164 ± 48	342 ± 43	4.91 ± 0.49	4.88 ± 0.88	4.33 ± 1.55*	0.14 ± 0.10
AGE-AG	8	163 ± 56	328 ± 54	4.80 ± 0.33	4.56 ± 0.64	5.32 ± 2.81*	0.14 ± 0.08

Values are mean ± SD. *$P < 0.01$ *vs* BSA group, **$P < 0.05$ *vs* AGE-low group. Serum AGE levels were measured by ELISA assay using specific antibodies against carboxymethyllysine. Nerve AGE levels were determined by the autofluorescence at the specific emission/excitation filters. AG, aminoguanidine; AGE, advanced glycation end-products; BSA, bovine serum albumin. AGE-high, rats treated with high-dose AGE (200 mg/kg) injection for 12 weeks. AGE-low, rats treated with low-dose AGE (20 mg/kg) injection for 12 weeks. AGE-AG, rats treated with low-dose AGE with aminoguanidine (50 mg/kg/day) for 12 weeks.

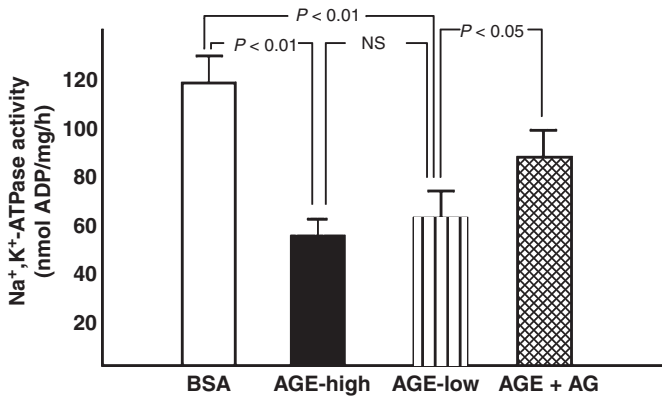

Figure 2 | Ouabain-sensitive Na^+,K^+-ATPase activity of the sciatic nerve in experimental rats. Na^+,K^+-ATPase activity was significantly reduced in the advanced glycation end-products (AGE)-low and the AGE-high groups compared with the bovine serum albumin (BSA)-treated group. The AGE-aminoguanidine (AG) group showed a slight but significant improvement of this enzyme activity. Each group consisted of eight animals.

emission 370/440 nm[15]. AGE was expressed by the intensity of fluorescence as arbitrary units per unit of protein content measured by the Bradford method[14].

Serum AGE levels were determined by a competitive enzyme-linked immunoassay (ELISA) using specific antibodies to carboxymethyllysine (CML) as described previously[8,12]. Briefly, test samples (50 μL) were added to each well as a competitor for 50 μL of AGE antiserum (1:2000) or purified antibodies (1:250–1:1500), followed by incubation for 2 h at room temperature with gentle shaking on a horizontal rotary shaker. Results were expressed as B/B0, calculated as (B/B0 = experimental OD − background OD)/(total OD − background OD). The immunoreactivity of each fraction was read from the calibration curve (CML-BSA) and was expressed as CML units (AU/mL), with 1U corresponding to the amount of antibody reactive material found in CML-BSA at a protein concentration of 1 μg/mL.

Measurement of Na^+,K^+-ATPase Activity

For measurement of Na^+,K^+-ATPase activity, sciatic nerves were excised and the perineurium was removed. The activity of Na^+,K^+-ATPase of ouabain-sensitive fraction was determined by the spectrophotometric enzymatic method as described[16].

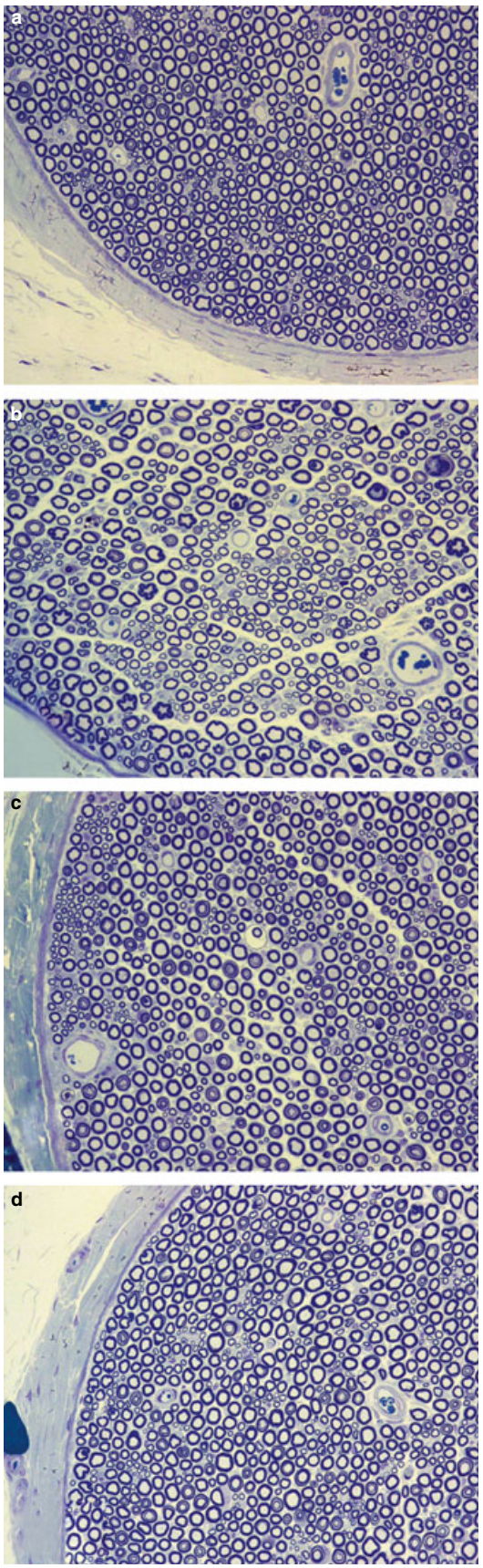

Figure 3 | Cross-sectional view of tibial nerves stained with toluidine blue in experimental animals. Compared with the sections from (a) bovine serum albumin (BSA)-treated animals, advanced glycation end-products (AGE)-treated animals showed interstitial edema (b, AGE-high; c, AGE-low). (d) Aminoguanidine treated group (AGE-AG) showed almost normal appearance.

Motor Nerve Conduction Velocity

Motor nerve conduction velocity (MNCV) was examined in all experimental animals. For these measurements, animals were anesthetized with ether. Body temperature was kept constant at 37°C on a thermostatically-controlled heating mat, and was continuously monitored with an anal temperature probe. Using a standard method, MNCV was measured in the left sciatic posterior tibial nerve using an evoked response stimulator (MS92 electromyogram device; Medelec, London, UK)[16,17].

Morphometric Analysis

At the end of the experiments, the right tibial nerves were excised and fixed overnight at 4°C in 2.5% glutaraldehyde buffered with 0.05 mmol/L sodium cacodylate (pH 7.3). The samples were then postfixed in 1% osmium tetroxide and dehydrated through an ascending series of ethanol concentrations. They were embedded in epon. One-micron thick semithin transverse nerve sections were stained with toluidine blue. For morphometric analysis, fascicular area, myelinated fiber density, myelinated fiber number, fiber size and fiber occupancy were measured at a magnification of ×2000 by a computer-assisted image analyzing system (NIH image, Version 1.6; NIH, Bethesda, MD, USA). The mean values of myelinated fiber size, measured as the area delineated by the outer myelin border, were calculated from at least 800 fibers randomly selected from 6–8 frames of each nerve fascicle. Fiber occupancy was calculated by dividing total fascicular area by the number of myelinated fiber × mean fiber size in each rat. Tissues affected by fixation artifacts were excluded from analysis.

To evaluate the changes of endoneurial microvessels, the number of capillaries in each tibial nerve fascicle was counted on semithin sections at a magnification of ×1000 and expressed as number of capillaries per unit area. For evaluation of endoneurial vascular changes, ultrathin sections were obtained with ultramicrotome and stained with uranyl acetate and lead citrate. They were examined by a JEOL 2000 electron microscope (Nihon Denshi, Tokyo, Japan). Five to eight microvessels were observed for the qualitative changes of endothelial cells and morphometric analysis of vessels. Vascular area size, endothelial cell area, luminal patency ratio relative to the whole vascular area and basement membrane thickness were measured as previously described[18].

Immunocytochemistry

A midportion of the sciatic nerve was fixed in 10% formalin overnight and embedded in paraffin. Four micron-thick sections were deparaffinized with xylene and processed for immunohistochemical evaluation of the expression of 8-hydroxy-2′-deoxyguanosine (8OHdG) and an activated form of nuclear factor-kappa B (p65) (NF-κBp65). Antibodies to 8OHdG (N45.1, mouse monoclonal; JICA, Nihon-Yushi, Shizuoka, Japan) and NF-κBp65 (rabbit polyclonal; Santa Cruz Biotechnology, San Francisco, CA, USA) were obtained commercially. Before the application of the primary antibody, deparaffinized

Table 2 | Morphometric analysis of myelinated fibers in tibial nerve of experimental animals

Group	*n*	Total fascicular area (mm^2)	Myelinated fiber density (#/mm^2)	Myelinated fiber number (#/fasc)	Mean fiber size (μm^2)	Index of circularity	Mean fiber occupancy (%)
BSA	6	0.19 ± 0.03	10 583 ± 1010	2047 ± 339	51.6 ± 2.8	0.88 ± 0.01	54.0 ± 5.2
AGE-high	6	0.20 ± 0.04	9619 ± 1350	1987 ± 602	46.8 ± 5.1	0.91 ± 0.03	44.8 ± 6.3*
AGE-low	6	0.17 ± 0.03	10 050 ± 1314	1674 ± 437	48.6 ± 5.6	0.89 ± 0.01	48.4 ± 5.1
AGE-AG	6	0.19 ± 0.03	11 160 ± 1006	2058 ± 199	47.2 ± 4.1	0.89 ± 0.00	52.4 ± 2.7

Values are mean ± SD. *$P < 0.01$ *vs* BSA group.

AG, aminoguanidine; AGE, advanced glycation end-products; BSA, bovine serum albumin. AGE-high, rats treated with high-dose AGE (200 mg/kg) injection for 12 weeks. AGE-low, rats treated with low-dose AGE (20 mg/kg) injection for 12 weeks. AGE-AG, rats treated with low-dose AGE with aminoguanidine (50 mg/kg/day) for 12 weeks.

Table 3 | Morphometric analysis of endoneurial microvessels in tibial nerve of experimental animals

Group	*n*	Vascular density (#/mm^2)	Average vascular area (μm^2)	Endothelial area (μm^2)	Luminal patency ratio	Basement membrane thickness (μm)	Vacuolar changes of endothelial cells	Vacuolar mitochondria
BSA	6	61.6 ± 4.7	230 ± 95	31.7 ± 16.3	0.43 ± 0.12	1.45 ± 0.70	0.87 ± 0.32	2.40 ± 1.46
AGE-high	6	60.7 ± 15.4	148 ± 11	24.0 ± 16.4	0.45 ± 0.07	1.36 ± 0.47	2.50 ± 0.81*	4.30 ± 2.27*
AGE-low	6	70.7 ± 23.5	181 ± 12	30.7 ± 21.4	0.50 ± 0.06	1.27 ± 0.54	1.71 ± 0.40*	2.75 ± 0.43
AGE-AG	6	74.1 ± 21.7	212 ± 34	34.9 ± 53.1	0.51 ± 0.04	1.32 ± 0.49	1.06 ± 0.28**	2.51 ± 0.75

Values are mean ± SD. *$P < 0.01$ *vs* BSA group, **$P < 0.05$ *vs* AGE-low group.

AG, aminoguanidine; AGE, advanced glycation end-products; BSA, bovine serum albumin. AGE-high, rats treated with high-dose AGE (200 mg/kg) injection for 12 weeks. AGE-low, rats treated with low-dose AGE (20 mg/kg) injection for 12 weeks. AGE-AG, rats treated with low-dose AGE with aminoguanidine (50 mg/kg/day) for 12 weeks.

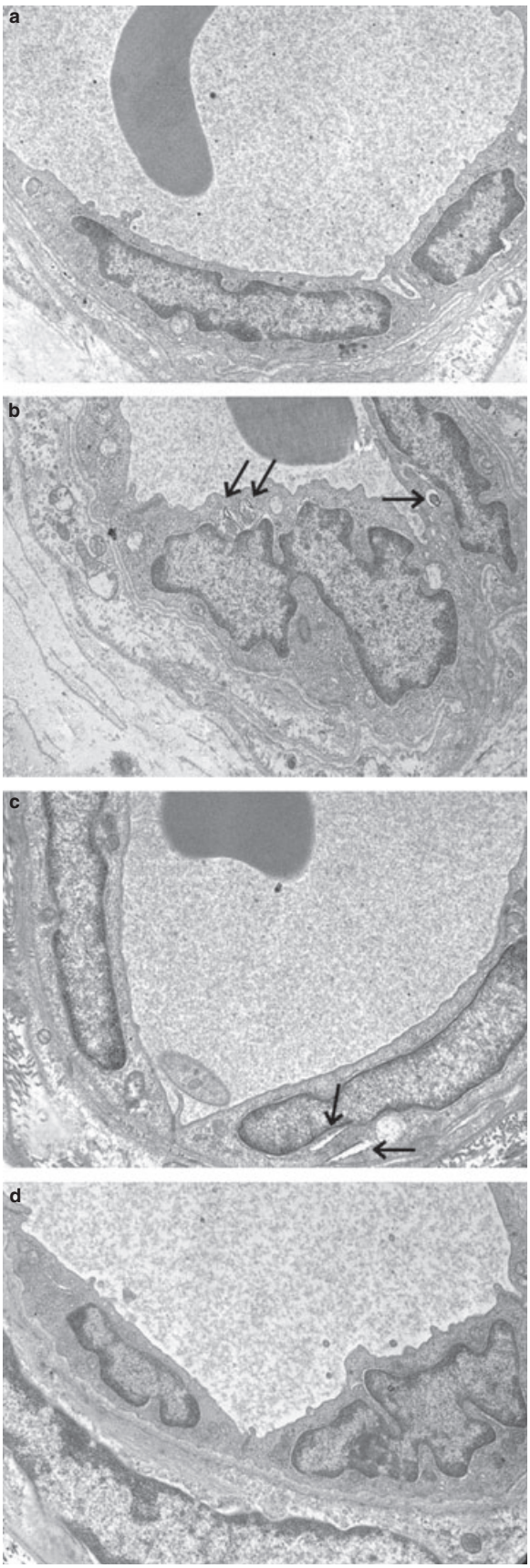

Figure 4 | Electron microscopic view of endothelial cells in experimental animals. (a) In normal rats, endothelial cells were flat, tightly adhered to neighboring cells. The endothelial cells in advanced glycation end-products (AGE)-treated animals (b, AGE-high and c, AGE-low) were swollen (double arrows) and contained many vacuolated bodies derived from mitochondria (arrow). (d) These changes were less prominent in aminoguanidine-treated animals (original magnification ×8000).

sections were pretreated with microwave irradiation (Milestone Srl., Sorrisole, Italy) for 3 × 5 times in citrate buffer for antigen retrieval. Antibodies to 8OHdG and NF-κBp65 were then applied to the sections overnight at 4°C. The sections were then incubated with secondary biotinylated antibody and streptavidin-biotin reagent (Histofine SAB-PO kit; Nichirei, Tokyo, Japan). The antigen–antibody complex was visualized by 3,3′-diaminobenzidine. For the objective evaluation, four samples from each animal group were mounted on a single slide and stained under the same conditions. Clear nuclear positive reactions of 8OHdG and NF-κBp65 were identified as positive. The frequency of positive cells was expressed as a percentage of total nuclei. At least 200–350 cells were counted in each section. Tissue sections affected by artifacts were discarded for analysis.

Statistical Analysis

Results were expressed as mean ± SD. Comparisons were made using a one-way ANOVA, followed by post-hoc Bonferroni's corrections. Unpaired Student's *t*-test was used for the comparison between AGE-low and AGE-AG groups. Statistical significance was considered when *P*-values were <0.05.

RESULTS

Bodyweight and Blood Glucose Level

During the observation period, fasting blood glucose and mean bodyweight were not significantly different among all the groups (Table 1). No animals died during the experiment.

AGE Concentrations in Serum and Nerve

The mean AGE concentration as reflected by CML values in serum was 2.1 and 3.0-fold higher in the AGE-low and the AGE-high groups, respectively, compared with those in the BSA group ($P < 0.01$ for both). AG treatment did not significantly influence the values of AGE in the serum (Table 1). Tissue concentration of AGE in the sciatic nerve determined by autofluorescence did not differ among the groups.

Motor Nerve Conduction Velocity

At the end of the experiments, AGE-treated rats showed slowed motor nerve conduction velocity (MNCV; Figure 1). The AGE-low group showed 14% decrease in average MNCV compared with BSA-treated animals ($P < 0.01$). This decrease was further augmented to 26% in the AGE-high group ($P < 0.01$ *vs* both BSA group and AGE-low dose group). AG treatment significantly improved the slowed MNCV ($P < 0.05$ *vs* AGE-low group).

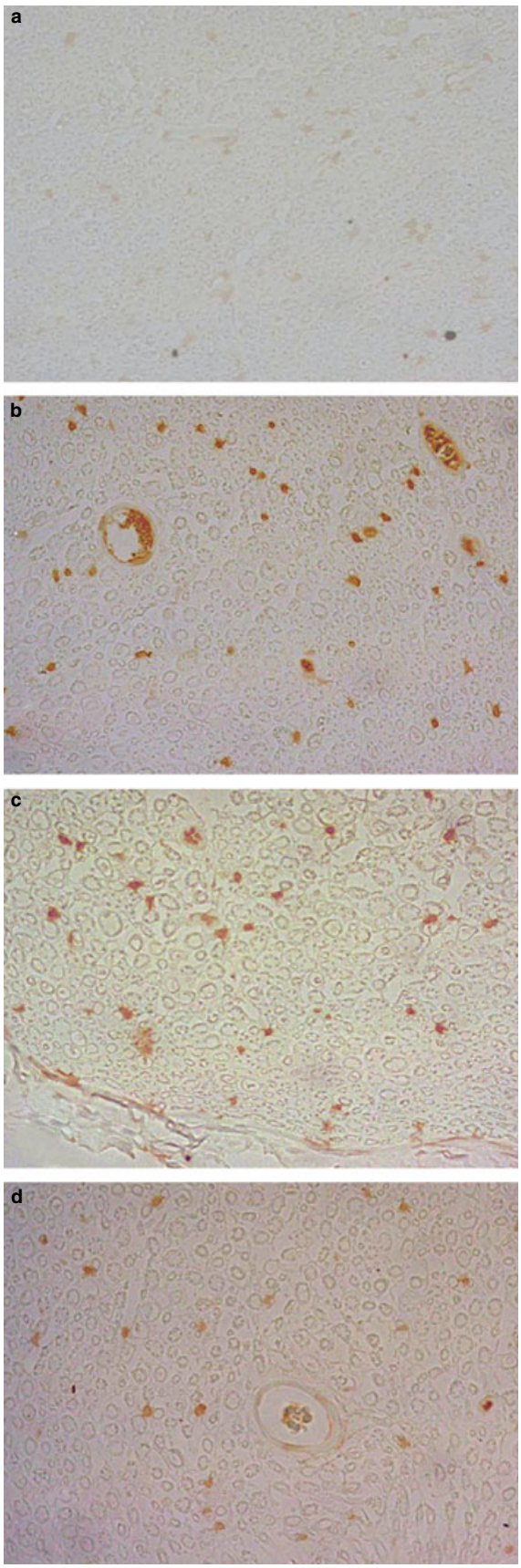

Figure 5 | Cross-sectional view of immunostained sections with 8-hydroxy-2′-deoxyguanosine (8OHdG). (a) There were only a few cells faintly stained in the bovine serum albumin (BSA)-treated group. By contrast, clear positive reactions were shown in the nuclei of endothelial cells and Schwann cells in (b) the advanced glycation end-products (AGE)-high and (c) the AGE-low group. (d) Aminoguanidine-treated group (AGE-AG) showed a reduction of cells with positive reactions.

Na^+,K^+-ATPase Activity

Ouabain-sensitive Na^+,K^+-ATPase activity in AGE-treated animals was significantly decreased (Figure 2). The AGE-high group showed 40% reduction ($P < 0.01$ *vs* BSA group) and the AGE-low group showed 33% reduction ($P < 0.01$ *vs* BSA group). There was no significant difference between the AGE-low and AGE-high groups' levels. AG treatment significantly improved this reduction by 60% ($P < 0.05$ *vs* AGE-low group).

Nerve Structure

On tibial nerve cross sections, AGE-treated animals showed interstitial edema (Figure 3). AG treatment appeared to prevent the edema (Figure 3). There was no significant difference, however, in mean total fascicular area, myelinated fiber density, myelinated fiber number and mean myelinated fiber size between AGE-treated animals and the controls, although there was a trend toward smaller values of average myelinated fiber size in the AGE-high group ($P = 0.10$, AGE-high *vs* BSA group) (Table 2). By contrast, the fiber occupancy was significantly reduced in the AGE-high group compared with the BSA group ($P < 0.05$), indicating the presence of endoneurial edema in the AGE-high group. The differences between the AGE-low group and the BSA group, or between the AGE-low group and the AG-treated group were not significant, although average values were decreased in the AGE-low group ($P = 0.09$, AGE-low *vs* BSA group; $P = 0.13$ AGE-low *vs* AGE + AG group).

Vascular density, mean vascular area, endothelial area, luminal patency rate and basement membrane thickness did not differ among the groups (Table 3). However, vacuolation of cytoplasm and mitochondria was frequently observed in the endoneurial microvessels in animals treated with AGE-high (Figure 4), and AG-treatment inhibited such changes (Figure 4 and Table 3).

Immunohistochemistry of 8 OHdG and NF-κB (p65)

Immunohistochemical staining showed clear evidence of oxidative stress related DNA injury in AGE-treated nerves showing positive nuclear reactions of 8OHdG (Figure 5). In the AGE-high group, positive reactions were found in the nuclei of endothelial cells of endoneurial microvessels and Schwann cells. The AGE-low group also showed scattered cells positive for 8OHdG. The expression of 8OHdG was significantly inhibited in AG-treated rats. Quantitation of positive cells confirmed these findings (Table 4). Positive reactions of NF-κBp65 in the nuclei

Table 4 | Quantitative analysis of positive reactions to 8-hydroxy-2′-deoxyguanosine and nuclear factor kappa-B in the sciatic nerve of experimental animals

Group	*n*	8-hydroxy-2′-deoxuguanosine (%)	Activated nuclear factor-κB (%)
BSA	6	3.4 ± 1.0	2.0 ± 0.6
AGE-high	8	23.5 ± 8.2*,***	7.5 ± 0.7*,****
AGE-low	6	12.5 ± 2.8*	5.2 ± 0.6*
AGE-AG	6	2.8 ± 1.2**	1.8 ± 0.5**,****

Values are mean ± SD. *$P < 0.01$ *vs* BSA group, **$P < 0.01$ *vs* AGE-low group, ***$P < 0.05$ *vs* AGE-low group.
AG, aminoguanidine; AGE, advanced glycation end-products; BSA, bovine serum albumin. AGE-high, rats treated with high-dose AGE (200 mg/kg) injection for 12 weeks. AGE-low, rats treated with low-dose AGE (20 mg/kg) injection for 12 weeks. AGE-AG, rats treated with low-dose AGE with aminoguanidine (50 mg/kg/day) for 12 weeks.

were also detected in the AGE-treated groups more commonly than in the BSA group (Figure 6 and Table 4). AG treatment suppressed this reaction.

DISCUSSION

The current study showed that elevated systemic AGE concentrations caused functional, biochemical and structural changes in the peripheral nerves of rats. The features are comparable to the neuropathic changes encountered in diabetic animal models[19–21]. Because AGE accumulation was not detected in peripheral nerve tissues in AGE-injected animals, the above neuropathic changes are likely to be mediated not by direct AGE reactions on the nerve fibers, but by the exposure of microvessels to exogenous AGE, thus eliciting endoneurial ischemia and altered vascular permeability. Because endoneurial microvessels did not show significant morphometric changes comparable to those found in diabetes, such as basement membrane thickening or alterations of vascular lumina, it appears that functional alterations of microvessels precede structural changes without accumulation of tissue AGE. The findings are consistent with reports which described effects of exogenously administered AGE on renal glomeruli and aortic tissues, recapitulating diabetic glomerulosclerosis or atheroma plaque formation[10,11].

Elevation of serum AGE is not limited to diabetic patients, as it is also detected in uremic patients[23–25]. Acceleration of vascular injury or worsening of chronic complications in diabetic

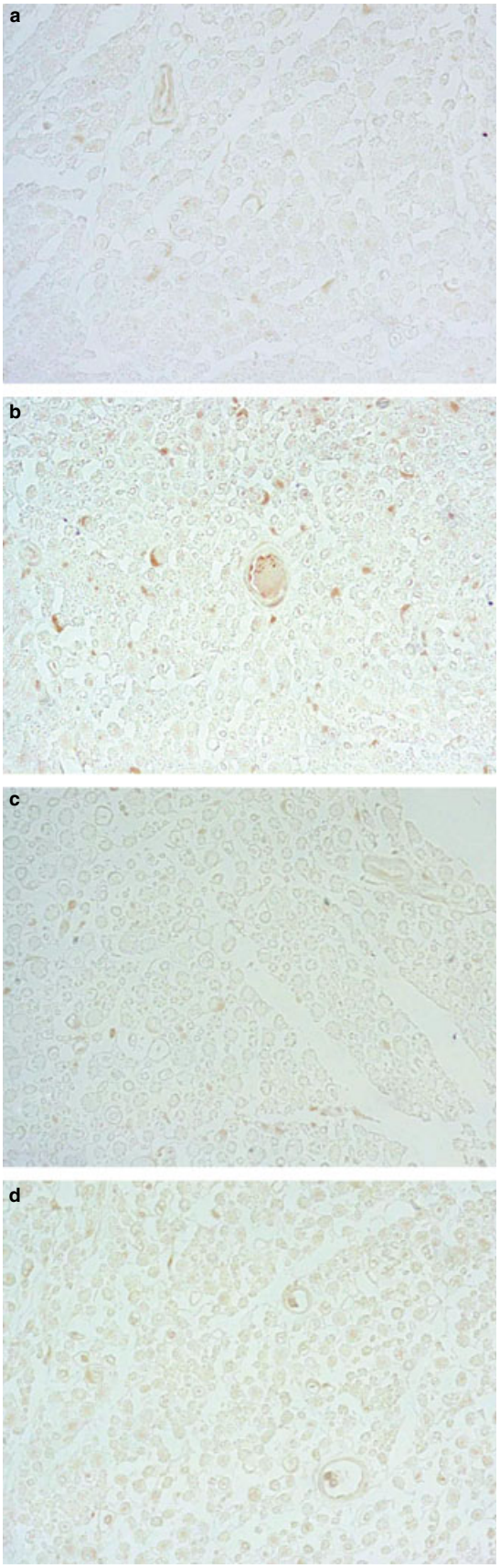

Figure 6 | Cross-sectional view of immunostained sections with nuclear factor-kappaB (NF-κB). (a) There was no clear positive reaction in the bovine serum albumin (BSA)-treated group. (b) By contrast, some nuclei of endothelial cells and Schwann cells in the advanced glycation end-products (AGE)-high group were definitely positive for NF-κB. (c) The reaction in the AGE-low group was equivocal. (d) Aminoguanidine-treated group (AGE-AG) did not show apparent positive reaction.

patients with uremia might be attributed not only to hyperglycemia but also to the toxic effects of AGE. The current findings further extend the contention that the severe delay in MNCV in uremic patients can be ascribed to elevated AGE concentrations in the blood[26,27]. The improvement of MNCV in uremic patients after dialysis of hemodiafiltration might be related to the decrease in AGE levels[28]. The dose-dependent severity of neuropathy in our AGE-injected animals might further indicate the importance of monitoring on the serum AGE concentration as a predictor for complications.

In the present study, we used BSA-derived AGE due to the availability of large amounts of AGE. One may wonder if the effects might be confounded by immunological reactions as a result of species difference. This is unlikely, because BSA-treated animals did not show apparent neuropathic changes. AGE constitute various chemical structures which show fluorescence or non-fluorescence. We confirmed that our AGE contained structures reactive to antibodies to both CML and non-CML, including imidazole or carboxyethyllysine (data not shown)[29]. Takeuchi *et al.* found that AGE derived from glyceraldehydes were toxic to neuronal cells and Schwann cells *in vitro*, causing apoptosis with activation of NF-κBp65, whereas CML did not show any deleterious effect[30,31]. It was also shown that glyceraldehyde-derived AGE have a strong affinity to RAGE[32]. Because we did not examine the effects of pretreatment with antibodies to specific components of AGE before injecting AGE, it was not possible to identify which component of AGE was the cause for neuropathic changes. Use of a specific type of AGE might clarify which component of AGE is most injurious to nerve tissues in future studies.

Most of the neuropathic changes we found in the present study might be accounted for by endoneurial vascular dysfunction caused by exogenous AGE. It has well been shown that neurovascular dysfunction is implicated in the early neuropathic changes in diabetic animal models[33–35]. In the diabetic condition, various metabolic cascades induced by hyperglycemia including polyol pathway, protein kinase C activity and oxidative stress as well as non-enzymatic glycation, all operate for the vascular impairment[33–35]. In the current study, we can assume that exposure of endothelial cells to AGE first exerts proinflammatory reactions of endoneurial vessels with enhanced expression of vasoactive substances, expression of cell adhesion molecules for leukocytes such as vascular cell adhesion molecule (VCAM) or intercellular cell adhesion molecule (ICAM), or secretion of cytokines such as tumor necrosis factor alpha (TNFα) or VEGF[22,36]. Oxidative stress is also generated after exposure of endothelial cells to AGE[37,38]. As a consequence, the nerve tissues might be followed by endoneurial ischemia with hyperpermeable milieu, resulting in reduced Na^+,K^+-ATPase activity and MNCV. This contention might be in part supported by a high expression of 8OHdG and NF-κBp65 in endothelial cells and Schwann cell nuclei detected in AGE-treated animals. The vacuolated cytoplasm and mitochondria frequently detected in AGE-treated animals might be in keeping with this view. Recent studies using RAGE transgenic mice showed that the levels of RAGE expression are important for the nerve tissue injury and sensory impairments through activation of NF-κBp65 in the diabetic condition[6,7].

In the present study, aminoguanidine effectively corrected MNCV and Na^+,K^+-ATPase activity. These effects are consistent with the results on the *in vivo* effects of AG or OPB 9195 on neuropathic changes in diabetic rats[15–17,39,40]. In these studies, tissue or serum AGE levels were significantly inhibited by treatment with these compounds, together with inhibition of 8OHdG expressions[15–17]. Interestingly, in the present study, we could not find any decrease of serum AGE in AG-treated animals, suggesting that the effects of AG were mediated by the inhibition of biological reactions of AGE/RAGE, thereby reducing oxidative stress and proinflammatory reactions. It should be cautioned, however, that aminoguanidine effects might also be ascribed to its direct inhibitory action of inducible NO synthase that can be induced by AGE[41].

In summary, the present study confirmed that AGE is a potent inciting factor for the development of neuropathy, and the inhibition of AGE/RAGE interaction might be a right target for the future treatment of diabetic neuropathy.

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