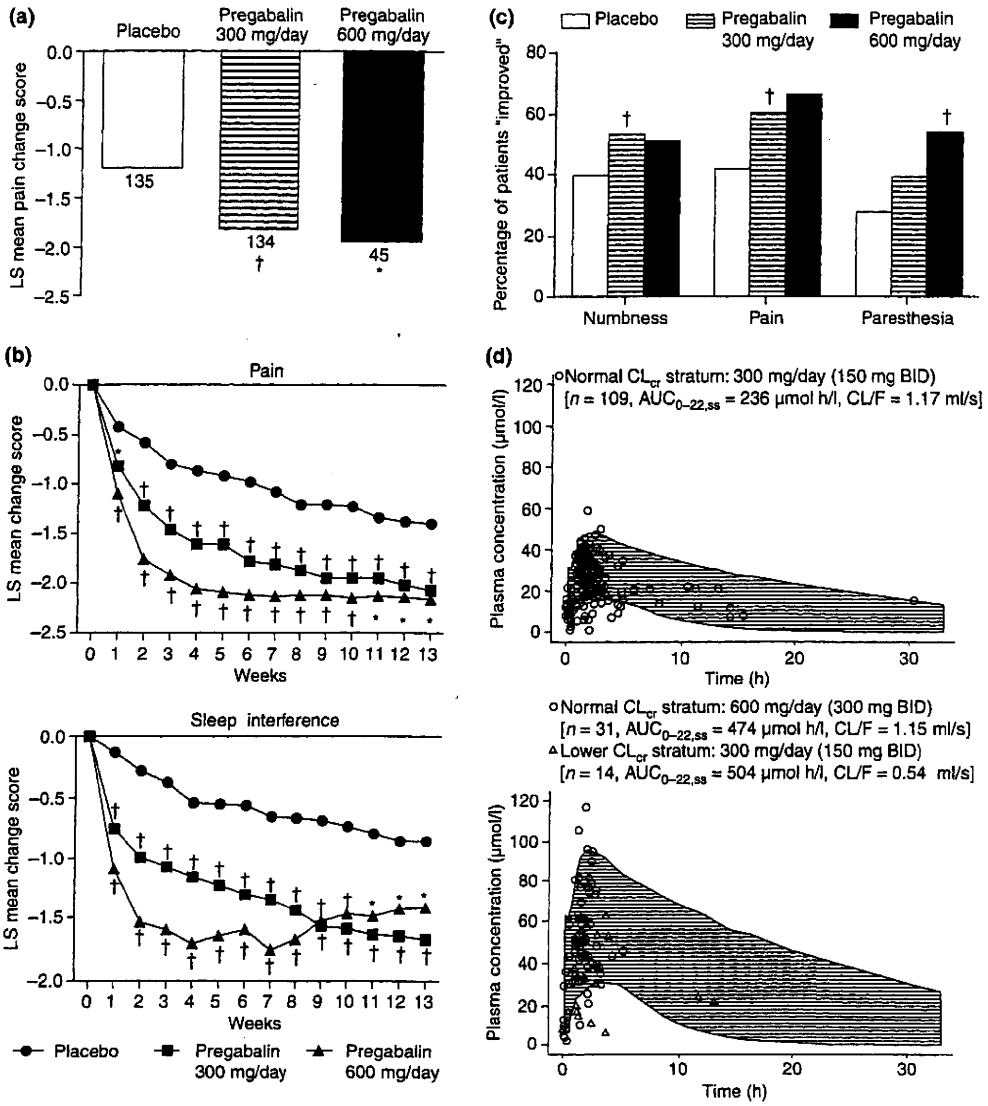

FIGURE 1 (a) Least-squares (LS) mean pain score change from baseline to end-point. Values below bars indicate sample sizes. (b) Change from baseline in weekly mean pain and sleep interference scores. (c) Percentage of patients rating themselves as 'improved' on symptoms of numbness, pain and paraesthesia. (d) Predicted plasma pregabalin concentration (hatched region) vs. time profile at steady state and observed plasma pregabalin concentrations (circles and triangles) by dose and creatinine clearance (CL_{cr}). The upper limit of the region indicates 97.5 percentile points of simulated 500 individuals with CL_{cr} = 1.0 ml/s (150 mg twice daily in the top panel and 300 mg twice daily in the bottom panel), and the lower limit indicates the 2.5 percentile points of those with CL_{cr} = 1.82 ml/s (150 mg twice daily in the top panel and 300 mg twice daily in the bottom panel). *P < 0.05, †P < 0.01. $AUC_{0-12,SS}$, steady-state area under the concentration–time curve from 0 to 12 h. BID, twice daily CL/F, oral clearance.

(CI), −1.09 to −0.17; P = 0.0075] and −0.74 (95% CI, −1.39 to −0.09; P = 0.0254) for the 300 and 600 mg/day pregabalin groups, respectively.

Secondary efficacy measures

Weekly pain scores for both pregabalin groups were significantly superior to the placebo group from weeks 1 through 13 (Fig. 1b). Overall, from baseline to end-point, the proportion of responders was higher in the 300 (29.1%) and 600 mg/day groups (35.6%) relative to the placebo group (21.5%), but no significant differences were found.

The mean sleep interference scores at the study end-point were significantly improved in the 300 and 600 mg/day groups compared with placebo (P < 0.0001 for 300 mg/day and P = 0.0273 for 600 mg/day vs. placebo). The improved sleep interference scores were significant at week 1 and continued throughout the study (Fig. 1b).

The mean values of the sensory, affective, total, visual analogue scale and Present Pain Intensity scores of the SF-MPQ

in the 300 and 600 mg/day groups were significantly superior to those observed in the placebo group ($P < 0.05$), with the exception of the affective score at 600 mg/day ($P = 0.0707$). On the MOS–Sleep Scale, the 300 mg/day group showed significant improvement on several subscales, including sleep disturbance ($P = 0.0019$), quantity of sleep ($P = 0.0177$) and overall Sleep Problems Index ($P = 0.0269$) compared with the placebo group; the 600 mg/day group was significantly superior to placebo on sleep adequacy ($P = 0.0173$).

Patient impressions of subjective symptoms (numbness, pain and paraesthesia) were favourable for pregabalin (Fig. 1c). Patients treated with 300 mg/day reported significant improvements in numbness ($P = 0.0072$) and pain ($P = 0.0019$), while patients treated with 600 mg/day reported a significant improvement in paraesthesia ($P = 0.0093$). There was a significant difference in the PGIC scores favouring the 600 mg/day group compared with placebo ($P = 0.0075$), and in the CGIC scores favouring both pregabalin groups compared with placebo (300 mg/day, $P = 0.0148$; 600 mg/day, $P = 0.0063$). Evaluation of health survey scores using the SF-36 revealed that pregabalin 600 mg/day was significantly superior to the placebo group on social functioning and vitality ($P < 0.05$).

Safety

A summary of the most common treatment-related adverse events and their discontinuation rates is provided in Table 2. The incidence of patients with one or more treatment-related adverse events in placebo, 300 and 600 mg/day groups was 36, 57 and 80%, respectively. Somnolence (26%), dizziness (24%), peripheral oedema (13%) and increased weight (11%) occurred most frequently in patients treated with pregabalin and appeared to be dose related. In all treatment groups, most of the adverse events were reported as mild or moderate.

Severe treatment-related adverse events were observed in two patients [one patient (diabetic nephropathy, 300 mg/day group) and one patient (somnolence, 600 mg/day group)], but were confirmed to have resolved after discontinuation of the study treatment.

Serious adverse events were observed in nine patients (three patients in the placebo group, four patients in the 300 mg/day group and two patients in the 600 mg/day group). All the serious adverse events observed in the pregabalin groups (six patients) were judged to have no causal relationship with the study drug. No severe or serious adverse events related to laboratory values, vital signs, electrocardiograms and neurological and ophthalmological examinations were observed in the pregabalin groups. Mean change in body weight from baseline to the final assessment was greater in the 300 and 600 mg/day groups compared with the placebo group (0.09, 1.59 and 1.72 kg, respectively). Blood glucose levels and glycated haemoglobin concentrations remained unchanged from baseline to the final assessment in patients treated with either placebo or pregabalin.

The proportion of discontinued patients owing to adverse events (all causes) was highest in the 600 mg/day group (28.9%, 13 patients), followed by the 300 mg/day group (12.7%, 17 patients) and the placebo group (5.2%, seven patients). The adverse events considered to be mostly responsible for discontinuation were dizziness (two patients in the placebo group, three patients in the 300 mg/day group and five patients in the 600 mg/day group] and somnolence (two patients in the 600 mg/day group).

Population pharmacokinetics

The steady-state predicted and observed plasma pregabalin concentrations by dose and CL_{cr} are shown in Fig. 1d. Most of the observed pregabalin concentrations in patients with $CL_{cr} > 1.0$ ml/s were within each predicted range. In patients with $0.5 < CL_{cr} \leq 1.0$ ml/s who received pregabalin 150 mg twice daily (300 mg/day), most of the concentrations fell within the predicted range of 300 mg twice daily (600 mg/day). Steady-state area under the concentration–time curve from 0 to 12 h

Table 2 Treatment-related adverse events and discontinuations occurring in ≥3% of any treatment group

	Placebo ($n = 135$)		Pregabalin 300 mg/day ($n = 134$)		Pregabalin 600 mg/day ($n = 45$)	
	n (%)	Discontinuation n (%)	n (%)	Discontinuation n (%)	n (%)	Discontinuation n (%)
Somnolence	11 (8.1)	0	28 (20.9)	0	18 (40.0)	2 (4.4)
Dizziness	9 (6.7)	2 (1.5)	26 (19.4)	3 (2.2)	17 (37.8)	5 (11.1)
Peripheral oedema	6 (4.4)	0	17 (12.7)	0	6 (13.3)	0
Weight increased	3 (2.2)	0	15 (11.2)	1 (0.7)	5 (11.1)	0
Constipation	1 (0.7)	0	4 (3.0)	0	2 (4.4)	0
Oedema	1 (0.7)	0	3 (2.2)	0	2 (4.4)	1 (2.2)
Face oedema	0	0	5 (3.7)	0	1 (2.2)	0
Blood creatine phosphokinase increased	0	0	2 (1.5)	0	2 (4.4)	0
Hot flush	1 (0.7)	0	1 (0.7)	0	2 (4.4)	0
Muscular weakness	0	0	0	0	2 (4.4)	0

during multiple oral dose treatment with pregabalin 150 mg twice daily (300 mg/day) in patients with impaired kidney function ($0.5 < CL_{cr} \leq 1.0$ ml/s) was the same as that observed during multiple oral dose treatment with pregabalin 300 mg twice daily (600 mg/day) in patients with $CL_{cr} > 1.0$ ml/s (504 vs. 474 μmol h/l). In addition, the clearance of pregabalin in patients with impaired kidney function was about half of the clearance in patients with $CL_{cr} > 1.0$ ml/s (0.54 vs. 1.15 ml/s).

Discussion

The results from this trial demonstrate that pregabalin is efficacious in treating neuropathic pain associated with diabetic peripheral neuropathy in Japanese patients. Both the 300 and 600 mg/day groups showed statistical improvement over placebo in reducing mean pain scores. This effect was observed as early as the first week and was maintained throughout the study. The proportion of patients responding to treatment (≥50% improvement in mean pain score from baseline) was higher (albeit not significant) in the 300 (29.1%) and 600 mg/day (35.6%) pregabalin groups relative to the placebo group (21.5%). These responder rates are somewhat lower than those reported in previously published studies (39 and 47% for 300 and 600 mg/day, respectively [23]), despite comparable placebo responder rates (22%). The reason for the potentially lower treatment response is unknown, but with regard to the proportion of patients with ≥30% improvement in mean pain score from baseline (36, 49 and 56% for placebo, 300 and 600 mg/day, respectively), (another well-known efficacy measure considered to be clinically important [24]), there were no major differences from those reported previously in published studies (37, 55 and 62% for placebo, 300 and 600 mg/day, respectively [23]).

Secondary efficacy measures also supported the superiority of pregabalin over placebo. Scores from the SF-MPQ were significantly improved over placebo, as were the sleep interference scores. Among the MOS-Sleep subscales, sleep adequacy, sleep disturbances, sleep quantity and sleep problems showed improvement at either the 300 and the 600 mg/day doses. PGIC and CGIC also improved in pregabalin-treated patients.

This is the first study to report on patient impressions of symptom improvement in numbness and paraesthesia, which are cardinal symptoms of diabetic peripheral neuropathy. Significant improvements were observed at either doses, albeit on different symptoms. The reason for this differential effect is unclear, but the overall improvement illustrates the efficacy of pregabalin in treating a variety of neuropathic symptoms beyond pain. Finally, health evaluation using the SF-36 revealed that pregabalin 600 mg/day was superior to placebo in social functioning and vitality, a positive finding partly consistent with a previous study [14].

The frequency and severity of adverse events in pregabalin-treated patients were similar to those reported in previous studies [12–17]. The most common adverse events were somnolence, dizziness, peripheral oedema and weight gain, all of which appeared to be dose related and were reported generally as mild to moderate. The serious adverse events in patients treated with pregabalin were deemed unrelated to the study drug. Dizziness and somnolence were the most frequent adverse events leading to treatment discontinuation, but each represented less than 5% of the pregabalin-treated patients. In this study, pregabalin dosage adjustment was made based on calculated creatinine clearance. Population pharmacokinetic analysis confirmed that the magnitude of the dose adjustment was accurate.

Depression has been documented in a significant proportion of Japanese patients with diabetes and appears to be associated with the presence of DPN, among other factors [8]. Diabetic peripheral neuropathy also coincides with sleep disturbances, activity limitations and a decrease in quality of life [4–9]. In this study, pregabalin appeared to alleviate some of these disturbing sequelae. The impact of pregabalin on depression and activity limitations was not evaluated, but they remain important outcome variables for future study.

Mean pain score in the placebo group continued to decrease throughout the treatment period, and approximately 20% of the patients in the placebo group achieved a ≥50% reduction in their pain score from baseline to end-point. Although this proportion of patients is comparable to the placebo responder rate in other pregabalin trials in diabetic peripheral neuropathy[23], it is nevertheless substantial and reduces the likelihood of detecting a significant separation between the placebo and treatment groups. The placebo response in neuropathic pain trials is recognized as an important issue that merits the consideration of alternative trial designs [25,26].

Finally, this study did not include an active comparator. At present in Japan, the aldose reductase inhibitor, epalrestat, is approved to treat the underlying pathogenesis of diabetic peripheral neuropathy, but its predominant effect appears to be on improving nerve function [27,28]. Further study should compare the efficacy of pregabalin with epalrestat or other medications (e.g. mexiletine) and evaluate the combination of pregabalin with other therapeutic agents in the treatment of this painful condition.

Competing interests

Jo Satoh has received research funding, lecture fees or consultancy fees from Astellas, Banyu, Daiichi-Sankyo, Dainippon-Sumitomo, Eli Lilly, Novo Nordisk, Ono, Pfizer, Sanofi-Aventis and Takeda. Soroku Yagihashi received a consultation fee from Pfizer for this study, and has been paid by Pfizer, Novartis, Takeda, Ono and Novo Nordisk for giving lectures on their educational programmes or at scientific meetings. Masayuki Baba has received research funding, lecture fees or consultancy fees from Astellas, Boehringer Ingelheim, Daiichi-Sankyo, Dainippon-Sumitomo, Eisai, Eli Lilly, Kyowa-Kirin, Ono, Otsuka, Pfizer, Takeda, Tanabe-Mitsubishi and Teijin. Makoto Suzuki, Akio Arakawa, Tamotsu Yoshiyama and Satoshi Shoji are full-time employees of Pfizer Japan Inc.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. List of All Clinical Investigators.

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Research Report

Increased susceptibility to ischemia and macrophage activation in STZ-diabetic rat nerve

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ABSTRACT

Ischemic vulnerability in diabetic nerve plays a paramount role in the development of diabetic neuropathy, yet little is known of the underlying mechanism. Diabetes enhances the inflammatory response to ischemia and reperfusion. We investigated pathological characteristics of nerve fibers and endoneurial macrophages along the length of sciatic–tibial nerves before and after ischemia (60 to 90 min) and reperfusion (6 h to 7 days) in 8 weeks of STZ-induced diabetic rats. Without ischemia, diabetic nerves revealed significantly increased the density of Iba-1-positive endoneurial macrophages when compared with controls. Most of macrophages appeared slim and triangular in shape, but in diabetic nerves, some were rounded with bromodeoxyuridine (BrdU) incorporation, suggesting proliferating macrophages. Seventy-five minutes of ischemia is the minimal ischemic time to cause pathological changes in diabetic nerves. Following 90 min of ischemia and 6 h of reperfusion in diabetic rats, the number of Iba-1-positive endoneurial macrophages was increased significantly at the thigh level of sciatic nerve when compared with those before ischemia. Endoneurial macrophages in diabetic nerves increased in number further significantly after 24 and 48 h of reperfusion and underwent morphological alterations; swollen and rounded including phagocytosis. After 90 min of ischemia and 7 days of reperfusion, severe pathological alterations, e.g., demyelination and endoneurial edema at proximal nerves and axonal degeneration distally, were observed in diabetic nerves, while control nerves showed normal morphology. We conclude that macrophage proliferation occurs in STZ-diabetic nerves. The acute inflammatory response after ischemia and reperfusion was intensified in diabetic nerves. Activation of resident macrophages and infiltration by recruited macrophages could be casually linked to ischemic susceptibility in diabetic nerve.

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Abbreviations: BrdU, bromodeoxyuridine; GFP, green fluorescent protein; Iba-1, ionized calcium-binding adaptor molecule 1; STZ, streptozotocin

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1. Introduction

Diabetes enhances ischemic/reperfusion injury in various tissues. We first demonstrated that peripheral nerves in STZ-diabetic rat are susceptible to acute ischemia by either arterial ligation or microsphere embolization (Nukada, 1986, 1992, 1993). A brief or mild ischemia, insufficient to cause nerve fiber damage in normal nerve, results in endoneurial edema, demyelination, axonal degeneration, and necrosis in STZ-diabetic sciatic and tibial nerves. Zochodne and his colleagues demonstrated this property by applying endothelin-1, the most potent vasoconstrictor, in the epineurium of sciatic nerve in STZ-diabetic rats (Zochodne et al., 1996; Zochodne and Cheng, 1999).

We also found aggravated reperfusion injury electrophysiologically and morphologically in STZ-diabetic nerve; delayed recovery of compound muscle action potential, greater endoneurial edema, and prominent axonal degeneration when compared with those in controls (Baba et al., 2006; Nukada et al., 2002). Low and his colleagues confirmed reperfusion exaggerated morphological pathology in STZ-diabetic nerve (Wang et al., 2004). They also showed enhanced inflammatory response nuclear factor-kappa B (NF-κB) activation after reperfusion in STZ-diabetic nerve (Wang et al., 2006). Similar vulnerability to ischemia and reperfusion has been reported in various tissues of diabetes, e.g., brain, heart, and kidney (Anzawa et al., 2006; Di Filippo et al., 2005; Ding et al., 2004; Hearse et al., 1978; Melin et al., 2003; Panagia et al., 2005; Thakker et al., 2008; Yue et al., 2005), and both acute and chronic hyperglycemia aggravate ischemic brain damage (Capes et al., 2001; Gisselsson et al., 1999; Martin et al., 2006; Muranyi et al., 2003; Nedergaard, 1987).

The macrophage has been emerged as an important player in the pathogenesis of both diabetes and diabetic complications. Macrophage accumulation is a feature of diabetes and is associated with development of vascular complications, including both macro- and microangiopathy (Boyle, 2007; Fernandez-Real and Pickup, 2008; Kolb and Mandrup-Poulsen, 2005; Odegaard and Chawla, 2008; Schenk et al., 2008; Tesch, 2007; Toso et al., 2008; Wellen and Hotamisligil, 2005). Macrophages mediate diabetic injury through a variety of mechanisms, including production of reactive oxygen species and cytokines. Reperfusion nerve injury is also a state where oxidative stress has been implicated (Anderson et al., 1997; Frangogiannis et al., 1998; He et al., 1999, 2003; Wang et al., 2005, 2008). Diabetes exaggerates inflammatory responses after ischemia and reperfusion: increased leukocyte–endothelial cell adhesion, albumin extravasation, and oxidant production by endothelial cells in post-capillary venules (Panes et al., 1996; Salas et al., 1998, 1999).

In the current study, we assessed pathological characteristics along the length of sciatic and tibial nerves in STZ-diabetic rats before and after ischemia and reperfusion. We also addressed the hypothesis that macrophage activation and proliferation could be enhanced in reperfused diabetic nerve.

2. Results

Mean body weights of diabetic and control rats at onset were 231±8 (n=46) and 231±8 (n=46) g, respectively (p>0.05). STZ treatment was associated with a significant attenuation of weight gain at the time of experiment; 343±7 g in diabetic rats and 491±7 g in controls (p<0.0001). STZ-treated rats displayed significant elevation in blood glucose; 29.9±0.5 mmol/l in diabetic rats and 5.5±0.1 mmol/l in controls (p<0.0001). When glucose level was above the scale (>33.3 mmol/l), it was calculated as 33.3 mmol. Motor nerve conduction velocity in sciatic–tibial nerves was reduced in diabetic nerve, being 43.3±0.8 and 53.6±1.0 m/s in diabetic rats and controls respectively (p<0.0001). Results of nerve conduction studies during and after reperfusion in STZ-diabetic rats have been detailed previously (Baba et al., 2006).

2.1. *Nerve pathology*

After 8 weeks of STZ-induced diabetes, there was no morphological change in sciatic–tibial nerves. Reperfusion after 60 min of ischemia did not cause any pathological abnormalities in both diabetic and control nerves. However, following 75 min of ischemia and 7 days of reperfusion, diabetic rats revealed focal or multifocal lesions consisting of axonal degeneration, which is the hallmark of an acute ischemic injury (Nukada and Dyck, 1984), at lower thigh and upper calf levels of sciatic and tibial nerves in 4 out of 6 nerves (Fig. 1), whereas nerve morphology was normal in controls.

After 90 min of ischemia and 7 days of reperfusion, diabetic rats exhibited invariably severe pathological abnormalities in sciatic and tibial nerves. At the upper thigh level of sciatic nerve in diabetic rats, the most proximal level evaluated, isolated demyelinated, or thinly myelinated nerve fibers and macrophages with myelin debris were found (Fig. 2A, B). Minimal endoneurial edema was also observed at this level (Fig. 2C, D). At the lower thigh level of diabetic sciatic and tibial nerves, endoneurial edema was more obvious and demyelinated fibers and macrophages were still observed (Fig. 2E, F).

At the upper calf level of tibial nerves after 90 min of ischemia and 7 days of reperfusion in diabetic rats, clusters of demyelinated fibers, macrophages with myelin debris, and severe endoneurial edema with intra-myelinic edema were found (Fig. 3). Demyelinated fibers were often located either near the vessel or macrophages. Control nerves did not reveal demyelinated nerve fibers. Reperfused diabetic nerves also exhibited frequent axonal degeneration (Fig. 3C, D). The density (/mm^2) of nerve fibers with axonal degeneration was significantly increased at the upper calf level in diabetic nerve than in controls; 24.0±6.5 and 1.2±2.8, p<0.0001, respectively.

At the lower calf and ankle levels of diabetic tibial nerve, axonal degeneration was prominent. Nerve fibers with axonal degeneration were co-existed with normal myelinated fibers, and focal lesions of axonal degeneration were found though not prominent as at proximal levels (Fig. 4A, B). Diffuse axonal degeneration was also seen in some diabetic rats (Fig. 4C). In contrast, there was normal morphology in control nerves after 90 min of ischemia and 7 day of reperfusion (Fig. 4D). Because of consistent severe pathology, we used 90 min of ischemia for the study of acute inflammatory response after ischemia and reperfusion.

2.2. *Endoneurial macrophages*

Immunohistochemical expression of Iba-1 was observed at thigh, knee, and calf levels of sciatic and tibial nerves before

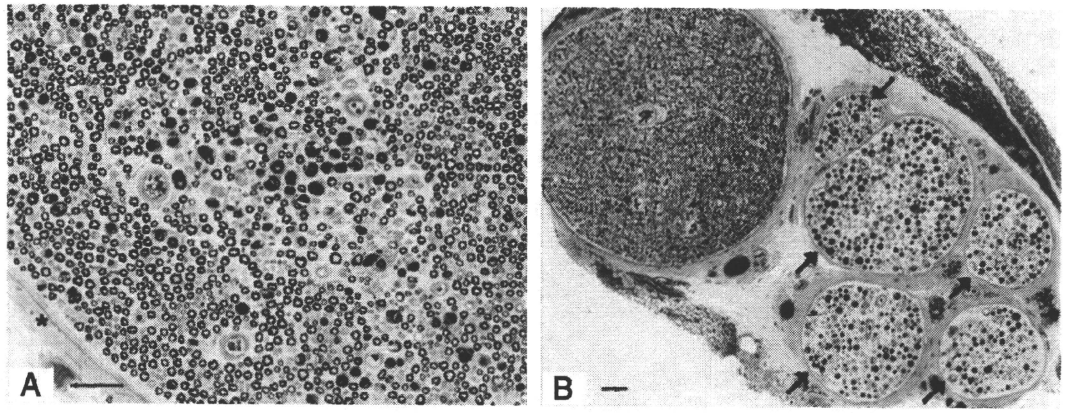

Fig. 1 – Transverse epoxy sections of sciatic and tibial nerves in diabetic rats after 75 min of ischemia and 7 days of reperfusion, showing characteristic pathology in acute ischemic nerve injury. (A) Sciatic nerve at the lower thigh level reveals a focal lesion of axonal degeneration of myelinated nerve fibers at the central fascicular region ("ischemic core"). (B) Tibial nerves at the upper calf level exhibits distinct contrast of myelinated nerve fiber density between a large fascicule on the left and five small fascicules (arrows). Diffuse nerve fiber degeneration is found in small fascicles (arrows), whereas the number of myelinated nerve fibers is preserved in the large fascicle. *Perineurium. Scale bars: 50 μm for both panels.

and after 90 min of ischemia in both diabetic and control nerves. After 8 weeks of STZ-induced diabetes, endoneurial macrophages detected by Iba-1 antibody were distributed throughout the endoneurium. These macrophages appeared slim and triangular and located often close to endothelial cells of endoneurial microvessels in both diabetic and control nerves (Fig. 5A, B). Without an ischemic injury, we found Iba-1-positive macrophages that had incorporated BrdU into their nuclei in diabetic nerves (Fig. 6), but not in controls. These BrdU-positive macrophages in diabetic nerve were swollen and rounded. BrdU did not co-localize with Schwann cell marker S-100, thus providing their identity as proliferating macrophages.

The density (/mm^2) of Iba-1-positive endoneurial macrophages along the length of sciatic and tibial nerves was significantly greater in diabetic rats than in controls: 42.9±2.9 and 34.0±2.6, p=0.03 at the thigh level; 44.3±3.5 and 26.7±3.8, p=0.004 at the knee level; and 30.8±2.1 and 21.9±1.9, p=0.008 at the calf level, in diabetic nerve and controls, respectively. In diabetic nerves, the density of Iba-1-positive endoneurial macrophage was significantly greater at thigh and knee levels than at calf level (thigh vs. calf: p=0.004, and knee vs. calf: p=0.005). Control nerves also revealed the highest density of Iba-1 macrophages at thigh level of sciatic nerve, although not at significant level statistically. Mean fascicular area of sciatic and tibial nerves was not significantly different between diabetic and control nerves.

After 90 min of ischemia and 6 h of reperfusion in diabetic nerves, the density (/mm^2) of endoneurial Iba-1-positive macrophages increased significantly at thigh level, but not at knee and calf levels, when compared with those before ischemia: 42.9±2.9 and 57.6±2.6, p=0.001 at the thigh; 44.3±3.5 and 46.2±2.9, p=0.68 at the knee; and 30.8±2.1 and 34.2±2.3, p=0.29 at the calf, before ischemia and after 6 h of reperfusion, respectively (Fig. 7). Cell bodies of Iba-1-positive endoneurial macrophages often began to enlarge (Fig. 5C, D). BrdU incorporation was detected in these rounded macrophages, and these changes were more prominent after 24 h of reperfusion. Nerve fiber morphology was normal, and the mean fascicular area at each level was not significantly different from pre-ischemia.

After 90 min of ischemia and 24 h of reperfusion in diabetic nerves, the density (/mm^2) of Iba-1-positive endoneurial macrophages was significantly increased further at thigh and knee levels, but not at the calf level, when compared with those after 6 h of ischemia: 57.6±2.6 and 71.5±2.7, p=0.002 at the thigh; 46.2±2.9, and 58.2±3.2, p=0.01 at the knee; and 34.2±2.3 and 36.8±3.2, p=0.52 at the calf, after 6 h and 24 h of reperfusion, respectively (Fig. 7). Morphologically, there were empty axons, endoneurial edema and intramyelinic edema at lower thigh and upper calf levels of diabetic nerves. These pathological features were more prominent after 48 h of reperfusion.

After 90 min of ischemia and 48 h of reperfusion, most macrophages were rounded and appeared like phagocytic cells in diabetic nerves (Fig. 5E). Some of these macrophages at thigh and kneel levels in diabetic nerves showed phagocytosis of myelin debris and lipid droplets (Fig. 5F). After 48 h of reperfusion, the density (/mm^2) of Iba-1 endoneurial-positive cells further increased significantly at all levels along the length of sciatic–tibial nerves when compared with those after 24 h of reperfusion: 71.5±2.7 and 109.0±3.4, p<0.0001 at the thigh; 58.2±3.2, and 149.9±10.1, p<0.0001 at the knee; and 36.8±3.2 and 103.7±8.7, p<0.0001 at the calf, after 24 h and 48 h of reperfusion, respectively (Fig. 7). In sciatic and tibial nerves from control rats, Iba-1-positive endoneurial macrophages increased, although not at significant level statistically; p values >0.05 at all levels and all time points (Fig. 7). After 90 min of ischemia and 72 h or later of reperfusion in diabetic nerves, the number of Iba-1-positive macrophages increased continuously and the most of macrophages showed phagocytosis. Following 7 days of reperfusion in diabetic nerves, ED1- and ED2-positive endoneurial macrophages with phagocytosis were also seen in diabetic nerves.

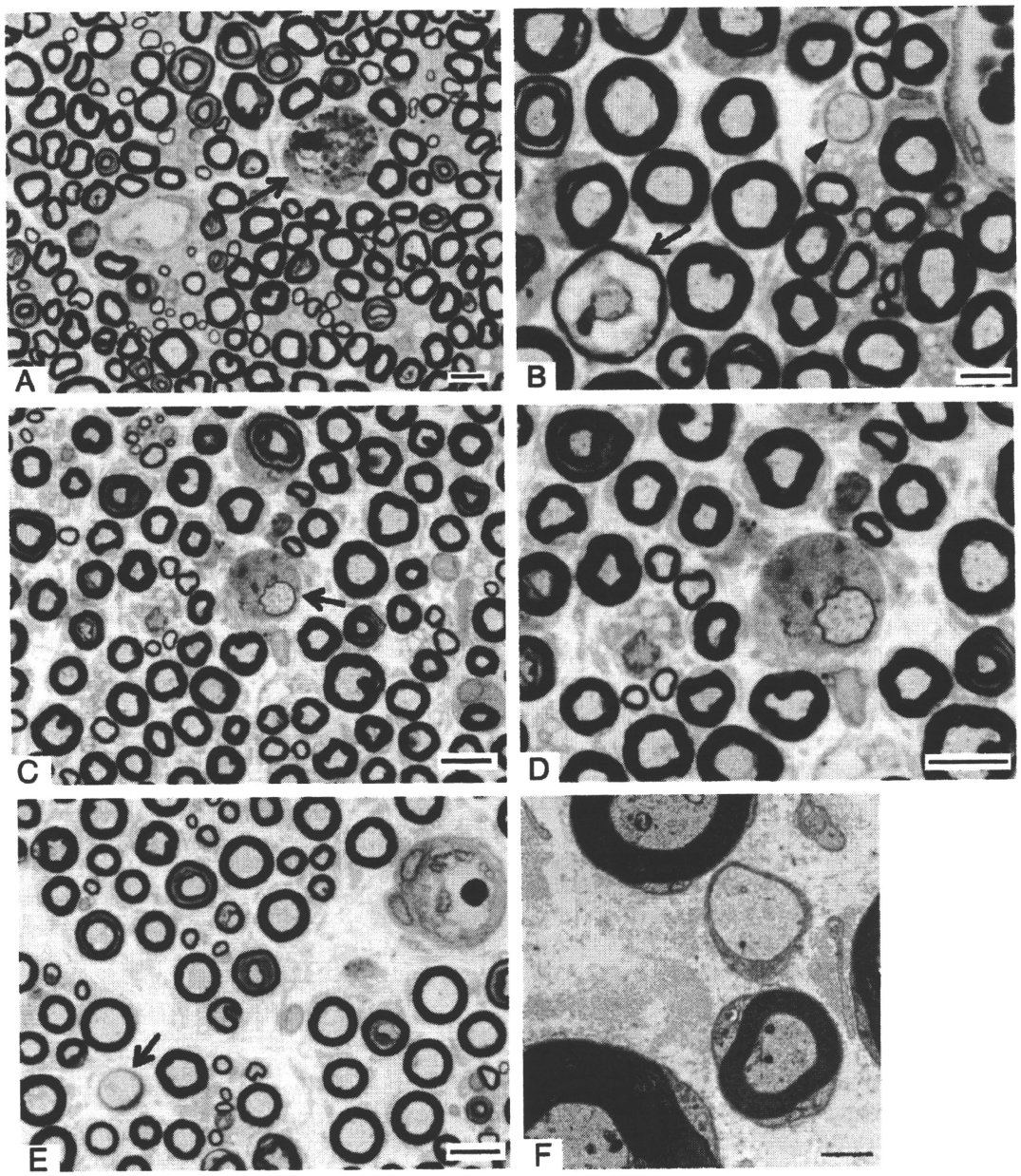

Fig. 2 – Nerve pathology after 90 min of ischemia and 7 days of reperfusion at the upper thigh (A–D) and lower thigh (E, F) levels of sciatic and tibial nerves from diabetic rats. (A) Macrophage with myelin debris (arrow) was surrounded by normal myelinated nerve fibers. (B) Demyelinated nerve fiber was located near the endoneurial vessel (arrowhead). Intra-myelinic edema was also observed (arrow). (C, D) Myelinated nerve fiber with disproportionately thin myelin and extensive cytoplasm around axon (arrow) suggests macrophages invading Schwann cells, although it is often difficult to distinguish Schwann cells containing myelin debris from macrophages by light microscopy. (E) At the lower thigh level of tibial nerve, demyelination (arrow) and endoneurial edema were seen. (F) Demyelinated nerve fiber with naked axon shown in E was confirmed by electron micrograph. Scale bars: 10 μm (A–E); 2 μm (F).

3. Discussion

This study confirmed that the ischemic threshold to cause abnormal nerve morphology in diabetic nerves is 75 min. Seventy-five minutes of ischemia could result in axonal degeneration in STZ-diabetic nerve. After 90 min of ischemia, severe nerve pathology was consistently observed along the length of sciatic and tibial nerves in diabetic rats, e.g., demyelination, activated macrophages, endoneurial edema at thigh and knee levels, and axonal degeneration distally. In control nerves, 4–5 h of severe ischemia needs to induce similar morphological changes (Gray et al., 2003; Nukada and McMorran, 1994; Nukada et al., 1997). In our previous studies of reperfusion nerve injury, we demonstrated demyelinated fibers particularly at perivascular region, endoneurial edema, intra-myelinic edema, and axonal degeneration at proximal nerve segments and panfascicular necrosis at distal levels after 5 h of ischemia

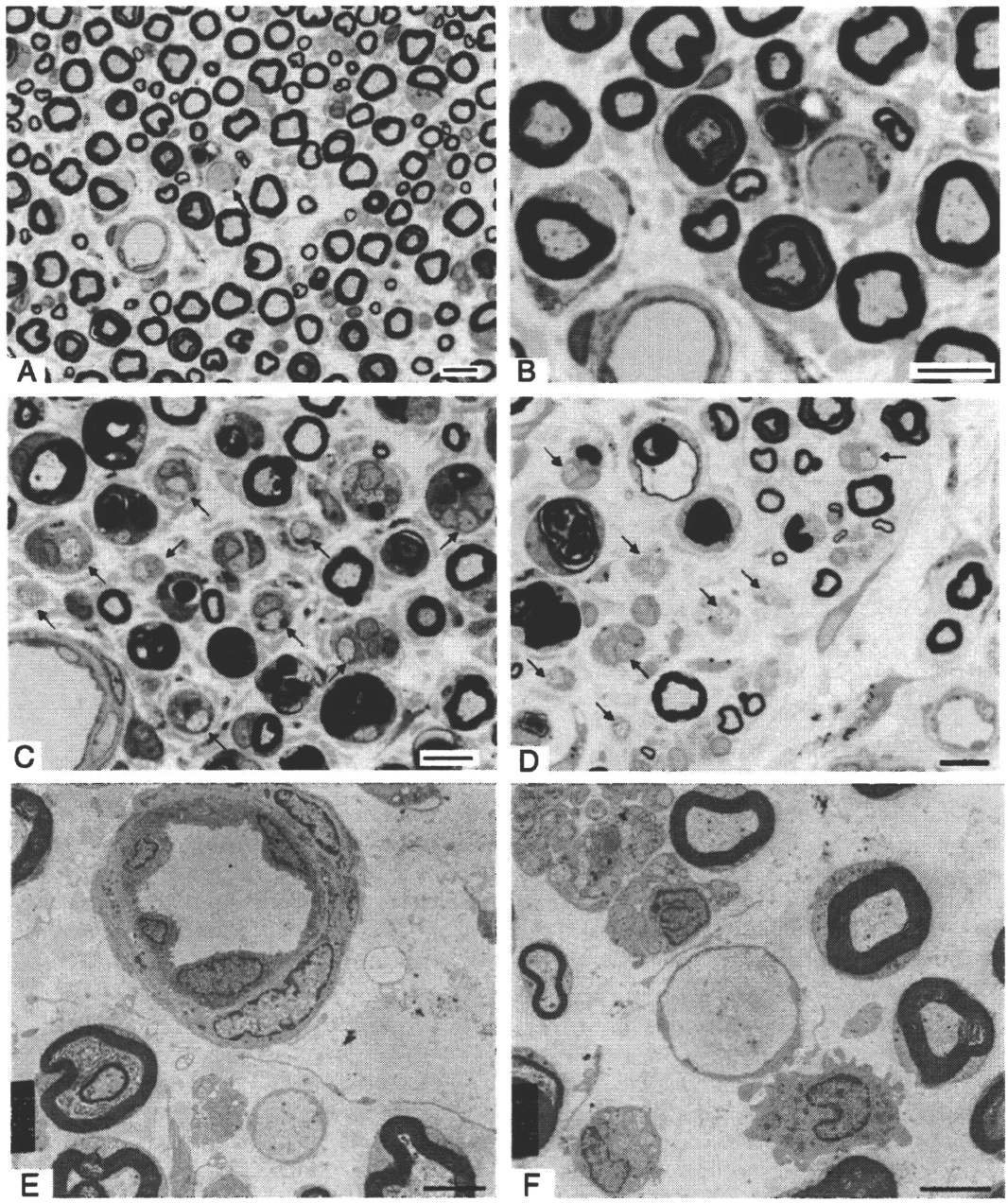

Fig. 3 – Nerve pathology after 90 min of ischemia and 7 days of reperfusion at the upper calf level of tibial nerve from diabetic rats showing demyelination, endoneurial edema, and axonal degeneration. (A) Demyelinated nerve fiber (arrow) was accompanied by a macrophage with myelin debris. (B) High magnification of a demyelinated fiber and a macrophage shown in A. (C) A cluster of nerves fibers with demyelination (arrows) and axonal degeneration were observed. It is often hard to distinguish naked axons from axons with one or two layers of myelin by light microscope. (D) Nerve fibers with demyelination (arrows) and axonal degeneration were observed with severe endoneurial edema and intra-myelinic edema. (E, F) Electron micrographs of demyelinated nerve fibers and endoneurial edema from the nerve shown in A and B. Note a monocyte beside a demyelinated fiber. Scale bars: 10 μm (A–D); 5 μm (E, F).

and 7 days of reperfusion in non-diabetic rats (Nukada and McMorran, 1994; Nukada et al., 1997). These pathological changes at thigh and knee levels are caused by reperfusion, while morphological changes at ankle level are induced by no-reflow phenomenon (Nukada et al., 1997). In the current study, we showed similar pathological changes in diabetic nerves after 90 min of ischemia, although the duration of ischemia is not long enough to cause no-reflow phenomenon at distal levels.

After 8 weeks of STZ-induced diabetes, the density of Iba-1-positive endoneurial macrophages increased significantly in sciatic and tibial nerves when compared with those in control nerves. Most of these endoneurial macrophages were slim and

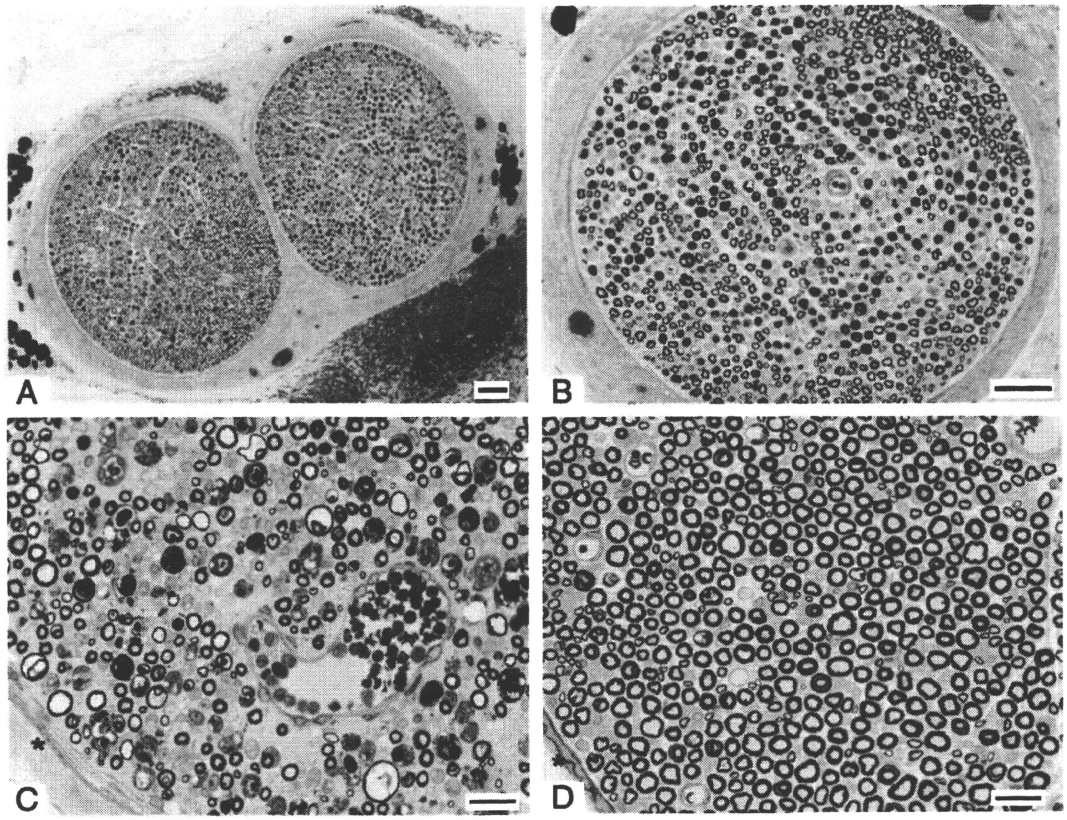

Fig. 4 – Nerve pathology after 90 min of ischemia and 7 days of reperfusion at the lower calf/ankle level of tibial nerve from diabetic (A–C) and control (D) rats. Axonal degeneration was prominent at this level in diabetic nerves. (A, B) Nerve fibers with axonal degeneration were scattered among normal myelinated fibers, although there are multifocal lesions to a certain degree. Approximately a half of myelinated nerve fibers shows axonal degeneration. (C) Myelinated nerve fibers exhibit various stages of axonal degeneration. Note accumulation and adhesion of circulating white blood cells in the endoneurial venule. (D) Control nerve reveals normal nerve morphology. *Perineurium. Scale bars: 50 μm (A, B), 20 μm (C, D).

triangular in shape, but some of them were rounded and colabelled with BrdU. It is also noted that endoneurial macrophages were not distributed evenly along the length of sciatic and tibial nerves. In diabetic rats, the density of endoneurial macrophages was significantly greater at thigh and knee levels than at calf level. A similar trend was seen in control nerves, although not significant statistically. These data indicate that hyperglycemia, per se, results in macrophage proliferation at thigh and knee levels of the diabetic nerve. Macrophage proliferation may occur at this level because of anatomic watershed zone of poor perfusion (Dyck et al., 1972; Dyck, 1989; Nukada and Dyck, 1984). A substantial increase in tissue macrophages is a common feature of type 2 diabetic vascular complications, including atherosclerosis, retinopathy, and nephropathy (Ehses et al., 2008; Tesch, 2007; Wellen and Hotamisligil, 2005). Various metabolic abnormalities secondary to hyperglycemia promote macrophage accumulation and activation within diabetic tissue. Subsequently macrophages mediate diabetic injury through a variety of mechanisms, i.e., expressing a plethora of regulatory cytokines and secreting free radicals. In STZ-diabetic nerve, an increased number of ED-1-positive endoneurial macrophages were inhibited significantly by pioglitazone, peroxisome proliferator-activated receptor γ (PPARγ)-ligand, treatment for 12 weeks (Yamagishi et al., 2008).

After 90 min of ischemia and 6, 24, and 48 h of reperfusion, the density of Iba-1 endoneurial macrophages increased significantly in diabetic nerves, but not in control nerves. Following 6 h of reperfusion, the number of endoneurial macrophages increased significantly at the thigh level, but not at knee and calf levels, of diabetic sciatic nerve when compared with those before ischemia. Subsequently, macrophage proliferation extended distally along the length of sciatic and tibial nerves in diabetic rats. After 24 h of reperfusion, the density of macrophages increased significantly at thigh and knee levels, when compared with those at 6 h. Following 48 h of reperfusion, the density of endoneurial macrophages increased significantly, in further, to 2.5-fold at thigh and 3.4-fold at knee and calf levels compared with pre-ischemic level in diabetic nerves. In our previous study, a similar trend of increased number of endoneurial macrophage was found in non-diabetic nerve after a longer period of ischemia. After 5 h of ischemia and 48 h of reperfusion, IC-7-positive endoneurial macrophages increased nearly 4-fold, and macrophage-associated inflammatory demyelination was observed after reperfusion (Nukada et al., 2000). Because the most severe ischemia was achieved at mid- and lower-thigh, and knee levels in the current model of an ischemic injury (Dyck et al., 1984; Nukada and Dyck, 1984; Nukada and McMorran, 1994; Nukada et al., 1997), macrophage proliferation was originally generated by reperfusion

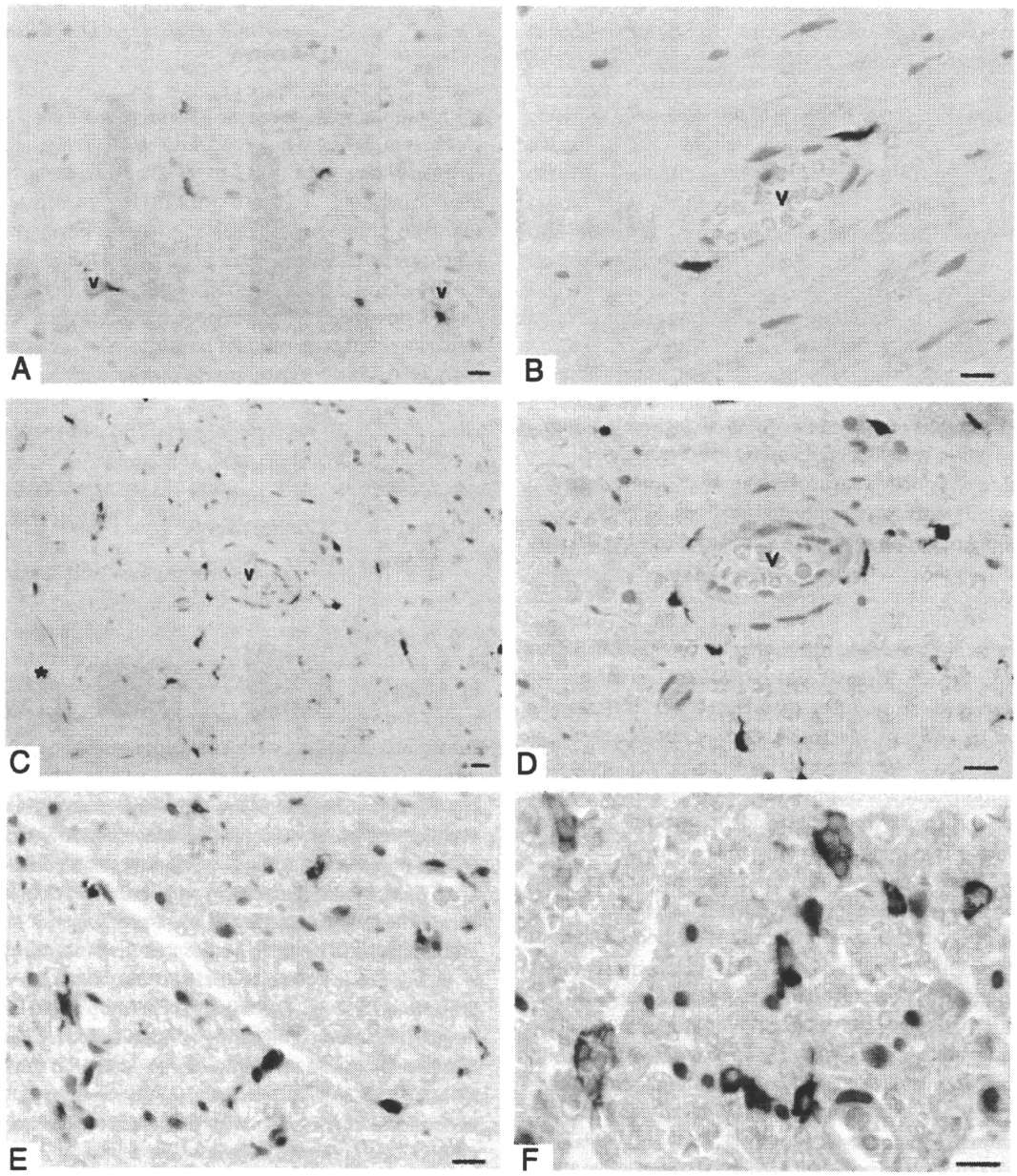

Fig. 5 – Endoneurial macrophages identified by the polyclonal macrophage antibody against Iba-1 in diabetic nerve before (A, B) and after 90 min of ischemia and 6 h (C, D) and 48 h (E, F) of reperfusion at the thigh level of sciatic and tibial nerves. (A, B) Before ischemia, endoneurial macrophages are small or skinny with a triangular or longitudinal shape. They are often positioned around endoneurial vessels. (C, D) After 90 min of ischemia and 6 h of reperfusion, the number of Iba-1-positive endoneurial macrophages increased, and cell bodies of many Iba-1-positive macrophages were enlarged or rounded. (E, F) After 90 min of ischemia and 48 h of reperfusion, Iba-1-positive endoneurial macrophages reveal phagocytosis with lipid debris. Blue nuclear counterstain with hematoxylin. v: Endoneurial vessel. *Perineurium. Scale bars: 10 μm for all panels.

injury at the thigh level of sciatic nerve and reached at distal levels 48 h later.

Endoneurial macrophages are crucially involved in the pathogenesis of peripheral neuropathies. In addition to infiltrating hematogenous macrophages, a population of local resident macrophages has long been recognized in the peripheral nerve (Griffin and George, 1993; Griffin et al., 1993). In the current study, it is impossible to distinguish resident endoneurial macrophages from infiltrating haematogenous macrophages. Muller and his colleagues have demonstrated elegantly a crucial role of resident endoneurial macrophages using bone marrow chimeric mice carrying green fluorescent protein (GFP) transgenic bone marrow, allowing the differentiation of resident (GFP^-) and invading hematogenous (GFP^+) macrophages (Mueller et al., 2001, 2003). They reported that the peripheral nervous system is able to generate an intrinsic macrophages reaction by resident macrophages (Muller et al., 2008). Only in the case of more severe nerve damage, an additional influx of hematogenous macrophages is

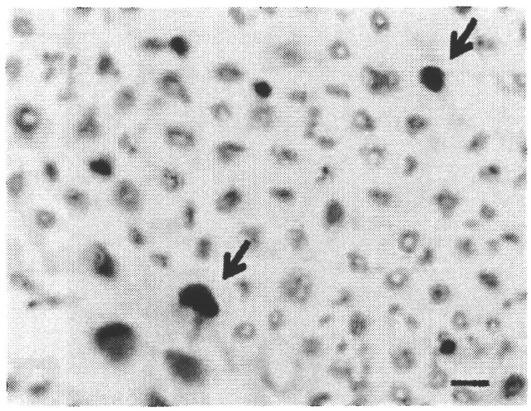

Fig. 6 – After 8 weeks of diabetes without an ischemic injury BrdU incorporation was detected among Iba-1-positive endoneurial macrophages at the thigh level of sciatic nerve, suggesting proliferating macrophages (arrows). Note these BrdU-colabelled macrophages were rounded. Scale bar: 20 μm.

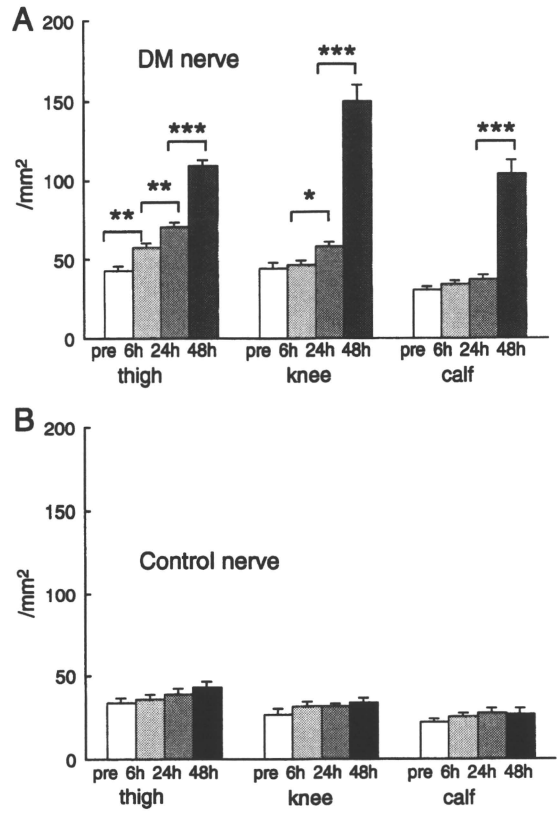

Fig. 7 – Quantification of Iba-1-positive endoneurial macrophage before and after 90 min of ischemia and 6, 24, and 48 h of reperfusion at thigh, knee, and calf levels of sciatic and tibial nerves from diabetic (A) and control (B) rats. (A) After 6 h of reperfusion, the density of Iba-1-positive endoneurial macrophages in diabetic nerves increased significantly at thigh level, but not at knee and ankle levels, when compared with pre-ischemia level. The density of macrophages in diabetic nerves increased significantly at thigh and knee levels after 24 h of reperfusion and at all three levels after 48 h when compared with 6 h and 24 h, respectively. (B) The density of Iba-1-positive endoneurial macrophages in control nerves did not significantly differ before and after ischemia and reperfusion. Statistically significant differences denoted by *$p<0.05$, **$p<0.01$, ***$p<0.0001$. ANOVA: in diabetic nerves, proximal; $p<0.0001$, mid; $p=0.0002$, distal; $p=0.001$, and in controls, proximal; $p=0.16$, mid; $p=0.40$, distal; $p=0.45$.

initiated. Proliferating resident macrophages were peaked 3 days after crush injury in sciatic nerve, and the influx of hematogenous macrophages begins around day 4 (Mueller et al., 2001). Myelin phagocytosis by resident macrophages was demonstrated as early as 2 days after injury and was found to be one of the first features of endoneurial macrophage activation. These collective data support the notion that the resident macrophages may orchestrate the inflammatory response that occurs in the acute phase of reperfusion nerve injury. Iba-1-positive macrophages after 6 to 48 h of reperfusion injury in diabetic rats may be much earlier than post-traumatic infiltration of hematogenous macrophages.

Intracellular oxidative stress has been proposed as a unifying explanation for the development of diabetic vascular complications (Cameron and Cotter, 2008; Jay et al., 2006; Pacher and Szabo, 2006; Pop-Busui et al., 2006; Zochodne, 2007) and of reperfusion injury (Frangogiannis et al., 1998) including reperfusion nerve injury (Anderson et al., 1997; He et al., 1999, 2003; Wang et al., 2005). It could be speculated that the oxidative stress from hyperglycemia and reperfusion injury could be particularly harmful. The exaggerated inflammatory response after ischemia and reperfusion shown in the current study may result in morphological susceptibility to ischemia and reperfusion in diabetic nerves, although the role of the enhanced inflammatory response needs to be clarified. Diabetic subjects may suffer from severe neuropathic damage even after normally tolerable ischemia and reperfusion. Pathways controlling macrophage activation can potentially be targeted to improve recovery from ischemic nerve injury in diabetic subjects.

4. Experimental procedures

4.1. *Animals*

Male Wistar rats (7–8 weeks old) were purchased from CLEA Japan Inc., Tokyo, Japan. Rats were fasted overnight prior to i.v. injection of either 40 mg/kg STZ (Sigma, St Louis, MO, USA) in 0.1 mol/l citrate buffer or buffer alone. Following injection, rats were returned to their cages, maintained under standard 12-h light–dark cycle, and given free access to food and water for the remainder of the study. Rats were considered diabetic if non-fasting blood glucose concentration was >19.4 mmol/l (350 mg/dl) at 7 days after the STZ injection and the time of experiment. All animal protocols in this study were approved by the Committee on Ethics in the Care and Use of Laboratory Animals, University of Otago, and the Committee for Animal Experimentation, Hirosaki University, and conform to National Institute of Health guidelines stated in "Principles of laboratory animal care" (NIH publication no. 85-23, revised 1985).

4.2. *Ischemia and reperfusion injury*

The surgery to produce an ischemic/reperfusion injury was performed 8 weeks after the STZ injection. The methods for inducing ischemia/reperfusion have been detailed previously (Nukada and McMorran, 1994). In brief, rats were anesthetized with pentobarbital (0.4 mg/100 g BW i.p.) and were placed on a heating pad. Major arteries supplying the right hindlimb, abdominal aorta, right common iliac artery, right femoral, and right superficial circumflex iliac arteries were occluded by microvascular clips (TSK-1 40 g, Kyowa, Tokyo, Japan). Reperfusion was achieved by release of these vascular clips. Control group received sham surgery only. The right hindlimb temperature was kept 35±1 °C during surgical procedures. Duration of ischemia was 60, 75, and 90 min. The duration of ischemia has been determined from our previous studies (Baba et al., 2006; Nukada and McMorran, 1994; Nukada et al., 1997, 2002).

4.3. *Morphological evaluation*

The morphology of sciatic and tibial nerves in right hindlimb were evaluated (1) without an ischemic injury in diabetic (n=8) and control (n=8) rats; (2) after 90 min of ischemia and 6, 24, 48, and 72 h and 7 days of reperfusion; and (3) after 60, and 75 min of ischemia and 7 days of reperfusion in diabetic (n=12) and control (n=12) rats. The group of 90-min ischemia consisted of 32 rats each from diabetic and control group. Bromodeoxyuridine (BrdU, 50 mg/kg BW i.p.) was injected 60 min before nerves were taken. Nerves were processed using previously described methods (Nukada and Dyck, 1984). Right sciatic and tibial nerves were dissected in continuity from the pelvic level to the ankle. The entire length of nerve was taken in continuity and cut into consecutive segments: pelvic to upper thigh (approx. 5 mm), mid-thigh (10 mm), lower-thigh (5 mm), knee (10 mm), upper calf (5 mm), calf (10 mm), and lower calf/ankle (5 mm) levels. Each 10-mm nerve segment at thigh, knee, and calf levels was processed for paraffin blocks of immunohistochemical examination. Adjacent 5-mm segments were fixed for epon-embedded blocks. Nerves for epon blocks were fixed with 2.5% glutaraldehyde in 0.025 M cacodylate buffer, pH 7.40, overnight, and then postfixed in 1% osmium tetroxide, dehydrated, and cut into consecutive 2- to 3-mm blocks before embedding in epoxy resin. Semi-thin sections (1-μm in thickness) were stained with methylene blue.

4.4. *Immunohistochemistry*

Immunohistochemical studies were performed before and after 90 min of ischemia. Nerve segments for immunohistochemical evaluation were fixed in 10% buffered neutral formalin. Sections were stained using the avin–biotin–peroxidase complex method (Histofine, Nitirei, Tokyo, Japan) as previously described (Nukada et al., 2000). After deparaffinization, the specimens were microwaved in 0.01 M citrate buffer, pH 6.0, for three 5-min periods. Endogenous peroxidase was blocked by 3% H_2O_2 followed by washing in PBS. Sections were then incubated for 15 min with 10% normal goat serum in PBS to block non-specific staining and incubated the primary antibody overnight 4 °C. Subsequently, incubation with the secondary antibody was performed for 30 min. The detection of the reaction was carried out with 3′3′diaminobenzidine tetrahydrochloride (DAB, 0.5 mg/ml, Sigma, USA). Concurrent controls included replica sections in which bridging antibody substituted for nonbinding control antibody, and positive control sections from rat spleen, liver, and thymus. The following anti-rat antibodies were used: Iba-1 (ionized calcium-binding adaptor molecule 1, Wako), ED1 and ED2 (Serotec) that recognize macrophages, and S100 (Abcam, Cambridge, UK) reacts with Schwann cells. Mouse anti-rat BrdU was colabelled to identify cellular activity.

Double labelling was performed in two steps: (1) first antigen retrieval 5 min in citrate buffer in microwave over three times, followed by overnight incubation with primary antibody. The reaction was developed with alkaline phosphates using New Fucsin (Nichirei, Tokyo, Japan) as a chromogan; (2) overnight incubation with BrdU and development with peroxidase and DAB as a chromogen. Three types of negative controls were designed: rats without BrdU administration, nonbinding primary antibody, and the positive control.

4.5. *Morphometry*

For morphometric analysis, the entire transverse fascicles were examined for the localization of immunoreactive cells. The total fascicular area of transverse sections was determined using a NIH image analysis, and the number of positively stained endoneurial cells was counted manually in a blinded fashion. The density of these positive cells (/mm^2) was calculated at thigh, knee, and calf levels of sciatic and tibial nerves.

4.6. *Nerve conduction study*

Nerve conduction study in sciatic–tibial nerves was made before ischemia as previously described (Baba et al., 2006). Motor conduction velocity and compound muscle action potential (CMAP) were measured using needle near-nerve stimulating and recording electrodes. The CMAP was recorded from the dorsum of the hind paw while simulating at the level of the sciatic notch. The right hindlimb temperature was kept 35±1 °C using a heating pad.

4.7. *Statistics*

Data are presented as means±SEM. Statistical differences between groups were analyzed by unpaired Student's t-test and one-way ANOVA as appropriate (GraphPad InStat, San Diego, CA). Data that did not follow normal Gaussian distribution were analyzed by a nonparametric Mann–Whitney test. Values of $p<0.05$ were considered statistically significant.

Acknowledgments

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ORIGINAL ARTICLE

Methylcobalamin effects on diabetic neuropathy and nerve protein kinase C in rats

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ABSTRACT

Background Methyl-base-attached cobalamin (Methycobalamin) (MC) has a special affinity for nerve tissues to promote myelination and transport of axonal cytoskeleton. It is not known, however, how MC influences on peripheral nerve in experimental diabetic neuropathy.

Materials and methods We studied the effects of MC on expressions and activities of protein kinase C (PKC) in peripheral nerve of streptozotocin-induced diabetic rats. Wistar rats, 8 weeks of age, were rendered diabetic by streptozotocin (40 mg kg^{-1}, iv) and followed for 16 weeks. A half of diabetic animals were treated with MC (10 mg kg^{-1} per every other day, im) after the induction of diabetes. Normal Wistar rats were served as control.

Results At the end, untreated diabetic animals developed significant delay of nerve conduction velocity (NCV), and MC treatment normalized the NCV. Nerve PKC activity was significantly suppressed in untreated diabetic rats, while the activity was normalized in treated animals. While PKCα located in Schwann cells, PKCβI α and βII distributed in axoplasm, vascular walls and macrophages. The decreased PKC activity in diabetic nerve was associated with reduced expression of membrane PKCα and increased membrane expression of PKCβII, and MC treatment corrected these changes. Diabetic nerve contained an increased number of macrophages and 8-hydroxydeoxyguanosine-positive cells in the endoneurium, the latter of which was significantly suppressed by MC treatment. Elevated nerve polyol levels in diabetic nerve were partially corrected by MC treatment.

Conclusions This study suggested that correction of impaired neural signalling of PKC and oxidative stress–induced damage may be a major attribute to the beneficial effects of MC on diabetic nerve.

Keywords Diabetic rat, methylcobalamin, oxidative stress, peripheral neuropathy, protein kinase C.

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Introduction

Neuropathy is a common and intractable complication of patients with diabetes. The pathogenesis is not fully clarified, and the effective treatment regimen is still under exploration. Vitamin B12 (cobalamin) is an important biofactor promoting versatile metabolic cascades for cellular activity and survival in both haematopoietic and nervous tissues [1,2]. In particular, methyl-base-attached cobalamin (methylcobalamin; MC) is shown to have threefold stronger affinity for nerve tissues compared with other types of cobalamins such as hydoxycobalamin or cyanocobalamin [2,3]. In addition, MC provides a basis for transmethylation into nerve tissues that promotes conversion of homocysteine to methionine, exerting myelination, neuronal differentiation and replication, and cellular activity [4,5]. Transmethylating action of MC may also be implicated in anti-atherogenic action on endothelial cells or smooth muscle cells of vascular walls [6,7]. It is thus proposed that MC should separately be considered from other cobalamins when its action is discussed on neurovascular diseases [4,5,8–10].

Methycobalamin has widely been used in Asian countries in patients with diabetic neuropathy [11–13]. In experimental diabetic animals, we as well as others could demonstrate beneficial effects on the peripheral nerve structure and functions [14–16]. The effects of MC on experimental diabetic neuropathy were suggested to be mediated through the activation of Na,K-ATPase activity [16]. However, precise mechanisms of how MC protects peripheral nerve tissues are yet to be clear. Recently, it was shown that alteration in protein kinase C (PKC) was involved in development of experimental diabetic neuropathy [17] and as such clinical trial of PKC inhibitor was conducted for the treatment of diabetic neuropathy [18,19]. In fact, alterations in PKC activity were associated with the changes in Na,K-ATPase activity and peripheral nerve dysfunction in

streptozotocin (STZ)-induced diabetic animals [20,21]. It is yet to be clear whether isoforms of PKC are differentially altered in diabetic nerve and whether MC treatment influences on the different isoforms of PKC. In this study, we, therefore, examined PKC expressions in peripheral nerve in experimental diabetic rats and evaluated the effects of MC treatment.

Materials and methods

Male Wistar rats, 8 weeks of age, were made diabetic by intravenous injection of STZ (Sigma Ltd., St Louis, MI, USA) (40 mg kg^{-1}, iv). Diabetes was identified by continuous glycosuria and high blood glucose exceeding 14 mM 1 week after STZ injection. Diabetic rats were randomly divided into two balanced groups based on comparable levels of body weight and hyperglycaemic levels (See Table 1). Thereafter, one diabetic group (a half of diabetic animals) was treated with intramuscular injection of MC (Eisai Co., Tokyo, Japan) (10 μg kg^{-1}) into the thigh area every other day for following 16 weeks. Dose of MC was determined by the previous reports that showed improvements in nerve conduction and structure in STZ-induced diabetic rats treated with MC [14–16]. All the animals were monitored with body weight and blood glucose during experimental period. One day before killing, motor and sensory nerve conduction velocities (MNCVs and SNCVs) were examined on the left lower limb. At the time of killing under anaesthesia with pentobarbital (Abbot Co., Chicago, IL, USA), blood was withdrawn from the right atrium and centrifuged for analysis of blood glucose and lipid levels by autoanalyzer. The sciatic nerve was extirpated for biochemical and immunohistochemical analyses. A portion of the sciatic nerve was frozen for the measurements of sorbitol, fructose and PKC activity as well as protein expressions of various types of PKC isoforms. Remaining sciatic nerve was fixed in formalin solution for immunohistochemical examinations.

All animal experimentations followed the Guideline for Animal Experimentation of Hirosaki University (Approval number M0015). The protocol of investigation also conformed to the Guide for the Care and Use of Laboratory Animals as published by NIH (NIH Publication No. 85-23, revised 1996).

Nerve conduction velocity (NCV)

All animals were anesthetized with isoflurane (Abbot Co.) and placed on a thermostatically controlled heated mat. The temperature near the sciatic nerve was kept constant at 37 °C by monitoring with an electronic thermometer (PC-9400 Delta; Sato Keiryoki MFG, Tokyo, Japan).

For motor nerve conduction velocity (MNCV), the left sciatic-tibial nerve was stimulated at ankle using needle electrodes (MS92 electromyogram device; Medelec, London, UK) and then at sciatic notch, and the waves were recorded from the second

Table 1 Laboratory findings and nerve carbohydrate levels in experimental animals at the end of experiment

Animal group	Number of animals	Body weight (g)		Blood sugar(mM)		Blood total cholesterol (mM)	Blood triglyceride (mM)	Blood homocysteine (nmol/mL)	Nerve sorbitol (nmol/mg protein)	Nerve fructose (nmol/mg protein)
		Initial	End	Initial	End					
Nondiabetic control	5	326 ± 12	462 ± 6	5·99 ± 0·17	7·60 ± 0·71	2·35 ± 0·15	2·51 ± 0·49	5·95 ± 0·45	8·14 ± 1·2	41·6 ± 2·45
Diabetic (untreated)	5	300 ± 17	352 ± 19*	30·0 ± 1·44*	27·9 ± 1·71*	3·03 ± 0·14*	3·26 ± 0·19	3·58 ± 0·44*	108 ± 10·8*	324 ± 38·2*
Diabetic (MC-treated)	5	301 ± 12	379 ± 6*	29·1 ± 2·01*	28·6 ± 1·71*	2·79 ± 0·14*	3·59 ± 1·00	2·44 ± 0·10*	86·5 ± 6·03*,**	257 ± 36·1*

MC, methylcobalamin.
Values are given as mean ± SE.
*$P < 0·01$ (vs. nondiabetic control rat).
**$P < 0·05$ (vs. untreated diabetic rats).

interosseous muscle of the foot [21]. The distance between the two stimulating sites was divided by their latency differences, yielding the value of MNCV. For detection of SNCV, the digital nerve was stimulated first at interdigital metatarsal site and then at ankle. The initial deflection point of H-reflex was identified as the latency for SNCV. The distance between the two stimulating sites was divided by the difference in proximal and distal latency, yielding SNCV. An average of at least five recordings for each was used for measurements.

Tissue carbohydrate levels

Tissue accumulation of sorbitol and fructose was measured in nerve homogenates by HPLC as previously described [20,21]. Briefly, tricyclic acid (TCA) and internal standard (D-sorbitol and D-fructose) were added to the nerve homogenate, followed by centrifugation at 10 000 *g* for 5 min at 4 °C to obtain the supernatant fraction. Sorbitol and fructose in the supernatant fraction were converted to sorbitol acetate derivative and fructose acetate derivative for the measurement by HPLC (HP1050; Hewlett Packard, Palo Alto, CA, USA) and mass spectrometry (TSQ; Finnigan Mat, San Jose, CA, USA).

PKC activity

Protein kinase C activities were assayed by the method described previously [20]. Excised nerve was homogenized and centrifuged at 50 000 *g* for 30 min at 4 °C. Supernatant was collected and used as cytosolic fraction. The pellet was resuspended in 0·6 mL homogenization buffer containing 1% Triton X-100 and stored on ice for 1 h. Resuspended solution was centrifuged at 50 000 *g* for 30 min at 4 °C, after which supernatant was used as membrane fraction. Phosphorylation assay was carried out in a reaction mixture [20 mmol Tris pH 7·5, 1 mmol $CaCl_2$, 10 mmol $MgCl_2$, 33 μmol octapeptide (RKRTLRRL), 5 mmol EGTA, 10 μmol γ-^{32}P-ATP (5–10 × 105 cpm] (Perkin Elmer Life Sciences, Boston, MA, USA) in the presence or absence of 6·4 μg mL^{-1} diorein and 96 μg mL^{-1} phosphatidylserine. The reaction was started by the addition of 30 μL cytosol or membrane fraction, incubated at 30 °C for 10 min and terminated by the spotting the reaction mixture onto P-81 paper (Whatman, Maidstone, Kent, UK). P-81 paper was washed by 75 mmol phosphate buffer 4 times for 15 min. Radioactivity was counted by liquid scintillation spectrometer (Aloka, Tokyo, Japan).

Western blot analysis

Western blot analysis was performed using supernatant proteins of nerve homogenates that were extracted as cytosol and membrane fraction for PKC isoforms.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Xcell SureLock system (Invitrogen, San Diego, CA, USA) in the reducing condition. Aliquots of 100 μg samples of protein were dissolved in the sample buffer and loaded onto the Novex Tris-glycine PreCast Gel (Invitrogen). The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA, USA) using a wet transfer unit of Xcell SureLock system. For blocking, blot membranes were incubated with 5% skimmed milk in PBS-T (phosphate-buffered saline–Triton-X 100) for overnight at 4 °C. After washing with PBS-T, membrane was incubated with antibodies to PKC-α, -βI and -βII (Santa Cruz BioTech Inc., Santa Cruz, CA, USA) and β-actin (Santa Cruz) for 1 h at room temperature. The dilution of all antibodies was 1 : 1000. A final incubation was carried out with peroxidase-conjugated anti-rabbit or anti-goat IgG (Santa Cruz) for 45 min at room temperature. Immunodetection was performed by ECL system (Amersham-Pharmacia, Buckinghamshire, UK). Quantitative analysis of exposed films was performed using NIH image software (Version 1.61; Bethesda, MD, USA).

Immunohistochemical evaluation

Four-micrometre-thick sections of formalin-fixed tissues were deparaffinized and pretreated with methanol containing 0·3% H_2O_2 to eliminate endogenous peroxidase activity. Antibodies to PKC-α, -βI and -βII (Santa Cruz BioTech Inc., Santa Cruz, CA, USA) were applied to the sections overnight at 4 °C. To demonstrate macrophage and to evaluate oxidative stress–induced DNA damage, antibodies to rat macrophage (Iba-1; ionized calcium-binding adaptor molecule-1) (Wako Ltd., Osaka, Japan) and to 8-hydroxydeoxyguanosine (8OHdG) (Nihon Yushi, Kumamoto, Japan) were used, respectively.

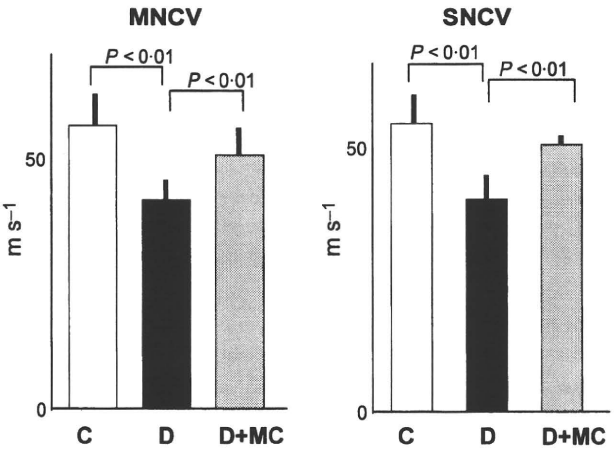

Figure 1 Nerve conduction studies on experimental animals. There was a significant delay of motor and sensory nerve conduction velocity (MNCV and SNCV) in untreated diabetic rats (D) compared with normal control rats (C). The delay of MNCV and SNCV was significantly improved by methylcobalamin treatment (D+MC). The number of animals was five in each group. Bar stands for SE.

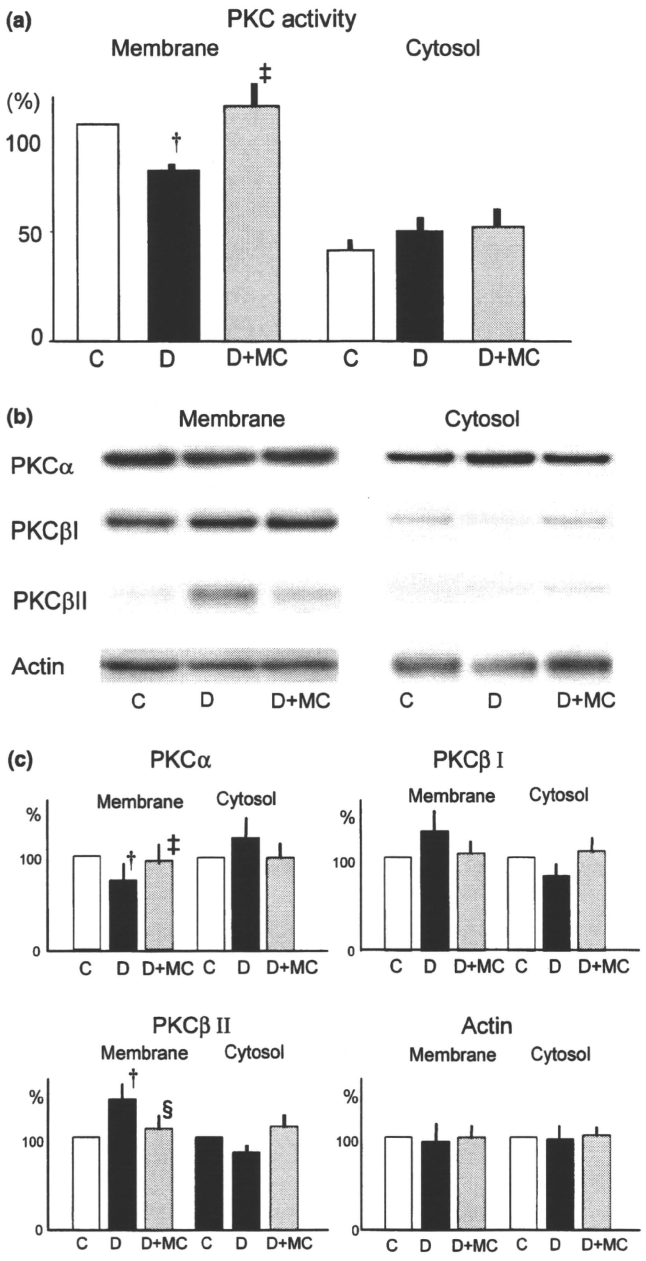

Figure 2 Protein kinase C (PKC) activity and expression of PKC isoform in endoneurial tissues of peripheral nerve in experimental rats. (a) There was significant reduction in membrane PKC activity in diabetic rats (D) compared with normal controls (C). Methylcobalamin-treated group (D+MC) showed normalization of PKC activity. Cytosolic PKC activity was not significantly altered in diabetic rats. (b) PKC isoform expressions of endoneurial tissues of peripheral nerve in experimental rats. There was a decrease in PKCα expression in membrane fraction in diabetic rats and methylcobalamin treatment corrected this decrease. PKCα in cytosolic fraction was not altered in diabetic rats, and methylcobalamin treatment did not influence on the expression. There was no significant change in membranous expression of PKCβI, although there was a slight decrease in cytosolic fraction. In contrast, there was an increase in membranous PKCβII expression in diabetic rats, and methylcobalamin treatment inhibited this change, whereas there was no change in cytosolic fraction. (c) Densitometric analysis shows the average value of each group, and bar stands for SE. The number of animals was five in each group. C, control rats; D, diabetic rats; D+MC, methylcobalamin-treated diabetic rats. †$P < 0{\cdot}01$ vs. C, ‡$P < 0{\cdot}01$ vs. D, §$P < 0{\cdot}05$ vs. D.

After the application of first antibodies, sections were incubated with secondary and tertiary agents using a streptavidin-biotin-peroxidase detection kit (Histofine SAB-PO Kit; Nichirei, Tokyo, Japan). N,N'-Diaminobenzidine was used to visualize peroxidase deposition. Specificity of the staining was confirmed by (i) omission of the first antibody during the process of the immunostaining, (ii) replacement of the first antibody by non-immune rabbit sera and (iii) absorption of the antibody, when available, by excessive antigens to antibodies.

Population of macrophage and 8OHdG-positive cells

For the count of 8OHdG-positive and Iba-1-positive cells, transverse sections of the sciatic nerve stained immunohistochemically were incorporated into NIH image analysis. Positive cells for 8OHdG and Iba-1 were identified as strongly positive reaction whose intensity was more than 10 times of background brown intensity. The number of macrophages was counted on the sections of individual animals and expressed as cell number per unit area.

Statistical analysis

Data were expressed as mean ± SE. Statistical analysis was carried out on a Macintosh computer (Apple Inc., Cupertino, CA, USA) using a commercially available statistical program (StatView, version 4.11 J; Huilinks, Tokyo, Japan). Comparison of the values among the groups was made using one-way ANOVA, followed by Bonferroni's correction for multiple comparisons. *P*-values <0·05 were considered to be significant.

Results

Laboratory findings

The laboratory data are summarized in Table 1. At the end of the experiment, average body weight in diabetic group was smaller compared with that in nondiabetic group. MC treatment did not influence these values. Blood glucose concentrations were significantly greater in diabetic group, and MC treatment did not influence the levels. Serum lipid levels of total cholesterol and triglyceride were both elevated in diabetic

