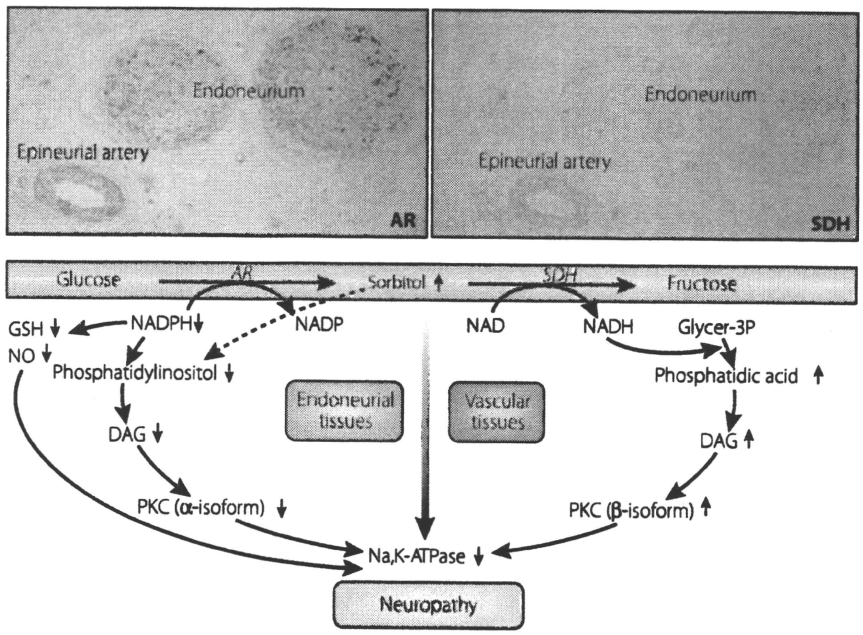

Figure 3 | Tissue-specific regulation of polyol pathway and its metabolic cascade to diabetic neuropathy. Major regulating enzymes of the polyol pathway are differentially expressed in the epineurial artery and endoneurial tissues. Aldose reductase (AR) is strongly expressed in both the endoneurium and the wall of the epineurial artery, whereas expression of sorbitol dehydrogenase (SDH) is equivocal in the endoneurium, but clearly positive for the wall of the epineurial artery (see reference 120, with kind permission from Springer Science + Business Media: Virchows Arch, Vol. 439, 2001, page 48. Enhanced *in situ* expression of aldose reductase in peripheral nerve and renal glomeruli in diabetic patients; Kasajima H, Yamagishi SI, Sugai S, Yagihashi N, Yagihashi S, Figure 2). Hence, hyperglycemia in nerve tissues exerts conversion from glucose to sorbitol by AR, thereby causing the depletion of reduced glutathione (GSH) and nitric oxide (NO) consequent from the overconsumption of nicotinamide adenine di-nucleotide phosphate (NADPH). Concurrently, intracellular *myo*-inositol is depleted to cause phosphatidylinositol (PI) depletion, which further suppresses diacylglycerol (DAG) production and finally protein kinase C (PKC) activity. As a consequence, Na,K-ATPase activity will be reduced to result in functional and structural changes of neuropathy. In contrast, the second portion of the polyol pathway regulated by SDH is activated in the vascular wall in the hyperglycemic condition. As a result of redox changes of NAD/NADH, conversion from glyceraldehyde-3-phosphate (Glycer-3P) to phosphatidic acid will be promoted. Then enhanced synthesis of DAG results in increased PKC activity. In our studies, major isoforms that underwent changes in the diabetic condition are PKCα in the nerve and PKCβ in the epineurial artery (reference 122).

ischemia/reperfusion injury, there emerges a new perspective that ischemia/reperfusion might be involved in the progression or exacerbation of neuropathy to which ARI is effective[77,78].

Glycation and Advanced Glycation End-products

Glycation has long been implicated in the pathogenesis of diabetic neuropathy[30,79,80]. Every component of nerve tissues can be excessively glycated in diabetic nerves. In fact, deposition of advanced glycation end-products (AGE) was shown in human and animal diabetic nerves, in every component of peripheral nerve tissues[30,80]. The deposition was found in the stromal collagens, axoplasms of nerve fibers and Schwann cells, as well as endoneurial vessels[81]. The intensity of AGE deposition detected by carboxymethyllysine immunoreactions correlated well with reduced myelinated nerve fiber density[81]. Hence, AGE was considered to exert injurious processes in the endoneurium through direct toxicity to nerve tissues together with endoneurial microangiopathy (Figure 5). *In vitro*, Schwann cells underwent apoptotic processes with release of tumor necrosis factor (TNF)-α, as well as other inflammatory cytokines, when exposed to a high AGE environment[82]. Axonal cytoskeletons of tubulin and neurofilaments were glycated to stagnate axonal transport, resulting in distal fiber degeneration[30]. Glycation of basement membrane collagen, laminin and fibronectin also caused impairment of regenerative efforts in diabetic nerves[83,84].

Transgenic mice with enhanced expression of the receptor for AGE (RAGE) in endothelial cells showed augmented neuropathic changes in the diabetic condition, exemplified by delayed NCV and more severe structural changes[85]. In this setting, it can be speculated that AGE exerts biological reactions after binding with RAGE expressed on endothelial cells and Schwann cells, leading to the functional and structural phenotype of neuropathy. During this process, intracellular oxidative stress mediated by NADPH oxidase activation might be elicited and then activate transcription of nuclear factor-κB (NF-κB)[86,87]. Bierhaus *et al.* reported that the activation of NF-κB was associated with the alteration of pain sensation in STZ-induced hyperglycemic mice[88]. Diabetic mice lacking the RAGE gene were

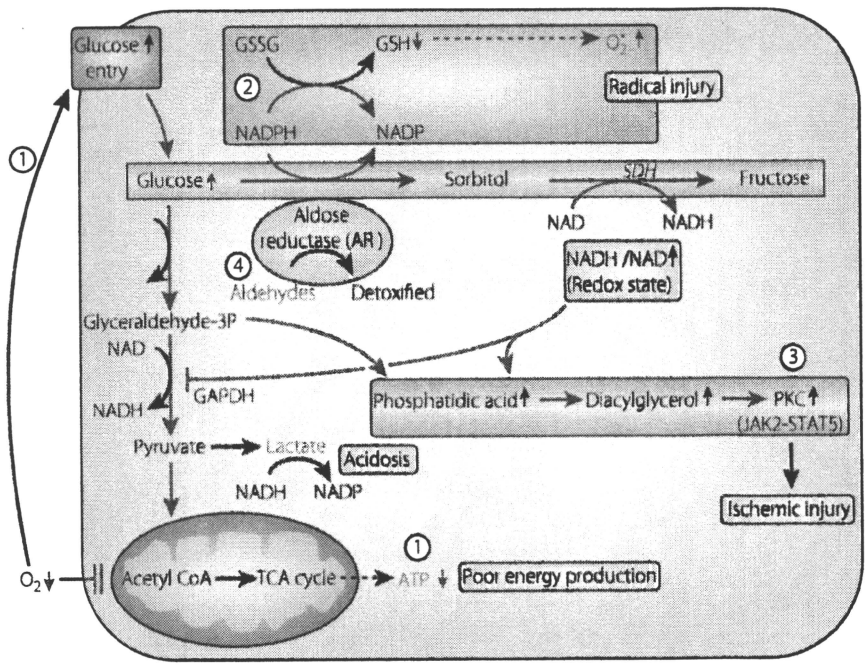

Figure 4 | Implication of aldose reductase in ischemia/reperfusion injury. Recently, a new role of aldose reductase in ischemia/reperfusion and inflammatory injury was proposed. When a cell becomes ischemic, glucose uptake is enhanced to compensate energy depletion (①). However, because mitochondria are impaired to produce ATP as a result of oxygen depletion, surplus glucose enters the collateral pathway to sorbitol and phosphatidic acid. From the former, aldose reductase is activated to cause glutathione deficiency and redox deviation, as in the hyperglycemic condition (②). As a result, free radical injury and protein kinase C (PKC) activation ensue to aggravate ischemic injury (③). Once reperfusion starts, oxygen radicals accumulate aldehydes, which are also substrates of aldose reductase, and enhance radical injury (④) (adapted from reference 69 and modified by the author).

protective against the induction of neuropathy[89]. Thus, these findings support the crucial role of AGE in the development of diabetic neuropathy.

Indirect evidence that suggests the role of AGE in neuropathy might be the effects of aminoguanidine on experimental diabetic neuropathy[47,90–92]. This compound was found to inhibit the formation of AGE, concurrently with the improvement of endoneurial blood flow[90], NCV, Na,K-ATPase activity and myelinated fiber structure[91,92]. It should be of note that aminoguanidine effects might also be mediated by its alternate action as an inducible nitric oxide synthase (iNOS) inhibitor or an anti-oxidative function[93].

In our most recent study, we showed that animals given AGE exogenously showed significant NCV delay resembling that found in experimental diabetic neuropathy (Figure 6)[94]. With delayed NCV, nerve Na,K-ATPase activity was reduced and myelinated nerve fibers underwent reduction of fiber size. In this setting, vascular reactions in response to exogenous AGE elicited functional impairment of peripheral nervous systems. In fact, endothelial cells showed a high expression of NF-κBp65 together with swollen and vacuolar changes at the ultrastructural levels. From these findings, AGE action mediated by binding with RAGE causes activation of NF-κB and thereby its downstream signals[88,95,96]. Although preliminary clinical trials of anti-glycation agent, benfotiamine, showed some efficacy for diabetic neuropathy[97], there is still no effective compound that can suppress the AGE formation *in vivo* and improve diabetic neuropathy in humans.

Oxidative Stress

As a cause of diabetic neuropathy, the generation of free radicals is proposed to be a major factor through increased glycolytic process[98,99]. In fact, there are numerous data that showed oxidative stress-induced tissue injury in the peripheral nerve in experimental diabetes[45,63,88,92,95,98]. Based on this background, attempts have been made to inhibit neuropathy with anti-oxidants[100,101]. In particular, α-lipoic acid has been used for the suppression of oxidative stress in experimental diabetic rats and it was found that it improved NCV delay, nerve blood flow and nerve structure[102–104].

Concurrent with the generation of free radicals during the glycolytic process, mitochondria have a crucial role in cellular death by activation of specific signals and the endonuclease system[105,106]. Hyperglycemia-induced mitochondrial changes include the release of cytochrome C, activation of caspase 3, altered biogenesis and fission, resulting in programmed cell death[105,107]. Excessive entry of glucose causes surplus transport of electrons to generate oxidants in mitochondria, leading to

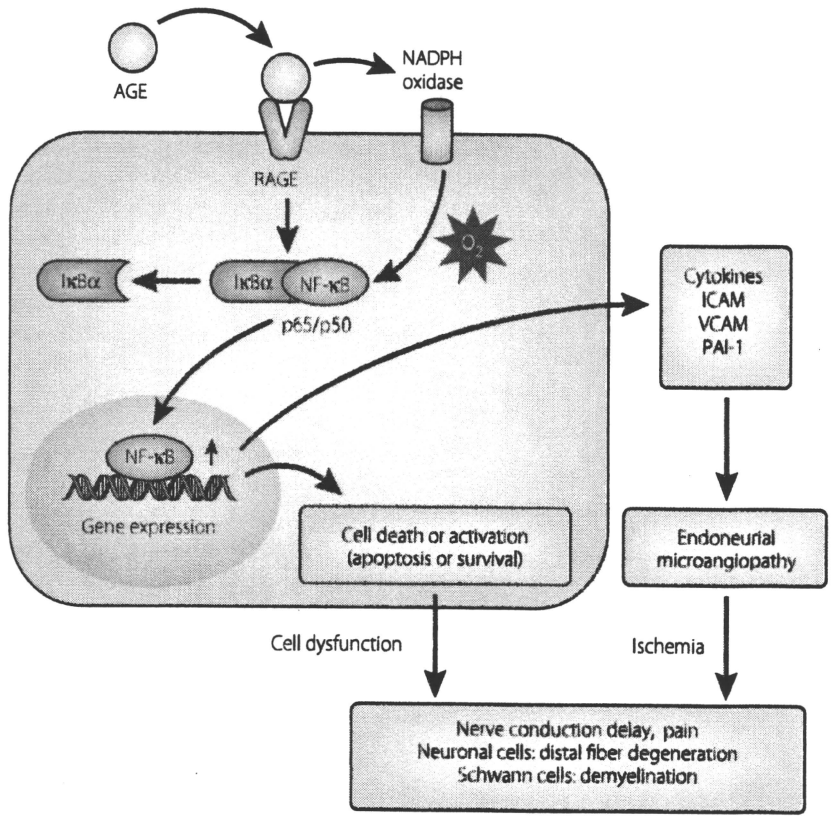

Figure 5 | Advanced glycation end-products (AGE) and receptor for AGE (RAGE) reactions in the pathogenesis of diabetic neuropathy. Nerve tissues, such as Schwann cells, nerve fibers and endothelial cells of vasa nervosum all express RAGE. When AGE bind with RAGE, the reaction generates oxidative stress mainly through the activation of NADPH oxidase. Complexes of IκBα-nuclear factor-(NF)-κB will be separated into each fraction of IκBα and NFκB, the latter of which translocates into the nucleus as a transcription factor to activate genes related to cell death or survival. As a result, both microangiopathic processes and neural dysfunction ensue, resulting in the manifestation of pain or nerve conduction delay.

reduced mitochondrial action potentials (MMP) with poor energy synthesis of ATP[108,109]. Neurotrophic support is also impaired by mitochondrial damage to cause reduced neurotrophin-3 (NT-3) and nerve growth factor (NGF)[108]. It is interesting that a small amount of insulin, that does not alter systemic blood glucose levels, was shown to improve the impaired mitochondrial membrane potential and delayed nerve conduction in STZ-diabetic rats[109].

As already alluded to, both the polyol pathway and AGE formation produce a large amount of oxidants, and ARI treatment suppresses the oxidative nerve injury[110–112]. In addition to mitochondria, other organelles, such as the Golgi apparatus and endoplasmic reticulum (ER), might also be regarded as an important source of free radicals, resulting in not only apoptosis, but cell death from autophagy[113]. Indeed, nitro-oxidative stress in conjunction with hyperglycemia exerts poly ADP-ribose polymerase (PARP) activation[114], resulting in cellular dysfunction and cell death, which can be prevented by PARP inhibitor[115]. Serum from type 2 diabetic patients accelerates neuroblastoma cell death by increased autophagic processes with activation of cell death signals[116]. α-Lipoic acid was found to be beneficial to some extent to alleviate neuropathic symptoms in diabetic patients[117]. However, to confirm whether this compound is in fact effective to inhibit the progression of the disease, further confirmation is required.

PKC Activity

PKC is central in nerve function and a key in the pathogenesis of diabetic neuropathy[118,119]. However, the alterations are complicated in nerve tissues and their supportive endoneurial vascular system, as the major enzymes of collateral glycolytic pathway are different between these two tissues[120] (Figure 3). Such inhomogeneous tissue composition might explain the inconsistent findings on PKC activity in diabetic nerves. Nakamura *et al.* did not find any significant change of PKC activity in the homogenized whole peripheral nerve tissues in STZ diabetic rats, although PKC-β specific inhibitor improved NCV delay and nerve blood flow[121]. In contrast, in our studies on STZ-induced diabetic mice, we separated the tissues into endoneurium and epineurium for the measurement of PKC activity, the latter of which is rich in microvessels[122]. We found that the former showed decreased PKC activity with significantly decreased

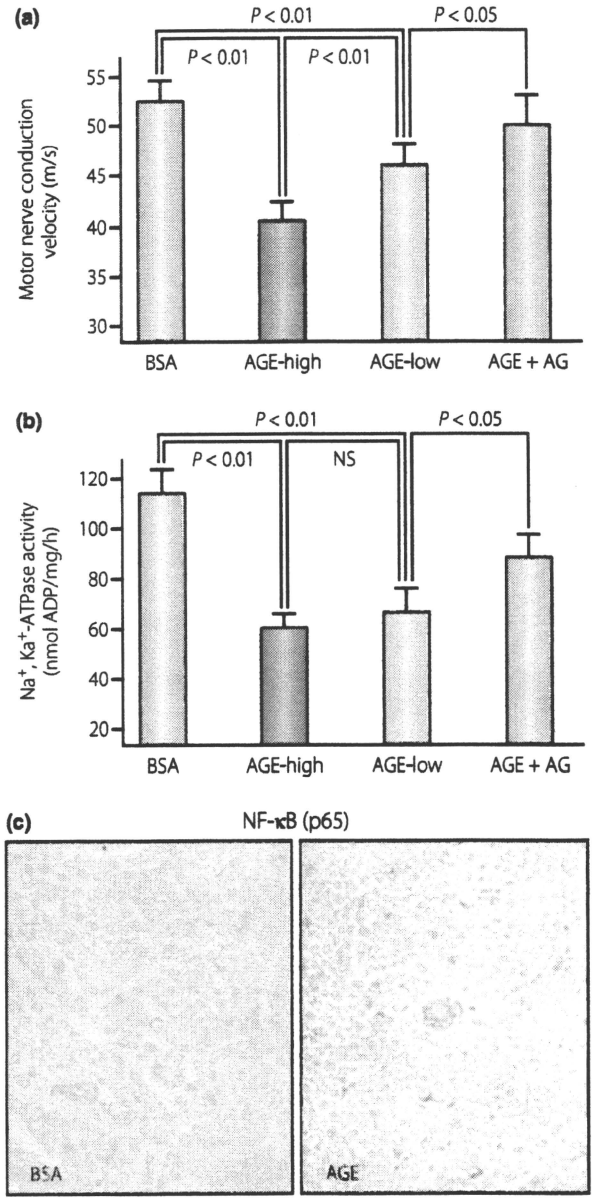

Figure 6 | Neuropathy in normal rats given exogenous advanced glycation end-products (AGE). When AGE were given exogenously, normal rats showed neuropathic changes, similar to those found in experimental diabetic animals. Rats given AGE showed (a) a significant delay of motor nerve conduction velocity and (b) suppression of nerve Na,K-ATPase activity, whereas no effects were detected in bovine serum albumin (BSA)-treated rats. Such suppression was corrected by co-treatment with aminoguanidine, an inhibitor of glycation and nitric oxide. (c) On the sections, AGE-treated rats showed strong expression of nuclear factor-κB on the nuclei of endothelial cells of microvessels and Schwann cells (quoted from reference 94).

membranous expression of the PKC-α isoform, as we already stated earlier about polyol pathway, whereas the latter showed increased PKC activity with enhanced expression of PKC-β (Figure 3). The results of epineurial tissues were consistent with the changes in other systemic vascular tissues. In keeping with this finding, hyperglycemia caused reduced PKC activity in cultured Schwann cells exposed to high glucose[123].

Hence, the application of PKC-β-specific inhibitor is expected to be useful for the treatment of diabetic vascular complications. Experimental studies showed beneficial effects of PKC-β-specific inhibitor on neuropathic changes in STZ-induced diabetic rats[121,124,125]. Despite extensive efforts, however, clinical trials were not successful due, in part, to the high improvement rate in the placebo group[126]. Other isoforms of PKC were also implicated in the causation of diabetic neuropathy and inhibitors for these isoforms have been explored[127,128].

Proinflammatory Processes

There is emerging evidence that nerve tissues in diabetes undergo a pro-inflammatory process that presents symptoms and enhances the development of neuropathy[129,130]. Indeed, diabetic nerves contain macrophages, occasionally lymphocytes and release increased TNF-α or interleukins (IL) in humans and animals[129,131,132] (Figure 7). Inhibition of cytokine release or macrophage migration was associated with the improvement of NCV delay and structure in STZ-diabetic rats treated with *N*-acetylcysteine[133] or pioglitazone[134]. The arachidonic acid pathway is activated to increase in cyclooxygenase (COX)-2 concentrations in the peripheral nerves of STZ diabetic rats in which inhibition of COX-2 corrected nerve blood flow and NCV delay[135]. To further confirm this data, COX-2 gene-deficient mice were protective for NCV delay and neuropathic deficits after STZ-induced hyperglycemia[136]. The pro-inflammatory condition activated the stress-kinase, mitogen-activated protein (MAP)-kinase, in diabetic nerves, which was also suppressed by treatment with pioglitazone[134]. Thus, MAP-kinase is considered to be a potential target for a new treatment of diabetic neuropathy[137,138]. In this process, NF-κB is activated to lead the cell to cell death or proliferation[139,140]. Because a pro-inflammatory reaction is induced by the polyol pathway hyperactivity or increased AGE formation as well, it should be clear to what extent the pro-inflammatory process is a single initiating or influential factor for the development of neuropathy. Ischemia reperfusion might also accelerate the inflammatory processes to which diabetic nerves are susceptible[77,78].

With increasing information about the role of inflammation, approaches to suppress the pain symptoms or neuropathy itself are now carried out with the specific target of cytokines or cell signals[141–143].

Cellular and Trophic Factors

The lack of neurotrophins plays an important role in the pathogenesis of diabetic neuropathy[144–149]. In fact, the production of NGF was suppressed in the skin and substitution of NGF ameliorated neuropathic changes of small fibers and autonomic pathology in diabetic animals[150,152]. NT-3, brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF)

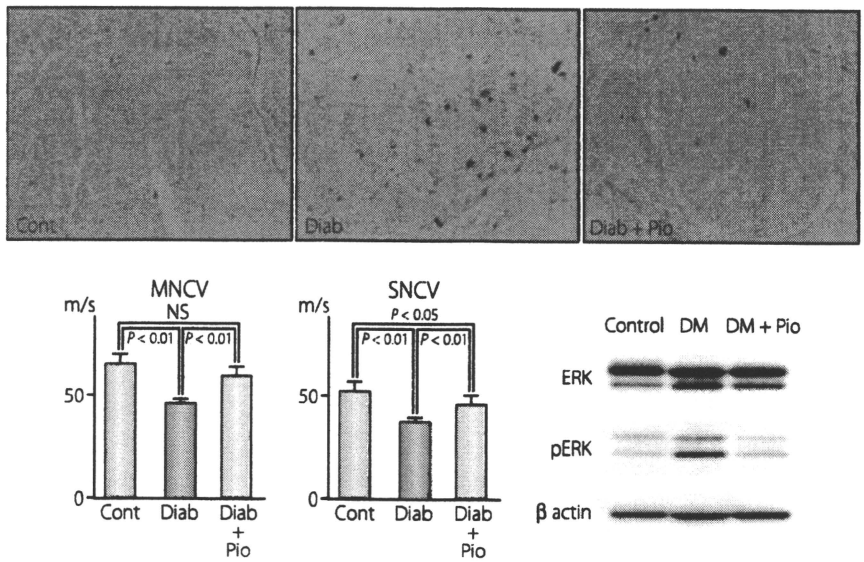

Figure 7 | Pro-inflammatory reactions and experimental diabetic neuropathy. In the sciatic nerve of STZ-induced diabetic rats, there were many macrophages stained positive for ED1 (upper center). Migration of macrophages was inhibited when diabetic rats were treated with pioglitazone (upper right). Pioglitazone treatment also corrected the delay of motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV), and activation of extracellular signal-regulated kinase (ERK), one of mitogen activated protein kinases (MAPK) (adapted from reference 134).

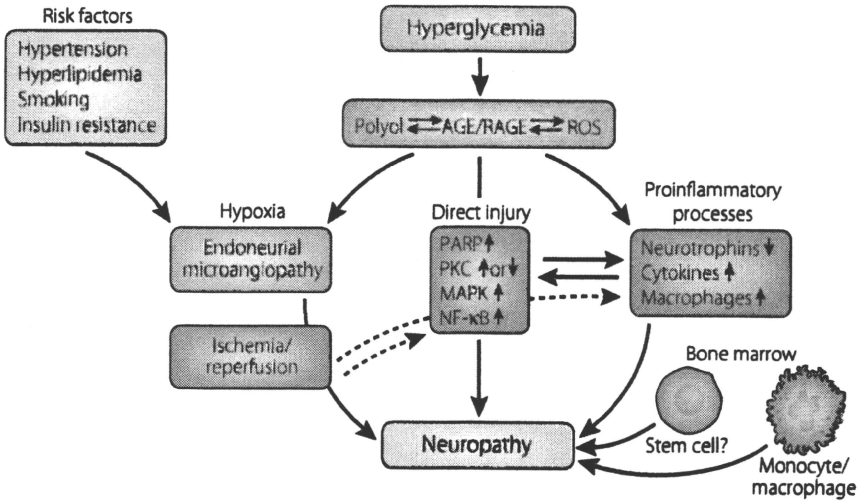

Figure 8 | Summary of pathogenetic mechanisms of diabetic neuropathy. Long-term hyperglycemia causes downstream metabolic cascades of polyol pathway hyperactivity, advanced glycation end-products (AGE)/receptor for AGE (RAGE) reactions and increased reactive oxygen species (ROS). They compromise both endoneurial microvessels and neural tissues themselves through activation of poly-ADP-ribose polymerase (PARP), alterations of protein kinase C (PKC) and an increase in mitogen-activated protein kinase (MAPK), as well as activation of nuclear factor-(NF)-κB, resulting in functional and structural changes of peripheral neuropathy. Metabolic aberrations in the nerve elicit pro-inflammatory reactions, inducing release of cytokines, suppression of neurotrophins and migration of macrophages, and promote the development of neuropathy. Recently, cellular factors derived from the bone marrow were found to produce chimeric cells in peripheral nerves of diabetic animals to elicit nerve injury. There is also the possibility that other cellular components from the bone marrow have an influence on the nerve pathology in diabetes. In addition, ischemia/reperfusion might also accelerate nerve injury, in part mediated by inflammatory reactions. Risk factors represented by hypertension, hyperlipidemia, smoking and insulin resistance are also important contributors to the development of neuropathy.

were also decreased in the muscle tissues in diabetic patients[153]. NT-3 was shown to protect the NCV delay and perception threshold in diabetic animals[154], but the results were not always positive[155,156]. Unfortunately, application of NGF in a clinical trial did not succeed in the correction of neuropathy, in part because of the emergence of pain[157]. Efforts have now been

made to more efficiently deliver or produce trophic factors at the target tissues by introducing gene therapy or cell transplantations[59,159–162].

Recent studies have shown a new insight into the pathogenesis of neuropathy. In diabetic nerves, there were chimeric cells that were a combination of resident Schwann cells or neuronal cells and migrated proinsulin-producing cells derived from bone marrow[163]. Although the significance of such chimeric cells is yet to be known, they eventually undergo apoptotic cell death, thus injuring the constitutive cells, leading to neuropathic changes. Much remains to be further investigated to confirm such intriguing cells and to clarify their significance.

Direction of Treatment

Based on the proposed mechanisms of neuropathy so far (Figure 8), efforts have been continuously made to develop effective means for the treatment of neuropathy. However, to date, there are only a few agents available in limited countries; ARI (epalrestat) in Japan and α-lipoic acid (thioctic acid) in Germany. Other agents, such as benfotiamine as an anti-glycation agent, PKC-β-inhibitor (ruboxitaurine) or NGF were unsuccessful at the final stage of randomized clinical trials. Nevertheless, there are still ongoing trials that we hope will be successful in future. Very recently, it was shown that autonomic neuropathy in the bone marrow impaired activation and migration of endothelial precursor cells (EPC), which might determine the fate of vascular complications[164]. It also becomes clear that the vagus nerve conveys signals for regeneration of islet β-cells[165], which might be disturbed in diabetic patients. These novel findings reinforce the importance of diabetic neuropathy for patient care and direction of treatment in diabetes. In particular, early inhibition of causative factors is extremely important not only to halt, but to reverse, the lesions. However, once the lesions are developed, as stated earlier, a variety of factors are exerted to accelerate the neuropathy. In this setting, the combination of several inhibitors might be required.

Neuropathy has long been regarded merely as a disorder of the most distal portion of the body. Effects of hyperglycemia on the nervous system have now been shown to be a much more serious condition. Neuropathy itself is an important trigger for systemic abnormalities in diabetic patients. Much more investigation on the nerve changes in the pancreas, liver and related organs is required for a better understanding of the whole body in diabetic patients and to develop effective treatment of this disease.

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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.

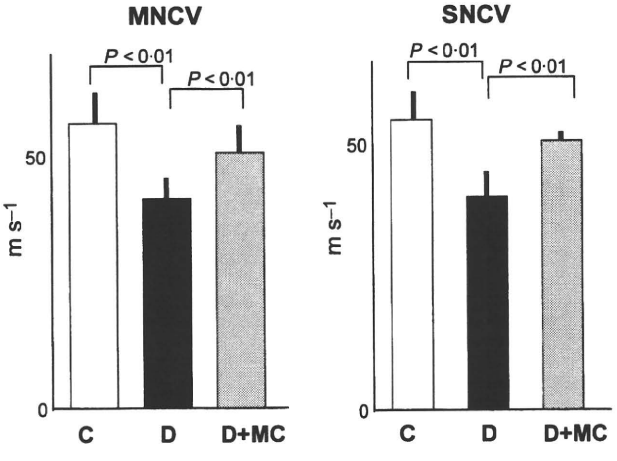

Figure 1 Nerve conduction studies on experimental animals. There was a significant delay of motor and sensory nerve conduction velocity (MNCV and SNCV) in untreated diabetic rats (D) compared with normal control rats (C). The delay of MNCV and SNCV was significantly improved by methylcobalamin treatment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

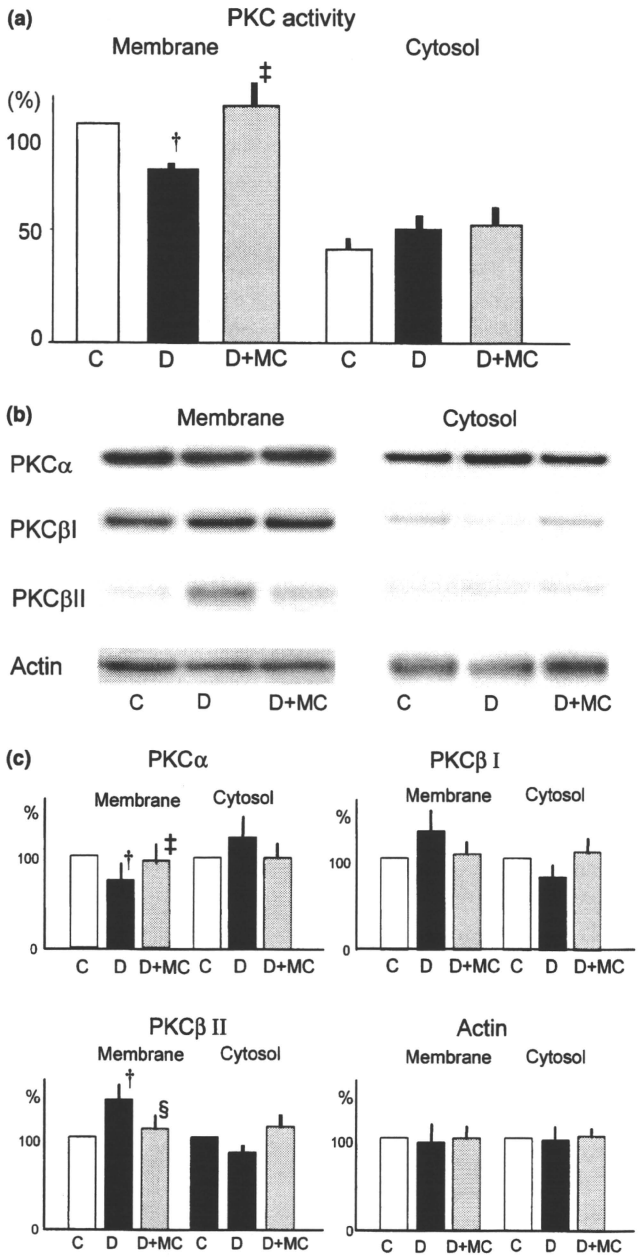

Figure 2 Protein kinase C (PKC) activity and expression of PKC isoform in endoneurial tissues of peripheral nerve in experimental rats. (a) There was significant reduction in membrane PKC activity in diabetic rats (D) compared with normal controls (C). Methylcobalamin-treated group (D+MC) showed normalization 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 Macintosch 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

group compared with those in controls, and MC treatment did not influence the values. Blood homocysteine concentrations were significantly reduced in diabetic group compared with that in normal control group, and MC treatment did not significantly influence the values although the average value was less in the treated group than that in untreated group.

Nerve sorbitol was increased more than 12-fold in diabetic group compared with normal controls, and MC treatment slightly but significantly suppressed this elevation ($P < 0.05$) (Table 1). Nerve fructose was increased ninefold in diabetic group compared with controls. MC treatment slightly decreased the average value, but it was not significant.

Nerve conduction velocity

Diabetic group showed 26% reduction in MNCV and 30% reduction in SNCV compared with those in normal control rats, respectively ($P < 0.01$ for both)(Fig. 1). MC treatment improved both MNCV by 15% ($P < 0.01$ vs. untreated diabetic rats) and SNCV by 22% ($P < 0.01$ vs. untreated diabetic rats).

PKC alterations

Endoneurial nerve homogenates were separated into membrane and cytosolic fractions. PKC activity of the membrane fraction was significantly reduced to 75% level in diabetic rats compared with the level of control rats ($P < 0.01$), while MC treatment showed a significant recovery of the activity to supranormal levels ($P < 0.01$ vs. untreated diabetic rats) (Fig. 2a). On the other hand, PKC activity of the cytosolic fraction was not significantly altered in diabetic rats.

Western blot analysis disclosed that PKCα was reduced in the membrane fraction from diabetic rats, while PKCα in the cytosolic reaction tended to increase (Fig. 2b). MC treatment corrected these changes in both membrane and cytosolic fractions. There was an elevated expression of PKCβII in the membrane fraction in diabetic rats. The cytosolic fractions of PKCβI appeared to be reduced. MC treatment corrected these changes. Comparison of the average values obtained from densitometric analysis among the groups confirmed these changes, although the changes in PKCβI were not significant (Fig. 2c).

Immunohistochemistry

Immunohistochemically, PKCα and PKCβ (both I and II) were differentially expressed in the nerve. PKCα was expressed in the Schwann cells, whereas PKCβI and II located in the membrane of axoplasm, macrophages and the wall of endoneurial microvessels (Fig. 3). There was a trend towards less expressions of PKCα and more intensified expressions of PKCβII in diabetic rats compared with those in normal control rats.

8-Hydroxydeoxyguanosine-positive cells were marked in diabetic rats compared with normal control rats, and this expression was suppressed in MC-treated diabetic rats (Fig. 4a). In normal control rats, there were no or only a few endoneurial Iba-1-positive macrophages (Fig. 4b). In contrast, diabetic rats showed an abundance of Iba-1-positive cells. MC treatment slightly suppressed the migration of macrophages, but it was not significant. Quantitative analysis confirmed the earlier findings (Fig. 4c).

Discussion

This study confirmed a significant NCV delay and decreased PKC activity in peripheral nerve in STZ-induced diabetic rats. The reduction in PKC activity was associated with reduced expression of membranous PKCα [21,22]. In contrast, membranous PKCβII expression was increased in diabetic rats, but this change was not reflected by total PKC activity. MC treatment effectively corrected the PKC activity and expression of PKCα, together with improved NCV. It is noteworthy that the localization of PKC isoform was quite distinct between PKCα of Schwann cells and PKCβ of axoplasm.

Protein kinase C is crucial to mediate cellular signalling and to stimulate cell activity, protein synthesis and cell movement [23,24]. PKC consists of many isoforms; α, βI, βII, γ, δ and others. Increased PKC activity owing to augmented PKCβ expressions has consistently been demonstrated in cardiovascular system in diabetic subjects [25,26], and as such clinical trial of PKCβ inhibitor for the treatment of diabetic neuropathy was conducted [18,19]. It was not successful, however, in part because of the fact that the role of PKC in neuropathic processes is yet to be established. In fact, PKC activity in diabetic nerves is differently reported in previous studies as elevated [27,28], reduced [20,21] or not altered [29]. Our present results may explain the reason for such contradictory results by differential expression of PKC isoform. First, we confirmed our previous findings of decreased PKC activity in the endoneurial fraction of STZ-induced diabetic nerve [21,22,30], while epineurial vascular system showed elevated PKC activity with PKCβ overexpression [30]. Localization of PKCα in Schwann cells found in this study was consistent with the data that cultured Schwann cells exposed to high glucose showed decreased PKC activity with reduced expression of PKCα [31]. On the other hand, increased phosphorylation of PKCβ in dorsal root ganglion cells that was found in STZ-diabetic rats with elevated pain sensation [27] may also be in agreement with our results of the strong immunoreactions of PKCβ in axoplasmic membrane in diabetic rats. It is therefore likely that although total nerve PKC activity may be suppressed, the beneficial effects of PKCβ inhibitor previously reported [29] may be ascribed to the amelioration of nerve blood flow as well as direct action of PKCβ in the soma of ganglion cells and axons shown in this study.

In previous studies, the altered PKC activity was closely correlated with increased polyol pathway in diabetic nerve.

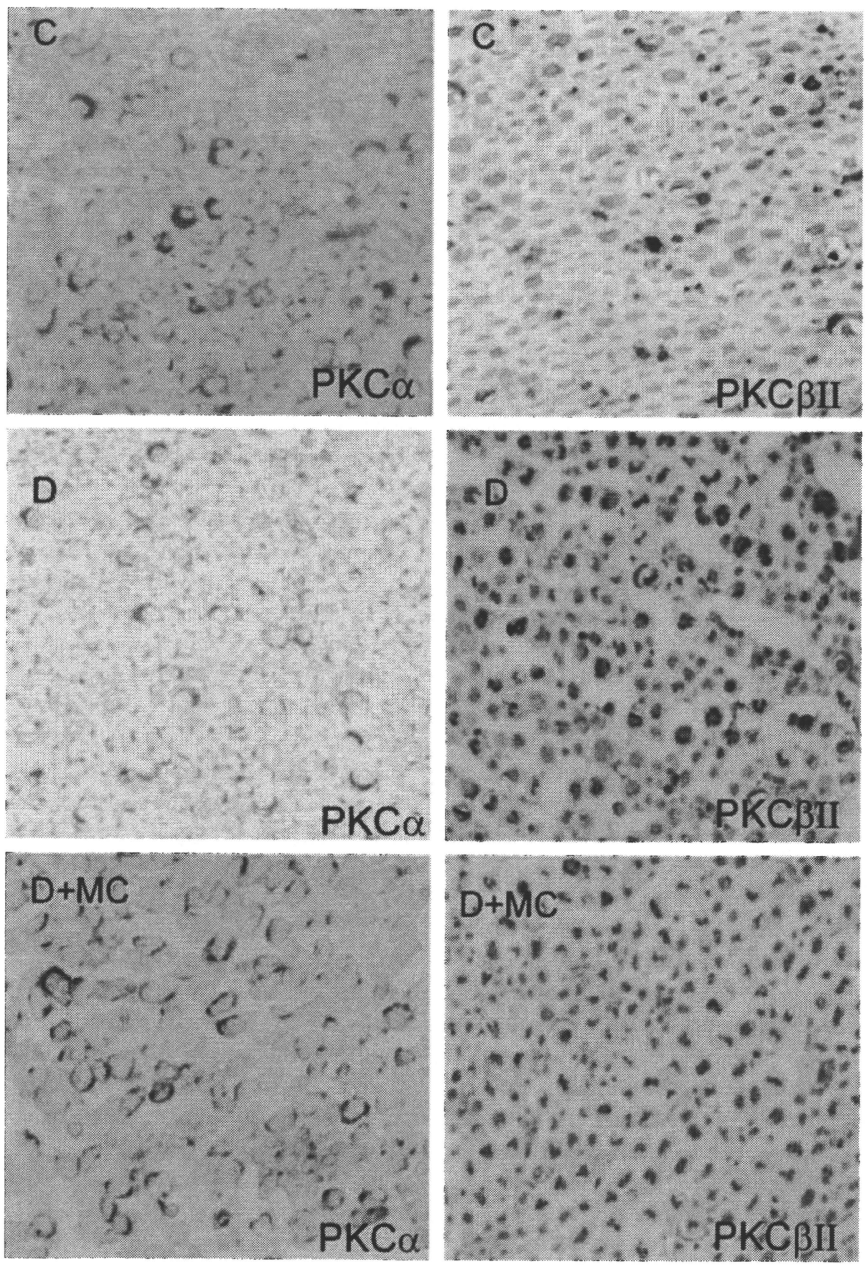

Figure 3 Topography of protein kinase C (PKC) isoform expressions as revealed by immunohistochemistry. PKCα isoform was diffusely localized in the Schwann cell cytoplasm in the peripheral nerve. There was no significant alteration in diabetic nerve, and methylcobalamin treatment did not influence the reactions. On the other hand, PKCβII isoform was localized in the axoplasm of nerve fibres. The membranous reaction of PKCβII isoform was intensified in the axoplasm in diabetic rats, and the changes were less marked in methylcobalamin-treated diabetic rats. C, normal control rats; D, untreated diabetic rats; D+MC, methylcobalamin-treated diabetic rats.

In transgenic mice overexpressing human aldose reductase (AR), PKC activity was severely suppressed, and this change was corrected by AR inhibitor [20,30]. In contrast, pioglitazone improved PKC activity without affecting polyol levels [21]. Instead, it corrected the oxidative stress–induced damage and macrophage migration in the endoneurium [21].

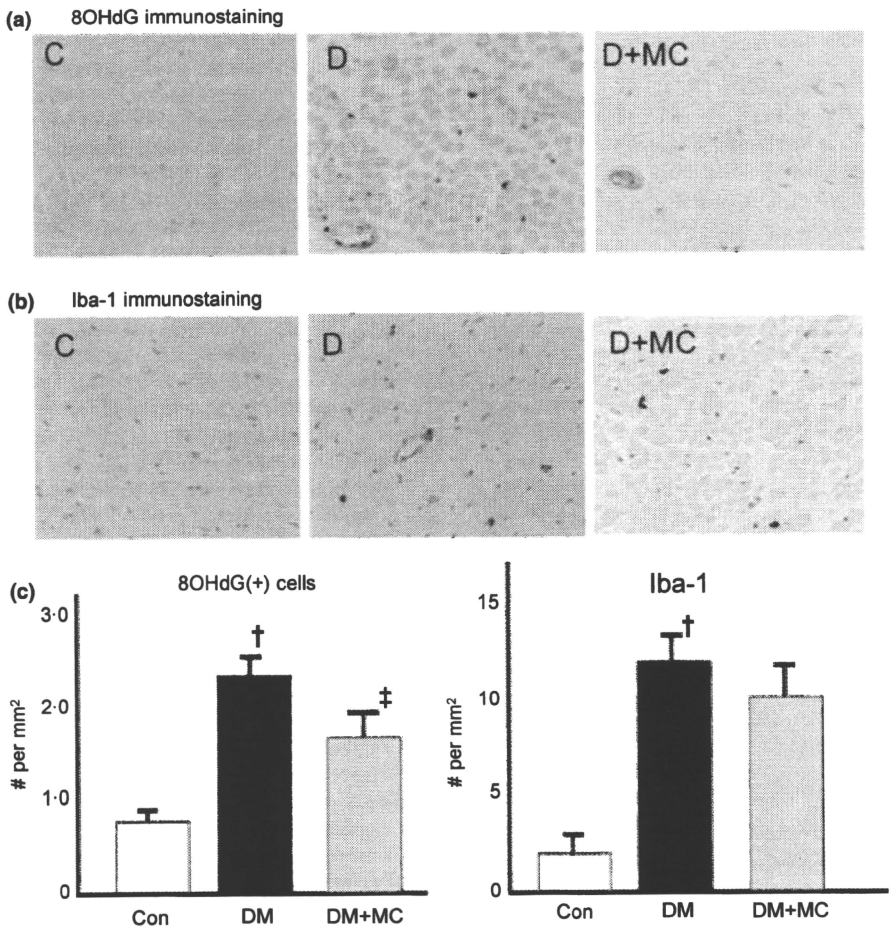

Figure 4 Expression of oxidative stress–induced DNA damage and macrophage migration in the peripheral nerve in experimental animals. (a) As revealed by immunohistochemistry, diabetic rats showed increased number of 8-hydroxydeoxyguanosine (8OHdG)-positive cells in the nerve, and this increase was significantly suppressed in methylcobalamin-treated diabetic rats. (b) In the peripheral nerve, there were an increased number of macrophages positive with Iba-1 immunoreactions in diabetic rats, and there was no significant influence of methylcobalamin treatment on the macrophage migration in untreated diabetic rats. (c) Quantitative estimation confirmed the findings. C, normal control rats; D, untreated diabetic rats; D+MC, methylcobalamin-treated diabetic rats. Bar stands for SE. †$P < 0{\cdot}01$ vs. C, ‡$P < 0{\cdot}01$ vs. D.

In this study, sorbitol accumulation was only partially inhibited by MC treatment, whereas 8OHdG-positive cells were significantly reduced. It was shown that sorbitol accumulates in tissues not only under hyperglycaemia but also ischaemic/hypoxic milieu [32,33]. In previous studies, sorbitol levels in the sciatic nerve were not influenced by MC despite the significant improvement in NCV in STZ-diabetic rats [16]. It is therefore likely that the recovery of PKC activity in MC-treated animals may not be directly dependent on inhibition of polyol pathway but rather relate to the changes in oxidative stress–induced damage. In this setting, antioxidant effects of MC may be mediated by the synthesis of cysteine donated from transmethylation of methionine [34,35]. Alternatively, activation of metabolic signals as well as protein synthesis by stimulation of nucleic acid metabolism elicited by MC may also have contributed to the improvement in PKC activity [36,37]. The consequence of recovered PKC alterations may directly or indirectly prevent the distal axonal fibre loss in the skin and enhance nerve fibre regeneration. Indeed, recent experimental studies demonstrated increased nerve fibre regeneration after crush injury of the sciatic nerve by MC treatment through the activation of cellular signalling of Akt and MAP kinase [38]. Upregulation of growth factor expressions like IGF-1 in the nerve by MC treatment also resulted in amelioration of neuropathic changes in STZ-diabetic rats [39].

Homocysteine levels in STZ-induced diabetic rats are known to be low in contrast to the elevation in human type 2 diabetic subjects [7,8,10,40]. The reason why homocysteine values are low in STZ-diabetic rats is not clear. Nevertheless, the values were further reduced in MC-treated animals. Hence, it is likely that there is a dynamic regulatory system between homocysteine and methionine by MC treatment, although the processes may be different between nerve and blood.

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Contributions of authors

Mizukami H conducted the experiment and summarized the data (40% contribution), Ogasawara S helped all the experiment with animal care and conducted biochemical analysis (30% contribution), Yamagishi SI measured nerve function and PKC (10% contribution), Takahashi K conducted immunohistochemistry and helped animal care (10% contribution), and Yagihashi S designed the experiment and wrote the manuscript (10% contribution).

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