[19] Thornalley PJ. Pharmacology of methylglyoxal: formation, modification of proteins and nucleic acids, and enzymatic detoxification--a role in pathogenesis and antiproliferative chemotherapy. Gen Pharmacol 1996; 27: 565-73.

[20] Thornalley PJ, Westwood M, Lo TW, McLellan AC. Formation of methylglyoxal-modified proteins *in vitro* and *in vivo* and their involvement in AGE-related processes. Contrib Nephrol 1995; 112: 24-31.

[21] Fukunaga M, Miyata S, Liu BF, Miyazaki H, Hirota Y, Higo S, *et al.* Methylglyoxal induces apoptosis through activation of p38 MAPK in rat Schwann cells. Biochem Biophys Res Commun 2004; 320: 689-95.

[22] Takeuchi M, Yamagishi S. Alternative routes for the formation of glyceraldehyde-derived AGEs (TAGE) *in vivo*. Med Hypotheses 2004; 63: 453-5.

[23] Sekido H, Suzuki T, Jomori T, Takeuchi M, Yabe-Nishimura C, Yagihashi S. Reduced cell replication and induction of apoptosis by advanced glycation end products in rat Schwann cells. Biochem Biophys Res Commun 2004; 320: 241-8.

[24] Suzuki T, Sekido H, Kato N, Nakayama Y, Yabe-Nishimura C. Neurotrophin-3-induced production of nerve growth factor is suppressed in Schwann cells exposed to high glucose: involvement of the polyol pathway. J Neurochem 2004; 91: 1430-8.

[25] Yamagishi S, Uehara K, Otsuki S, Yagihashi S. Differential influence of increased polyol pathway on protein kinase C expressions between endoneurial and epineurial tissues in diabetic mice. J Neurochem 2003; 87: 497-507.

[26] Uehara K, Yamagishi S, Otsuki S, Chin S, Yagihashi S. Effects of polyol pathway hyperactivity on protein kinase C activity, nociceptive peptide expression, and neuronal structure in dorsal root ganglia in diabetic mice. Diabetes 2004; 53: 3239-47.

[27] Sorkin LS, Xiao WH, Wagner R, Myers RR. Tumour necrosis factor-alpha induces ectopic activity in nociceptive primary afferent fibres. Neuroscience 1997; 81: 255-62.

[28] Soulis-Liparota T, Cooper ME, Dunlop M, Jerums G. The relative roles of advanced glycation, oxidation and aldose reductase inhibition in the development of experimental diabetic nephropathy in the Sprague-Dawley rat. Diabetologia 1995; 38: 387-94.

[29] Hamada Y, Araki N, Horiuchi S, Hotta N. Role of polyol pathway in nonenzymatic glycation. Nephrol Dial Transplant 1996; 11 Suppl 5: 95-8.

[30] Phillips SA, Mirrlees D, Thornalley PJ. Modification of the glyoxalase system in streptozotocin-induced diabetic rats. Effect of the aldose reductase inhibitor Statil. Biochem Pharmacol 1993; 46: 805-11.

[31] Yabe-Nishimura C, Nishinaka T, Iwata K, Seo HG. Up-regulation of aldose reductase by the substrate, methylglyoxal. Chem Biol Interact 2003; 143-144: 317-23.

[32] Dan Q, Wong R, Chung SK, Chung SS, Lam KS. Interaction between the polyol pathway and non-enzymatic glycation on aortic smooth muscle cell migration and monocyte adhesion. Life Sci 2004; 76: 445-59.

[33] Dan Q, Wong RL, Yin S, Chung SK, Chung SS, Lam KS. Interaction between the polyol pathway and non-enzymatic glycation on mesangial cell gene expression. Nephron Exp Nephrol 2004; 98: e89-e99.

[34] Lander HM, Tauras JM, Ogiste JS, Hori O, Moss RA, Schmidt AM. Activation of the receptor for advanced glycation end products triggers a p21(ras)-dependent mitogen-activated protein kinase pathway regulated by oxidant stress. J Biol Chem 1997; 272: 17810-4.

[35] Nishinaka T, Yabe-Nishimura C. EGF receptor-ERK pathway is the major signaling pathway that mediates upregulation of aldose reductase expression under oxidative stress. Free Radic Biol Med 2001; 31: 205-16.

[36] Sugimoto K, Nishizawa Y, Horiuchi S, Yagihashi S. Localization in human diabetic peripheral nerve of N(epsilon)- carboxymethyllysine-protein adducts, an advanced glycation endproduct. Diabetologia 1997; 40: 1380-7.

[37] Haslbeck KM, Schleicher ED, Friess U, Kirchner A, Neundorfer B, Heuss D. N(epsilon)-Carboxymethyllysine in diabetic and non-diabetic polyneuropathies. Acta Neuropathol 2002; 104: 45-52.

[38] Ryle C, Donaghy M. Non-enzymatic glycation of peripheral nerve proteins in human diabetics. J Neurol Sci 1995; 129: 62-8.

[39] Baynes JW. Role of oxidative stress in development of complications in diabetes. Diabetes 1991; 40: 405-12.

[40] Arai K, Maguchi S, Fujii S, Ishibashi H, Oikawa K, Taniguchi N. Glycation and inactivation of human Cu-Zn-superoxide dismutase. Identification of the *in vitro* glycated sites. J Biol Chem 1987; 262: 16969-72.

[41] Yoshida K, Hirokawa J, Tagami S, Kawakami Y, Urata Y, Kondo T. Weakened cellular scavenging activity against oxidative stress in diabetes mellitus: regulation of glutathione synthesis and efflux. Diabetologia 1995; 38: 201-10.

[42] Williamson JR, Chang K, Frangos M, Hasan KS, Ido Y, Kawamura T, *et al.* Hyperglycemic pseudohypoxia and diabetic complications. Diabetes 1993; 42: 801-13.

[43] Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001; 414: 813-20.

[44] Yu T, Robotham JL, Yoon Y. Increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology. Proc Natl Acad Sci USA 2006; 103: 2653-8.

[45] Vincent AM, Olzmann JA, Brownlee M, Sivitz WI, Russell JW. Uncoupling proteins prevent glucose-induced neuronal oxidative stress and programmed cell death. Diabetes 2004; 53: 726-34.

[46] Desco MC, Asensi M, Marquez R, Martinez-Valls J, Vento M, Pallardo FV, *et al.* Xanthine oxidase is involved in free radical production in type 1 diabetes: protection by allopurinol. Diabetes 2002; 51: 1118-24.

[47] Inoguchi T, Sonta T, Tsubouchi H, Etoh T, Kakimoto M, Sonoda N, *et al.* Protein kinase C-dependent increase in reactive oxygen species (ROS) production in vascular tissues of diabetes: role of vascular NAD(P)H oxidase. J Am Soc Nephrol 2003; 14: S227-32.

[48] Mullarkey CJ, Edelstein D, Brownlee M. Free radical generation by early glycation products: a mechanism for accelerated atherogenesis in diabetes. Biochem Biophys Res Commun 1990; 173: 932-9.

[49] Bierhaus A, Chevion S, Chevion M, Hofmann M, Quehenberger P, Illmer T, *et al.* Advanced glycation end product-induced activation of NF-kappaB is suppressed by alpha-lipoic acid in cultured endothelial cells. Diabetes 1997; 46: 1481-90.

[50] Russell JW, Golovoy D, Vincent AM, Mahendru P, Olzmann JA, Mentzer A, *et al.* High glucose-induced oxidative stress and mitochondrial dysfunction in neurons. FASEB J 2002; 16: 1738-48.

[51] Cotter MA, Cameron NE. Effect of the NAD(P)H oxidase inhibitor, apocynin, on peripheral nerve perfusion and function in diabetic rats. Life Sci 2003; 73: 1813-24.

[52] Schmeichel AM, Schmelzer JD, Low PA. Oxidative injury and apoptosis of dorsal root ganglion neurons in chronic experimental diabetic neuropathy. Diabetes 2003; 52: 165-71.

[53] Coppey LJ, Gellett JS, Davidson EP, Dunlap JA, Lund DD, Yorek MA. Effect of antioxidant treatment of streptozotocin-induced diabetic rats on endoneurial blood flow, motor nerve conduction velocity, and vascular reactivity of epineurial arterioles of the sciatic nerve. Diabetes 2001; 50: 1927-37.

[54] Cheng C, Zochodne DW. Sensory neurons with activated caspase-3 survive long-term experimental diabetes. Diabetes 2003; 52: 2363-71.

[55] Ziegler D, Sohr CG, Nourooz-Zadeh J. Oxidative stress and antioxidant defense in relation to the severity of diabetic polyneuropathy and cardiovascular autonomic neuropathy. Diabetes Care 2004; 27: 2178-83.

[56] Hoeldtke RD, Bryner KD, McNeill DR, Hobbs GR, Riggs JE, Warehime SS, *et al.* Nitrosative stress, uric Acid, and peripheral nerve function in early type 1 diabetes. Diabetes 2002; 51: 2817-25.

[57] Deuther-Conrad W, Loske C, Schinzel R, Dringen R, Riederer P, Munch G. Advanced glycation endproducts change glutathione redox status in SH-SY5Y human neuroblastoma cells by a hydrogen peroxide dependent mechanism. Neurosci Lett 2001; 312: 29-32.

[58] Loske C, Neumann A, Cunningham AM, Nichol K, Schinzel R, Riederer P, *et al.* Cytotoxicity of advanced glycation endproducts is mediated by oxidative stress. J Neural Transm 1998; 105: 1005-15.

[59] Cellek S, Qu W, Schmidt AM, Moncada S. Synergistic action of advanced glycation end products and endogenous nitric oxide leads to neuronal apoptosis *in vitro*: a new insight into selective nitrergic neuropathy in diabetes. Diabetologia 2004; 47: 331-9.

[60] Weimbs T, Stoffel W. Topology of CNS myelin proteolipid protein: evidence for the nonenzymatic glycosylation of extracytoplasmic domains in normal and diabetic animals. Biochemistry 1994; 33: 10408-15.

[61] Vlassara H, Brownlee M, Cerami A. Excessive nonenzymatic glycosylation of peripheral and central nervous system myelin components in diabetic rats. Diabetes 1983; 32: 670-4.

[62] Vlassara H, Brownlee M, Cerami A. Recognition and uptake of human diabetic peripheral nerve myelin by macrophages. Diabetes 1985; 34: 553-7.

[63] Ryle C, Leow CK, Donaghy M. Nonenzymatic glycation of peripheral and central nervous system proteins in experimental diabetes mellitus. Muscle Nerve 1997; 20: 577-84.

[64] Cullum NA, Mahon J, Stringer K, McLean WG. Glycation of rat sciatic nerve tubulin in experimental diabetes mellitus. Diabetologia 1991; 34: 387-9.

[65] Williams SK, Howarth NL, Devenny JJ, Bitensky MW. Structural and functional consequences of increased tubulin glycosylation in diabetes mellitus. Proc Natl Acad Sci USA 1982; 79: 6546-50.

[66] Pekiner C, Cullum NA, Hughes JN, Hargreaves AJ, Mahon J, Casson IF, *et al.* Glycation of brain actin in experimental diabetes. J Neurochem 1993; 61: 436-42.

[67] Federoff HJ, Lawrence D, Brownlee M. Nonenzymatic glycosylation of laminin and the laminin peptide CIKVAVS inhibits neurite outgrowth. Diabetes 1993; 42: 509-13.

[68] Luo ZJ, King RH, Lewin J, Thomas PK. Effects of nonenzymatic glycosylation of extracellular matrix components on cell survival and sensory neurite extension in cell culture. J Neurol 2002; 249: 424-31.

[69] Ozturk G, Sekeroglu MR, Erdogan E, Ozturk M. The effect of nonenzymatic glycation of extracellular matrix proteins on axonal regeneration *in vitro*. Acta Neuropathol 2006; 112: 627-32.

[70] Stern DM, Yan SD, Yan SF, Schmidt AM. Receptor for advanced glycation endproducts (RAGE) and the complications of diabetes. Ageing Res Rev 2002; 1: 1-15.

[71] Brett J, Schmidt AM, Yan SD, Zou YS, Weidman E, Pinsky D, *et al.* Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. Am J Pathol 1993; 143: 1699-712.

[72] Soulis T, Thallas V, Youssef S, Gilbert RE, McWilliam BG, Murray-McIntosh RP, *et al.* Advanced glycation end products and their receptors co-localise in rat organs susceptible to diabetic microvascular injury. Diabetologia 1997; 40: 619-28.

[73] Yan SD, Schmidt AM, Anderson GM, Zhang J, Brett J, Zou YS, *et al.* Enhanced cellular oxidant stress by the interaction of advanced glycation end products with their receptors/binding proteins. J Biol Chem 1994; 269: 9889-97.

[74] Wautier JL, Zoukourian C, Chappey O, Wautier MP, Guillausseau PJ, Cao R, *et al.* Receptor-mediated endothelial cell dysfunction in diabetic vasculopathy. Soluble receptor for advanced glycation end products blocks hyperpermeability in diabetic rats. J Clin Invest 1996; 97: 238-43.

[75] Vincent AM, Perrone L, Sullivan KA, Backus C, Sastry AM, Lastoskie C, *et al.* Receptor for advanced glycation end products activation injures primary sensory neurons *via* oxidative stress. Endocrinology 2007; 148: 548-58.

[76] Huttunen HJ, Kuja-Panula J, Sorci G, Agneletti AL, Donato R, Rauvala H. Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products (RAGE) activation. J Biol Chem 2000; 275: 40096-105.

[77] Huttunen HJ, Kuja-Panula J, Rauvala H. Receptor for advanced glycation end products (RAGE) signaling induces CREB-dependent chromogranin expression during neuronal differentiation. J Biol Chem 2002; 277: 38635-46.

[78] Haslbeck KM, Schleicher E, Bierhaus A, Nawroth P, Haslbeck M, Neundorfer B, *et al.* The AGE/RAGE/NF-(kappa)B pathway may contribute to the pathogenesis of polyneuropathy in impaired glucose tolerance (IGT). Exp Clin Endocrinol Diabetes 2005; 113: 288-91.

[79] Toth C, Rong LL, Yang C, Martinez J, Song F, Ramji N, *et al.* RAGE and experimental diabetic neuropathy. Diabetes 2007; Nov 26 [Epub ahead of print].

[80] Srivastava AK. High glucose-induced activation of protein kinase signaling pathways in vascular smooth muscle cells: a potential role in the pathogenesis of vascular dysfunction in diabetes (review). Int J Mol Med 2002; 9: 85-9.

[81] Cameron NE, Cotter MA, Jack AM, Basso MD, Hohman TC. Protein kinase C effects on nerve function, perfusion, Na(+), K(+)-ATPase activity and glutathione content in diabetic rats. Diabetologia 1999; 42: 1120-30.

[82] Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang J, *et al.* Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes. J Clin Invest 1995; 96: 1395-403.

[83] Basta G, Lazzerini G, Massaro M, Simoncini T, Tanganelli P, Fu C, *et al.* Advanced glycation end products activate endothelium through signal-transduction receptor RAGE: a mechanism for amplification of inflammatory responses. Circulation 2002; 105: 816-22.

[84] Vlassara H. The AGE-receptor in the pathogenesis of diabetic complications. Diabetes Metab Res Rev 2001; 17: 436-43.

[85] Vlassara H, Palace MR. Diabetes and advanced glycation endproducts. J Intern Med 2002; 251: 87-101.

[86] Amano S, Kaji Y, Oshika T, Oka T, Machinami R, Nagai R, *et al.* Advanced glycation end products in human optic nerve head. Br J Ophthalmol 2001; 85: 52-5.

[87] Del Carro U, Fiorina P, Amadio S, De Toni Franceschini L, Petrelli A, Menini S, *et al.* Evaluation of polyneuropathy markers in type 1 diabetic kidney transplant patients and effects of islet transplantation: neurophysiological and skin biopsy longitudinal analysis. Diabetes Care 2007; 30: 3063-9.

[88] Misur I, Zarkovic K, Barada A, Batelja L, Milicevic Z, Turk Z. Advanced glycation endproducts in peripheral nerve in type 2 diabetes with neuropathy. Acta Diabetol 2004; 41: 158-66.

[89] Sell DR, Lapolla A, Odetti P, Fogarty J, Monnier VM. Pentosidine formation in skin correlates with severity of complications in individuals with long-standing IDDM. Diabetes 1992; 41: 1286-92.

[90] McCance DR, Dyer DG, Dunn JA, Bailie KE, Thorpe SR, Baynes JW, *et al.* Maillard reaction products and their relation to complications in insulin-dependent diabetes mellitus. J Clin Invest 1993; 91: 2470-8.

[91] Beisswenger PJ, Moore LL, Brinck-Johnsen T, Curphey TJ. Increased collagen-linked pentosidine levels and advanced glycosylation end products in early diabetic nephropathy. J Clin Invest 1993; 92: 212-7.

[92] Garay-Sevilla ME, Regalado JC, Malacara JM, Nava LE, Wrobel-Zasada K, Castro-Rivas A, *et al.* Advanced glycosylation end products in skin, serum, saliva and urine and its association with complications of patients with type 2 diabetes mellitus. J Endocrinol Invest 2005; 28: 223-30.

[93] Hwang JS, Shin CH, Yang SW. Clinical implications of N epsilon-(carboxymethyl)lysine, advanced glycation end product, in children and adolescents with type 1 diabetes. Diabetes Obes Metab 2005; 7: 263-7.

[94] Meerwaldt R, Graaff R, Oomen PH, Links TP, Jager JJ, Alderson NL, *et al.* Simple non-invasive assessment of advanced glycation endproduct accumulation. Diabetologia 2004; 47: 1324-30.

[95] Lutgers HL, Graaff R, Links TP, Ubink-Veltmaat LJ, Bilo HJ, Gans RO, *et al.* Skin autofluorescence as a noninvasive marker of vascular damage in patients with type 2 diabetes. Diabetes Care 2006; 29: 2654-9.

[96] Yagihashi S, Kamijo M, Baba M, Yagihashi N, Nagai K. Effect of aminoguanidine on functional and structural abnormalities in peripheral nerve of STZ-induced diabetic rats. Diabetes 1992; 41: 47-52.

[97] Kihara M, Schmelzer JD, Poduslo JF, Curran GL, Nickander KK, Low PA. Aminoguanidine effects on nerve blood flow, vascular permeability, electrophysiology, and oxygen free radicals. Proc Natl Acad Sci USA 1991; 88: 6107-11.

[98] Wada R, Sugo M, Nakano M, Yagihashi S. Only limited effects of aminoguanidine treatment on peripheral nerve function, (Na+,K+)-ATPase activity and thrombomodulin expression in streptozotocin-induced diabetic rats. Diabetologia 1999; 42: 743-7.

[99] Coppey LJ, Gellett JS, Davidson EP, Dunlap JA, Yorek MA. Effect of treating streptozotocin-induced diabetic rats with sorbinil, myo-inositol or aminoguanidine on endoneurial blood flow, motor nerve conduction velocity and vascular function of epineurial arterioles of the sciatic nerve. Int J Exp Diabetes Res 2002; 3: 21-36.

[100] Coppey LJ, Gellett JS, Davidson EP, Yorek MA. Preventing superoxide formation in epineurial arterioles of the sciatic nerve from diabetic rats restores endothelium-dependent vasodilation. Free Radic Res 2003; 37: 33-40.

[101] Sugimoto K, Yagihashi S. Effects of aminoguanidine on structural alterations of microvessels in peripheral nerve of streptozotocin diabetic rats. Microvasc Res 1997; 53: 105-12.

[102] Birrell AM, Heffernan SJ, Ansselin AD, McLennan S, Church DK, Gillin AG, *et al.* Functional and structural abnormalities in the nerves of type 1 diabetic baboons: aminoguanidine treatment does not improve nerve function. Diabetologia 2000; 43: 110-6.

[103] Freedman BI, Wuerth JP, Cartwright K, Bain RP, Dippe S, Hershon K, *et al.* Design and baseline characteristics for the aminoguanidine Clinical Trial in Overt Type 2 Diabetic Nephropathy (ACTION II). Control Clin Trials 1999; 20: 493-510.

[104] Nakamura S, Makita Z, Ishikawa S, Yasumura K, Fujii W, Yanagisawa K, *et al.* Progression of nephropathy in spontaneous diabetic rats is prevented by OPB-9195, a novel inhibitor of advanced glycation. Diabetes 1997; 46: 895-9.

[105] Wada R, Nishizawa Y, Yagihashi N, Takeuchi M, Ishikawa Y, Yasumura K, *et al.* Effects of OPB-9195, anti-glycation agent, on experimental diabetic neuropathy. Eur J Clin Invest 2001; 31: 513-20.

[106] Vasan S, Zhang X, Kapurniotu A, Bernhagen J, Teichberg S, Basgen J, *et al.* An agent cleaving glucose-derived protein crosslinks *in vitro* and *in vivo*. Nature 1996; 382: 275-8.

[107] Yang S, Litchfield JE, Baynes JW. AGE-breakers cleave model compounds, but do not break Maillard crosslinks in skin and tail collagen from diabetic rats. Arch Biochem Biophys 2003; 412: 42-6.

[108] Kass DA, Shapiro EP, Kawaguchi M, Capriotti AR, Scuteri A, deGroof RC, *et al.* Improved arterial compliance by a novel advanced glycation end-product crosslink breaker. Circulation 2001; 104: 1464-70.

[109] Demiot C, Tartas M, Fromy B, Abraham P, Saumet JL, Sigaudo-Roussel D. Aldose reductase pathway inhibition improved vascular and C-fiber functions, allowing for pressure-induced vasodilation restoration during severe diabetic neuropathy. Diabetes 2006; 55: 1478-83.

[110] Hammes HP, Du X, Edelstein D, Taguchi T, Matsumura T, Ju Q, *et al.* Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy. Nat Med 2003; 9: 294-9.

[111] Pomero F, Molinar Min A, La Selva M, Allione A, Molinatti GM, Porta M. Benfotiamine is similar to thiamine in correcting endothelial cell defects induced by high glucose. Acta Diabetol 2001; 38: 135-8.

[112] Stracke H, Hammes HP, Werkmann D, Mavrakis K, Bitsch I, Netzel M, *et al.* Efficacy of benfotiamine versus thiamine on function and glycation products of peripheral nerves in diabetic rats. Exp Clin Endocrinol Diabetes 2001; 109: 330-6.

[113] Sanchez-Ramirez GM, Caram-Salas NL, Rocha-Gonzalez HI, Vidal-Cantu GC, Medina-Santillan R, Reyes-Garcia G, *et al.* Benfotiamine relieves inflammatory and neuropathic pain in rats. Eur J Pharmacol 2006; 530: 48-53.

[114] Stracke H, Lindemann A, Federlin K. A benfotiamine-vitamin B combination in treatment of diabetic polyneuropathy. Exp Clin Endocrinol Diabetes 1996; 104: 311-6.

[115] Haupt E, Ledermann H, Kopcke W. Benfotiamine in the treatment of diabetic polyneuropathy--a three-week randomized, controlled pilot study (BEDIP study). Int J Clin Pharmacol Ther 2005; 43: 71-7.

[116] Cameron NE, Gibson TM, Nangle MR, Cotter MA. Inhibitors of advanced glycation end product formation and neurovascular dysfunction in experimental diabetes. Ann N Y Acad Sci 2005; 1043: 784-92.

[117] Lee AY, Chung SS. Contributions of polyol pathway to oxidative stress in diabetic cataract. FASEB J 1999; 13: 23-30.

[118] Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes 2005; 54: 1615-25.

[119] Babaei-Jadidi R, Karachalias N, Ahmed N, Battah S, Thornalley PJ. Prevention of incipient diabetic nephropathy by high-dose thiamine and benfotiamine. Diabetes 2003; 52: 2110-20.

[120] Marshall SM. The podocyte: a potential therapeutic target in diabetic nephropathy? Curr Pharm Des 2007; 13(26): 2713-20.

[121] Moulder JE, Fish BL, Cohen EP. Treatment of radiation nephropathy with ACE inhibitors and AII type-1 and type-2 receptor antagonists. Curr Pharm Des 2007; 13(13): 1317-25.

[122] Victor VM, Rocha M. Targeting antioxidants to mitochondria: a potential new therapeutic strategy for cardiovascular diseases. Curr Pharm Des 2007; 13(8): 845-63.

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Correction of protein kinase C activity and macrophage migration in peripheral nerve by pioglitazone, peroxisome proliferator activated-γ-ligand, in insulin-deficient diabetic rats

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Abstract

Pioglitazone, one of thiazolidinediones, a peroxisome proliferator-activated receptor (PPAR)-γ ligand, is known to have beneficial effects on macrovascular complications in diabetes, but the effect on diabetic neuropathy is not well addressed. We demonstrated the expression of PPAR-γ in Schwann cells and vascular walls in peripheral nerve and then evaluated the effect of pioglitazone treatment for 12 weeks (10 mg/kg/day, orally) on neuropathy in streptozotocin-diabetic rats. At end, pioglitazone treatment improved nerve conduction delay in diabetic rats without affecting the expression of PPAR-γ. Diabetic rats showed suppressed protein kinase C (PKC) activity of endoneurial membrane fraction with decreased expression of PKC-α. These alterations were normalized in the treated group. Enhanced expression of phosphorylated extracellular signal-regulated kinase detected in diabetic rats was inhibited by the treatment. Increased numbers of macrophages positive for ED-1 and 8-hydroxydeoxyguanosine-positive Schwann cells in diabetic rats were also corrected by the treatment. Pioglitazone lowered blood lipid levels of diabetic rats, but blood glucose and nerve sorbitol levels were not affected by the treatment. In conclusion, our study showed that pioglitazone was beneficial for experimental diabetic neuropathy via correction of impaired PKC pathway and proinflammatory process, independent of polyol pathway.

Keywords: diabetic neuropathy, peroxisome proliferator activated receptor-γ, proinflammatory process, protein kinase C, thiazolidinedione.

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Neuropathy is a common complication of diabetes that affects nearly 50% of diabetic patients (Shaw and Zimmet 1999; Vinik and Mehrabyan 2004). Most of the patients suffer from intractable signs and symptoms like pain, paresthesia as well as severe autonomic failure, eventually resulting in shortened life expectancy (Vinik and Mehrabyan 2004; Boulton *et al.* 2005). Satisfactory treatments are yet to be available in spite of numerous clinical trials, due in part to the fact that the pathogenesis of diabetic neuropathy is yet to be clear. Polyol pathway hyperactivity, increased non-enzymatic glycation, excessive oxidative stress, and altered protein kinase C (PKC) activity are all invoked in its pathogenesis (Yagihashi 1995; Sima and Sugimoto 1999; Vinik and Mehrabyan 2004). Recent studies disclosed involvement of PKC-mitogen activated protein (MAP)-kinase and nuclear transcription factors such as nuclear factor kappa B (NF-κB) in the injurious process of diabetic neuropathy (Tomlinson 1999; Bierhaus *et al.* 2004; Vincent and Feldman 2004). Impaired cell signaling from these factors related to cell function and structure is now regarded to be a promising target for an effective treatment of diabetic neuropathy (He and King 2004; Obrosova *et al.* 2004).

Peroxisome proliferator-activated receptor is a nuclear receptor that activates cellular metabolism leading to cell growth and differentiation (Murphy and Holder 2000; Hihi *et al.* 2002). Thiazolidine-derivative, thiazolidinedione, is a

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Abbreviations used: 8OHdG, 8-hydroxydeoxyguanosine; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MNCV, motor nerve conduction velocity; NCV, nerve conduction velocity; NF-κB, nuclear factor kappa B; pERK, phosphorylated ERK; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; SNCV, sensory nerve conduction velocity; STZ, streptozotocin.

potent ligand to this receptor and is shown to improve insulin sensitivity, thus being used as an anti-diabetic agent for type 2 diabetes. The ligand to PPAR-γ is also known to have anti-inflammatory and antioxidant effects and found to be beneficial for macrovascular complications in diabetes (Hihi *et al.* 2002; Verrier *et al.* 2004). In this setting, it has been proposed that thiazolidinedione modulates cell activity or cell survival through regulation of key molecules of cell signaling like NF-κB (Duez *et al.* 2001; Verrier *et al.* 2004).

In our previous survey, diabetic patients treated with troglitazone, a prototype of PPAR-γ ligand, although now retreated from the market because of its hepatotoxicity, showed less symptoms of neuropathy compared with non-treated group (Satoh *et al.* 2003). It has also been reported that PPAR-γ-ligand ameliorates peripheral nerve function and structure in diabetic animal models but precise mechanisms why they improved neuropathic changes are not well clarified (Qiang *et al.* 1998; Shibata *et al.* 2000). In this study, we therefore explored the effects of pioglitazone, PPAR-γ-ligand, on the development of experimental diabetic neuropathy and attempt to identify factors involved in this process.

Materials and methods

Animals

Male Wistar strain rats 8 weeks of age were made diabetic by intravenous injection of streptozotocin (STZ) (Sigma Co. Ltd., St Louis, MO, USA) (40 mg/kg, i.v.) diluted with 0.1 mol/L citrate buffer. Diabetes was identified by continuous glycosuria and high blood glucose exceeding 14 mmol/L 1 week after STZ injection. Thereafter, a group of diabetic rats was treated orally with pioglitazone (10 mg/kg/day) (kindly donated from Takeda Co., Osaka, Japan) and followed for 12 weeks. All the animals were monitored with body weight and blood glucose during the 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 anesthesia with pentobarbital (Abbot Co., Chicago, IL, USA), blood was withdrawn from the right atrium and centrifuged at 1500 *g* 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 PKC isoforms, MAP-kinase, and PPAR isoforms. Remaining nerve was fixed in formalin solution for structural 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

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

For MNCV, the left sciatic-tibial nerve was electrically stimulated first at the site of ankle using needle electrodes (MS92 electromyogram device; Medelec, London, UK) and then at the site of sciatic notch and the waves were recorded from the second interosseous muscle of the foot (Yagihashi *et al.* 2001). Supramaximal electrical stimulation of 0.1 ms pulses was identified as the period of latency. The latency differences derived from two stimulating sites were divided by the distance between the stimulating sites, yielding the value of MNCV. For detection of SNCV, the digital nerve was stimulated first at the interdigital metatarsal site and then at the site of the ankle. The initial deflection point of H-reflex was identified as the latency for SNCV. The difference of proximal and distal latency was divided by the distance between the stimulating sites, 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 (Yagihashi *et al.* 2001). Briefly, nerve tissues were homogenized in distilled water. Tricyclic acid and internal standard (D-sorbitol and D-fructose) were added to the homogenate, followed by centrifugation at 10 000 *g* for 5 min at 4°C to obtain the supernatant fraction. To remove tricyclic acid, the supernatant fraction was washed with ethyl ether. 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) using a Cadenza CD-C18 COLUMN (75 × 2.0 mm, internal diameter 3 mm; Imtakt, Kyoto, Japan).

Preparation for protein expressions in peripheral nerve

Desheathed nerve tissues were transferred to a tube containing 1.0 mL homogenization buffer [20 mmol Tris–HCl (pH 7.5), 330 mmol sucrose, 0.5 mmol EGTA, 2 mmol EDTA, 2 μg/mL aprotinin, 25 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride] and homogenized with a Polytron. Homogenate was centrifuged at 50 000 *g* for 30 min at 4°C. The pellet was resuspended in 0.6 mL homogenization buffer containing 1% Triton X-100 and stored on ice for 1 h. The protein expression of PPAR, PKC, and MAP-kinase was examined by western blot analysis using each specific antibody. For comparison of PPAR isoform expressions, liver and subcutaneous fatty tissues were also treated in a similar way.

Protein kinase C activity

Protein kinase C activities were assayed by the method described previously (Yagihashi *et al.* 2001). Excised nerve was homogenized in 1.0 mL of the same buffer as used for other protein expressions 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, and 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 diorein and 96 μg/mL 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 four 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 for PPAR isoforms and MAP-kinase and those that were extracted as cytosol and membrane fraction for PKC isoforms. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis 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 [2.5% 2-mercaptoethanol, 62.5 mmol Tris–HCl, 10% glycerol, 2% sodium dodecyl sulfate, 0.0025% bromophenol blue, 50 mmol reducing agent (dithiothreitol), pH 6.8] and loaded onto the Novex Tris–glycine PreCast Gel (Invitrogen). After completion of the migration, the proteins were transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA) in a transfer buffer (25 mmol Tris, 0.2 mol/L glycine, and 20% methanol) using a wet transfer unit of Xcell SureLock system. For blocking, blot membranes were incubated with 5% skimmed milk in phosphate buffered saline–Triton X-100 (137 mmol NaCl, 2.7 mmol KCl, 1.5 mmol KH_2PO_4, 8.0 mmol Na_2HPO_4, pH 7.4, and 1% Triton-X 100) for overnight at 4°C. After washing with phosphate buffered saline–Triton X-100 membrane was incubated with antibodies to PPAR-α (Santa Cruz BioTech Inc., Santa Cruz, CA, USA), PPAR-γ (RDI, Flanders, NJ, USA), extracellular signal-regulated kinase (ERK)-I and II (Santa Cruz BioTech Inc.), phosphorylated ERK (pERK)-I and -II (Santa Cruz BioTech Inc.), JNK-I (Santa Cruz BioTech Inc.), PKC-α, -βI, and -βII specific antibodies (Santa Cruz BioTech Inc.), and β-actin (Santa Cruz BioTech Inc.) for 1 h at 25°C. 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 BioTech Inc.) for 45 min at 25°C. 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

For immunohistochemical analysis, 4-μm thick transverse sections of formalin-fixed nerve tissues were deparaffinized and pre-treated with methanol containing 0.3% H_2O_2 to eliminate endogenous peroxidase activity. Antibodies to PPAR-γ (RDI) and PPAR-α (Santa Cruz BioTech Inc.), as well as PKC-α, -βI, -βII antibodies (Santa Cruz BioTech Inc.) were applied to the sections overnight at 4°C. To demonstrate the migration of macrophage, antibody to specific rat macrophage (CD68, clone ED1) (Dako Cytomation, Tokyo, Japan) was used. The effect of oxidative stress-induced DNA damage was examined by detection of 8-hydroxydeoxyguanosine (8OHdG) using antibody to 8OHdG (Nihon Yushi, Jika, Shizuoka, Japan) (*Wada et al. 2001*). After the application of first antibodies, the 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 at the antigenic sites, and these sections were counterstained with hematoxylin. 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 excessive antigens, (iii) absorption of the antibody by non-immune rabbit sera, and (iv) absorption of the antibody by excessive antigens of PKC-α, -βI, and -βII. Some nerve samples from groups of rats were distorted during tissue sampling and therefore discarded for the structural investigations.

Population of macrophage and 8OHdG-positive cells

For the evaluation of 8OHdG-positive and ED-1 positive cells, transverse sections of the sciatic nerve stained immunohistochemically were incorporated into NIH image analysis. Positive cells for 8OHdG and ED-1 were identified as strongly positive reaction which intensity was more than five times compared with background intensity evaluated by NIH image analysis (Wada *et al.* 2001; Wang *et al.* 2005) . The number of macrophages were counted on the sections of individual animals and expressed as cell number per unit area. Only the cells with nuclei were selected.

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 4.11J; Huilinks, Tokyo, Japan). Comparison of the values among the groups was carried out using one-way ANOVA, followed by Bonferroni's correction for multiple comparisons. p-values < 0.05 were considered to be significant.

Results

Laboratory data

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 non-diabetic group. Pioglitazone-treatment did not influence these values. Blood glucose concentrations were significantly greater in diabetic group and pioglitazone 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 pioglitazone treatment reduced the levels of triglyceride but not of cholesterol (Table 1).

Nerve conduction velocity

At 12 weeks of diabetes, diabetic group showed 30% reduction of MNCV and 35% reduction of SNCV compared with those in normal control rats, respectively ($p < 0.01$ for both) (Fig. 1). Pioglitazone treatment improved both MNCV

Table 1 Body weight, concentrations of blood glucose, lipids, and nerve carbohydrates in experimental animals

Group	Number of animals (n)	Body weight (g)		Blood glucose (mmol/L)		Blood lipids		Nerve sorbitol (nmol/mg protein)	Nerve fructose (nmol/mg protein)
		Initial	End	Initial	End	T-cholesterol (mmol/L)	Triglyceride (mmol/L)		
Control	9	270 ± 9	491 ± 16	6.8 ± 0.4	6.9 ± 0.4	2.26 ± 0.11	2.60 ± 0.31	3.5 ± 0.24	17.9 ± 1.79
Diabetic	10	247 ± 3*	352 ± 13*	30.4 ± 1.2*	27.5 ± 0.1*	2.88 ± 0.11*	3.80 ± 0.63**	44.0 ± 2.15*	170.2 ± 5.19*
Diabetic + Pioglitazone	7	249 ± 4*	345 ± 18*	30.2 ± 1.3*	27.7 ± 1.2*	2.34 ± 0.06†	1.72 ± 0.24‡	48.7 ± 4.21*	162.8 ± 5.12*

Values are mean ± SE. *$p < 0.01$ versus control, **$p < 0.05$ versus control, †$p < 0.05$ versus diabetic, ‡$p < 0.01$ versus diabetic rats. Initial is the point of starting the treatment.

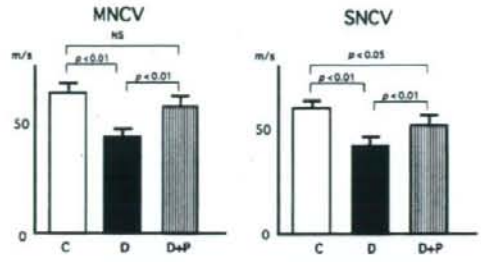

Fig. 1 Nerve conduction studies on experimental animals. There was a significant delay of motor and sensory nerve conduction velocity (MNCV and SNCV) in untreated diabetic rats (D) compared with normal control rats (C). The delay of both MNCV and SNCV was significantly improved by pioglitazone treatment (D + P). The number of animals was nine in control, ten in diabetic, and seven in pioglitazone-treated group.

by 15% ($p < 0.01$ vs. untreated diabetic rats) and SNCV by 25% ($p < 0.01$ vs. untreated diabetic rats).

Nerve carbohydrate metabolites

Nerve sorbitol was increased more than 12-fold in diabetic group compared with normal controls and pioglitazone treatment did not influence the levels (Table 1). Similarly, nerve fructose was increased ninefold in diabetic group compared with controls and again pioglitazone treatment did not influence the level.

Peroxisome proliferator-activated receptor expressions

Immunohistochemistry revealed the localization of PPAR-γ in Schwann cells of myelinated fibers and endothelial cells of endoneurial vessels in non-diabetic control nerves (Fig. 2). There was no positive reaction for PPAR-α. By western blot analyses, while the liver contained both PPAR-α and PPAR-γ, only PPAR-γ was detected in peripheral nerve and subcutaneous fatty tissues (Fig. 2). Expression levels of PPAR were not affected by pioglitazone-treatment.

Protein kinase C alterations

We separated homogenates of the endoneurial tissues into membrane and cytosolic fractions. PKC activity of the membrane fraction was significantly reduced by 35% level in diabetic rats compared with the level of control rats, while pioglitazone-treated group showed a significant recovery of the activity to near normal levels (Fig. 3a). On the other hand, PKC activity of the cytosolic fraction was not significantly altered in diabetic rats but increased in pioglitazone-treated diabetic rats compared with the levels of control and untreated diabetic rats.

Western blot analysis disclosed that PKC-α was reduced in the membrane fraction from diabetic rats, while PKC-α in the cytosolic fraction was increased, although the protein content

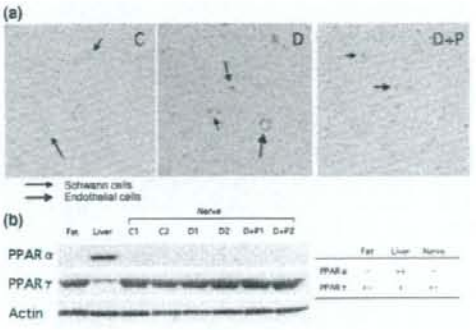

Fig. 2 (a) Expression of PPAR in peripheral nerve tissues and the effect of diabetes. Immunohistochemically, PPAR-γ was specifically expressed on the Schwann cell cytoplasm (arrow) and endothelial cells of endoneurial microvessels (thick arrow) in non-diabetic control nerves. There was no positive reaction of PPAR-α in peripheral nerve tissues (not shown). (b) By western blot analysis, PPAR-γ was expressed in peripheral nerve, liver, and fatty tissue, while PPAR-α was contained only in liver. Diabetic condition did not alter the PPAR-γ expression in the nerve. C, control rats; D, diabetic rats; D + P, pioglitazone-treated diabetic rats (C1, C2, D1, D2, D + P1, and D + P2 represent the individual animal number and P for D + P group).

in the cytosolic fraction was much less than in the membrane fraction (Fig. 3b). Pioglitazone treatment corrected these changes in both membrane and cytosolic fractions. There was an elevated expression of PKC-βII in the membrane fraction in diabetic rats and pioglitazone-treatment improved this change. The membrane and cytosolic fractions of PKC-βI did not alter in diabetic rats and pioglitazone treatment did not affect the expressions. Comparsion of the average values obtained from densitometric analysis among the groups confirmed these changes (Fig. 3c).

Mitogen-activated protein kinase

The expression levels of ERK-I and -II in diabetic rats were comparable with those in normal control rats, whereas the levels of pERK-I and -II were significantly elevated in diabetic rats and this increase was corrected by pioglitazone treatment (Fig. 4). There was no significant alteration of the expression level in JNK-I (data not shown).

Macrophage and 8OHdG-positive cells in peripheral nerve

Immunohistochemistry exhibited only a few endoneurial ED-1-positive macrophages in normal control rats (Fig. 5a). In contrast, diabetic rats showed an abundance of ED-1-positive cells often with vacuolar appearance. Such cells appeared to be less marked in pioglitazone-treated diabetic rats. Similarly, 8OHdG-positive cells were marked in diabetic rats compared with normal control rats and this expression was suppressed in pioglitazone-treated diabetic rats (Fig. 5b). Quantitative analysis confirmed the above findings (Fig. 5c).

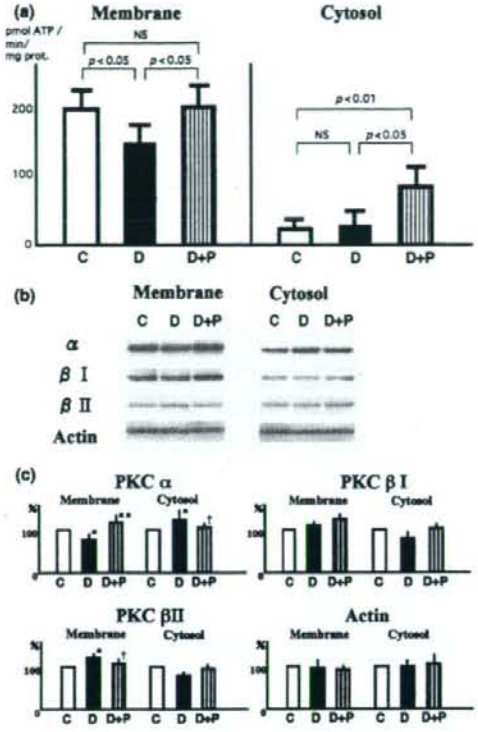

Fig. 3 (a) PKC activity of endoneurial tissues of peripheral nerve in experimental rats. There was significant reduction of membrane PKC activity in diabetic rats (D) compared with normal controls (C). Pioglitazone-treated group (D + P) showed normalization of PKC activity. Cytosolic PKC activity was not significantly altered in diabetic rats but there was elevation of cytosolic PKC activity in pioglitazone-treated diabetic rats. The number of animals was seven in each group. (b) PKC isoform expressions of endoneurial tissues of peripheral nerve in experimental rats. There was a decrease in PKC-α expression in membrane fraction in diabetic rats and pioglitazone treatment corrected this decrease. PKC-α in cytosolic fraction was increased in diabetic rats and pioglitazone treatment corrected this change. There was no significant change in PKC-βI, but an increase in membranous PKC-βII expression in diabetic rats and pioglitazone inhibited this change, whereas there was no change in cytosolic PKC-βII expression. (c) Densitometric analysis shows the average value of each group and bar stands for SE. The number of animals was seven in each group. C, control rats; D, diabetic rats; D + P, pioglitazone-treated diabetic rats. $^{*}p < 0.01$ versus C, $^{**}p < 0.01$ versus D, and $^{\dagger}p < 0.05$ versus D.

Discussion

We have shown that PPAR-γ-ligand, pioglitazone, significantly improved nerve conduction velocity (NCV) in

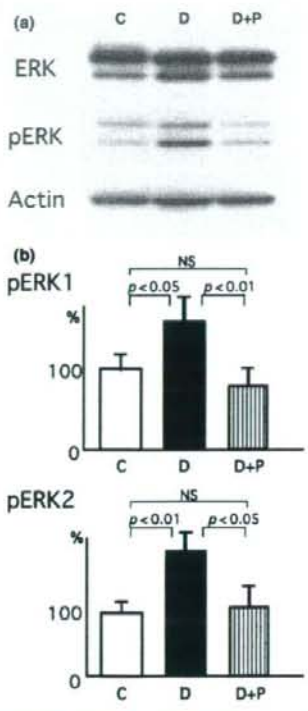

Fig. 4 MAP-kinase expression in experimental rats. There was no significant change in the expression of non-phosphorylated ERK among groups. In contrast, expression of phosphorylated ERK (pERK)-I and -II was enhanced in diabetic rats. Pioglitazone treatment inhibited the elevation of pERK-I and -II. The number of animals was seven in each group. C, control rats; D, diabetic rats; D + P, pioglitazone-treated diabetic rats.

insulin-deficient STZ-diabetic rats, indicating beneficial effects of pioglitazone on experimental diabetic neuropathy. It is of note that the effects were independent of the levels of hyperglycemia or accumulated polyols in nerve tissues but associated with correction of PKC activity and pERK. The recovery of PKC activity was associated with restoration of membrane PKC-α expression. In addition, pioglitazone treatment suppressed the proinflammatory condition possibly elicited by oxidative stress-induced injury and migration of macrophages in diabetic nerve. These results provide a new facet of the multifactorial cause of diabetic neuropathy and a novel direction of the treatment for neuropathic complications of human diabetes.

Thiazolidinedione was originally developed as an anti-diabetic agent acting as an insulin sensitizer to promote adipocyte differentiation and glucose utilization (Olefsky 2000; Evans *et al.* 2004). This agent has pleiotropic effects on cellular function, cell survival, and proliferation in the nervous system through binding with PPAR-responsive element via retinoid receptor (transactivation) (Nishijima *et al.* 2001; Wada *et al.* 2006). It also exerts neuro-glial cells to protect neuronal cell death by suppressing the gene activation to repress the transcription factor of NF-κB or activated protein-1 (transrepression) (Lennon *et al.* 2002; Heneka *et al.* 2005). In fact, correction of cellular signals of PKC activity and MAP-kinase in line with NF-κB is considered to be the main action of thiazolidinedione (Straus *et al.* 2000; Hung *et al.* 2001), serving as a potent anti-inflammatory agent (Duez *et al.* 2001; Verrier *et al.* 2004). The experimental approach has been conducted to circumvent a variety of degenerative neuronal disorders including Alzheimer's disease (Coombs *et al.* 2000; Landreth and Heneka 2001), experimental allergic encephalomyelitis (Feinstein *et al.* 2002), amyotrophic lateral sclerosis (Kiaei *et al.* 2005), and ischemic damage of the brain (Zhao *et al.* 2005; Ou *et al.* 2006).

There are only two reports in the literature that showed beneficial effects of thiazolidinedione on neuropathic changes in diabetic animals models (Qiang *et al.* 1998; Shibata *et al.* 2000). Qiang *et al.* (1998) reported that troglitazone improved NCV and fiber atrophy in STZ rats in which suppression of blood tumor necrosis factor-α and lipid peroxides was thought to be a key process for the thiazolidinedione effects. Our results may well be in keeping with the above data showing less migration of macrophages and less numbers of cells positive with 8OHdG *in situ* in pioglitazone-treated diabetic nerve compared with those in untreated rats. Shibata *et al.* (2000) reported an improvement of NCV in diabetic ZDF rats treated with a new thiazolidine-derivative. It is not clear, however, whether the NCV change was caused by this compound or blood glucose control itself as the treated animals also showed better metabolic controls. The current study is the first to demonstrate the expression of PPAR-γ, but not PPAR-α in the Schwann cells. The presence of PPAR-γ suggests that improvement of neuropathy may be ascribed to a direct action of pioglitazone on the nerve as well as endoneurial vessels, although other factors may also be involved.

The correction of altered MAP-kinase or PKC activity is an important mechanism that occurs in improvement of neuropathic changes in diabetic animals when treated with aldose reductase inhibitor or anti-oxidants (Tomlinson 1999; Purves *et al.* 2001; Ho *et al.* 2006). The metabolic changes correlate with amelioration of nerve Na,K-ATPase activity and NCV (Kim *et al.* 1991; Yagihashi *et al.* 2001) and lead to inhibition of nerve fiber atrophy with improved cytoskeletal protein synthesis (Scott *et al.* 1999). Our findings suggest that transactivating effects of pioglitazone mainly contribute to the activation of PKC activity with increased expression of PKC-α isoform that finally resulted in the alleviated nerve function. Indeed, activation of PKC activity is crucial for neuronal differentiation of PC12 cells when the

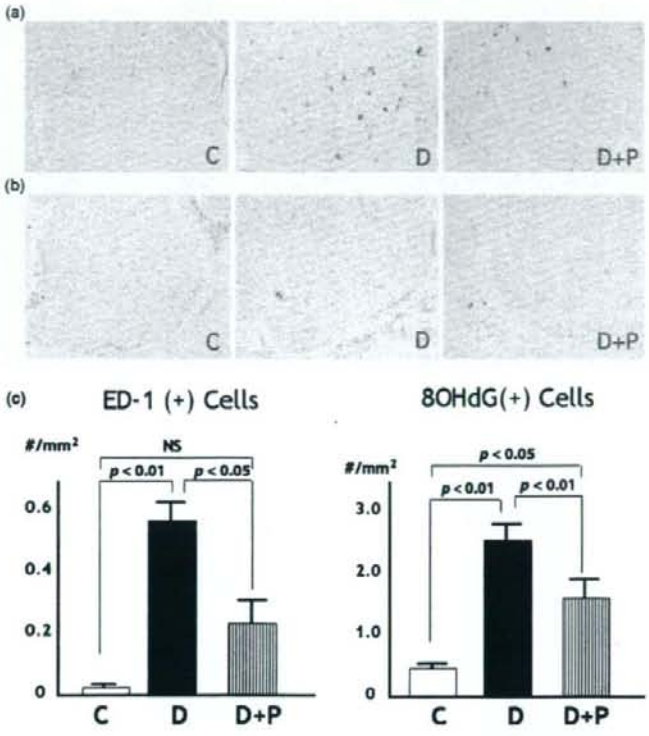

Fig. 5 (a) Immunohistochemical detection of ED-1-positive macrophages in sciatic nerve of experimental rats. In normal controls (C), only a few positive cells were noted. There was a considerable increase in ED-1-positive cells in diabetic rats (D). Pioglitazone-treated rats (D + P) showed only a few positive cells. (b) Immunohistochemical detection of 8OHdG in sciatic nerve of experimental rats. There was an only weak reaction of Schwann cells to 8OHdG in normal control rats (C), whereas diabetic rats (D) contained many Schwann cells positive to 8OHdG and this reaction was suppressed in pioglitazone-treated rats (D + P). (c) Quantitative analysis confirmed the increases in ED-1- and 8OHdG-positive cells in diabetic rats (D) compared with normal control rats (C). These changes were suppressed in pioglitazone-treated group (D + P). The number of animals was five in each group.

cells are stimulated by nerve growth factor which action is mediated by PPAR-γ after its binding with nerve growth factor receptor (Fuenzalida *et al.* 2005).

Protein kinase C activity in diabetic nerves is differently reported in previous studies as elevated (Roberts and McLean 1997; Kim *et al.* 2003), reduced (Kim *et al.* 1991) or not altered (Nakamura *et al.* 1999). We consider that different tissue sampling or different methods for the measurement may have yielded the discrepancy. In our studies on diabetic transgenic mice over-expressing human aldose reductase, we showed clearly different PKC alterations between endoneurial and vessel-rich epineurial tissues and between membrane and cytosolic fractions. Similar to the current results, endoneurial membrane fraction displayed reduced PKC activity with reduced expression of PKC-α isoform (Yamagishi *et al.* 2003; Uehara *et al.* 2004). Recent studies on the effects of rosiglitazone in diabetic Zucker rats also demonstrated tissue-specific alterations of PKC activity, in which PKC-β was activated in microvessels but not consistently so in the retina (Sotiropoulos *et al.* 2006). They further showed that rosiglitazone enhanced PKC activity in microvessels and the retina, but not in mononuclear cells (Sotiropoulos *et al.* 2006).

Increased migration of macrophages or oxidative-stress induced DNA damage appeared to be characteristic in peripheral nerve of STZ-diabetic rats. These changes were clearly inhibited by pioglitazone treatment. As the elevated sorbitol and fructose levels were not affected, the effects could be independent of polyol pathway. AGE-RAGE system or oxidative stress is suggested to elicit an inflammatory process in diabetic nerve (Bierhaus *et al.* 2001; Chawla *et al.* 2001; Wada and Yagihashi 2005), in which activation of MAP-kinase and NF-κB is central for neural tissue damage (Bierhaus *et al.* 2001; Wada and Yagihashi 2005). Our results indicated that MAP-kinase may not be directly connected with the alteration of total PKC activity in diabetic nerve. It can be speculated that proinflammatory processes may activate MAP-kinase related to PKC-βII activation (He and King 2004; Sotiropoulos *et al.* 2006), while impaired cellular signaling of phopshoinositide metabolism may lead to suppressed PKC activity with reduced membrane PKC-α expression (Kim *et al.* 1991; Yamagishi *et al.* 2003; Uehara *et al.* 2004). Under such circumstances, pioglitazone raised PKC activity to promote cell activity and inhibited phosphorylation of MAP-kinase by transrepression. Supranormal levels of PKC activity in the cytosolic fraction

in pioglitazone-treated diabetic nerve could support this contention, although precise mechanisms are not clear.

Strong expression of PPAR-γ in endothelial cells suggests that improvement of neuropathic changes by pioglitazone could also be the results of correction of neurovascular dysfunction. Indeed, oxidative stress as well as proinflammatory stimuli on neurovascular system is known to have a strong impact on the development of neuropathy (Cameron *et al.* 2001; Yorek 2003). It should also be taken into account that correction of lipid levels by pioglitazone may also have an impact on the nerve function. High lipid levels may exert enhancement of oxidative stress, resulting in neurovascular deficits in diabetes. In this study, we did not specifically examine the parameters of vascular system like nerve blood flow or vascular structure. Therefore, this possibility as well as an impact of hyperlipidemia needs to be evaluated by future investigations.

In conclusion, we explored the effects of pioglitazone on experimental diabetic neuropathy. PPAR-γ was expressed in the peripheral nerve and pioglitazone improved the peripheral nerve function. The changes were associated with correction of PKC activity and inhibition of proinflammatory processes independent of polyol pathway. We expect that these findings provide a new direction for the treatment of diabetic neuropathy in humans.

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References

Bierhaus A., Schiekofer S., Schwaninger M. *et al.* (2001) Diabetes-associated sustained activation of the transcription factor nuclear factor-κB. *Diabetes* **50**, 2792–2808.

Bierhaus A., Haslbeck K. M., Humpert P. M. *et al.* (2004) Loss of pain perception in diabetes is dependent on a receptor of the immunoglobulin superfamily. *J. Clin. Invest.* **114**, 1741–1751.

Boulton A. J., Vinik A. I., Arezzo J. C. *et al.* (2005) Diabetic neuropathies: a statement by the American Diabetes Association. *Diabetes Care.* **28**, 956–962.

Cameron N. E., Eaton S. E., Cotter M. A. and Tesfaye S. (2001) Vascular factors and metabolic interactions in the pathogenesis of diabetic neuropathy. *Diabetologia* **44**, 1973–1988.

Chawla A., Barak Y., Nagy L., Liao D., Tontonaz P. and Evans R. M. (2001) PPAR-γ dependent, and independent effects on macrophage-gene expression in lipid metabolism, and inflammation. *Nature Med.* **7**, 48–52.

Coombs C. K., Johnson D. E., Karlo C., Cannady S. B. and Landreth G. E. (2000) Inflammatory mechanisms in Alzheimer's disease: inhibition of β-amyloid-stimulated proinflammatory responses and neurotoxicity by PPARγ agonists. *J. Neurosci.* **20**, 558–567.

Duez H., Fruchart J. C. and Staels B. (2001) PPARs in inflammation, atherosclerosis and thrombosis. *J. Cardiovasc. Risk* **8**, 187–194.

Evans R. M., Barish G. and Wang Y.-X. (2004) PPARs and the complex journey to obesity. *Nature Med.* **10**, 1–7.

Feinstein D. L., Galea E., Gavrilyuk V. *et al.* (2002) Peroxisome proliferator-activated receptor-γ agonists prevent experimental autoimmune encephalomyelitis. *Ann. Neurol.* **51**, 694–702.

Fuenzalida K. M., Aguilera M. C., Piderit D. G., Ramos P. C., Contador D., Quiñoñes V., Rigotti A., Bronfman F. C. and Bronfman M. (2005) Peroxisome proliferator-activated receptor γ is a novel target of the nerve growth factor signaling pathway in PC12 cells. *J. Biol. Chem.* **280**, 9604–9609.

He Z. and King G. L. (2004) Microvascular complications of diabetes. *Endocrinol. Metab. Clin. North Am.* **33**, 215–238.

Heneka M. T., Sastre M., Dumitrescu-Ozimek L. *et al.* (2005) Acute treatment with PPARγ agonist pioglitazone and ibuprofen reduces glial inflammation and Ab1-42 levels in APPV717I transgenic mice. *Brain* **128**, 1442–1453.

Hihi A. K., Michalik L. and Wahli W. (2002) PPARs: transcriptional effectors of fatty acids and their derivatives. *Cell Mol. Life Sci.* **59**, 790–798.

Ho E. C., Lam K. S., Chen Y. S. *et al.* (2006) Aldose reductase-deficient mice are protected from delayed motor nerve conduction velocity, increased c-Jun NH_2-terminal kinase activation, depletion of reduced glutathione, increased superoxide accumulation, and DNA damage. *Diabetes* **55**, 1946–1953.

Hung M. C., Hayase K., Yoshida R., Sato M. and Imaizumi K. (2001) Cerebral protein kinase C and its mRNA level in apolipoprotein E-deficient mice. *Life Sci.* **69**, 1419–1427.

Kiaei M., Kipiani K., Chen J., Calingasan N. Y. and Beal M. F. (2005) Peroxisome proliferator-activated receptor-γ agonist extends survival in transgenic mouse model of amyotrophic lateral sclerosis. *Exp. Neurol.* **191**, 331–336.

Kim J., Rushovich E. H., Thomas T. P., Ueda T., Agranoff B. W. and Greene D. A. (1991) Diminished specific activity of cytosolic protein kinase C in sciatic nerve of streptozocin-induced diabetic rats and its correction by dietary myo-inositol. *Diabetes* **40**, 1545–1554.

Kim H., Sasaki T., Maeda K., Koya D., Kashiwagi A. and Yasuda H. (2003) Protein kinase Cβ selective inhibitor LY333531 attenuates diabetic hyperalgesia through ameliorating cGMP level of dorsal root ganglion neurons. *Diabetes* **52**, 2102–2109.

Landreth G. E. and Heneka M. T. (2001) Anti-inflammatory actions of peroxisome proliferator-activated receptor γ agonists in Alzheimer's disease. *Neurobiol. Aging* **22**, 937–944.

Lennon A. M., Ramaugé M., Dessouroux A. and Pierre M. (2002) MAP kinase cascades are activated in astrocytes and preadiocytes by 15-deoxy-Δ^{12-14}-prostaglandin J_2 and the thiazolidinedione ciglitazone through peroxisome proliferator activator receptor γ-independent mechanism involving reactive oxygen species. *J. Biol. Chem.* **277**, 29681–29685.

Murphy G. J. and Holder J. C. (2000) PPAR-γ agonists: therapeutic role in diabetes, inflammation, and cancer. *Trends Pharmacol. Sci.* **21**, 469–472.

Nakamura J., Kato K., Hamada Y. *et al.* (1999) A protein kinase C-β-selective inhibitor ameliorates neural dysfunction in streptozotocin-induced diabetic rats. *Diabetes* **48**, 2090–2095.

Nishijima C., Kimoto K. and Arakawa Y. (2001) Survival activity of troglitazone in rat motoneurones. *J. Neurochem.* **76**, 383–390.

Obrosova I. G., Li F., Abatan O. I., Forsell M. A., Komjati K., Pacher P., Szabo C. and Stevens M. J. (2004) Role of poly(ADP-ribose) polymerase activation in diabetic neuropathy. *Diabetes* **53**, 711–720.

Olefsky J. M. (2000) Treatment of insulin resistance with peroxisome proliferator-activated receptor γ agonists. *J. Clin. Invest.* **106**, 467–472.

Ou Z., Zhao X., Labiche L. A., Strong R., Grotta J. C., Herrmann O. and Aronowski J. (2006) Neuronal expression of peroxisome prolifer-

ator-activated receptor-γ (PPARγ) and 15Δ-prostaglandin J2-mediated protection of brain after experimental cerebral ischemia in rat. *Brain Res.* **1096**, 196–203.

Purves T., Middlemas A., Agthong S., Jude E. B., Boulton A. J., Fernyhough P. and Tomlinson D. R. (2001) A role for mitogen-activated protein kinases in the etiology of diabetic neuropathy. *FASEB J.* **15**, 2508–2514.

Qiang X., Satoh J., Sagara M., Fukuzawa M., Masuda T., Sakata Y., Muto G., Muto Y., Takahashi K. and Toyota T. (1998) Inhibitory effect of troglitazone on diabetic neuropathy in streptozotocin-induced diabetic rats. *Diabetologia* **41**, 1321–1326.

Roberts R. E. and McLean W. G. (1997) Protein kinase C isozyme expression in sciatic nerves and spinal cords of experimentally diabetic rats. *Brain Res.* **754**, 147–156.

Satoh J., Yagihashi S. and Toyota T. (2003) The possible role of tumor necrosis factor-α in diabetic polyneuropathy. *Exp. Diabesity Res.* **4**, 65–71.

Scott J. N., Clark A. W. and Zochodne D. W. (1999) Neurofilament and tubulin gene expression in progressive experimental diabetes: failure of synthesis and export by sensory neurons. *Brain* **122**, 2109–2118.

Shaw J. E. and Zimmet P. Z. (1999) The epidemiology of diabetic neuropathy. *Diabetes Rev.* **7**, 245–252.

Shibata T., Takeuchi S., Yokota S., Kakimoto K., Yonemori F. and Wakitani K. (2000) Effects of peroxisome proliferator-activated receptor-α and -γ agonist, JTT-501, on diabetic complications in Zucker diabetic fatty rats. *Br. J. Pharmacol.* **130**, 495–504.

Sima A. A. and Sugimoto K. (1999) Experimental diabetic neuropathy: an update. *Diabetologia* **42**, 773–788.

Sotiropoulos K. B., Clermont A., Yasuda Y. *et al.* (2006) Adipose-specific effect of rosiglitazone on vascular permeability and protein kinase C activation: novel mechanism for PPARγ agonist's effects on edema and weight gain. *FASEB J.* **20**, E367–E380.

Straus S. E., Pascual G., Li M., Welch J. S., Ricote M., Hsiang C.-H., Sengchanthalangsy L. L., Ghosh G. and Glass C. K. (2000) 15-Deoxy-$\Delta^{12\text{-}14}$-prostaglandin J_2 inhibits multiple steps in the NF-κB signaling pathway. *Proc. Natl Acad. Sci. USA* **97**, 4844–4849.

Tomlinson D. R. (1999) Mitogen-activated protein kinases as glucose transducers for diabetic complications. *Diabetologia* **42**, 1271–1281.

Uehara K., Yamagishi S., Otsuki S., Chin S. and Yagihashi S. (2004) Effects of polyol pathway hyperactivity on protein kinase C activity, nociceptive peptide expression, and neuronal structure in dorsal root ganglia in diabetic mice. *Diabetes.* **53**, 3239–3247.

Verrier E., Wang L., Wadham C., Albanese N., Hahn C., Gamble J. R., Chatterjee V. K. K., Vadas M. A. and Xia P. (2004) PPARγ agonists ameliorate endothelial cell activation via inhibition of diacylglycerol-protein kinase C signaling pathway. Role of diacylglycerol kinase. *Circ. Res.* **94**, 1515–1522.

Vincent A. M. and Feldman E. L. (2004) New insights into the mechanisms of diabetic neuropathy. *Rev. Endocr. Metab. Disord.* **5**, 227–236.

Vinik A. I. and Mehrabyan A. (2004) Diabetic neuropathies. *Med. Clin. North Am.* **88**, 947–999.

Wada R. and Yagihashi S. (2005) Role of advanced glycation end products and their receptors in development of diabetic neuropathy. *Ann. NY Acad. Sci.* **1043**, 598–604.

Wada R., Nishizawa Y., Yagihashi N. *et al.* (2001) Effects of OPB-9195, anti-glycation agent, on experimental diabetic neuropathy. *Eur. J. Clin. Invest.* **31**, 513–520.

Wada K., Nakajima A., Katayama K. *et al.* (2006) Peroxisome proliferator-activated receptor γ-mediated regulation of neural stem cell proliferation and differentiation. *J. Biol. Chem.* **281**, 12673–12681.

Wang Y., Schmeichel A. M., Iida H. *et al.* (2005) Ischemia-reperfusion injury causes oxidative stress and apoptosis of Schwann cell in acute and chronic experimental diabetic neuropathy. *Antioxidants Redox Signal.* **7**, 1513–1520.

Yagihashi S. (1995) Pathology and pathogenetic mechanisms of diabetic neuropathy. *Diabetes Metab. Rev.* **11**, 193–225.

Yagihashi S., Yamagishi S. I., Wada R., Baba M., Hohman T. C., Yabe-Nishimura C. and Kokai Y. (2001) Neuropathy in diabetic mice overexpressing human aldose reductase and effects of aldose reductase inhibitor. *Brain* **124**, 2448–2458.

Yamagishi S., Uehara K., Otsuki S. and Yagihashi S. (2003) Differential influence of increased polyol pathway on protein kinase C expressions between endoneurial and epineurial tissues in diabetic mice. *J. Neurochem.* **87**, 497–507.

Yorek M. A. (2003) The role of oxidative stress in diabetic vascular and neural disease. *Free Radic. Res.* **37**, 471–480.

Zhao Y., Patzer A., Gohlke P., Herdegen T. and Culman J. (2005) The intracerebral application of the PPARγ-ligand pioglitazone confers neuroprotection against focal ischaemia in the rat brain. *Eur. J. Neurosci.* **22**, 278–282.

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Pathology and pathogenetic mechanisms of diabetic neuropathy: Correlation with clinical signs and symptoms

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Abstract

Drastic increase in diabetic patients poses serious problems in the care of neuropathy so that there needs to explore the pathogenesis and to establish the effective treatment. Recent clinical and basic studies revealed characteristic pathophysiology of diabetic neuropathy and some clue to the direction of the treatment. The pathology of diabetic neuropathy is characterized by progressive nerve fiber loss that gives rise to positive and negative clinical signs and symptoms such as pain, paresthesia and loss of sensation. The nerve fiber loss takes the form of pan-modal pattern with proximo-distal gradient. Endoneurial microangiopathic change is also a constant feature of peripheral nerve pathology and negatively correlates with nerve fiber density. The vascular change and distal nerve fiber loss of small caliber, in particular, at the site of epidermis, commence even in subjects with impaired glucose tolerance and precede loss of nerve fibers in the nerve trunk of lower extremities. Pathogenetic mechanisms underlying the progressive nerve fiber loss seem to be multifactorial, including polyol pathway, glycation, reactive oxygen species, and altered protein kinase C activity. Clinical trials based on this background confirmed that fundamental treatment is in fact beneficial for the prevention and halting of this intractable disorder.

Keywords: Diabetes; Neuropathy; Pathology; Pathogenetic mechanisms

1. Introduction

Functional and structural impairments of peripheral nervous system are generally defined as diabetic neuropathy and its diagnosis is based on the exclusion of other diseases that may involve nervous system [1]. In fact, diabetic neuropathy is the most common complication of diabetes reaching 45–50% prevalence compared to 25–30% of retinopathy and 20% of nephropathy [2]. Recent advances in clinical evaluation and pathological studies on diabetic neuropathy revealed systematic changes of peripheral nervous systems. Signs and clinical symptoms can mostly be attributed to functional and structural alterations detected in peripheral nerves and many studies on experimental diabetic animal models have explored the pathogenetic mechanisms of this disorder [3–5]. In this communication, pathological background of diabetic neuropathy and underlying mechanisms at the molecular levels are briefly summarized. Recent results of clinical trials targeted for the pathogenetic factors are also introduced.

2. Pathological background and its clinical relevance

Most pathological studies to date on diabetic neuropathy were conducted on autopsy materials or

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biopsied samples of sural nerve obtained from diabetic patients with established neuropathy. From these systematic studies, it is well established that peripheral nerve pathology in diabetic patients is characterized by progressive nerve fiber loss with pan-modal fiber size pattern [3,6] (Fig. 1). In particular, the nerve fiber degeneration is length-dependent and conspicuous in distal portion. It has recently been shown that small fibers are preferentially affected in early stages of diabetic patients followed by the involvement of large fibers related to reduced nerve conduction velocity or decreased vibration threshold [7,8]. As an early sign, there is a loss of intra-epidermal nerve fibers that can be detected by immunohistochemistry [8,9]. Such distal fiber loss is typically found in the skin of calf in the subjects with impaired glucose tolerance (IGT) in the absence of significant change in the thigh, indicating the implication of length of nerve fibers in fiber degeneration [8,9]. At this early stage, microangiopathic changes are also detected before there is an apparent nerve fiber loss [10,11].

With this structural background, we can consider that the symptoms and signs are based on the structural alterations (Fig. 2). When fibers undergo active nerve fiber degeneration or impaired regeneration, those fibers exert exciting impulses, thus inducing subjective symptoms such as pain or paresthesia (positive symptoms). Once nerve fibers are lost, then the loss of sensation will take place. With increasing loss of fibers, the area of sensory loss or its severity will be augmented (negative symptoms). In this stage, every care should be paid for the foot care not to be amputated. Thus, the symptoms should be divided into positive symptoms that the patients feel awful and complain of and into negative symptoms that the patients may not complain. The latter is particularly important for the necessity of foot care.

One particular characteristic of diabetic neuropathy is the presence of endoneurial microangiopathy that parallels with nerve fiber loss [3,6]. The figure is typical of microangiopathy showing thickened and multiplicated basement membranes and swollen endothelial cells, similar to those encountered in other site of diabetic patients, including skin, eye, kidney, and muscles. Our studies well demonstrated significant correlation of basement membrane thickness of endoneurial vessels with reduced myelinated nerve fiber density, suggesting close correlation of microangiopathy with the development of diabetic neuropathy [3].

The progressive nerve fiber loss may be attributed to ongoing axonal degeneration of Wallerian type as well as dying back type or secondary to severe demyelina-

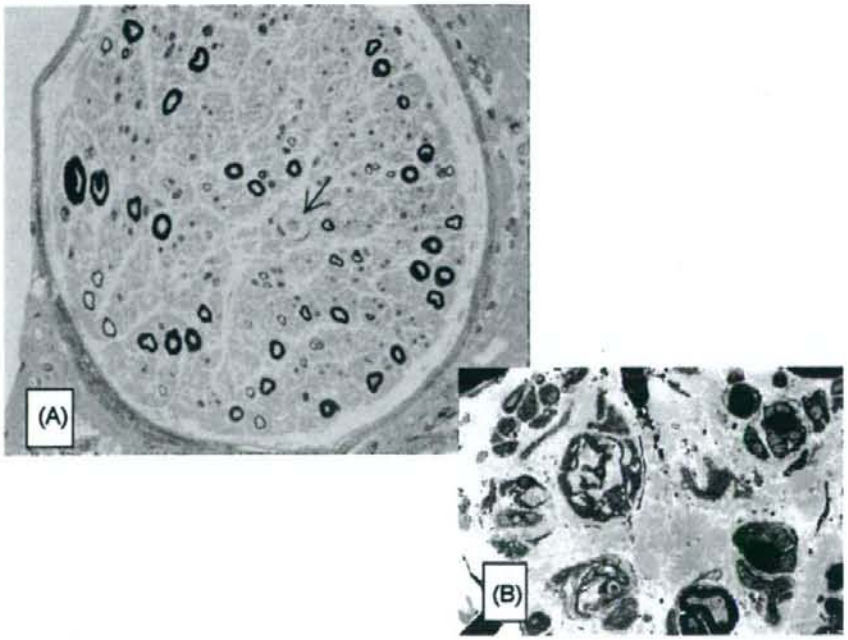

Fig. 1. Pathological findings of sural nerve obtained from diabetic patients with clinically overt neuropathy. There is a marked loss of myelinated nerve fibers of both large and small caliber. Endoneurial vessels show typical microangiopathic changes exemplified by thickened wall (arrow) (A). There is also a marked loss of unmyelinated fibers at EM level (B).

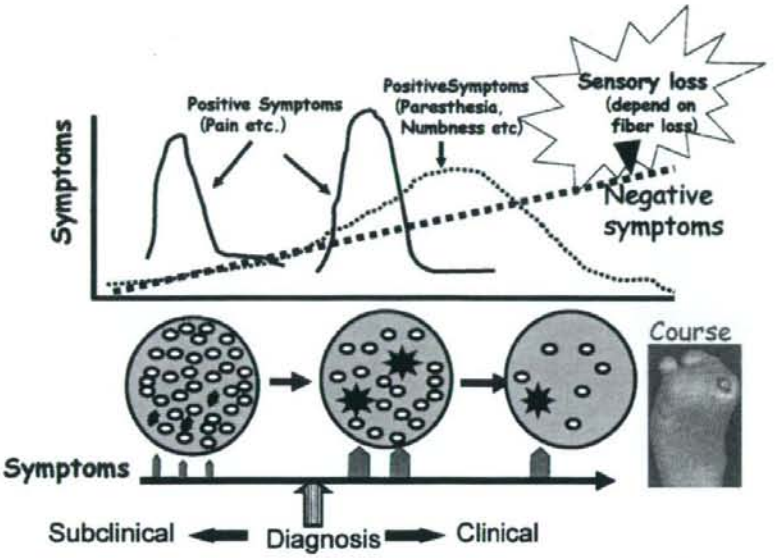

Fig. 2. Natural history of diabetic neuropathy and clinical signs and symptoms with pathological background. With increasing stage of neuropathy, there is a progressive loss of nerve fibers that convey sensation. When the fibers undergo degeneration or impaired remyelination, they release impulse of positive symptoms. With progression of disease, negative symptoms of sensory loss are increased.

tion. If regeneration fails, degenerated fibers will be lost. In diabetic nerve, all the complicated degenerative processes are found depending on the nature of injuries. When the nerves are under active degenerative processes due to local injury or vascular causes, those fibers may yield positive symptoms that can be alleviated only by symptomatic treatment. On the other hand, to prevent progressive nerve fiber loss and inhibit the development of neuropathy, fundamental treatment based on the pathogenetic mechanisms become essential.

3. Neuropathy in IGT

There are several lines of evidence that showed the presence of neurological involvement at the stage of IGT. The symptoms are the type of small fiber neuropathy pathologically characterized by loss of intradermal nerve fibers [8,9]. The suggestion of an early presence of neuropathy in IGT is also supported by the Japanese epidemiological survey of IGT patients that showed high prevalence of subjects with loss of Achilles tendon reflex, reduced vibration perception threshold, or even reduced nerve conduction velocity. However, it still remains unknown that such complexes should clinically be called as neuropathy. Life style changes are recently found to ameliorate the skin nerve pathology, thus reinforcing the importance of life style intervention in the prevention and treatment of diabetic neuropathy [12].

4. Pathogenesis of neuropathy

As alluded earlier, it is essential to prevent or halt the development of diabetic neuropathy based on the information about the pathogenesis. Long term hyperglycemia elicits enhanced polyol pathway, increased nonenzymatic glycation of structural proteins, increased oxidative stress as well as altered protein kinase C activity and polyADP-ribose polymerase (PARP) activation that are all interrelated for the cause and development of neuropathy [3–5] (Fig. 3). These in turn activate or suppress the protein kinase C activity or activate MAP kinase activity, resulting in functional and structural derangement of peripheral nervous system. It is yet to be clear, however, how these mechanisms are related each other or independently operate.

5. Role of polyol pathway

Polyol pathway is a simple metabolic pathway converting glucose to sorbitol by enzyme of aldose reductase (AR), then converting sorbitol to fructose by sorbitol dehydrogenase (SDH). Under normoglycemic condition, glucose is metabolized to oxidative glycolytic pathway with phosphorylated oxidation through

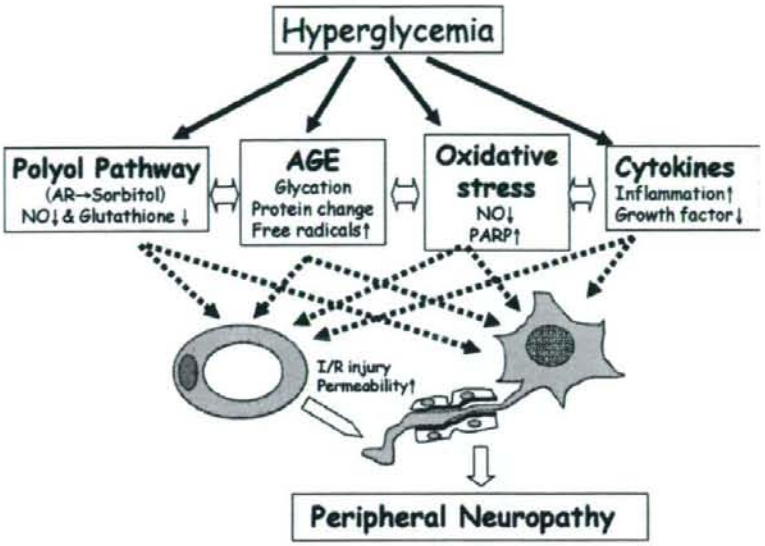

Fig. 3. Multifactorial etiology of diabetic neuropathy. Hyperglycemia exerts increased polyol pathway, enhanced AGE formation, increased oxidative stress as well as cytokine release. These factors are complicatedly interactive or independently operate for the cause and development of diabetic neuropathy directly affecting nerve tissues or through nutrient vascular tissues.

mitochondria. They produce ATP energy by citric acid cycle. Under hyperglycemia, excessive glucose is converted to sorbitol by AR. During this process, NADPH is consumed as a coenzyme. Consumption of NADPH in turn elicits impaired synthesis of NO or reduced glutathione, resulting in vascular insufficiency and overproduction of free radicals. Immunodistribution of AR in Schwann cells thus supports the role of polyol pathway in the nerve damage under hyperglycemia.

In humans, expression levels of AR in tissues are determined in part by gene polymorphism, gene promoter function or other epigenetic regulations, indicating the presence of complication-prone subjects related to AR gene. Measurement of erythrocyte AR protein levels by enzyme-linked immunoasssay in fact demonstrated a greater prevalence of diabetic complications in patients with high AR protein levels than those with low AR protein levels [13]. Furthermore, intraepidermal nerve fibers were degenerated more severely in patients with high AR levels compared to those with low levels of AR [14].

6. Results from studies on AR-transgenic animals

To explore detailed mechanisms how polyol pathway is involved in diabetic neuropathy and to develop specific inhibitors for human AR, transgenic mouse model overexpressing human AR is now available. Under galactose load, this model exhibits significant delay of motor nerve conduction velocity and nerve fiber atrophy with tissue accumulation of polyol (galactitol). In this setting, neither blood glucose levels nor insulin levels are altered. Hyperactivity of polyol pathway indeed causes neuropathic changes independent of hyperglycemia or hypoinsulinemia [15]. On the other hand, suppression of endoneurial Na,K-ATPase and protein kinase C (PKC) activity is major metabolic sequelae in this model when diabetes is induced by streptozotocin [16]. Similar to galactosemic condition, slowed nerve conduction and nerve fiber atrophy are typical neuropathic changes in this model when diabetes is induced, to much more severe extent, compared to diabetic littermate control mice [16]. The reduced PKC activity in nerve tissues may be paradoxical to other diabetic microvascular tissues like retina or kidney. Our fractionated analysis of PKC expression clearly demonstrated the difference in the activity of PKC and protein expression of PKC isoforms between endoneurial and epineurial vascular tissues, in which PKC activity is increased in endoneurium while it is decreased in vascular tissues in diabetes [17] (Fig. 4). In this setting, membrane fraction of PKC-α is markedly decreased in nerve, while membrane fraction of PKC-β is increased in vascular tissues, thus

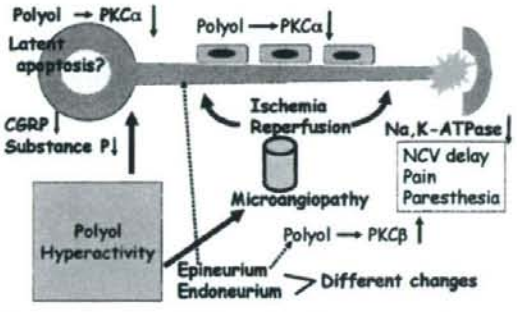

Fig. 4. Mechanisms of how polyol pathway causes neuropathy. Increased polyol pathway affects differently nerve fibers and supplying vascular tissues. In nerve tissues, polyol hyperactivity causes suppression of PKC activity by the inhibition of membranous PKC-α expression while it causes increased PKC activity by elevation of membranous PKC-β expression in vascular wall. These dichotomous processes eventually elicit decreased Na,K-ATPase activity of nerve tissues and delay of nerve conduction.

indicating again the tissue-specific regulation of polyol pathway and its cellular signalling. In addition, there is a possibility that various isoforms of PKC are complicatedly altered in disease conditions.

7. Results from clinical trials

For the treatment of diabetic neuropathy, two basic directions may be raised; one for the symptomatic treatment for signs and symptoms that the patients complain, another for the fundamental treatment based on the pathogenetic mechanisms. Symptomatic treatment may not be effective for the prevention or halting of the disease but should be good for pain or to alleviate other subjective symptoms, while fundamental treatment may not be effective to relieve the symptoms but able to halt or inhibit the progression of the disease.

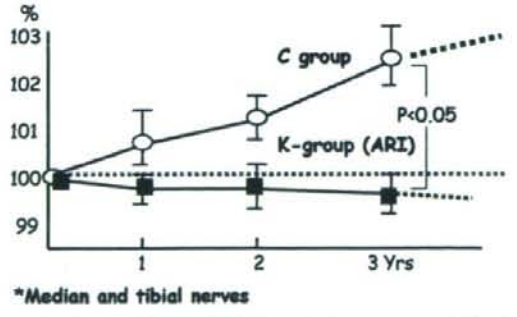

Fig. 5. Effects of 3-year inhibition of aldose reductase (AR) by epalrestat on peripheral nerve function in diabetic patients. Without treatment with AR-inhibitor, non-treated group showed constant deterioration as shown by prolonged F-wave latency while the latency of treated group remained normal. The average level of HbA1c was 7.4 in treated diabetic group and 7.5 in untreated control group.

For symptomatic treatment, a variety of drugs have been developed for the pain; anti-epileptics, anti-convulsants, or Na-channel blockers as well as opioids, SSRI, etc. On the other hand, only aldose reductase inhibitor (ARI) is available for the fundamental treatment. Other drugs, such as anti-glycation agent, protein kinase C inhibitors, anti-oxidants, or PARP inhibitors are now under way of clinical trials. Recently, we could demonstrate that 3-year inhibition of AR in fact could inhibit the progression of neuropathy exemplified by significant decrease in the number of patients with negative symptoms and delay of nerve conduction velocity compared to those of untreated group [18] (Fig. 5). Importantly, we could not demonstrate any effects of ARI on the incidence of patients with positive symptoms. Similar results are also reported by Hotta and his group with much larger scale in Japan [19].

8. Concluding remarks

Recent studies demonstrated that neuropathy is treatable and preventable disease and early diagnosis is essential for this condition. To this end, exploration of pathogenesis of diabetic complications is most important, and such efforts will eventually lead to better outcome in diabetic patients in long-run.

References

[1] A.J. Boulton, et al., Diabetic neuropathies: a statement by the American Diabetes Association, Diabetes Care 28 (2005) 956–962.
[2] J.E. Shaw, P.Z. Zimmet, The epidemiology of diabetic neuropathy, Diabetes Rev. 7 (1999) 245–252.
[3] S. Yagihashi, Pathology and pathogenetic mechanisms of diabetic neuropathy, Diabetes Metab. Rev. 11 (1995) 193–225.
[4] A.A. Sima, K. Sugimoto, Experimental diabetic neuropathy: an update, Diabetologia 42 (1999) 773–788.
[5] D.R. Tomlinson, Mitogen-activated protein kinases as glucose transducers for diabetic complications, Diabetologia 42 (1999) 1271–1281.
[6] P.J. Dyck, C. Giannini, Pathologic alterations in the diabetic neuropathies of humans: a review, J. Neuropathol. Exp. Neurol. 55 (1996) 1181–1193.
[7] P.J. Dyck, P.J. Dyck, T.S. Larson, P.C. O'Brien, J.A. Velosa, Patterns of quantitative sensation testing of hypoesthesia and hyperalgesia are predictive of diabetic polyneuropathy: a study of three cohorts. Nerve growth factor study group, Diabetes Care 23 (2000) 510–517.
[8] C.J. Sumner, S. Sheth, J.W. Griffin, D.R. Cornblath, M. Polydefkis, The spectrum of neuropathy in diabetes and impaired glucose tolerance, Neurology 60 (2003) 108–111.

[9] M. Polydefkis, J.W. Griffin, J. McArthur, New insights into diabetic polyneuropathy, JAMA 290 (2003) 1371–1376.
[10] S. Thrainsdottir, R.A. Malik, L.B. Dahlin, P. Wiksell, K.F. Eriksson, I. Rosen, et al., Endoneurial capillary abnormalities presage deterioration of glucose tolerance and accompany peripheral neuropathy in man, Diabetes 52 (2003) 2615–2622.
[11] R.A. Malik, S. Tesfaye, P.G. Newrick, D. Walker, S.M. Rajbhandari, I. Siddique, et al., Sural nerve pathology in diabetic patients with minimal but progressive neuropathy, Diabetologia 48 (2005) 578–585.
[12] A.G. Smith, J. Russell, E.L. Feldman, J. Goldstein, A. Peltier, S. Smith, et al., Lifestyle intervention for pre-diabetic neuropathy, Diabetes Care 29 (2006) 1294–1299.
[13] C. Nishimura, T. Saito, T. Ito, Y. Omori, T. Tanimoto, High levels of erythrocyte aldose reductase and diabetic retinopathy in NIDDM patients, Diabetologia 37 (1994) 328–330.
[14] A. Hirai, H. Yasuda, M. Joko, T. Maeda, R. Kikkawa, Evaluation of diabetic neuropathy through the quantitation of cutaneous nerves, J. Neurol. Sci. 172 (2000) 55–62.
[15] S. Yagihashi, S. Yamagishi, R. Wada, K. Sugimoto, M. Baba, H.G. Wong, et al., Galactosemic neuropathy in transgenic mice for human aldose reductase, Diabetes 45 (1996) 56–59.
[16] S. Yagihashi, S.I. Yamagishi, R. Wada, M. Baba, T.C. Hohman, C. Yabe-Nishimura, et al., Neuropathy in diabetic mice overexpressing human aldose reductase and effects of aldose reductase inhibitor, Brain 124 (2001) 2448–2458.
[17] S. Yamagishi, K. Uehara, S. Otsuki, S. Yagihashi, Differential influence of increased polyol pathway on protein kinase C expressions between endoneurial and epineurial tissues in diabetic mice, J. Neurochem. 87 (2003) 497–507.
[18] M. Baba, K. Kimura, T. Suda, S. Yagihash, Aomori Diabetic Study (ADNS) Group, Three-year inhibition of aldose reductase on development of symptomatic neuropathy in diabetic patients, J. Peripher. Nerv. Syst. 11 (2006) 176–178.
[19] N. Hotta, Y. Akanuma, R. Kawamori, K. Matsuoka, Y. Oka, M. Shichiri, et al., Long-term clinical effects of epalrestat, an aldose reductase inhibitor, on diabetic peripheral neuropathy: the 3-year, multicenter, comparative Aldose Reductase Inhibitor-Diabetes Complications Trial, Diabetes Care 29 (2006) 1538–1544.

糖尿病の胃腸障害

Gastrointestinal disturbance in diabetes

診断の指針 / 治療の指針

安田 斎
YASUDA Hitoshi

はじめに

糖尿病では，消化器系の全臓器が障害を受けることが明らかになっており，糖尿病外来患者の75％に消化器症状を認めたとの報告がある．しかし，糖尿病に特徴的な症状は少ない．糖尿病における消化器機能障害は，高血糖自体あるいは糖尿病合併症により惹起される．とくに糖尿病自律神経障害の関与は大きい．一方，糖尿病では，免疫機構の障害により，食道カンジダ症や消化管の化膿性合併症などの消化器感染症が起こる頻度が高いことが報告され，ヘリコバクターピロリ感染の糖尿病患者における頻度についての論争などもあるが，本稿では，糖尿病自律神経障害に関連した胃腸機能異常の病態と治療について概説する．

1. 胃の消化運動機序

食物は経口摂取された後，胃での消化のプロセスを経て，小腸へ排出され吸収される．摂取直後に胃底部が弛緩することで，食物はいったん貯えられた後，前庭部の蠕動運動によって胃酸およびペプシンと混和・粉砕される．粥状に粉砕された食物は幽門を通過し，胃内容が空になるまで排出運動が持続し，十二指腸で膵液・胆汁と混和され，上部空腸で主な栄養素の吸収が行われる．正常の胃では，胃近位部は嚥下により弛緩する．この弛緩は受容性弛緩と呼ばれ，嚥下物が増加するに従いさらに弛緩して，胃内圧を高めることなく嚥下物を収容・貯留することができる仕組みになっている．この受容性弛緩は迷走神経抑制線維により調節されており，糖尿病で障害されていると考えられている．

2. 糖尿病胃不全麻痺

糖尿病における胃の機能異常は，広く糖尿病胃症と呼ばれており，胃排出遅延の“ある場合”と“ない場合”がある．胃排出遅延は自律神経障害によって惹起されると考えられ，1型糖尿病患者の50％，2型糖尿病患者の30％で観察される．重症の病態は糖尿病胃不全麻痺と呼ばれる．一般的に，胃排出遅延の程度と消化器症状の強さには相関関係は乏しい．これは，併存する感覚神経障害のために消化器症状が明瞭でないことに起因すると思われる．実際，悪心嘔吐などの症状が強いものでは胃不全麻痺の存在する確率は高いが，消化器症状がなくても胃不全麻痺が存在しうることを銘記すべきである．

3. 糖尿病胃症の血糖コントロールに及ぼす影響

糖尿病胃症の臨床的問題点は，消化器症状よりも，むしろ血糖コントロールに対する影響である．胃不全麻痺患者では，大量の胃内残存物が徐々に排出されるので，食後数時間以上経ってから予期しない時刻に栄養吸収，続いて血糖上昇が起こる．また，胃からの排出も不規則であるため血糖は不安定となる．この場合，インスリン治療者では，血糖上昇が起こらないうちにインスリン効果が発現することになるので，低血糖になったり，逆に，胃からの排出が遅れると予想外の高血糖になる危険性がある．

他方，軽度の胃排出遅延の場合には，逆に血糖上昇が緩徐であるために血糖調節が容易になりうる．さらに，血糖降下薬の投与下では，薬物吸収および効果発現が緩徐になりうるので注意が必要である．

4. 糖尿病胃症の薬物治療

胃排出遅延を伴う糖尿病胃症の治療の一般的な対策として，胃内容物は一定の大きさ以下になるまで胃内に残存するので，十分な咀嚼に努めることが有用である．消化管運動機能異常は血糖調節と密接に関連し，また血糖値上昇が同機能低下と関連するため，両者の悪循環を断ち切ることが必要となる．しかし，消化管運動機能低下の状態では薬物吸収も遅延するので，その薬理効果も予想よりも遅れた時期に出現するので注意が必要である．

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薬物療法としては，消化管運動機能賦活薬が主に使用される．海外ではメトクロプラミド，ドンペリドンなどが比較的よく使用されているが，わが国ではクエン酸モサプリド，塩酸イトプリド，ナパジシル酸アクラトニウムなどが用いられる．モチリン受容体刺激作用を有するエリスロマイシンは強い胃収縮作用を有することが知られているが，保険適用はない．糖尿病胃不全麻痺に効果を示す薬物は多いが，保険適用とした薬剤はないのが現状である．

5. 小腸・大腸の運動機能異常の異常と対策

糖尿病患者で最も頻繁に認められる消化器症状は便秘であり，大腸運動機能低下に起因する．自律神経障害があると大腸通過時間の延長が認められ，とくにS状結腸から直腸での通過遅延が高度になる．腸管壁内神経叢の異常に基づく感覚低下に起因する排便反射低下や，食事療法による摂食量の制限で糞便塊が減少し，便秘に拍車をかける．

糖尿病下痢は糖尿病患者に生じる胃，大腸の基礎疾患を伴わない下痢であり，除外診断になる．ほとんどの患者が体性神経障害と自律神経障害を併せ持ち，自律神経障害によって引き起こされると考えられる．

突然の便意のために失禁することもあり，夜間に多く発症することが特徴的である．糖尿病下痢は，数日間の下痢の後，腸管内容物の完全な排除に伴って自然緩解し，便秘あるいは正常排便の時期を経て再発することが多く，下痢と便秘を繰り返すのが特徴である．下痢があっても腹痛は著明ではない．自然緩解と再発までの間隔は必ずしも一定ではなく，薬物療法の有効性が自然緩解によるものかどうかを判定することが困難な場合がある．

糖尿病下痢では腸管通過時間が遅延しており，健常者でみられる空腹期に繰り返し規則的に出現する小腸の第3相の収縮運動が消失していることが多い．そのため，腸管内細菌叢の異常増殖が起こり，下痢が起こると考えられる．通常，数回の下痢により腸管内容物の排泄が完了すると，腸管内細菌叢が減少し下痢が止まり，その後，腸管運動機能低下のために便秘が起こる．下痢の発現には自律神経障害による二次性の腸液や電解質の吸収障害も関係していると考えられる．

6. 糖尿病患者の便秘に対する治療

糖尿病患者の便秘症の治療は非糖尿病患者と基本的に同じである．緩下剤などの通常の治療に反応しない場合，メトプラミドの追加投与が推奨される．また，間欠的であれ，持続的であれ，慢性下痢を呈する糖尿病患者の治療は，まず，鑑別診断から開始し，場合によっては種々の薬物による治療的診断を推奨する専門家もいる．

治療戦略として，最初，抗生剤から開始する．有効であっても耐性・菌交代現象を考慮して抗生剤の長期投与には注意する．無効であれば，腸管の蠕動と腸液分泌を抑制する塩酸ロペラミドにて対症療法を行う．症状が持続する場合は治療を続け，症状が消失すれば治療を中止する．この際，場合によっては乳酸菌製剤や消化酵素薬などを併用する．対症療法が効果のない患者では，小腸や大腸での吸収障害に有効なα2受容体刺激薬であるクロニジンを投与する．効果が得られない場合は，腸粘膜の分泌を減少させ，吸収を増強させる持続性ソマトスタチン・アナログの酢酸オクトレオチドの投与を試みる．なお，下痢の後に便秘が続く交代性の下痢には便秘の防止を優先し，軟便傾向に調整するのが望ましい．

おわりに

糖尿病患者にみられる消化管機能異常は，消化器症状の発現にとどまらず，血糖調節動態に深く関与する．さらに，血糖調節異常が消化管運動機能異常を引き起こす．また，消化器症状を示さず消化管機能異常を有していることも多いので，糖尿病患者で説明しがたい血糖コントロールの不良状態を観察した場合には，糖尿病胃不全麻痺などの消化管機能異常の存在を考慮して，病態の精査・治療にあたる必要がある．