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Original Contribution

Triglyceride, nonesterified fatty acids, and prediabetic neuropathy: role for oxidative–nitrosative stress

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ABSTRACT

Peripheral neuropathy develops in human subjects with prediabetes and metabolic syndrome before overt hyperglycemia. The contributions of impaired glucose tolerance and insulin signaling, hypertriglyceridemia and/or increased nonesterified fatty acids (NEFA), and hypercholesterolemia to this condition remain unknown. Niacin and its derivatives alleviate dyslipidemia with a minor effect on glucose homeostasis. This study evaluated the roles of impaired glucose tolerance versus dyslipidemia in prediabetic neuropathy using Zucker fatty (*fa/fa*) rats and the niacin derivative acipimox, as well as the interplay of hypertriglyceridemia, increased NEFA, and oxidative–nitrosative stress. Sixteen-week-old Zucker fatty rats with impaired glucose tolerance, obesity, hyperinsulinemia, hypertriglyceridemia, hypercholesterolemia, and increased NEFA displayed sensory nerve conduction velocity deficit, thermal and mechanical hypoalgesia, and tactile allodynia. Acipimox (100 mg kg^{-1} day^{-1}, 4 weeks) reduced serum insulin, NEFA, and triglyceride concentrations without affecting glucose tolerance and hypercholesterolemia. It alleviated sensory nerve conduction velocity deficit and changes in behavioral measures of sensory function and corrected oxidative–nitrosative stress, but not impaired insulin signaling, in peripheral nerve. Elevated NEFA increased total and mitochondrial superoxide production and NAD(P)H oxidase activity in cultured human Schwann cells. In conclusion, hypertriglyceridemia and/or increased NEFA concentrations cause prediabetic neuropathy through oxidative–nitrosative stress. Lipid-lowering agents and antioxidants may find a use in the management of this condition.

Evidence for the importance of factors other than overt hyperglycemia, i.e., insulin resistance and impaired insulin signaling in the peripheral nervous system [1–4], hypertension [5], and increased body mass index/obesity [5], in diabetic peripheral neuropathy is emerging. Several studies revealed significant positive associations between the presence and/or severity of diabetic peripheral neuropathy and dyslipidemia and, in particular, total cholesterol [5], LDL cholesterol [5], and triglycerides [5–7]. A multifactorial etiology of diabetic neuropathy is supported by a higher incidence of neuropathic changes in human subjects with impaired glucose tolerance [8,9] and metabolic syndrome [10], although the existence of an association between impaired fasting glucose or impaired glucose tolerance and neuropathy is not uniformly accepted [11,12]. The mechanisms of neuropathic changes preceding overt diabetes are unknown, and their exploration is complicated by the lack of animal models that develop prediabetes first and then spontaneously transit to overt diabetes. For this reason, the mechanisms underlying prediabetes per se as well as end-organ damage associated with this condition are studied in high-fat diet fed mice [13–15] and Zucker fatty (*fa/fa*) rats [16–20] that maintain metabolic abnormalities characteristic of the prediabetic condition, i.e., hyperinsulinemia, impaired glucose tolerance in the absence of overt hyperglycemia, hypertriglyceridemia, and/or increased nonesterified fatty acid abundance, as well as hypercholesterolemia, during their whole life span. Both models exhibit nerve conduction deficit, small sensory nerve fiber dysfunction, and clearly manifested oxidative–nitrosative stress in the peripheral nerve and vasa nervorum [21–25] and are, therefore, suitable for dissection of the relative contributions of these phenomena to peripheral neuropathy in prediabetes. The Zucker *fa/fa* rat, with genetically predetermined obesity, hyperinsulinemia, and other aforementioned metabolic abnormalities, is the preferred model for this kind of study, as many pharmacological interventions, including those alleviating oxidative stress, have been reported to interfere with the prediabetic condition per se in high-fat diet fed rodents [26–28].

Dissecting the relative contributions of impaired glucose tolerance/overt hyperglycemia vs dyslipidemia to complications associated with type 2 diabetes or prediabetes is quite challenging, as a vast majority of therapeutic agents affect both carbohydrate and lipid metabolism.

Abbreviations used: HDL, high-density lipoprotein; HSC, human Schwann cells; IRβ, insulin receptor β; IRS-1, insulin receptor substrate 1; LDL, low-density lipoprotein; MNCV, sciatic motor nerve conduction velocity; NEFA, nonesterified fatty acids; ROS, reactive oxygen species; SNCV, hindlimb digital sensory nerve conduction velocity; VLDL, very low density lipoprotein.

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Niacin reduces triglyceride concentration by inhibiting hepatic synthesis of fatty acids and triglycerides and hepatic VLDL secretion as well as release of fatty acids from adipose tissue [29,30]. In a clinical study in 468 participants including 125 with diabetes mellitus [31], niacin improved lipid profile, without affecting blood glucose control, in human subjects with and without diabetes. The niacin derivative acipimox (6-methyl-1-oxidopyrazin-1-ium-2-carboxylic acid), which can be used in lower doses and has less marked adverse side effects, was also reported to alleviate dyslipidemia, but not hyperglycemia, when administered to type 2 diabetic subjects long term [32]. Furthermore, acute administration of acipimox reduced hyperinsulinemia and nonesterified fatty acid (NEFA) concentrations in subjects with metabolic syndrome [33]. We therefore used acipimox for gaining new insights into the roles of impaired glucose tolerance, insulin sensitivity, and dyslipidemia in the development of prediabetic neuropathy using the Zucker fatty rat model. Identification of a causative role of elevated triglyceride and NEFA, together with the current knowledge on the roles of both factors in reactive oxygen species (ROS) generation [34,35], consequently led us to the evaluation of the interplay of hypertriglyceridemia, fatty acidemia, and oxidative–nitrosative stress in the peripheral nerve, using material from the aforementioned in vivo study, as well as in cultured human Schwann cells (HSC).

Methods

Reagents

Unless otherwise stated, all chemicals were of reagent-grade quality and were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). The cholesterol quantitation kit for assessment of serum total cholesterol concentration was obtained from MBL International (Woburn, MA, USA). The triglyceride quantification kit and HDL and LDL/VLDL-cholesterol assay kit for measurements of serum triglyceride and VLDL/LDL-cholesterol concentrations were purchased from Abcam (Cambridge, MA, USA). The HR Series NEFA-HR(2) kit for assessment of serum NEFA concentrations was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The Ultra Sensitive rat insulin ELISA kit from Crystal Chem., Inc. (Downers Grove, IL, USA), was used for measurements of serum insulin concentrations. For Western blot analyses of variables of insulin signaling, rabbit monoclonal (4B8) anti-insulin receptor β (IRβ) antibody, rabbit monoclonal (59G8) anti-insulin receptor substrate 1 (IRS-1) antibody, rabbit polyclonal (Ser307) anti-phospho-IRS-1 antibody, and rabbit polyclonal antibodies for total and phosphorylated Akt were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). The OxiSelect nitrotyrosine ELISA kit for nitrotyrosine assay in the sciatic nerve was obtained from Cell Biolabs (San Diego, CA, USA). Dihydroxyethidium and MitoSOX for assessment of oxidative stress variables in cell culture experiments were purchased from Invitrogen (Carlsbad, CA, USA).

Animals

The experiments were performed in accordance with regulations specified by the National Institutes of Health *Principles of Laboratory Animal Care,* 1985 revised version, and Pennington Biomedical Research Center Protocol for Animal Studies. Ten-week-old male Zucker fatty (*fa/fa*) and Zucker lean rats (Charles River, Wilmington, MA, USA) were fed a standard rat chow (PMI Nutrition International, Brentwood, MO, USA) and had access to water ad libitum. At 16 weeks of age, all the rats were weighed. Blood samples for glucose measurements were taken from the tail vein. Zucker fatty and Zucker lean rats were randomly divided into groups maintained with or without acipimox treatment, 100 mg kg^{-1} day^{-1}, for another 4 weeks. Glucose tolerance test (2 g glucose, ip, after 12-h fasting) and measurements of serum insulin, NEFA, triglyceride, total cholesterol, VLDL/LDL-cholesterol, motor nerve conduction velocity, sensory nerve conduction velocity, thermal and mechanical algesia, and tactile response thresholds were conducted in 16-week-old Zucker fatty and Zucker lean rats before acipimox treatment, as well as in 20-week-old untreated and acipimox-treated Zucker fatty and Zucker lean rats at the end of the experiment. After completion of functional studies, the rats were sedated with CO_2 and immediately sacrificed by cervical dislocation. Sciatic nerves were rapidly removed, frozen in liquid nitrogen, and stored at −80 °C before assessment of variables of insulin signaling and oxidative–nitrosative stress.

Specific methods

Measurements of serum insulin, total and VLDL/LDL cholesterol, triglyceride, and NEFA were performed in accordance with the manufacturers' instructions. Sciatic motor and hindlimb digital sensory nerve conduction velocities (MNCV and SNCV), thermal algesia (Hargreaves method), mechanical algesia (Randall–Selitto test), tactile response thresholds (flexible von Frey filament test), and sciatic nerve nitrotyrosine concentrations were evaluated as we described previously [36–39]. Nitrotyrosine, a stable "footprint" of peroxynitrite ($ONOO^-$) action, was chosen over other variables of oxidative stress, for several major reasons. First, evidence for the important role of peroxynitrite in cell injury and pathological conditions associated with oxidative stress is emerging [40,41]. Second, peroxynitrite is the major contributor to the pathogenesis of diabetes and diabetic complications [40–43]. Third, a shortened lag time to plasma $ONOO^-$ production (pholasin test) was identified as an independent risk factor for the severity of diabetic polyneuropathy [6]. Fourth, our recent experimental study demonstrated diabetes-associated nitrotyrosine accumulation in all major cell targets for diabetic peripheral neuropathy, including endothelial and Schwann cells of the peripheral nerve; neurons, astrocytes, and oligodendrocytes of the spinal cord; and neurons and glial cells of the dorsal root ganglia [38]. Furthermore, sciatic nerve nitrotyrosine concentrations were inversely correlated with motor and sensory nerve conduction velocities and myelin thickness. Variables of insulin signaling in the sciatic nerve were assessed by Western blot analysis. We employed 7.5% sodium dodecyl sulfate–polyacrylamide gels for IRβ, phospho-IRS-1, and IRS-1 and 10% gels for phospho-Akt and Akt. The electrophoresis was conducted for 2 h. Protein bands were visualized with the Amersham ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK). Membranes were then stripped and reprobed with β-actin antibody to verify equal protein loading. The data were quantified by densitometry (Quantity One 4.5.0 software; Bio-Rad Laboratories, Richmond, CA, USA). All other details of Western blot analysis were described by us in detail previously.

Cell culture experiments

Schwann cells play a key role in the pathology of various inflammatory, metabolic, and hereditary polyneuropathies, including diabetic peripheral neuropathy [44]. Previous studies demonstrated that cultured HSC (cell line No. 1700; ScienCell, Carlsbad, CA, USA) manifest increased superoxide production, accumulation of nitrated and poly(ADP-ribosyl)ated proteins and 4-hydroxynonenal adducts, inducible nitric oxide synthase overexpression, 12/15-lipoxygenase overexpression and activation, and downregulation of taurine transporter, as well as impaired insulin signaling early (1–7 days) after exposure to high glucose [45–49]. They therefore represent a good cell culture model for studying mechanisms of diabetes-associated oxidative–nitrosative stress in the peripheral nerve. Our pilot experiments revealed that, like other cell types [50], HSC produce ROS shortly after exposure to elevated concentrations of NEFA, consistent with a recent report for other immortalized Schwann cells [35]. We, therefore, used HSC to compare the effects of NEFA, in the concentration range