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Increased autophagy in peripheral nerves may protect Wistar Ottawa Karlsburg W rats against neuropathy

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ABSTRACT

Objective: Wistar Ottawa Karlsburg W (RT1^u) rats (WOKW) develop obesity, dyslipidemia, moderate hypertension, hyperinsulinemia and impaired glucose tolerance prone to induce peripheral neuropathy (PN). Autophagy has been shown to prevent neurodegeneration in the central and peripheral nervous system. We analyzed the potential protective role of autophagy in an established rat model in preventing PN.
Methods: We examined electrophysiology (motor-and sensory/mixed afferent conduction velocities and the minimal F-wave latency) and morphology, including ultrathin sections, myelin sheath thickness (g-ratio) and immunohistochemical markers of autophagy and inflammation in the sciatic nerve of five-month-old, male WOKW as compared to Wistar derived, congenic LEW.1W control rats, characterized by the same major histocompatibility complex as WOKW rats (RT1^u). Moreover, the expression of axonal and synaptic proteins (NF68, GAP43, MP0), autophagy- (Atg5, Atg7, LC3), and apoptosis (cleaved caspase-3)-related markers was measured using Western blot.
Results: No abnormalities in nerve electrophysiology and morphology were found in WOKW compared to LEW.1W rats. However, autophagosomes were more frequently apparent in sciatic nerves of WOKW rats. In Western blot analyses no significant differences in expression of neuronal structural proteins were found, but autophagy markers were up-regulated in WOKW compared to LEW.1W sciatic nerves. Immunostaining revealed a greater infiltration of Iba1/ED-1-positive macrophages, CD-3-positive T-cells and LC3-expression in sciatic nerves of WOKW rats.
Conclusions: Our results indicate that WOKW rats show an up-regulated autophagy and a mild inflammatory response but do not develop overt neuropathy. We suggest that autophagy and inflammatory cells may exert a protective role in preventing neuropathy in this rat model of the metabolic syndrome but the mechanism of action is still unclear.

Introduction

Wistar Ottawa Karlsburg W (RT1^u) rats (WOKW) develop obesity, dyslipidemia, hypertension, hyperinsulinemia, impaired glucose tolerance but not diabetes (Klöting et al., 2006; van den Brandt et al., 2000, 2002). Therefore, the WOKW rats have become a useful animal model to study the pathophysiology of the human metabolic syndrome (MS).

Recent data have shown that metabolic alteration as reflected by the WOKW model may be risk factors for a peripheral neuropathy (PN) (Miscio et al., 2005; Smith and Singleton, 2006). Patients with metabolic syndrome have been claimed to develop peripheral neuropathic changes similar to those occurring in patients with overt diabetes including reduction of motor and sensory nerve responses, small nerve fiber injury, pain and microvascular dysfunction (Pittenger et al., 2005;

Abbreviations: Atg5, autophagy protein 5; Atg7, autophagy protein 7; CD3, cluster of differentiation 3; CD68/ED-1, cluster of differentiation 68; CMAP, compound muscle action potential; CNS, central nervous system; CY3, cyanine 3; D, dermis; E, epidermis; FITC, fluorescein isothiocyanate; GAP43, growth associated protein 43; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; HbA1c, hemoglobin-A1c; Iba-1, ionized calcium binding adapter molecule 1; IENFD, intraepidermal nerve fiber density; LC3, microtubule associated protein light chain 3; LEW.1 W, Lewis 1.W; MF, myelinated fiber; MP0, myelin protein zero; MS, metabolic syndrome; NCV, nerve conduction velocity; NF68, neurofilament 68; NF200, neurofilament 200; PNS, peripheral nervous system; PN, peripheral neuropathy; PGP9.5, protein gene product 9.5; SC, Schwann cell; SNAP, sensory nerve action potential; UF, unmyelinated fiber; WOKW, Wistar Ottawa Karlsburg W.

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Zhou et al., 2011; Ziegler et al., 2008, 2009). In MS patients with normoglycemia, the associated neuropathy has been linked to hyperinsulinemia and insulin sensitivity (Miscio et al., 2005).

A number of observations support a potential causal role of the MS: insulin receptors are present in neurons of PNS and CNS and activate PI3K/Akt signaling pathways involved in cell survival, growth and proliferation, protein synthesis, and glucose metabolism (Kim and Feldman, 2012). The neuronal insulin resistance observed in the MS was shown to cause a loss of insulin-mediated neurotrophism and neuronal injury (Kim and Feldman, 2012).

To date there is no definitive evidence proving that MS with normoglycemia is causing overt metabolic neuropathy. Here, we searched for signs of PN in an established animal model of MS in adult WOKW rats by addressing nerve function and morphology including autophagy markers. Autophagy is an evolutionary conserved process implicated in degradation and recycling of aggregated proteins and damaged organelles (Levine and Klionsky, 2004). Autophagy is critically important for neurons, where damaged organelles and altered proteins cannot be redistributed to daughter cells and accumulate causing neurotoxicity. In the central nervous system (CNS), failure in autophagy causes protein aggregation inside the affected neurons, neurotoxicity, neuronal atrophy, degeneration, and cell death and has been linked to Parkinson's, Huntington', and Alzheimer's disease (Cherra and Chu, 2008; Komatsu et al., 2006; Lynch-Day et al., 2012). On the other hand, upregulation of autophagy in protein clearance was shown to prevent neurodegeneration in the CNS including retinal ganglion cells or in hypoglycemic peripheral nerves (Mohseni, 2011; Rodríguez-Muela et al., 2012). A beneficial effect of activating autophagy on the clearance of altered and toxic proteins inside neurons has opened a new therapeutic option in neuroprotection. We therefore extended our study to examine the relation between autophagy and nerve pathology in WOKW rats with MS. We show that WOKW rats do not develop overt neuropathy, but show macrophage and T-cell infiltration as well as strongly enhanced autophagy.

Material and methods

Animals

Five-month old WOKW (n = 12) and LEW.1W (n = 12) male rats were obtained from the Department of Laboratory Animal Science of the University of Greifswald (Karlsburg, Germany). Rats were adapted to the local animal facilities (4 rats per group and cage) and maintained at 21 ± 1 °C on a 12:12 h light/dark cycle (5 AM/5 PM). All rats had free access to water and were fed with regular food (Global Rodent T.2018.R12 from Harlan Teklad). The body weight was recorded for each group (WOKW mean 712 g; LEW.1W mean 486 g).

Experiments followed the international guidelines animal care and the study protocols were approved by the Regierungspräsidium Leipzig, the local authority for animal care (TVV 25/12). All rats were sacrificed via a CO_2 overdose at the end of the experiment.

Blood glucose, HbA1c and serum insulin concentration

Blood glucose and HbA1c concentrations were measured in whole blood taken from the ventral caudal vein using an Opticum Omega glucometer (GlucoMen, Menarini Diagnostics, Berlin, Germany) and HbA1c levels were analyzed by a standard procedure on an automated chemical analyzer in the Institute of Laboratory Medicine and Clinical Chemistry. Serum insulin was determined using enzyme immunoassay kit (Rat Insulin ELISA, Mercodida AB, Uppsala, Sweden).

Sciatic nerve electrophysiology

The nerve conduction velocity was measured as described elsewhere (De Koning and Gispen, 1987; Hartung et al., 1988). In brief, the motor and sensory/mixed afferent nerve conduction velocity (mNCV or s/mix.aff. NCV) and the minimal F-weave latency in the left sciatic nerve of WOKW (n = 9) and LEW.1W (n = 7) rats were measured under inhalation of anaesthesia with 2.5% isoflurane and N_2O at a constant body temperature of 36.5 °C using a Neurosoft-Evidence 3102 electromyograph (Schreiber und Tholen Medizintechnik GmbH Stade, Germany). The sciatic notch was used as the proximal stimulation point (S1) with a first pair of needle electrodes (DISA near nerve electrodes) (Hartung et al., 1988). These electrodes served also as recording electrodes for the sensory/mixed afferent nerve potentials. Ankle region of the sciatic nerve was used as the distal stimulation point (S2) by insertion of second pair of steel needle electrodes. Recording electrodes for the compound muscle action potentials (CMAP) were placed between digits 2 and 3 of the left foot. The mNCVs were calculated by dividing the distance between the two stimulation points by the differences in latencies of the M response (CMAP) and the H reflex after proximal and distal stimulation. The s/mix.aff. NCV was calculated by dividing the distance by the latency. Moreover, the compound muscle -and sensory nerve action potential (CMAP and SNAP) amplitudes were automatically calculated using the Neurosoft-Evidence 3102 electromyograph software.

Transmission electron microscopy

Sciatic nerves of WOKW and LEW.1W rats (n = 3) were fixed in 2% glutaraldehyde with 1% paraformaldehyde in 0.1 M PBS at 4 °C for 2 h, and postfixed in 1% buffered osmium tetroxide (1.5 h, 4 °C). Cross-sections of the sciatic nerves were transferred into 70% acetone, treated with 1% phosphotungstic acid and 1% uranyl acetate (20 °C, 1 h) and further dehydrated in acetone. Then they were embedded in resin (Durcopan® ACM Fluka, Sigma-Aldrich, Steinheim Germany) and polymerized at 60 °C for 48 h. Semithin sections were stained with 1% toluidine blue solution; ultrathin sections were mounted on copper grids, in contrast with uranyl acetate and lead citrate, and analyzed using an EM 900 electron microscope (Zeiss, Jena, Germany). The thickness of myelin sheaths was measured as described previously (Nowicki et al., 2012).

Immunostaining

Rats (n = 5 per group) were perfused via the left heart ventricle, first with 250 ml phosphate buffered saline (PBS, pH 7.4) containing 6250 u/l heparin (Sigma, Taufkirchen, Germany), and then 250 ml 4% formaldehyde in 0.1 M PBS. All perfusion buffers were warmed to 37 °C. Dissected sciatic nerves and hind foot skin biopsies were postfixed in the same fixative for 4 h, rinsed with PBS, transferred into 30% buffered sucrose solution, and stored at 4 °C until sectioning. The 10-μm-thick frozen cross nerve and 30-μm-thick skin sections from the hind foot were mounted on gelatinized glass slides. After buffer rinse, sections were incubated with rabbit polyclonal microglia/macrophage cytoplasmatic calcium adaptor Iba-1 antibody for the detection of macrophages (1:200; WAKO Chemicals USA, Richmond, VA) or with rabbit polyclonal CD3 for detection of T-cells (1200; Dako Cytomation, Hamburg, Germany) in double staining with the mouse monoclonal antibody against neurofilament 200 (NF200; 1:500; Sigma Aldrich, Taufkirchen, Germany). For the identification of intraepidermal small nerve fibers polyclonal antibody against protein gene product (PGP) 9.5 (1:1000; Abcam) was used. The autophagy was visualized using anti-mouse LC3 antibody (1:100; nanoTools, clone 5F10, Munich, Germany) in double immunostaining with the rabbit polyclonal antibodies against Iba-1 (1:200), Schwann cell specific S100 (1:200, ab76729; Abcam, MA, USA) and PGP9.5 (1:1000) at 4 °C overnight. After buffer rinse, Cy3-conjugated goat anti-mouse IgG (Dianova) or/with FITC-conjugated goat anti-rabbit IgG was diluted 1:1000, and sections were incubated at room temperature for another 2 h. Sections were mounted with Dako Glycergel (Dako Cytomation) containing 10 μg/ml DAPI (Serva, Heidelberg, Germany) for nuclear staining and