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EXPERIMENTAL DIABETES IN NEONATAL MICE INDUCES EARLY PERIPHERAL SENSORIMOTOR NEUROPATHY

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Abstract—Animal models of diabetes do not reach the severity of human diabetic neuropathy but relatively mild neurophysiological deficits and minor morphometric changes. The lack of degenerative neuropathy in diabetic rodent models seems to be a consequence of the shorter length of the axons or the shorter animal life span. Diabetes-induced demyelination needs many weeks or even months before it can be evident by morphometrical analysis. In mice myelination of the peripheral nervous system starts at the prenatal period and it is complete several days after birth. Here we induced experimental diabetes to neonatal mice and we evaluated its effect on the peripheral nerve 4 and 8 weeks after diabetes induction. Neurophysiological values showed a decline in sensory nerve conduction velocity at both time-points. Morphometrical analysis of the tibial nerve demonstrated a decrease in the number of myelinated fibers, fiber size and myelin thickness at both time-points studied. Moreover, aldose reductase and poly(ADP-ribose) polymerase activities were increased even if the amount of the enzyme was not affected. Thus, type 1 diabetes in newborn mice induces early peripheral neuropathy and may be a good model to assay pharmacological or gene therapy strategies to treat diabetic neuropathy. **© 2014 IBRO. Published by Elsevier Ltd. All rights reserved.**

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Abbreviations: AF, amyelinated fibers; AR, aldose reductase; CMAP, compound muscle action potential; DPN, diabetic peripheral polyneuropathy; EDTA, ethylenediaminetetraacetic acid; MF, myelinated fibers; NADPH, nicotinamide adenine dinucleotide phosphate; NCV, nerve conduction velocity; PAR, poly(ADP-ribosyl)ated proteins; PARP, poly-ADP-ribose polymerase; SC, Schwann cells; SNAP, compound sensory nerve action potentials; STZ, streptozotocin.

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Key words: diabetic neuropathy, neonatal mice, myelination, aldose reductase activity, PARP activity.

INTRODUCTION

Peripheral polyneuropathy (DPN) is one of the most common long-term secondary complications of diabetes mellitus and affects both somatic sensory and autonomic nerves caused by hyperglycemia/hypoinsulinemia. Between the characteristic pathological features of human DPN are axonal degeneration, nerve fiber loss, segmental demyelination and problems in remyelination. The prevalence in humans is about 60% of the patients but electrophysiological alterations can be demonstrated in almost 100% of diabetic patients even if the pathology is subclinical (Aguilar-Rebolledo, 2005).

The molecular mechanisms involved in the pathogenesis of diabetic complications are multiple. Among them, acceleration of the polyol pathway due to the high levels of glucose was one of the first factors suggested. This pathway is composed of two enzymes, aldose reductase (AR) that is highly expressed in Schwann cells (SC) and converts glucose to sorbitol using NADPH as a cofactor, and sorbitol dehydrogenase that converts sorbitol to fructose using NAD^+. High levels of sorbitol induce damage in nervous tissue and, if NAPDH is not available in SC, the amount of reduced glutathione decreases and there is an increased susceptibility to intracellular oxidative stress (Suzuki et al., 1999; Oka and Kato, 2001; Brownlee, 2005). Slowed conduction of action potentials is triggered by both, polyol accumulation which decreases Na^+, K^+-ATPase activity and leads to intra-axonal sodium accumulation (Mandersloot et al., 1978), and by paranodal and segmental demyelination (Sima and Sugimoto, 1999). On the other hand, under hyperglycemic conditions there is an increase of cell metabolism and of reactive oxygen species production that may alter the mitochondrial membrane potential. Under these conditions the enzyme poly-ADP-ribose polymerase (PARP) is activated, a fundamental mechanism in the development of diabetic complications, including endothelial dysfunction, cardiomyopathy, retinopathy, nephropathy and neuropathy (Garcia Soriano et al., 2001; Pacher et al., 2002; Obrosova et al., 2005a; Drel et al., 2006).

Understanding the biochemical mechanisms underlying DPN requires the use of appropriate animal models that reproduce the main features of the human disease (Sullivan et al., 2008). Among them,

streptozotocin (STZ) administration to induce type 1 diabetes is one of the most used models for diabetic neuropathy (Kimura et al., 2005; Hong and Kang, 2008; Jang et al., 2010; Sanchez-Zamora et al., 2010). STZ is a drug with diabetogenic properties due to its specific toxicity to pancreatic beta cells. Two procedures have been described to induce diabetes with this antibiotic, using either a single high dose or multiple low doses of STZ. The latter treatment induces subtoxic effects on beta cells and resembles type 1 diabetes due to slow progressive hyperglycemia and lymphocytic infiltration in Langerhans islets. Animals with STZ-induced diabetes present thermal hyperalgesia, reduced intraepidermal innervations and hypoalgesia at the long-term as neuropathic signs (Cameron et al., 2001; Calcutt, 2004; Li et al., 2005). They also show decreased nerve conduction velocity (NCV) (Calcutt, 2004; Obrosova et al., 2005a; Obrosova, 2009). Among the genetic animal models that have been characterized as appropriate models for diabetic neuropathy, the B6Ins2Akita mice and the BB/Wor (bio-breeding Worcester) mice for diabetes type 1 present deficits in conduction velocity (Choeiri et al., 2002; Stevens et al., 2004; Kamiya et al., 2005; Sullivan et al., 2007), and the most used db/db and ob/ob mice show decreased thermal sensibility and conduction velocity as models for type 2 diabetes (Sullivan et al., 2007; Vareniuk et al., 2007; Vincent et al., 2007).

Unfortunately, animal models of diabetes do not commonly reach the severity of human DPN. Animal nerves usually show relatively mild neurophysiological deficits and minor morphological changes, thus limiting the relevance of experimental studies (Sharma and Thomas, 1987; Wright and Nukada, 1994). The lack of degenerative neuropathy in diabetic rodent models seems to be a consequence of the short life span of rodents or the physically shorter axons. Degenerative neuropathy is minimal even in larger animals like dogs or primates with the exception of cats (Mizisin et al., 2007). Demyelination-induced by diabetic neuropathy is hardly detected in adult animal models of diabetes. As an alternative, combination of diabetes and injury has been used to exacerbate the sensorymotor neuropathy since diabetic patients and animal models show impaired nerve regeneration (Kennedy and Zochodne, 2000; Homs et al., 2011). However, in this case it is difficult to distinguish the contribution of diabetic neuropathy from the influence due to nerve injury, and therapeutic strategies that may work in DPN may not necessarily be effective in nerve regeneration and vice versa.

Type 1 diabetes in humans can develop as early as in infanthood and often affects young children and adolescents. Here, by inducing experimental diabetes to neonatal mice, we aimed to produce hyperglycemia during the period when the PNS is most actively myelinating and to study whether experimental diabetes in newborn mice was able to cause severe DPN. We analyzed how diabetes induced during the first week of age affects the peripheral nerve by biochemical, electrophysiological and morphological studies at 4 and 8 weeks after diabetes induction.

EXPERIMENTAL PROCEDURES

Animals

Outbread Swiss mice Hsd:ICR(CD-1) were used. Male newborn mice received 3 intraperitoneal injections of 40-mg/kg of STZ (dissolved in 0.1 mol/l citrate buffer, pH 4.5, immediately before administration) at post-natal day 3 (P3), P4 and P8. Control animals were injected with citrate buffer at the same ages. Body weight and blood glucose were measured weekly after weaning with a Glucometer Elite (Bayer, Leverkusen, Germany). Breading and weaned mice were fed *ad libitum* with a standard diet (2018S Teklad Global, Harlan Laboratories; 17% calories from fat) and kept under a light–dark cycle of 12 h (lights on at 8:00 am). Mice were euthanized at 4 and 8 weeks after the STZ administration. Animal care and experimental procedures were approved by the Biosafety and the Animal and Human Experimentation Ethics Committees of the Universitat Autònoma de Barcelona.

AR activity in sciatic nerve protein extracts

AR activity was spectrophotometrically measured following the decrease of NADPH during the reduction of glyceraldehydes (both from Sigma, St Louis, MO, USA) as published (Sato, 1992). Briefly, the reaction was started by adding 100 mM NADPH to 100 μg of sciatic nerve protein extract in 100 mM sodium phosphate buffer containing 1 mM D-L-glyceraldehyde and 400 mM lithium sulfate. Absorbance of NADPH at 340 nm was measured at 37 °C before and 5, 10 and 15 min after addition of NADPH. Results are expressed as nanomoles of $NADP^+$ generated per minute per milligram of protein.

Western blot analysis

Sciatic nerves were dissected from non-diabetic and 4- or 8-week diabetic ICR. All the animals were above 250 mg/dl of blood glucose level. Samples were sonicated and homogenized in lysis buffer (50 mM Tris–Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodiumdeoxycholate, 50 mM sodium floride, 1 mM sodium orthovanadate, 10 mM sodium glycerophosphate, 5 mM pirophosphate and protease inhibitor cocktail (Roche Diagnostics, Basel, Germany)). Protein concentration was determined by binchoninic acid (BCA) Protein Assay (Pierce, Rockford, IL, USA), and 15 μg was separated on 10% acrylamide gels. Proteins were transferred in polyvinylidene fluoride (PVDF) membranes and incubated with anti-AR (1:500, kindly provided by T.G.Flynn, Queen's University, Ontario, Canada), anti-poly-ADP-ribose modified proteins (1:1,000; Biomol International, Plymouth Meeting, PA, USA), anti-tubulin (1:1,000, Sigma, St Louis, MO). Anti-rabbit (DakoCytomation, Glostrup, Denmark) or anti-mouse (GE Healthcare) conjugated to horseradich peroxidase (HRP) was used as secondary antibody (1:10,000). Band intensities were quantified by GeneSnap software for Gene Genius Bio Imaging

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