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ScienceDirect

Experimental Neurology 201 (2006) 399–406

Experimental Neurology

www.elsevier.com/locate/yexnr

Reduced expression of endothelin B receptors and mechanical hyperalgesia in experimental chronic diabetes

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Received 23 March 2006; accepted 17 April 2006
Available online 27 June 2006

Abstract

Diabetic neuropathy is one of the major complications of diabetes mellitus. Small nerve fibers degenerate early in the disease, leading to symptoms ranging from hyperalgesia to loss of pain and temperature sensation. However, the cellular and molecular mechanisms responsible for abnormal pain perception in diabetes have not been identified. Both type-A and type-B endothelin receptors (ETAR and ETBR, respectively) are present in sensory nerves and appear to regulate neuropathic and inflammatory pain. In this study, we compared the expression of endothelin receptors and nociceptive responses in normal and experimentally diabetic rats. Diabetic animals exhibited both an increase in the withdrawal responses to high threshold stimuli (mechanical hyperalgesia) and to light touch stimuli (tactile allodynia). Immunohistochemical and Western blot analysis revealed that diabetic rats have significantly reduced expression of ETBR in sciatic nerves, while no changes were observed in dorsal root ganglia (DRG). In contrast, the expression of ETAR in either sciatic nerves or DRG of diabetic rats was not altered. Importantly, ETBR-deficient transgenic rats showed alterations in pain perception similar to those observed in diabetic rats. These results suggest that changes in the expression of ETBR in peripheral nerve may contribute to the development of mechanical hyperalgesia and tactile allodynia in chronic diabetes.

Keywords: Diabetes; Neuropathic pain; Peripheral nerve; Endothelin B receptor; Tactile allodynia; Mechanical hyperalgesia

Introduction

Diabetic patients often develop sensory neuropathy. Distal symmetric sensory neuropathy, the predominant form, can give rise to either a painless syndrome, with loss of sensation to touch, pain or temperature, or to a painful disorder, depending on the type of nerve fibers affected. The painful disorder is characterized by hyperalgesia and/or allodynia, i.e., pain perception in response to non-painful stimuli. Studies in experimental animal models of diabetes have also identified abnormal nociceptive responses, including early thermal hyperalgesia that, with time, turns into hypoalgesia, mechanical hyperalgesia, thermal and tactile allodynia, increased C fiber activity and reduced sensitivity to opioids (Calcutt, 2002). Although the cellular and molecular mechanisms responsible for increased pain perception in diabetes are unclear, alterations in endothelin signaling may play a key role in this pathology.

Endothelins (ETs) comprise a family of peptides, ET-1, ET-2 and ET-3, that regulate blood flow, cell proliferation, secretion, ion transport (Rubanyi and Polokoff, 1994) and pain perception. Three ET receptor subtypes, ETAR, ETBR and ETCR, have been cloned. ET receptors are expressed in both CNS and PNS in neurons and glial cells including astrocytes, microglia and non-myelinated Schwann cells (Van den Buuse and Webber, 2000; Pomonis et al., 2001). Recent evidence suggests that ET receptors transduce nociceptive information. Activation of ETAR in afferent primary fibers initiates nociceptive responses

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doi:10.1016/j.expneurol.2006.04.034

(Gokin et al., 2001), possibly through increases in intracellular calcium and activation of tetrodotoxin-resistant sodium channels (Zhou et al., 2001, 2002).

Increased concentration and activity of ET-1 has been reported both in diabetic patients and in experimental diabetes (Hopfner and Gopalakrishman, 1999; Khan and Chakrabarti, 2003). Exposed sciatic nerve vasa nervorum from diabetic rats display an enhanced and prolonged vasoconstriction in response to ET-1, followed by increased fiber loss, multifocal axonal degeneration and loss of motor nerve excitability (Zochodne and Cheng, 1999). Interestingly, administration of ETAR antagonists attenuates tactile allodynia in streptozotocin-induced diabetic rats (Jarvis et al., 2000). While these results suggest that ETAR may contribute to the development of peripheral nerve abnormalities in experimental diabetes, the possible role of ETBR has not been addressed.

We have recently reported that ETBR regulate the proliferation of Schwann cells, and that this regulation is altered in nerve explants from diabetic rats and Schwann cells cultured in high glucose-containing media (Berti-Mattera et al., 2001; Almhanna et al., 2002). These results suggest that ETBR may regulate nerve regeneration, consistent with previous observations supporting a protective role for ETBR following neuronal injury (Siren et al., 2002; Yagami et al., 2002).

In this study we examined the distribution and expression of ETAR and ETBR in sciatic nerves and dorsal root ganglia (DRG) from experimentally diabetic rats displaying enhanced nociception. Further, we evaluated nociceptive responses in animals that genetically lack ETBR. Our observations suggest that changes in ETBR levels may contribute to diabetic neuropathic pain.

Materials and methods

Experimental animals and induction of diabetes

Lewis rats (male) weighing ~200 g were obtained from Charles River (Wilmington, MA). Diabetes was induced in animals fasted for 24 h by a single intraperitoneal injection of streptozotocin (Sigma; 60 mg/kg body weight). After 48 h, animal weight and serum glucose concentration were monitored weekly and also determined at the time of death. Animals exhibiting clear signs of poor health, as evidenced by significant weight loss (more than 30–35% of starting weight), were not included in the study. Expression of ET receptors was analyzed 9 months after induction of experimental diabetes (chronic stage of this disease). Diabetic animals displayed decreased body weight (diabetic 304 ± 48 g; normal 609 ± 58 g), increased blood glucose (diabetic 325 ± 30 mg/dl; normal 67 ± 1 mg/dl) and glycosylated hemoglobin levels (diabetic: 9 ± 1.1% and normal: 4.0 ± 0.2%) (mean ± SEM; $n = 6–8$). After euthanasia, sciatic nerves and DRG from diabetic and age-matched normal rats were dissected, and quickly frozen for subsequent immunoblotting and immunohistochemical studies. Studies were also performed in transgenic rats that are deficient in ETBR. In contrast to homozygous ETBR-deficient rats (ETB*sl/sl*) which die shortly after birth due to congenital aganglionosis, the DBH-ETB;ETB$^{sl/sl}$ transgenic animals expressing the ETBR under the control of the human dopamine-β-hydroxylase (DBH) promoter live into adulthood (Gariepy et al., 1998). Adult DBH-ETB;ETB$^{sl/sl}$ rats express ETBR in adrenergic tissues such as adrenal medulla and sympathetic ganglia, but lack expression of this receptor driven by the endogenous promoter in the eye, lung and kidney (Gariepy et al., 1998; Ivy et al., 2002). The DBH-ETB;ETB$^{sl/sl}$ rats are obtained by crossing heterozygous ETB*sl*/+ with DBH-ETB;ETB$^{+/+}$ rats (transgenic rats harboring a wild-type ETB cDNA under the control of the human DBH promoter). In our studies we used 5-month-old DBH-ETB;ETB$^{sl/sl}$ rats (as the ETBR-deficient animal model) and 5-month-old DBH-ETB;ETB$^{+/+}$ rats (as their normal counterparts). In some experiments, 4- to 5-month-old DBH-ETB;ETB$^{sl/sl}$ and DBH-ETB;ETB$^{+/+}$ were rendered diabetic by streptozotocin injection as described above. Experimental protocols were reviewed and carried out according to the guidelines of the Institutional Animal Care and Use Committee at Case Western Reserve University.

Immunohistochemical and immunofluorescence microscopy in DRG and sciatic nerve sections

Ten-micron sections of pooled L4 and L5 DRG or sciatic nerve (at least three sections separated by 300 μm were taken from each of 3–4 animals per experimental group) were collected on gelatin-coated glass slides, and stored at −80°C until use. Tissue sections were fixed in 4% paraformaldehyde/0.1 M sodium phosphate for 30 min at room temperature. The sections were rinsed in PBS and permeabilized in 0.1% Triton X-100/0.4% Tween/0.5% BSA/PBS at room temperature, and incubated overnight at 4°C with rabbit polyclonal antisera against rat ETAR or ETBR (10 μg IgG/ml; Alomone labs, Israel) in the same buffer. Some sections were incubated overnight at 4°C with rabbit anti-GFAP (Accurate) in dilution buffer consisting of 20% goat serum, 0.1% Triton X-100/PBS. After rinsing in PBS, tissues were incubated in 1:300 biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch) in the same dilution buffer for 1 h at room temperature. After washing, sections were incubated in 1:750 streptavidin Cy2 (green) or Cy3 (red) (Jackson ImmunoResearch) in PBS, for 1 h at room temperature. In some cases, antibody binding was detected with a visible DAB substrate (Vectastain). Negative controls were obtained by incubating samples in the absence of primary antisera. Sections were mounted in 2% *n*-propyl gallate in 1:1 glycerol:PBS to reduce fading and images were collected on a Leica DMR fluorescence microscope and captured on a Hamamatsu C4742–95 digital camera.

Immunoblotting analysis

DRG (pooled L4 and L5) and sciatic nerves were homogenized on ice in 9 volumes of lysis buffer containing 20 mM Tris/HCl pH 8.0, 0.1 M NaCl, 0.5 mM EDTA, 10% glycerol, 1% NP-40 and 10% protease inhibitor cocktail (Sigma). Insoluble material was removed by centrifugation (15,000 × *g* for 5 min at 4°C), and the supernatants were