ABSTRACT

A clearer understanding of the mechanisms underlying the development and progression of diabetic neuropathy is likely to indicate new directions for the treatment of this complication of diabetes. In the present study we investigated the expression of cannabinoid CB1 receptors in models of diabetic neuropathy. PC12 cells were differentiated into a neuronal phenotype with nerve growth factor (NGF) (50 ng/ml) in varying concentrations of glucose (5.5–50 mM). CB1 receptor expression was studied at the mRNA level by reverse transcriptase-polymerase chain reaction (RT-PCR) and at the protein level via immunohistochemical and Western blot analysis. CB1 expression was also compared in dorsal root ganglia (DRG) removed from streptozotocin-induced diabetic rats versus control animals. Total neurite length induced by NGF was reduced in cells cultured in 20 to 50 mM glucose at day 6 ($P < 0.01$ versus 5.5 mM; $n = 6$). Cell viability assays conducted in parallel on day 6 confirmed that the total cell numbers were not significantly different among the various glucose concentrations ($P = 0.86$; $n = 12$). RT-PCR, immunohistochemical, and Western blot analysis all revealed down-regulation of the CB1 receptor in cells treated with high glucose ($P < 0.05$; $n = 4–5$ for each), and in DRG removed from diabetic rats compared with controls ($P < 0.01$; $n = 5$ for immunohistochemistry, and $n = 3$ for Western blot). These results suggest that high glucose concentrations are associated with decreased expression of CB1 receptors in nerve cells. Given the neuroprotective effect of cannabinoids, a decline in CB1 receptor expression may contribute to the neurodegenerative process observed in diabetes.

The number of adults with diabetes worldwide is estimated to rise from 171 million in 2000 to 366 million in 2030 (Wild et al., 2004), and at least 50% of those will develop neuropathy. Distal symmetrical polyneuropathy, the most common form of diabetic neuropathy, is characterized by neurodegeneration of peripheral nerve endings, resulting in acute pain, sensorimotor deficits, and an increased risk of limb amputation (Vinik et al., 2006). A treatment that can delay the onset of neuropathy and slow its progression is urgently needed to improve the quality of life in patients with diabetes and reduce demands on health resources.

In diabetic patients small fiber (C and A$\delta$) neuropathy is responsible for the early hyperalgesia and allodynia and the late hypoalgesia, impairment of warm thermal perception, and skin blood flow. The majority of small diameter unmyelinated C-fibers and thinly myelinated A$\delta$-fibers are nociceptive and constitutively express the vanilloid receptor (TRPV1), a nonselective cation channel sensitive to the pungent vanilloid capsaicin (Caterina et al., 1997). TRPV1 receptors are activated endogenously by noxious heat ($>42^\circ$C), acidic pH ($<6.0$), and the endocannabinoid, anandamide (Zygmunt et al., 1999; Smith and McQueen, 2001), causing central and peripheral release of substance P and calcitonin gene-related peptide (CGRP). Capsaicin-evoked CGRP release from sensory neurons is inhibited by anandamide (Richardson et al., 1998). This effect is mediated by cannabinoid CB1 receptors, which are coexpressed with TRPV1 in nociceptive afferents (Ahuwalia et al., 2000). Activation of CB1 receptors suppresses neuropeptide release via inhibition of Ca$^{2+}$ channels and activation of K$^+$ conductance (Ahuwalia et al., 2003a). Thus, anandamide can either inhibit or stimulate sensory neurotransmission, via the CB1 or TRPV1 receptor, respectively.

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ABBREVIATIONS: TRPV1, transient receptor potential vanilloid 1; CGRP, calcitonin gene-related peptide; CB, cannabinoid; PKC, protein kinase C; PC12, pheochromocytoma cell line; NGF, nerve growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, reverse transcriptase; PCR, polymerase chain reaction; BSA, bovine serum albumin; STZ, streptozotocin; FAAH, fatty acid amide hydrolase; SR141716, rimonabant.
There is some evidence to suggest that the balance of CB1- versus TRPV1-mediated responses is tipped unfavorably toward TRPV1 in diabetes. Kamei et al. (2001) have reported that the thermal hyperalgesia and allodynia observed in diabetic mice is due to sensitization of TRPV1 receptors. This sensitization can occur via protein kinase C (PKC)-mediated phosphorylation of TRPV1, which lowers the activation threshold for proton and heat-induced currents (Premkumar and Ahern, 2000; Numazaki et al., 2002). Up-regulation of the TRPV1 receptor protein also occurs in response to hyperglycemia (Rashid et al., 2003; Hong and Wiley, 2005).

We hypothesize that a decline in CB1 receptor expression contributes to the neurodegenerative process observed in diabetic neuropathy. In the present study, using in vitro and in vivo models of diabetic neuropathy, we investigated whether neuronal CB1 receptor expression is down-regulated in the presence of high glucose.

Materials and Methods

Cell Culture Model of Hyperglycemia

Rat adrenal pheochromocytoma PC12 cells (ECACC) were grown as nonadherent cells in RPMI 1640 medium (Sigma Chemical, Poole, Dorset, UK) supplemented with 10% (v/v) fetal calf serum (heat inactivated), penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively; Invitrogen, Carlsbad, CA) and 1-glutamine (2 mM; Invitrogen). They were maintained at 37°C in a humidified atmosphere of 5% CO2 and split every 3 to 4 days. For neuronal differentiation, PC12 cells were plated in 6- or 24-well dishes coated with poly-L-lysine (0.02%; Sigma Chemical) at a density of 1 × 10^5 cells/ml, with the addition of nerve growth factor (NGF) (50 ng/ml; Sigma Chemical) and varying concentrations of glucose as an in vitro model of diabetic neuropathy after establishment by Lelkes et al. (2001). Dulbecco’s modified Eagle’s medium (low glucose, 5.5 mM; Sigma-Aldrich)-NGF medium was supplemented with sterile β-glucose (Sigma Chemical) solutions to achieve final glucose concentrations of 5.5 to 50 mM, simulating hyperglycemic conditions. To observe potential effects of simply increasing osmolality of the culture medium, β-mannitol (30 and 50 mM) was added to some of the wells containing 5.5 mM glucose. Osmolality of the culture media was measured in an osmometer (model 3D3; Advanced Instruments Inc., Norwood, MA). Medium was replaced every 2 days, and cultures were maintained for 6 days. Aspirated medium was collected and analyzed for glucose concentrations using a Glucose (GO) Assay Kit (Sigma Chemical), whereby the glucose present was oxidized to gluconic acid and hydrogen peroxide using glucose oxidase enzyme (12.5 units). In the presence of peroxidase (2.5 units), hydrogen peroxide reacted with o-dianisidine (125 µM) to form a colored product that was made more stable by addition of sulfuric acid (12%). The color intensity of each sample was measured in a Spectronic BioMate-3 spectrophotometer at 540 nm and compared with that of a glucose intensity of each sample was measured in a Spectronic BioMate-3 spectrophotometer at 540 nm and compared with that of a glucose standard.

Morphometric Assessment of PC12 Cell Differentiation

Neurite length was determined from photomicrographs taken on day 5 from three random fields/well, using MetaMorph software (Molecular Devices, Sunnyvale, CA). The length of individual neurites was added together for each cell, and total neurite length was expressed as a percentage of cell body diameter.

Cell Viability (MTT) Assay

Viable cells were measured using a colorimetric assay based on the ability of live cells to reduce a tetrazolium-based compound to a blue formazan product. Cells were incubated with MTT (0.5 mg/ml; Sigma Chemical) for 4.5 h at 37°C, after which the wells were aspirated and the formazan crystals were dissolved using dimethyl sulfoxide at 37°C for 5 min. After transfer to a 96-well plate, samples were read in a Dynex spectrophotometer at 550 nm.

Measurement of CB1 Receptor mRNA Expression

RNA was extracted from PC12 cells using TRI Reagent (Sigma Chemical). cDNA synthesis was carried out at 48°C for 45 min by incubating total RNA (2 µg/sample) with 1 U of avian myeloblastosis virus reverse transcriptase, 0.4 mM concentrations of dNTPs, 2 µM concentrations of random nonamer oligodeoxynucleotides, and avian myeloblastosis virus buffer (final concentration, 50 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 10 mM MgCl2, 0.5 mM spermidine, 10 mM dithiothreitol) (all from Promega, Madison, WI) in a final volume of 25 µl. The reverse transcriptase was inactivated by heating at 95°C for 2 min before proceeding to the PCR step and 5 µl of the cDNA from the RT reaction was used for PCR amplification using a Hybrid PCR Express thermal cycler (Thermo Electron Corporation, Waltham, MA). Each cDNA was added to a PuReTaq Ready-To-Go PCR Bead containing –2.5 U of PuReTaq DNA polymerase, 200 µM concentrations of 4NTPs, and reaction buffer (10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM MgCl2, stabilizers, and BSA), together with 125 pmol of specific primers for CB1 (sense: 5’-ATGAACTGATCCTAGATGGCCTTGGA-3’; antisense: 5’-GGTTCCTCCCCACACTGGATTGTG-3’; MGW-Biotech AG, Germany) (Zhuang et al., 1998) or rat β-actin (R&D Systems Europe Ltd., Abingdon, Oxfordshire, UK), to a final volume of 25 µl. The amplification profile for CB1 and β-actin was as follows: 95°C for 30 s, 62°C for 1 min, and 68°C for 2 min for 30 cycles, followed by strand extension at 68°C for 7 min. The reaction products were then loaded on a 1.5% agarose gel containing 1 µg/ml ethidium bromide, and electrophoresed for 60 min at 100 V, before visualizing under UV light and quantifying using GeneTools (Syngene, Cambridge, UK).

Experimental Animals

Male Sprague-Dawley rats were housed in the animal facility of the University of Michigan Unit for Laboratory Animal Medicine, which was maintained at 22°C and 55% relative humidity, with an automatic 12-h light/dark cycle. The animals received a standard laboratory diet and tap water ad libitum. All experiments were approved by the University of Michigan Committee on Use and Care of Animals according to the National Institutes of Health guidelines. Diabetes mellitus was induced by a single i.p. injection of streptozotocin (STZ) (45 mg/kg) to rats weighing 180 to 200 g that had been fasted overnight to maximize the effectiveness of STZ treatment. STZ solution was prepared fresh by dissolving it in 0.1M citrate buffer, pH 5.5. Age-matched control rats were injected with citrate buffer alone. The diabetic condition was assessed by glucose levels greater than 300 mg/dl (16.7 mM). Rats meeting this criterion were used experimentally 4 to 8 weeks after STZ induction.

Immunohistochemical and Immunofluorescence Analyses

PC12 Cells. Cells cultured on coverslips were washed briefly with PBS solution (PBS × 1 with 0.3% BSA and 10 mM EDTA) and then fixed with 4% paraformaldehyde for 30 min. The cells were incubated with 10% normal blocking serum (donkey) for 20 min to suppress nonspecific binding of IgG. After a 5-min PBS wash, the specimens were incubated for 2 h with 0.3 µg/ml primary antibody and rabbit anti-rat TRPV1 receptor or goat anti-rat CB1 receptor (both from Santa Cruz Biotechnology Inc., Santa Cruz, CA), followed by washing with three changes of PBS for 10 min each. Cells were then incubated with 0.3 µg/ml fluorochrome-conjugated secondary antibody and fluorescein isothiocyanate-conjugated donkey anti-goat IgG (1:80), or rhodamine-conjugated donkey anti-rabbit IgG (1:80) (both from Santa-Cruz Biotechnology Ltd.) and then washed with three changes of PBS for 10 min each. Coverslips were mounted with Mowiol (Sigma Chemical) on glass slides.
Rat DRG. Fixed paraffin-embedded serial sections (4–6 μm) of DRG from control and diabetic rats were deparaffinized and incubated for 2 h in 0.3% Triton X-100-PBS-0.1% Tween to permeabilize cellular membranes. Sections were incubated for 4 h at room temperature with 10% normal blocking serum (donkey) in PBS-0.1% Tween. After washing with PBS, sections were incubated with primary antibody overnight at 4°C. Primary antibodies used were rabbit anti-rat CB1 receptor (1:300; Sigma Chemical) and mouse anti-rat peripherin (for C- and Aδ-fibers, 1:500; Sigma Chemical). After washing in one change of PBS-0.1% Tween and two changes of PBS for 5 min each, the sections were exposed to rhodamine-conjugated donkey anti-rabbit IgG (1:80) and Alexa Fluor 633-conjugated goat anti-mouse (1:400) (Invitrogen, Eugene, OR), for 120 min in the dark at room temperature. Again, sections were washed in one change of PBS-0.1% Tween and two changes of PBS for 5 min each before applying Mowiol and coverslips. All slides were examined by confocal laser scanning microscopy (Zeiss LSM-510 system).

Western Blotting

PC12 Cells. For immunoprecipitation, cultured PC12 cells were lysed in ice-cold Nonidot P-40 buffer solution containing a cocktail of protein phosphatase and protease inhibitors [20 mM Tris HCl (pH 8), 137 mM NaCl, 10% glycerol, 1% Nonidot P-40, 2 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml antipain, and 1 μg/ml pepstatin A] to avoid dephosphorylation and degradation of proteins and transferred to clean Eppendorf tubes. Samples were agitation and spun down at 12,000g at 4°C for 20 min. The supernatant was then assayed for total protein concentration using the Bradford Protein Assay Kit (Bio-Rad, Herford Hampstead, UK) with bovine serum albumin as a standard. Samples of equal protein quantity with equal concentration were submitted to precipitation with anti-CB1 antibody (Sigma Chemical) followed by incubation with protein A Sepharose (Sigma Chemical). For Western immunoblotting, the precipitates were dissolved in SDS-mercaptoethanol sample buffer and separated in 10% SDS-polyacrylamide gel and blotting, the precipitates were dissolved in SDS-mercaptoethanol sample buffer and separated in 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The nitrocellulose membrane was blocked with 5% nonfat dry milk for 4 h and then incubated with anti-CB1 antibody (Cayman Chemical, Ann Arbor, MI) overnight. The membrane was then probed with horseradish peroxidase-conjugated secondary antibody for 1 h and developed using the West Dura Supersignal chemiluminescence kit (Pierce Chemical, Rockford, IL). The corresponding bands were scanned and quantified with ImageJ software (National Institutes of Health, Bethesda, MD), and normalized to β-actin band intensity.

Data Analysis

Data are expressed as means ± S.E.M. Statistical analysis was performed using an unpaired Student’s t test for comparison of two groups or a one-way analysis of variance followed by Fisher’s post hoc subgroup testing for multiple treatment data. The null hypothesis was rejected at P < 0.05.

Results

Effects of Glucose on Neurite Outgrowth. We found a reduction in total neurite length induced by nerve growth factor in PC12 cells cultured in 20 to 50 mM glucose on day 6 (P < 0.01 versus 5.5 mM; n = 70–79 from six independent cultures) (Fig. 1, A and B). This effect was due to raised glucose levels, used to mimic diabetic conditions, rather than to any hyperosmolality effects as mannitol (30 and 50 mM) gave results similar to that of the physiological 5.5 mM glucose control (P = 0.79); no significant differences were observed in osmolality values between glucose and the mannitol osmotic controls (30 mM: 320 ± 1 and 320 ± 1 Osm/kg H2O, P = 1.00; 50 mM: 342 ± 1 and 341 ± 0 Osm/kg H2O; P = 0.37; unpaired Student’s t test). Figure 1C shows that the cell culture model is one of approximately maintained elevated glucose concentrations. The average fall in glucose levels from aspirated medium is 3.1 ± 0.8 mM across the concentration range after 2 days in cell culture, but there is not a severe peak-to-trough variation in glucose. There were no significant effects of glucose or mannitol on cell viability (P = 0.86, n = 12) (data not shown).

Effects of Glucose on CB1 Receptor Expression. An inverse relationship between CB1 expression and glucose concentration was observed when we examined RNA and protein levels of the receptor. Figure 2 shows that there was a reduction in CB1 mRNA expression in PC12 cells cultured in 50 mM glucose (P < 0.05 versus 5.5 mM band intensity; n = 4 independent cultures). The soma and neurite outgrowths of PC12 cells express CB1 receptors, with immunofluorescence associated predominantly on the neurite tips and cell body membrane (Fig. 3, B and C). Immunohistochemical analysis revealed down-regulation of CB1 receptor protein in cells treated with high glucose (30 and 50 mM) (Fig. 3, D–F). This finding was further confirmed via Western blot analysis of CB1 receptor protein (Fig. 3G), whereby the Western blot analysis of plasma membrane protein in cells treated with high glucose (30 and 50 mM) gave results similar to that of the physiological 5.5 mM glucose control (P = 0.79); no significant differences were observed in osmolality values between glucose and the mannitol osmotic controls (30 mM: 320 ± 1 and 320 ± 1 Osm/kg H2O, P = 1.00; 50 mM: 342 ± 1 and 341 ± 0 Osm/kg H2O; P = 0.37; unpaired Student’s t test). Figure 1C shows that the cell culture model is one of approximately maintained elevated glucose concentrations. The average fall in glucose levels from aspirated medium is 3.1 ± 0.8 mM across the concentration range after 2 days in cell culture, but there is not a severe peak-to-trough variation in glucose. There were no significant effects of glucose or mannitol on cell viability (P = 0.86, n = 12) (data not shown).

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In the present study, we provide evidence of altered expression of cannabinoid CB1 receptors in experimental models of diabetic neuropathy. Increasing concentrations of glucose were found to decrease expression of CB1 receptors at the RNA and protein levels in PC12 cells, which have been previously used as a cell culture model of diabetic neuropathy (Lelkes et al., 2001). These results were corroborated in DRG from diabetic rats, in which the number of CB1-positive neurons was decreased to approximately half that of control animals, and the density of CB1 receptors was reduced by 60% in diabetic versus control DRG. Previous studies reported that 25 to 57% of DRG neurons are CB1-positive in rats (Ahluwalia et al., 2000; Bridges et al., 2003). The decrease in number of CB1-positive neurons might reflect a cessation of CB1 synthesis in neurons that normally express CB1 (a phenotypic switch) or simply the fact that the level of expression was undetectable because of reduced receptor density. CB1 receptors mediate, in part, the neuroprotectant properties of cannabinoids (reviewed by van der Stelt and Di Marzo, 2005), and any decrease in expression is expected to contribute to the development and progression of the neurodegeneration associated with diabetes.

There is considerable evidence supporting a neuroprotective role of cannabinoids. Much of the research thus far has focused on the protective role of cannabinoids in the central nervous system in response to chronic neurodegenerative diseases, including Alzheimer's disease and multiple sclerosis, or injury associated with stroke or brain trauma (reviewed by Bahr et al., 2006). The exact mechanisms involved in their neuroprotectant effects remain unclear but involve both CB1 receptor-independent (antioxidant) and -dependent mechanisms (inhibition of Ca2+ influx, reduced glutamate release and excitotoxicity, vasodilatation, increased NGF production and neurotrophic support, and hypothermia). The loss of CB1 receptors has been demonstrated in a number of neurodegenerative diseases, including Huntington's disease (Denovan-Wright and Robertson 2000; Glass et al., 2004) and
Parkinson’s disease (Silverdale et al., 2001). Moreover, De-
ovoan-Wright and Robertson (2000) found that the decrease
in CB1 mRNA occurred before the onset of the motor-related
Huntington’s disease-like symptoms in mice and preceded
neural degeneration, suggesting that abnormalities in can-
nabinoid signaling play a significant pathogenetic role. We
hypothesized that a decline in CB1 receptor expression con-
tributes to the neurodegenerative process observed in dia-
betic neuropathy. In the present study, CB1 receptor protein
appears to be down-regulated in nerve cells grown in condi-
tions mimicking hyperglycemia and in neurons from diabetic
rats. A study recently published by Duarte et al. (2007)
revealed decreased CB1 mRNA expression in the hippocam-
pus of diabetic rats, concomitant with an increase in CB1
protein density. Although the mRNA data are in agreement
with our findings, differences in neuronal CB1 protein ex-
pression between hippocampus (increased in Duarte et al.,
2007) and DRG (decreased in the present study) from STZ-
induced diabetic rats may reflect site-specific signaling path-
ways involved in mRNA translation, e.g., activation of the
key rate-limiting translational pathway mammalian target
of rapamycin in hippocampal neurons (Duarte et al., 2007).
Clearly, the effects of altered receptor expression on CB1
signaling remain to be established in functional studies.

A major consequence of a decline in CB1 expression is
likely to be increased TRPV1 receptor signaling. Up-regula-
tion of TRPV1 receptors on sensory nerves contributes to the
thermal hyperalgesia and allodynia observed in diabetic mice
(Kamei et al., 2001; Rashid et al., 2003). We previously con-
ducted behavioral measurement in the rat STZ model of
diabetes and observed hyperalgesia in these diabetic rats
compared with controls (Hong et al., 2004). In 2005, Hong
and Wiley published data showing enhanced function of
TRPV1 in diabetic rats, involving increased receptor phos-
phorylation via protein kinase C, oligomerization to active
form, and recruitment to cell surface plasma membrane.
PKC activation in diabetes could result from the enhanced
release of chemical mediators (e.g., bradykinin) under isch-
emic conditions, following hyperglycemia-induced oxidative
stress. Indeed, inhibitors of PKC have been shown to de-
crease the hyperalgesia and C-fiber hyperexcitability found
in diabetic rats (Ahlgren and Levine, 1994). Interestingly,
although PKC can sensitize TRPV1 receptors, it down-regu-
lates the activity of CB1 receptors (Garcia et al., 1998). El-
lington et al. (2002) examined anandamide-induced inhibi-
tion of capsaicin-evoked CGRP release in rat paw skin from
control and diabetic rats. They found that anandamide in-
hibited CGRP release only in skin from control animals, and
furthermore, actually stimulated CGRP release in skin from diabetic rats when tested at higher concentrations. These data suggest that anandamide action at TRPV1 receptors overcomes the inhibitory actions mediated by CB1 receptors in diabetics. Even if TRPV1 activation evokes synthesis and release of anandamide, as has been demonstrated in cultured rat neurons (Ahluwalia et al., 2003b), this will only serve to enhance TRPV1 signaling under conditions in which CB1 receptors are down-regulated. Thus, there seems to be differential regulation of CB1 versus TRPV1 expression and/or function in diabetes.

Higher circulating endocannabinoid levels (anandamide and 2-arachidonoylglycerol) have been demonstrated in obese patients with type 2 diabetes (Matias et al., 2006a), and the same authors reported up-regulation of endocannabinoids in postmortem eye tissues of patients with diabetic retinopathy (Matias et al., 2006b). In obese postmenopausal women with fasting hyperinsulinemia, elevated levels of anandamide and 2-arachidonoylglycerol were found to be associated with decreased CB1 receptor expression in adipose tissue (Engeli et al., 2005). The mechanistic link between glucose concentration and CB1 receptors is likely to be oxidative stress. Obesity is strongly correlated with increased oxidative stress, and, because high glucose enhances the production of cellular reactive oxygen species (Brownlee, 2001), the oxidative burden is even greater when obesity is coupled with insulin resistance (Van Guilder et al., 2006). Raised levels of endocannabinoids are likely to result from decreased enzymatic degradation, as Engeli et al. (2005) reported that fatty acid amide hydrolase (FAAH) gene expression was down-regulated in adipocytes from obese women. In turn, the raised ligand concentrations may down-regulate CB1 receptor expression via a negative feedback loop. As previously mentioned, a decline in CB1 receptors in the presence of elevated endocannabinoid levels might tip the balance toward TRPV1 activation. Furthermore, FAAH can attenuate TRPV1 activation (Milts et al., 2006), so that any reduction in FAAH levels would further enhance signaling via TRPV1 receptors.

There is currently no single treatment for neuropathy that is effective in all diabetic patients: a clearer understanding of the mechanisms underlying the development and progression of diabetic neuropathy are likely to indicate new directions for the treatment of this complication of diabetes. When interpreting the current data, one has to consider the limitations of using a cell culture model of diabetic neuropathy. Numerous in vitro models have previously been used to examine the cellular mechanisms involved in the pathophysiology of diabetic complications, including human SH-SY5Y neuroblastoma cells (Shindo et al., 1996) and rat DRG (Russell et al., 1999). In the current study, the neural crest-derived rat pheochromocytoma cell line PC12 was chosen as the in vitro model for diabetic neuropathy after establishment by Lelkes et al. (2001) and because these cells are known to express both CB1 receptors (Bisogno et al., 1998) and TRPV1 receptors (Someya et al., 2004). Elevated glucose levels significantly attenuate NGF-induced neurite outgrowth and are associated with increased levels of oxidative stress in PC12 cells (Lelkes et al., 2001), thus reproducing some of the phenomena of diabetic neuropathy. Although data from in vitro studies involving animal cells cannot be directly extrapolated to human disease, the fact that we also demonstrated reduced CB1 expression in DRG neurons of a rat model of diabetes ex vivo adds credence to our findings. Of course, due consideration must be given when we compare “in vitro high glucose” and “in vivo diabetic” states, as we had a 9-fold difference in glucose levels between cell treatment groups, which remained elevated during the culture, whereas the rat diabetic model showed a 5-fold elevation in plasma glucose compared with controls, and these levels would fluctuate throughout the animals’ lives. Others have reported plasma glucose levels in the STZ-induced diabetic rat to range from 25 to 50 mM (mean ± S.E.M. 34.5 ± 3.05 mM) (Purves et al., 2001), thus making the concentrations used in the present in vitro study reasonable. The pathophysiological relevance of our observations remains to be established in vivo, by treating, for example, diabetic rats with CB1 receptor agonists and measuring sensory nerve morphology and function.

A potential caveat in using CB1 receptor agonists to prevent neurodegeneration in diabetes comes from a recent report revealing that administration of anandamide causes glucose intolerance in rats (Bermúdez-Siva et al., 2006). This appears to result from a reduction of glucose-dependent insulin secretion from the pancreas, which has implications in...
type 2 diabetes. Conversely, one must also consider the potential adverse effects on the nervous system if the selective CB1 antagonist SR141716 (rimonabant) is prescribed for obese type 2 diabetic individuals. Recently, rimonabant has been studied in patients with type 2 diabetes. Although a reduction in body weight and improved metabolic risk factors were observed in the treatment group (Scheen et al., 2006), no data have yet been presented on nerve function in clinical trials of rimonabant.

In conclusion, we have demonstrated that high glucose concentrations are associated with decreased expression of CB1 receptors in nerve cells, which may contribute to the pathogenesis of diabetic neuropathy. Given the neuroprotective effect of cannabinoids, CB1 receptors may be an appropriate therapeutic target in preventing the neurodegenerative process in diabetes.

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