 doi: 10.1111/j.1471-4159.2008.05220.x

The TRPV1 receptor is associated with preferential stress in large dorsal root ganglion neurons in early diabetic sensory neuropathy

Shuangsong Hong, Laura Agresta, Chunfang Guo and John W. Wiley

Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan, USA

Abstract

Chronic diabetic neuropathy is associated with peripheral demyelination and degeneration of nerve fibers. The mechanism(s) underlying neuronal injury in diabetic sensory neuropathy remain poorly understood. Recently, we reported increased expression and function of transient receptor potential vanilloid 1 (TRPV1) in large dorsal root ganglion (DRG) neurons in diabetic sensory neuropathy. In this study, we examined the effects of TRPV1 activation on cell injury pathways in this subpopulation of neurons in the streptozotocin-induced diabetic rat model. Large DRG neurons from diabetic (6–8 weeks) rats displayed increased oxidative stress and activation of cell injury markers compared with healthy controls. Capsaicin (CAP) treatment induced decreased labeling of MitoTracker Red and increased cytosolic cytochrome *c* and activation of caspase 3 in large neurons isolated from diabetic rats. CAP treatment also induced oxidative stress in large diabetic DRG neurons, which was blocked by pre-treatment with caspase or calpain inhibitor. In addition, both μ-calpain expression and calpain activity were significantly increased in DRG neurons from diabetic rats after CAP treatment. Treatment with capsazepine, a competitive TRPV1 antagonist, markedly reduced these abnormalities *in vitro* and prevented activation of cell injury in large DRG neurons in diabetic rats *in vivo*. These results suggest that activation of the TRPV1 receptor activates pathways associated with caspase-dependent and calpain-dependent stress in large DRG neurons in STZ-diabetic rats. Activation of the TRPV1 receptor may contribute to preferential neuronal stress in large DRG neurons relatively early in diabetic sensory neuropathy.

Keywords: calpain, diabetic neuropathy, dorsal root ganglion, neuronal injury, rat, transient receptor potential vanilloid 1.

J. Neurochem. (2008) **105**, 1212–1222.

Diabetic neuropathy is one of the most frequent long-term complications of diabetes mellitus and is a significant cause of morbidity and mortality in diabetic patients. The most common peripheral neuropathy is sensory, in which patients can experience pain in the early to mid-phases of the disease process that progresses to diminution of pain, position, and vibratory sensation overtime (Boulton and Malik 1998; Leinninger *et al.* 2004). Like other neuropathic pain states, the pathogenesis of diabetic sensory neuropathy remains poorly understood. Recently, increased oxidative stress has been implicated in the onset and development of diabetes and its late complications including neuropathy (Vincent *et al.* 2004b). In order to better understand the pathophysiology of diabetic neuropathy, it will be important to determine the cellular pathways that induce oxidative stress and cell injury in sensory neurons in the early stages of the disease.

In the peripheral nervous system, the primary sensory dorsal root ganglion (DRG) neurons with large cell bodies (diameter > 35 μm) are typically associated with myelinated A-β fibers and transmit proprioception and vibration sensation, whereas neurons with small cell bodies (diameter < 25 μm) are typically associated with unmyelinated C-fibers or thinly myelinated A-δ fibers that are responsible for conducting nociceptive stimuli (Lawson 2002). Diabetic sensory neuropathy is conceptually considered a mixed neuropathy involving both large and small, myelinated and unmyelinated nerve fibers. Compared with these nerve fibers, the soma of the primary afferent neurons is more vulnerable

Received November 16, 2007; revised manuscript received December 25, 2007; accepted December 28, 2007.

Address correspondence and reprint request to Shuangsong Hong, Department of Internal Medicine, University of Michigan, 1150 W. Medical Center Dr, MSRB II, Room 2562, Ann Arbor, MI 48109, USA. E-mail: hongss@med.umich.edu

Abbreviations used: AMC, 7-amino-4-methylcuomarin; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; CAP, capsaicin; CZP, capsazepine; DHE, dihydroethidium; DRG, dorsal root ganglion; IR, immunoreactivity; MPT, mitochondrial permeability transition; NAC, *N*-acetyl-L-cysteine; NF200, neurofilament 200; STZ, streptozotocin; TRPV1, transient receptor potential vanilloid 1.

© 2008 The Authors
Journal Compilation © 2008 International Society for Neurochemistry,

to damage under physiological stress or tissue injury because of the absence of a blood barrier (McHugh and McHugh 2004). Several reports have demonstrated neuronal apoptosis in streptozotocin (STZ)-induced diabetic rats or in cultured DRG neurons harvested from diabetic rats (Russell *et al.* 1999; Zochodne *et al.* 2001; Schmeichel *et al.* 2003). Thus, DRG neurons are a primary target of neural complications and may be preferentially affected in diabetic sensory neuropathy.

The transient receptor potential vanilloid 1 (TRPV1) is highly expressed in DRG neurons. TRPV1 is a Ca^{2+}-permeable, non-selective cation channel, gated by noxious heat, low pH, capsaicin (CAP), and endogenous ligands (Caterina *et al.* 1997). Physiologically, TRPV1 is essential in modulation of pain sensation and development of thermal hypersensitivity following tissue injury or inflammation (Caterina *et al.* 2000; Davis *et al.* 2000). Repeated activation of the TRPV1 receptor causes the overload of intracellular Ca^{2+}, resulting in oxidative stress and apoptotic cell injury (Shin *et al.* 2003; Kim *et al.* 2005). A recent report suggests that activation of TRPV1 contributes to pancreatic beta cell stress and may play a role in the pathogenesis of diabetes mellitus (Razavi *et al.* 2006). Increased expression and function of the TRPV1 receptor has been reported in large DRG neurons in the STZ-induced diabetic rats (Hong and Wiley 2005).

Based on these observations, we examined the hypothesis that increased expression of the TRPV1 receptor in large DRG neurons is associated with enhanced oxidative stress and activation of cell injury pathways relatively early in the natural history of diabetic sensory neuropathy. Markers of oxidative stress and cellular injury were examined both *in vitro* and *in vivo* in STZ-induced diabetic and control rats in the absence or presence of the competitive TRPV1 receptor antagonist capsazepine (CZP). We observed that increased expression of the TRPV1 receptor in large DRG neurons in diabetic rats (diabetic for 6–8 weeks) was associated with activation of oxidative stress and neuronal injury which was prevented by treatment with CZP.

Materials and methods

Experimental animals

Male Sprague–Dawley rats were housed in the animal facility that was maintained at 22°C with an automatic 12 h light/dark cycle. Diabetes mellitus was induced by a single intraperitoneal injection of STZ at a dose of 45 mg/kg body weight to rats weighing 200–220 g. Age-matched control rats received injections of saline vehicle. Blood glucose levels greater than 300 mg/dL (16.7 mM) 3 days after STZ injection were considered diabetic. Rats meeting this criterion were used experimentally 6–8 weeks after induction of diabetes. All experiments were approved by the University of Michigan Committee on Use and Care of Animals according to National Institutes of Health guidelines.

Isolation of DRG neurons

The lumbar DRGs (L1-L6) were incubated at 37°C in 0.3% collagenase (Worthington type 2, Calbiochem, San Diego, CA, USA) in minimal essential medium (Gibco, Carlsbad, CA, USA) supplemented with 16.5 mM $NaHCO_3$ and 28.2 mM glucose minimal essential medium for 50 min and then in 0.1% trypsin (type 1; Sigma, St. Louis, MO, USA) for 10 min. After titration in minimal essential medium containing 10% fetal bovine serum, cells were plated on cover glasses that were previously coated with calf collagen. Isolated neurons from control or diabetic rat (n = 8 rats in each group) were incubated in 95% air + 5% CO_2 at 37°C prior to treatment with CAP (1 μM) for 4 h.

Administration of capsazepine *in vivo* and *in vitro*

TRPV1 antagonist CZP (30 mg/kg; Sigma) was administered either intraperitoneally or by hind-paw injection in 20% dimethyl sulfoxide/1% ethanol/1% Tween 80/78% saline in a volume of 0.2 mL right after STZ-injected rats develop high levels of blood glucose (> 300 mg/dL) as described previously (Walker *et al.* 2003). Some STZ-injected rats were administered vehicle alone as controls. The CZP was administered weekly after the first subcutaneous application to sustain the effects *in vivo*. For experiments using acutely dissociated DRG neurons, the plated cells were treated with CAP (1 μM) for 4 h in the presence or absence of TRPV1 antagonist CZP (10 μM), calpain inhibitor I (50 μM; Santa Cruz Biotech, Santa Cruz, CA, USA), pan caspase inhibitor Z-VAD-fmk (100 μM; Santa Cruz Biotech), or reactive oxygen species-specific inhibitor *N*-acetyl-L-cysteine (NAC, 200 μM; Sigma).

Measurement of reactive oxygen species

Oxidative stress was measured by analyzing dihydroethidium (DHE) oxidation to Eth (Russell *et al.* 2002) and immunofluorescence for 8-hydroxy-2′-deoxyguanosine (8-OHdG), an oxidized nucleoside of DNA (Schmeichel *et al.* 2003). Isolated DRG neurons were incubated with 2 μM DHE (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C, rinsed and then immediately viewed under a microscope.

Staining cells with CMXRos

MitoTracker Red CMXRos staining (Molecular Probes, Carlsbad, CA, USA) was performed to monitor the transmembrane potential of mitochondria using the method described previously (Shibayama-Imazu *et al.* 2006). After treatment with CAP, DRG neurons were incubated with MitoTracker Red CMXRos (0.25 μg/mL; Molecular Probes) and then cells were incubated for a further 15 min at 37°C. After several washes with culture medium, cells were fixed in 4% *p*-formaldehyde in 0.1 M phosphate buffer, pH 7.4, and mounted on a glass slide.

Calpain and caspase activity assay

Calpain activity was assayed spectrophotometrically using the calpain-specific membrane-permeable peptide substrate Suc-LLVY-7-amino-4-methylcoumarin (AMC) as previously described (Liu-Snyder *et al.* 2006). Briefly, isolated DRG neurons from control and diabetic rats were incubated with 130 μM of the calpain substrate for 1 h at 37°C. Then, neuron pellets were collected by centrifugation at 1000 *g* and re-suspended in phosphate-buffered

© 2008 The Authors

saline and transferred to 100 µL quartz cuvettes. Activity was measured under linear conditions as a function of AMC hydrolysis using excitation and emission wavelengths of 355 and 444 nm, respectively. Caspase 3 like activity was measured in lysates of DRG neurons using substrates *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin as described (Lau *et al.* 2006). Sample fluorescence was quantified at 355 nm excitation and 460 nm emission.

Mitochondria and cytosolic fraction preparation

Dorsal root ganglion mitochondria was isolated using a method described (Kaur *et al.* 2007). Lumbar DRGs were collected and homogenized with five volumes of ice-cold isolation buffer (20 mM HEPES-KOH, pH 7.5, 215 mM mannitol, 75 mM sucrose, 10 mM KCl, 1.5 mM NaCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride). The tissue homogenate was spun at 1000 *g* for 10 min in an Eppendorf microcentrifuge at 4°C. The pellet was re-suspended in isolation buffer and spun again at 14 000 *g* for 20 min in order to pellet the mitochondria. Both the pellet and supernatant were collected for western blot analysis of cytochrome *c*.

Immunohistochemistry

Lumbar DRGs were removed and fixed for 2–3 h in 4% *p*-formaldehyde in 0.1 M phosphate buffer. Transverse sections through the DRG (10 µm) were cut on a cryostat. Sections were permeabilized with 0.3% Triton X-100 and blocked with 10% normal goat serum overnight. In some experiments, isolated DRG neurons plated on coated cover glasses were fixed and subjected to immunostaining. Primary antibodies used were monoclonal anti-NF200 (N52 clone), anti-cytochrome *c* (Sigma), anti-peripherin (Chemicon, Temecula, CA, USA); polyclonal anti-caspase 3 (Cell Signaling, Danvers, MA, USA), anti-8-OHdG (Genox Corp., Baltimore, MD, USA), and anti-TRPV1, anti-caspase-activated DNase, anti-µ-calpain (Santa Cruz Biotech). Secondary antibodies Alexa Fluor 350, 488, and 594 from Molecular Probes were used with 2-h incubation. Images were acquired using a Zeiss Axioplan microscope or a Zeiss LSM510 confocal microscope (Zeiss, Thornwood, CA, USA) in the Microscopy and Image Analysis Laboratory at the University of Michigan.

Western blot analysis

Lumbar DRGs were isolated and homogenized in ice-cold lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EGTA, 50 mM NaF, 1.5 mM $MgCl_2$, 10% v/v glycerol, 1% v/v Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and 'Complete' protease inhibitor mixture (Roche Diagnostics, Basel, Switzerland). Proteins were separated on 8% Tris–HCl gels and transblotted to polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked with 10% dry milk for 12 h and incubated with the primary antibodies at 4°C, overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) for 1 h at 25°C and developed using the West Dura Supersignal chemiluminescence kit (Pierce, Rockford, IL, USA).

Data analysis

In each rat, 1500–2000 total DRG neurons were counted blindly. Neurons were judged to be positive if they had mean brightness values greater than the corresponding control background value stained with the secondary antibody alone. The area of individual neurons was determined by tracing the cell margins on a computer screen. According to the measured area, neurons were classified as large with cell area larger than 1200 μm^2. The mean staining intensity was determined by National Institute of Health Image-J software for each cell body by subtraction of the control background staining and was normalized to cell area (100 μm^2). Statistical analyses were performed using non-parametric Mann–Whitney test by GraphPad InStat 3 (GraphPad Software Inc., San Diego, CA, USA). Differences with $p < 0.05$ were considered significant.

Results

Diabetes was associated with activation of caspase-dependent cell injury in DRG neurons *in vivo*

After STZ injection for 72 h, 75% of the rats developed high levels of blood glucose (mean level = 482 ± 32 mg/dL), whereas the control rats receiving vehicles had normal levels (mean level = 92 ± 10 mg/dL). In diabetic rats (diabetic for 6–8 weeks), the number of activated/cleaved caspase 3 immunoreactivity (IR)-positive DRG neurons increased significantly *in situ* compared with controls and these apoptotic cells colocalized with the TRPV1 receptor, whereas the number of caspase 3 IR-positive neurons was not altered in TRPV1 IR-negative large neurons (Fig. 1a–c). Immunoblot assays showed an increased level of the caspase-activated DNase, a cytosolic nuclease responsible for oligonucleosomal DNA fragmentation at the late stage of apoptosis, in DRGs of diabetic rats (Fig. 1d and e), suggesting an increased apoptotic neuronal injury as a result of diabetes.

It has been reported that the preferential derangement of cytoskeletal neurofilament 200 (NF200), a major determinant of large myelinated fibers, is an early morphological feature of tissue injury (Posmantur *et al.* 1996). As shown in Fig. 1f and g, diabetes was associated with a significant decrease in the percentage of large NF200 IR-positive but not small peripherin IR-positive neurons in DRGs compared with controls. Immunoblot assays also revealed a decrease in the protein level of NF200 in DRGs in diabetic rats (Fig. 1d). These data suggest that cytoskeletal NF200 was altered in diabetic DRGs and support the interpretation that early diabetes may involve preferential injury to this subpopulation of DRG neurons.

TRPV1 was associated with an increase in oxidative stress in diabetic rats

We reported previously that the TRPV1 (vanilloid receptor 1) expression was differentially increased in large DRG neurons in diabetic rats compared with healthy controls by immunohistochemistry studies (Hong and Wiley 2005). In TRPV1 IR-positive neurons, the neurons also IR-positive for NF200 (large neurons) was 23.8 ± 1.5% in diabetic rats and 11.7 ± 2.3% in control rats, respectively ($p < 0.05$). In this study, we examined whether TRPV1 activation could induce

© 2008 The Authors

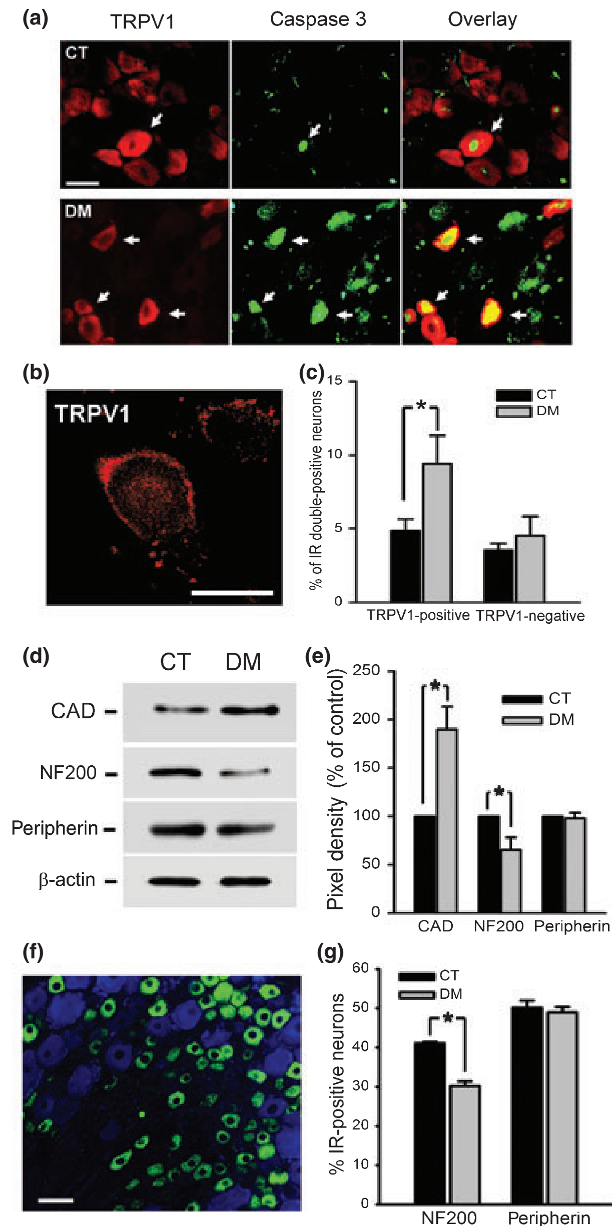

Fig. 1 DRG neurons from diabetic rats demonstrated an increase in caspase-dependent cell injury. (a) Double immunofluorescence showed an increase in cleaved caspase 3 labeling (green) in TRPV1 IR-positive (red) DRG neurons in diabetic (DM) rats compared with controls (CT). The neurons IR-positive for both caspase 3 and TRPV1 were indicated by white arrows. (b) Confocal image of cultured DRG neurons that express TRPV1 receptor mainly at cell membrane. (c) Bar graph showed an 85 ± 6% increase in the number of neurons that were IR-positive for both TRPV1 and cleaved caspase 3 in TRPV1 IR-positive neurons in diabetic rats (n = 5). (d and e) Immunoblot analysis of protein levels of caspase-activated DNase (CAD), NF200, and peripherin in DRGs (n = 4). (f) The large NF200 (blue, marker for large A-fiber neurons) IR-positive neurons and small peripherin (marker for small C-fiber neurons) IR-positive neurons did not colocalize in DRGs. (g) Bar graph showing the percentage of DRG neurons IR-positive for NF200 or peripherin in control and diabetic rats (n = 10). Scale bar: 40 μm. *$p < 0.05$.

the accumulation of reactive oxygen species in this subpopulation of neurons in diabetes *in vitro*. As shown in Fig. 2a and b, the labeling intensity of 8-OHdG in large DRG neurons (cell area > 1200 μm^2) was significant higher in diabetic rats compared with controls. CAP treatment, which activates the TRPV1 receptor, induced a significant increase in the labeling intensity of 8-OHdG in large DRG neurons isolated from diabetic rats compared with the untreated as measured by confocal microscopy imaging ($p < 0.05$), while CAP treatment caused a modest increase in 8-OHdG intensity in large neurons from controls ($p = 0.07$). TRPV1 antagonist CZP pre-treatment prevented the induction of 8-OHdG in diabetic DRG neurons (Fig. 2b). Consistent with this, the percentage of DHE-positive neurons also increased significantly in DRGs in diabetic rats compared with controls. After CAP treatment, the percentage of DHE-positive neurons was significantly increased and this increase was prevented by pre-treatment with CZP, calpain inhibitor I or pan caspase inhibitor Z-VAD-fmk (Fig. 2c). After CAP treatment, the number of DHE-positive neurons was increased to 39.4 ± 4.6% from 18.9 ± 2.1% in diabetic rats ($p < 0.05$), while this percentage significantly decreased to 20.9 ± 1.4% after CZP pre-treatment, to 20.6 ± 3.3% after calpain inhibitor I pre-treatment, or to 18.7 ± 1.7% after pan caspase inhibitor pre-treatment. Incubation with TRPV1 antagonist CZP alone without CAP did not affect the number of DHE-positive neurons compared with the untreated. In addition, to investigate CAP-induced oxidative stress in DRG neurons, the reactive oxygen species-specific inhibitor NAC (200 μM) was pre-incubated with acutely dissociated DRG neurons before CAP treatment. NAC pre-treatment significantly reduced the number of DHE-positive neurons compared with CAP-treated diabetic neurons ($p < 0.05$; Fig. 2c). To confirm the presence of increased oxidative stress in DRG neurons in diabetic rats *in situ*, DRG sections from diabetic rats were subjected to 8-OHdG staining and colocalized with TRPV1 (Fig. 2d). The percentage of DRG neurons IR-positive for both 8-OHdG and TRPV1 increased significantly in diabetes compared with controls (Fig. 2d). In addition, the percentage of neurons IR-positive for both 8-OHdG and NF200 was significantly increased in DRGs from diabetic rats, suggesting the oxidative stress is preferentially increased in large, NF200 IR-positive neurons in early diabetic rats (Fig. 2e).

Capsaicin-induced collapse of mitochondrial transmembrane potential and activation of caspase-dependent cell injury in DRG neurons

Mitochondrial transmembrane potential was evaluated by staining DRG neurons with MitoTracker Red CMXRos, a lipophilic cationic dye shown to rapidly accumulate in normal mitochondria as a result of the negative transmembrane potential of the organelles (Shibayama-Imazu *et al.* 2006). The labeling intensity of MitoTracker Red CMXRos

© 2008 The Authors

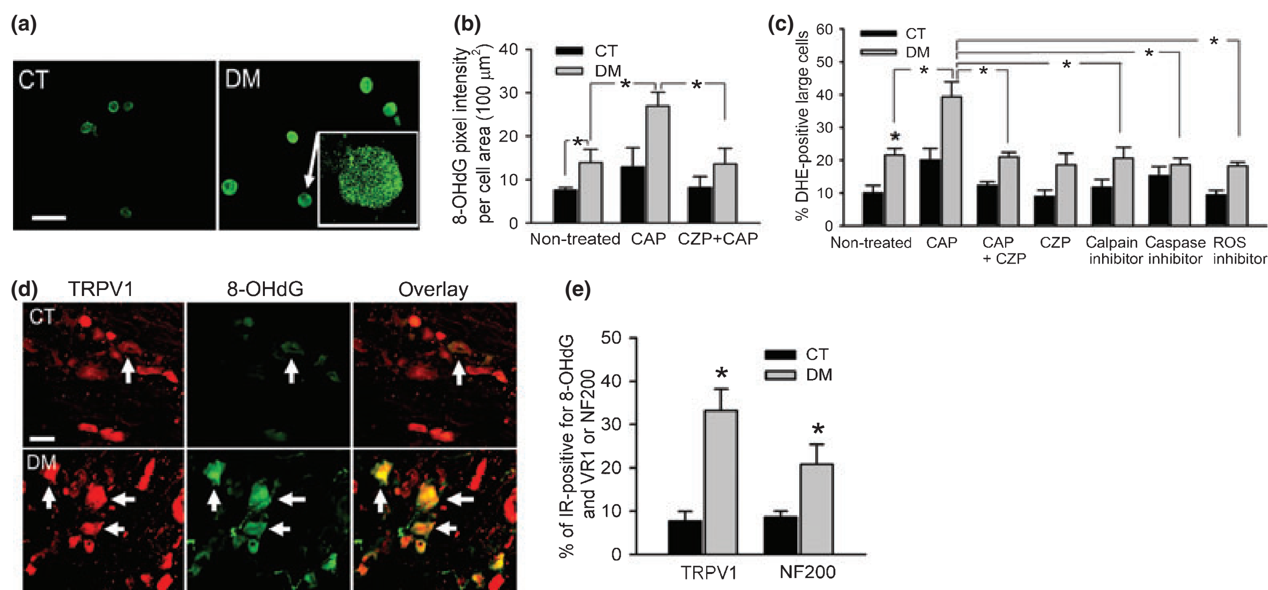

Fig. 2 Oxidative stress was enhanced in large DRG neurons in diabetic rats. (a) Immunofluorescence staining showed increased labeling intensity of 8-OHdG in large DRG neurons (cell diameter > 40 μm and cell area > 1200 μm^2) isolated from diabetic rats after capsaicin treatment. The inset was a typical image of 8-OHdG staining viewed under the confocol microscope. Scale bar: 100 μM. (b) Pixel intensity measurement of 8-OHdG labeling showed that the increase in 8-OHdG staining was prevented in diabetic DRG neurons with pretreatment with TRPV1 antagonist capsazepine. (c) Capsaicin treatment increased the percentage of DHE-positive DRG neurons isolated from diabetic rats. Pretreatment with capsazepine, calpain inhibitor I, pan caspase inhibitor or reactive oxygen species inhibitor *N*-acetyl-L-cysteine (NAC) blocked the increased labeling of DHE evoked by capsaicin in large DRG neurons from diabetic rats. (d) Double immunofluorescence showing increased 8-OHdG labeling (green) in TRPV1 IR-positive neurons (red) *in situ* in DRGs from diabetic rats. Scale bar: 40 μm. (e) Bar graph showing the increased number of 8-OHdG IR-positive neurons in large, TRPV1-expressing DRGs from diabetic rats *in situ*. $^*p < 0.05$.

was significant lower in isolated large DRG neurons that expressed the TRPV1 receptor in diabetic rats compared with controls (50 TRPV1 IR-positive large neurons counted per rat). CAP treatment caused a significant decrease in the labeling intensity of MitoTracker Red CMXRos in large, TRPV1-expressing DRG neurons from diabetic DRG neurons but not controls. CZP pre-treatment prevented the decrease in the mitochondrial membrane potential in large DRG neurons isolated from diabetic rats. In contrast, the MitoTracker Red CMXRos staining was not altered in DRG neurons isolated from control rats after CAP treatment compared with untreated neurons (Fig. 3a). We next examined the activation of caspase 3 in DRG neurons after CAP treatment by immunofluorescence staining. In TRPV1 IR-positive cells, the presence of cleaved/activated caspase 3 staining was significantly more intense in neurons after CAP treatment in diabetic rats (Fig. 3b). We also examined caspase activity in DRG neurons from control and diabetic rats. Caspase activity was significantly increased in diabetic rats compared with controls in isolated DRG neurons and in CAP-treated DRG neurons (Fig. 3c). Moreover, the levels of cytochrome *c* in DRG neurons after CAP treatment increased significantly in DRG neurons from diabetic rats compared with controls as shown by immunofluorescence staining (Fig. 3d). Western blot analysis also showed that cytochrome *c* was significantly increased in the cytosolic fraction of DRGs from diabetic rats compared with controls (Fig. 3e and f), consistent with the previous reports (Srinivasan *et al.* 2000; Di Noia *et al.* 2006).

Capsaicin treatment caused greater Ca^{2+}-dependent cell injury in DRG neurons in diabetic rats

Calpains, including widely expressed μ-calpain, are Ca^{2+}-dependent neuronal cysteine proteases that are known to be involved in the proteolysis of a number of important proteins and plays a important role in neuronal cell death (Lankiewicz *et al.* 2000). After CAP treatment, large DRG neurons that were IR-positive for TRPV1 displayed a significant increase in the labeling intensity of μ-calpain in diabetic rats compared with controls (Fig. 4a). To assess calpain activity, DRGs from control and diabetic rats were treated with CAP in the presence of calpain substrate Suc-LLVY-AMC. As shown in Fig. 4b, CAP-activated Suc-LLVY-AMC hydrolysis was significantly increased in DRGs isolated from diabetic rats compared with controls. Consistent with this, CAP treatment also induced a significant increase in the level of μ-calpain protein in isolated DRG neurons from diabetic rats compared with controls (Fig. 4c and d), while the

© 2008 The Authors
Journal Compilation © 2008 International Society for Neurochemistry,

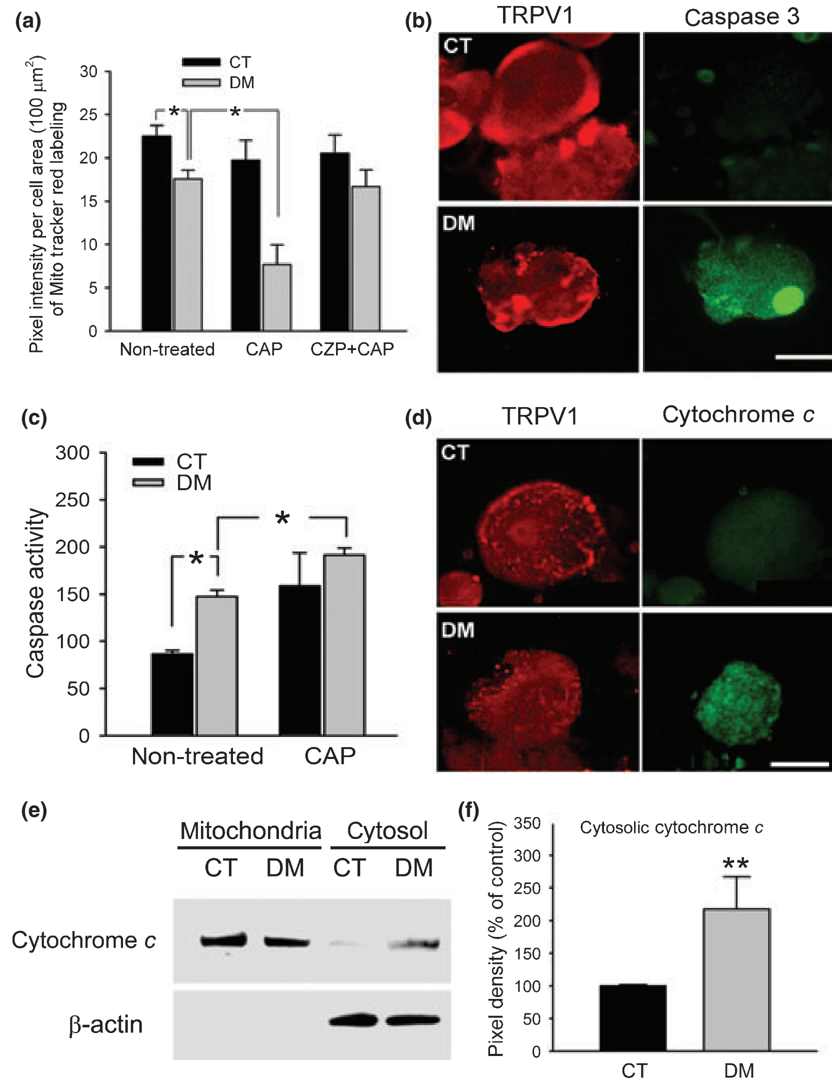

Fig. 3 Capsaicin treatment induced shift of mitochondrial transmembrane potential and increased cell injury in DRG neurons isolated from diabetic rats. (a) Bar graph depicting the changes in the labeling intensity of MitoTracker Red in DRG neurons treated with capsaicin and/or capsazepine in diabetic rats ($n = 5$). (b) Cleaved caspase 3 was activated and increased in DRG neurons from diabetic rats after capsaicin treatment. (c) Bar graph showing increased caspase activity in DRG preparations from diabetic rats when compared with controls before and after capsaicin treatment ($n = 4$). (d) Increased cytochrome *c* labeling in DRG neurons IR-positive for TRPV1 in diabetic rats after capsaicin treatment. Scale bar: 40 μm. (e) Western blot analysis of the expression level of cytochrome *c* in crude mitochondria and cytosol fraction in DRGs from control and diabetic rats. (f) Pixel graph showing cytochrome *c* was significantly increased in the cytosolic fraction of DRGs from diabetic rats compared with controls. The cytochrome *c* band density was normalized to β-actin. $^*p < 0.05$, $^{**}p < 0.01$.

cytoskeletel NF200 was reduced after CAP treatment in diabetic rat DRGs, suggesting the initiation of Ca^{2+}-dependent cell injury pathway in diabetic DRGs.

Capsazepine treatment, *in vivo*, prevented the reduction in large NF200 IR-positive DRG neurons in diabetic rats

To investigate the effects of TRPV1-mediated neuronal injury *in situ*, CZP was delivered to the rat hind paw and intraperitoneally prior to and after STZ-injection. After 2 months of CZP treatment, DRGs from control and diabetic rats were isolated and processed for staining with NF200 and peripherin. No differences were observed between hind paw and intraperitoneal injection and the data were pooled for analysis. In diabetic rats not receiving CZP treatment, the number of large-sized DRG neurons decreased significantly as described above. In rats that received CZP treatment, the percentage of large-sized, NF200 IR-positive DRG neurons was not significantly different compared with healthy controls. Treatment with CZP had no effect on the percentage of neurons in control rats (Fig. 5a). The percentage of small DRG neurons, as measured by peripherin immunofluorescence staining, did not change with CZP treatment both in

© 2008 The Authors

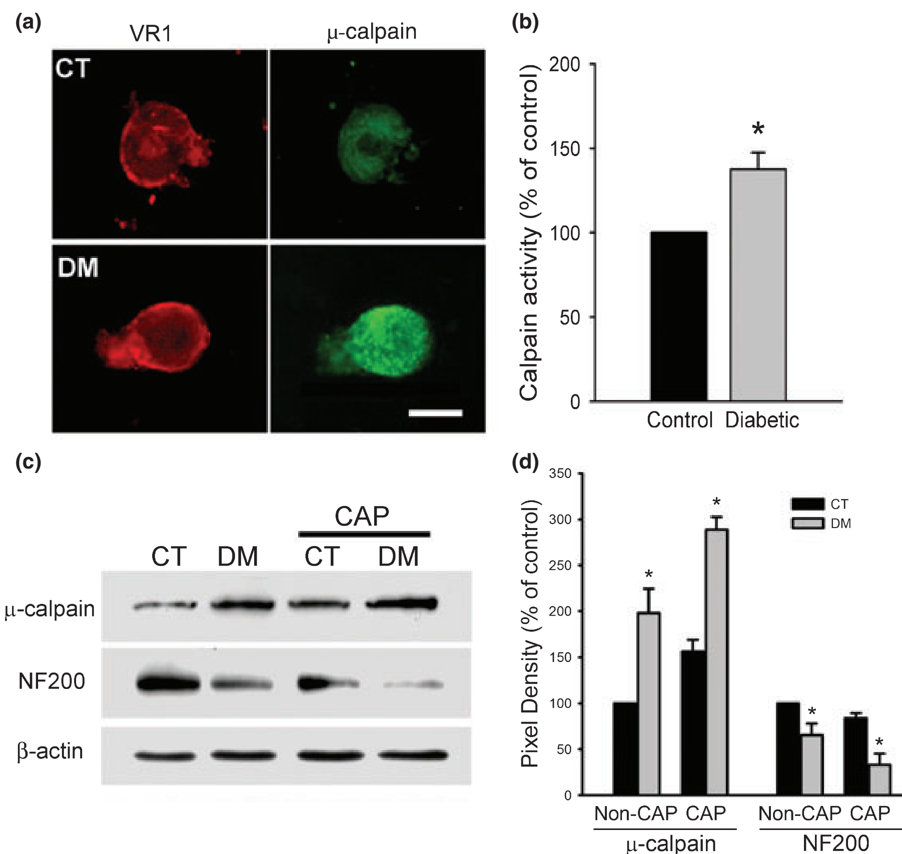

Fig. 4 Increased Ca^{2+}-dependent protease, μ-calpain, in DRGs from diabetic rats. (a) Double immunofluorescence staining showed increased μ-calpain labeling in TRPV1 IR-positive neurons after capsaicin treatment. Scale bar: 40 μm. (b) Increased calpain activity in DRG neurons isolated from diabetic rats after capsaicin treatment. (c and d) Increased level of μ-calpain and decreased level of NF200 in DRGs in diabetic rats after capsaicin treatment ($n = 4$). $^*p < 0.05$.

control and diabetic rats (Fig. 5b). These data provide additional support that activation of the TRPV1 receptor-ion channel complex contributes to neuronal injury in early diabetes.

Discussion

Oxidative stress and programmed cell death have been proposed to play a role in the natural history of diabetic neuropathy (Russell *et al.* 1999; Srinivasan *et al.* 2000; Cheng and Zochodne 2003; Schmeichel *et al.* 2003; Vincent *et al.* 2004a,b). However, our understanding of the mechanisms underlying neuronal injury in diabetic sensory neuropathy is incomplete. We believe that this is the first report demonstrating activation of TRPV1 receptor induces preferential activation of oxidative stress and neuronal injury pathways in large DRG neurons at a relatively early stage of diabetes. In diabetic rats (diabetic for 6–8 weeks), oxidative stress and cell injury markers were significantly increased *in vivo* in large NF200 IR-positive DRG neurons that express TRPV1. CAP-evoked TRPV1 activation resulted in loss of the mitochondrial membrane potential, accumulation of reactive oxygen species, and induction of both apoptotic and calpain-dependent cell injury in DRG neurons from diabetic rats. The competitive TRPV1 receptor antagonist CZP inhibited these effects *in vitro* and markedly reduced activation of cell injury mechanisms in large DRG neurons in diabetic rats *in vivo*.

TRPV1 is a ligand-gated non-selective cation channel and its activation leads to Ca^{2+} influx and membrane depolarization (Caterina *et al.* 1997; Shin *et al.* 2003). Enhanced activity of TRPV1 is known to place neurons at increased risk for stimulation of calpain (calcium)-dependent neuronal injury. Although TRPV1-mediated cell death has been reported in a variety of cell types (Jambrina *et al.* 2003; Shin *et al.* 2003; Kim *et al.* 2005), the majority of these investigations employ either malignant or transformed cells and the role of TRPV1 has not been examined in diabetic sensory neuropathy. It is interesting that TRPV1-mediated neuroprotection against excitotoxicity that is attributed to the endogenous desensitization of TRPV1 has been reported recently in the brain (Veldhuis *et al.* 2003). However, our previous study demonstrates that TRPV1 desensitization is impaired in DRG neurons in diabetic rats and activation of

© 2008 The Authors

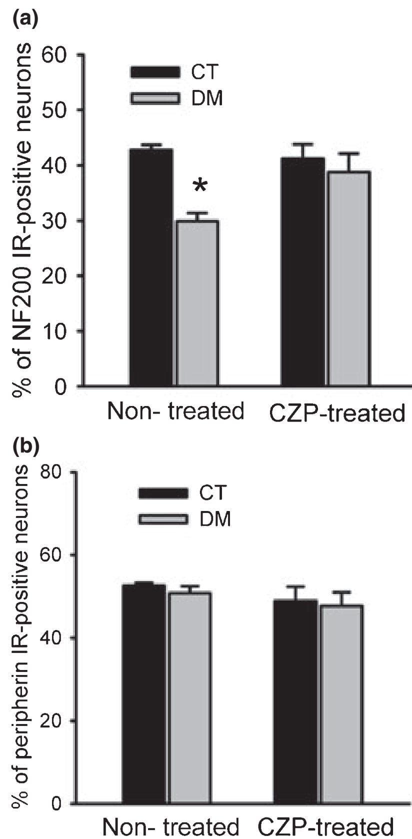

Fig. 5 Capsazepine pretreatment prevented the decrease in large NF200 IR-positive DRG neurons in diabetic rats *in vivo*. (a) Bar graph showing the percentage of large NF200 IR-positive neurons in DRGs in diabetic rats before and after capsazepine treatment ($n = 6$). (b) No change in small, peripherin IR-positive neurons was observed in diabetic rats with or without capsazepine treatment. $^*p < 0.05$.

TRPV1 by CAP triggers an increase in TRPV1 currents with a longer duration compared with controls (Hong and Wiley 2005). Alternatively, the endogenous level of TRPV1 agonists might be increased in diabetes to activate more TRPV1 channels open and Ca^{2+} influx. For example, the endogenous CB1 agonist cannabinoid anandamide, which also activates TRPV1, was increased in diabetes (Bloomgarden 2006). Moreover, our previous and current studies show that the expression of TRPV1 increased significantly in large myelinated A-β fiber (NF200 IR-positive) DRG neurons in diabetic rats (Hong and Wiley 2005), possibly acting as modulators to transmit pain signals or maintain the peripheral and central sensitization in neuropathy states. This is consistent with the recent findings that TRPV1 expression increases in DRG neurons in other neuropathic pain models like nerve injury and that silencing TRPV1 reduces neuropathic pain (Christoph *et al.* 2006; Biggs *et al.* 2007). In the current study, we report that activation of TRPV1 by CAP induces oxidative stress and activation of apoptotic cascade in large TRPV1-expressing DRG neurons from diabetic rats, which was inhibited by calpain inhibitor, caspase inhibitor and reactive oxygen species-specific inhibitor. Treatment with the TRPV1 antagonist CZP reduced CAP-induced cell cytotoxicity in diabetic DRG neurons *in vitro* and prevented the reduction in NF200-immunoreactive large DRG neurons *in vivo*. These observations are in agreement with a neuroprotective role for the TRPV1 antagonist CZP reported previously (Veldhuis *et al.* 2003; Kim *et al.* 2005). Our findings may have pathophysiological significance and support a mechanistic role for the TRPV1 receptor in diabetic sensory neuropathy.

A substantial body of research supports that mitochondria undergo structural and functional changes that contribute to activation of cell injury pathways. Reduction of mitochondrial transmembrane potential is an early step of programmed cell death. Our data support the concept that the development of neuronal injury in DRGs in diabetes occurs, at least in part, through the alterations of mitochondria following activation of TRPV1 receptor. Specifically, the mitochondrial transmembrane potential was preferentially depolarized in large DRG neurons expressing TRPV1 after CAP treatment in diabetic rats, which may be related to an overload of intracellular Ca^{2+} that depolarizes the mitochondrial transmembrane potential and eventually leads to the opening of mitochondrial permeability transition (MPT) pores (Bernardi 1999). In addition, the elevation of intracellular Ca^{2+} also triggers the formation of reactive oxygen species by uncoupling electron transport from ATP production in mitochondria (Vincent *et al.* 2004a). Although these free radical reactions are essential for host defense mechanisms, the overproduction of free radicals may cause tissue injury and programmed cell death (Pop-Busui *et al.* 2006). Our data indicate that the increase in oxidative stress as measured by 8-OHdG and DHE is associated with TRPV1 activation because CAP-induced accumulation of reactive oxygen species and the increase in caspase activity were reversed by pre-treatment with the TRPV1 antagonist CZP in these neurons. The release of cytochrome *c* was also observed in large DRG neurons isolated in diabetic rats after CAP treatment *in vitro* and in the cytosolic fraction of DRG neurons in diabetes *in situ*. Consistent with our data, morphological changes such as mitochondrial swelling caused by TRPV1 activation has been observed in sensory neurons (Szoke *et al.* 2002). Thus, it is likely that TRPV1-mediated cell injury in diabetic sensory neuropathy involves accumulation of reactive oxygen species and opening of MPT pores that consequently leads to mitochondrial dysfunction, substantial swelling of the mitochondria with rupturing outer membrane and releasing apoptosis-inducing factors such as cytochrome *c*, and subsequent activation of caspase 3.

In addition to the caspase-dependent cell injury pathway, our data support a calpain-mediated cell injury pathway that may be involved in diabetic peripheral neuropathy. Calpain, a Ca^{2+}-dependent cysteine protease, has been implicated in a variety of disorders, including Parkinson's disease, Alzhei-

© 2008 The Authors
Journal Compilation © 2008 International Society for Neurochemistry,

mer's disease, multiple sclerosis, and spinal cord injury (Harwood *et al.* 2005). We observed TRPV1-associated elevation of calpain activity and μ-calpain expression in diabetic DRG neurons compared with controls. It is likely that TRPV1 activation induces an increase in intracellular calcium, activates Ca^{2+}-activated calpain, and eventually leads cell injury in diabetic DRG neurons. First, calpain can cleave its specific substrates which can directly or indirectly lead to cell rupture (Huang and Wang 2001). Second, calpain may cleave important regulators of apoptosis such as proapoptotic Bid and anti-apoptotic Bcl-X_L (Nakagawa and Yuan 2000; Mandic *et al.* 2002), suggesting a crosstalk between the calpain-dependent and caspase-dependent cell injury pathways (Raynaud and Marcilhac 2006). Consequently, calpain-mediated hydrolysis of these proteins leads to changes in their activity or intracellular localization. In addition, activation of calpain in the large diabetic DRG neurons may increase the permeability of plasma membrane (Huang and Wang 2001), making it more susceptible to the damage exerted by other factors, such as osmotic force. Because calpain substrates represent some of the most important cellular proteins including ion pumps and channels, structural proteins, and intracellular enzymes (Liu *et al.* 2004b), the TRPV1 receptor may represent an attractive therapeutic target to regulate calpain activation and downstream events. A recent study suggests that expression of calpain-10 is specifically activated in diabetes. Over-expression of calpain-10 resulted in mitochondrial dysfunction through the cleavage of complex I of the electron transport chain and activation of MPT (Arrington *et al.* 2006). Additional studies are required to investigate whether calpain-10 activation is also mediated by TRPV1 receptor in diabetic sensory neuropathy.

Recent studies using morphometric analysis support the provocative observation that diabetic peripheral neuropathy may be associated with preferential loss of large DRG neurons in long-term diabetic rats (Kishi *et al.* 2002). In contrast, other studies using morphometric methods have not observed loss of DRG neurons in the time frame of diabetes (diabetic for 6–8 weeks) that we studied in the current experiments (Zochodne *et al.* 2001; Cheng and Zochodne 2003). This suggests the intriguing possibility that activation of oxidative stress and cell injury pathways relatively early in diabetes is not irrevocably linked to concurrent loss of neurons. It is possible that the apparent loss in large DRG neurons may reflect the preferential effects of diabetes-associated hyperosmolarity and concomitant shrinkage of the soma in this subpopulation of neurons and, therefore, not represent an actual dropout of DRG neurons. Although shrinkage of large myelinated axons has been observed in STZ-induced diabetes, the decrease of large, NF200 IR-positive neurons observed in diabetic rats is not likely attributable to cell shrinkage because the expression of NF200 does not correlate with axonal caliber (Sayers *et al.* 2003). The decrease of NF200 in DRGs in early diabetic rats (6–8 weeks) we observed is consistent with the previous reports that also demonstrate a reduction of neurofilament heavy chain in axons at early stages of diabetes possibly because of reduced synthesis of transcripts (Mizisin *et al.* 1999; Sayers *et al.* 2003). In 2–3 months diabetic rats, the neurofilament phosphorylation is triggered and thereafter induces expression and accumulation of neurofilament in DRGs (Sayers *et al.* 2003). Neurofilaments are important cytoskeletal proteins that act together to form and maintain cell shape and facilitate the transport of particles and organelles within the cytoplasm (Liu *et al.* 2004a). Abnormalities of neurofilament synthesis and transport have been suggested to contribute to the axonal atrophy and reduction of nerve conduction velocity in diabetes (Scott *et al.* 1999; Sayers *et al.* 2003). Furthermore, neurofilaments are a major target in conditions of elevated oxidative stress (Leski *et al.* 2001). Increased oxidative stress may degrade the neurofilaments in large DRG neurons in diabetic rats. Activation of calpain observed in these neurons can also cleave neurofilaments such as NF200 (Stys and Jiang 2002). The preferential cell stress observed in large NF200 IR-positive DRG neurons in diabetes is consistent with a recent study that large NF200 IR-positive neurons are more vulnerable compared with small DRG neurons in response to injury (Guseva and Chelyshev 2006). Thus, our data support that neuronal stress does occur in large DRG neurons relatively early in diabetic neuropathy at this time frame (diabetic for 6–8 weeks). However, the cell injury does not translate into a parallel loss of neurons. This suggests that primary neurons are relatively 'protected' from cell death (defined as loss or drop out of cell bodies), either by the robust expression of cytoprotective mechanisms or reduced expression of key regulatory components of cell death, such as caspase 8.

In summary, our data suggest that increased expression and activation of the TRPV1 receptor in the subpopulation of large DRG neurons is associated with the activation of oxidative stress and neuronal injury pathways at a relatively early stage of diabetes.

Acknowledgements

This work was supported by Grant R01DK052387 and R01DK056997 to JWW from National Institute of Health, by Pilot/Feasibility study Grant to SH from Michigan Diabetes Research and Training Center, and by ANMS/SmartPill Grant to SH from the American Neurogastroenterology and Motility Society.

References

Arrington D., Van V. T. and Schnellmann R. (2006) Calpain 10: a mitochondrial calpain and its role in calcium-induced mitochondrial dysfunction. *Am. J. Physiol. Cell Physiol.* **291**, 1159–1171.

© 2008 The Authors
Journal Compilation © 2008 International Society for Neurochemistry,

Bernardi P. (1999) Mitochondrial transport of cations: channels, exchangers, and permeability transition. *Physiol. Rev.* **79**, 1127–1155.

Biggs J. E., Yates J. M., Loescher A. R., Clayton N. M., Boissonade F. M. and Robinson P. P. (2007) Changes in vanilloid receptor 1 (TRPV1) expression following lingual nerve injury. *Eur. J. Pain* **11**, 192–201.

Bloomgarden Z. T. (2006) Weight control in individuals with diabetes. *Diabetes Care* **29**, 2749–2754.

Boulton A. J. and Malik R. A. (1998) Diabetic neuropathy. *Med. Clin. North Am.* **82**, 909–929.

Caterina M. J., Schumacher M. A., Tominaga M., Rosen T. A., Levine J. D. and Julius D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389**, 816–824.

Caterina M. J., Leffler A., Malmberg A. B., Martin W. J., Trafton J., Petersen-Zeitz K. R., Koltzenburg M., Basbaum A. I. and Julius D. (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* **288**, 306–313.

Cheng C. and Zochodne D. W. (2003) Sensory neurons with activated caspase-3 survive long-term experimental diabetes. *Diabetes* **52**, 2363–2371.

Christoph T., Grunweller A., Mika J., Schafer M. K., Wade E. J., Weihe E., Erdmann V. A., Frank R., Gillen C. and Kurreck J. (2006) Silencing of vanilloid receptor TRPV1 by RNAi reduces neuropathic and visceral pain in vivo. *Biochem. Biophys. Res. Commun.* **350**, 238–243.

Davis J. B., Gray J., Gunthorpe M. J. *et al.* (2000) Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* **405**, 183–187.

Di Noia M. A., Van D. S., Palmieri F., Yang L. M., Quan S., Goodman A. I. and Abraham N. G. (2006) Heme oxygenase-1 enhances renal mitochondrial transport carriers and cytochrome C oxidase activity in experimental diabetes. *J. Biol. Chem.* **281**, 15687–15693.

Guseva D. and Chelyshev Y. (2006) The plasticity of the DRG neurons belonging to different subpopulations after dorsal rhizotomy. *Cell. Mol. Neurobiol.* **26**, 1223–1232.

Harwood S. M., Yaqoob M. M. and Allen D. A. (2005) Caspase and calpain function in cell death: bridging the gap between apoptosis and necrosis. *Ann. Clin. Biochem.* **42**, 415–431.

Hong S. and Wiley J. W. (2005) Early painful diabetic neuropathy is associated with differential changes in the expression and function of vanilloid receptor 1. *J. Biol. Chem.* **280**, 618–627.

Huang Y. and Wang K. K. (2001) The calpain family and human disease. *Trends Mol. Med.* **7**, 355–362.

Jambrina E., Alonso R., Alcalde M., del Carmen R. M., Serrano A., Martinez A., Garcia-Sancho J. and Izquierdo M. (2003) Calcium influx through receptor-operated channel induces mitochondria-triggered paraptotic cell death. *J. Biol. Chem.* **278**, 14134–14145.

Kaur P., Radotra B., Minz R. W. and Gill K. D. (2007) Impaired mitochondrial energy metabolism and neuronal apoptotic cell death after chronic dichlorvos (OP) exposure in rat brain. *Neurotoxicology* **28**, 1208–1219.

Kim S. R., Lee d. Y., Chung E. S., Oh U. T., Kim S. U. and Jin B. K. (2005) Transient receptor potential vanilloid subtype 1 mediates cell death of mesencephalic dopaminergic neurons in vivo and in vitro. *J. Neurosci.* **25**, 662–671.

Kishi M., Tanabe J., Schmelzer J. D. and Low P. A. (2002) Morphometry of dorsal root ganglion in chronic experimental diabetic neuropathy. *Diabetes* **51**, 819–824.

Lankiewicz S., Marc L. C., Truc B. N., Krohn A. J., Poppe M., Cole G. M., Saido T. C. and Prehn J. H. (2000) Activation of calpain I converts excitotoxic neuron death into a caspase-independent cell death. *J. Biol. Chem.* **275**, 17064–17071.

Lau A., Arundine M., Sun H. S., Jones M. and Tymianski M. (2006) Inhibition of caspase-mediated apoptosis by peroxynitrite in traumatic brain injury. *J. Neurosci.* **26**, 11540–11553.

Lawson S. N. (2002) Phenotype and function of somatic primary afferent nociceptive neurones with C-, Adelta- or Aalpha/beta-fibres. *Exp. Physiol.* **87**, 239–244.

Leinninger G. M., Vincent A. M. and Feldman E. L. (2004) The role of growth factors in diabetic peripheral neuropathy. *J. Peripher. Nerv. Syst.* **9**, 26–53.

Leski M. L., Bao F., Wu L., Qian H., Sun D. and Liu D. (2001) Protein and DNA oxidation in spinal injury: neurofilaments – an oxidation target. *Free Radic. Biol. Med.* **30**, 613–624.

Liu Q., Xie F., Siedlak S. L., Nunomura A., Honda K., Moreira P. I., Zhua X., Smith M. A. and Perry G. (2004a) Neurofilament proteins in neurodegenerative diseases. *Cell Mol. Life Sci.* **61**, 3057–3075.

Liu X., Van V. T. and Schnellmann R. G. (2004b) The role of calpain in oncotic cell death. *Annu. Rev. Pharmacol. Toxicol.* **44**, 349–370.

Liu-Snyder P., Borgens R. B. and Shi R. (2006) Hydralazine rescues PC12 cells from acrolein-mediated death. *J. Neurosci. Res.* **84**, 219–227.

Mandic A., Viktorsson K., Strandberg L., Heiden T., Hansson J., Linder S. and Shoshan M. C. (2002) Calpain-mediated Bid cleavage and calpain-independent Bak modulation: two separate pathways in cisplatin-induced apoptosis. *Mol. Cell. Biol.* **22**, 3003–3013.

McHugh J. M. and McHugh W. B. (2004) Diabetes and peripheral sensory neurons: what we don't know and how it can hurt us. *AACN. Clin. Issues* **15**, 136–149.

Mizisin A. P., Calcutt N. A., Tomlinson D. R., Gallagher A. and Fernyhough P. (1999) Neurotrophin-3 reverses nerve conduction velocity deficits in streptozotocin-diabetic rats. *J. Peripher. Nerv. Syst.* **4**, 211–221.

Nakagawa T. and Yuan J. (2000) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. *J. Cell Biol.* **150**, 887–894.

Pop-Busui R., Sima A. and Stevens M. (2006) Diabetic neuropathy and oxidative stress. *Diabetes Metab. Res. Rev.* **22**, 257–273.

Posmantur R. M., Kampfl A., Liu S. J., Heck K., Taft W. C., Clifton G. L. and Hayes R. L. (1996) Cytoskeletal derangements of cortical neuronal processes three hours after traumatic brain injury in rats: an immunofluorescence study. *J. Neuropathol. Exp. Neurol.* **55**, 68–80.

Raynaud F. and Marcilhac A. (2006) Implication of calpain in neuronal apoptosis. A possible regulation of Alzheimer's disease. *FEBS J.* **273**, 3437–3443.

Razavi R., Chan Y., Afifiyan F. N. *et al.* (2006) TRPV1+ sensory neurons control beta cell stress and islet inflammation in autoimmune diabetes. *Cell* **127**, 1123–1135.

Russell J. W., Sullivan K. A., Windebank A. J., Herrmann D. N. and Feldman E. L. (1999) Neurons undergo apoptosis in animal and cell culture models of diabetes. *Neurobiol. Dis.* **6**, 347–363.

Russell J. W., Golovoy D., Vincent A. M., Mahendru P., Olzmann J. A., Mentzer A. and Feldman E. L. (2002) High glucose-induced oxidative stress and mitochondrial dysfunction in neurons. *FASEB J.* **16**, 1738–1748.

Sayers N. M., Beswick L. J., Middlemas A., Calcutt N. A., Mizisin A. P., Tomlinson D. R. and Fernyhough P. (2003) Neurotrophin-3 prevents the proximal accumulation of neurofilament proteins in sensory neurons of streptozocin-induced diabetic rats. *Diabetes* **52**, 2372–2380.

Schmeichel A. M., Schmelzer J. D. and Low P. A. (2003) Oxidative injury and apoptosis of dorsal root ganglion neurons in chronic experimental diabetic neuropathy. *Diabetes* **52**, 165–171.

Scott J. N., Clark A. W. and Zochodne D. W. (1999) Neurofilament and tubulin gene expression in progressive experimental diabetes: failure of synthesis and export by sensory neurons. *Brain* **122**(Pt. 11), 2109–2118.

© 2008 The Authors

Shibayama-Imazu T., Sonoda I., Sakairi S., Aiuchi T., Ann W. W., Nakajo S., Itabe H. and Nakaya K. (2006) Production of superoxide and dissipation of mitochondrial transmembrane potential by vitamin K(2) trigger apoptosis in human ovarian cancer TYK-nu cells. *Apoptosis* **11**, 1535–1543.

Shin C. Y., Shin J., Kim B. M., Wang M. H., Jang J. H., Surh Y. J. and Oh U. (2003) Essential role of mitochondrial permeability transition in vanilloid receptor 1-dependent cell death of sensory neurons. *Mol. Cell. Neurosci.* **24**, 57–68.

Srinivasan S., Stevens M. and Wiley J. W. (2000) Diabetic peripheral neuropathy: evidence for apoptosis and associated mitochondrial dysfunction. *Diabetes* **49**, 1932–1938.

Stys P. K. and Jiang Q. (2002) Calpain-dependent neurofilament breakdown in anoxic and ischemic rat central axons. *Neurosci. Lett.* **328**, 150–154.

Szoke E., Czeh G., Szolcsanyi J. and Seress L. (2002) Neonatal anandamide treatment results in prolonged mitochondrial damage in the vanilloid receptor type 1-immunoreactive B-type neurons of the rat trigeminal ganglion. *Neuroscience* **115**, 805–814.

Veldhuis W. B., van der S. M., Wadman M. W. *et al.* (2003) Neuroprotection by the endogenous cannabinoid anandamide and arvanil against in vivo excitotoxicity in the rat: role of vanilloid receptors and lipoxygenases. *J. Neurosci.* **23**, 4127–4133.

Vincent A. M., Olzmann J. A., Brownlee M., Sivitz W. I. and Russell J. W. (2004a) Uncoupling proteins prevent glucose-induced neuronal oxidative stress and programmed cell death. *Diabetes* **53**, 726–734.

Vincent A. M., Russell J. W., Low P. and Feldman E. L. (2004b) Oxidative stress in the pathogenesis of diabetic neuropathy. *Endocr. Rev.* **25**, 612–628.

Walker K. M., Urban L., Medhurst S. J., Patel S., Panesar M., Fox A. J. and McIntyre P. (2003) The VR1 antagonist capsazepine reverses mechanical hyperalgesia in models of inflammatory and neuropathic pain. *J. Pharmacol. Exp. Ther.* **304**, 56–62.

Zochodne D. W., Verge V. M., Cheng C., Sun H. and Johnston J. (2001) Does diabetes target ganglion neurones? Progressive sensory neurone involvement in long-term experimental diabetes. *Brain* **124**, 2319–2334.

© 2008 The Authors