DISTAL DEGENERATIVE SENSORY NEUROPATHY IN A LONG TERM TYPE 2 DIABETES RAT MODEL

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ABSTRACT

Objective: Peripheral neuropathy associated with Type 2 diabetes mellitus (DPN) is not widely modelled. We describe unique features of DPN in Type 2 Zucker diabetic fatty (ZDF) rats.

Research Design and Methods: We evaluated the structural, electrophysiological, behavioural and molecular features of DPN in ZDF rats and littermates over 4 months of hyperglycemia. The status of insulin signaling transduction molecules that might be interrupted in Type 2 diabetes, and selected survival, stress and pain related molecules was emphasized in dorsal root ganglia (DRG) sensory neurons.

Results: ZDF rats developed slowing of motor sciatic-tibial and sensory sciatic digital conduction velocity, and selective mechanical allodynia with preserved thermal algesia. Diabetic sural axons, preserved in number, developed atrophy but there was loss of large calibre dermal and small calibre epidermal axons. In diabetic rats, insulin signal transduction pathways in lumbar DRGs were preserved or had trends toward upregulation: mRNA levels of insulin receptor β subunit (IRβ), IRS-1 (insulin receptor substrate -1) and IRS-2. The numbers of neurons expressing IRβ protein were also preserved. There were trends toward early rises of mRNA levels of HSP27 (Heat shock protein 27), the α2δ1 calcium channel subunit and PI3-K (phosphoinositide 3-kinase) in diabetes. Others were unchanged including NFκ-B (p50/p105) and RAGE (receptor for advanced glycosylation endproducts) as was the proportion of neurons expressing HSP 27, NFκ-B and RAGE protein.

Conclusions: ZDF Type 2 diabetic rats develop a distal degenerative sensory neuropathy accompanied by a selective long term pain syndrome. Neuronal insulin signal transduction molecules are preserved.
The WHO (World Health Organization) estimates that 366 million people worldwide (up from 171 million in 2000) will suffer from diabetes by the year 2030 (1). Most have Type 2 diabetes and about half have evidence of peripheral polyneuropathy (DPN). Attention toward the mechanisms of DPN has largely focused on Type 1 models, for example, after streptozotocin (STZ)-induced β cell damage in rats and mice. In long term Type I models, however, important insights into the human disease have emerged. These have suggested a chronic neurodegenerative condition with gradual loss of distal motor and sensory terminals and features of neuropathic pain (2-7). The upregulation of selected “stress” related neuronal molecules may or may not accompany frank neuronal loss, depending on the model (7;8).

Work using Type 2 diabetic animal models has been carried out, but less frequently (9-11). These models are more expensive to maintain and only rarely are carried out to longer time points. One important question is whether this model exhibits features of DPN that include a progressive degenerative disorder manifested as selective loss and abnormality of distal terminal axons. Neuropathic pain is a critical element of the DPN phenotype but whether both thermal hyperalgesia and mechanical allodynia develop concurrently has been debated. Type 1 DPN models also exhibit heightened expression of insulin receptors in lumbar DRG and sensitivity to its action, even at low subhypoglycemic doses (12-15). Whether insulin signalling transduction machinery is preserved or downregulated in a Type 2 DPN model where initial high circulating insulin levels occur, is of interest. Finally, it is uncertain whether alterations in select neuronal indices of neurotoxic stress observed in Type 1 diabetes, also occur in a longer term Type 2 model.

In this work, we examine a long term model of Type 2 DPN, that of the Zucker Diabetic Fatty (ZDF) rat first described in work by Peterson and by Shaw and colleagues (16;17). Male obese Zucker diabetic fatty (ZDF; fa/fa or ZDF/Drt-fa; Charles River Labs) are homozygous for a missense mutation causing a non-functional leptin receptor (fa/fa) and control littermates are heterozygous (fa/+ ) lean males (18). ZDF rats develop obesity, initial hyperinsulinemia (insulin resistance) then pancreatic failure with overt diabetes after 8-10 weeks (19). Several papers have described neurological features including slowing of conduction velocity (10;11;20-22), alterations in sensory testing (10;21) and differences from the long term STZ model in autonomic ganglia (23). Here, we evaluate the impact of Type 2 diabetes on the phenotype and expression patterns of sensory neurons. The findings suggest a distal degenerative disorder resembling that of Type 1 DPN, with selective pain behaviour and preserved insulin signal transduction machinery.

METHODS

Model diabetes. Rats were purchased from Charles River Laboratories (Wilmington, MA) and shipped to the University of Calgary or University of Manitoba at 6 weeks of age. Cohorts were obese diabetic male ZDF/crl-lepr/fa and control male lean fa/+ . The onset of hyperglycemia was determined by weekly urine testing after receipt and developed at approximately 8 weeks of age. The rats were raised on Purina rat chow 5008. Blood glucose levels were measured using a glucose strip tester (One Touch Ultra, Lifescan, Johnson and Johnson, Burnaby, BC). All invasive procedures were carried out under pentobarbital anaesthesia (65 mg/kg). The protocols were reviewed and approved by the University of Calgary and University of
Manitoba Animal Care Committees using the Canadian Council of Animal Care guidelines.

**Electrophysiology, pain behaviour testing.** Motor conduction in sciatic-tibial fibers was performed by stimulating at the sciatic notch and knee while recording the M-wave (compound muscle action potential) from the tibial-innervated dorsal interossei foot muscles. Digital sciatic-tibial sensory conduction was made by stimulation of the digital nerves of the paw and recording over the sciatic nerve at the level of the popliteal fossa. All stimulating and recording used platinum subdermal needle electrodes (Grass Instruments, Astro-Med Inc., West Warwick, RI,) with near nerve temperature kept constant at 37 °C ± 0.5 °C using a heating lamp.

Mechanical withdrawal frequencies were measured using calibrated (4-26 g) von Frey monofilaments (Stoelting, Wood Dale, IL, USA) applied to the dorsum of the paw to a rat habituated within a plexiglass cage with holes in the flooring to allow application. Each paw was probed three times for approximately 1 second to the plantar surface with enough force to cause slight flexion of the monofilament and the amount of withdrawal was graded at 0 (none)-2 (maximum) for each probe. Summing these grades bilaterally yielded values ranging from 0 (no withdrawal to any of the probes) to 6 (maximum withdrawal to each probe): corresponding then to withdrawal percentages of 0%-100%. For thermal sensitivity, the latency of withdrawal of the hindpaw to an applied heat using the Hargreaves apparatus stimulus (24) was measured and the sum of the right and left latency calculated.

**Morphometry.** Sural nerves were harvested and processed as described in previous work (2). Samples were fixed in 2.5% glutaraldehyde in 0.025 M cacodylate buffer overnight. Semithin (1µm) sections of sural nerve and lumbar L4-6 DRGs were cut on an ultramicrotome (Reichert, Vienna, Austria) and were stained with toluidine blue. Morphometric analysis was carried out using a Zeiss Axioskop at 1000x magnification. Computer-assisted image analysis allowed for the determination of number and caliber of intact myelinated fibers. All morphological measurements were performed using Scion Image v.4.0.2 (Scion Corporation, Frederick, MD) by a single microscopist blinded to origin of the samples.

**Immunohistochemistry.** L5 DRGs were harvested, fixed in Zamboni’s preparation and mounted in O.C.T (Optimal Cutting Temperature; Tissue-Tek, Sakura-Finetek, Torrance, CA). Cryostat sections (14µm for DRG samples) were immunostained with primary antibodies: rabbit polyclonal antibody against insulin receptor β subunit (IR-β (C-19) 1:100 Santa Cruz); rabbit polyclonal antibody to HSP27 (1:100; Stressgen Biotechnologies, San Diego CA); rabbit polyclonal antibody to human RAGE (RAGE; 1:100; Gift from Dr. A. M. Schmidt, Columbia University NY); rabbit polyclonal antibody to NF-κB (NFκB p50 subunit (H-119) 1:100 Santa Cruz). For skin sections (25 µm thickness) we used a rabbit polyclonal antibody to PGP9.5 (PGP9.5; 1:1000 Cedarlane, UK). Nonspecific staining was blocked using 1% goat serum (excepting PGP 9.5 that used 10%). Secondary antibodies were anti-goat and rabbit 488 (1:400; Invitrogen, Burlington, Ontario) and donkey anti goat 488 (1:200; Invitrogen).

All analysis was conducted with the examiner blinded to the identity of the samples being studied. Neurons were counted in three sections through the midportion of the DRG to determine the total neuron numbers per transverse section,and the total number and percentage of neurons labeled with HSP27, RAGE and IR-β. Neurons with an arbitrary measurement of luminosity>70 were classified as immunoreactive. We also classified neurons as none-low (0-100), moderate (101-200) or high (>200) using
Adobe photoshop software version 7.0 (Adobe, San Jose, CA). Specifically for NF-kB p50 we examined three sections through the midportion of the DRG and determined the mean total neuron number per transverse section, the mean number of neurons with nuclei per transverse section and the total number (and percentage) of neurons labeled with NF-kB p50 subunit with luminosity>70 (including that of the cytoplasm, nucleus or both). A caveat to the analysis was that nuclei in general were more likely to be identified if labelled, probably falsely elevating the overall percent of positive nuclei in both groups. We calculated the percentage of positive nuclei out of the total number of nuclei identified for both groups. For footpads dermal analysis was carried out after labelling with an antibody to Nf200, the heavy subunit of neurofilament (mouse monoclonal anti-Nf200; 1:200, Sigma, St. Louis MO). Six fields per section and four sections per animal underwent quantitative analysis: Both live images and computer-captured images were used for counting but live images were preferred for the analysis. A mean value per rat and per test group was calculated.

The epidermal fibers were counted in six sections per rat (five papillae fields were examined in each section for a total of 30 papillae per rat) and we determined the number of epidermal fibers including axons directed vertically or horizontally (separately or combined). We measured the areas (mm²) for each papilla using Image J software (NIH, Bethesda, MD). Images were captured using an Olympus laser scanning Confocal microscope equipped with epifluorescence 60x magnification and the scanning step size was 1µm.

**qRT-PCR.** Real-time quantitative RT-PCR was performed on the ABI Prism7000 sequence detection system (Applied Biosystems, Foster City, CA). Total RNA was extracted from rat lumbar L3 and L6 DRG using Trizol reagent according to the manufacturer’s protocol (Invitrogen, Inc., Burlington, Ontario, Canada). One (1) µg of DNAsel 1-treated total RNA was used to synthesize first strand DNA utilizing SuperScript II First-strand Synthesis Kit (Invitrogen, Inc). Random hexamers (0.9 µg) were utilized as per the manufacturer’s protocol. First strand DNA (0.05 µl) was then used for PCR reactions. Quantification of the amplified product was done on a cycle-by-cycle basis through the acquisition of a fluorescent signal generated by binding of the fluorophore SybrGreen I (Invitrogen, Carlsbad, CA) to double-stranded DNA. The cycle number was determined at which the fluorescence signal crossed a fixed threshold (threshold cycle [CT]). The volume of the PCR mixture was 30 µl comprising 1U Taq DNA polymerase mixture (Invitrogen, Carlsbad, CA), 3 mM MgCl₂, 1.25 µM concentrations of primers of interest (Invitrogen, Carlsbad, CA), and 5 µl of cDNA. The amplification programs were performed as follows: (a) heating at 95°C for 10 min; (b) 40 cycles of 95°C for 15 s and 60°C for 1 minute. A melting curve was acquired by heating the product and the fluorescent signal was collected as the last step to look for a signal from nonspecific products, particularly from primer dimers. The standards, as well as the samples tested, were run in duplicate. Amplification of the product was visualized in the quantification curve analysis. The primer sequences used were designed in Primer Express 2.0 (Applied Biosystems, Foster City, CA) and are given in Table 1. The unknown samples are compared against the calibrator sample to give relative gene expression using the Comparative Cₜ Method 2⁻ΔΔCₜ. Final results were analyzed by choosing cyclophilin as the housekeeping gene (HKG) and separately analyzing both the L3 and the L6 ganglia. As an additional control we also completed all assays using a second HKG, the 18s subunit of ribosomal RNA (rRNA). In the case of mRNAs
demonstrating changes or trends toward changes we were able to confirm that similar directions of change were also observed when using 18S rRNA. In the case of both HSP27 and IRS-2, upregulation was approximately 2-fold or greater in DRG from diabetic rats irrespective of the HKG or ganglia chosen, a consistent result we interpreted as meaningful.

**Analysis.** Data were calculated as mean ± standard error of the mean (SEM). Data were analyzed by one way ANOVA with Tukey’s multiple comparison posthoc test or using two tailed Student’s t-test comparisons (unless otherwise stated). In all tests, statistical significance was set at α 0.05.

**RESULTS**

**Model.** Diabetic rats developed significant and progressive hyperglycemia from baseline to 1 and 4 months of diabetes duration. At one month, ZDF diabetic rats had a greater weight than controls, but by the 4 month endpoint, nondiabetic lean control rats had a greater weight. Weights and glucose levels are given in Table 2.

**Progressive conduction slowing.** Diabetic rats developed progressive and significant slowing of both motor sciatic-tibial and sciatic digital sensory conduction velocities between the 1 and 4 month endpoints. There was a trend toward slower motor and sensory conduction in diabetic rats compared to nondiabetic littermates by 1 month that was significant by 4 months. Results are given in Figures 1a and 1b. The amplitudes of the compound muscle action potentials and sensory nerve action potentials did not differ between diabetics and nondiabetic controls (data not shown).

**Selective development of mechanical allodynia.** Diabetic rats demonstrated mechanical allodynia at the 4 month endpoint as illustrated by higher withdrawal scores to von Frey hair stimulation at the 2 highest of the 5 stimulus intensities studied (Figure 1c). The thermal withdrawal latency did not differ between diabetic and nondiabetic rats at the 4 month endpoint (Figure 1d). Overall the findings confirmed a selective phenotype of pain behaviour in diabetic rats.

**Retraction of distal sensory terminals.** The number of myelinated axons, or fibers (MFs) in the sural nerve was comparable between diabetic and nondiabetic rats (Figure 2a). Diabetic sural nerve axons demonstrated axonal atrophy with shifts of MFs to smaller axon size categories (Figure 2b). The mean axonal area (µm²) was 15.0± 1.0 (n=5) in controls and 11.9±1.1 (n=7) in diabetics; p=0.04, one tailed Student’s t-test).

In contrast to sural axon counts, analysis of large axon innervation of the dermis of the foot pad using an antibody directed against neurofilament (Nf200) illustrated reductions in diabetic rats (Figure 2c,d). The most terminal portions of the sensory axons in the epidermis were labelled with PGP 9.5. In diabetic rats there was a reduction of the density of epidermal axons (Figure 3a,b).

**Sensory neuron expression of insulin receptor signal transduction pathway.** In other models of DPN and after axotomy injury to a peripheral branch of a sensory neuron, expression of insulin receptors (IRs) increases and renders the associated signalling pathways more sensitive to the trophic properties of the peptide. We measured overall mRNA levels of three key elements of the insulin receptor signal transduction pathway by qRT-PCR: IRβ, IRS-1 and IRS-2 (Figure 4a). The mRNA levels of L6 DRGs had nonsignificant trends toward higher IRβ in diabetics than nondiabetics. The mRNA levels of IRS-1 also demonstrated a nonsignificant trend toward higher levels in diabetic L6 DRGs. For IRS-2 there were trends toward rises in its relative mRNA expression in L6 ganglia and a significant rise in its expression in the L3 ganglia. To address the proportion of neurons expressing IRβ, one of the subunits of the insulin receptor, we
counted the proportion of sensory DRG neurons with low, medium or high intensity expression by immunohistochemistry. There was no difference in the percentage of neurons expressing IRβ at the varying ranges of intensity (Figure 4b,c).

**Sensory neuron expression of selected survival and stress-related pathways.** There are a number of survival and protective molecules that are upregulated in other models of DPN including HSP 27, PI3K and Akt. RAGE and NF-κB have been linked both to cell damage but also survival and are also upregulated in other models of DPN (25;26). To address their expression we measured overall mRNA levels and counted sensory neurons expressing selected marker proteins. We identified trends toward rises in RAGE, NF-κB [p50/p105] (borderline trend), and the α2δ-1 calcium channel subunit mRNAs in L6 ganglia (but not L3) of diabetic rats (Figure 5a). NF200, CGRP, caspase-3, Akt and GLUT4 mRNAs were not significantly different between diabetic and control animals (data not shown). There was a significant rise in the mRNA expression of PI3 K in L6. For HSP27 there were rises in its relative mRNA expression in the L3 ganglia and a trend toward an increase in the L6 ganglia.

The percentage of L5 DRG sensory neurons expressing HSP27 and RAGE proteins at low, medium and high intensity levels detected using immunohistochemistry were comparable between diabetic and nondiabetic rats (Figure 5b,c). For NF-κB p50 subunit, there were nonsignificant trends toward a rise in the percentage of neurons expressing this protein in either the L5 DRG cytoplasm or nucleus (Figure 5b,c).

**DISCUSSION**

In this work, we first confirmed that a long term type 2 diabetic animal model exhibits progressive nerve conduction slowing, an electrophysiological feature of DPN in rats, mice, other models and humans. Secondly, we confirm that pain behaviour is a feature of this model, as in humans, but that it was both modality specific and present at a long term endpoint. Thirdly, we demonstrate that DPN is a distal neurodegenerative disorder with involvement of dermal and epidermal axons but preservation of proximal components of the sensory axis. Fourthly, we show that insulin signal transduction pathways are preserved or upregulated in the sensory neurons of this model. Finally, we suggest that there may also be early alterations in mRNAs of selected neuronal “stress” and survival pathways in this model.

The electrophysiological findings confirm the place of this model among others with similar findings. Unlike the STZ-induced Type I-like model, both hyperglycemia and conduction slowing develop more gradually. Progressive declines in CMAPs and SNAPs were not observed, but have only been consistently identified in STZ mouse models (4;27).

In previous investigations, Oltman and colleagues (11) compared ZDF, lean littermates and also the Zucker parent strain that develops insulin resistance, hypertension and dyslipidemia without hyperglycemia. ZDF diabetic rats had declines in motor nerve conduction velocity, declines in nerve blood flow and changes in epineurial arteriole vascular relaxation. Insulin levels in the ZDF rats were 4-5 times that of lean controls and remained elevated to at least thrice normal levels by approximately 30 weeks of age, an age comparable with our endpoint measures of insulin receptors and IRS. While we did not measure insulin levels, these data were obtained on rats from the identical supplier and strain (Charles River). They indicate that our findings in insulin signal transduction molecules occurred in the setting of elevated, not reduced insulin levels.

Several papers have also addressed other Type 2 diabetic models in rats and mice. The BBZDR/Wor rat (9) had more mild
electrophysiological changes and structural changes of nerves than related BB/Wor Type 1 rats despite similar levels and durations of hyperglycemia. Further Type 2 models have included the TSOD mouse exhibiting severe axon and myelin changes after 14 months of diabetes (28), the Goto-Kakizaki (GK) rat that demonstrated motor nerve conduction slowing, mild axonal atrophy, low level demyelination and axonal degeneration at 18 but not 2 months of diabetes (29), the db/db mouse with slowed sciatic nerve conduction velocity, axonal atrophy and epidermal axon fiber loss up to 6 months of age (30;31) the leptin deficient (ob/ob) mouse with motor nerve conduction slowing, thermal hyperalgesia, mechanical allodynia and loss of epidermal axons at 11 weeks of age (32).

The diabetic rats exhibited substantial mechanical allodynia without evidence of thermal hyperalgesia. While controversial, several investigators have found a similar pattern of more robust mechanical allodynia than thermal hyperalgesia (33;34). Courteix and colleagues observed thermal hyperalgesia at 4 weeks in STZ-diabetic rats but analyzed tail immersion, potentially targeting more distal tail cutaneous axons (35). Calcutt has argued that some of these discrepancies may arise from testing methodology and the duration of diabetes (36). Dobretsov and colleagues have suggested that hyperalgesia may relate better to impaired insulin signalling than hyperglycemia (37). In the current study, despite a long term endpoint (4 months), pain behaviour had not disappeared in favour of simple sensory loss, despite significant, but incomplete epidermal denervation. By contrast, the long term STZ-diabetic mouse models exhibit earlier mechanical and thermal hyperalgesia eventually followed by loss of sensation (27) and a separate investigation of ZDF rats identified loss of thermal sensation, albeit with a different food source and a slightly longer duration (28 weeks compared to 24 weeks)(21). Thus, we think it unlikely that thermal testing and mechanical testing have inherently different sensitivity. Thermal stimuli do however, penetrate more deeply than the epidermis and may trigger responses from dermal nociceptive afferents that are better preserved.

We also noted a trend toward upregulation of mRNAs for the α2δ-1 calcium channel subunit, an auxiliary VGCC protein that modulates calcium channel activity and is widely expressed in most DRG sensory neurons (38). This is particularly relevant given the binding of gabapentin and pregabalin to this channel in mediating analgesia during neuropathic pain (39). Other specific ion channels linked to diabetic neuropathic pain, not examined here include Cav3.2 T-type calcium channels, and several sodium channels: Nav1.3 (upregulation), Nav1.7 (upregulation, increased tyrosine phosphorylation), Nav1.6 and Nav1.8 (both downregulated but with increased serine/threonine phosphorylation; Nav 1.6 also had increased tyrosine phosphorylation) (40), Nav1.9 (upregulated) and β3 (upregulated) (41;42).

Sural myelinated axon counts did not identify evidence of more proximal perikaryal or axon dropout. In the skin, however, we provide evidence of both dermal and epidermal axon loss. Epidermal axon loss has been described in several DPN models including the long term STZ-diabetic mouse (3;43); dermal loss has only been reported previously in mice, along with loss of Merkel cells (44;45). We deliberately investigated larger calibre axons that express the neurofilament marker and confirmed that DPN is not exclusively confined to small axons in this Type 2 model. While microvascular changes are a recognized feature of ZDF rats, we chose not to emphasize its role in DPN in this work. Changes in nerve blood flow do not provide a satisfying explanation of the phenotype
identified and the issue has been extensively discussed elsewhere (46).

We chose qRT-PCR as a sensitive measure of early changes that might exist in the mRNA population in lumbar DRG in response to diabetes. qRT-PCR can be extraordinarily sensitive to artifact such that we chose several types of controls. Samples without expression of a “housekeeping gene” (HKG) were excluded, and since diabetes might alter HKG expression, we chose two separate markers not known to be altered by diabetes: 18S rRNA and cyclophilin, emphasizing the latter. We were skeptical of results with a less than 2-fold change in expression. While we might expect L3 and L6 ganglia (L5 were used for immunohistochemistry and we did not pool ganglia, an approach sometimes used) to show parallel changes in expression, they do have different innervation territories, a possible reason for different patterns of change. It is important to recognize that mRNA changes, especially mild ones, may simply represent trends in gene expression without changes in overall protein content. The overall changes we observed in mRNA levels and protein were concordant: mild trends toward mRNA upregulation but generally preserved proportions of protein expression in neurons.

Our list of molecules was highly selected based on previous work in Type 1 models but each had a specific rationale. We had the most confidence in the changes seen in IRS-2 and HSP-27 since the direction of change was consistent in both ganglia, greater than 2-fold, seen with two HKGs and statistically significant in at least one ganglia level. Regrettably we did not have a suitable antibody to address protein expression of IRS-2 whereas HSP-27 protein expression was observed in a similar proportion of sensory neurons between diabetic and control animals. In previous work we have identified rises in HSP-27 mRNA by ISH in long term Type 1 STZ diabetic rats and by immunohistochemistry in motor neurons of Type 1 diabetic mice (2;4).

**HSP27 (heat shock protein 27)** is a molecular chaperone that refolds of denatured proteins, and promotes the survival of injured neurons. It does this by inhibiting cytochrome c mediated apoptosis(47), stabilizing mRNA, and stabilizing the neuron cytoskeleton(48;49).

Overall, our ZDF rats had relatively mild DPN, without declines in mRNAs for Nf200 or CGRP, features of Type 1 models (2;50). Had our ZDF rats been taken out to longer time points, it seems possible that these critical structural proteins would have been altered by diabetes. This would be a difficult undertaking however, since ZDF rat mortality significantly rises by the 4-5 month timepoint evaluated in this study.

The preservation and trends toward upregulation of insulin signal transduction machinery illustrate that such pathways remain available for input despite the gradual loss of ligand known to occur with pancreatic β cell failure. The upregulation of IRβ resembles the changes noted in other Type 1 models of DPN where ligand deficiency may play a role in inducing a counter-regulatory enhancement of insulin receptor expression. Alternatively, axotomized axons also upregulate their expression of IRβ and the trend toward change may reflect a response to distal axon loss. It is uncertain if the rises in IRβ and IRS-2 we observed contribute to or compensate for neuronal abnormalities.

**ACKNOWLEDGEMENTS**

Brenda Boake provided expert secretarial assistance. D.W.Z. is supported as a Scientist of AHFMR. The work was supported by CIHR, CDA and AHFMR.
REFERENCES


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### TABLE 1. PRIMER SEQUENCES

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<th>Gene</th>
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### TABLE 2. WEIGHTS

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<td>4.5± 0.2 (15)</td>
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<tr>
<td>Control 4 months</td>
<td>447±8 (18)</td>
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<tr>
<td>Diabetic baseline</td>
<td></td>
<td>4.5± 0.3 (15)</td>
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<tr>
<td>Diabetic 1 month</td>
<td>334±6*(23)</td>
<td>10.0± 0.6 (15)</td>
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<tr>
<td>Diabetic 4 months</td>
<td>397±10**(19)</td>
<td>12.6 ± 0.9 (15)</td>
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ANOVA p<0.0001;  
*diabetic vs. control p<0.01; **diabetic vs. control p<0.001  
(Tukey’s multiple comparison test post ANOVA)
FIGURE LEGENDS

Figure 1. Electrophysiological recordings and measurements of pain sensation
a: Measurements of conduction velocity in sciatic-tibial sensory axons. ZDF Type 2 diabetic rats exhibited slowing of conduction at 4 months after the onset of diabetes (*p<0.001 diabetic vs. control; controls n=13, month 1, 11 month 4; diabetics; n=4 month1, 15 month 4).
b: Measurements of conduction velocity in sciatic-tibial motor axons. ZDF Type 2 diabetic rats exhibited slowing of conduction at 4 months after the onset of diabetes (*p<0.001 diabetic vs. control; controls n=14, month 1, 11 month 4; diabetics n=4 month1, 15 month 4).
c: Measurements of sensitivity to mechanical stimulation in the sole of the hindpaw. ZDF Type 2 diabetic rats (DI) had a greater sensitivity to von Frey hair stimulation at 26g and 15g than control littermates, indicating allodynia (*p<0.02 diabetic vs. control; control n=8; diabetic n=14).
d: Measurements of sensitivity to thermal stimulation in the sole of the hindpaw. There was no difference in the withdrawal latencies between diabetics and controls (control n=8; diabetic n=14).

Figure 2. Sural myelinated axons and dermal axons
a: Morphometric analysis of sural myelinated axons shows no difference in the number of sural axons between diabetic and controls (controls n=5; diabetic n=7).
b: The histogram shows a shift in the calibre of diabetic myelinated axons to smaller size categories. The mean axonal area was smaller in diabetics compared to controls (data not shown, see text) (controls n=5; diabetic n=7).
c: Examples of dermal axons in the footpads of rats labeled with an antibody to the heavy subunit of neurofilament (Nf200). Controls are in the three upper panels and diabetics in the three lower panels. Note that the diabetic samples have fewer dermal axons identified. [Bar= 50 \( \mu \)m].
d: Analysis of dermal axon counts. All counts were carried out blinded to the identity of the samples. Diabetic ZDF rats had less dermal axons than nondiabetic controls (*p=0.05; n=4/group).

Figure 3. Epidermal innervation
a: Examples of epidermal axons in the footpads of rats labeled with an antibody to PGP 9.5, a panaxonal marker. Controls are in the upper three panels and diabetics in the lower three panels. Note that the diabetic samples have fewer epidermal axons identified. [Bar= 33 \( \mu \)m].
b: Analysis of epidermal axon density. All counts were carried out blinded to the identity of the samples. The gray bars indicate the density of vertically oriented axons (perpendicular to the epidermal surface) and the black bars indicate all epidermal axons irrespective of trajectory. Diabetic ZDF rats had less epidermal axons than nondiabetic controls (*p<0.01; n=6/group).
Figure 4. Insulin signal transduction pathway.
a: Analysis of DRG mRNA expression for insulin pathway signal transduction moieties. There were nonsignificant trends toward greater expression of IRβ subunits and IRS-1 in ZDF diabetic rats. In the L3 ganglia, there was a significant rise in the mRNA expression of IRS-2 (p<0.0001; n numbers for IRβ C=5L3, 5L6; D=7L3, 6L6; for IRS-1 C=5 L3, 6L6; D=7 L3, 5L6; for IRS-2 C=5L3, 3L6; D=6L3, 5L6).
b: Profile counts of neurons expressing IRβ subunit in the L5 DRG. There was no significant difference in the percentage of neurons with low, medium or high level expression between diabetic ZDF and nondiabetic rats (n=4/group).
c: Examples of L5 DRG sensory neurons expressing IRβ. [Bar=50 µm]

Figure 5. Survival and stress pathways.
a: Analysis of DRG mRNA expression for survival, stress and pain molecules involving neurons. Significant rises were identified in HSP27 mRNAs of L3 neurons (*p=0.01) and PI3-K of L6 neurons (*p=0.02) of diabetics with nonsignificant trends in the same direction for RAGE, α2δ-1 and NFκB (n numbers for HSP27 C=5L3, 4L6; D=5L3, 5L6; for PI3K C=5 L3, 6L6; D=5 L3, 5L6; for RAGE C=3L3, 6L6; D=7L3, 5L6; for α2δ-1 C=3L3, 6L6; D=5L3,5L6; for NFκB C=5L3, 3L6; D=7L3,5L6).
b: Profile counts of neurons expressing HSP27, RAGE or NFκB in the L5 DRG. There were no significant differences in the percentage of neurons with low, medium or high level expression between diabetic ZDF and nondiabetic rats. For NFκB, the percentage of all nuclei that were labeled is indicated (see caveat concerning percent positive nuclei in Methods) and the percentage of neurons in which cytoplasmic labeling was identified is indicated (n=4/group).
c: Examples of L5 DRG sensory neurons expressing HSP27, RAGE and NFκB. [Bar=50 µm].
Figure 1c

Withdrawal latency to thermal stimulation
Figure 2c

CONTROL

DIABETIC
Figure 2d
Loss of dermal axons in footpads of Type 2 ZDF diabetic rats

Number of dermal axons

Control  Diabetic
Figure 3a
Control

Diabetic
Figure 3b

Epidermal innervation

Density of epidermal axons (#/sq mm)

Nondiabetic  Diabetic

*  *

Distal Degenerative Neuropathy
Figure 4b

Insulin receptor beta expression L5

- Control
- Diabetic

Percentage of neurons

Low | Medium | High
Figure 4c

Diabetic

Control
Figure 5a

HSP27

NFκB

α2δ-1

PI3K

RAGE

Relative mRNA expression

L6 L3

Control Diabetic

Control Diabetic

Control Diabetic

Control Diabetic

Control Diabetic
Figure 5b

**HSP27**

- Control
- Diabetic

**RAGE**

- Control
- Diabetic

**NFκB**

- Control
- Diabetic

Percentage of neurons

Low  | Medium | High
---|---|---

Percentage of neuron cytoplasm or nuclei labelled:

- % nuclei
- % cytoplasm

\[ \text{Percentage of neurons} \]

\[ \text{Percentage of neuron cytoplasm or nuclei labelled} \]
Figure 5c

Diabetic  Control

HSP-27

RAGE

NFkB