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Oxidative Injury and Neuropathy in Diabetes and Impaired Glucose Tolerance

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Abstract

Clinical studies suggest that impaired glucose tolerance (IGT) is associated with the development of neuropathy. The aim of the current study was to determine if neuropathy developed in the female Zucker Diabetic Fatty (ZDF) rat, an animal model of IGT and type 2 diabetes. The ZDF rat develops impaired glucose tolerance (IGT) when fed a control diet, and frank diabetes when fed a high fat diet. Following 10 weeks of hyperglycemia, sensory nerve action potentials (SNAP) and compound motor action potentials (CMAP) were reduced and sensory conduction velocities were slowed (distal > proximal) in the tail and hind limb in ZDF animals with IGT and frank diabetes ($p<0.01$). Neuropathy was coupled with evidence of increased reactive oxygen species (ROS) and cellular injury in dorsal root ganglion (DRG) neurons from IGT animals. Our study supports the hypothesis that neuropathy develops in an animal model of IGT and is associated with evidence of oxidative injury in DRG and peripheral nerves.

Keywords

neuropathy; diabetes; impaired glucose tolerance; oxidative stress; axons; dorsal root ganglia; Schwann cells

INTRODUCTION

Impaired glucose tolerance (IGT) results in micro-and macrovascular complications in human patients (Singleton et al., 2003). Clinical studies indicate that IGT is a common cause of painful sensory neuropathy (Singleton et al., 2001; Singleton et al., 2001; Singleton et al., 2003; Smith et al., 2006). The nature of neuropathy resulting from IGT is unclear and may be due to episodic increases in serum glucose, or to a generalized metabolic disturbance ("metabolic syndrome") including dyslipidemia and inflammation. While oxidative stress-induced injury in the

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peripheral nervous system is well characterized in rodent models of diabetes (for review see (Vincent and Feldman, 2004), it is unclear if similar changes occur during IGT.

The Zucker Diabetic Fatty (ZDF) rat used in this study is a model of IGT and type 2 diabetes, exhibiting insulin resistance, hyperinsulinemia, dyslipidemia (Wahle and Radcliffe, 1976; Bray, 1977; Godbole and York, 1978) and other characteristics of metabolic syndrome. Rats homozygous for a glycine 269 to proline substitution in the leptin receptor are obese, while heterozygous animals are lean, and serve as control animals in experimental studies (Clark et al., 1983). Homozygous ZDF rats develop hyperphagia, hyperglycemia, hypercholesterolemia and hyperinsulinemia with insulin resistance at approximately 4–5 weeks of age (Clark et al., 1983; Johnson et al., 1990) (www.harlan.com). Male ZDF rats spontaneously progress from IGT to frank diabetes. Female ZDF rats are arrested at IGT demonstrated by abnormal glucose tolerance (Noland et al., 2007; Oltman et al., 2006; Turner et al., 2007). Diabetes is induced in female ZDF rats by diet manipulation. Feeding the female the GMI 13004 diet, composed of 48% fat and 16% protein, will induce frank diabetes within 14–21 days. Female ZDF rats fed a high fat diet (ZDF-HF, diabetic) gain weight for approximately 30 days followed by weight loss compared to female ZDF rats fed a normal diet (ZDF-N, IGT); however, both animals are severely obese compared to lean control rats.

Diabetic neuropathy (DN) is reported in ZDF rats and demonstrated by decreased motor nerve conduction velocity (MNCV), decreased sensory nerve conduction velocity (SNCV) and decreased levels of calcitonin gene-related peptide (Shibata et al., 2000; Shimoshige et al., 2000; Oltman et al., 2005; Li et al., 2006). All of these studies were performed in male diabetic ZDF rats; therefore this study is the first comparison of diabetes and IGT in the female ZDF rat.

Evidence from multiple sources indicates that oxidative stress due to hyperglycemia-induced generation of reactive oxygen species (ROS) is an important mechanism leading to both the development and progression of DN (Cameron et al., 1993; Nagamatsu et al., 1995; Hohman et al., 1997; Tomlinson, 1998; Stevens et al., 2000; Obrosova, 2002; Russell et al., 2002; Schmeichel et al., 2003; Vincent and Feldman, 2004). There is evidence of increased systemic oxidative stress in the ZDF rat (Chinen et al., 2007; Oltman et al., 2005; Oltman et al., 2006; Serkova et al., 2006; Sonta et al., 2004); however, an association between oxidative stress and the development of neuropathy is not yet established in the ZDF rat with IGT. We hypothesize that hyperglycemia induced ROS leads to injury of dorsal root ganglion (DRG) neurons. In support of this idea, we demonstrated *in vitro* that either inhibition of the glucose-induced generation of ROS at the level of the mitochondrial electron transfer chain or stabilization of the inner mitochondrial membrane potential blocks sensory neuron injury (Russell et al., 2002; Leinninger et al., 2004; Vincent and Feldman, 2004).

The current study explores the development of DN in female ZDF rats with either IGT or diabetes. We find that a significant polyneuropathy develops both in animals with IGT as well as in diabetic animals and that there is a positive association between neuropathy and oxidative injury in the peripheral nervous system. These data confirm the presence of DN in an animal model of IGT, supporting the recent contention that IGT contributes to DN in human patients.

METHODS

Materials

Antibodies against 4-hydroxynonenal (HNE) and malondialdehyde (MDA) were purchased from Abcam (Cambridge MA), 3-nitrotyrosine (3-NT) from Cayman Chemicals (Ann Arbor MI), and AlexaFluor 488 from Molecular Probes (Carlsbad CA). Anti-caspase-3 antibody was purchased from Pharmingen (San Diego CA). All other chemicals were purchased from Sigma

Chemical Co (St. Louis MO). Electrodes for electrophysiology were purchased from Oxford Instruments and a Viking IV recording machine (Nicolet Biomedical VQ, Madison WI) was used.

Animals

Female ZDF *fa/fa* rats (GMI ZDF drt/fa) were purchased from Genetic Models Inc. (Indianapolis, IN) and, at 8 weeks of age, were fed either a control diet (Purina 5008 lab chow, Purina, Richmond, IN) or a high fat GMI 13004 diet (Genetic Models, Inc., Indianapolis, IN) consisting of 48% fat and 16% protein. Animals were divided into 3 groups of 8 animals: 1) lean control animals fed a high fat GMI 13004 diet (lean control), 2) ZDF rats fed a normal Purina 5008 diet (ZDF-N), and 3) ZDF rats were fed a high fat GMI 13004 diet (ZDF-HF). Rats were housed in a pathogen-free environment, with continuous access to food (see above) and water on a 12-hour light—dark schedule and were cared for following the University of Michigan Committee on the Care and Use of Animals guidelines.

Blood glucose was monitored weekly using a standard glucometer (HemoCue Glucose Analyzer, Lake Forest CA). Concurrently, blood was collected for serum insulin, cholesterol, free fatty acid and triglyceride determinations. Insulin values were determined by ELISA per the manufacturer's protocol (Alpco, Salem NH). Triglyceride, free fatty acid and cholesterol values were determined by colorimetric assay per the manufacturer's protocol (Wako Chemicals, Richmond VA). All were performed in accordance with national guidelines.

Oral Glucose Tolerance Tests

An oral glucose tolerance test was performed by administering an oral glucose load (2g/kg glucose by gavage) and measuring blood glucose at 0, 30, 60 and 120 minutes after dosing. Blood was collected from the tail vein and analyzed as described above.

Nerve Conduction Studies (NCS)

Rats were anesthetized with ketamine 60 mg/kg and acepromazine 0.75 mg/kg by intraperitoneal injection. The hind limbs were kept injection free to avoid nerve or muscle injury. NCS were performed in the tail and left hind limb using platinum electrodes adjacent to the nerve to obtain near nerve recordings. The G1 (active) and G2 (indifferent) recording electrodes were separated by a distance of 10 mm. Tail NCS were recorded over a 9 cm distance measured from the base of the tail. Proximal nerve conduction velocities were measured over the proximal 5 cm from the base of the tail, and distal nerve conductions over the terminal 4 cm. Orthodromic tail compound muscle action potentials (CMAP) were obtained by recording at the tip of the tail and stimulating with the cathode 4 and 9 cm proximal to the G1 recording electrode. In the hind limb, CMAP responses were recorded from the dorsum of the foot with stimulation at the malleolus and proximally at the sciatic notch. Digital sensory responses were obtained by stimulating the second digit of the hind limb and recording 3.0 cm proximal to the cathode in the foot. All sensory responses were averaged to obtain a stable waveform. CMAP and sensory nerve action potential (SNAP) amplitudes were measured from onset of the negative portion of the waveform to the peak of the negative response. Temperatures and stimulus intensities were as previously described (Russell JW, 2006).

Tissue Harvest

Animals were deeply anesthetized with sodium pentobarbital 50 mg/kg i.p. and fixed by intracardiac perfusion with 500 ml of 4% paraformaldehyde 0.1 M phosphate buffer (PBS, 0.1M, pH 7.2). DRG and sciatic nerves were dissected and post-fixed overnight in the same fixative. The tissue was rinsed in PB containing 5, 10 and 20% sucrose prior to cryoembedding in OCT.

Neuron Counting and Pixel Intensity Measurements

Cryosections (3 microns) from lumbar DRG were placed in phosphate buffered saline (PBS, 0.1M, pH 7.2) for 5 min, followed by 10 min in 0.1% TX100/PBS containing 2% nonfat dry milk and 1% normal serum to reduce non-specific adherence of antibodies. Sections were incubated with anti-HNE or anti-MDA polyclonal antibodies at 1:1000 for 24 h at 22°C in a humid chamber. Sections were rinsed 3 times with PBS followed by 1 h incubation with the appropriate secondary IgG conjugated to AlexaFluor 488. The sections were rinsed and coverslips applied with Prolong mounting media (Invitrogen, Carlsbad CA). Images of the DRG were obtained using a Spot-RT digital camera and MetaMorph software using equal gain, exposure time and acquisition between slides. Only neurons with clearly identifiable nuclei were counted. Sections were separated by 30 μm to avoid double counting. All neurons in two consecutive DRG sections were counted (>100 neurons were counted per animal per experiment) and the results averaged for the two sections and then for each animal. Two separate experiments were performed (total number of sections > 4). The pixel intensity, neuronal area, and diameter were recorded. Area vs. intensity histograms were prepared for comparison between the groups. Neuron number is expressed as the number of neurons/μm^2 of DRG section.

Detection of Cellular Injury

DRG sections were incubated with rabbit anti-CM1 antibody (1:5000 of a 0.410 mg/ml working stock) as described above (Russell et al., 1999). The nuclear chromatin was counterstained with 1 mg/ml bis-benzamide in PBS. The number of apoptotic neurons was determined using a random grid counting system (Russell et al., 1999) on a Zeiss axiophot 2 microscope using a 40 X objective. Five fields per slide were counted on 2 slides, and new sections from each animal examined four times to ensure inter-observation reliability. Only neurons with clearly identifiable nuclei were counted. Nuclei were viewed with an ultraviolet filter. Each field was viewed in differential interference contrast (DIC) mode, under an ultraviolet filter and a rhodamine filter. Antibody specificity was confirmed by immunoabsorption with peptide which when applied to tissue sections, immunoreactivity was not observed.

To determine the percentage of cells with damaged DNA, DRG and sciatic nerve sections were processed for TUNEL labeling (digoxygenin-dUTP) as previously described (Russell et al., 1999). For each experiment, positive controls included treating sections with DNase 1 and negative controls with PBS substituted for TdT. Determination of TUNEL positive neurons and SC was performed as previously described (Russell et al., 1999).

Statistical Analysis

Assumptions about the Gaussian distribution of data and rules for transformation of nonnormative data were made as previously described (Russell et al., 1999; Russell et al., 2002). Comparison of dependent variables was performed using factorial analysis of variance (ANOVA) with 95% confidence intervals. An observer blinded to the experimental condition made measurements. Bar graphs illustrate the mean ± standard error of the mean.

RESULTS

Weight and Glucose Regulation in the ZDF Rat

Circulating insulin levels in the ZDF-HF and ZDF-N animals were elevated compared to lean control animals that partially express the leptin receptor. The ZDF female rat developed hyperinsulinemia and hyperlipidemia (Table 1). In ZDF-HF animals, free fatty acid levels were not significantly increased compared to baseline (3.83 ± 0.23 mg/dl) 5 weeks but fell by the end of the study. Furthermore, in ZDF-HF animals, cholesterol levels did not increase at 5

weeks (42.11 ± 4.54 mg/dl), but doubled at 10 weeks. In contrast, ZDF-N animals showed a two-fold increase in free fatty acids and a five-fold increase in cholesterol levels by the end of the study. In comparison to changes in lipid levels, insulin levels increased five-fold in ZDF-HF animals and only one and a half fold in ZDF-N animals after 10 weeks.

Female ZDF animals progressively became obese irrespective of the type of diet they are fed (ZDF-HF and ZDF-N). In contrast, lean control animals gained weight normally even on a high fat diet, but did not become obese. Lean control animals were significantly smaller on average compared to ZDF-N and ZDF-HF animals both at baseline and at 10 weeks ($p<0.001$); however, there was no significant difference in the weights of ZDF-N and ZDF-HF animals. Female ZDF animals developed diabetes when fed a high fat diet (ZDF-HF, Fig. 1B). ZDF animals fed a control diet (ZDF-N) developed impaired IGT and abnormal glucose tolerance (Fig. 1C). Lean control animals fed a control diet maintained euglycemia (Fig. 1C).

Diabetes and IGT are Associated with Neuropathy in the ZDF Rat

In ZDF female animals with diabetes (ZDF-HF) or IGT (ZDF-N), there was a decrease in SNAP amplitudes, measured in the distal digit, and sensory conduction velocity (SCV) compared to lean controls (Fig 2A and B). Measurements were made in animals following 10 weeks on their respective diets (8 weeks of diabetes). NCS abnormalities were more severe in ZDF-HF animals than ZDF-N animals, but both were significantly affected compared to lean controls. In general, sensory conduction velocities were more severely affected than amplitudes, especially in the digit. Both were abnormal and consistent with an axonal polyneuropathy (Fig. 2A and B)

In addition to the sensory abnormalities, motor amplitude was reduced in the tail and hind limb, proximal motor conduction velocity (MCV) was slowed, and distal latency was prolonged in ZDF-HF and ZDF-N animals (Fig. 3A–C). NCV slowing was greater in the sciatic nerve than the smaller dorsal tail nerve/muscle group. Both tail and hind limb CMAP amplitudes were more severely reduced in ZDF-HF animals than in ZDF-N animals but the differences between the two groups were not statistically significant. In ZDF-HF animals, the distal motor latencies (DML) were more severely affected compared to the proximal conduction velocity, consistent with a length-dependent distal axonal neuropathy, similar to that observed in human diabetic neuropathy. In contrast, the minimal F-wave latency that measures proximal nerve and nerve root conduction was essentially unchanged between the different groups (Fig. 3C).

Reduced tail distal sensory conduction studies at the end of the study showed a modest association with the corresponding serum glucose (R=0.4), cholesterol levels (R=0.5), and insulin levels (R=0.5). Reduced hind limb proximal motor conduction studies showed a similar association with serum glucose (R=0.4) and cholesterol (R=0.7), but in contrast to sensory responses, there was also a stronger association with triglyceride (R=0.8) and free fatty acid (R=0.6) levels. The reduction in the tail distal sensory nerve conduction velocity showed a moderate association with evidence of PCD and specifically, DRG TUNEL staining (R = 0.6). The tail distal sensory amplitude was mildly to modestly associated with serum glucose (R = 0.4), cholesterol (R = 0.3), triglycerides (R = 0.4), free fatty acids (R = 0.4), insulin (R = 0.5), TUNEL (R = 0.2). The hind limb distal motor amplitude was moderately associated with serum glucose (R = 0.7), cholesterol (R = 0.5), triglycerides (0.3), free fatty acids (0.6), insulin (R = 0.6), TUNEL (R = 0.6).

Diabetes Induces Formation of ROS in DRG Neurons

In L5 DRG neurons from ZDF animals, there was an increase in MDA (Fig. 4 A–C and G) and HNE (Fig. 4 D–F and H) immunoreactivity consistent with evidence of oxidative stress (Fig. 4). MDA immunohistochemistry of DRG resulted in a mean pixel density of 989.6 ± 27.4

in lean control DRG sections and 2,887.8 ±80.4 DRG sections from ZDF-HF animals at identical time points. Minimal background reactivity and autofluorescence were detected. DRG sections that were not processed for immunohistochemistry were used to set the thresholds used in this study. In the largest DRG neurons (>1,500 μm^2) from ZDF rats, mean MDA (Fig. 4 A–C and G)levels were greatest in diabetic (253.3 ± 9.1), compared to ZDF-N (209.0 ± 7.2) and lean control (194.9 ± 18.5) animals.

Diabetes and IGT Induce Neuronal Cellular Injury

In order to determine if impaired glucose regulation is associated with cellular injury, activated caspase-3 immunoreactivity (Fig. 5) and TUNEL (Fig. 6) were measured in DRG neurons and SCs. In ZDF-HF rats, 7% of DRG neurons and 4% of SC showed evidence of caspase-3 cleavage, compared to less than 2% in neurons ($p<0.01$) and 0.1% in SC from controls, and 5% in neurons and 3% in SC from ZDF-N animals ($p<0.01$). 20% of neurons and 8% of SC were TUNEL-positive in ZDF-HF animals, compared to 1% in neurons and <2% in SC in controls ($p<0.001$). In ZDF-N animals there were 14% positive neurons and 5% positive SC.

Quantitation of DRG Neuronal Size and Number

Quantitation of DRG neuronal size and number was performed. The number of largest neurons (>1,500 μm^2) in diabetic ZDF-HF animals decreased by 15% compared to controls ($4.45.10^{-5}/\mu m^2$ compared to $5.22.10^{-5}/\mu m^2$) and a 17% decrease was detected in the IGT ZDF-N animals compared to lean controls ($4.43.10^{-5}/\mu m^2$ compared to $5.22.10^{-5}/\mu m^2$). There was no statistically significant difference in the overall number of neurons, large or small, in any group (Table 2).

DISCUSSION

The female ZDF rat used in the current study is an excellent model for both IGT and type 2 diabetes. These animals become obese and develop hyperglycemia, dyslipidemia hyperinsulinemia and are insulin resistant (Wahle and Radcliffe, 1976; Bray, 1977; Godbole and York, 1978). Both the diabetic ZDF-HF and IGT ZDF-N animals develop a sensorimotor neuropathy with evidence of oxidative stress and subsequent neuronal damage. These metabolic and neuropathic derangements mirror those observed in type 2 diabetic patients. The current study confirms previous reports of DN in the ZDF rat; however, we report here for the first time that animals with IGT develop a significant axonal neuropathy.

It has been recognized for almost 50 years that neuropathy may be the initial presentation of early diabetes (Ellenberg, 1958). More recently, it has been demonstrated that diabetic microvascular complications including neuropathy occur concurrently with impaired glucose regulation (IGT and impaired fasting glucose) well in advance of diabetes diagnosis (Singleton et al., 2001; Singleton et al., 2001; Singleton et al., 2003). Up to 50% of patients with idiopathic sensory neuropathy have IGT or impaired fasting glucose (Singleton et al., 2001; Singleton et al., 2001) significantly higher than the prevalence in an age-matched general population. In human patients, neuropathy associated with IGT presents as a distal symmetrical sensory polyneuropathy with prominent neuropathic pain, similar to early diabetic neuropathy (Singleton et al., 2001; Singleton et al., 2001; Singleton et al., 2003; Russell JW, 2006; Smith et al., 2006). Electrophysiology findings in human patients reveal a mixed distal sensorimotor axonal polyneuropathy, similar to our data, supporting our hypothesis that the female ZDF-N rat is a good animal model of IGT and neuropathy.

DN in human patients is primarily a sensory neuropathy. Reduction of both SNAP amplitudes and sensory conduction velocity confirms the presence of a sensory neuropathy in the female ZDF-N rat. These reductions parallel those reported by Li et al, who also detected thermal and

mechanical allodynia and reductions in the neurotransmitter CGRP in male ZDF rats (Li et al., 2006). The decreases in motor conduction velocity and CMAPs detected in the female ZDF rat are also similar to those reported in male ZDF rats (Shibata et al., 2000; Shimoshige et al., 2000; Oltman et al., 2005; Li et al., 2006). Previous examinations of DN in the ZDF rat were performed only in frankly diabetic male animals, (Shibata et al., 2000; Shimoshige et al., 2000; Oltman et al., 2005; Li et al., 2006). We detect similar deficits in the female ZDF-N animals.

The cause of neuropathy in IGT is uncertain. Both type 1 and 2 diabetes, are associated with systemic oxidative stress paralleling that observed in complication prone tissues (Hayden and Tyagi, 2004; Maiese et al., 2007; Wright et al., 2006). In the current study, changes in both sensory and motor conduction velocities show the strongest association with increased serum glucose and cholesterol, although similar associations were observed for the corresponding amplitudes. Association between motor conduction abnormalities and triglyceride, free fatty acid levels, and insulin suggests that impaired nerve conductions in the ZDF animal model may be similar to that observed in humans with metabolic syndrome. These patients are at risk for diabetes and vascular complications (Alberti and Zimmet, 1998). This syndrome is also associated with insulin insensitivity and provides a further mechanism for induction of polyneuropathy including the neuronal and SC damage observed in this study. Insulin and insulin-like growth factor reverse DN in models of type 1 diabetes (Brussee et al., 2004; Xu et al., 2004; Toth et al., 2006) and insulin receptors are located within peripheral nerves of healthy rats (Sugimoto et al., 2000). Therefore, a relative reduction in insulin, insulin-like growth factor levels, or insulin insensitivity may be associated with induction of an axonal neuropathy and is implicated in mitochondrial dysfunction in sensory neurons (Huang et al., 2003; Brussee et al., 2004). In this study, ZDF animals with IGT or diabetes are hyperinsulinemic and hyperglycemic, consistent with reduced insulin sensitivity and/or signaling. However, insulin insensitivity alone is unlikely to explain the DRG neuroaxonal degeneration observed in this study. There is no evidence of neuroaxonal dystrophy in sympathetic ganglia or ileal mesenteric nerves in the ZDF rat, unlike the insulinopenic STZ rat (Schmidt et al., 2003).

Neurons and SC in the peripheral nervous system of ZDF rats may be more susceptible to oxidative stress, resulting from a series of events: impaired glycemic control, impaired mitochondrial function, and reduced insulin sensitivity. In this study, both diabetic (ZDF-HF) and IGT (ZDF-N) animals demonstrate an increase in markers of oxidative stress in DRG neurons compared to control animals. MDA and HNE are lipid adducts and among the most common markers of oxidative stress in biological systems (Vincent and Feldman, 2004). In this study, immunoreactivity for MDA and HNE was minimal in DRG neurons of lean control animals, with clear increases in DRG neurons from the diabetic ZDF-HF and IGT ZDF-N animals. This data supports the concept that increased oxidative stress occurs with IGT as well as frank diabetes and may contribute to the neuro-axonal dysfunction associated with neuropathy. Increased immunoreactivity was detected across all neuronal sizes in diabetic and IGT animals; however, levels of MDA were clearly increased in the largest DRG neurons, a population of cells reported to be lost in diabetic animals (Schmeichel et al., 2003). The role of oxidized lipids in inducing cellular damage is an ongoing area of investigation. Comparison of animal models exhibiting both hyperglycemia and dyslipidemia with either condition alone is the next step in dissecting the contribution of these metabolic derangements to the development of DN.

Evidence of oxidative stress is well documented in animal models of type 1 diabetes (Cameron et al., 1993; Nagamatsu et al., 1995; Hohman et al., 1997; Tomlinson, 1998; Stevens et al., 2000; Obrosova, 2002; Schmeichel et al., 2003). Under hyperglycemic conditions, increased metabolic flux in the mitochondria, coupled with loss of mitochondrial proton pump regulation

results in increased formation of ROS including peroxynitrite, superoxide and hydroxyl radicals (Brownlee, 2001; Vincent et al., 2002; Brownlee, 2005). These ROS are associated with membrane lipid peroxidation, nitration of proteins and DNA damage (for review see (Vincent and Feldman, 2004). Mitochondrial oxidative injury coupled with loss of insulin and neurotrophic support contributes to mitochondrial inner membrane depolarization in sensory neurons, ballooning of mitochondria, release of cytochrome c into the cytosol, and activation of caspases in DRG neurons (Russell et al., 2002; Schmeichel et al., 2003 Srinivasan et al., 2000; Cheng and Zochodne, 2003; Vincent et al., 2004) all of which are associated with neuronal or axonal injury (Sasaki et al., 1997; Fujimura et al., 1999; Russell et al., 1999; Kishi et al., 2002; Huang et al., 2003). In the current study, both DRG neurons and SC show evidence of caspase activation and positive TUNEL labeling. In parallel with evidence of cellular ROS, evidence of apoptosis is greater in DRG neurons than in SC. These findings support previous observations that SC are partially protected from cellular injury in mixed neuronal, SC, and axonal populations, potentially by increased glutathione antioxidant defense in SC (Berent-Spillson et al., 2004; Berent-Spillson and Russell, 2007) or differences in regulation of Bcl family members and other critical signaling pathways (Delaney et al., 2001).

A small decrease in the mean number of large DRG neurons was observed in both diabetic and IGT animals compared to controls; however, these differences were not statistically significant. An area of controversy is whether classical apoptosis in the peripheral nervous system is responsible for the neuropathic deficits observed in both animal models and human patients. Multiple studies report activation of caspases in DRG neurons both *in vitro* and *in vivo* (Russell et al., 1999; Srinivasan et al., 2000; Kishi et al., 2002; Russell et al., 2002; Cheng and Zochodne, 2003; Schmeichel et al., 2003; Vincent et al., 2004). Others detect some loss of DRG (Russell et al., 1999; Zochodne et al., 2001; Kishi et al., 2002) and in particular there is a statistically significant loss of large DRG neurons (Kishi et al., 2002). Using rigorous counting techniques, Zochodne et al, concluded that there was no significant loss of neurons in the DRG of diabetic animals (Zochodne et al., 2001); although this study did detect a 14.5% decrease in the mean number of neurons (determined by nuclei). There is clear evidence that not all neurons are affected equally and, as with human patients, not all rodents develop the same degree of neuropathy or the same degree of neuronal injury. Even though there is evidence of a small DRG neuronal loss, the number of DRG neurons showing evidence of oxidative injury is greater than the measured loss of neurons. This may occur because activation of caspases does not invariably result in neuronal death, or because of an intrinsic capacity for repair within the neuron (Sayers et al., 2003). However, overall the pathology points to a chronic dysfunction of neurons under diabetic conditions.

In summary, our study demonstrates the presence of neuropathy in animal models of type 2 diabetes and IGT. Our results suggest that impaired glucose regulation coupled with dyslipidemia induce neuropathic injury. There is evidence of nerve conduction slowing, increased oxidative stress, and activation of cell death pathways that affect both DRG neurons and SC, although DRG neurons are more severely compromised. While all three parameters are less severely affected in IGT compared to diabetic animals, there are clear abnormalities in IGT animals compared to controls. Our findings indicate that even mild, intermittent hyperglycemia coupled with other metabolic derangements can lead to peripheral nervous system injury and the development of neuropathy. Our studies support the idea that IGT may cause neuropathy in humans and highlight the importance of screening for diabetes in patients presenting with “idiopathic” sensory neuropathy.

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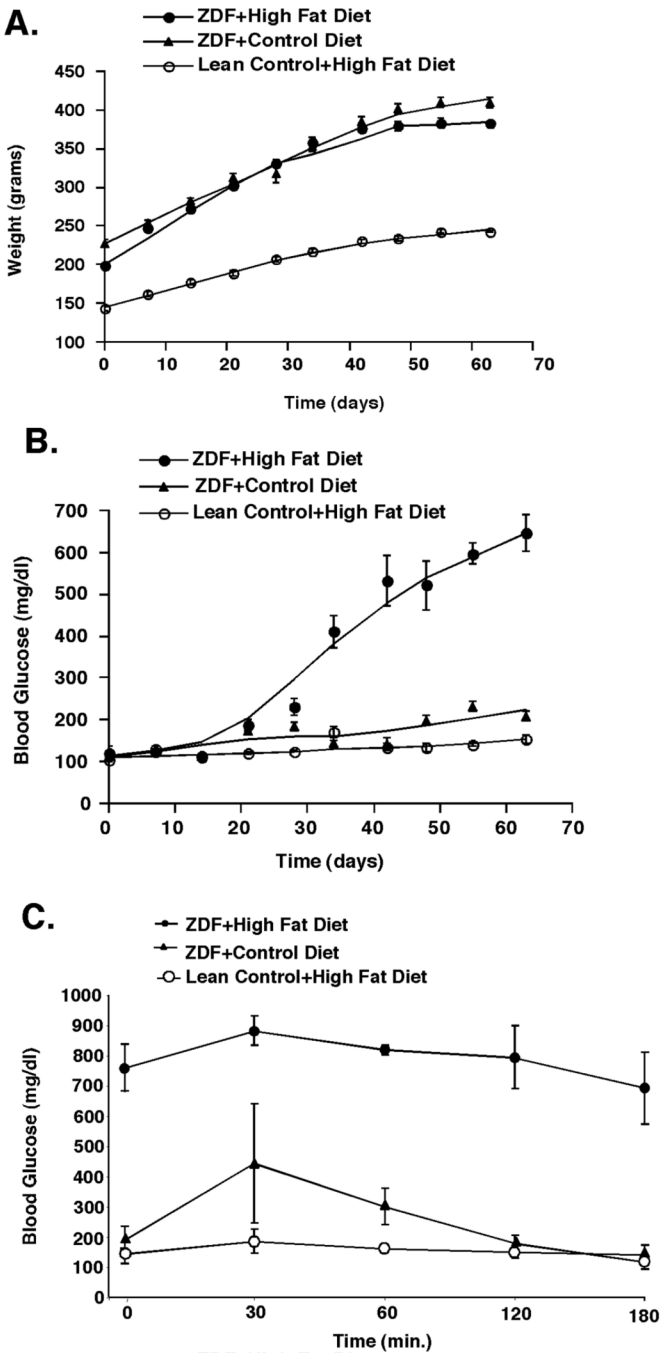

Fig 1. Female ZDF rats develop obesity and hyperglycemia

Female ZDF-HF or ZDF-N animals become progressively obese. Lean control animals do not become obese (A). ZDF-HF females develop diabetes. In contrast, ZDF-N animals develop IGT, but are not continuously hyperglycemic. Controls remain normoglycemic (B). IGT is observed in ZDF females after glucose tolerance testing (C). The blood glucose peaked at 30 min, rising to 820 mg/dl in ZDF-HF, and 444 mg/dl in ZDF-N animals. In contrast, glucose levels remained normal after a GTT in control animals.

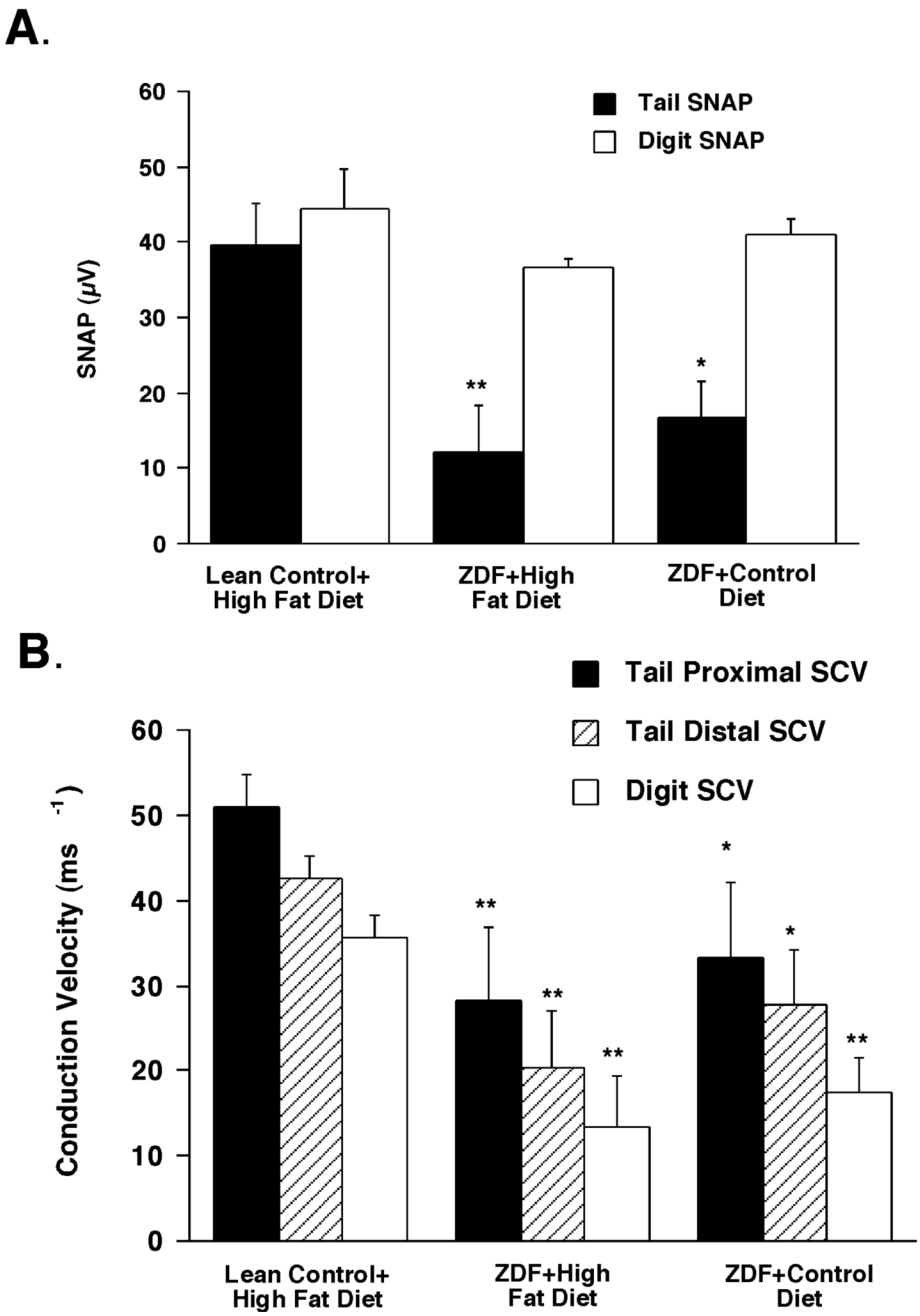

Fig 2. Level of hyperglycemia in ZDF rats is associated with reduction in sensory nerve action potentials and conduction velocities

Mean tail and first digit SNAP amplitudes are severely reduced in diabetic (ZDF-HF) and IGT (ZDF-N) animals (A). SNAP amplitudes obtained from the distal tail are more severely reduced than digit SNAP amplitudes, consistent with a length dependent axonal neuropathy. Amplitudes are smaller in ZDF-HF than ZDF-N animals. Tail and digit mean sensory conduction velocities (SCVs) are more severely reduced in ZDF-HF than ZDF-N animals (B). Control animals had normal NCS. * = $p<0.05$, ** = $p<0.01$ compared to lean control.

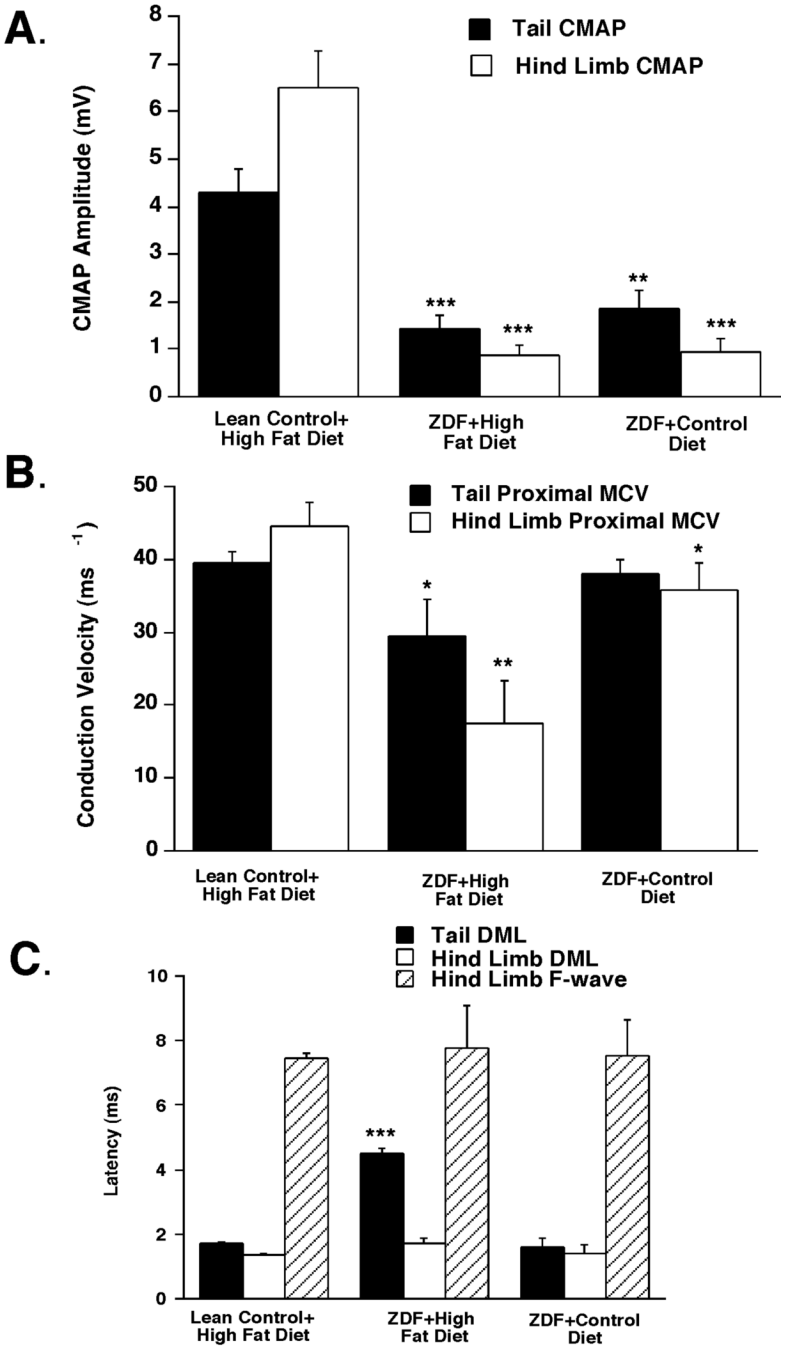

Fig 3. Hyperglycemia in ZDF rats reduces motor action potential responses

The tail and sciatic/peroneal CMAP amplitudes (A), sciatic/peroneal MCVs and tail MCVs (B) are severely reduced, and tail DML prolonged in both ZDF-HF and ZDF-N animals, consistent with a length dependent process. There was also no significant difference in the hind limb F-wave responses in diabetic animals, consistent with preserved motor axons in proximal nerves.

* = $p<0.05$, ** = $p<0.01$ *** = $p<0.001$ compared to lean control.

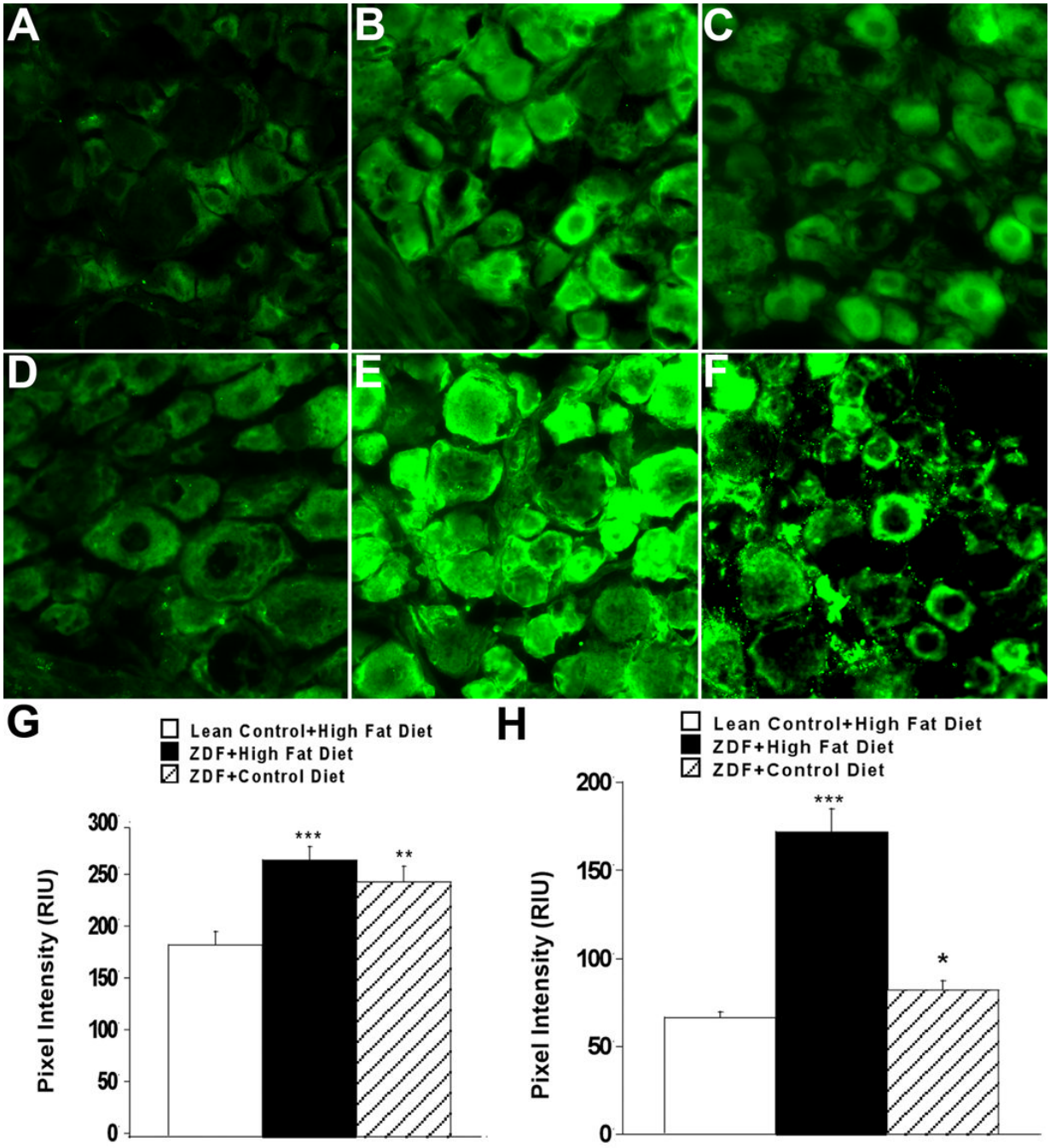

Fig 4. Markers of oxidative stress in DRG neurons from lean control ZDF-HF and ZDF-N animals

Levels of MDA (A–C and G), or HNE (D–F and H) were measured in over 100 DRG neurons in each animal. Levels of MDA and HNE were increased in both IGT as well as diabetic animals, consistent with evidence of early oxidative injury associated with hyperglycemia. * = $p<0.05$, ** = $p<0.01$ *** = $p<0.001$ compared to lean control.

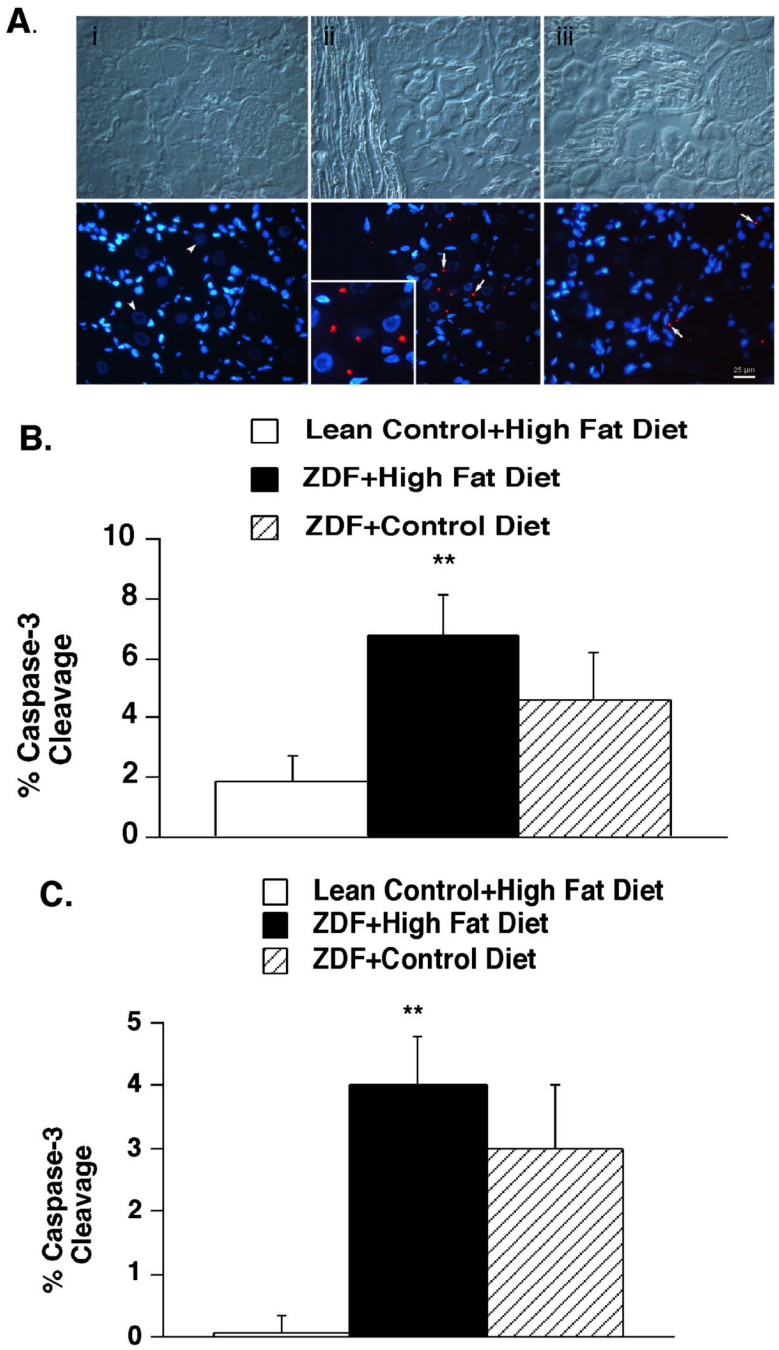

Fig 5. Caspase-3 cleavage is increased in DRG and SC from ZDF-HF and ZDF-N rats

Caspase cleavage in DRG sections (A). The upper row of panels indicates DRG DIC images from: lean control animals on a high fat diet (i), diabetic animals (ZDFHF) (ii), IGT animals (ZDFN) (iii). The lower panel shows the corresponding DRG sections stained for cleaved (active) caspase-3 (red), and nuclear chromatin (blue), indicated by white arrow heads (see inset in middle panel). There is no caspase-3 staining in (i), but increased cleaved caspase-3 in the cytoplasm (white arrows) in neurons from diabetic (ii) compared to IGT neurons (iii). Caspase-3 cleavage (active caspase-3) is measured in DRG neurons (B) and SC (C). ** = $p<0.01$ compared to lean control.

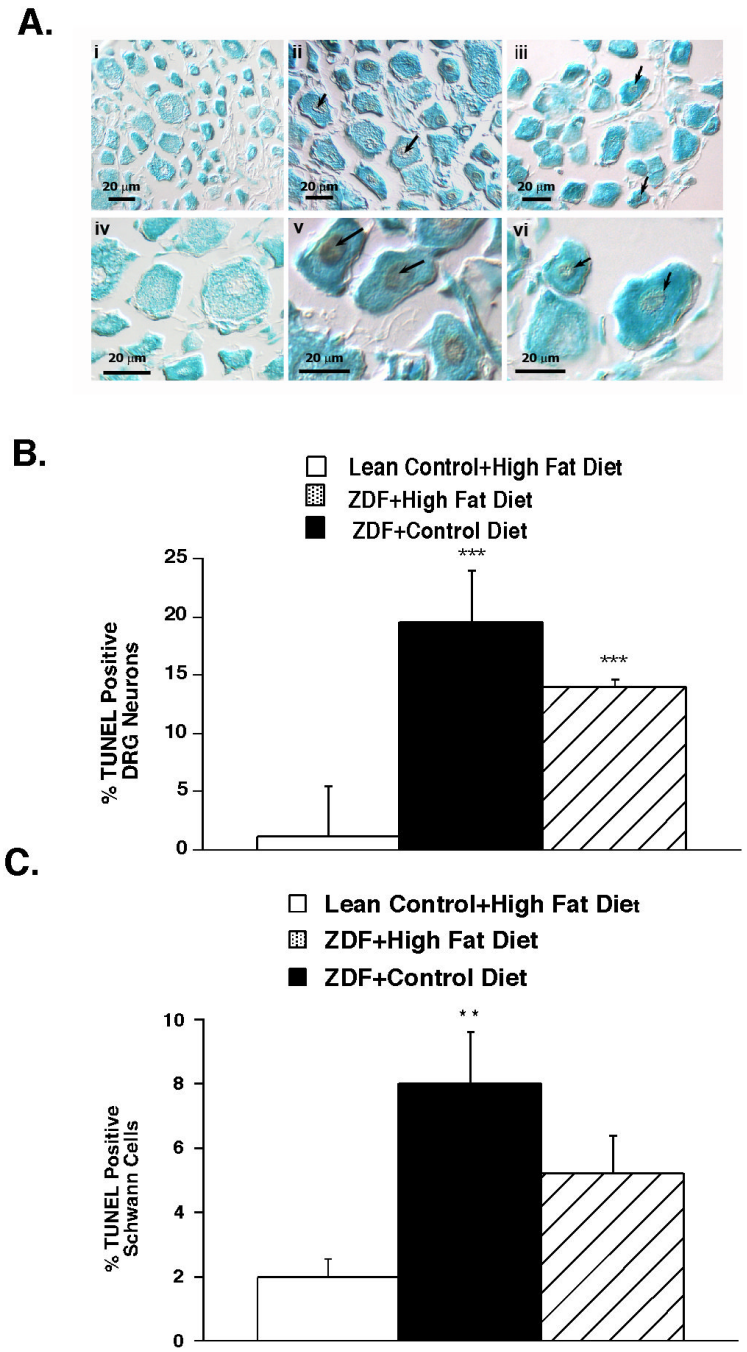

Fig 6. Apoptotic changes are increased in DRG from ZDF-HF and ZDF-N rats

DRG nuclei were stained using the TUNEL technique (A). The figure represents composite immunohistochemical staining overlaying the DIC image. i–iii: Low magnification images. iv–vi: High magnification images. i and iv: TUNEL positive neurons are rare in control sections. ii and v: DRG from diabetic animals (ZDF-HF) showing numerous apoptotic neuronal nuclei (arrow), interspersed with non-apoptotic neurons (TUNEL staining negative) consistent with PCD in single cells. iii and vi. DRG from IGT animals (ZDF-N) showing apoptotic nuclei (arrows). TUNEL staining is measured in DRG neurons (B) and SC (C).

** = $p<0.01$ *** = $p<0.001$ compared to lean control.

Table 1

Changes in Insulin and Lipid Measurements in Control and ZDF Animals

	Study Start			Study End		
	CTL	**ZDFN**	**ZDFHF**	**CTL**	**ZDFN**	**ZDFHF**
Insulin (ng/ml)	0.92±0.13	2.76±0.46	0.78±0.01	0.065±0.01	4.04±0.98	3.92±0.74
Cholesterol (mg/dl)	34.40±6.37	79.15±16.00	56.80±10.85	25.16±1.37	455.10±81.76	141.53±19.74
Free Fatty Acids (mg/dl)	1.66±0.19	4.63±0.60	3.20±0.31	0.99±0.11	10.05±1.09	3.13±0.34
Triglycerides (mg/dl)	37.50±2.54	301.50±21.5	216.2±23.50	1074.41±118.87	2415.38±289.06	582.97±76.01

Table 2

DRG Neuronal Size and Number

	Lean control	ZDF-HF	ZDF-N
numer of neurons > 1,500 um^2	$4.45.10^{-5}$	$5.22.10^{-5}$	$4.43.10^{-5}$

The number of large DRG neurons decreased in the ZDF rats compared to lean controls but these differences were not statistically significant.