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Pioglitazone inhibits the development of hyperalgesia and sensitization of spinal nociceptive neurons in type 2 diabetes

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Abstract

Thiazolidinedione drugs (TZDs) such as pioglitazone are FDA-approved for the treatment of insulin resistance in type 2 diabetes. However, whether TZDs reduce painful diabetic neuropathy (PDN) remains unknown. Therefore we tested the hypothesis that chronic administration of pioglitazone would reduce PDN in Zucker Diabetic Fatty (ZDF^{fa/fa}) rats. Compared to Zucker Lean (ZL^{fa/+}) controls, ZDF developed: (1) elevated blood glucose, HbA1c, methylglyoxal and insulin; (2) mechanical and thermal hyperalgesia at the hindpaw; (3) increased avoidance of noxious mechanical probes in a mechanical conflict avoidance behavioral assay, the first report of a measure of affective-motivational pain-like behavior in ZDF; and (4) exaggerated lumbar dorsal horn immunohistochemical expression of pressure-evoked phosphorylated extracellular signal-regulated kinase (pERK). Seven weeks of pioglitazone (30 mg · kg⁻¹ · d⁻¹ in food) reduced blood glucose, HbA1c, hyperalgesia, and pERK expression in ZDF. This is the first report to reveal hyperalgesia and spinal sensitization in the same ZDF animals, both evoked by a noxious mechanical stimulus that reflects pressure pain frequently associated with clinical PDN. As pioglitazone provides the combined benefit of reducing hyperglycemia, hyperalgesia, and central sensitization, we suggest that TZDs represent an attractive pharmacotherapy in patients with type 2 diabetes-associated pain.

Keywords

ZDF; PPAR gamma; painful diabetic neuropathy; pain

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DISCLOSURES:

The authors declare there are no conflicts of interest.

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Introduction

Approximately one-third of patients with diabetes experience pain^{1, 17, 47}, commonly referred to as painful diabetic neuropathy (PDN)¹⁰². A majority of preclinical PDN studies focus on the streptozotocin (STZ) model of type 1 diabetes⁸. However, 90% of diabetic patients are type 2 (World Health Organization), the prevalence of PDN is greater in patients with type 2^{1, 100}, and pain mechanisms in type 1 versus type 2 likely differ^{39, 85–87}. Therefore we chose to study a genetic model of type 2 PDN, the Zucker Diabetic Fatty (ZDF^{fa/fa}) rat¹³.

Hyperglycemia develops within 6–10 wks of age^{6, 13, 49, 92} in ZDF but not Zucker Lean (ZL^{fa/+}) controls, and is followed by behavioral correlates of PDN, including hypersensitivity to mechanical^{6, 25, 69, 78, 92, 101, 112} and thermal^{25, 49, 78, 80} somatosensory stimulation. However, conflicting studies report either hypoalgesia^{89, 92} or hyperalgesia^{78, 112} in similarly aged ZDF. This raises concerns regarding examination of sensory thresholds at just a single age without regard to the developmental stage of diabetes^{6, 49, 78, 89, 112}, or omission of ZL controls when multiple ages are assessed⁶⁹. These deficiencies in the literature led us to rigorously evaluate multiple pain-like behaviors in ZDF and ZL at various ages in a well-controlled study.

We hypothesized that spinal cord plasticity contributes to pain in type 2 diabetes because: (1) hyperalgesic db/db mice exhibit dorsal horn increases in both phosphorylated extracellular signal-regulated kinase (pERK)^{16, 109}, a marker of nociceptive neuron activation²⁶ in the spinal dorsal horn^{37, 63, 114}, and the astrocyte activation marker GFAP^{16, 52, 77, 109}; (2) intrathecal injection of either the ERK phosphorylation inhibitor U0216¹⁰⁹ or the astrocyte toxin L- α -aminoadipate⁵² reverses hyperalgesia; and (3) augmented NMDA and AMPA receptor expression and function in the spinal cord⁵⁰ may contribute to hyperalgesia in ob/ob mice⁴⁴. A recent report indicated that spinal neurons in ZDF exhibit central sensitization in response to non-noxious hindpaw stimulation⁸⁷. However, concurrent evaluation of pain-like behavior was not performed. We go beyond these studies by evaluating spinal plasticity in PDN using a clinically relevant noxious pressure stimulus to evoke not only expression of pERK in the spinal dorsal horn but also behavioral hyperalgesia in the same subjects.

Thiazolidinedione drugs (TZDs) such as pioglitazone (Actos®) are approved by the U.S. Food and Drug Administration for the treatment of type 2 diabetes. TZDs also reduce the molecular and behavioral sequelae of neurological disease^{22, 40}, as well as neuropathic pain associated with brain^{84, 111}, spinal cord^{58, 71} or nerve^{11, 30, 55, 63, 96} injury. For example, we recently reported that pioglitazone reduced hypersensitivity to non-noxious mechanical stimulation in rats with traumatic nerve injury⁶³. However, it still remains unclear whether TZDs reduce the neuropathic pain associated with type 2 diabetes⁹⁷. In addition, behavioral signs of PDN in rodents are more frequently associated with hypersensitivity to noxious, rather than non-noxious stimulation. To address these gaps, we tested the hypothesis that chronic administration of Actos® would prevent the development of noxious pressure-evoked hyperalgesia and spinal central sensitization in ZDF rats.

Materials and Methods

Subjects

Experiments were carried out in accordance with the Institutional Animal Care and Use Committee at the University of Kentucky (Approved Protocol # 2009–0429). All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, in accordance with the International Association for the Study of Pain¹¹⁵ and the National Institutes of Health Office of Laboratory Animal Welfare Guide for the Care and Use of Laboratory Animals.

Male ZL and ZDF rats ([http://www.criver.com/products-services/basic-research/find-a-model/zucker-diabetic-fatty-\(zdf\)-rat](http://www.criver.com/products-services/basic-research/find-a-model/zucker-diabetic-fatty-(zdf)-rat), Charles River, Wilmington, MA) aged 4–19 weeks were used for all experiments. “Obese” ZDF rats are homozygous for the loss-of-function “fatty” mutation in the leptin receptor (*fa/fa*) that results in the development of type 2 diabetes¹³. “Lean” ZL rats are heterozygous (*fa/+*), do not develop a diabetic phenotype, and are genetic controls for ZDF. All rats were housed in a temperature- and humidity-controlled room on a 12 hour light – 12 hour dark cycle with lights on from 7:00am to 7:00pm. Prior to any behavioral or dietary manipulations, all rats were provided water and Formulab 5008 (TestDiets, Purina Mills, Richmond, IN) chow *ad libitum*. Formulab 5008 (formerly Purina 5008) food yields reliable diabetic symptoms including hyperglycemia, hyperlipidemia, impaired glucose tolerance and insulin insensitivity as reported by Charles River.

Measurement of Blood Glucose & HbA1c

Blood was collected at sacrifice for the measurement of MG-AGE and insulin at 19 wks of age in the Characterization of PDN study, and for the measurement HbA1c both before (12 wks) and after (19 wks) drug treatment in the Pioglitazone Administration study. Blood glucose was measured at weekly intervals in both studies. The experimental designs for these studies are described below.

Rats were lightly restrained in a towel and the distal tail wiped with an alcohol swab. A small nick was made at the distal tip of the tail using a #11 scalpel blade. Initial bleeding was wiped clean with gauze and subsequent drops of blood were either loaded into a room temperature HbA1c cartridge and analyzed using a DCA Vantage Analyzer (Siemens, Munich, Germany), or placed on a glucose test strip in triplicate and inserted into a glucose monitor (TrueTrack, Walgreens, Deerfield, IL). To avoid perturbations in pain-like behavior elicited by fasting or exogenous glucose administration in a tolerance test^{18, 19}, non-fasted blood glucose was measured. A random blood glucose level greater than 200 mg/dL (11.1 mmol/L) was defined as hyperglycemia⁸¹. We measured blood glucose at the same time each week to minimize circadian-induced fluctuations.

Pain-Like Behavior: Stimulus-Evoked

Fluctuations in noise, vibrations, temperature, and other distractors in the behavioral testing room were minimized to optimize reliable measurements between cohorts of animals tested during different behavioral sessions. To further reduce variability and acclimate animals to

the different testing apparatuses, two weeks of training were performed prior to behavioral measures reported at 8 weeks of age.

Heat hyperalgesia was assessed by placing the animals on a heated surface ($52.5 \pm 1^\circ\text{C}$) within an acrylic enclosure (Hotplate; Columbus Instruments, Columbus, OH). The time until a hindpaw withdraw response (e.g. jumping, licking, flicking) occurred was recorded. The animal was immediately removed after the withdraw response or at a cutoff of 30 s to avoid tissue injury. Three trials, with an inter-trial interval of at least 5 min, were averaged for each time point.

Mechanical hyperalgesia was measured using the Randall Selitto method⁷⁶ with slight modification to accommodate an electronic device with a force transducer (IITC Life Science Inc., Woodland Hills, CA). The thoracic body and head were lightly restrained in a towel with both hindpaws exposed. To start, one hindpaw was placed into the calipers with the blunted-point of the force transducer placed on the plantar surface between the 2nd and 3rd digits. The force exerted on the surface of the hindpaw by the blunted-point was gradually and uniformly increased until a withdraw reflex occurred. Animals were not required to vocalize. The gram force required to elicit a withdraw response was recorded. Because patients report increased responsiveness to repeated mechanical stimuli⁷⁰, we used multiple trials per time point to assess pressure hyperalgesia. The force until paw withdraw was measured three times for each hindpaw and then averaged together, as there was no significant difference in left versus right withdraw thresholds. The combined average of hindpaw withdraw thresholds is reported at each timepoint.

Cold hyperalgesia was assessed by placing the animal on a cooled surface ($4^\circ\text{C} \pm 0.5^\circ\text{C}$) within an acrylic enclosure (Coldplate; IITC Life Science Inc., Woodland Hills, CA) for five minutes. The combined number of nociceptive responses (e.g. jumping, licking, flicking) for both hindpaws during the 5 min period is reported³⁶.

Pain-Like Behavior: Affective-Motivational

To assess the affective-motivational component of pain, we utilized a novel Mechanical Conflict Avoidance System (MCS)⁴⁵. MCS gives animals the option to remain in a start chamber containing an aversive light stimulus or choose to cross a chamber containing an aversive mechanical stimulus in the form of height-adjustable, blunted probes. If animals chose to leave the light chamber and cross the probes in the stimulus chamber, they then had the option to enter a darkened reward chamber. MCS behavioral testing began at 17 wks of age and consisted of three stages: familiarization, training, and testing. The latency to exit the light chamber was used as a measure of avoidance of the mechanical stimulus, and therefore a measure of affective-motivational pain-like behavior.

Familiarization – 1 day—Animals were placed in the light chamber with the light off; access to the stimulus chamber was restricted by a closed guillotine door. After 15 s of darkness, the light was turned on. After 20 s of light exposure, the guillotine door was raised and the animal was free to explore the entire apparatus, including the dark chamber, for 5 min. The animal was then returned to the homecage.

Training – 4 days—Animals were trained to move from the light chamber, through the stimulus chamber at a probe height of 0 mm (i.e. no probes), and into the dark chamber. Animals were placed in the light chamber with the light off; access to the stimulus chamber was restricted by a closed guillotine door. After 15 s of darkness, the light was turned on. After 20 s of light exposure, the guillotine door was raised and a timer started to measure the latency to exit the light chamber. If the animal did not exit the light chamber within 30 s, the guillotine door was closed and the animal was returned to its homecage. Upon entering the dark chamber, a second guillotine door was closed to restrict the animal to the dark chamber. Animals remained in the dark chamber for 45 s to reinforce the crossing of the stimulus chamber and then were returned to the homecage. Each animal underwent four training trials on each of the four training days.

Testing – 3 days—Assessment of the latency to exit in the presence of varying degrees of mechanical stimulus (1, 3, 4 mm) was initiated at 18 wks of age. Each testing day began with 1 trial in the absence of a stimulus (0 mm) followed by 3 trials with the probes raised to a predetermined height: 1 mm (d 1), 3 mm (d 2), 4 mm (d 3). The training procedure described above was also used for testing in either the absence (trial 1) or presence (trials 2–4) of mechanical probes in the stimulus chamber. Only one probe height was tested per day. Assessment at the 0 mm probe height was used to confirm that animals were still trained to cross the stimulus chamber.

Animals remained in their home cage for at least 10 min between training and testing trials. All four paws were required to leave the light chamber to determine the latency to exit. The average of 4 trials for each training day or 3 trials for each testing probe height is reported for each animal.

pERK Quantification via Immunohistochemistry

Upon general anesthesia with isoflurane (5% induction, 1.5% maintenance), the plantar surface between the 2nd and 3rd digits of the left hindpaw was stimulated with 110 g of constant force for 30 s per minute over a 5 min period. These stimulus parameters were chosen to mimic those used to elicit withdraw responses to noxious pressure as described above for mechanical hyperalgesia testing. Ten minutes after initiating pressure stimulation, animals were perfused through the left ventricle with 250 ml of room temperature 0.1 M phosphate buffered saline (PBS) with heparin (10,000 USP units/L) followed by 250 ml of ice-cold fixative (10% phosphate buffered formalin). The lumbar spinal cord was removed and post-fixed overnight in 10% phosphate buffered formalin and then cryoprotected in 30% sucrose in 0.1 M PBS for several days. Transverse sections (30 μ m) from L4-L5 were cut on a freezing microtome and collected in 0.1 M PBS. The sections were washed three times in 0.1 M PBS and then pretreated with blocking solution (3% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS) for 1 h. Sections were then incubated in blocking solution containing the primary antibodies rabbit anti-pERK (1:250, #4370, Cell Signaling Technology, Danvers, MA) and Alexa Fluor 488 conjugated mouse anti-NeuN (1:200, MAB377X, EMD Millipore, Billerica, MA) overnight at room temperature on a slow rocker in the dark. The sections were washed three times in 0.1M PBS, and incubated in goat anti-rabbit secondary antibody (1:800, Alexa 568, Molecular Probes, Grand Island, NY) for 90

min, washed in 0.1M PBS, 0.01M PBS, then 0.01M PB, and non-sequentially mounted onto Superfrost Plus slides, air dried, and cover-slipped with Prolong Gold with DAPI mounting medium (Molecular Probes). 4–6 high quality sections were randomly selected for quantification.

All images were captured on a Nikon Eclipse TE2000-E microscope using a 10x objective and analyzed using NIS-Elements Advanced Research software. We focused our quantification of the number of pERK immunopositive cell profiles within lamina I-II, where the majority of C-fibers and nociceptive peripheral afferents terminate within the dorsal horn^{4, 14}. Colabeling of pERK with NeuN is expressed as the percentage of the total number of pERK profiles in laminae I-II also expressing NeuN. Each spinal cord slice was analyzed by an observer blinded to treatment. The average of n=5–6 animals per group is reported.

Quantification of Methylglyoxal-Derived Advanced Glycation End-Products (MG-AGEs)

MG-AGEs were quantified using a competitive ELISA according to the manufacturer's instructions (STA-811, Cell BioLabs, San Diego, CA). This ELISA uses a primary antibody that recognizes the hydroimidazolone (H1) moiety created by the modification of protein residues by methylglyoxal². Whole blood taken from the left ventricle prior to transcatheter perfusions was collected in serum separator tubes (SSTTM, BD Biosciences, Franklin Lakes, NJ) and allowed to clot for 30 min. Clotted blood in SSTs was centrifuged at 5000 x g for 10 min at 4°C and the serum was transferred to fresh microcentrifuge tubes and stored at –80°C until MG-H1 ELISA analysis. Serum samples were diluted 1:2 in 0.1 M PBS to obtain concentrations in the span of the standard curve.

Insulin Quantification via ELISA

Non-fasted insulin levels were quantified by ELISA according to the manufacturer's recommendations (EMD Millipore; EZRMI-13K; Darmstadt, Germany) from serum obtained as described above in serum separator tubes (SSTTM, BD Biosciences) at time of sacrifice.

Pioglitazone Incorporation into Food

Actos® (pioglitazone hydrochloride; Takeda Pharmaceuticals U.S.A., Inc., Deerfield, IL) was obtained from the University of Kentucky pharmacy in 30 mg tablets of 25% purity, and was incorporated into Formulab 5008 rat chow (TestDiets, Purina Mills, Richmond, IN). Average body weight and food consumption⁸³ were used to determine the concentration of Actos in chow required to achieve a dosing of 30 mg pioglitazone / kg body weight / day. For ZDFs the concentration of Actos in chow was 0.16% (catalog no. 5W01) and for ZLs the concentration was 0.207% (catalog no. 5W02). Food was provided *ad libitum* and consumption and body weights were monitored in order to calculate actual dosing.

Experimental Design: Characterization of PDN

To determine the time course of development of pain-like behavior, we monitored ZL and ZDF rats weekly from 4 to 18 weeks of age with n=12 per group. Weekly measurement of pain-like behaviors occurred on Monday thru Friday in the following order: coldplate,

pressure, hotplate. Assessment of pain-like behaviors occurred prior to determination of blood glucose and mass at 2–4pm on Fridays. Animals were not fasted in order to minimize the potential effects of fasting on pain-like behavior. At least one day separated coldplate and hotplate determinations to avoid cross-modality sensitization. After behavioral and metabolic outcomes were assessed at 18 wks, the left hind paws of control and diabetic rats were stimulated, using the same pressure device used to determine behavioral hyperalgesia, in order to evoke pERK. Next, cardiac blood was taken prior to transcatheter perfusion and fixation, and then tissues were harvested for subsequent assays.

Experimental Design: Pioglitazone Administration

Rats were divided into the following treatment groups using a 2×2 experimental design: ZL Vehicle (n=10), ZL Pioglitazone (n=10), ZDF Vehicle (n=10), ZDF Pioglitazone (n=9). Vehicle food was defined as unaltered Formulab 5008 chow.

Pain-like behaviors, blood glucose, and weight were measured weekly from 10 to 19 wks of age. HbA1c was measured at 12 and 19 wks. Vehicle or pioglitazone food treatment began after assessment of behavioral and metabolic outcomes at 12 wks and all further testing was performed by an observer blinded to drug treatment group. Food consumption was measured 3 times per week by calculating the mass difference between food added to the cage and food remaining in the cage then dividing by 2, as rats were housed in pairs, and the number of days that had lapsed between food additions. After outcome measurements were obtained during week 19, animals were pressure-stimulated to evoke pERK, perfused and fixed with buffered formalin for immunohistochemical analyses of spinal cords.

One rat from the ZDF Pioglitazone group was removed from all analyses prior to beginning pioglitazone administration due to an abnormally high baseline blood glucose level of 514 mg/dL (more than double all other animals). Because the phenotype for coldplate responses in ZDF was modest, we did not assess the effect of pioglitazone on cold hypersensitivity.

Statistical Analysis

Mass, glucose, hotplate, pressure, coldplate, and MCS were compared using a repeated measures two-way ANOVA followed by Holm-Sidak multiple comparison correction. To compare ZL vs. ZDF, the outcome measures insulin, MG-AGE, HbA1c, and area-under-the-curve (AUC; calculated via the trapezoidal method) were analyzed using an unpaired, two-tailed t-test. To compare ZL vs. ZDF and vehicle vs. pioglitazone, the outcomes HbA1c and AUC were analyzed via a two-way ANOVA followed by Holm-Sidak multiple comparison correction. Ipsilateral vs. contralateral or pioglitazone vs. vehicle comparisons of pERK immunohistochemistry in ZL vs. ZDF were analyzed by two-way ANOVA followed by Holm-Sidak multiple comparison correction. A value of $\alpha=0.05$ was used to determine statistical significance. All data were analyzed and graphed using Prism 6.0 (GraphPad, La Jolla, CA) and are presented as mean $\pm$ SEM.

Results

The ZDF Rat is a Model of Progressive Painful Diabetic Neuropathy

When compared to control heterozygotes or wild-types, only ZDF rats homozygous for the *fatty* (*fa/fa*) leptin receptor mutation develop cardinal symptoms of glucose-intolerant diabetes¹³ including hyperphagia^{69,83}, hyperinsulinemia^{51, 65, 92}, obesity⁶⁹, hyperglycemia^{69, 73, 101}, elevated HbA1c^{51, 92}, and increased MG-AGE⁸⁹. In the first experiment we characterized the progression of hyperglycemia and pain-like behavior in diabetic ZDF and control ZL rats. As illustrated in Figure 1A–C, ZDF had elevated mass [strain x time; $F(14, 308) = 8.249$; $P < 0.0001$] from 4 to 18 wks [$p < 0.01$], elevated blood glucose [strain x time; $F(14, 308) = 46.31$; $P < 0.0001$] from 8 to 18 wks [$p < 0.0001$], and elevated serum insulin [$p = 0.0009$] at 19 wks.

Advanced glycation end-products (AGEs) such as methylglyoxal (MG) derived hydroimidazolone (MG-H1) are associated with diabetic pain in patients⁹³. MG-AGEs result from the accumulation of MG³⁵, a metabolite of glucose found in blood that is elevated in type 2 diabetes⁴¹ and exacerbated in patients and mice with PDN⁵. Therefore we measured MG-AGE levels and hemoglobin glycation in the form of HbA1c, an AGE considered by clinicians to be the ‘gold standard’ diagnostic for diabetes⁸¹. Figure 1D indicates that 19 wk old ZDFs had elevated blood levels of MG-AGE [$p < 0.05$]. In a separate group of subjects, Figure 1E indicates that 12 wk-old ZDFs had elevated blood levels of HbA1c [$p < 0.0001$]. Thus, consistent with the literature, we found that ZDFs exhibited: (1) obesity after weaning that persisted until the conclusion of the study; (2) hyperglycemia beginning at 6 wks of age that progressively increased throughout the study; (3) hyperinsulinemia, elevated MG-AGE, and elevated HbA1c levels at 12 wks.

To rigorously evaluate the ZDF rat as a behavioral model of PDN, we measured not only evoked responses to noxious stimuli over an extended time course, but also signs of affective pain. First, to assess stimulus-evoked hyperalgesia, we measured responses to noxious mechanical and thermal stimuli. Figure 2A–B illustrates that ZDFs were hypersensitive to a noxious hotplate [strain; $F(1, 22) = 14.2$; $P = 0.0011$] at 15, 17, and 18 weeks of age [$p < 0.05$], exhibiting an overall decrease in heat response threshold from 14 to 18 wks [AUC; $p < 0.05$]. Figure 2C–D illustrates that ZDFs were hyperresponsive to noxious mechanical pressure [strain x time; $F(10, 220) = 6.045$; $P < 0.0001$] from 15 to 18 wks [$p < 0.001$], exhibiting an overall decrease in pressure threshold from 14 to 18 wks [AUC; $p < 0.0001$]. Figure 2E–F illustrates that ZDFs exhibited more hindpaw responses in a coldplate test [strain x time; $F(10, 220) = 2.372$; $P = 0.0110$] at 18 wks [$p < 0.01$], with an overall increase from 14 to 18 wks [AUC; $p < 0.001$].

Preclinical research targeting the mechanisms of PDN primarily relies on stimulus-evoked behavioral outcomes⁶⁶. However, PDN symptoms include not just pressure and thermal hyperalgesia but also aching, paresthesia/dysesthesia, and affective pain⁵³. To our knowledge there are no reports of affective pain in a rodent model of type 2 diabetes⁴⁷. To address this gap and determine whether ZDFs possess the affective-motivational component of PDN that is most relevant to diabetic patients, we utilized a mechanical conflict-avoidance system (MCS)^{20, 45, 64} as shown in Figure 3A. Figure 3B illustrates that

illustrates that during the four training trials when no pins were present (0 mm probe height), latency to exit the light chamber was similar between ZL and ZDF rats ($p > 0.05$), indicating no differences in light aversion or learned motivation/ability to find the dark chamber. Despite contrasting results in an open field test, where we (unpublished findings; R.B. Griggs *et al.*, 2013) and others³⁸ found diminished locomotor activity in ZDF, exploration in a novel open field environment is not equitable to behavioral training to seek the dark chamber in MCS. Raising the probe height produced an increase in the latency to exit the light chamber [strain x probe height; $F(6, 102) = 4.262$; $P = 0.0007$] in ZL at 4 mm (vs. 1 mm; $p = 0.0051$) and in ZDF at both 3 mm ($p < 0.0001$) and 4 mm ($p < 0.0001$) heights, indicating aversion to the mechanical stimulus in both control and diabetic rats. Importantly, the latency to exit the light chamber was higher in ZDF compared to ZL at both 3 mm ($p = 0.0493$) and 4 mm ($p < 0.0001$) heights, indicating that diabetes potentiates the avoidance of a noxious mechanical stimulus.

Central sensitization is defined as the increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input¹⁰⁷. As an example, expression of phosphorylated extracellular signal-regulated kinase (pERK) in the dorsal horn is exacerbated by light touch stimulation after peripheral nerve injury⁶³ or inflammation²⁷ but not uninjured controls, and is therefore an important marker of spinal central sensitization²⁶. To test the hypothesis that diabetes results in central sensitization, we evaluated noxious pressure-evoked pERK expression in dorsal horn neurons. We chose pressure as the evoking stimulus because it is a hallmark pain modality in PDN patients⁷⁰. Figure 4A shows that unilateral stimulation of the tibial receptive field produced unilateral pERK expression within the superficial laminae of the dorsal horn. pERK was restricted to the side ipsilateral to stimulation and was predominantly located within the medial extent of the dorsal horn, respecting previously-described somatotopic boundaries of tibial nerve anatomy^{14, 94}. Figure 4B shows representative images indicating that pERK predominantly colabeled with NeuN, an established immunohistochemical marker for neurons⁴². The percentage of pERK cell-profiles that colabeled with NeuN was (mean $\pm$ SEM): ZL contralateral (81.5 ± 6.5), ZL ipsilateral (88.3 ± 3.6), ZDF contralateral (80.5 ± 3.1), ZDF ipsilateral (92.1 ± 1.3). The degree of colocalization was similar between ZL and ZDF at either contralateral [$p = 0.93$] or ipsilateral [$p = 0.89$] dorsal horns. Figure 4C illustrates that pressure increased pERK in ZL [ipsilateral vs. contralateral; $p < 0.0001$] and ZDF rats [$p < 0.0001$]. Diabetes exacerbated this increase [ZDF vs. ZL; ipsilateral only; $p = 0.001$], and there was an interaction between Strain and Stimulation [$F(1, 20) = 8.051$; $P = 0.0102$].

Chronic Administration of Oral Pioglitazone Reduces Pathological Signs of Diabetes

Numerous reports indicate that oral administration of pioglitazone or rosiglitazone reduces hyperglycemia in db/db mice⁹⁵, ZDF rats⁷³, and humans^{3, 72, 108} as well as HbA1c levels in ZDF¹⁰⁵ and diet-induced type 2 diabetes³⁴. To confirm that pioglitazone alleviated signs of type 2 diabetes in ZDF, we measured blood glucose and HbA1c. The average dose of pioglitazone ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) received by ZL (35.53 ± 1.94) and ZDF (32.19 ± 2.52) was similar over the 7 wk treatment period, and was comparable to previous studies in db/db mice (4 wks of $30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$)⁹⁵ and ZDF rats (6 wks of $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$)¹⁰⁵. As illustrated in Figure 5A, pioglitazone decreased blood glucose in ZDF [$F(1, 18) = 16.51$; P

= 0.0007] but not in ZLs [F (1, 18) = 3.51; P=0.077] from 13 to 19 wks of age. Figure 5B illustrates that blood levels of HbA1c were elevated in ZDF rats at 19 wks of age [$p < 0.0001$], even greater than observed at 12 wks (Fig 1E). Pioglitazone normalized HbA1c levels in ZDF to the level of ZL [$p < 0.0001$].

Chronic Oral Pioglitazone Inhibits the Development of PDN and Reduces Evoked pERK

Hyperalgesia develops in ZDF at approximately 14 wks of age (Fig 2). To test the hypothesis that pioglitazone prevents the development of hyperalgesia, we initiated its administration at 12 wks of age. As illustrated in Figure 6A–B, ZDF exhibited lower heat response thresholds as compared to ZL [strain x time; F (8, 144) = 2.039; P = 0.0458] from 13 to 19 wks. Pioglitazone temporarily normalized heat thresholds in ZDF [F (1, 17) = 22.09; P = 0.0002] from 13 to 15 wks [$p < 0.01$], and then heat hypersensitivity returned at 16 wks. Figure 6C–D illustrates that ZDF exhibited a decrease in pressure response thresholds compared to ZL [strain x time; F (8, 144) = 2.178; P = 0.0324] from 11 to 19 wks [$p < 0.05$]. Pioglitazone normalized pressure thresholds in ZDF [drug; F (1, 17) = 104.9; P < 0.0001] throughout the duration of treatment, from 13 to 19 wks [$p < 0.05$]. Further studies are needed to investigate the differential effect of pioglitazone on heat and noxious mechanical sensitivity. In addition, we speculate that pioglitazone would reduce cold hypersensitivity as we recently reported that pioglitazone reduced the development of⁶³ and reversed³⁰ hyperresponsivity to acetone in the spared nerve injury model of neuropathic pain.

Because we began pioglitazone administration prior to the development of hyperalgesia, further intervention studies are needed to determine if the mechanism of pioglitazone antihyperalgesia is independent from its reduction of hyperglycemia. This may indeed be the case, since we found that a single intraperitoneal injection of pioglitazone, given after the development of hyperalgesia in ZDF, *reversed* heat hypersensitivity without changing blood glucose levels (unpublished data, R.R. Donahue et al, 2014).

To determine whether chronic pioglitazone treatment reduces spinal nociceptive neuron activation, we quantified pERK expression after noxious mechanical stimulation of the hindpaw. Figure 7A–B illustrates that noxious pressure evoked greater pERK in vehicle-treated ZDF as compared to ZL [$p = 0.0006$]. Pioglitazone but not vehicle normalized the expression of pERK in ZDF [$p = 0.011$] but not ZL [$p = 0.96$]. There was an interaction between Strain and Drug [F (1, 19) = 6.048; P = 0.0237].

Discussion

ZDFs Develop Multiple Types of Pain-Like Behavior

Consistent with previous reports, ZDFs developed mechanical^{6, 25, 49, 69, 78, 80, 92, 101, 112}, heat^{25, 49, 78, 80}, and cold hyperalgesia⁸⁰ at 14 to 18 wks of age^{25, 69, 101}, results which we replicated in two separate cohorts. In contrast, a comparatively small number of ZDF studies report either mechanical hypoalgesia⁸⁹, heat hypoalgesia^{89, 92, 101}, no difference in heat⁶ or pressure⁷³ response thresholds, and/or pressure hyperalgesia prior to hyperglycemia⁷⁹.

Discrepancies in heat responses could be due to differences in the stimulus type: we used a hotplate while other studies used an infrared beam directed at the tail ⁹² or hindpaw ^{6, 89, 101}.

A recent review of preclinical models of PDN highlights the need to establish indices of spontaneous/affective pain ⁴⁷. One study used conditioned place preference (CPP), an assay that has re-emerged ⁹¹ as a leading preclinical measure of tonic pain in rats ⁴³ and mice ³², to reveal affective pain in type 1 diabetic STZ mice in the absence of an evoking stimulus ¹⁰³. Here, we discovered that type 2 diabetes exacerbates the avoidance of a noxious mechanical stimulus. Unlike CPP, where presumed relief of *non-evoked* affective pain reinforces a chamber preference, MCS utilizes the avoidance of an *evoked* stimulus as a measure of the motivational component of pain. Our results demonstrate for the first time the existence of a motivational-affective component of PDN in a preclinical model of type 2 diabetes.

ZDFs Develop Central Sensitization in Spinal Dorsal Horn Neurons

The dorsal horn is a key site of nociceptive integration ⁴ including central sensitization after tissue or nerve injury ¹⁰⁷. A recent electrophysiological study in ZDF spinal cord slices indicated that non-noxious mechanical stimulation increased afterdischarges and spontaneous activity of dorsal horn neurons ⁸⁷. However, the authors did not investigate pain-like behavior and they analyzed dorsal horn neurons at 32–34 wks, after ZDF aged 26 wks are reported to be hypoalgesic ⁴⁷. Thus, at this advanced diabetic stage, it is unclear whether central sensitization contributed to *painful* diabetic neuropathy. In our study using younger ZDF, we resolved this uncertainty by evaluating both behavioral hypersensitivity and stimulus-evoked pERK in the same subjects.

We are the first to demonstrate both pain-like behavior and elevated spinal neuron activation in ZDF. Both of these results were observed in response to a *noxious* mechanical stimulus, and this fits with the clinical manifestations of PDN: up to 71% of patients ⁷⁰ report hyperalgesia in response to application of a similar, static, noxious pressure stimulus ¹⁰⁶ used in the current study on ZDF. By contrast, Schuelert *et al* reported that spinal neurons were not sensitized to noxious mechanical stimuli ⁸⁷. Albeit, our results provide strong evidence in support of the hypothesis proposed by Schuelert *et al* that central sensitization contributes to hyperalgesia in type 2 diabetes ⁸⁷. If true, then therapies aimed at reducing central sensitization could alleviate PDN. Indeed, spinal administration of MEK inhibitors, which prevent phosphorylation of ERK, attenuated hyperalgesia in db/db ¹⁰⁹ and STZ ¹² models.

Pioglitazone Reduces Pathological Signs of PDN in ZDF by Actions at PPAR γ

Several lines of evidence lead us to speculate that activation of *spinal* peroxisome proliferator-activated receptor gamma (PPAR γ) mediated the antihyperalgesic actions of pioglitazone reported here in ZDF. First, spinal sites are rich in PPAR γ expression ^{11, 55, 61} and pioglitazone crosses the blood-brain barrier ⁵⁶. Second, intrathecal administration of PPAR γ antagonists inhibits the antihyperalgesic effect of both repeated systemic or single intrathecal administration of PPAR γ agonists ^{11, 30, 63}. Alternatively, non-PPAR γ mechanisms can be proposed for the antihyperalgesic effects of pioglitazone in PDN.

Pioglitazone inhibits HMGB1-RAGE signaling in spinal neurons¹⁰⁴ and reduces plasma RAGE³⁴, both of which are implicated in the pathogenesis of neuropathic pain in models of traumatic nerve injury²⁴ and PDN^{33,77}. Whether pioglitazone reduces the contribution of spinal gliosis and/or HMGB1-RAGE signaling to PDN remains an important direction of future studies.

Our current results suggest that pioglitazone decreases central sensitization to reduce PDN in type 2 diabetes. In support of this idea, chronic oral pioglitazone (at the same 30 mg · kg⁻¹ · d⁻¹ dose used in the current study) reduced non-noxious stimulus-induced mechanical allodynia and spinal pERK in a traumatic nerve injury model of neuropathic pain⁶³. Our results extend this finding by showing that pioglitazone reduces spinal pERK following a different stimulus (*noxious* pressure) and in a different neuropathic pain model that is associated with disease (type 2 diabetes).

Our studies aimed to evaluate whether ZDF exhibited hyperalgesia alongside spinal sensitization and whether this could be reduced by a TZD, and therefore the focus was not on peripheral mechanisms. However, several studies suggest that pioglitazone can inhibit peripheral mechanisms of chronic pain: 1) PPAR γ is expressed in sciatic nerve¹¹⁰; 2) TZDs decreased hyperalgesia, proinflammatory cytokine expression, and macrophage infiltration in sciatic nerve after nerve injury^{55,96}; 3) Pioglitazone reduced macrophage infiltration and pERK expression into the sciatic nerve of STZ rats¹¹⁰; 4) Pioglitazone reduced pERK expression in sciatic nerve of db/db mice¹⁵; and 5) Rosiglitazone and resolvin D1 coadministration to the site of hindpaw incision in db/db mice promotes a M2 macrophage phenotype and reduces mechanical hypersensitivity⁸². Future studies in preclinical models of type 2 PDN could investigate the effect of local TZD administration to the peripheral nerve^{31,96} on: macrophage polarization³¹; TNF α , which is elevated in patients with PDN^{74,99}; nerve conduction velocity, which is decreased in type 2 diabetes⁶; and pain-like behaviors.

Pioglitazone Reduces PDN Independent of its Anti-Hyperglycemic Action

Although we found that hyperglycemia preceded pain-like behavior as it does in type 2 diabetic patients¹, we propose that pioglitazone reduces PDN independent of its simultaneous reduction of blood glucose. This is based on several lines of evidence. First, pioglitazone and other PPAR γ agonists reduce neuropathic pain-like behavior in normoglycemic animals after various routes of administration including: repeated oral^{55,63} or intraperitoneal^{31,55,63,96}; local injection to the injured sciatic nerve⁹⁶ or incised paw³¹; or a single spinal^{11,30,62,71} or brain⁶² injection. Second, numerous drugs can reduce PDN without reducing hyperglycemia in patients with PDN, including tapentadol⁸⁸ or the FDA-approved PDN medications duloxetine and pregabalin^{59,90}. Furthermore, the antioxidant taurine⁴⁹, aldose reductase inhibitors^{9,66,75}, insulin growth factor^{112,113}, or poly-ADP-ribose inhibitors^{21,67,68} reduce hyperalgesia in animal models of PDN without inhibiting hyperglycemia. Consistent with these findings, pioglitazone normalized peripheral nerve conduction velocities without affecting hyperglycemia in the STZ model¹¹⁰. Third, mechanical hyperalgesia that is not associated with hyperglycemia has been reported in ZDFs, which suggests that hyperalgesia can be independent from blood glucose levels⁷⁹.

Finally, it is important to note that normalization of blood glucose alone cannot reverse the neurotoxic effects resulting from long-term hyperglycemia⁹⁸ that contribute to PDN¹⁰².

The results from this pretreatment study are clinically important as they suggest that pre-diabetic patients could benefit from adjuvant treatment with pioglitazone or an alternative PPAR γ agonist in addition to recommended lifestyle changes such as diet and exercise. This is supported by preclinical studies indicating that TZD treatment prior to or coinciding with peripheral nerve injury are quite effective at reducing hyperalgesia^{55, 63, 96}. Further study is needed to definitively dissociate the antihyperglycemic and antihyperalgesic effects of targeting PPAR γ with pioglitazone. For example, alternative agonists such as 15d-PGJ2 that alleviate neuropathic pain¹¹ without alteration of glucose or HbA1c could be tested in ZDF. Also, we could test a “control” drug that normalizes glucose metabolism without affecting hyperalgesia or investigate whether administration of glucose to pioglitazone-treated ZDF could reinstate hyperalgesia.

Conclusions and Future Directions

The current results are the first to demonstrate in a preclinical model of type 2 diabetes that pioglitazone prevents the development of not only pain-like behavior but also noxious stimulation-evoked central sensitization – within the same subjects. We go beyond the use of non-noxious von Frey hairs to evaluate spinal sensitization in ZDF⁸⁷ or nerve injury⁶³ by evoking spinal pERK and hyperalgesia using a clinically relevant pressure stimulus⁷⁰. Our results extend the potential efficacy of pioglitazone from resolving neuropathic pain after traumatic nerve injury to the growing problem of PDN.

Alternative strategies using other TZDs or related therapies may bypass the human safety risks of pioglitazone⁴⁸. First, the non-TZD PPAR γ agonists FK614⁶⁰, MBX-102^{10, 29}, or MDG548⁴⁶ reduce insulin insensitivity, hyperglycemia, and inflammation and are neuroprotective⁴⁶. Second, TZDs^{28, 111} or the novel ligand TT01001⁹⁵ reduce diabetic mitochondrial dysfunction by targeting the mitochondrial protein mitoNEET, which is thought to alleviate other neurological diseases^{23, 111}. Finally, a multiple drug strategy, such as coadministration of pioglitazone with canagliflozin¹⁰⁵ or geraniol³⁴ might reduce diabetes without the adverse effects of adipocyte differentiation, fat deposition, and weight gain associated with pioglitazone-only treatment. PDN patients may also benefit from other diabetic drugs such as metformin, which reduces peripheral neuropathic pain⁵⁷ and PDN in STZ rats^{7, 54}, as well as decreases hyperglycemia, HbA1c, and MG in patients with type 2 diabetes⁴¹. Future clinical studies could investigate whether the above treatments or other PPAR γ -directed therapies alleviate *both* hyperglycemia and neuropathic pain, as simultaneous inhibition is necessary to maximize quality of life in type 2 diabetic patients.

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Perspective

This is the first preclinical report to demonstrate that: 1) ZDF exhibit hyperalgesia and affective-motivational pain concurrent with central sensitization; and 2) Pioglitazone reduces both hyperalgesia and spinal sensitization to noxious mechanical stimulation within the same subjects. Further studies are needed to determine the anti-PDN effect of TZDs in humans.

Highlights

- The ZDF model of type 2 diabetes exhibits signs of motivational-affective pain
- Central sensitization of dorsal horn neurons coincides with mechanical hyperalgesia
- Pioglitazone inhibits hyperalgesia and central sensitization in type 2 diabetes
- PPAR γ -directed therapies may independently alleviate hyperglycemia and PDN
- TZDs may be beneficial for patients presenting with both diabetes and chronic pain

Fig. 1. ZDF rats develop a type 2 diabetes phenotype

(A) Body mass (n=12) and (B) blood glucose levels (n=12), serum (C) insulin (n=6) at 19 wks, (D) methylglyoxal-derived advanced glycation end-products (MG-AGE; n=3) at 19 weeks and (E) HbA1c at 12 weeks of age (n=10) in Zucker Diabetic Fatty (ZDF) and Zucker Lean (ZL) controls. * $p < 0.05$, ZDF vs. ZL.

Fig. 2. ZDF rats develop hyperalgesia

Paw withdraw responses to (A–B) heat, (C–D) pressure, and (E–F) cold stimuli in ZL and ZDF rats. Area under the curve (AUC) analyses for 14–18 wks are shown. n=12 per group. * $p < 0.05$, ZDF vs. ZL.

Fig. 3. Diabetes increases the avoidance of mechanical probes

(A) Diagram of the mechanical conflict avoidance system (MCS) used as a measure of affective-motivational pain in ZL and ZDF rats. MCS behavioral testing began at 17 wks of age and consisted of three stages: familiarization (1 d), training (4 d), and testing (3 d). *Familiarization:* Animals were initially placed in the light chamber and allowed free access to explore the entire MCS apparatus. *Training:* Animals were trained to move from the light chamber, through the stimulus chamber with mechanical probes set to a height of 0 mm (i.e. no probes), and into the dark chamber. Each animal underwent four training trials on each of the four consecutive training days. *Testing:* Animals were placed in the light chamber and allowed to cross the stimulus chamber at probe heights of 1 mm, 3 mm, and 4 mm. Each animal was tested for 3 trials at each probe height with only one probe height tested on each of the three testing days. (B) The latency to exit the light chamber (latency) in ZL and ZDF rats is shown for the 4 d of training (left) and 3 d of testing (right). The latency was similar in the absence of mechanical probes (probe height = 0 mm) during training and at a 1 mm probe height during testing. Raising the testing probe height increased the latency in ZL at 3 mm and ZDF at 3 and 4 mm. The latency was greater in ZDFs compared to ZLs at the 3 and 4 mm probe height. $n=9-10$ per group. * $p < 0.05$. † $p < 0.05$ vs. ZL at 1mm. ‡ $p < 0.05$ vs. ZDF at 1 mm.

Fig. 4. Diabetes exacerbates noxious pressure-induced activation of dorsal horn neurons
Pressure-evoked pERK expression and colabeling with NeuN in the L4/5 dorsal horn at 18 wks of age in ZL and ZDF rats. Representative images of pERK (**A**) in the stimulated (ipsilateral) or unstimulated (contralateral) side and (**B**) colabeling with the neuronal marker NeuN in the medial portion of the ipsilateral lumbar dorsal horn after pressure stimulation. (**C**) Quantification of cell-like profiles labeled with pERK in laminae I-II. $n=6$ per group. † $p<0.05$ vs. ZL ipsilateral. ‡ $p<0.05$ vs. ZDF ipsilateral. * $p<0.05$. Scale bars = 100 μm .

Fig. 5. Pioglitazone reduces pathological signs of type 2 diabetes

Effect of vehicle or pioglitazone (administered in food, yellow bar) on blood levels of (A) glucose (n=9–10 per group) from 11 to 19 wks and (B) HbA1c (n=9–10 per group) at 19 wks of age in ZL and ZDF rats. ‡ p<0.05 vs. both ZL groups. † p<0.05, ZDF Pioglitazone vs. ZDF Vehicle. * p<0.05.

Fig. 6. Pioglitazone attenuates pain-like behavior

Effect vehicle or pioglitazone in food (yellow bar) on the development of (A–B) heat and (C–D) mechanical hyperalgesia (n=9–10 per group). Pioglitazone inhibits the development of heat and mechanical hyperalgesia in ZDF. ‡ p<0.05 ZDF Vehicle vs. all other groups. * p<0.05.

Fig. 7. Pioglitazone attenuates dorsal horn neuron activation

Noxious pressure-evoked expression of pERK in the lumbar superficial dorsal horn of ZL and ZDF rats at 19 wks of age following a 7 wk treatment with vehicle or pioglitazone administered in chow. (A) Representative images of pERK immunostaining. (B) Pioglitazone reduced pERK expression in ZDFs to the level of ZLs. $n=5-6$ per group. * $p<0.05$. Scale bar = 100 μm .