

Protection of Sensory Function and Antihyperalgesic Properties of a Prosaposin-derived Peptide in Diabetic Rats

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Background: Short-term diabetes causes sensory disorders in rats ranging from thermal hypoalgesia to exaggerated behavioral responses to other sensory stimuli. As impaired neurotrophic support may promote sensory nerve disorders during diabetes, the authors investigated whether TX14(A), a neurotrophic peptide derived from prosaposin, was able to ameliorate nerve disorders in diabetic rats.

Methods: TX14(A) was delivered by intraperitoneal or intrathecal injection to control or streptozotocin-diabetic rats in either single or multiple (three times weekly) dose regimens. Efficacy was measured against diabetes-induced disorders of sensory nerve conduction velocity, paw withdrawal latency to radiant heat, tactile response thresholds to von Frey filaments, and flinching after paw formalin injection.

Results: Prolonged TX14(A) treatment of diabetic rats prevented the progressive decline in large sensory fiber conduction velocity in the sciatic nerve, development of paw thermal hypoalgesia, and increased flinching after paw formalin injection. The effect on formalin hyperalgesia persisted for 48 h but not 72 h after injection. No effects were noted in control rats. A single injection of TX14(A) 30 min before testing did not alter thermal response latencies in control or diabetic rats but prevented formalin hyperalgesia in diabetic rats. Tactile allodynia and the prolonged paw thermal hyperalgesia to radiant heat after intrathecal delivery of substance P were also dose-dependently ameliorated in diabetic rats by a single injection of TX14(A), whereas no effects were observed on the responses to these tests in control rats.

Conclusions: TX14(A) exhibits both neuroprotective and acute antihyperalgesic properties in diabetic rats without altering normal nociceptive function. (Key words: Neuropathy; pain; prosaptide.)

DIABETES is the most frequent cause of peripheral neuropathy in the developed world. Early disorders of nerve function in recently diagnosed diabetic patients include slowed conduction velocities that may be accompanied by paresthesias, dysesthesias, tactile allodynia, or spontaneous pain and an increase in pain perception thresholds.¹⁻³ Diabetic rats also display a range of sensory disorders that may model those seen in newly diagnosed

diabetic patients.⁴⁻⁸ Because short-term hyperglycemia in rats is not sufficient to induce the overt nerve degeneration or attempted regeneration that occurs as neuropathy progresses in diabetic patients,⁹ diabetic rats are useful for examining how hyperglycemia or its neurochemical consequences may induce sensory dysfunction in the absence of neurodegeneration and also for establishing the efficacy of potential therapeutic agents.

There is accumulating evidence of impaired neurotrophic support to peripheral nerves during hyperglycemia, and it has been speculated that neurotrophic factor supplementation may prove useful for treating diabetic neuropathy. Of the few studies published to date, nerve growth factor has proved effective in maintaining, or even exaggerating, neuropeptide levels in peripheral nerve of diabetic rodents and preventing thermal hypoalgesia^{10,11} but has not been shown to influence disorders of large sensory fiber function. In contrast, neurotrophin-3 selectively protects large sensory fibers.¹² The selectivity of individual neurotrophins may restrict their usefulness in a neuropathy such as diabetes that disrupts both large and small fibers.

We have recently demonstrated that small sensory fiber dysfunction, as indicated by thermal hypoalgesia, was prevented in rats with paclitaxel-induced neuropathy by concurrent treatment with prosaptides.¹³ Prosaptides are small peptide analogs of the region of the saposin C domain of prosaposin that possesses neurotrophic activity.^{14,15} Prosaptides also attenuated development of a range of nerve disorders in diabetic rats when treatment was begun at the onset of hyperglycemia. These included large-fiber conduction slowing and axonal atrophy and small-fiber disorders such as substance P depletion and thermal hypoalgesia.¹⁶ The potential action of prosaptides as therapeutic neurotrophic factors for treating peripheral neuropathies may be augmented by an acute antihyperalgesic property, as illustrated by studies in rats with tumor necrosis factor α -induced hyperalgesia.¹⁷ The present study was initiated to determine whether prosaptide TX14(A) could halt the progression of established sensory nerve disorders in diabetic rats and to define the acute antihyperalgesic properties of TX14(A) in normal and diabetic rats.

Materials and Methods

Animals and Peptide

All experiments were performed on adult (starting weight, 200–240 g) female Sprague-Dawley rats (Harlan

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Industries, San Diego, CA) using procedures approved by the local Animal Subjects Committee. Diabetes was induced by a single intraperitoneal injection of streptozotocin (50 mg/kg in 0.9% sterile saline; Sigma, St. Louis, MO) after an overnight fast. Hyperglycemia was confirmed 2 days later and also before behavioral testing by measurement of tail vein blood glucose concentration (Ames Glucostix; Myles Inc., Elkhart, IN) and at the time of death by spectrophotometric assay of plasma glucose concentration (glucose assay kit, Sigma, St. Louis, MO). Rats were allowed free access to food and water and were maintained on paper bedding with a 12-h light-dark cycle. Prosaptide TX14(A) (TXLIDNNATEEILY; where X = D-alanine) was synthesized to 98% purity (AnaSpec, San Jose, CA), and efficacy was confirmed by stimulation of neurite outgrowth and activation of intracellular signaling pathways in cultured neuroblastoma cells.^{15,18}

Study Design

To examine the effect of prolonged multidose treatment with prosaptide TX14(A) on sensory dysfunction in diabetic rats, TX14(A) was administered three times weekly by subcutaneous injection in 250 μ l of phosphate-buffered saline vehicle beginning either 4 weeks after the onset of hyperglycemia and continuing for a further 4 weeks, or for the duration of an 8-week period of diabetes. The three times weekly regimen was chosen based on prior studies using neurotrophic factors in hyperglycemic rats,¹² and the doses were based on prior studies in rats with diabetic or paclitaxel-induced neuropathy.^{13,16} Control and diabetic rats that did not receive TX14(A) were treated with an equivalent volume of phosphate-buffered saline vehicle.

For single dose studies, TX14(A) or vehicle was administered to previously untreated control or diabetic rats by either intraperitoneal injection or intrathecally in a volume of 10 μ l phosphate-buffered saline *via* a spinal catheter¹⁹ that had been implanted 3 or 4 days earlier during recovery halothane anesthesia (4% in oxygen for induction, 2% for maintenance).

Nerve Conduction

Nerve conduction studies were performed on rats anesthetized with halothane, as described above, 4 weeks after the onset of hyperglycemia (immediately prior to the onset of treatment with TX14(A) or vehicle) and after 8 weeks of hyperglycemia (24–48 hours after the final injection of TX14(A) or vehicle). A thermistor probe was placed adjacent to the left sciatic nerve *via* a skin incision and blunt separation of the connective tissue fascia between the biceps femoris and gluteus maximus muscles. The incision was closed, and local temperature was maintained at 37°C using a heat lamp and temperature controller during stimulation of the sciatic nerve (5V, 0.05-ms single square wave pulses) at

the sciatic notch or ankle. Evoked responses were recorded with needle electrodes placed in the interosseous muscles of the ipsilateral foot, amplified ($\times 100$) with a P15 AC Amplifier (Grass Instruments, Quincy, MA), and displayed on a 5110 Storage Oscilloscope and 5D10 Waveform Digitizer (Tektronix Inc., Beaverton, OR). The difference in H-wave latency was measured as the time required for sensory conduction to travel from ankle to notch. This procedure was repeated three times for each rat, and the median latency differences was used for calculating sensory nerve conduction velocity (SNCV) by dividing the distance between the stimulation sites by the latency difference.

Formalin Test

The formalin test was performed using 50 μ l of a 0.5% formalin solution injected into the dorsal surface of the right hind paw. We have previously demonstrated that this dose of formalin evoked submaximal flinching in control rats and revealed significant hyperalgesia during phases Q and 2 in diabetic rats.²⁰ Subsequent studies in control rats used 50 μ l of 5.0% formalin solution to obtain maximal behavioral responses. Flinches were counted for 1-min periods at 5-min intervals for 60 min with phase 1 defined as flinching during minutes 1–2, the Q phase as the sum of flinches counted during minutes 5–6, 10–11, 15–16, and 20–21; and phase 2 at all time points thereafter.

Tactile Responses

Rats were transferred to a testing cage with a wire mesh bottom and allowed to acclimate for 10–15 min. von Frey filaments (Stoelting, Wood Dale, IL) were used to determine the 50% mechanical threshold for foot withdrawal.²¹ A series of filaments, starting with one that possessed a buckling weight of 2.0 g, was applied in sequence to the plantar surface of the right hind paw with a pressure that caused the filament to buckle. Lifting of the paw was recorded as a positive response, and the next lightest filament was chosen for the next measurement. Absence of a response after 5 s prompted use of the next filament of increasing weight. This paradigm was continued until four measurements had been made after an initial change in the behavior or until five consecutive negative (15 g) or four positive (0.25 g) responses had occurred. The resulting sequence of positive and negative scores was used to interpolate the 50% response threshold.

Thermal Responses

To determine responses to an acute thermal nociceptive stimulus, rats were placed in an open-top Plexiglas cylinder on top of a Thermal Stimulation System (UARD, San Diego, CA) with a surface temperature of 30°C. After a 5-min acclimation period, a mobile radiant heat source was maneuvered below the plantar surface of the right

Table 1. Physiologic Parameters

Group	N	Body Weight (g)		SNCV (m/s)		Thermal Response Latency (s)	
		Week 4	Week 8	Week 4	Week 8	Week 4	Week 8
Control	8	262 ± 31	273 ± 27	59.9 ± 3.7	62.3 ± 4.0	12.5 ± 1.5	13.3 ± 2.0
Diabetic	9	183 ± 19	176 ± 15	50.9 ± 5.8	45.9 ± 4.7*	13.2 ± 3.0	17.8 ± 2.9*
Diabetic + TX14(A)	9	186 ± 18	171 ± 19	50.9 ± 5.4	50.9 ± 8.0	12.0 ± 2.4	14.2 ± 4.0

Changes in body weight, sensory nerve conduction velocity (SNCV), and paw thermal response latency between weeks 4 and 8 of diabetes and the effect of thrice-weekly treatment with 200 $\mu\text{g/kg}$ TX14(A) for this period. Data are mean \pm SD. Within-group comparisons between weeks 4 and 8 by paired *t* test.

* *P* < 0.05 versus week 4.

hind paw, and heat was applied (to increase surface temperature to 38.5°C in 20 s) until the limb was moved. The time latency between initiation of heating and withdrawal response was recorded automatically by movement sensors. An initial measurement was made to acustom the animal to the measurement conditions, and then three subsequent measurements were made, each 5 min apart. The median response time of the three measurements was used for analysis.

Substance P Test

Rats were implanted with spinal catheters during halothane anesthesia and allowed to recover for 3–4 days before use. Thermal response tests were performed on both hind paws as previously described. Tests were performed before and at time points after intrathecal delivery of substance P (3 or 30 nmol) in a total volume of 10 μl phosphate-buffered saline.²²

Statistical Analyses

All measurements were performed by investigators who were unaware of the treatment received by individual animals to preclude observer bias. Statistical analyses were performed using the Instat software package (Graphpad Software, San Diego, CA). Physiologic parameters and formalin-evoked flinch responses at a single time point were compared between multiple groups by one-way analysis of variance, and, where the *F* test indicated *P* less than 0.05, between-group differences were identified using the Student-Newman-Keuls *post hoc* test. Within a group, changes over time between two points were compared by paired *t* test (SNCV and thermal response latency). Comparisons between a defined start value and a number of subsequent time points were made for parametric data (thermal response latency during the intrathecal substance-P test) by repeated-measures analysis of variance with individual differences from the start value identified by Dunnett's test and for nonparametric data (tactile response threshold) by the Friedman test followed by Dunn's *post hoc* test. Parametric data are presented as mean \pm SD and nonparametric data as median \pm 95% confidence intervals.

Results

Prolonged Treatment with TX14(A)

Two separate experiments were performed. In the first, one group of control and two groups of untreated diabetic rats were maintained for 4 weeks. One of the diabetic groups then received 200 $\mu\text{g/kg}$ TX14(A) three times weekly for 4 further weeks, whereas the other diabetics and the control groups received vehicle. TX14(A) treatment did not alter diabetic hyperglycemia at the end of the study (control = 7.1 ± 1.1 mm; untreated diabetic = 37.6 ± 2.9 mm; TX14(A)-treated diabetic = 37.6 ± 5.9 mm) or reverse loss of body weight in diabetic rats (table 1). However, it did halt the progressive slowing of SNCV between weeks 4 and 8 and prevented development of thermal hypoalgesia (table 1).

After 8 weeks of diabetes, flinching frequency after injection of 0.5% formalin into the hind paw was exaggerated during phases Q and 2 in vehicle-treated rats (both *P* < 0.05) compared with controls (fig. 1). Treatment with TX14(A) for the last 4 weeks of diabetes, with the last treatment occurring 24 h before the formalin test, prevented hyperalgesia during phases Q and 2 so that values were not different from control and significantly (both *P* < 0.05) lower than vehicle-treated diabetic rats. A single intraperitoneal injection of 200 $\mu\text{g/kg}$ TX14(A) given to previously vehicle-treated diabetic rats 30 min before the formalin test also prevented hyperalgesia in both of these phases (both *P* < 0.05 vs. untreated diabetic rats).

In a second study, groups of control and diabetic rats received three-times-weekly injections of vehicle or 200 $\mu\text{g/kg}$ TX14(A) for the duration of 8 weeks of diabetes. Flinch responses to paw injection of 0.5% formalin were measured in vehicle and TX14(A)-treated control rats 24 h after the final injection. No significant difference was found in flinching between the two groups (fig. 2). Vehicle-treated diabetic rats exhibited significantly (*P* < 0.01) exaggerated flinching during phase 2 compared with vehicle-treated control rats. Suppression of the exaggerated flinching of diabetic rats by TX14(A) treatment persisted for 48 h after the last injection but had disappeared within 72 h (fig. 2).

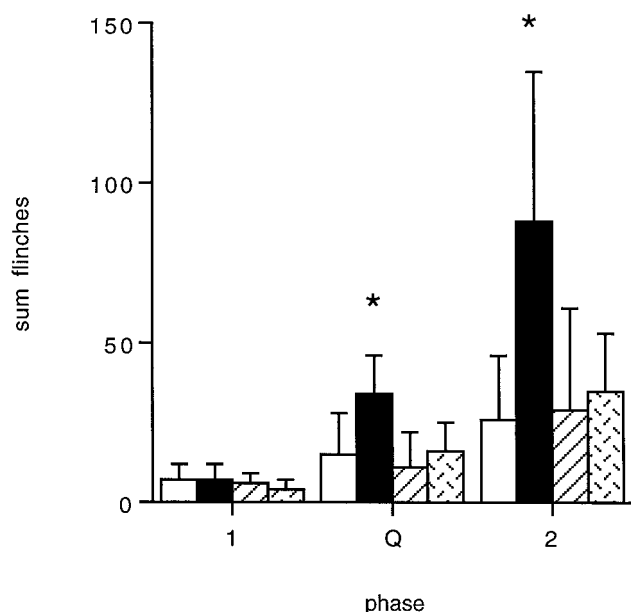


Fig. 1. Sum flinches during phases 1, Q, and 2 of the response to 0.5% paw formalin injection in control rats (open bars), untreated diabetic rats (filled bars), diabetic rats treated with 200 µg/kg TX14(A) for the last 4 weeks of diabetes (striped bars), and diabetic rats treated with a single bolus injection of 200 µg/kg TX14(A) 30 min before the formalin test (hatched bars). $n = 5-7$ per group. Data are mean \pm SD. * $P < 0.05$ versus all other groups by one-way analysis of variance and Student-Newman-Keuls *post hoc* test.

Short-term Treatment with TX14(A)

All single-treatment studies were performed on otherwise untreated streptozotocin-diabetic rats (blood sugar > 27.7 mM at the time of testing) and age-matched controls. To determine whether the prevention of thermal hypoalgesia seen in rats repeatedly treated with TX14(A) (table 1) arose solely from the final dose administered 24 h before the test, we measured thermal response latencies in otherwise untreated control and diabetic rats 24 h after a single intraperitoneal injection of 200 µg/kg TX14(A). There was no change in thermal response latency compared with preinjection values in either controls ($95.7 \pm 8.2\%$; $n = 6$) or diabetic rats ($95.4 \pm 5.8\%$; $n = 6$). To determine whether the acute antihyperalgesic effect of TX14(A) on the formalin test in diabetic rats (fig. 1) extended to formalin-induced responses in control rats, we gave control rats a single intraperitoneal injection of TX14(A) 30 min before performing the formalin test using either 0.5% or 5.0% formalin. TX14(A), at doses between 20 and 1,000 µg/kg, was without significant effect on any phase (table 2). Intrathecal delivery of 1 µg of TX14(A) 30 min before either 0.5% or 5.0% formalin tests was also without effect (table 2).

The rapid effect of TX14(A) on hyperalgesia during the formalin test in diabetic rats prompted studies of other behavioral indices of altered nociception in separate groups of diabetic rats. No effect of 1 mg/kg TX14(A) on the tactile responses of control rats was apparent over a

24-h period compared with vehicle-treated controls (fig. 3A). After 4 weeks of hyperglycemia, diabetic rats exhibited tactile allodynia that was ameliorated by intraperitoneal delivery of 1 mg/kg TX14(A), with an effect that was maximal 6 h after injection ($P < 0.01$ vs. preinjection values) and disappeared by 24 h (fig. 3B). The efficacy of a range of doses (30 µg to 10 mg/kg administered intraperitoneally) of TX14(A) in ameliorating tactile allodynia 6 h after injection was examined. The ED_{50} fell between 300 µg/kg and 1 mg/kg, with maximal efficacy occurring at 1 mg/kg ($82 \pm 28\%$ of maximal possible effect). Using the 1-mg/kg dose, we then investigated the effects of repeat dosing with TX14(A) and tactile testing in previously untreated diabetic rats. Where testing was performed at 1, 3, 6, 9, and 24 h after each of four injections of TX14(A) spaced either 48 or 72 h apart, the peak effect seen at the 6-h time point progressively declined (fig. 4). When the 50% response threshold at 6 h after the last of the four injections was compared with that of a group of diabetic rats that had received parallel injections but had not been tested after the first three injections, it was significantly ($P < 0.02$ by Mann-Whitney U test for nonparametric data) lower in rats that had undergone multiple testing.

In a separate group of rats, 4 weeks of diabetes (blood sugar level > 27.7 mM at the time of testing) did not significantly alter thermal response latencies (12.53 ± 1.01 s; $n = 8$) compared with age-matched controls (11.31 ± 0.99 s; $n = 8$). Cerebrospinal fluid collected at the time of spinal catheter implantation also showed

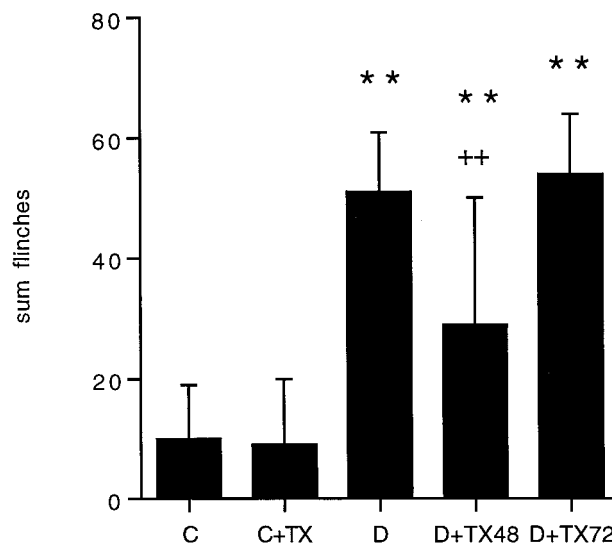


Fig. 2. Sum flinches during phase 2 of the response to 0.5% paw formalin injection in control rats (C); control rats treated with 1 mg/kg TX14(A) three times weekly for 8 weeks, and with the last injection 24 h before testing (C+TX); untreated diabetic rats (D); diabetic rats treated with 1 mg/kg TX14(A) three times weekly for 8 weeks, with the last injection either 48 h (D+TX48) or 72 h (D+TX72) before testing. $n = 5-12$ per group. Data are mean \pm SD. ** $P < 0.01$ versus C or C+TX and ++ $P < 0.01$ versus D by one-way analysis of variance and Student-Newman-Keuls *post hoc* test.

Table 2. Effect of TX14(A) on Formalin-evoked Flinching

Group	Administration Route	N	Phase 1	Q Phase	Phase 2
5% Formalin					
Control + PBS	IP	6	22 ± 6	26 ± 14	123 ± 42
Control + 20 µg TX14(A)	IP	5	21 ± 9	18 ± 7	110 ± 30
Control + 200 µg TX14(A)	IP	6	14 ± 8	27 ± 14	136 ± 38
Control + 1,000 µg TX14(A)	IP	6	18 ± 4	21 ± 24	145 ± 38
Control + PBS	IT	5	21 ± 13	20 ± 20	118 ± 36
Control + 1 µg TX14(A)	IT	5	26 ± 8	37 ± 18	122 ± 36
0.5% Formalin					
Control + PBS	IP	6	5 ± 10	10 ± 4	15 ± 8
Control + 20 µg TX14(A)	IP	5	6 ± 3	12 ± 13	22 ± 20
Control + 200 µg TX14(A)	IP	5	8 ± 7	17 ± 20	17 ± 15
Control + 1,000 µg TX14(A)	IP	5	6 ± 6	15 ± 5	17 ± 13
Control + PBS	IT	5	6 ± 2	5 ± 3	10 ± 7
Control + 1 µg TX14(A)	IT	5	9 ± 5	9 ± 6	9 ± 4

Sum flinches counted per phase after injection into the hindpaw of either 5% or 0.5% formalin in rats treated with TX14(A), either by intraperitoneal (IP) or intrathecal (IT) administration 30 min before formalin injection. Data are mean ± SD. No significant differences between groups were noted using one-way analysis of variance or unpaired *t* test as appropriate.

PBS = phosphate-buffered saline.

increased glucose levels (controls = 2.4 ± 0.4 vs. diabetics = 7.3 ± 1.8 mM; $P < 0.01$ by unpaired *t* test). No significant thermal hyperalgesia occurred in either control or diabetic rats in the 1-h period after intrathecal delivery of 3 nmol of substance P (data not shown). Intrathecal delivery of 30 nmol of substance P induced a transient thermal hyperalgesia in control rats that was significantly ($P < 0.05$) different from predelivery responses only at the 15-min time point (fig. 5A). Diabetic rats exhibited thermal hyperalgesia of similar magnitude, but this was prolonged such that response times remained significantly ($P < 0.05$) lower up to 60 min after delivery. Neither control nor diabetic rats treated with 1 mg/kg TX14(A) intraperitoneally showed any change in thermal response latency between measurements made before TX14(A) delivery and measurements made 30 min later, immediately before delivery of substance P (fig. 5B). TX14(A) did not modify the magnitude or duration of substance P-induced thermal hyperalgesia in control rats. However, in diabetic rats, the prolonged hyperalgesia was prevented after TX14(A) treatment such that significant ($P < 0.05$ vs. baseline) hyperalgesia occurred only at the 15-min time point.

Discussion

Prosaposin is the precursor of saposins A-D, which act as lysosomal activator peptides for sphingolipid hydrolases.²³ Prosaposin is also found in cerebrospinal fluid, seminal plasma, and milk, indicating the presence of alternative secretory pathways.²⁴ Prosaposin exhibits neurotrophic properties *in vitro*,¹⁴ and this activity is localized to the N-terminal region of the saposin C domain,¹⁵ whereas the ability to activate sphingolipid hydrolases is confined to the C-terminal region.²⁵ Peptides derived from the N-terminal region of saposin C, called

prosaptides, also act as neurotrophic factors in cell culture systems,^{15,18,26} and prosaptides, including TX14(A), prevented thermal hypoalgesia in a rat model of paclitaxel-induced neuropathy.¹³ In the present studies we extended observations of the *in vivo* properties of TX14(A) and demonstrated efficacy against progression of a range of sensory disorders of diabetic rats.

We recently reported that TX14(A) treatment for the duration of diabetes can protect against onset of structural and functional disorders of small and large fibers without having effects on control nerve.¹⁶ Our present findings indicate that it can also halt progression of an established deficit in large-fiber SNCV, indicating therapeutic benefit against a developing neuropathy. It remains to be seen whether higher doses than used in the present study may also reverse an established SNCV disorder. NCV slowing in diabetic rats has been related to glucose metabolism by aldose reductase,²⁷ possibly *via* effects on nerve blood flow,²⁸ axonal caliber,²⁹ or neurochemical disorders.³⁰ TX14(A) does not act as an aldose reductase inhibitor or prevent nerve ischemia in diabetic rats.¹⁶ However, it does protect against axonal atrophy¹⁶ and may replace deficient endogenous neurotrophic support to large fibers, although effects on other aspects of nerve metabolism cannot be discounted.

There are conflicting data regarding the effects of diabetes on paw thermal response latencies in rats, with hypoalgesia,^{5,31} hyperalgesia,³² and normal sensitivity³³ all reported. We previously found thermal hypoalgesia after 4 weeks of diabetes using a 52.5°C hot-plate test.³⁴ In the present study, two different experiments revealed only a trend toward thermal hypoalgesia at this time point using a directed heat source, perhaps because of the different methodology used. The progressive development of thermal hypoalgesia between weeks 4 and 8 of diabetes was prevented by three-times-weekly

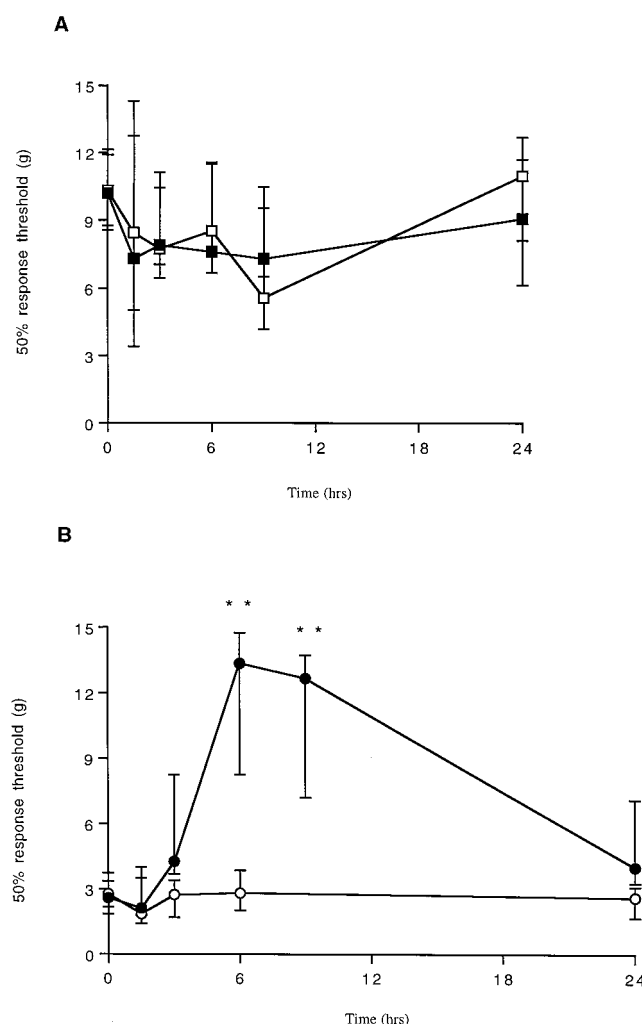


Fig. 3. Fifty percent response thresholds to von Frey filaments in (A) untreated (open squares) or 1 mg/kg TX14(A)-treated (filled squares) control rats and (B) untreated (open circles) or 1 mg/kg TX14(A)-treated (filled circles) diabetic rats. Data are median \pm 95% confidence limits. $n = 6$ per group. Values at time points after TX14(A) injection were compared with preinjection values by the Friedman test for nonparametric data and Dunn's *post hoc* test. *** $P < 0.01$ versus preinjection values.

TX14(A), with the last dose given 24 h before testing. A single injection of TX14(A) at the same dose did not alter thermal response latencies of diabetic rats when measured 24 h later. Repeated dosing appears to be required for efficacy of TX14(A), perhaps as a result of phenotypic regulation of thermal nociceptive fibers or central sensory pathways. The actions of TX14(A) on thermal response latencies differs from other neurotrophic factors such as nerve growth factor, which, while attenuating thermal hypoalgesia in diabetic animals, also rapidly reduced nociceptive thresholds in controls.^{10,35} We previously demonstrated that long-term treatment with prosaptides does not alter the thermal response latency of normal rats.^{13,16} Thus, the actions of TX14(A) appear to be directed selectively against diabetes-induced abnormal thermal nociception rather than normal thermal nociceptive processing.

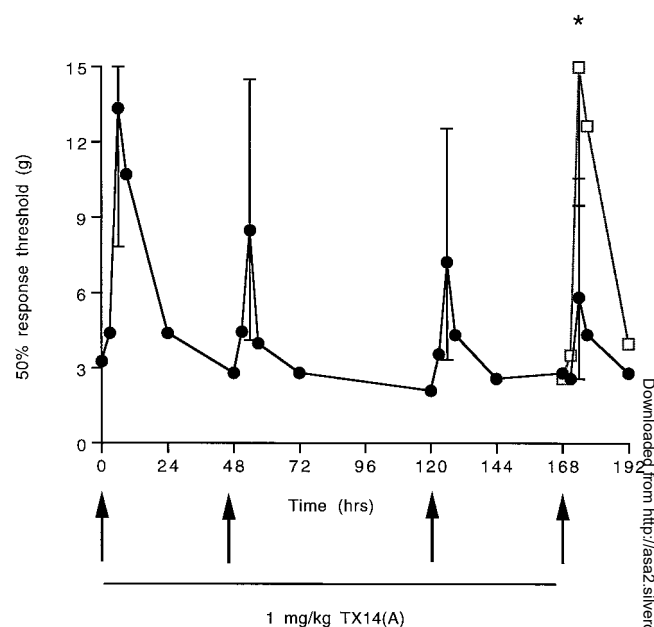


Fig. 4. Fifty percent response thresholds to von Frey filaments in diabetic rats after repeated injections of 1 mg/kg TX14(A) (filled circles). Animals that received equivalent injections of TX14(A) but were only tested after the final injection are shown by open squares. Data are median \pm 95% confidence limits. $n = 6$ per group. * $P < 0.02$ versus the repeatedly tested group at this time point by Mann-Whitney U test for nonparametric data.

The formalin test comprises behavior reflecting a combination of primary afferent input arising from developing peripheral inflammation and amplification of the afferent signal in the spinal cord. Diabetic rats show increased flinching that is prevented by treatment with insulin⁶ or aldose reductase inhibitors.^{20,34} Gangliosides and opiates are also effective, but their action is nonspecific as they exhibit equal or greater efficacy in control rats.^{8,36} TX14(A), delivered either as a single bolus dose 30 min before the test or *via* long-term administration, suppressed hyperalgesia in diabetic rats. After long-term dosing, the efficacy against hyperalgesia was complete at 24 h after the last injection, partial by 48 h, and had disappeared by 72 h. No effects of TX14(A) were noted on formalin-induced flinching in control rats, using either 0.5% formalin to reveal potential hyperalgesic actions or 5.0% formalin to provide maximal flinching and illustrate potential antihyperalgesic properties. There was also no effect when TX14(A) was delivered intrathecally to localize delivery to the spinal cord. Although we cannot exclude effects outside the dose and time ranges used, our data suggest that TX14(A) is selectively effective against diabetes-induced hyperalgesia during the formalin test and not against normal nociceptive processing.

The rapid-acting effect of TX14(A) on formalin-evoked hyperalgesia in diabetic rats prompted examination of efficacy against other behavioral sensory tests that show abnormalities during diabetes. TX14(A), at a dose that ameliorated formalin hyperalgesia within 30 min, was not effective against tactile allodynia. However, higher

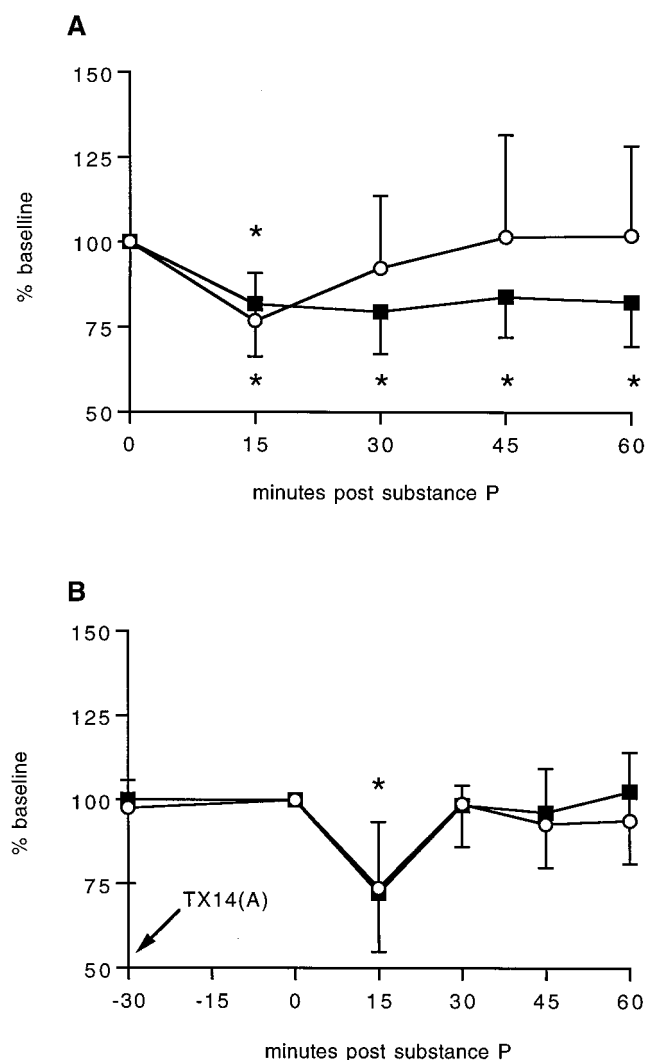


Fig. 5. Paw thermal response latency, expressed as a percentage of the latency immediately before intrathecal delivery of (A) 30 nmol substance P to control (open circles) and diabetic (closed squares) rats and (B) 30 nmol substance P to control (open circles) and diabetic (closed squares) rats after intraperitoneal injection of 1 mg/kg TX14(A). Data are mean \pm SD. $n = 5-8$ per group. Within each group, values at time points after substance P delivery were compared with pre-substance P (baseline) values by repeated-measures analysis of variance with Dunnett's *post hoc* test. * $P < 0.05$ versus pre-substance P values.

doses produced reversal of allodynia that was maximal 6 h after treatment. Repeated injections of TX14(A) did not diminish efficacy against tactile allodynia, although repeated tactile testing reduced the apparent efficacy. This may be a result of changes in paw sensitivity to the von Frey filaments, as noted in control rats.²¹ Thus, the dose and time profile for relief of tactile allodynia in diabetic rats by TX14(A) is distinct from the formalin test, possibly reflecting different pathophysiologies. How TX14(A) may attenuate hyperalgesia and allodynia in diabetic rats is unclear, as it does not act *via* agonism or antagonism of a range of receptors, including those responding to the following ligands: tumor necrosis factor α , bradykinin (B_2 receptor), calcitonin gene-related

peptide, acetylcholine (M_{1-3} receptors), histamine (H_1 receptor), opiates (δ , κ , or μ receptors), γ -aminobutyric acid, glutamate (kainate or *N*-methyl-D-aspartate receptors), glycine (strychnine-sensitive site), or flux through L-type calcium channels (J. S. O., unpublished observations).

The formalin and tactile tests both evoke primary afferent activity but may also incorporate central processing. We used intrathecal delivery of substance P, which initiates transient thermal hyperalgesia in normal rats,²² to focus on a defined spinal stimulus. The involvement of peptides in the exaggerated pain responses of diabetic rats is suggested by reports that NK-1 receptor antagonists alleviate mechanical hyperalgesia and tactile allodynia in such animals,^{38,37} although there is a paradoxical decrease in primary afferent levels, axonal transport and evoked release of substance P.^{38,39} The prolonged thermal hyperalgesia seen in diabetic rats after intrathecal delivery of 30 nmol of substance P is a new finding and suggests that nociceptive input is amplified at the spinal level by diabetes. The ability of TX14(A) to rapidly prevent the prolonged thermal hyperalgesia in diabetic rats without altering either the initial hyperalgesic period or the hyperalgesia seen in control rats argues against it acting directly as an NK-1 antagonist. Furthermore, altered pharmacokinetic characteristics³⁶ indicate that diabetic animals are likely to receive lower local doses of TX14(A) than controls after systemic administration. Although the possibility that TX14(A) may affect substance P-induced transient thermal hyperalgesia in control and diabetic rats at higher doses cannot be discounted, the present data suggest that TX14(A) acts at a spinal or supraspinal site to block a gain of function caused by diabetes.

In summary, TX14(A) had effects on disorders of large and small sensory fiber function in diabetic rats. The halting of progressive SNCV slowing and thermal hypoalgesia suggests potential use as an intervention therapy in developing diabetic neuropathy. Prevention of formalin- and substance P-induced hyperalgesia occurred within minutes of a single injection, whereas relief of tactile allodynia took hours to develop, and prevention of thermal hypoalgesia required multiple treatments. This suggests that TX14(A) may act concurrently through a variety of mechanisms ranging from fast-acting ion channel block or modification of intracellular signaling cascades to phenotypic regulation. The short circulating half-life of TX14(A) (10 min; J. S. O., unpublished observations) is accompanied by the ability to induce prolonged signaling cascades in neuronal and glial cells,^{18,40} suggesting that only a brief receptor occupancy is required to initiate protracted intracellular and physiologic consequences. The precise nature of the G protein-coupled prosaposin-prosaptide receptor²⁶ is not known, and its elucidation will help indicate potential sites of action for TX14(A) on abnormal sensory processing. The ability of

TX14(A) to rapidly correct abnormal sensory processing in diabetic rats without altering normal nociceptive function, coupled with neuroprotective actions that halt deterioration of peripheral nerve function, make prosapaptides of interest as potential agents for both rapid pain relief and prevention of progressive neuropathies.

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