Direct Role of Streptozotocin in Inducing Thermal Hyperalgesia by Enhanced Expression of TRPV1 in Sensory Neurons

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Abbreviation:
ADPR, adenine 5'-diphosphoribose; DPN, diabetic peripheral neuropathy; DRG, dorsal root ganglion; FDA, fluorescein diacetate; HA, hemagglutinin; HEK, human embryonic kidney; IDDM, insulin-dependent diabetes mellitus; PWL, paw withdrawal latency; NBM, neurobasal medium; PI, propidium iodide; PARP, poly ADP-ribose polymerase; ROS, reactive oxygen species; STZ, streptozotocin
Abstract

Streptozotocin (STZ) is a diabetogenic agent extensively used to induce diabetes and to study complications including diabetic peripheral neuropathy (DPN). While studying the influence of TRPV1 on DPN in the STZ-induced diabetic mouse model, we found that a proportion of STZ-treated mice was non-diabetic, but still exhibited hyperalgesia. In order to understand the mechanism underlying this phenomenon, dorsal root ganglion (DRG) neurons and stably TRPV1 expressing HEK293T cells were used to study the expression and the function of TRPV1. Incubation of DRG neurons with STZ resulted in a significant increase in the amplitude of capsaicin-induced TRPV1-mediated current and Ca$^{2+}$ influx as compared to vehicle-treated sister cultures. It was also found that STZ treatment induced higher levels of reactive oxygen species (ROS), which was abolished with concomitant treatment with catalase. Treatment of cells with H$_2$O$_2$ mimicked the effects of STZ. Western blot analysis revealed an increase in TRPV1 protein content and phospho p38 (p-p38) MAPK levels in DRG of STZ-injected diabetic and non-diabetic hyperalgesic mice as compared to control mice. Furthermore, in stably TRPV1 expressing HEK293T cells, STZ treatment induced an increase in TRPV1 protein content and p-p38 MAPK levels, which was abolished with concomitant treatment with catalase or p38 MAPK inhibitor. These results reveal that STZ has a direct action on neurons and modulates expression and function of TRPV1, a nociceptive ion channel that is responsible for inflammatory thermal pain.
Introduction

STZ, a glucosamine-nitrosourea compound obtained from *Streptomyces achromogenes*, is used as a common tool to induce insulin-dependent diabetes mellitus (IDDM) in rodents to study diabetes-induced complications. One such complication is diabetic peripheral neuropathy (DPN). DPN in rodents and humans is characterized by an early thermal and mechanical hyperalgesia (Courteix et al., 1996; Sugimoto et al., 2000). A direct effect of hyperglycemia contributing to hyperalgesia has been suggested by various studies (Courteix et al., 1996; Chen and Pan, 2002). However, treatment with insulin-like growth factor (IGF) or direct neuronal delivery of low doses of insulin, insufficient to reduce hyperglycemia, ameliorated diabetic neuropathy suggesting that mechanisms other than hyperglycemia may be involved in the pathogenesis of hyperalgesia (Zhuang et al., 1997; Brussee et al., 2004).

STZ is transported into beta cells of the pancreas through glucose transporter GLUT2 and causes DNA damage either by alkylation, by the generation of nitric oxide (NO) or by the generation peroxynitrite (Schnedl et al., 1994; Turk et al., 1993; Kröncke et al., 1995). The DNA strand breaks lead to the activation of the nuclear enzyme poly ADP-ribose polymerase (PARP), which synthesizes large amounts of the ADP-ribose polymer, using cellular nicotinamide adenine dinucleotide (NAD\(^+\)) as a substrate (Pieper et al., 1999; Delaney et al., 1995; Ledoux et al., 1988). A decrease in the intracellular NAD\(^+\) levels causes a depletion of ATP, a mechanism that can induce cell death (Berger, 1985). Failure of STZ to induce diabetes in PARP deficient mice suggests the important role played by PARP in STZ-induced beta cell necrosis (Burkart et al., 1999). Another proposed mechanism of STZ-induced cytotoxic effect is its selective inhibition of N-acetyl-beta-D-glucoaminidase (O-GlcNAcase), which is highly expressed in pancreatic beta cells as compared to neurons (Konrad et al., 2001). Inhibition of O-GlcNAcase results in increased glycosylation of proteins altering their
structure and function leading to the death of beta cells (Konrad et al., 2001). In pancreatic beta cells, STZ has been shown to produce superoxide anion by inhibiting the Krebs cycle, which will limit the generation of ATP, promoting the death of beta cells (Turk et al., 1993; Nukatsuka et al., 1990; Sofue et al., 1991). However, while the effects of STZ are believed to be specific to pancreatic beta cells, STZ administration has been shown to adversely affect renal, hepatic and muscle tissues (Brambilla et al., 1987; Petzold and Swenberg, 1978; Johnston et al., 2007).

Topical application of capsaicin, a Transient Receptor Potential Vanilloid 1 (TRPV1) agonist, improves sensory perception in humans with DPN (Forst et al., 2002). In animal models of diabetes, where DPN manifests as hyperalgesia, TRPV1 expression and function have been shown to be increased (Kamei et al., 2001; Hong and Wiley, 2005). The TRP family of ion channels is involved in sensing physical and chemical stimuli (Clapham, 2003). TRPV1 is a Ca\(^{2+}\) permeant non-selective cation channel expressed predominantly in unmyelinated C-fibers and thinly myelinated A\(\delta\) fibers (Julius and Basbaum, 2001). TRPV1 is activated by heat (>42 °C), capsaicin, protons, N-arachidonyl dopamine (NADA), anandamide and leukotrienes (Julius and Basbaum, 2001; Caterina et al., 1997). A role of TRPV1 beyond that of a simple temperature sensor is suggested by the diversity of its agonists and its expression in areas that are not subjected to higher temperatures (Huang et al., 2002; Mezey et al., 2000; Birder et al., 2001). Sensitization of TRPV1 by phosphorylation is mediated by numerous agents including, prostaglandins (PGs), bradykinin (BK), glutamate, serotonin, histamine, ATP, and trypsin (Julius and Basbaum, 2001). Modulation by substances associated with inflammation suggests that TRPV1 functions as a sensor of nociceptive inflammatory thermal pain and the finding is corroborated by a lack of thermal hyperalgesia in TRPV1 knock-out mice (Caterina et al., 2000; Davis et al., 2000).
This study was undertaken based on the observation that a proportion of STZ-injected mice did not become diabetic but exhibited hyperalgesia. Here, we sought to determine whether STZ can exert a direct effect on neurons contributing to the hyperalgesia by altering the expression and function of TRPV1.

Materials and Methods

Induction of diabetes

All procedures used in this study were approved by the animal care and use committee at Southern Illinois University, School of Medicine and conformed according to NIH and institutional guidelines. Mice were housed in standard cages and maintained on a 12-h light/dark cycle at an ambient temperature of 22 ± 1°C. Mice were housed in specific pathogen free barrier animal facility and rodent laboratory chow (Laboratory Diet 5001, Nutrition International, Inc., Brentwood, MO, USA) and drinking water were provided ad libitum. Mice were at least 6-10 weeks of age and weighed between 18-23 gms at the beginning of the experiments.

Age-matched single transgenic mice (Ins-HA.D2 mice, which express influenza hemagglutinin (HA) peptide were used and were kindly provided by Dr. Lo (Lo et al., 1992). Freshly prepared STZ (50 to 200 mg/kg) in saline (pH 4.5 with 0.1 N citrate buffer) was injected intraperitoneally as described previously (Kamei et al., 1991). Control mice received citrate buffered saline alone. Although Ins-HA.D2 mice are genetically altered, HA expression does not seem to affect glucose metabolism or neuronal function as measured by blood glucose levels and hot plate tests as compared to normal wild-type mice.
Glucose levels were determined with an OneTouch Ultra blood glucose monitoring system (Life Scan, CA) using whole-blood obtained from the tail. Diabetes was defined as blood glucose concentrations greater than 299 mg/dl (16.7 mM) (Kamei et al., 1991).

**Determination of thermal pain sensitivity**

All the mice used in this study were housed in the barrier facility. Mice were tested in the barrier facility on the days the cages were not cleaned to avoid factors that might influence the test measurements. Mice were placed individually on a Hot Plate Analgesia Meter (Harvard Apparatus, Boston, MA) maintained at a constant temperature of 52 ± 0.3°C. The paw withdrawal latency (PWL) is defined as the time taken for the animal to exhibit a distinct pain behavior either by a hind paw lick or a characteristic hind paw flick (whichever occurs first). Mice that did not respond within 20 seconds were removed from the hot plate to prevent tissue damage. We did not find a significant difference in PWL either with the duration of habituation (0.5-2 hrs). The experiments were conducted in a blind fashion by measuring the PWL in randomly chosen animals from diabetic or control groups. After completing the test, the ear tags were read to place them in the appropriate groups. There were slight sex differences, but were not statistically significant, therefore males and females were grouped together for further analyses.

**In vitro STZ treatment**

To assess the effect of STZ on TRPV1 expression, ROS production, or cell viability, cultured DRG neurons and stably TRPV1 expressing HEK293T cells (Puntambekar et al., 2005) were exposed to 10, 40, 100, 300, 400 and 1000 µM of STZ. STZ was prepared as a fresh stock solution by dissolving it in 0.1 N citrate buffer (pH 4.5) before adding it to the neuronal medium.
and incubated for 24-72 hrs. In the experiments that required the exposure of STZ longer than 24 hrs, the medium was replaced with appropriated doses of STZ. Always the experimental observations were compared with sister cultures that were treated with 0.1 N citrate buffer.

**Electrophysiology**

Primary DRG neuronal cultures were prepared from embryonic day 18 (E18) rat embryos. Adult pregnant rats were killed with an overdose of isoflurane. DRG were dissected and the neurons were dissociated by trituration with a fire-polished glass pipette in Hank's balanced salt solution (HBSS) (Ca$^{2+}$- and Mg$^{2+}$-free). Neurons were cultured in Neurobasal medium (NBM, Life Technologies, Buffalo, NY, USA), supplemented with L-glutamine and B27-supplement (Gibco, Invitrogen, Grand Island, N.Y) and grown on poly-D-lysine-coated glass coverslips in 24-well plates. Neurons were incubated at 37 °C in a humidified atmosphere of 5% CO$_2$. Neurons were used from 3 days after plating.

For whole-cell patch clamp current recordings, the bath solution contained (in mM): 140 Nagluconate, 2.5 KCl, 10 HEPES, 1 MgCl$_2$, 1.5 EGTA, pH adjusted to 7.35 with NaOH and the pipette solution contained (in mM): 140 Kgluconate, 5 KCl, 10 HEPES, 2 MgCl$_2$, 10 EGTA, 2 ATP, 0.25 GTP, pH adjusted to 7.35 with NaOH. Ca$^{2+}$-free extracellular solution was used to avoid desensitization and tachyphylaxis of capsaicin-induced currents. The junction potential between the patch pipette and the bath solutions was cancelled before the giga-ohm seal was formed. The tip of the drug application pipettes was placed within 100 µm of the neurons. Currents were recorded using a WPC-100 patch-clamp amplifier (E.S.F. Electronic, Goettingan, Germany). Data were digitized (VR-10B; InstruTech, Great Neck, NY) and stored in videotapes or directly stored in the computer using a Lab View (National Instruments, Austin, TX) interface. For analysis, data were filtered at 2.5 kHz (−3 dB frequency with an eight-pole
low-pass Bessel filter; LPF-8; Warner Instruments, Hamden, CT) and digitized at 5 kHz. Current amplitudes were measured using Channel 2 Software (kindly provided by Michael Smith, Australian National University, Canberra, Australia). The traces and graphs were plotted using Origin Software (OriginLab Corporation, Northampton, MA). Capacitance of the cell was measured manually by using the read-out in the WPC-100 amplifier.

**Measurement of reactive oxygen species (ROS)**

ROS production was detected using the dye 2', 7'-dichlorofluorescein diacetate (DCF-DA, Invitrogen, Molecular Probes, OR,). DCF-DA, a non-fluorescent cell-permeant compound, is cleaved by endogenous esterases and the de-esterified product becomes fluorescent upon oxidation by ROS. Cells were incubated with DCF-DA (20 µM) at 37°C for 20 minutes and washed twice in HBSS to reduce non-specific fluorescence. Fluorescence measurements were carried out using an inverted microscope (DMI2RE2; Leica, Plymouth, MN) equipped with a camera (Retiga Ex, Roper Scientific, Ottobrunn, Germany) and the Lambda DG4 wave length switcher (Sutter Instrument Company, Novato, CA) and the data were analyzed using Scanalytics software (Scanalytics Inc., Fairfax, VA). The exposure time was kept to <1 s in order to avoid photo oxidation of the ROS sensitive dyes and for all treatments the exposure time was kept constant. At least three independent fields were chosen for each condition and 5-20 cells in a given field were used for quantification of the fluorescence signals. DCF fluorescence was measured and collected with an excitation $\lambda_{\text{exc}}$ of 488 nm and emission $\lambda_{\text{emi}}$ of 535 nm.

**Ca$^{2+}$ imaging**
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DRG neurons grown on glass coverslips were incubated with Fluo-4 AM (3 µM) (Molecular Probes, Eugene, OR) for 20 minutes at 37 °C and washed with physiological buffer containing the following (in mM): 140 NaCl, 5 HEPES, 2 CaCl₂, 1 MgCl₂, 2.5 KCl, 2 Lidocaine, pH 7.35. The experiments were carried out using a microscope (DMIRE2) attached to a camera (Retiga Ex) and the Lambda DG4 wave length switcher (Sutter Instruments). Fluo-4 was excited at 488 nm, and the emitted fluorescence was filtered with a 535 ± 25 nm bandpass filter and analyzed using the Scanalytics software. Multiple cells were selected and the fluorescence of individual cells was tracked. The ratio of the fluorescence change \( F/F₀ \) was plotted to represent the change in intracellular Ca²⁺ levels.

**Cell viability assay**

Cover slips containing embryonic DRG neurons were incubated with 15 µl fluorescein diacetate (FDA, 15 mg/ml) and 15 µl propidium iodide (PI, 4.6 mg/ml) for 3 minutes by adding them into 0.5 ml of NBM. Cells were washed twice with Ca²⁺ free buffer to prevent non-specific background fluorescence. Green fluorescence (\( \lambda_{exc} \) excitation 488 nm and emission \( \lambda_{emi} \) 520 nm wavelengths for FDA) for live and red fluorescence (\( \lambda_{exc} \) excitation 535 nm and emission \( \lambda_{emi} \) 590-615 nm wavelengths for PI) for dead cells were observed using a microscope (DMIRE2), equipped with a camera (Retiga Ex), and the Lambda DG4 wave length switcher (Sutter Instruments), and analyzed using Scanalytics software. At least 5-6 independent fields were chosen for analysis for each condition. The percent survival or viability of DRG neurons was calculated for different concentrations of STZ.

**Western blot**
Mice were sacrificed one week after STZ treatment and DRG were removed and placed in a lysis buffer (0.1% SDS, 1% Triton X-100, 1% deoxycholate, protease and phosphatase inhibitor cocktail, 1:100, Sigma, St. Louis, MO), homogenized and centrifuged. Stably TRPV1 expressing HEK 293T cells were cultured as described previously (Puntambekar et al., 2004). TRPV1 expressing HEK cells were scraped into a 400 µl lysis buffer and centrifuged. The protein concentration was measured by the bicinchoninic acid (BCA) assay. Proteins were separated by 10% SDS-PAGE and transferred to the nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were probed overnight with rabbit anti-p38, phospho-p38 (1:200, Santa Cruz biotechnology, Santa Cruz, CA), β-actin (1:200, Sigma), or goat anti-TRPV1 (1:100, Santa Cruz biotechnology) antibodies followed by incubation with horseradish peroxidase-conjugated (HRP) goat anti-rabbit IgG or rabbit anti-goat IgG (1:10,000, Santa Cruz biotechnology) for 1 hr. After incubation with enhanced chemiluminescence reagents (Santa Cruz biotechnology), membranes were scanned using the Hitachi genetic systems (Hitachi Software Engineering, Japan) and blots were analyzed using GeneTools Analysis Software (SynGene, Frederick, MD).

Data analysis

For behavioral experiments, mixed model analysis was performed using SAS/STAT software (Cary, NC), which included both fixed events (age and time after diabetes onset) and random events (number of subjects) with repeated measures of analysis of variance (ANOVA). The comparisons were made between control group and diabetic groups. Data are shown as mean ± S.E.M. (Standard Error of the Mean). Data are considered significant at p<0.05. For all other experiments, data are shown as mean ± S.E.M. Significance is tested using unpaired Student's t-test and the data are considered significant at p<0.05. DCF and Fluo-4 AM were
obtained from Molecular Probes, OR and B27-supplement was obtained from Gibco Invitrogen, Grand Island, NY. p38 MAPK inhibitor, SB203580 was obtained from Santa Cruz Biotechnology. All other chemicals used in this study were obtained from Sigma (St. Louis, MO).

Results

Alterations in thermal pain sensitivity in STZ-injected diabetic mice

STZ is commonly used to induce type 1 diabetes in rodents for the study of DPN because disease induction is both rapid and reliable. In this study, we observed the STZ-treated mice for a period of 7-9 weeks after the onset of diabetes. Ins-HA.D2 single transgenic mice were injected with a single dose of STZ (200 mg/kg). Following STZ treatment, blood glucose levels were significantly elevated (70-75% of the mice) by the first week after treatment and remained elevated for the course of the study (pre-injection, 157 ± 4.4; week 1 STZ, 503.5 ± 24.6; week 7 STZ, 574.7 ± 22.6 mg/dl, n=11) as compared to control vehicle-injected mice (pre-injection, 157 ± 6.1; week 1 control, 183.6 ± 2.2; week 7 control, 170 ± 2 mg/dl, n=6, p<0.001) (Fig. 1A).

As the disease progressed, the body weights of STZ-treated animals remained stagnant, whereas the body weights of control animals increased steadily (STZ week 1, 22.9 ± 0.5; STZ week 6, 21.2 ± 0.6, gms, n=11, p< 0.001; control week 1, 22.1 ± 0.2; control week 6, 26.2 ± 0.3 gms, n=6; (Fig. 1B).

Then, the mice were tested for thermal pain sensitivity by measuring the paw withdrawal latency (PWL) using a hot plate maintained at 52 ± 0.3°C. An early phase of thermal hyperalgesia occurred between 1 and 3 weeks following STZ treatment, which paralleled the onset of hyperglycemia (control week 2, 8.9 ± 0.5 s, n=19; diabetic week 2, 7.1 ± 0.4 s, n = 33, p <0.001) (Fig. 1C). Hyperalgesia was followed by a phase of hypoalgesia (control week 8, 9 ±
Together these results indicate that STZ-induced diabetic mice exhibited an initial phase of hyperalgesia followed by a phase of hypoalgesia.

**Alterations in thermal pain sensitivity in STZ-injected non-diabetic mice**

A proportion (20-25%) of STZ-injected mice did not become diabetic as indicated by the blood sugar levels (<300 mg/dl), but these mice exhibited thermal hyperalgesia. This was further confirmed by a non-diabetogenic dose of STZ (50 mg/kg). The blood glucose levels were slightly higher as compared to vehicle-injected mice (STZ-injected non-diabetic mice, 170 ± 8.8; week 2, 222.4 ± 24.3; week 6, 211.4 ± 19.6 mg/dl, n=13; vehicle-injected mice, 157 ± 6.1; week 2, 163.8 ± 3.9; week 6, 186 ± 1.6 mg/dl, n=6) (Fig. 2A). Body weights of STZ-injected non-diabetic and vehicle-injected mice increased steadily (STZ-injected non-diabetic mice week 1, 23 ± 1 and week 6, 25.2 ± 0.9 gms, n = 13) (vehicle-injected mice week 1, 22.1 ± 0.2; week 6, 26.2 ± 0.3 gms, n=6) (Fig. 2B). STZ-injected non-diabetic mice exhibited a phase of hyperalgesia (STZ-injected non-diabetic mice; 9.6 ± 0.5 s; week 2, 6.1 ± 0.3 s, n = 13, p<0.001) followed by a phase of normal PWL (STZ-injected non-diabetic mice week 7, 7.9 ± 0.6 s, n = 13; vehicle-injected mice week 8, 9 ± 0.8 s, n=6) (Fig. 2C). These results suggest that STZ treatment induces changes in thermal pain sensitivity, which is independent of hyperglycemia.

**Incubation of neurons with STZ increases TRPV1-mediated current responses**

In order to determine whether STZ has a direct action on neurons, cultured embryonic DRG neurons were incubated for 24 hrs with different concentrations of STZ (20 to 400 µM), and capsaicin (1 µM)-induced TRPV1-mediated whole-cell currents were recorded. There was a
dose-dependent increase in TRPV1 current at lower concentrations followed by a decrease at higher concentrations. Vehicle-treated sister cultures were used as controls. TRPV1-mediated currents were significantly higher at 20 µM (control, 1 ± 0.1, n = 10; STZ, 2.0 ± 0.1 fold, n = 10, p<0.004) and 100 µM STZ (control, 1 ± 0.1, n = 9; STZ, 2.5 ± 0.2 fold, n=9, p<0.04), but there was no significant change at 200 µM STZ (control, 1 ± 0.3, n = 10; STZ, 1.1 ± 0.2 fold, n=10), and a decrease in the current amplitude was observed at 400 µM STZ (control, 1 ± 0.2, n = 6; STZ, 0.6 ± 0.2 fold, n=6) (Fig. 3B). Typically, small-diameter (<25 µm) neurons responded to capsaicin. Average capacitance of DRG neurons in 100 µM STZ-treated neurons (19.2 ± 1.48 pF, n = 9) did not differ from that of control neurons (21.5 ± 1.7 pF, n = 11). Peak current amplitudes were expressed as current densities (pA/pF) using cell capacitance in order to normalize the differences in cell size, which was found to be significantly higher at 20 and 100 µM STZ concentrations (control, 1 ± 0.2, n = 10; 20 µM STZ, 1.7 ± 0.2 fold, n = 10, p<0.004) (control, 1 ± 0.2, n = 9; 100 µM STZ, 2.1 ± 0.2 fold, n = 9, p<0.05) but not at 200 and 400 µM STZ (Fig. 3C). These results demonstrate that lower concentrations of STZ (20 and 100 µM) induce an increase in TRPV1-mediated currents, whereas higher concentrations (>200 µM) cause a decrease, possibly as a result of reaching toxic concentrations. Elevated TRPV1 currents may be one of the mechanisms by which neuronal excitability increases, contributing to hyperalgesia observed in STZ-injected diabetic and non-diabetic mice.

To corroborate the finding that the treatment of STZ increases TRPV1 function, increases in intracellular Ca2+ in response to application of capsaicin (30 nM) were monitored in cultured DRG neurons loaded with Fluo-4 AM following STZ treatment. The advantage of the Ca2+ imaging technique is that it enables the study of multiple neurons at the same time. As described earlier, sister cultures were used in parallel to determine the effect induced by STZ. We observed a significantly higher TRPV1-mediated Ca2+ influx in embryonic DRG neurons.
incubated with STZ (40 µM) for 24, 48 and 72 hrs (Fig. 4 A, B) as compared to sister cultures treated with a citrate buffer (0.1 N) (Fold change in F/F₀: citrate buffer, 1 ± 0.01, n=49 cells; STZ (40 µM) 24 hrs, 1.7 ± 0.4, n=75 cells; p<0.05); 48 hrs, 3.3 ± 0.7 n=88 cells; 72 hrs, 1.3 ± 0.4, n=65 cells (Fig. 4C). These results suggest that chronic STZ treatment increases the function of TRPV1 either by an increase in the membrane expression of TRPV1 or an increase in TRPV1 sensitivity. However, longer exposure to STZ resulted in a decrease in Ca²⁺ influx, possibly as a result of toxicity as seen with current recordings (Fig. 3).

We noticed a decrease in TRPV1-mediated current when the neurons were exposed to higher concentrations of STZ (> 200 µM). Studies have suggested that 1 mM STZ induces pancreatic beta cell death in vitro (Konrad et al., 2001; Nukatsuka et al., 1990). Based on these studies, we hypothesized that STZ might alter the viability of DRG neurons. To test whether STZ can alter the viability of neurons, we performed a viability assay using live and dead cell staining dyes FDA and PI, respectively. Results of the viability assay suggest that increasing concentrations of STZ decreased the viability of embryonic DRG neurons (200 µM, 26% n=180; 400 µM, 27.4% n=114, and 1000 µM STZ, 16.4% n=64, viable cells) as compared to vehicle-treated sister cultures.

**STZ treatment induces reactive oxygen species (ROS) production in cultured DRG neurons in vitro.**

It has been suggested that acute and chronic treatments of STZ induce myopathy through the ROS-mediated mechanism (Johnston et al., 2007). Similarly, NGF induces TRPV1 expression through NADPH oxidase-dependent ROS pathway (Suzukawa et al., 2000; Puntambekar et al., 2005). Therefore, we determined whether ROS is involved in the STZ-induced increase in
TRPV1 currents. Initially, we tested whether a known ROS generating agent, such as hydrogen peroxide, (H₂O₂, 25 µM) could increase DCF fluorescence intensity in cultured DRG neurons treated for 24 hrs. As expected, H₂O₂ significantly increased DCF fluorescence (data not shown). Next, in order to determine the role of STZ, we pretreated the cultured embryonic DRG neurons with different concentrations of STZ for 24 hrs and changes in ROS were measured. We observed a higher DCF fluorescence intensity with increasing concentrations of STZ (citrate buffer, 1 ± 0.03, n= 170 cells; 100 µM STZ, 1.8 ± 0.04, n=105 cells, p< 0.0001; 400 µM STZ, 1.77 ± 0.05, n=75 cells, p< 0.0001) (Fig. 5). These results suggest that STZ treatment induces ROS production in cultured DRG neurons, which might play a role in increasing TRPV1-mediated current through ROS-mediated transcriptional or translational regulation. Although ROS levels were higher even at higher concentration of STZ (400 µM) treatment, TRPV1-mediated currents showed a reduction (Fig. 3), suggesting that higher levels of ROS may become toxic to neurons.

To further test that ROS is involved in STZ-induced TRPV1 expression and function, we pretreated cultured DRG neurons with H₂O₂ (25 µM) for 24 hrs and TRPV1-mediated whole cell currents were recorded. There was a significant increase in TRPV1-mediated currents as compared to citrate buffer-treated cells (buffer; 1 ± 0.1 fold, n=8; 25 µM H₂O₂, 1.7 ± 0.1 fold, n=8, p<0.05), whereas treatment with 50 µM H₂O₂, in fact resulted in a decrease in the current amplitude (50 µM H₂O₂, 0.63 ± 0.19 fold, n=8) suggesting a narrow effective range of ROS in modulating TRPV1 responses (Fig. 6A, B). Peak currents normalized to capacitance (pA/pF) were also found to be increased (buffer, 1 ± 0.1 fold, n=8; 25 µM H₂O₂, 2.0 ± 0.1 fold, n= 8, p<0.002) (Fig. 6C). To further confirm that ROS is involved in STZ-induced TRPV1 expression, cultured DRG neurons were concomitantly treated with catalase (200 units/ml), a ROS
scavenger. TRPV1-mediated whole-cell currents recorded from the neurons incubated with STZ (100 µM) + catalase or H₂O₂ (25 µM) + catalase did not increase the current amplitude (buffer, 1 ± 0.2, n= 9; STZ + catalase, 0.96 ± 0.06 fold, n = 10; buffer, 1 ± 0.2 fold, n = 7; H₂O₂ + catalase, 1.1 ± 0.2, n=8). Peak current amplitudes expressed as current densities (pA/pF) were not altered following treatment with catalase (STZ + catalase, 0.94 ± 0.09 fold, n=8; H₂O₂ + catalase, 1.07 ± 0.18 fold, n=8) (Fig. 6A, B). Average capacitance values of DRG in STZ+catalase treated neurons (18.5 ± 0.67 pF, n = 8) did not differ from control (18.1 ± 0.7 pF, n = 10). These data implicate that STZ-induced ROS levels are involved in the increase in TRPV1-mediated current.

**Increase in TRPV1 and p-p38 MAPK levels in diabetic and non-diabetic hyperalgesic mice**

Activation of MAP kinases increases the expression of proteins either by transcriptional or posttranslational mechanisms. DRG obtained from STZ-treated diabetic or non-diabetic mice one week after injection of STZ were probed with TRPV1 and p-p38 MAPK antibodies. Our results demonstrate an increase in TRPV1 levels (expressed as a ratio of β-actin levels) in both diabetic (1.73 ± 0.25 fold; n = 3, p<0.05) and non-diabetic hyperalgesic DRG (1.52 ± 0.07 fold, n = 3, p<0.05) (Fig. 7) as compared to vehicle-treated mice. Also, the p-p38 MAPK levels (expressed as a ratio of total p38 MAPK levels) were elevated in STZ-treated diabetic (1.45 ± 0.14 fold; n = 3, p<0.05) and non-diabetic hyperalgesic mice (1.43 ± 0.05 fold; n = 3, p<0.05) as compared to control mice (Fig. 7). Total p38 MAPK levels remained constant after STZ-treatment indicating that STZ caused an increase in the phosphorylation of p38 MAPK rather than increasing the levels of the substrate.
Activation of STZ-ROS-p38 MAPK pathway promotes TRPV1 expression

Previous studies have suggested that JNK and p38 MAPK are strongly activated by ROS or by a mild oxidative shift of the intracellular thiol/disulfide redox state (Abe et al., 1996; Hehner et al., 2000). A similar mechanism is also involved in the nerve growth factor (NGF)-induced increase in TRPV1 expression, in which p38 MAPK is activated by ROS, thereby contributing to hyperalgesia (Puntambekar et al., 2005; Ji et al., 2002). Experiments were conducted using stably TRPV1 expressing HEK293T cells to determine whether the increase in TRPV1 expression induced by chronic STZ treatment is mediated by the ROS-p38 MAPK pathway. Cells were treated with STZ, ROS generating and scavenging agents and probed for TRPV1 and p-p38 MAPK. Consistent with our previous results, STZ (100 µM)-treated cells exhibited an elevated TRPV1 expression (1.52 ± 0.13 fold, n = 4, p<0.01) (Fig. 8B). Removal of ROS by catalase (200 units/ml) abolished this increase (1.08 ± 0.02 fold, n=4, p<0.05). p-p38 MAPK was also found to be increased in STZ-treated cells (1.25 ± 0.04 fold, n = 4, p<0.01) but did not increase when treated simultaneously with catalase (0.98 ± 0.04 fold, n=4, p<0.05) (Fig. 8B). In support of our findings, we successfully reproduced these results with a ROS generating agent, H₂O₂ (25 µM) (TRPV1: H₂O₂ 1.4 ± 0.1 fold, n=4, p<0.01 ; H₂O₂ + catalase, 1.01 ± 0.09 fold, n=4, p<0.05 ) (p-p38 MAPK: H₂O₂,1.48 ± 0.09 fold, n=4, p<0.01 ; H₂O₂ + catalase, 0.92 ± 0.06 fold, n=4, p<0.05) (Fig. 8C) suggesting that STZ-induced TRPV1 expression is mediated by the ROS-p38 MAPK pathway. In order to confirm that the effect is mediated by p38 MAPK, we incubated the cells with a p38 MAPK inhibitor, SB203580 (20 µM). The increase in TRPV1 and p-p38 MAPK levels was significantly reduced following treatment with the inhibitor (TRPV1, 1.08 ± 0.11 fold, n=3, p<0.05; p-p38 MAPK, 0.95 ± 0.08 fold, n=3, p<0.05) (Fig. 8D).
Discussion

STZ is a glucosamine nitrosourea compound with diabetogenic properties purported to cause a selective destruction of pancreatic beta cells. STZ-induced diabetic mice exhibit two phases of thermal pain sensitivity, an initial phase of hyperalgesia and a late phase of hypoalgesia. However, a proportion (~20-25%) of STZ-treated mice did not become diabetic, but became hyperalgesic as compared to the vehicle-injected group. It was further confirmed that hyperalgesia was observed in non-diabetogenic doses of STZ. However, we could not find a correlation between the degree of hyperalgesia and the dose of STZ. Possible reasons for the failure of STZ to induce diabetes are: (1) inability to damage all pancreatic beta cells due to insufficient concentration; (2) faster recovery of damaged pancreatic beta cells; (3) decreased bioavailability due to rapid breakdown by the liver enzymes or rapid excretion by the kidneys.

A normal gain of body weights of STZ-injected non-diabetic mice and a decrease in the body weights of diabetic mice suggest that STZ-injected non-diabetic mice do not suffer from metabolic derangement. Both time-course and the degree of thermal hyperalgesia were similar in all STZ-treated mice regardless of their blood glucose levels following STZ injection. Other investigators have not reported this phenomenon, perhaps because of the common practice of excluding non-diabetic mice from studies. However, it has been reported in a study that ~40-60% of rats injected with STZ were non-diabetic but exhibited mechanical hyperalgesia and insulinopenia (Romanovsky et al., 2004). It was suggested that insulinopenia itself or consequences of insulinopenia that is independent of hyperglycemia might contribute to mechanical hyperalgesia. A direct effect of STZ was ruled out as a possible causative factor of mechanical hyperalgesia because insulin-treated STZ-injected diabetic rats became normoglycemic and exhibited attenuated pain sensation (1).
TRPV1 plays a role in both diabetic and inflammatory hyperalgesia (Hong and Wiley, 2005; Caterina et al., 2000). STZ destroys pancreatic beta cells by elevating the ROS levels (Turk et al., 1993; Nukatsuka et al., 1990; Sofue et al., 1991). In another study, moderate increases in ROS levels have been shown to function as secondary messengers that can influence redox-sensitive signal transduction pathways (Suzukawa et al., 2000). In our in vitro studies, STZ-treated neurons exhibited an increase in TRPV1-mediated currents and an increase in the levels of ROS. By recording TRPV1-mediated whole cell currents in cultured embryonic DRG neurons treated with ROS generating and scavenging agents, we have shown that STZ induces TRPV1 expression through the ROS-mediated pathway. We further observed that STZ also caused an increase in the phosphorylated form of p38 MAPK suggesting that the increase in the TRPV1 protein expression may involve the ROS-p38 MAPK pathway. However, at higher concentrations of STZ and higher levels of ROS, the effect of toxicity is seen as a decrease in TRPV1 expression and a reduction in cell viability.

Peripheral nerve injury leads to an increase in the expression of BDNF and the $\alpha_{2\delta}$ Ca$^{2+}$ channel subunit through activation of p38 MAPK (Zhou et al., 1999; Luo et al., 2001). A similar mechanism is also involved in NGF-induced increase in TRPV1 expression, in which p38 MAPK is activated by ROS, thereby contributing to hyperalgesia (Puntambekar et al., 2005; Ji et al., 2002). Ji et al. (Ji et al., 2002) have proposed that p38 MAPK activates the translational factor eIF4E, via Mnk1/2 (MAPK-interacting kinase 1 and 2) and the phosphorylation of which results in an increased affinity of eIF4E for capped RNA influencing translation. Different oxidative stress-inducing stimuli such as, relatively low concentrations of hydrogen peroxide, UV light, $\gamma$-irradiation, and interleukin-1 have been shown to activate the activator protein-1 (AP-1) a transcription factor (Angel and Karin, 1991; Devary et al., 1991; Meyer et al., 1993). Similarly, STZ-ROS-p38 MAPK pathway could increase the activity of transcription factors like
AP-1, inducing transcription of TRPV1 or may increase the translation of TRPV1 through eIF4E. This could lead to an increase in membrane expression and function of TRPV1, thereby contributing to the hyperalgesia observed in STZ-injected non-diabetic mice. Nevertheless, a similar mechanism cannot be ruled out in STZ-injected diabetic mice. In HEK cells stably transfected with TRPV1, treatment of STZ caused a similar increase in TRPV1 and p-p38 MAPK expression suggesting that the effect we observe is posttranslational rather than posttranscriptional.

It has been suggested that STZ is transported into beta cells through the glucose transporter GLUT2 (Schnedl et al., 1994). It is intriguing that the predominant glucose transporter expressed in the peripheral nervous system is GLUT3. STZ-treatment may result in PARP activation, possibly through its direct damaging effects of DNA. PARP activation results in an increased production of poly ADP-ribose, from which adenine 5'-diphosphoribose (ADPR) is generated using cellular NAD+ as a substrate (Pieper et al., 1999; Delaney et al., 1995; Ledoux et al., 1988). ADP-ribose is a potent activator of TRPMelastatin 2 (TRPM2), which has been suggested to play a role in sensing oxidative stress (Fonfria et al., 2004).

Together, our results suggest that STZ exerts a direct action on neurons altering the expression and function of TRPV1 via the ROS-p38 MAPK pathway-mediated translational regulation. Of course, we cannot rule out the possibility that STZ might exert its effects on other nociceptive ion channels such as the voltage-gated sodium channels and the mechano-sensitive channels. Therefore, future studies are needed to address the extent of modulation of other ion channels by direct action of STZ (Hayase et al., 2007). To our knowledge, this is the first report suggesting that STZ might have a direct effect on neurons; thus, caution should be exercised in interpreting data as diabetes- or hypoglycemia-induced, while using STZ as a diabetogenic agent.
References


Footnotes

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Figure 1. Altered thermal pain sensitivity in STZ-induced diabetic mice. A. Blood glucose levels were significantly higher in diabetic mice (filled triangles) as compared to control mice (open triangles). B. The body weights of diabetic mice (filled triangles) remained constant, whereas the body weights of control mice (open triangles) steadily increased. C. Diabetic mice (filled triangles) show significant changes in PWL: exhibited a period of hyperalgesia between weeks 1 and 4 and then a phase of hypoalgesia as compared to vehicle-injected mice (open triangles). Asterisks represent the significance levels (*p<0.05; **p<0.01).

Figure 2. Altered thermal pain sensitivity in STZ-injected non-diabetic mice
A. A proportion of STZ-injected mice ((filled triangles)) did not show elevated blood glucose levels and were similar to vehicle-injected mice (open triangles). B. Body weights of both STZ-injected non-diabetic mice (filled triangles) and vehicle-injected mice (open triangles) steadily increased. C. STZ-injected non-diabetic mice (filled triangles) exhibited a phase of thermal hyperalgesia as compared to vehicle-injected mice (open triangles). Asterisks represent the significance levels (*p<0.05; **p<0.01).

Figure 3. TRPV1-mediated currents following incubation of cultured DRG neurons with STZ. A. Incubation with lower concentrations of STZ (20 and 100 µM) for 24 hrs increased capsaicin (1µM)-evoked currents, recorded from small
to medium diameter DRG neurons as compared to 0.1 N citrate buffer-treated DRG neuronal sister cultures. But at higher concentrations of STZ (>200 µM) capsaicin-evoked currents decreased. B, C. Summary graphs show the fold change in peak current amplitude and current densities in STZ-treated neurons. Number in the parenthesis represents the number of cells and the asterisks represent the significance levels (*p<0.05; **p<0.001; compared to control).

**Figure 4. Treatment of STZ increases TRPV1-mediated Ca\(^{2+}\) influx in cultured DRG neurons.** Selected DRG neurons in a coverslip were individually tracked for changes in intracellular Ca\(^{2+}\) levels in response to application of capsaicin (30 nM) A. Representative images showing capsaicin-induced increase in intracellular Ca\(^{2+}\) after STZ (40 µM) treatment as compared to 0.1 N citrate buffer treatment. The inset shows the enlarged image of one of the neurons pointed with arrows. B. Representative traces of changes in fluorescence intensity in a group of STZ-treated neurons (right, n=9) and buffer-treated neurons (left, n=11). C. Summary graph depicting the fold increase in fluorescence intensity (F/F\(_{o}\)) in STZ-treated neurons as compared to buffer-treated cultures. Number in the parenthesis represents the number of cells and the asterisks represent the significance levels (*p<0.05; **p<0.01; compared to control). Scale bar is 100 µm.

**Figure 5. Generation of ROS following incubation of cells with STZ.**
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STZ- or vehicle-treated DRG neurons for 24 hrs were preloaded with DCF-DA (20 µM) and DCF fluorescence intensity was measured. Summary graph depicting a significant increase in DCF fluorescence intensity in STZ (20 to 1000 µM)-treated DRG neurons as compared to vehicle-treated neuronal sister cultures. Number in the parenthesis represents the number of DRG neurons and the asterisk represents significance level (*p<0.001 compared to control).

**Figure 6.** STZ-induced increase in TRPV1 current is mimicked by H$_2$O$_2$ and abolished by concomitant treatment with catalase. **A.** Representative traces show that the incubation of neuronal cultures with ROS generator H$_2$O$_2$ (25 µM) increased the capsaicin-evoked current amplitude as compared to currents recorded from neurons in sister cultures. Higher concentration of H$_2$O$_2$ (50 µM) caused a decrease in current amplitude. The increase in current amplitude induced by STZ and H$_2$O$_2$ was reversed by concomitant treatment with catalase. **B, C.** Summary graphs show the change in current amplitude and current densities after incubation of cultures with H$_2$O$_2$ or STZ or along with catalase. Asterisks represent the significance levels (*p<0.05; **p<0.01; compared to control).

**Figure 7.** TRPV1 and p38 MAPK levels in DRG of STZ-treated diabetic and non-diabetic hyperalgesic mice. DRG collected from control, STZ-treated diabetic and non-diabetic mice one week after STZ or vehicle treatment were probed for TRPV1 and p-p38 MAPK by Western blots. Representative Western
blots show elevated TRPV1 and p-p38 MAPK levels in diabetic and non-diabetic hyperalgesic mice. Summary graph shows fold increase in TRPV1 and p-p38 MAPK levels calculated as a ratio of β-actin and total p38 MAPK levels, respectively. Asterisk represents the significance levels (*p<0.05 compared to control).

Figure 8. STZ-induced increase in TRPV1 levels is mediated by ROS-p38 MAPK pathway. Stably TRPV1-transfected HEK 293T cells were treated with STZ (100 µM) or H₂O₂ (25 µM) and along with catalase or p38 MAPK inhibitor (SB203580) for 24 hrs and Western blots were performed. A. TRPV1 expression in transfected HEK cells and no TRPV1 was detected in non-transfected HEK cells. B. STZ-induced an increase in TRPV1 and p-p38 MAPK levels, which was blocked by concomitant treatment with catalase (200 units/ml). C. H₂O₂-induced increase in TRPV1 and p-p38 MAPK levels were blocked by treatment with catalase. D. STZ-induced increase in TRPV1 and p-p38 MAPK levels were blocked by treatment with a p38 MAPK inhibitor SB203580 (20 µM). Right panels show the summary graphs of TRPV1 and p-p38 MAPK levels. Asterisks represent the significance levels (*p<0.05 compared to STZ treatment; **p<0.01 compared to control).
Fig. 3

A. 24 hrs Incubation

- Control
- 0.1 N citrate buffer
- 20 μM STZ
- 100 μM STZ
- 200 μM STZ
- 400 μM STZ

1 μM Capsaicin

B. Fold change in peak current

- Control
- 0.1 N citrate buffer
- 20 μM
- 100 μM
- 200 μM
- 400 μM

C. Fold change in current density

- Control
- 0.1 N citrate buffer
- 20 μM
- 100 μM
- 200 μM
- 400 μM

* indicates p < 0.05
** indicates p < 0.01
Fig. 6

A 24 hr Incubation
0.1 N citrate buffer
1μM capsaicin

B

Fold change in peak current

C

Fold change in current density

Control 25 μM H₂O₂ 50 μM H₂O₂ 100 μM STZ STZ+Catalase

H₂O₂ H₂O₂ STZ 100 μM STZ+Catalase

0.0 0.5 1.0 1.5 2.0 2.5 3.0

Fold change in peak current

0.0 0.5 1.0 1.5 2.0 2.5 3.0

Fold change in current density

30 sec

100 pA