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RESEARCH

Alpha-lipoic acid modulates the diabetes mellitus-mediated neuropathic pain via inhibition of the TRPV1 channel, apoptosis, and oxidative stress in rats

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Abstract

Diabetes mellitus (DM) is a chronic syndrome involving neuropathic pain. Increased oxidative stress in DM is assumed to increase free reactive oxygen radicals (ROS) and causes diabetic damage. The sciatic nerve (ScN) and dorsal root ganglion (DRG) both contain high levels of the TRPV1 channel, which is triggered by capsaicin and ROSs and results in increased Ca^{2+} entry into the neurons. Alpha-lipoic acid (ALA) is considered an important part of the antioxidant system. To better characterize the protective effects of ALA on the DM-induced neuronal through TRPV1 modulation, we investigated the role of ALA on DM-induced neuropathic pain, oxidative ScN, and DRG damage in diabetic rats. Forty adult Wistar albino female rats were divided into four groups as control, ALA (50 mg/kg for 14 days), streptozotocin (STZ and 45 mg/kg and single dose), and STZ + ALA. Rats were used for the pain tests. After obtaining the DRGs and ScN, they were used for plate reader, patch-clamp, and laser confocal microscope analyses. We observed the modulator role of ALA on the thresholds of mechanical withdrawal pain (von Frey test) and hot sensitivity pain (hot plate test) in the STZ + ALA group. The treatment of ALA decreased STZ-induced increase of TRPV1 current densities, intracellular free Ca^{2+} concentrations (Fura-2 and Fluo − 3/AM), ROS, caspase 3, caspase 9, mitochondrial membrane potential, and apoptosis values in the ScN and DRG neurons, although its treatment induced the increase of cell viability and body weight gain. The treatment of ALA acted a neuroprotective role on the TRPV1 channel stimulation-mediated Ca^{2+} influx, neuropathic pain, and neuronal damage in diabetic rats. The neuroprotective role of ALA treatment can be explained by its modulating the TRPV1 channel activity, intracellular Ca^{2+} increase-induced oxidative stress, and apoptosis.

Keywords Alpha-lipoic acid · Apoptosis · Neuropathic pain · Oxidative stress · TRPV1 channel

Abbreviations

ALA	Alpha-lipoic acid
CAP	Capsaicin
CPZ	Capsazepine
$[Ca^{2+}]_c$	Cytosolic free calcium ion concentration
Ctr	Control
DM	Diabetes mellitus
DiNP	Diabetic neuropathic pain
DRG	Dorsal root ganglion
ROS	Reactive oxygen species
ScN	Sciatic nerve
SDU	Suleyman Demirel University
STZ	Streptozotocin
TRP	Transient receptor potential
TRPV1	Transient receptor potential vanilloid 1
WC	Whole cell

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Introduction

Diabetes mellitus (DM) is one of the most significant long-term health issues in the world. Serious cellular losses and damages may occur via the adverse action of the diabetic conditions (Rochette et al. 2015). Diabetic neuropathic pain (DiNP) is one of the complications of DM (Nemenov et al. 2022). The molecular mechanisms of DiNP are still not understood. However, there are possible mechanisms for the induction of DiNP. It provides evidence of increased production of reactive free oxygen species (ROS) associated with hyperglycemia and causing oxidative stress in many tissues (Zhang et al. 2013; Koneri et al. 2014). The excessive generations of ROSs are eliminated in neurons by the antioxidant systems. In the event of oxidant antioxidant balance deterioration, oxidative stress occurs, resulting in neuron damage and death (Rochette et al. 2015). The brain and neurons have high oxygen consumption and polyunsaturated fatty acid (PUFAs) content, whereas they have low antioxidant levels (Halliwell 2006). Hence, the brain and neurons are more sensitive to oxidative damage.

Alpha-lipoic acid (ALA) is a substance of some foods, and it is also synthesized in the body. ALA is abundantly distributed in the cytosol and extracellular regions, and it is easily transported through cell membranes because it is soluble in both water and lipids (Ghibu et al. 2009; Shaygannia et al. 2018). This molecule is considered to be protective against increased ROS generation with mitochondrial dysfunction (Park et al. 2014). By acting as a strong antioxidant, ALA has a direct antioxidant action in ROS reduction reactions. It is also among the main components of the antioxidant system that works for the control of ROS generation and apoptosis induction (Tibullo et al. 2017; Zhang et al. 2022). ALA can be used to prevent several pains such as diabetes and chemotherapy-induced neuropathic pain due to its direct and indirect antioxidant and cation channel blocker properties (Joseph et al. 2008; Zhang et al. 2020). Hence, numerous clinical studies confirmed the idea that ALA causes a marked reduction in diabetes-induced Ca^{2+} influx, apoptosis, and ROS generations (Ametov et al. 2003; Mijnhout et al. 2012; Garcia-Alcala et al. 2015).

The Ca^{2+} permeable transient receptor potential vanilloid type 1 (TRPV1) channel is a multimodal cation channel, and is activated with low pH and harmful heat, also with capsaicin (CAP), a component of red-hot chili pepper (Caterina et al. 1997; Shimizu et al. 2014). Capsazepine (CPZ) is an antagonist of TRPV1 (Kahya et al. 2017; Carrasco et al. 2018). Results of numerous studies indicated that the abnormalities such as ROS and apoptosis in dorsal root ganglion (DRG) and sciatic nerve (ScN) of diabetic experimental animals are directly related for the induction of DiNP. Expression levels of TRPV1 channels are high in neurons such as DRG and ScN (Vandewauw et al. 2013; Uslusoy et al. 2017). Additionally, oxidative stress activates the TRPV1 channels in a variety of cells and neurons, including the DRG and ScN (Nazıroğlu 2017; Uslusoy et al. 2017; Ertilav et al. 2021). Repeated activation of TRPV1 has resulted in increased cytosolic free Ca^{2+}, apoptosis, and oxidative stress via the increase of caspase 3 and caspase 9, although its activation was decreased by the treatment of antioxidants such as *Hypericum perforatum*, selenium, and melatonin (Özdemir et al. 2015; Kahya et al. 2017; Yazğan and Nazıroğlu 2017). In two previous studies with MCF-7 breast cancer and human glioblastoma cell lines, the modulator role of ALA on TRPA1 and TRPV1-mediated Ca^{2+} influx, ROS, and apoptosis has been reported (Nur et al. 2017; Deveci et al. 2019). In recent studies, the modulator roles of ALA and antioxidant noopept via inhibition of TRPV1 channel on DiNP were also reported in the streptozotocin (STZ)-induced diabetic rats (Zhang et al. 2020; Düzova et al. 2021).

The detailed molecular mechanisms underlying the antioxidant, anti-apoptotic, and anti-neuropathic effects of ALA via the inhibition of TRPV1 are not known yet. Also, it has been shown that ALA induced a protective role in the cellular damages and DM caused by increased intracellular Ca^{2+} influx-mediated oxidative processes (Piotrowski et al. 2001; Bishara et al. 2002). Hence, ALA may modulate Ca^{2+} entry via stimulation of the TRPV1 channel, and this condition may reduce levels of apoptosis and oxidative stress in the ScN and DRG of diabetic rats. It should be investigated in ScN and DRG of diabetic rats in light of these considerations. So, we investigated the effects of ALA injection on the TRPV1 stimulation-mediated DiNP, Ca^{2+} influx, apoptosis, and ROS in the DRG and ScN of STZ-induced rats.

Materials and methods

Chemicals

ALA, Fluo − 3/AM, and Fura-2-acetoxymethyl ester (Fura-2AM) were obtained from Calbiochem (Taufkirchen, Germany). STZ, HEPES, sucrose, collagenase (Type IV), and N-acetyl Asp-Glu Val-Asp-7 amino-4 methylcoumarin (ACDEVD-AMC) were obtained from Sigma-Aldrich Inc. (Darmstadt, Germany). Dulbeccos modified Eagles medium (DMEM) was purchased from Gibco (Istanbul, Türkiye). N-acetyl Leu-Glu His-Asp-7 amino-4 methylcoumarin (AC-LEHD-AMC) was purchased from Bachem (Bubendorf, Switzerland). 2',7' –dichlorofluorescin diacetate (DCFH-DA) were purchased from ThermoFisher Scientific (Istanbul, Türkiye). All others reagents were analytical grade.

Experimental animals

The current study used 40 female Wistar albino mature (10–12-week-old) rats weighing 190–200 g each. The Suleyman Demirel University (SDU), Local Experimental Animal Ethical Committee (Protocol number: 2017-05-04. Date: 24.08.2017) gave its approval to all study protocols and animal care. According to animal studies, STZ therapy made female rats more prone to the development of diabetes than their male counterparts (Vital et al. 2006; Choi et al. 2013). Hence, we used female rats in the current study. The SDU Animal Welfare Act, the Guide for the Care of Laboratory Animals, and other applicable regulations were followed in the upkeep and use of the animals. Two experimental animals were housed in each cage, which had a temperature of 22 °C and a humidity level of 60%. Experimental animals were kept under control and kept in a 12-hour cycle of darkness and light. Commercial feed and tap water were made available to the experimental animals.

Experimental groups

The 40 rats were equally divided into four groups as follow;

- Control (Ctr) Group (n = 10): Placebo (0.9% w/v physiologic saline water) was intraperitoneally (ip) administered to experimental animals in this group.
- STZ Group (n = 10). Experimental animals in this group received ip STZ with a single dose (45 mg/kg). Blood glucose levels were measured with a glucometer 4–5 days after the STZ injection to the rats (Sözbir and Nazıroğlu 2015; Yazğan and Yazğan 2022; Aydın and Nazıroğlu 2023).
- ALA Group (n = 10): ALA (50 mg/kg/day) was ip given to experimental animals in this group for 14 days (Hussein et al. 2012; Ghibu et al. 2019).
- STZ + ALA Group (n = 10): After the induction of DM via STZ injection, ALA (50 mg/kg/day) was ip injected to experimental animals in this group for 14 days.

We waited 14 days for the induction of DiNP after the induction of DM via the STZ injection (Düzova et al. 2021). Then, the rats in the ALA and STZ + ALA groups received ALA for the 14 days. All experiments were completed at the 32-33th day (4–5 + 14 + 14) of the study. After induction of pain tests, all experimental animals were dissected by either asphyxiation or cervical dislocation in accordance with SDU Experimental Animal legislation. The ScN and DRG nerve cell samples were isolated from the rats. Then, the ScN and DRG cell samples were used for calcium signaling (Fura-2), patch-clamp, ROS, apoptosis, mitochondrial determination, caspase 3, and caspase 9 analyses. In the laser scan confocal microscope analyses (LSM-800, Zeiss, Ankara, Turkiye), we used the whole DRG neurons, and they were seeded in the 35 mm dishes with bottom glass (Mattek Corporation, Ashland, MA, USA).

The STZ-mediated diabetic rat model is mostly used rodent model for investigating the mechanisms of DiNP and searching the new treatments. The full etiology of DM and STZ-mediated painful DiNP is unclear. However, the DiNP is induced by the injured peripheral nerve conduction and degeneration of nerve fibers (Cunha et al. 2009). The induction of STZ-mediated DiNP is tested in rats by von Frey and hot plate tests (Kahya et al. 2017; Ahmad et al. 2022; Khan et al. 2023). Hence, we used the von Frey and hot plate tests in the current study for the induction of STZ-mediated DiNP.

Administration of a pancreatic beta cell toxin (STZ) is a valuable way for the induction of Type 1 DM. Long time (4–5 days) but low dose (20–35 mg/kg) of STZ injection are required in the rats for the induction of DM (Akinlade et al. 2021; Ahmad et al. 2022). The development of acute DM symptoms and high glucose levels can occur within 2–5 days with a single high dose (45–60 mg/kg) intraperitoneal injection of STZ (Kahya et al. 2017; Khan et al. 2023). Therefore, in the current study, we selected the STZ dose.

Preparation of ALA, agonist, and antagonist solutions

CAP was prepared from 0.1 M stock solution in DMSO and stored at − 33 °C. The final concentration of DMSO was < 0.001%. After adding CAP (10 μM) and CPZ (100 μM) to the standard solutions, the pH values of the solutions were adjusted properly before use and checked after completing the experiments. ALA was dissolved in 20% DMSO and 80% sterile physiological saline (0.9% NaCl). For example, 0.5 ml of DMSO was added to 20 mg of ALA and mixed well. Then it was completed to 2 ml with sterile physiological saline (0.9% NaCl). This solution was applied to 200 g two animals.

Von frey and hot plate tests

Rats were housed in suspended wire mesh cages, and the measurement of DiNP was performed using calibrated von Frey filaments (20PC Aesthe Model, NO. 160,615, Muromachi Kikai Co., Ltd. Tokyo, Japan). The rear paw lifted as a sign of responsiveness (Dogrul et al. 2011). To measure the paw withdrawal latency to thermal nociceptive stimuli, a heat-controlled plate (Varioma, Thermo Fischer Inc., Langenselbold, Germany) for the hot plate test was employed at a constant temperature of 55.0 ± 0.6 °C. Every rat was put on a hot plate, and the reaction time was recorded until

the rat either licked its hind paws or leaped (the "up and down" approach). To avoid tissue injury during the hot plate test, a 60-second cut-off time was employed (Dogrul et al. 2011). The withdrawal threshold was established by gradually boosting and lowering the stimulus intensity.

Preparation of DRG and ScN samples

ScN from the right leg and DRG neurons from the T13 and L5 levels were carefully extracted (Supplement 1. Video). Separately, the ScN and DRGs were collected were placed in 1ml DMEM. The neurons with additional trypsin type III enzyme and collagenase (Type IV) were placed in an incubator with 37 °C and 5% CO_2 in a shaking bath for 45 min to separate connective tissue. The soybean trypsin inhibitor (1.25 mg/ml, type II-S1, Sigma) was used for stopping the enzymatic digestion. Then, a 1500 g centrifuge was used to spin the cell suspension. With the use of a sterile syringe, medium and large ScN and DRG were dissociated into smaller sizes (Özdemir et al. 2015; Uslusoy et al. 2017). The ScN and DRGs were seeded on the sterile 25T flasks with filter caps (1×10^6 neurons). In the laser scan confocal microscope analyses, we used the whole DRG neurons in the 35 mm dishes with bottom glass (Mattek Corporation).

Electrophysiology (patch-clamp)

The patch-clamp technique in the whole-cell mode of DRG neurons was studied using the EPC10 patch-clamp set and patch-master program (HEKA, Lamprecht, Germany) (Yazğan and Nazıroğlu 2017). In the puller (P-97, Sutter Instrument, Novato, CA, USA), the pipettes were made from borosilicate glass with filament. Access cell resistances of whole cell recording electrodes were 3–7 MΩ. The extracellular (patch chamber) solution was prepared by using the chemicals (in mM): NaCl (145), $MgCl_2$ (1), $CaCl_2$ (1), KCl (5), HEPES (10), D-glucose (10) (pH: 7.4). For preparing the Na^+-free extracellular solution, we used 150 mM N-methyl-D-glucamine ($NMDG^+$) instead of Na^+, and the pH was adjusted with HCl. The osmolarity of the solution was kept between 300 and 310 mOsmol/l. The intracellular (pipette) solution was prepared by using the chemicals (in mM): cesium glutamate (145), NaCl (8), EGTA (8), $MgCl_2$ (1), and HEPES (10). pH (7.2) was adjusted with CsOH. The MAXC-program (http://www.stanford.edu/~cpatton/maxc.html) was used for the preparations of the physiologic Ca^{2+} concentrations of the intracellular. All experiments were performed at room temperature (20 ± 3 °C). After inducing the whole cell configuration, we waited till to returning basal (control) levels of the currents. For testing the death or live cell currents, we used $NMDG^+$. When we observed inward current in the neurons after the application of $NMDG^+$, we performed the extracellular CAP (10 µM) application. TRPV1 channel was inhibited by adding the extracellular CPZ (100 µM). For the expression of patch-clamp current results as pA/pF, the maximal current amplitude (pA) in a DRG was divided by the cell capacitance (pF). N numbers in the patch-clamp experiments were 3–4 DRGs.

Measurement of cytosolic free calcium ion concentration in the ScN and DRG by using the Fluo 3/AM

The fluorescent indicator dye (Fura-2-AM) was used to assess the concentration of free calcium ion concentration ($[Ca^{2+}]_c$) in the cytosol. The ScN and DRG neurons were loaded with 5 µM Fura-2-AM for 45 min. A spectrofluorometer (Carry Eclipsys, Varian Inc, Sydney, Australia) with excitation wavelengths (340 and 380 nm) and emission wavelength (505 nm) was used to record the fluorescence of ScN and DRG suspensions at 37°C. The 340/380 nm fluorescence ratio was used to measure changes in the $[Ca^{2+}]_c$ and was calibrated using the Grynkiewicz' method (Grynkiewicz et al. 1985).

The integral of the increase in $[Ca^{2+}]_c$ for the first 220 s following the addition of CAP (10 µM) was used to assess Ca^{2+} release in the ScN and DRG. The release of Ca^{2+} is expressed in nanomolar levels (Espino et al. 2011). In some experiments, the neurons were exposed to CPZ (100 µM) for 30 min before the CAP stimulation.

The imaging analyses of cytosolic free calcium ion concentration in the DRGs by using by using the Fluo - 3/AM

The fluorescent indicator dye (Fluo - 3⁄AM) was used to analyze the concentration of free calcium ion concentration ($[Ca^{2+}]_c$) in the cytosol of green whole DRG images. The whole DRGs in the dishes with bottom glass were loaded with 1 µM Fluo - 3⁄AM for 45 min. The extracellular solution with Ca^{2+} was also used in the Fluo - 3⁄AM experiments for washing the DRGs. The fluorescence intensity changes of Fluo 3⁄AM in a laser scan confocal microscope (LSM-800, Zeiss, Ankara, Turkey) attached with 20x objective were expressed by using the arbitrary unit (a.u.) (Düzova et al. 2021). The neurons were stimulated at 405 nm in the LSM-800 by argon laser. The DRGs were treated by CPZ (100 µM) to inhibit Ca^{2+} influx before stimulation of TRPV1 (CAP and 10 µM).

Intracellular ROS production and mitochondrial membrane potential (ΔΨM) measurements

A non-fluorescent, non-charged dye called DCFH-DA can easily pass through cell membranes. Upon oxidation to DCF once within the cell, DCFH-DA turns fluorescent, with the fluorescence being proportional to ROS production. Succinctly, the ScN and DRG neurons (10^3 neuron/ml) were washed with serum-free RPMI-1640 medium and incubated with 2 µM DCFH-DA at 37 °C for 25 min (Uslusoy et al. 2017). The fluorescence intensity of DCF was measured (excitation 488 nm and emission 543 nm) in an automatic microplate reader (Infinite PRO 200; Tecan Austria GmbH, Groedig, Austria). Fold-increase (experimental/Ctr) unit was used for the expression of ROS.

The JC-1 probe was used to measure the production of ΔΨM (Rothe et al. 1988). 4 µM JC-1 was incubated in the medium of the ScN and DRG at 37 °C for 45 min. Using a microplate reader (Infinite PRO 200) and a single excitation wavelength of 488 nm with dual emission of green (520 nm) and red (596 nm), JC-1 fluorescence change was recorded. The ratio of red to green fluorescence is used to calculate the value of ΔΨM. Fold-increase (experimental/Ctr) unit was used for the expression of ΔΨM.

Determination of apoptosis

The apoptosis assay was performed using a commercial kit and the guidelines offered by Biocolor Ltd. (Northern Ireland). The APOPercentage dye, which is actively transported into ScN and DRG when an apoptotic cell loses its asymmetry, can be used to color apoptotic neurons red in order to identify apoptosis using microplate reader (Infinite PRO 200) (Uğuz and Nazıroğlu 2012).

Determination of caspase 3 and 9 activities

For the determinations of caspase 3 and 9 activities, CAP stimulated or resting the ScN and DRG were washed once with 1xPBS (Espino et al. 2011; Düzova et al. 2021). Fifteen microliters of the neuron suspensions (10^3 neurons/ml) were added to the microplate and mixed with the appropriate peptide substrate dissolved in the standard reaction buffers of caspase 3 [100 mM HEPES, pH 7.25, 10% sucrose, 0.1% CHAPS, 5mM DTT, 0.001% NP40, and 0.04 ml of caspase 3 substrate (AC-DEVD-AMC)] and caspase 9 [0.1 M MES hydrate, pH 6.5, 10% PEG, 0.1% CHAPS, 5 mm DTT, 0.001% NP40, and 0.1 mM of caspase 9 substrate (AC-LEHD-AMC)]. In the Infinite PRO 200, the substrate cleavages were measured with excitation wavelength of 360 nm and emission at 460 nm. Fold-increase (experimental/Ctr) unit was used for the expression of caspase 3 and 9 activities.

Assay for cell viability

For the cell viability analyses, we used 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in the DRG and ScN neurons as described elsewhere (Uğuz and Nazıroğlu 2012). The absorbance values of MTT were recorded by microplate reader (Infinite PRO 200) at 490 nm. Fold-increase (experimental/Ctr) unit was used for the expression of MTT results.

Statistical analyses

All results were expressed as means ± standard deviation (SD) and p values less than $p \leq 0.05$ were regarded as significant. To determine the effect of treatment, data were analyzed using one-way ANOVA. Post-hoc test was used for finding a significant difference. The presence of significance was assessed with the Tukey HSD test. The SPSS statistical program was used to analyses the data (version 17.0 software, SPSS Inc. Chi, USA).

Results

The injection of ALA modulated body weight and DiNP induction in STZ-induced rats

The body weight changes of rats were remarkably ($p \leq 0.05$) decreased in the STZ group as compared to the groups of Ctr and ALA, although they were increased in the STZ + ALA group by the ALA injection (Fig. 1A). The induction of DiNP was weekly tested in the rats by using the hot plate tests (Fig. 1B1) and von Frey needles (Fig. 1C1) (Zhang et al. 2020; Düzova et al. 2021). The hot sensitivity pain threshold (Fig. 1B) and mechanical sensitivity pain threshold (Fig. 1C) values were lower in the STZ group than in the Ctr and ALA groups ($p \leq 0.05$). However, the treatment of ALA increased the values in the Ctr and ALA groups by the treatment of ALA ($p \leq 0.05$) (Fig. 1C). The lowest values were noted during the fourth week of the STZ treatment for changes in body weight, hot sensitivity pain threshold level, and mechanical withdrawal pain threshold value.

Effects of ALA administrations on $[Ca^{2+}]_c$ via TRPV1 activation in the ScN and DRG of diabetic rats

It is widely recognized that neuropathic pain is induced by an increase in excessive Ca^{2+} influx. After observing an

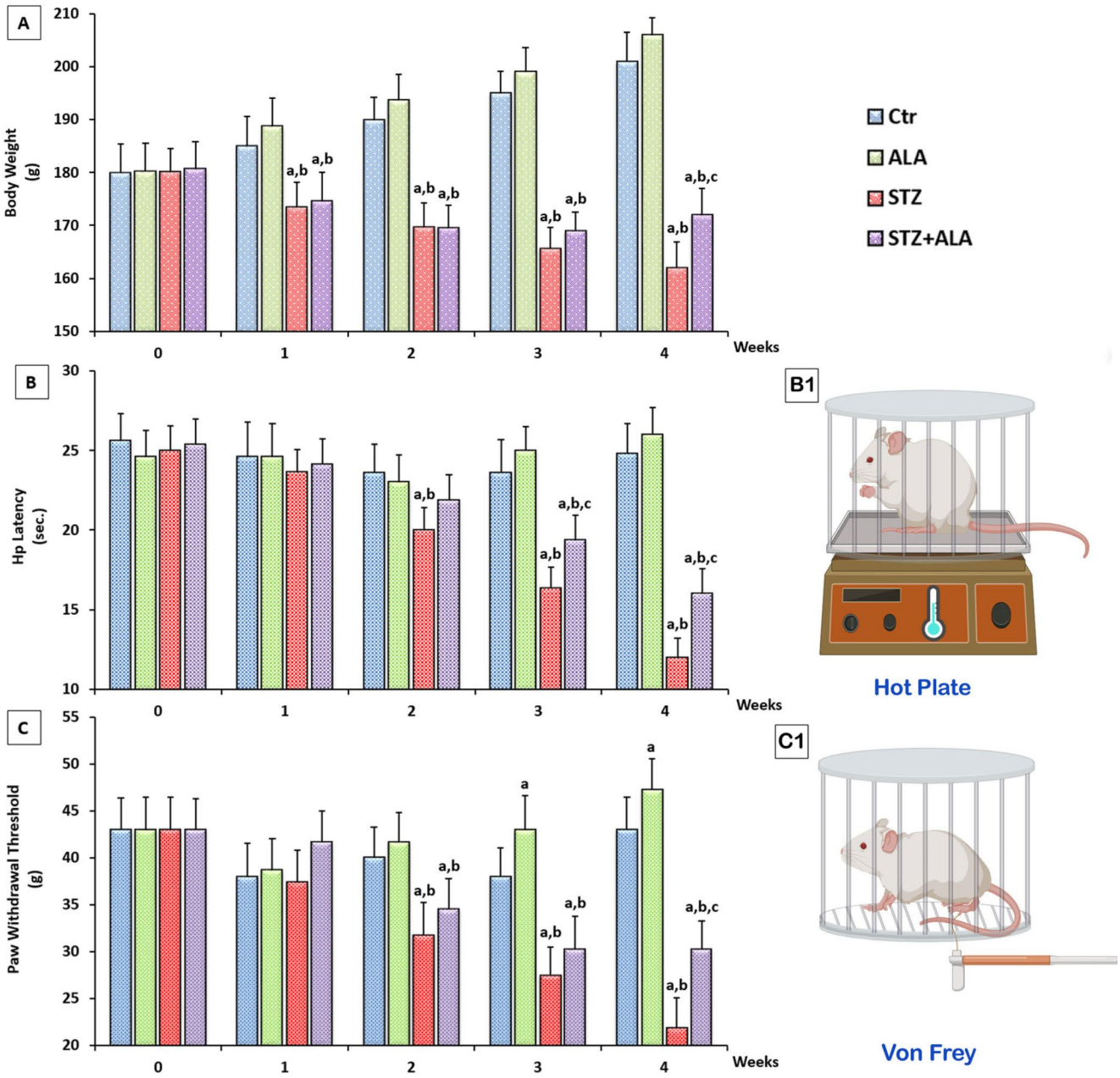

Fig. 1 Effect of ALA (50 mg/kg for 14 days) treatment on body weight change **(A)**, paw withdraw thresholds of hot plate **(B** and **B1)** and von Frey tests **(C** and **C1)** in Ctr, ALA, STZ, and STZ + ALA groups of rats. (n = 10 and mean ± SD). ($^{a}p \leq 0.05$ vs. Ctr group, $^{b}p \leq 0.05$ vs. ALA group, $^{c}p \leq 0.05$ vs. STZ group)

increase in pain intensity, we made the hypothesis that the activation of TRPV1 in the neurons would raise the Ca^{2+} concentration. Hence, it has been investigated whether the activation of TRPV1 channels is related to the STZ-induced diabetes model as well as the channel caused increase in cytosolic stress parameters due to excessive Ca^{2+} influx. For this, TRPV1 channel activator CAP and inhibitor CPZ were investigated by measuring the $[Ca^{2+}]_c$. The concentrations of Ca^{2+} in ScN (Fig. 2A1 and 2A2) and DRG (Fig. 2B1 and 2B2) were increased in the STZ group as compared to the Ctr and ALA groups ($p \leq 0.05$). After the treatment of ALA and CPZ, the level of $[Ca^{2+}]_c$ in the ScN and DRG was lower in the STZ + ALA and STZ + CPZ groups than in the STZ groups ($p \leq 0.05$).

Effects of ALA administrations on TRPV1 channel current density in the DRG of diabetic rats

The treatment of CAP (10 µM) induced TRPV1 currents in the DRG neurons of Ctr and STZ without ALA (Fig. 3B C,

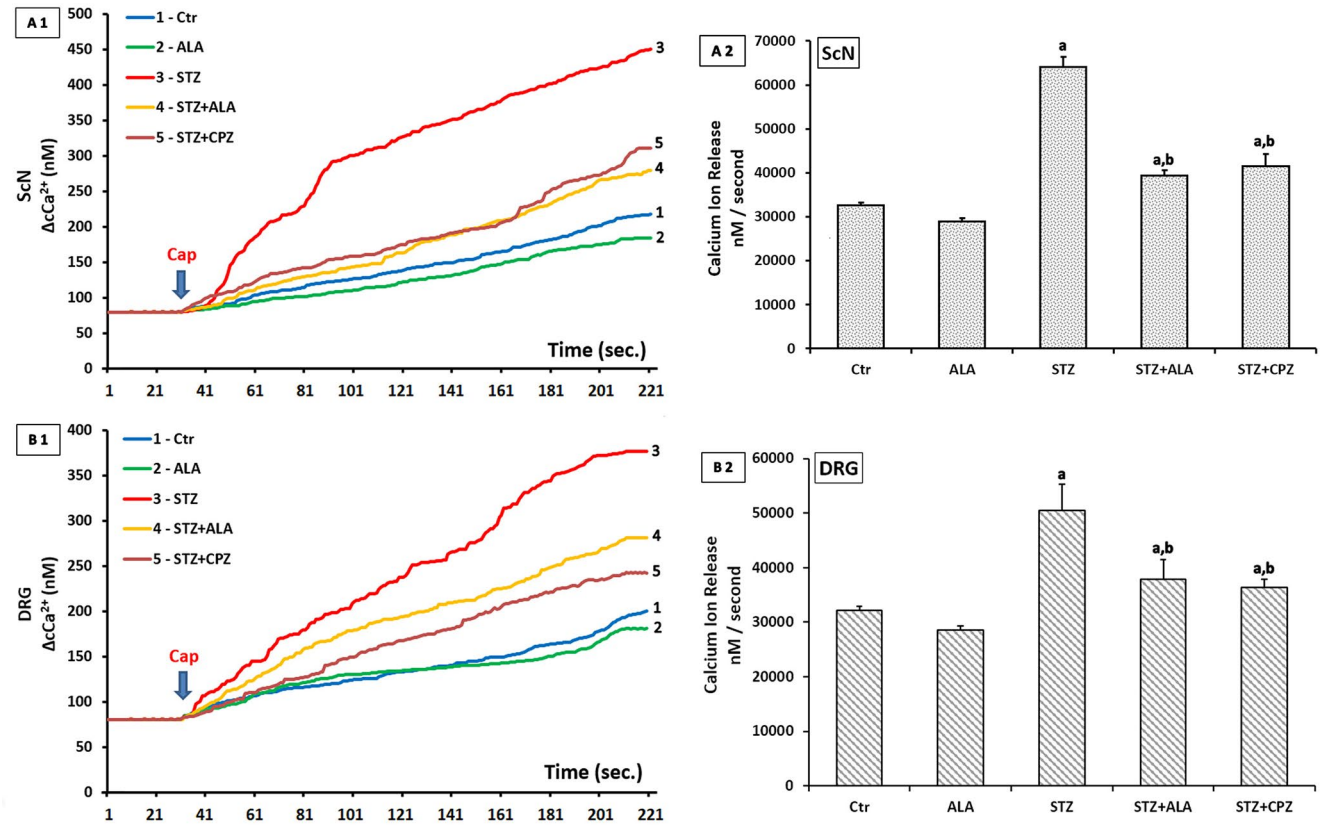

Fig. 2 The injection of ALA (50 mg/kg for 14 days) decreased the cytosolic free calcium concentration ($[Ca^{2+}]_c$) through TRPV1 channel activation in the ScN and DRGs of rats with DM (STZ). (n = 10 and mean ± SD). The experimental animals were applied intraperitoneal ALA and STZ. ScN (**A1** and **A2**) and DRGs (**B1** and **B2**) were dissected from Ctr and treated experimental animals. Fura-2-loaded ScNs and DRGs were stimulated with CAP (10 μM) in the presence of normal extracellular calcium (1.2 mM) for 220 s, although the pre-incubation of CPZ (100 μM for 30 min) was used in the neurons of STZ + CPZ groups for the inhibition of $[Ca^{2+}]_c$ (TRPV1 activity). The TRPV1 stimulator (CAP) was added between 20 and 30 s. Therefore, there was CAP stimulation in the five groups during the 190–200 s. ($^a p \leq 0.05$ vs. Ctr and ALA groups, $^b p \leq 0.05$ vs. STZ group)

3D, and 3E). We found no differences in the TRPV1 currents in the DRG of Ctr in the absence of CAP (Fig. 3A). The CAP-induced TRPV1 currents were reversibly blocked by the treatment of TRPV1 blocker (100 μM CPZ) and $NMDG^+$ (Fig. 3B C). The TRPV1 current densities of the DRGs were significantly ($p \leq 0.05$) increased in the group of STZ + CAP as compared to the groups of Ctr, Ctr + CAP, Ctr + CAP + CPZ, and STZ + CAP + CPZ (Fig. 3F). We found no differences in the TRPV1 currents in the DRGs of ALA + CAP (Fig. 3D) and STZ + ALA + CAP groups (Fig. 3E). The TRPV1 current densities in the DRGs were remarkably ($p \leq 0.05$) diminished in the ALA + CAP and STZ + ALA + CAP groups as compared to the STZ + CAP group by the treatment of ALA (Fig. 3F). Figure 3G displayed a whole-neuron patch-clamp arrangement.

STZ-induced the increase of $[Ca^{2+}]_c$ was decreased via modulation of TRPV1 in the DRGs by the treatment of ALA

In addition to the electrophysiology (patch-clamp) and Fura-2 analyses, we performed Fluo 3 /AM for investigating the relationship between ALA and the TRPV1-mediated $[Ca^{2+}]_c$ concentration in the DRGs. For recording the Fluo - 3/AM imaging, the TRPV1 channel in the DRGs was stimulated by CAP (10 μM), although it was inhibited by CPZ (100 μM). The CAP Stimulations induced an increase in the $[Ca^{2+}]_c$ in the DRGs (Fig. 4A and B, and 4 C) of four groups (Ctr, ALA, STZ, and STZ + ALA), because of the stimulation of Ca^{2+}-permeable TRPV1 ($p \leq 0.05$). The concentration of $[Ca^{2+}]_c$ in the DRG neurons were higher in the STZ group than in the Ctr and ALA groups, although they were effectively diminished in the neurons by the groups of STZ + ALA ($p \leq 0.05$).

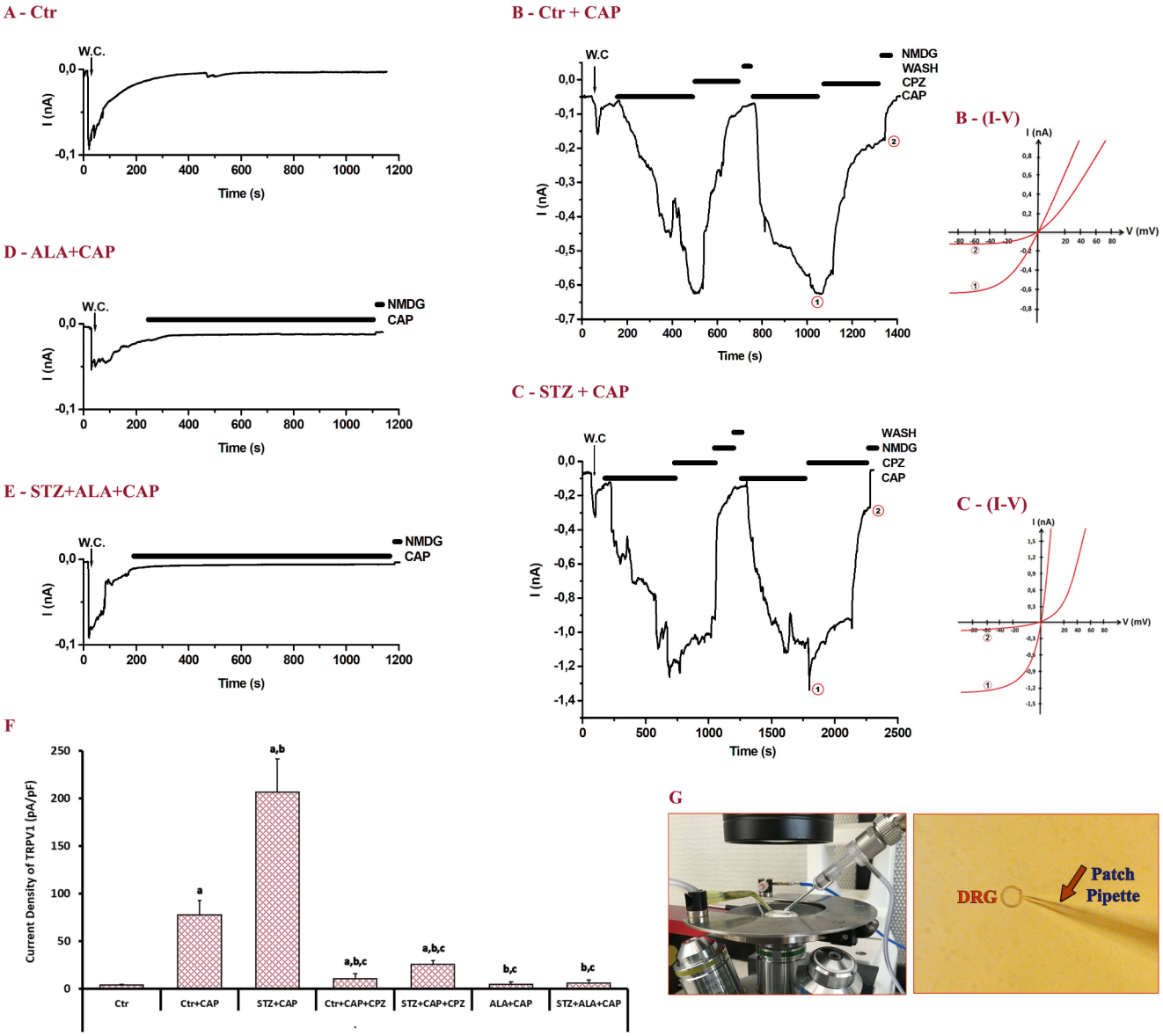

Fig. 3 Effects of ALA (50 mg/kg for 14 days) administration on TRPV1 channel activation in the DRG of rats with DM (STZ). (n = 3–4 and mean ± SD). The holding potential was kept at − 60 mV. The DRGs were extracellularly (in patch chamber bath) stimulated by CAP (10 μM), although they were inhibited by CPZ (100 μM). **A**. Ctr group (without CAP). **B**. Ctr + CAP group (with CAP). **C**. STZ + CAP group. **D**. ALA + CAP group. **E**. STZ + ALA + CAP group. **B. I-V** and **C. I-V**: Current voltage relationships of whole-cell currents in the presence of various extracellular cations, activators, and inhibitors as indicated. **(F)** The mean values of current densities (pA/pF). **(G)** whole cell (W.C.) record and patch-clamp chamber. ($^{a}p \leq 0.05$ vs. Ctr. group, $^{b}p \leq 0.05$ vs. Ctr + CAP group, $^{c}p \leq 0.05$ vs. STZ + CAP group)

The results of Fluo - 3/AM were obviously evidenced that the STZ-caused increase of $[Ca^{2+}]_c$ in DRGs was decreased via inhibition of TRPV1 by the treatment of ALA.

The treatment of ALA attenuated STZ-induced apoptotic markers

The cell viability (MTT) values were significantly ($p \leq 0.05$) downregulated in the STZ as compared to the groups of Ctr and ALA (Fig. 5A). However, the MTT values were significantly ($p \leq 0.05$) upregulated in the DRGs of STZ + ALA and STZ + CPZ groups by the treatment of ALA and CPZ. The apoptosis levels (Fig. 5B), caspase 3 (Fig. 5C), and 9 (Fig. 5D) activities were increased ($p \leq 0.05$) in the ScN and DRG of STZ groups as compared to the groups of Ctr and ALA. In the ScN and DRG, the apoptosis levels, caspase 3 and 9 activities were diminished in the STZ + ALA and STZ + CPZ groups as compared to the STZ groups.

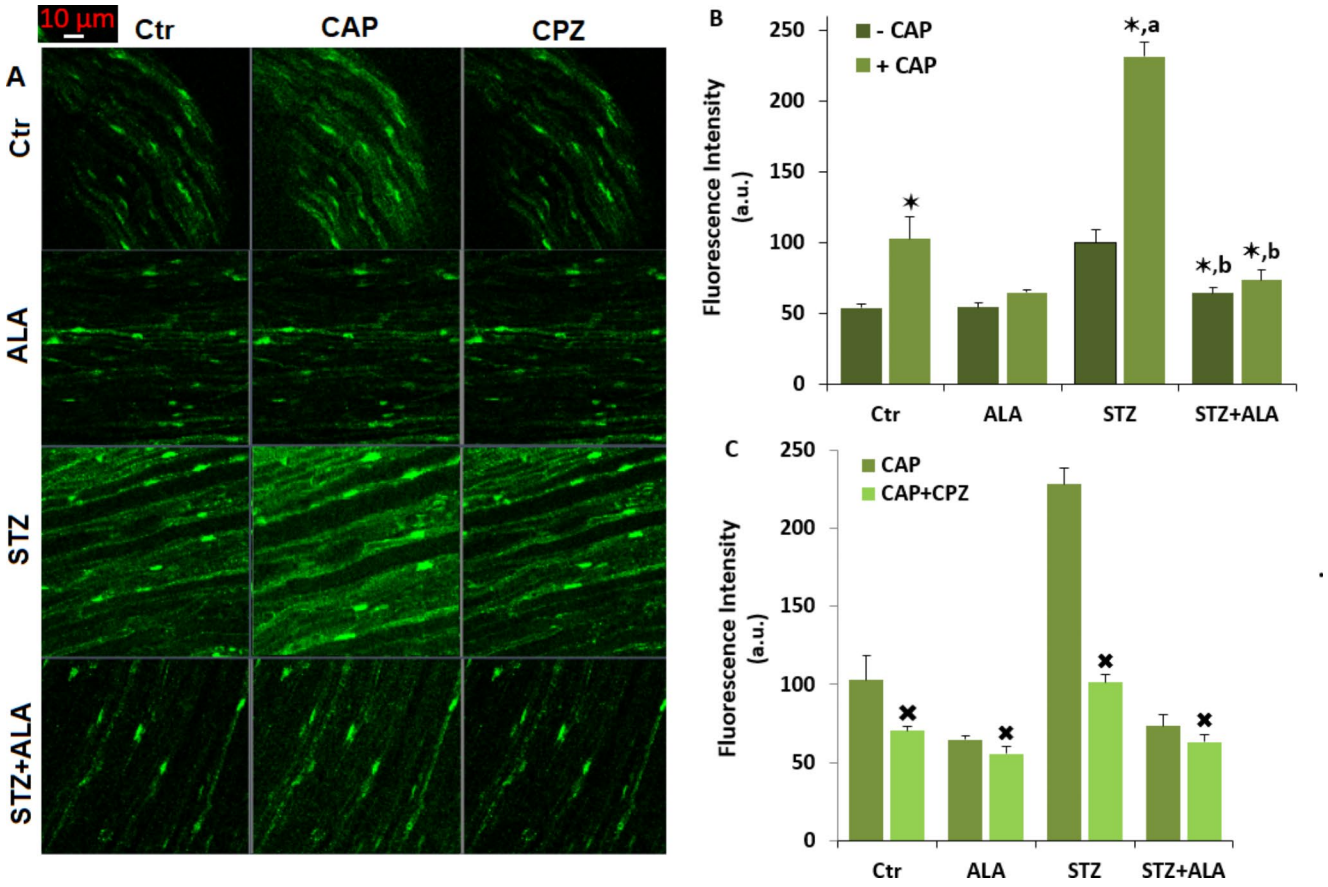

Fig. 4 The injection of ALA (50 mg/kg for 14 days) modulated DM (STZ)-caused the increase of $[Ca^{2+}]_c$ in the whole DRG. (Mean ± SD). The DRGs were stained with Fluo - 3⁄AM (1 µM for 45 min). After washing the DRGs with extracellular buffer, they were stimulated with CAP (10 µM for 5 min) and then they were inhibited by CPZ (100 µM for 5 min) in the LSM-800 with 20x objective. **A.** The representative images of the Fluo - 3⁄AM in the four groups [control (Ctr), ALA, STZ, and STZ + ALA. **B** and **C**. The mean fluorescence intensity changes as arbitrary unit (a.u.) in the four groups after the CAP and CPZ treatments. The scale bar in the images was kept as 10 µm. One represented image of each figure was selected from 10 DRGs of 10 independent experiments for each condition. ($^{a}p \leq 0.05$ vs. the groups of Ctr and ALA. $^{b}p \leq 0.05$ vs the groups of STZ. $*p \leq 0.05$ vs. without CAP stimulation (- CAP group). $^{\times}p \leq 0.05$ vs. CAP stimulation (+ CAP group)

The STZ-induced increases of ROS (DCFH-DA) and ΔΨM (JC-1) were decreased by the treatment of ALA and TRPV1 channel blocker

We also investigated the protective effects of ALA on ROS (DCFH-DA) and ΔΨM (JC-1) levels in the ScN and DRG. The levels of ROS (Fig. 6A) and ΔΨM (Fig. 6B) in the ScN and DRG were remarkably ($p \leq 0.05$) higher in the STZ groups than in the Ctr and ALA groups. However, ROS and ΔΨM levels in the neurons were diminished in the STZ + ALA and STZ + CPZ groups by the treatments of ALA and CPZ ($p \leq 0.05$).

Discussion

DM causes serious cell and tissue damage and losses. Most patients experience diabetic polyneuropathy, which causes sensory dysfunction. Numerous mechanisms are proposed regarding diabetic neuropathy and the molecular basis of pain caused by hyperglycemia (Umeda et al. 2006; Pabbidi et al. 2008; Uchida et al. 2011). As a compatible with the previous studies, the impaired insulin signal and Ca^{2+} balance through the activation of TRPV1 in the STZ groups triggered ΔΨM and oxidative stress in the ScN and DRG (Kahya et al. 2017; Zhang et al. 2020; Düzova et al. 2021). The findings of the present investigation showed that disturbance of Ca^{2+} homeostatic processes mediated by TRPV1 leads to disruption in $[Ca^{2+}]_c$. As a result, DM induced mitochondrial dysfunction, increased ROS production, and impaired cellular signaling pathways, such as DiNP and apoptosis. We suggest that these results may explain

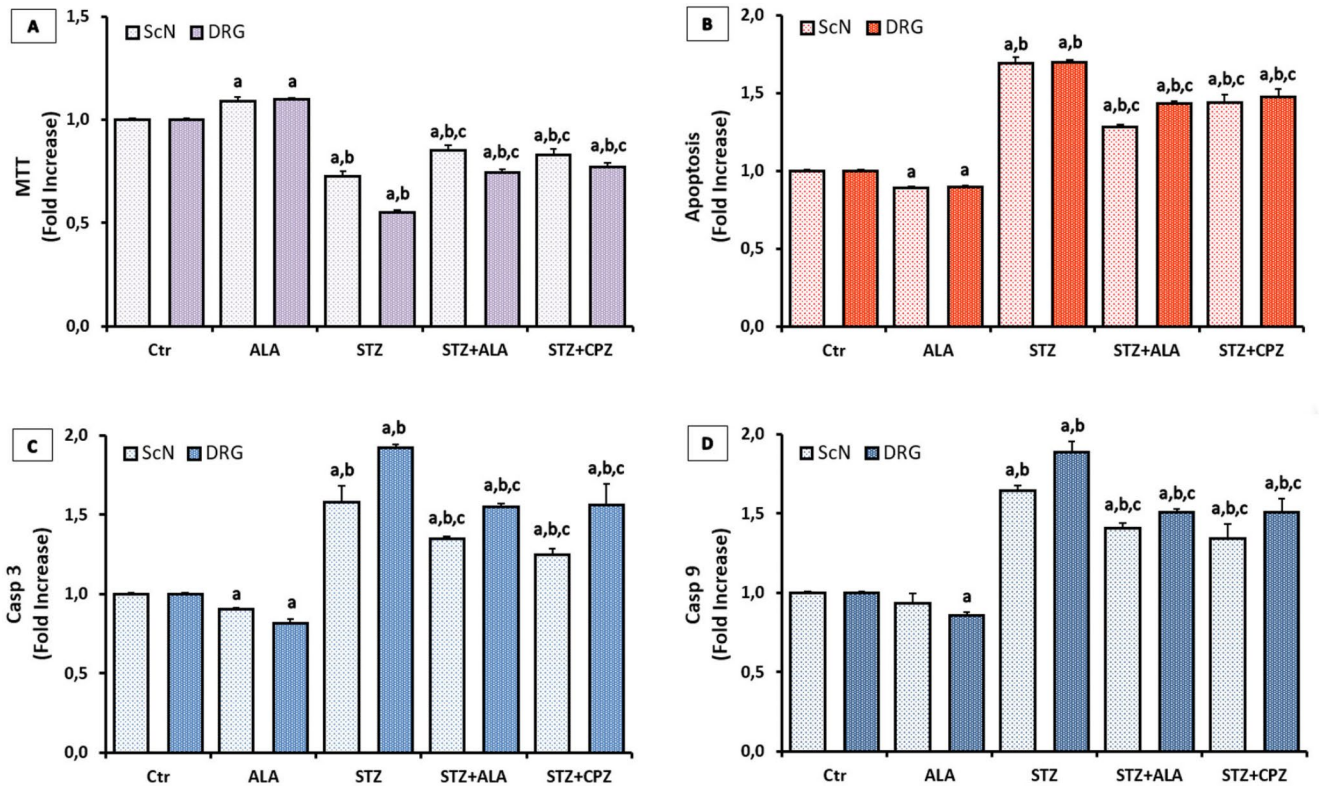

Fig. 5 Effects of ALA injections (50 mg/kg for 14 days) on the levels of MTT **(A)**, apoptosis **(B)**, caspase 3 **(C)**, and caspase 9 **(D)** levels in ScN and DRGs of rats with DM (STZ). Cell viability (**A**) was performed by using the MTT analyses. The levels of apoptosis (**B**), caspase 3 (**C**), and caspase 9 (**D**) were performed in the plate reader (PRO 200). The parameters were presented as mean ± SD of ten separate experiments and expressed as fold-increase (experimental/Ctr). ($^{a}p \leq 0.05$ vs. Ctr group, $^{b}p \leq 0.05$ vs. ALA group, $^{c}p \leq 0.05$ vs. STZ group)

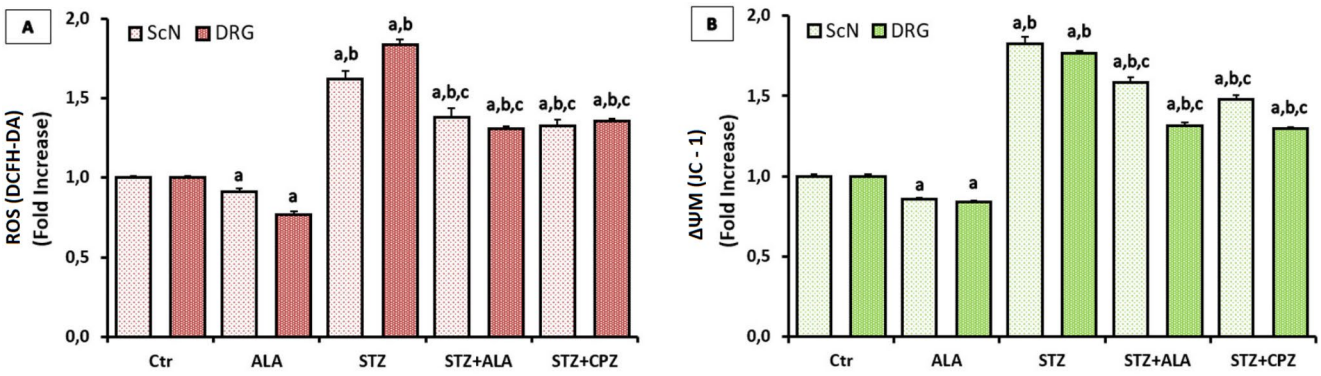

Fig. 6 Effects of ALA injections (50 mg/kg for 14 days) on the levels of ROS **(A)**, and JC-1 **(B)** levels in ScN and DRGs of rats. The levels of ROS (**A**), and mitochondrial membrane potential (JC-1) (**B**) were performed in the plate reader (PRO 200). The parameters were presented as mean ± SD of ten separate experiments and expressed as fold-increase (experimental/Ctr). ($^{a}p \leq 0.05$ vs. Ctr group, $^{b}p \leq 0.05$ vs. ALA group, $^{c}p \leq 0.05$ vs. STZ group)

the pathophysiological mechanisms of DiNP. It is recognized that the increase in ROS production associated with hyperglycemia is linked to degenerative processes in DM (Rochette et al. 2015). In the results of this study, we have shown that TRPV1 channels are activated by increased cellular oxidative stress. The results of diabetes groups confirm the theory that the increases of oxidative stress and ΔΨM may be responsible from TRPV1 activation-mediated the increase of Ca^{2+} influx (Uslusoy et al. 2017; Kahya et al. 2017).

The modulator roles of ALA treatment on TRPA1 and TRPV1 channel activities in tumor cell lines were reported (Nur et al. 2017; Deveci et al. 2019). Similar modulator results of ALA treatments were observed on the TRPA1/

TRPV1 channels in the rat trigeminal neuron (Gualdani et al. 2015) and a mouse model of cancer-induced pain (Antoniazzi et al. 2019). The protective role of ALA on TRPV1 channel stimulation in the DRG and ScN of diabetic rats has not been investigated till today. Hence, we tested the effects of ALA on $[Ca^{2+}]_c$, ROS, apoptosis, caspase 3, and 9 levels via TRPV1 stimulation in the DRG and ScN of diabetic rats. We observed that diabetic rats are characterized by increased ROS, apoptosis, and Ca^{2+} influx. However, the treatment with ALA was indicated protective actions on the ScN and DRG of rats with DM. In the results of a recent study, the TRPV1 expression levels were increased in the DRGs of mice by the treatment of STZ, although their expression levels were decreased by the treatment of ALA (Zhang et al. 2020). In accordance with the results, the cisplatin-induced increase of TRPV1 channel expression was decreased in the rat organ of Corti by the treatment of ALA (Mukherjea et al. 2008). The results confirmed that the treatment of ALA modulated the protein structure of TRPV1 via binding the TRPV1 in the DRGs. The findings were also supported by the previous non-diabetic rat models and various cell line studies (Nur et al. 2017; Deveci et al. 2019; Joseph et al. 2008; Melli et al. 2008).

In the current experiment, we found the stimulation of TRPV1 in DRG and ScN of diabetic rats by the stimulation of CAP, although its stimulation was inhibited by the treatment of CPZ. Similarly, it was reported that TRPV1 channel expressions increased in the sensory neuron of STZ-induced rat (Bishnoi et al. 2011). Repeated activation of TRPV1 has been shown to result in increased $[Ca^{2+}]_c$, apoptosis, and oxidative stress (Hong et al. 2008). It was reported that TRPV1 activation increased with CAP and oxidative stress (Chen et al. 2009). Activation of TRPV1 with CAP-induced Ca^{2+} influx and oxidative stress in the hippocampus and DRG of rats (Yazğan and Nazıroğlu 2017). The ALA treatment was effective in the treatment of hypoxia-induced oxidative stress and apoptosis in MCF-7 breast cancer cells by the induction of TRPV1 channel desensitization (Deveci et al. 2019). In this case, oxidative stress may act TRPV1 responses, including the increase of DiNP sensation in the DRG and ScN oxidative toxicity of diabetic rats (Chen et al. 2009).

ALA is considered to be protective against increased ROS production with mitochondrial dysfunction (Park et al. 2014). It has been reported that ALA can perform the "scavenger" function of hypochlorous acid, hydroxyl radicals, singlet oxygen, and peroxyl radicals (Tibullo et al. 2017). Numerous clinical studies have shown that ALA causes a marked reduction in diabetes complications (Ametov et al. 2003; Mijnhout et al. 2012; Garcia-Alcala et al. 2015). It was reported that ALA inhibited oxidative stress and CAP-induced increase of $[Ca^{2+}]_c$ in the tumor cells through the inhibition of ROS production (Nur et al. 2017). In the current study, we observed that the increases of $[Ca^{2+}]_c$, apoptosis, $\Delta\Psi M$, and ROS were induced by STZ, although they were regulated by the treatment of ALA. According to the findings of the current investigation, ALA injections have modulatory effects on the DM-induced mitochondrial abnormalities and apoptosis induction in the neurons of rats with DM.

ALA is a potent antioxidant that acts as an essential cofactor of mitochondrial enzymes involved in oxidative metabolism and has been used as a dietary supplement to treat high glucose and fat diet-induced obesity and reduce body weight (Prieto-Hontoria et al. 2013; Midaoui et al. 2015; Valdecantos et al. 2019). However, the effect of ALA treatment appears to be transient, since experimental animals immediately regain body weight after ALA treatment is terminated (Kim et al. 2004). Potential long-term positive effects of ALA administration on weight gain in rats were recently reported (Rabaglino et al. 2021). In the current study, the body weight gain was increased at 4th week of ALA treatment, although there were no changes of body weight gain at the 1st, 2nd, and 3rd weeks. This is in agreement with a recent study which had demonstrated that the short-term treatment of ALA induced mitochondrial enzymes changes in liver in support of significant improvement of whole-body lipid status (Valdecantos et al. 2019).

Conclusion

We induced an experimental DM model with STZ. In the model, the TRPV1 channel was involved in the Ca^{2+} entry-induced DRG and ScN death in rats. By regulating the channel currents of ALA therapy, the reduction of apoptosis and Ca^{2+} influx can be explained by the neuroprotective effect on nerve cells (Fig. 7). The TRPV1 channel in the DRG and ScN is inhibited by ALA, which suggests that it acts as a modulator against oxidative stress-induced Ca^{2+} mobilization apoptosis. Current results are particularly important and can be explained by the DM-induced cell death reduction and peripheral pain-reducing properties of ALA. It seems that TRPV1 channels may become an important pharmacological target in the treatment of DM-induced oxidative stress and DiNP.

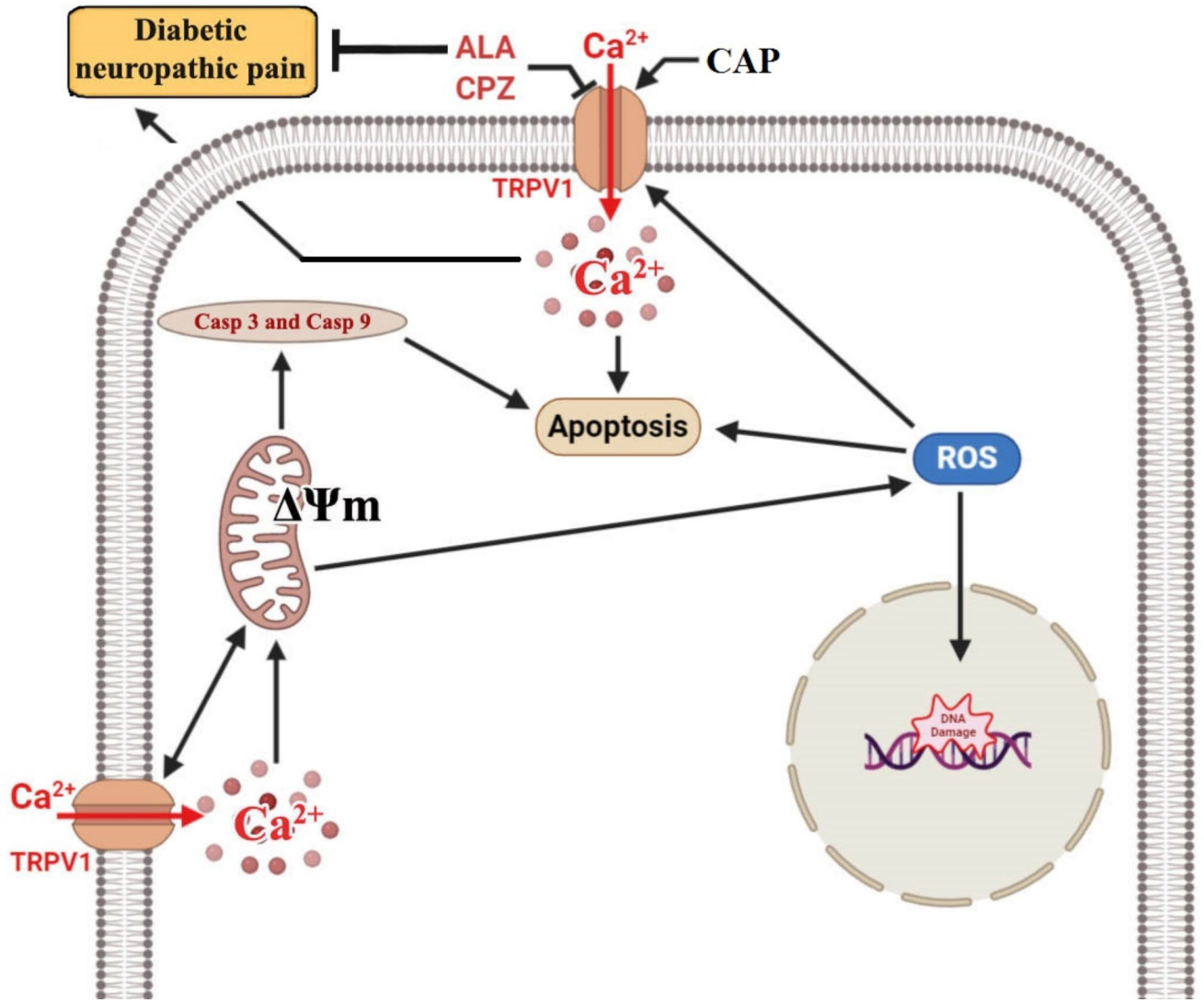

Fig. 7 The possible protective action of alpha-lipoic acid (ALA) on the molecular pathways of oxidative stress, apoptosis, and pain intensity in rat with diabetes mellitus (STZ). Increasing evidence suggests that although the treatments of ALA and CPZ suppress diabetic neuropathic pain via the inhibition of TRPV1, the generations of CAP and CPZ enhance it through the activation of TRPV1. In the ScN and DRG of diabetic rats, increased cytosolic free Ca^{2+} via the activation of TRPV1 produces an increase in mitochondrial membrane potential (ΔΨm). Consequently, the rise in ΔΨm leads to an excessive production of cytosolic ROS. Through the activation of caspase pathways like caspase 3 (casp 3) and 9 (casp 9), the STZ and TRPV1 activation-mediated increased Ca^{2+} influx results in apoptosis and cell death in the ScN and DRG. Treatments with ALA and TRPV1 antagonist (CPZ) modified the oxidative and apoptotic effects of STZ.

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Authors' contributions Dr. Betül Yazğan: Conceptualization, Methodology, Formal analysis, Data curation, Resources. Dr. Yener Yazğan: Conceptualization, Methodology, Formal analysis, Data curation, Resources, Writing – review & editing. Prof. Dr. Mustafa Nazıroğlu: Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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Data Availability The present analyses were performed in company (BSN Health), and row data are available from Dr. M. Nazıroğlu on reasonable request. All graphics, in the manuscript were prepared by a coauthor (Dr. Y Yazğan).

Declarations

Conflict of interest The authors report no conflicts of interest.

Ethics approval No data of human and animal participants. The author

has no ethical conflicts to disclose.

Consent to participate Not applicable.

Consent for publication The manuscript has not been submitted for publication elsewhere. All authors have read and have abided by the statement of ethical standards for manuscripts.

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