Inhibition of IκB Kinase (IKK) Protects Against Peripheral Nerve Dysfunction of Experimental Diabetes

Geeta Negi · Shyam S. Sharma

Abstract Nuclear factor-κB (NF-κB) has been reported as a critical component of signalling mechanisms involved in the pathogenesis of a number of inflammatory conditions. Previous reports have shown that anti-inflammatory agents have a protective role in experimental diabetic neuropathy. Here, we assessed whether the inhibition of NF-κB cascade via IκB kinase (IKK) exerts any neuroprotective effect in experimental diabetic neuropathy. IKK inhibitor SC-514 (1 and 3 mg/kg) was administered daily for 2 weeks starting after 6 weeks of streptozotocin-induced diabetes. Nerve conduction and blood flow were determined by Powerlab and LASER Doppler system, respectively. We evaluated the changes in NF-κB, iNOS, and COX-2 expression by Western blotting in sciatic nerve. We found that IKK inhibition with SC-514 increased nerve blood flow and conduction velocity and improved pain threshold in diabetic animals. SC-514 also reduced the expression of NF-κB and phosphorylation of IKKβ in the sciatic nerve. Treatment with SC-514 reduced the elevated levels of pro-inflammatory cytokines (TNF-α and IL-6), iNOS, and COX-2. SC-514 reduces the expression of NF-κB and its downstream inflammatory components which may be involved in the improvement in nerve functions and pain perception in diabetic neuropathy. From the data of the present study, we suggest that diminution in IKK can be exploited as a drug target to significantly reduce the development of long-term complications of diabetes, particularly neuropathy.

Keywords Diabetic neuropathy · NF-κB · IKK · IκB · SC-514

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Introduction

Diabetic neuropathy, the most common complication of long-standing diabetes mellitus, is associated with clinically significant morbidities. Being multifactorial in pathogenesis, several pathways have been implicated in diabetic neuropathy such as polyol pathway, oxidative stress, neuroinflammation, mitogen-activated protein kinase (MAPK), and poly ADP ribose polymerase (PARP) overactivation and activation of transcription factors, such as nuclear factor-κB (NF-κB). Diabetes and related complications have been found to be associated with altered levels of cytokines and chemokines which suggest a strong participation of inflammation in their pathophysiology. NF-κB is considered the master regulator of inflammatory responses, and it is now clear that many of the major pathways that are involved in pathophysiology of diabetic neuropathy are related to NF-κB activation. Furthermore, NF-κB regulates the transcription of many genes like iNOS, COX, and lipoxygenase which further propagate inflammation in peripheral nerves.

Under normal physiological condition, an inhibitory protein, IκB (inhibitor of NF-κB), binds to NF-κB thus sequestering it within cytosol in inactive form. Various stimulatory signals like TNF-α, cytokines, ROS, and bacterial and viral stimulants lead to the activation of a specific IκB kinase (IKK) complex that phosphorylates IκB. Phosphorylation of IκB leads to the IκB tagging with ubiquitin by an SKp1–Cullin–F-box (SCF)-type E3 ligase [1, 2]. Polyubiquitination marks IκB for rapid proteolytic degradation by the 26S proteasome. IκB degradation result in unmasking of nuclear localization signal of NF-κB which thus translocates into the nucleus and binds to promoter regions of target proinflammatory genes, thus initiates their transcription.

Thus, phosphorylation of IκB by IKK is a triggering event in NF-κB activation. IKK has been identified as a multisubunit complex containing catalytic subunits IKKα and IKKβ and regulatory subunit IKKγ (NEMO, NF-κB

essential modifier). Although IKKα and IKKβ share 50 % sequence identity, many *IKKα* and *IKKβ* knockout studies have shown that IKKβ is the prime regulator of NF-κB-dependent proinflammatory signal transduction whereas IKKα is required for activation of alternative noncanonical pathway [3-6]. Several IKKβ inhibitors have been developed and have shown therapeutic responses in animal models of different diseases and are in early clinical trials [7-9]. Recent findings demonstrate that IKK overactivity is one of the pathogenetic factors in the development of diabetes, obesity, and related complications [10, 11].

The present study explores the specific involvement of NF-κB cascade in peripheral nerve dysfunction that characterize neuropathic component of diabetes. For this, we used small molecule inhibitor SC-514 (4-Amino-[2′, 3′-bithiophene]-5-carboxamide) which is specific for a pivotal event in the NF-κB cascade, i.e., IκB phosphorylation.

Materials and Methods

Unless otherwise stated, all chemicals were of reagent grade and were purchased from Sigma (St Louis, Missouri, USA).

Induction of Diabetes and Experimental Design

All animal experiments were performed in accordance with Committee for the Purpose of Control and Supervision on Experimentation on Animals (CPCSEA) guidelines. Experimental protocols were approved by the Institute Animal Ethics Committee (approval number IAEC/10/71) of NIPER SAS Nagar, Punjab, India. Male Sprague Dawley rats (250–270 g) were used and fed on standard rat diet and water ad libitum. Diabetes was induced by single dose of streptozotocin (STZ, 55 mg/kg, i.p.). Blood samples were collected 48 hours after STZ administration. Rats with plasma glucose level more than 250 mg/dl were considered as diabetics and were further considered for the study. The experimental groups comprised of nondiabetic (ND) control rats, diabetic control rats (STZ-D), and diabetic rats treated with SC-514 (D+SC 1 and D+SC 3, respectively for 1 and 3 mg/kg, i.p., in 2 % DMSO in saline). Each group contained six to eight animals. The treatment was started 6 weeks after diabetes induction and was continued for 2 weeks. The functional experiments were performed and nerves were isolated for biochemical and protein expression studies 24 h after administration of last dose.

Nerve Function Studies

Nerve Blood Flow

Nerve blood flow (NBF) was measured using LASER Doppler system (Perimed, Jarfalla, Sweden) [12]. The sciatic nerve was exposed by giving incision on the left flank, and the LASER Doppler probe was applied just in contact with an area of sciatic trunk free from epi or perineurial blood vessels. Flux measurement was obtained from the same part of the nerve and for the same time period (over a 10-min period). The blood flow was reported in arbitrary perfusion units (PU).

Motor Nerve Conduction Velocity

Motor nerve conduction velocity (MNCV) was determined in the sciatic-posterior tibial conducting system using the Power Lab 8sp system (ADInstruments, Bellaviata, NSW, Australia) as previously reported [13]. Briefly, animals were anesthetized by 4 % halothane in a mixture of nitrous oxide and oxygen and anesthesia was maintained with 1 % halothane, using gaseous anesthesia system (Harvard apparatus, Kent, UK). Body temperature was monitored using a rectal probe and maintained with a homeothermic blanket throughout the experiment. The sciatic nerve was stimulated with 3 V proximally at the sciatic notch and distally at the ankle via bipolar electrodes. Receiving electrodes were placed on the foot muscle. The latencies of the compound muscle action potentials were recorded via bipolar electrodes from the first interosseous muscle of the hind paw and measured from the stimulus artifact to the onset of the negative M-wave deflection. MNCV was calculated by dividing the distance between the stimulating and recording electrode with the difference obtained by subtracting the distal latency from the proximal latency and expressed in meters per second.

Behavioral Studies

Thermal Hyperalgesia

Thermal hyperalgesia was assessed in the rat hind paws using a Hargreaves apparatus (Ugo Basile, Como, Italy) [14]. Rats were placed into individual transparent plexiglass testing chamber. Rats were acclimatized to their environments for at least 30 min before any testing was carried out. A heat stimulus was delivered using a 0.5-cm-diameter radiant heat source situated under the plantar surface of the paw. The radiant heat source was adjusted to result in basal latencies of 12–14 s. We used a cutoff time of 15 s. The trials were performed three times at 5-min intervals, and the paw withdrawal latencies were averaged.

Mechanical Hyperalgesia

Sensitivity to noxious mechanical stimuli was determined by quantifying the withdrawal threshold of the hind paw in response to mechanical stimulation using a von Frey anesthesiometer (IITC Life Science, California, USA) as described [15]. The force causing the withdrawal response was

recorded in grams. The test was repeated four to five times at ~5-min intervals on each animal, and the mean value was calculated.

Biochemical Parameters

Blood was collected from tail vein and plasma was separated at 5,000 rpm for 5 min at 4 °C. Plasma glucose level was estimated using GOD–POD kit (Accurex, Mumbai, India) as per manufacturer's instructions.

Nerves were homogenized in PBS buffer containing phenyl methane sulphonyl fluoride (PMSF) and protease inhibitor cocktail. After homogenization, it was sonicated and detergent was added to the homogenate. Homogenate was kept in ice-cold water for 30 min and then sonicated. The homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatant was used for estimations. Commercially available ELISA kits from eBiosciences (USA) for assaying TNF-α and IL-6 proteins were used. TNF-α and IL-6 levels were expressed as picograms per milligram of protein [16].

Immunohistochemistry

Sciatic nerves were fixed in 10 % buffered formaldehyde, dehydrated through graded concentrations of ethanol, embedded in paraffin, and sectioned. Sections (5-μm thick) were mounted on slides, cleared, and hydrated. Sections were treated with a buffered blocking solution (5 % goat serum). Then, sections were co-incubated with primary rabbit polyclonal IgG for NF-κB p65 subunit (rabbit polyclonal; Cell Signaling Technology) at a dilution of 1:400 at 4 °C for 12 h. Sections were washed with Tris–HCl buffer 0.5 M, pH 7.6, and incubated with horse radish peroxidase conjugated anti-rabbit secondary antibody, at room temperature for 1 h. Thereafter, sections were washed as before. Bound antibody was visualized by incubating the microsections with 3, 3-o-diaminobenzidine chromogen substrate solution in the dark, at room temperature for 10 min. Sections were washed with Tris–HCl buffer, counterstained with hematoxylin according to standard protocols and observed under light microscope (Leica, Solms, Germany) [13].

Western Blotting

Protein lysates were obtained by homogenizing sciatic nerves with lysis buffer containing 1 % Triton X-100, 150 mM NaCl, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 20 mM Tris (pH 7.5). Equal amounts of proteins were separated by SDS–PAGE and transferred to a nitrocellulose membrane (Pall Life Sciences, Florida, USA). After blocking with 3 % bovine serum albumin, membranes were incubated with primary rabbit polyclonal

IgG NF-κB (p65 subunit), IKKβ, phospho-IKKβ, iNOS (Cell Signaling Technology, Massachusetts, USA) (1:1,000), and COX-2 (Santa Cruz Biotechnologies, California, USA) (1:400) for 12 h at 4 °C. After washing, membranes were incubated with horse radish peroxidase/alkaline phosphatase conjugated secondary antibody (1:2,000) and bound antibody was visualized by enhanced chemilumniscence or by using a colored reaction with BCIP-NBT. The relative band densities were quantified by densitometry. Equal loading of protein was confirmed by measuring β-actin expression [17].

Statistical Analysis

Data were expressed as mean±SEM. Data were first analyzed by normality test to ascertain normal distribution. For comparing the differences between the two groups Student t test was used. For multiple comparisons, analysis of variance (ANOVA) was used. If ANOVA test showed significant difference, post hoc Tukey or Dunnet test was applied. Significance was defined as $p < 0.05$. All statistical analysis was performed using Jandel Sigma Stat 2, statistical software (Jandel Scientific, Erkrath, Germany).

Results

Body Weight and Plasma Glucose

STZ-injected rats displayed symptoms characteristic of type 1 diabetes like hyperglycemia and weight loss. Eight weeks post-STZ administration, diabetic rats had significantly lower body weight and higher plasma glucose levels as compared to nondiabetic rats ($p < 0.001$). IKK inhibition with SC-514 did not bring any significant changes in body weight or plasma glucose levels in treated diabetic group (Table 1).

Nerve Function Parameters

Eight weeks of diabetes resulted in a significant reduction in NBF ($p < 0.001$) and MNCV ($p < 0.01$) in the sciatic-tibial

Table 1 Effect of SC-514 (1 and 3 mg/kg) on body weight and plasma glucose levels in diabetic rats

Parameter	ND	STZ-D	D+SC 1	D+SC 3
Body weight (g)	387±12	213±16[###]	212±13	218±10
Plasma glucose levels (mg/dl)	99±9	396±15[###]	405±10	390±16

Results are expressed as Mean±SEM

ND nondiabetic, *STZ-D* diabetic, *D+SC 1* diabetic group treated with SC-514 1 mg/kg, D+SC 3 diabetic group treated with SC-514 3 mg/kg

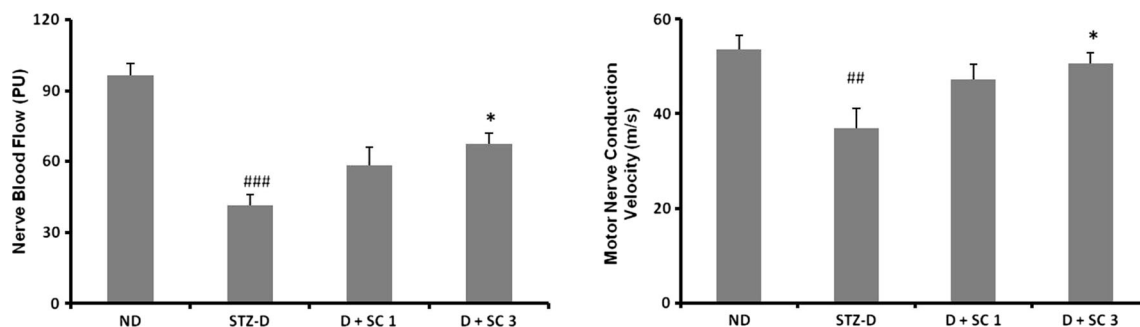[###] $p < 0.001$ vs. ND ($n=6$–7).

Fig. 1 Effect of SC-514 on nerve functions (nerve blood flow, NBF and motor nerve conduction velocity, MNCV) in diabetic rats. *ND* nondiabetic, *STZ-D* diabetic, *D+SC 1* and *D+SC 3* diabetic group treated with

SC-514 1 and 3 mg/kg, respectively. Results are expressed as mean± SEM; [##]$p<0.01$ and [###]$p<0.001$ vs. ND; *$p<0.05$ vs. STZ-D ($n=6$)

nerve conducting system of rats. Nerve function studies with SC-514 demonstrated that in SC-514 (3 mg/kg) treated diabetic group, the reduction in NBF and slowing of MNCV was reversed as compared to untreated diabetic rats ($p<0.05$). The lower dose of 1 mg/kg, however, did not produce significant improvement in these parameters (Fig. 1).

Nociceptive Parameters

After 8 weeks of diabetes, rats developed significant thermal ($p<0.01$) and mechanical hyperalgesia ($p<0.001$) compared with the control group. Both thermal and mechanical hyperalgesia in the hind paw was significantly attenuated with SC-514 treatment at the dose level 3 mg/kg ($p<0.05$) (Fig. 2).

TNF-α and IL-6 Levels

As TNF-α and IL-6 play central role in the inflammatory response in immediate, acute, and chronic inflammation, their levels were measured in this study by sandwich ELISA. Nerve homogenate from diabetic animals contained significantly

higher amount of TNF-α (31.9±2.4 pg/mg of protein, $p<0.05$) and IL-6 (34.5±1.8 pg/mg of protein, $p<0.01$) compared to normoglycemic animals (TNF-α, 14.8±3.2 pg/mg of protein and IL-6, 18.7±1.1 pg/mf of protein). With SC-514 treatment, there was a significant reduction ($p<0.05$) in proinflammatory cytokines levels (TNF-α 18.1±2.5 and 17.4± 3.7 with 1 and 3 mg/kg respectively; IL-6 24.4±2.5 and 22.4± 2.6 with 1 and 3 mg/kg respectively) (Fig. 3).

NF-κB Expression

Increased expression of NF-κB in sciatic nerves of diabetic animals indicates an overwhelmed NF-κB pathway which is subsequently associated with inflammatory neuronal death. We therefore investigated the neuroprotective potential of NF-κB inhibition in diabetic neuropathy. Western blot analysis in sciatic nerve homogenate revealed an increased NF-κB expression ($p<0.001$) in untreated diabetic rats, while SC-514-treated diabetic group displayed far less NF-κB levels (D+SC 1 $p<0.01$ and D+SC 3 $p<0.05$) (Fig. 4a).

Immunohistochemical analysis of sciatic nerve sections supports the immunoblotting studies as NF-κB-positive cells
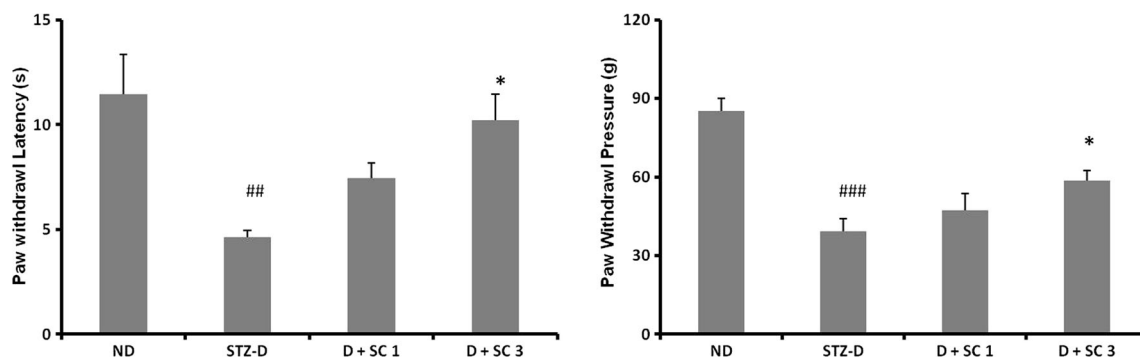

Fig. 2 Effect of SC-514 on thermal and mechanical hyperalgesia in diabetic rats. *ND* nondiabetic, *STZ-D* diabetic, *D+SC 1* and *D+SC 3* diabetic group treated with SC-514 1 and 3 mg/kg, respectively. Results

are expressed as mean±SEM; [##]$p<0.01$ and [###]$p<0.001$ vs. ND; *$p<0.05$ vs. STZ-D ($n=6$)
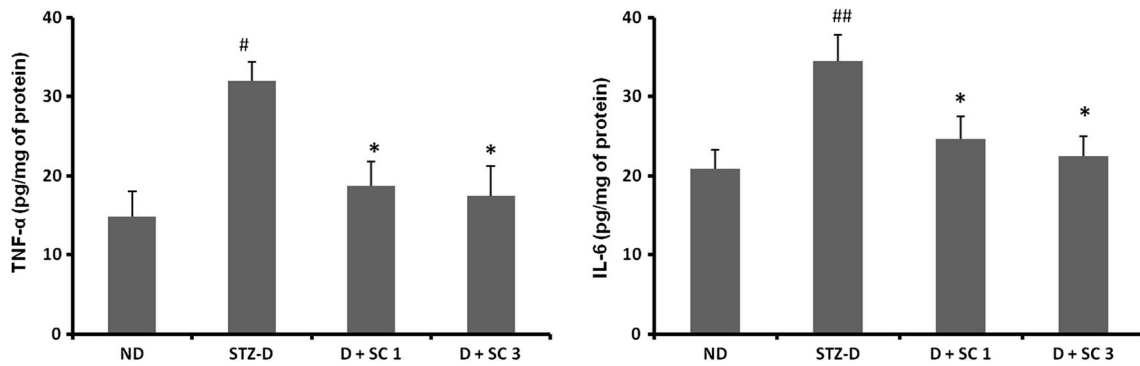
Fig. 3 Effect of SC-514 on TNF-α and IL-6 levels in diabetic rats. *ND* nondiabetic, *STZ-D* diabetic, *D+ SC 1* and *D+ SC 3* diabetic group treated with SC-514 1 and 3 mg/kg, respectively. Results are expressed as mean±SEM; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. ND; $^{*}p<0.05$ vs. STZ-D ($n=3$)

were more pronounced in the sciatic nerve microsections of diabetic rats than in nondiabetic control group ($p<0.001$). Similar to the Western blot results, 2-week treatment with SC-514 was able to reduce the percentage of NF-κB-positive cells ($p<0.05$) (Fig. 4b).

IKK Phosphorylation

Next, we analyzed the phosphorylation of IKKβ, which was increased significantly ($p<0.01$) in diabetic group indicating that it is activated to a greater extent in diabetes. Analogous to
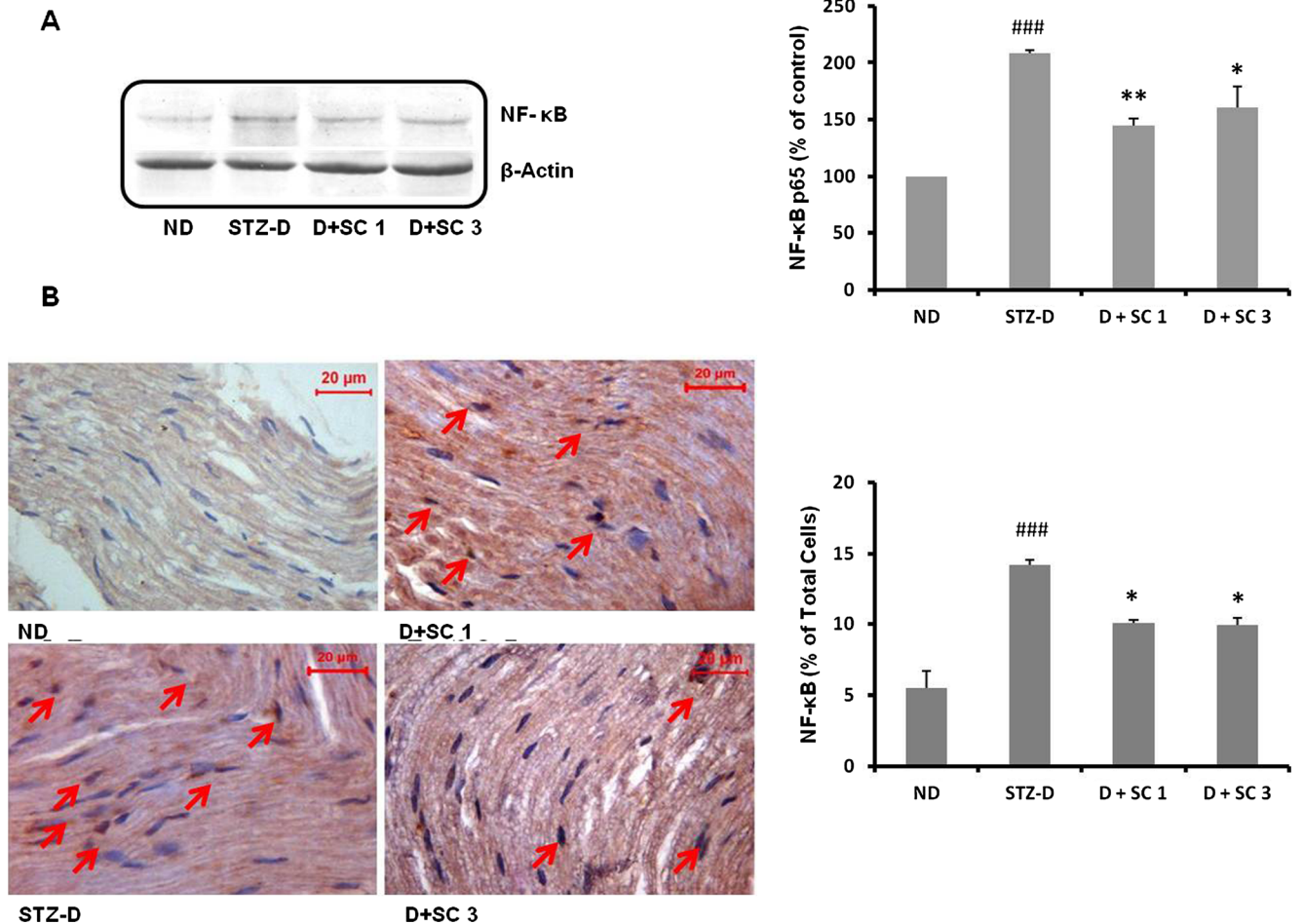

Fig. 4 Effect of SC-514 on NF-κB in diabetic rats measured by Western blotting (**a**). Equal loading was confirmed by β-actin. Change in the expression of NF-κB as observed by immunohistochemistry (**b**). *Arrows* indicate NF-κB-positive staining corresponding to the magnification images ×40. *Micron bar* shows a length of 20 μm. *ND* nondiabetic, *STZ-D* diabetic, *D+ SC 1* and *D+ SC 3* diabetic group treated with SC-514 1 and 3 mg/kg, respectively. Results are mean±SEM of three independent experiments. $^{\#\#\#}p<0.001$ vs. ND; $^{*}p<0.05$ and $^{**}p<0.01$ vs. STZ-D
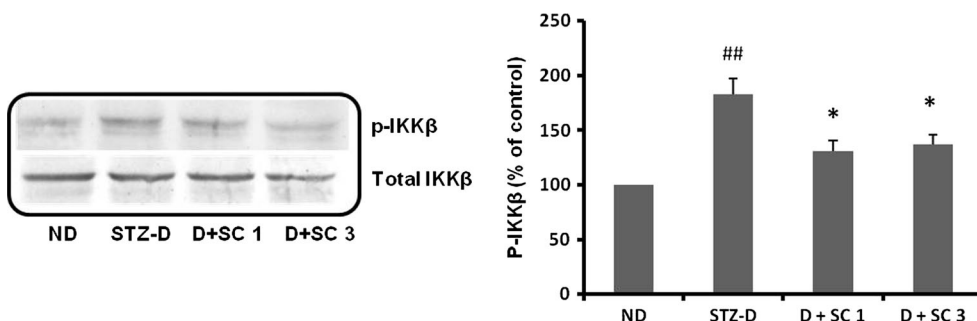
Fig. 5 Effect of SC-514 on IKK phosphorylation in diabetic rats measured by Western blotting. Equal loading was confirmed by β-actin. *ND* nondiabetic, *STZ-D* diabetic, *D+ SC 1* and *D+ SC 3* diabetic group treated with SC-514 1 and 3 mg/kg, respectively. Results are mean±SEM of three independent experiments. $^{##}p<0.01$ vs. ND; $^{*}p<0.05$ vs. STZ-D

NF-κB, IKKβ phosphorylation was inhibited by SC-514 in treated groups ($p<0.05$) (Fig. 5).

iNOS and COX-2 Expression

iNOS is NF-κB target gene that regulates inflammation and plays a role in neurodegeneration by generating a highly reactive species peroxynitrite. COX-2, another NF-κB target, has been shown to be a key enzyme in nerve injury in diabetes. Thus, to assess the effect of NF-κB inhibition on diabetes-associated inflammatory stress, we examined two enzyme markers of inflammation in the sciatic nerve. Diabetes resulted in a significant increase ($p<0.001$) in the expression of iNOS and COX-2. Treating diabetic rats with SC-514 significantly prevented the diabetes-induced increase in iNOS and COX-2 ($p<0.01$) levels in comparison with untreated diabetic rats (Fig. 6).

Discussion

In the recent years, experimental, epidemiological, and clinical data have suggested that inflammation is a determinant factor in the initiation and progression of diabetic neuropathy. There is a mounting consensus that hyperglycemia-induced NF-κB activation and subsequent events initiate as well as propagate the inflammatory changes in diabetes [18]. In the present study, we sought to delineate the role of IKK (a critical component of NF-κB activation cascade) in diabetic neuropathy, which can form novel target for drugs directed against inflammatory stress in various diabetic complications. IKK inhibitor, SC-514, improved nerve conduction and perfusion and normalized the altered sensory perception, thermal and mechanical hyperalgesia.

As discussed in previous sections, dissociation of inhibitory protein IκB from NF-κB is a prerequisite for its activation

Fig. 6 Effect of SC-514 on iNOS and COX-2 in diabetic rats measured by Western blotting. Equal loading was confirmed by β-actin. *ND* nondiabetic, *STZ-D* diabetic, *D+ SC 1* and *D+ SC 3* diabetic group treated with SC-514 1 and 3 mg/kg, respectively. Results are mean±SEM of three independent experiments. $^{###}p<0.001$ vs. ND; $^{**}p<0.01$ vs. STZ-D
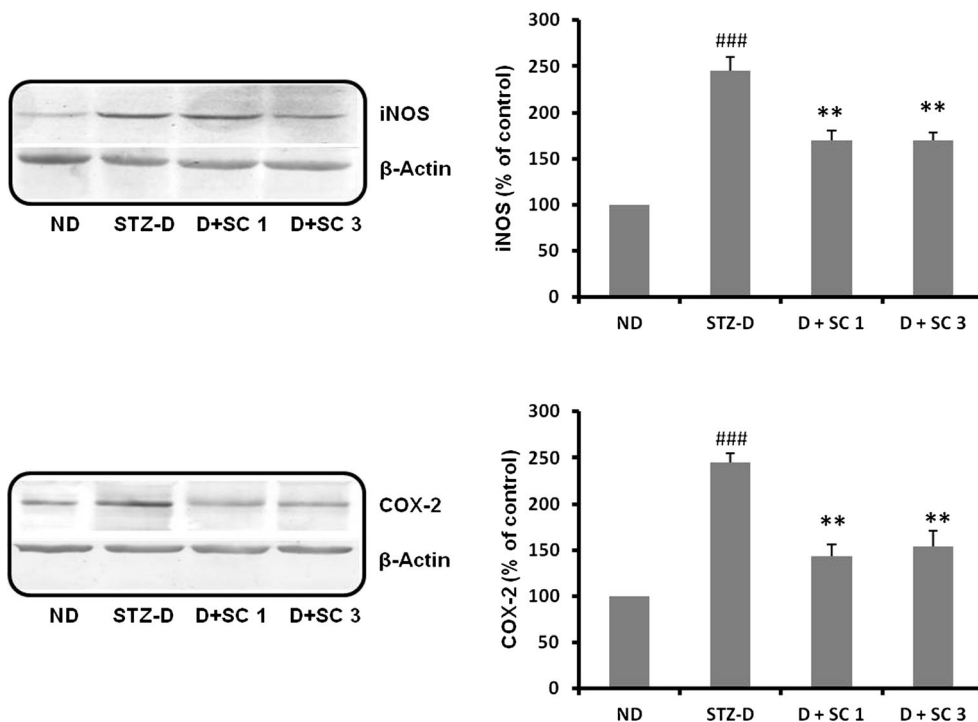
and its nuclear translocation. A number of detailed studies have shown that meeting point for the majority of NF-κB activating stimuli is multisubunit IKK which carries out the phosphorylation of IκB [19, 20]. SC-514 reduced the expression of NF-κB in our study in the sciatic nerve of treated animals. This concurs with other reports on various other disease models. Selective inhibition of IKK by SC-514 abrogates the phosphorylation and activation of p65 subunit of NF-κB [21, 22]. It not only inhibits the degradation of IκB but also promotes the export of nuclear NF-κB back to cytosol [23]. Thus, SC-514 not only reduces the p65 translocation into the nucleus but also serves to reduce the transcriptional effects of the NF-κB which has already reached the nucleus.

IKK is one of the first kinases activated in NF-κB cascade following exposure to activating stimuli. In vitro kinase assays in $Ikk\beta^{+/-c}$ells showed that IKK activity is rate limiting in NF-κB activation as marginal diminution of kinase activity resulted in significant decline in IκBα degradation and NF-κB activation [24]. IKK activation depends on its phosphorylation which brings about a conformational change [2]. In our study, we found that SC-514 inhibited the IKK phosphorylation in the treated group thereby preventing it from further phosphorylating IκBα.

Recently, considerable progress has been made in dissecting out the potential role of NF-κB in various nerve pathophysiologies. Particular stress has been placed on the involvement of NF-κB in neurodegeneration in the central nervous system. Brain tissues from patients and in vitro studies with cultured neurons have revealed increased NF-κB activity in cells undergoing neurodegeneration [25-27]. Although much less information is available on its role in diabetic insult to peripheral nervous system, NF-κB has been extensively studied for its role in nociceptive alterations. NF-κB activation is one of the earliest events occurring in sciatic nerve injury model and is critical for hyperalgesia. We found that hyperalgesia develops 8 weeks after induction of experimental diabetes in rats. This coincides with the reduction of nerve blood flow and motor nerve conduction velocity. NF-κB inhibition with SC-514 suppressed thermal and mechanical hyperalgesia and also improved the nerve function by increasing nerve perfusion. In other studies as well SC-514 has been shown to modulate pain perception via NF-κB inhibition. In ceramide-induced inflammatory pain in rats, SC-514 blocked the development of thermal hyperalgesia in a dose-dependent manner [28]. A recent study concluded that IKK is involved in the development of inflammatory hyperalgesia as IKK knockout mice exhibited a decreased pain [29]. Proinflammatory mediators TNF-α and IL-6 which are regulated by NF-κB have been strongly correlated to the peripheral nerve injury and pain [30]. Thus, we quantified the levels of proinflammatory mediators. SC-514 treatment diminished the increase in the levels of IL-6 and TNF-α in the treated group thus

decreasing altered nociceptive perception and improvement in nerve perfusion and conduction.

Many enzymes upregulated during inflammatory response such as iNOS and COX-2 are known transcriptional targets of NF-κB. Evidence that NF-κB regulates the transcription of various genes including COX-2 and iNOS is largely derived from indirect immunohistochemical and neuroanatomical studies [31]. However, in vitro conditions give a clear picture because two putative NF-κB motifs have been found on promoter region of both, *COX-2* and *iNOS* gene [32, 33]. iNOS and COX-2 both have been known to be major perpetrator of nerve injury in diabetes. Studies with diabetic rats showed that inhibition of iNOS/COX-2 by administration of the selective iNOS/COX-2 inhibitors exert anti-nociceptive effect [34-36]. Western blot analysis showed that SC-514 reduced the expression of iNOS and COX-2.

In conclusion, we have demonstrated that SC-514 significantly alleviated hyperalgesia and improved nerve functions in diabetic rats, highlighting the significance of IKK as a pharmacological target which may have therapeutic benefits in patients with painful diabetic neuropathy.

Conflict of Interest   All the authors have no competing interests.

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