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NF- κ B subunits are differentially distributed in cells of lumbar dorsal root ganglia in naïve and diabetic rats

L.N. Berti-Mattera², B. Larkin¹, Z. Hourmouzis¹, T.S Kern³, and R.E. Siegel¹

¹Department of Pharmacology, Case Western Reserve University 10900 Euclid Avenue
Cleveland, OH 44106

²Department of Pathology, Case Western Reserve University 10900 Euclid Avenue Cleveland,
OH 44106

³Department of Medicine Case Western Reserve University 10900 Euclid Avenue Cleveland, OH
44106

Abstract

Previous studies demonstrated that Nuclear Factor κ B (NF- κ B) activation is decreased in dorsal root ganglia (DRG) of rats having streptozotocin (STZ) - induced diabetes. DRG contain cell bodies of neurons that convey sensory signals from the periphery. To determine the relationship between diabetes-induced neuropathy and NF- κ B expression in DRG, behavioral, immunohistochemical, and biochemical studies were performed on naïve and 3-month diabetic rats. Behavioral studies confirmed that many diabetic rats develop tactile allodynia, or increased sensitivity to light touch, in the hind paws. Immunohistochemical studies on lumbar DRG that receive input from the affected regions revealed that p50 and p65, frequent NF- κ B subunit partners, are differentially localized. Intense p65 immunostaining was detected in the cytoplasm of small- and medium-sized neurons as well as in satellite cells. In contrast, p50 was localized in the cytoplasm of virtually all neurons. In many cases, prominent staining was also present in nuclei, a location consistent with transcription factor activation. Immunohistochemical and biochemical studies found that the nuclear to cytoplasmic ratio of p50 expression was significantly reduced in diabetic rats compared to that in naïve animals. Our findings raise the possibility that changes in NF- κ B activation in a subset of DRG neurons participates in mediating diabetes-induced sensory neuropathy.

Keywords

diabetes; neuropathy; DRG; sensory neurons; NF κ B; NF κ B subunit distribution

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Corresponding author: Ruth Siegel, Ph.D. Department of Pharmacology Case Western Reserve University 10900 Euclid Avenue Cleveland, OH 44106-4965 Tel: (216) 368-5554; FAX: (216) 368-1300 ruth.siegel@case.edu.

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NF- κ B is a family of transcription factors that plays multiple roles in diverse cell types [20]. In the nervous system, NF- κ B subunits are expressed in both neurons and non-neuronal cells [13, 17, 18, 21]. When inactive, the subunits reside in a latent form in the cytoplasm in a complex with I κ B (inhibitor of κ B). When activated, the subunits become dissociated from the inhibitor, are translocated to the nucleus, and bind to DNA to regulate target gene transcription. The most widely expressed NF- κ B complex in nuclei of cells in the CNS is a heterodimer composed of the p50 and p65 subunits. The p50/p65 complex has bifunctional effects; it participates in pathways that lead to cell survival or cell death. For example, NF- κ B acts in regulating neurodevelopment as well as memory formation. It is also neuroprotective and participates in pathways promoting myelination. In contrast, NF- κ B activation can participate in regulating the response to inflammation, nerve injury-induced apoptosis, and neurodegeneration [7, 23, 24, 30].

It has been hypothesized that changes in NF- κ B activation play a role in regulating sensory neuron function and survival in diabetes [7, 9]. In this disorder the regulation of blood glucose levels is abnormal, a change that may arise in part from disruption of immune signaling and prolonged inflammation. Uncontrolled diabetes leads to many complications including sensory neuropathy [27]. Although as many as 60% of all diabetics experience neuropathic pain, the molecular mechanisms leading to this complication are not well understood [32]. Sensory neurons and satellite cells in dorsal root ganglia (DRG) transmit sensory information (including pain) from the periphery to the CNS. Previous studies found that NF- κ B subunits are expressed in DRG [4, 22]. The fact that NF- κ B subunit protein levels and factor activation in DRG are reduced in diabetic rats supports a role for transcription factor-regulated signaling in mediating diabetic complications. In addition, treatment of diabetic rats with sulfasalazine (SFZ), an anti-inflammatory reagent that works partly by inhibiting the activation of NF- κ B [29], blocks the development of some characteristics of diabetic neuropathy. Notably, it inhibits the onset of tactile allodynia, defined as increased sensitivity to light touch, in the hind paws of these animals [4]. However, the identity of the cells expressing the transcription factor subunits and where changes in factor activation occur remains unknown.

To learn more about NF- κ B signaling in sensory ganglia we used immunohistochemical, biochemical, and behavioral approaches to examine the relationship between transcription factor expression and neuropathy in naïve and diabetic (streptozotocin-induced; STZ) rats. Surprisingly, our studies revealed that the NF- κ B p50 and p65 subunits in lumbar DRG are differentially distributed in neurons and satellite cells of both naïve and diabetic animals. Our findings highlight the importance of examining NF- κ B subunit expression in specific cell populations when assessing transcription factor function.

Materials and Methods

Diabetes was induced [31] in Lewis rats weighing ~200 g rats by a single intraperitoneal injection of streptozotocin (STZ, 60 mg/kg body weight, a procedure approved by the Animal Care and Use Committee of Case Western Reserve University). Body weight, blood glucose, and glycated hemoglobin (GHb) were monitored throughout the experimental time course. Diabetic rats were treated with insulin (0-3 units daily) as needed to maintain body

weight while maintaining chronic hyperglycemia. Animals exhibiting signs of poor health or blood glucose levels lower than 250 mg/dl were not included in the studies.

Sensitivity of the rats to tactile stimuli was assessed as previously described [3, 4] using a series of 6 Von Frey filaments (Stoelting, Chicago, IL) with logarithmically increasing stiffness (1.4-15 g). The statistical significance of differences in sensitivity among the experimental groups was analyzed by the Kruskal-Wallis non-parametric test. Where significance was observed, multiple comparisons between groups were analyzed by the Dunn's test.

For Western blotting, the animals were killed in accordance with Institutional guidelines. L4-L6 DRG from each animal were pooled and homogenized in 9 volumes of 2% SDS in 50 mM Tris/HCl, pH 8.0. Fractions enriched for the nucleus and cytoplasm were prepared using a kit (Panomics, Redwood City, CA) and processed for Western blotting as previously described [4]. Immunoreactivity was quantified by densitometry and normalized to the ERK2 signal of each sample.

For immunohistochemical studies, isolated L4 and L5 DRG were fixed by immersion in 4% formaldehyde in phosphate-buffered saline (PBS) for 2 h. The tissues were rinsed in PBS, incubated in 30% sucrose overnight at 4°C, and stored at -80°C until use. Cryostat sections (8 µm) were cut through the entire DRG and mounted on slides. The sections were processed for immunohistochemistry essentially as previously described [11]. The slides were incubated in buffer containing antibodies against p50 (1:100; Santa Cruz H-119, a rabbit polyclonal, or C-19, a goat polyclonal), p65 (1:100; Santa Cruz C-20; 1:50, Cell Signaling 3033), c-Rel (1:50; Santa Cruz C), calcitonin gene-related peptide (CGRP; 1:2000, Sigma C8198), or the 200 kDa neurofilament (NF200; 1:1000, Sigma N4142) for 1 h at room temperature. After rinsing in PBS, the sections were incubated with species-specific Alexa Fluor-conjugated secondary antibodies (1:1000; Invitrogen) for 1 h at room temperature. In double-label experiments, tissues were incubated sequentially with the different antibodies. Some sections were also incubated with isolectin B4 (1:100 Alexa Fluor 488-conjugated; Invitrogen). All stained sections were then mounted in DAPI-containing mounting medium and observed with a light (Nikon FX) or confocal (Zeiss 510) microscope. Staining specificity was established by omission of either the primary or secondary antibody, the use of multiple antibodies (p50 and p65), or preincubation of the antibody with antigen.

Staining intensity and identification of immunopositive cells was evaluated in at least 3 randomly chosen sections from 3 different animals. To determine the number of cells positive for a particular marker, at least 200 cells/animal were evaluated. To quantify intensity, images collected by confocal microscopy were exported to Photoshop in gray-scale format. No adjustments of contrast or brightness were made. Staining intensity in the nucleus and cytoplasm of cells with positive nuclei were measured using ImageJ software.

Statistical analysis: All values are expressed as means $\pm$ SEM. Significant difference between means was analyzed by ANOVA. Significance was defined as $p < 0.05$.

Results

Immunohistochemical studies demonstrated that p65 and p50, the most frequent NF- κ B subunit partners, are differentially expressed in neurons of lumbar DRG. As previously observed in naïve animals [15], p65 was distributed throughout the cytoplasm of many large, medium and small cells. The staining was strongest in ~30% of the small- and medium-sized neurons (Fig. 1A). In addition to its expression in the cytoplasm, intense p65 staining was observed at the perimeter of some neuronal nuclei.

In contrast to p65, strong p50 subunit staining was found in the cytoplasm of virtually all neurons of naïve rats (Fig. 1B). Furthermore, approximately 60% of the medium/small p50-positive neurons were also positive for p65. Most striking, however, was that at least 50% of the p50-positive neurons exhibited prominent nuclear staining, a location consistent with NF- κ B activation. A similar pattern of p50 distribution was seen using two different antisera. In addition, preincubation of the p50 and p65 antibodies with their corresponding antigen peptides completely abolished staining (not shown).

Distribution of the p65 and p50 subunits in satellite cells of naïve animals also differed. In these cells, p65 immunoreactivity was significantly more intense than that of p50. The p65 staining was strongest in the cytoplasm of the small satellite cells surrounding the faintly immunoreactive large neurons. The same pattern was found using an antibody against the phosphorylated, activated form of p65. In contrast to p65, the satellite cells displayed only weak staining for p50. These immunohistochemical findings show that the distribution of p50 and p65 only partially overlaps in both DRG neurons and satellite cells.

In addition to interacting with p65, p50 can heterodimerize with the NF- κ B c-Rel subunit. c-Rel distribution was thus examined immunohistochemically to gain a more complete understanding of NF- κ B subunit expression in lumbar DRG. These studies demonstrated that in contrast to p65 and p50, c-Rel staining was weak in naïve rats (not shown). In neurons, staining was found in the cytoplasm but was insignificant in nuclei. Very faint staining was also observed in satellite cells surrounding the neurons.

To identify the NF- κ B-positive DRG neurons, sections from naïve animals were co-stained with markers selective for different populations. Previous studies demonstrated that calcitonin gene-related peptide (CGRP) and isolectin B4 (IB4) are markers of Nerve Growth Factor (NGF) and Glial Cell Line-derived Neurotrophic Factor (GDNF)-sensitive small nociceptive neurons, respectively [12, 25]. In agreement with these reports, we observed that ~45% of small/ medium DRG neurons in naïve rats were positive for CGRP and IB4 (Fig. 2). In contrast, the largest neurons were rarely positive for these markers. Additional DRG cell populations were characterized by staining for the 200 kDa neurofilament (NF-200), a marker of cells with myelinated fibers. These studies found that ~45% of the p50-immunoreactive neurons also expressed NF-200 (not shown). Although the neurofilament staining was most prominent in large and medium-sized cells, immunoreactivity was also found in some smaller neurons and in fibers running through the ganglia.

Before evaluating the role of NF- κ B in diabetes-induced peripheral neuropathy, physiological parameters characteristic of experimental diabetes were examined. The

diabetic rats exhibited significantly lower body weights than their age-matched normal controls (naïve: 437 ± 17 g; diabetic: 277 ± 26 g; $n=6$). Moreover, blood glucose and GHb levels in the same animals were significantly elevated as compared to the naïve animals (GHb naïve: $2.9 \pm 0.2\%$; diabetic $11.5 \pm 0.9\%$; $n=6$).

STZ-treated animals also developed tactile allodynia, a characteristic symptom of sensory neuropathy, within 4-7 days after treatment [4]. This feature of sensory neuropathy was manifested by a decrease in the withdrawal threshold to stimulation with Von Frey filaments in the hind paws. In naïve animals, the 50% withdrawal threshold was 14.8 ± 0.8 g ($n=6$) while that of the diabetics was 3.1 ± 1.6 g ($n=6$). Having confirmed these diabetes-induced changes, immunohistochemical and biochemical studies were performed to examine whether alterations in NF- κ B signaling may underlie changes in sensory perception.

Since NF- κ B subunit translocation into the nucleus is a marker of its activation [16], immunohistochemical studies were performed to compare the nuclear/cytoplasmic p50 distribution in DRG from naïve and diabetic rats. These studies found differences in the pattern of p50 staining in the cytoplasm of neurons (Fig. 3). In naïve animals, the p50 subunit staining was distributed uniformly. In contrast, p50 staining in the cytoplasm of diabetic animals was occasionally clumped in patches, a condition that was most notable in large neurons (Fig. 3, C & D). Differences in nuclear p50 subunit staining were also observed. Whereas the staining appeared crisp and abundant in neurons of naïve rats, it was more diffuse and less intense in diabetic animals. These findings raise the possibility that the extent of NF- κ B activation is dependent on experimental condition. In contrast to the p50 subunit, however, changes in the distribution of p65 or c-Rel in either the cytoplasm or nucleus were not observed in the diabetic animals.

To quantitatively evaluate changes in p50 subunit localization, the nuclear/cytoplasmic ratio of p50 in naïve and diabetic rats was measured using histochemical and biochemical approaches. Analysis of p50 staining intensity revealed that this ratio was reduced in diabetic animals by approximately 20% (Fig. 3E). This change reflects an increase in the intensity of p50 staining in the cytoplasm and a decrease in the nucleus in diabetic compared to naïve rats, findings consistent with decreased neuronal NF- κ B activation in diabetics. Western blot analysis of the ratio of the p50 protein supported this finding (Fig. 3F). These biochemical studies found that the nuclear/cytoplasmic ratio decreased approximately 40% in diabetic compared to naïve animals. The apparent difference between the values obtained using histochemical and biochemical assays may reflect properties specific to the experimental approaches. Whereas the immunohistochemical studies assessed transcription distribution only in neurons, Western blots assessed p50 expression in all cells present in the DRG. This finding underscores the need to further identify the cells involved in the diabetes-induced changes.

Discussion and Conclusions

Our studies demonstrate that p50 and p65, two NF- κ B subunits that frequently heterodimerize to activate gene expression, are differentially distributed in neurons of lumbar DRG. Whereas p50 staining is strong in neurons of all sizes, p65 immunostaining is

intense in only a fraction of the small/medium neurons. These latter neurons are routinely identified by their immunoreactivity for IB4 and CGRP. In addition, other reports have demonstrated that small/medium neurons are involved in pain transmission, including mechanical hyperalgesia [1, 2, 28]. In agreement with these findings, we observed that 65 and 45% of small/medium neurons are IB4 and CGRP positive, respectively. Our results are thus consistent with the participation of some NF- κ B expressing neurons in the development of tactile allodynia.

The distribution of p50 and p65 in the expressing DRG neurons, however, is strikingly different. While p50 staining is strong in the nucleus and cytoplasm of more than half of these cells, p65 staining is intense only in the cytoplasm of a fraction of the small/medium-sized neuronal population. A similar difference in subunit distribution is observed in the large proprioceptive DRG neurons [2]. Whereas significant nuclear and cytoplasmic p50 staining is present in virtually all of these cells, p65 is weak or barely detectable in either location. We believe our findings are the first to report differences in the distribution of NF- κ B subunits in intact lumbar DRG, a difference sometimes found in neurons of other brain regions [14] and retinal ganglia [6].

Although a disparity between p50 and p65 subunit expression in the nucleus and cytoplasm of satellite cells is not detectable, the patterns of subunit staining differ in these cells. Whereas p50 subunit staining is weak, p65 subunit immunostaining is very prominent in the cytoplasm of cells surrounding the large neurons. Whether these subunits are expressed in the nucleus and the possibility that the nuclear/cytoplasmic ratio differs in naïve and diabetic animals could not be determined. It is tempting, however, to speculate that NF- κ B-activated responses in satellite cells are involved in pain modulation [8]. Indeed, several findings support the emerging role of satellite and Schwann cells in regulating responses to pain in the DRG [5, 26].

The fact that p50 is expressed in nuclei of neurons in DRG of naïve and diabetic rats raises the possibility that NF- κ B signaling is constitutively active. In contrast to p50 expression in many nuclei, however, the p65 subunit is virtually undetectable in this compartment. Other findings indicate that nuclear p50 alone is unlikely to reflect constitutive activation of the transcription factor. Although p50 can form homodimers that bind to DNA, these complexes do not directly stimulate gene transcription [10]. While the p50 subunit classically heterodimerizes with p65, it can also dimerize with c-Rel to stimulate gene transcription [19]. Recent studies have shown that p50/c-Rel heterodimers in cortical neurons promote neuronal survival, while p50/p65 might be involved in neuron apoptosis [23]. In the present study only weak c-Rel immunostaining was found that was uniformly distributed throughout the cytoplasm of cells of both naïve and diabetic animals. This finding precludes the contribution of this subunit in NF- κ B activation in normal and experimentally induced diabetic rats.

While we observed a significant decrease in the nuclear/cytoplasmic ratio of p50 in neurons from diabetic animals, there was a small increase (20%) in the proportion of small/medium neurons that display nuclear p50/p65 subunit co-staining in diabetic animals. These alterations suggest that changes in NF- κ B activation in some neurons may be partially

responsible for the manifestations of diabetic neuropathy. These immunohistochemical data concerning p65 distribution appears to differ from previous biochemical findings [4]. We believe this discrepancy reflects differences in experimental protocol. In our earlier studies, we examined subunit expression in nuclear-enriched fractions prepared from the entire DRG, containing neurons as well as satellite, Schwann, and endothelial cells. In the current studies we examined the relationship between subunit expression in the nucleus to that in the cytoplasm of identified neurons, an approach allowing us to follow subunit translocation in specific cells for changes in NF- κ B activation.

Regardless of p50's partner, the decline in its nuclear expression in some sensory neurons in diabetic animals suggests that NF- κ B-mediated signaling in lumbar DRG is altered in this condition. In addition, our results showing cell-specific NF- κ B subunit localization highlight the importance of complementing biochemical data obtained using lysates of DRG nuclei and cytosol with the immunohistochemical identification of NF- κ B subunit-positive cells. The present study also advances our behavioral findings to provide new insight into molecular mechanisms that underlie the pathophysiology of diabetic neuropathy.

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Research Highlights

- NFkB p50 and p65 subunits are differentially distributed in DRG neurons
- p50 is found in many neuronal nuclei; the nuclear/cytoplasmic ratio of p50 decreases in diabetes
- p65 is expressed mainly in the cytoplasm of small/medium-sized neurons
- p65 is also present in satellite cells surrounding large neurons
- p50 and p65 are found in medium/small DRG neurons expressing CGRP or IB4

Fig. 1.

Distribution of NF κ B subunits in DRG of naïve rats. Confocal images of a section co-stained for the p65 and p50 subunits shows that their distribution only partially overlaps. A, p65 staining is prominent in small neurons and satellite cells but is undetectable in most nuclei. B, p50 subunit staining is found in the cytoplasm of virtually all neurons and in some neuronal nuclei. Weaker immunostaining is observed in satellite cells. C, DAPI stained nuclei. Long arrow: small neurons; arrowhead: satellite cells; asterisk: nuclei. Scale bar = 20 μ m

Fig. 2. Colocalization of NF κ B subunits with a marker of sensory neurons in DRG of naïve rats. A, p65 stained section; B, IB4 distribution in the same section; C, DAPI stained merged image. D, p50 stained section; E, IB4 distribution in the same section, F, merged images. Scale bar = 20 μ m.

Fig. 3.

Distribution of p50 in naïve and diabetic rats. Low power confocal views of DRG sections of naïve (A) and diabetic (B) rats show similar patterns of subunit staining. Scale bar = 30 μ m. Higher power images show that p50 is more prominent in neuronal nuclei of naïve (C) than diabetic (D) rats. Clumps of subunit staining (arrow) were found only in diabetics. Scale bar in C and D = 15 μ m. E, Analysis of the ratio of p50 staining intensity in the nucleus and cytoplasm. Values represent the ratio in a representative experiment and are the mean $\pm$ SEM of 28 (naïve) or 27 cells (diabetic), $p = 0.02$. Similar data were obtained in four independent experiments. F, Biochemical analysis of p50 distribution. Top, Western blot of p50 levels in nuclear- and cytoplasm-enriched samples. ERK 2 was used as a loading control. Bottom. Quantitative analysis of p50 levels calculated from Western blots. Relative levels were quantified by assessing band density. Values are the mean $\pm$ SEM, $p = 0.01$ ($n=4$). Experiment was repeated 3 times.