Posttraumatic Activity of Signal Pathways of Nuclear Factor κB in Mature Sensory Neurons

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The aim of this study was to clear out whether injury to the peripheral nerve leads to activation of nuclear factor κB in mature spinal ganglia. Analysis of matrix RNA of nuclear factor κB-dependent genes (monocyte chemoattractant protein MCP-1 and inhibitor of nuclear factor κB IκBα) showed different levels of expression of these genes in the spinal ganglia *in vivo* after axotomy and *in vitro* after TNF-α stimulation. On the other hand, DNA-binding activity of nuclear factor κB increased in the spinal ganglia 6 h after axotomy and after 10-min incubation of sensory neuron culture with TNF-α. These data attest to possible involvement of nuclear factor κB in the posttraumatic regulation of gene transcription in spinal ganglion cells.

Key Words: *neuron; transcription nuclear factor κB; spinal ganglia*

Molecular and cellular changes in the spinal ganglia after peripheral nerve injury were described [1,8]. The development of inflammatory reaction around perikaryons of spinal ganglionic neurons can serve as the stimulatory factor in regeneration of the peripheral nerve [5]. Its molecular mechanisms are linked, among other things, with induction of cytokines IL-6 [7], TNF-α [9], *etc.* The nuclear transcription factor κB (NF-κB) is a classical messenger regulating cascades of reactions associated with various cytokines and cell death. NF-κB consists of two DNA-binding subunits p50 and p65. In inactive state, NF-κB forms a complex and is retained in the cytoplasm by the IκBα inhibitory protein (NF-κB inhibitor). Phosphorylation (after appropriate stimulation) by IkB-kinase and proteolysis yields active p50/p65 dimer that migrates into the nucleus and binds to a certain consensus DNA sequence in the regulatory sites of NF-κB-dependent genes. In the majority of cases, this promotes their activation and synthesis of a series of key proteins (including cytokines, growth factors, and metalloproteinases) involved in inflammation, immune response, proliferation, apoptosis, *etc.* [4].

We tried to clear out whether NF-κB was stimulated in mature spinal ganglia after injury to the peripheral nerve.

MATERIALS AND METHODS

The study was carried out on adult Wistar rats of both genders (n=30). The left sciatic nerve was crossed at the level of the hip middle in 20 animals narcotized with isofluorane under aseptic conditions. The right contralateral sciatic nerve was left intact. Intact (nonoperated) animals served as controls (n=10). Laminectomy was carried out in experimental rats 6, 12, 24, 48, 96, and 196 after the nerve injury and in intact animals under isoflurane narcosis and the spinal ganglia at the levels of L_{IV}-L_V on the left were isolated for subsequent extraction of RNA or nuclear protein fraction.

In order to prepare the primary culture of sensory neurons, the spinal ganglia were isolated under sterile conditions in adult rats at the L_{IV}-L_V level and plunged

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in Hanks saline (Sigma) on ice. The neurons were then dissociated and cultured as described previously [3]. In order to stimulate the neurons, they were cultured in plates in F-12 growth medium for 16 h, after which NF-α (30 ng/ml; Sigma) was added, the cells were incubated for 10, 30, 60, and 120 min more, and the neurons were collected for subsequent extraction of RNA or nuclear proteins.

Extraction of RNA was carried out according to the TRIzol protocol (Life Technologies). The RNA (1 μg) from each pool of spinal ganglia was subjected to reverse transcription in an amplifier (Biometra) using The Super Script™ III RNAse H Reverse Transcriptase (Life Technologies) according to the instruction at the following incubation program: 5 min at 25°C, 60 min at 50°C, and 15 min at 70°C.

Quantitative real-time PCR. Primer designs for real-time PCR were selected using the GeneBank gene sequences (rat-XM_234230 IκBα, rat monocyte chemoattractant protein-1 rat-NM_031530 MCP-1) using Primer 3 software in accordance with the options published by Corbett Research. Primer nucleotide sequences: IκBα: TGAGGAGAGCTATGACACGG (5') and TGGCCTCCAAACACACAGT (3') (Invitrogen) and Taqman samples CACGGAAGATGA GTTCCCTACGA (MWG-Biotech AG), MCP-1: CAC TCACCTGCTGCTACTC (5') and CTGCTGCTGGT-GATTCTCTT (3') (Invitrogen) and Taqman samples TC CCAATGAGTCGGCTGGAGAA (Applied Biosystems). The 5'-terminal of each sample was labeled with FAM and VIC fluorescent labels, respectively, while the 3'-terminals were labeled with TAMRA fluorescence quencher.

Quantitative real-time PCR was carried out simultaneously for all experimental samples and reference 18S rRNA gene loaded in three repeats under identical reaction conditions with identical components. In order to plot the control standard curve, 5 dilutions of splenic cDNA and matrix-free control with H_2O were included in each test. The matrix (2 μl) was added to the following mixture: 1.5 μl primer 1, 1.5 μl primer 2, 1 μl Taqman sample, 0, 25 μl primer 1 18S RNA, 0, 25 μl primer 2 18S RNA, 0, 25 μl Taqman sample 18S RNA; 25 μl Taqman Universal PCR Master Mix No AmpErase UNG (ABgene), and 16.25 μl nuclease-free H_2O. The samples were put into the amplifier (the Rotogene 3000 quantitative real-time PCR machine, Corbett Research) and processed according to the following protocol: 10 min at 95°C, 1 min at 60°C, 15 sec at 95°C, 45 cycles. After the experiment, the data were processed using the Rotogene software version 4.6 (Corbett Research). The concentrations of samples (copies/μl) were standartized by the level of 18S pRNA. The statistical significance of the data was evaluated using Student's *t* test. The resultant value was used to determine the content of the products of the studied genes in each sample.

Electrophoretic mobility shift assay of DNA fragments in gel. Electrophoretic mobility shift assay (EMSA) is used to evaluate DNA-binding capacity of proteins and to characterize their transcription activity. Isolation of nuclear extracts, preparation of labeled oligonucleotides, and analysis of protein binding to oligonucleotides labeled with radionuclides were carried out as described previously [10]. Cultured spinal ganglionar neurons ($2\text{-}4\times10^4$), whole spinal ganglia (n=6) from operated and control animals, and specimens of other tissues (rat spleen, adult rat brain, newborn rat brain) were analyzed. Protein concentration was measured by the BCA method (Pierce). DNA oligonucleotides (Invitrogen) containing NF-κB binding to mouse Ig κ-light chain site, were used as the sample (direct: 5-GGAGTTGAGGGGACTTTCCCAGGC-3, reverse: 5-CCTGGGAAAGTCCCCTCAACT-3). Oligonucleotides for AP-1 were 5-GGCGCTTGAT-GAGTCAGCCGGAA-3 (direct) and 5-TTCCGGCT-GACTCATCAAGCG-3 (reverse). Double-stranded consensus oligonucleotides were labeled (5'-terminal) with ^{32}P–[α-^{32}P]-dCTP (Amersham Pharmacia Biotech Ltd.) using Klenow enzyme (Roche Diagnostics Ltd.) according to manufacturer's instruction. Binding reaction was carried out as follows. Nuclear protein (1 μg) was mixed with 2 μl dilution buffer (20 mM HEPES pH 7.9, 60 mM KCl, 0.25 mM EDTA, 0.125 mM EGTA, 20% glycerol, and 1 mM DTT with protease inhibitors 10 μg/ml aprotinine, 10 μg/ml leupeptine, 20 μg/ml perstatine, 2 mM benzamidine, and 100 μM AEBSF) to a final volume of 7 μl. The mixture was incubated at 4°C for 20 min.

For competitive analysis, 25-fold excess of unlabeled cold double-stranded oligonucleotides was added to the corresponding reaction mixtures. The protein-oligonucleotide complexes were analyzed by electrophoresis in 6% PAAG (0.25× TBE buffer, 22.5 mM Tris-borate, 0.5 mM EDTA, pH 8.0) (Accugel 29:1 sequencing grade, National Diagnostics) in 0.25× TBE buffer. Dry gels were exposed with X-ray film (Hyperfilm, Amersham Pharmacia Biotech Ltd.) at -70° for 8-48 h.

RESULTS

Analysis of expression of NF-κB dependent genes in spinal ganglia *in vivo* and *in vitro*. Neurons of spinal ganglia expressed NF-κB-dependent genes (MCP-1 and IκBα) after axotomy *in vivo* and after stimulation with TNF-α *in vitro*. However, the expression of these genes differed in the ganglia after crossing of the sciatic nerve and in sensory neuron culture after stimulation with TNF-α. High expression of MCP-1 matrix RNA (mRNA) was observed 6 h after crossing of the

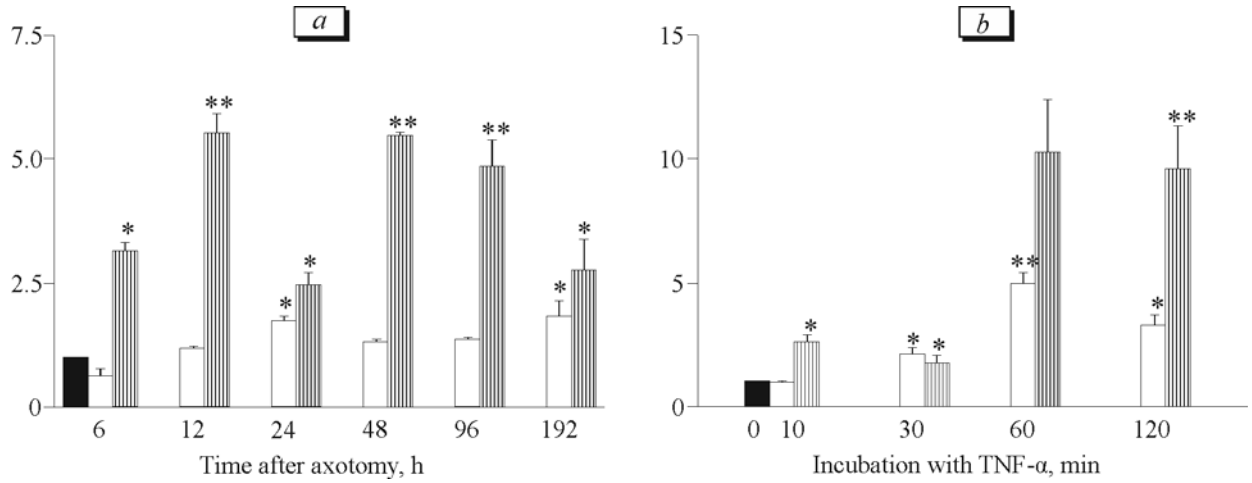

Fig. 1. Analysis of the expression of NF-κB-dependent genes IκBα (light bars) and MCP-1 (vertically hatched bars) in spinal ganglia. Different periods: *a*) after sciatic nerve crossing; *b*) after TNF-α (30 ng/ml) stimulation of sensory neuron culture for 10-120 min. Ordinate: changes in genes expression presented as the ratio of mRNA levels in spinal ganglia of damaged to contralateral side. **p*<0.05, ***p*<0.005 compared to the control (dark bars).

sciatic nerve (Fig. 1, *a*); it reached the maximum (5-fold surpassed the expression in the contralateral ganglia) 12 h after axotomy. The expression of IκBα mRNA in the ganglia increased only 2-fold 24 h after axotomy.

The synthesis and accumulation of MCP-1 mRNA after stimulation of spinal neuron culture with TNF-α were also more rapid and intense than the synthesis and accumulation of IκBα mRNA under the same conditions (Fig. 1, *b*). Incubation of sensory neurons with TNF-α for 1 h led to a 5-fold elevation of IκBα mRNA level and to a 10-fold increase of MCR-a mRNA level in comparison with unstimulated neurons.

Analysis of DNA-binding activity of NF-κB in mature sensory neurons of rats in response to

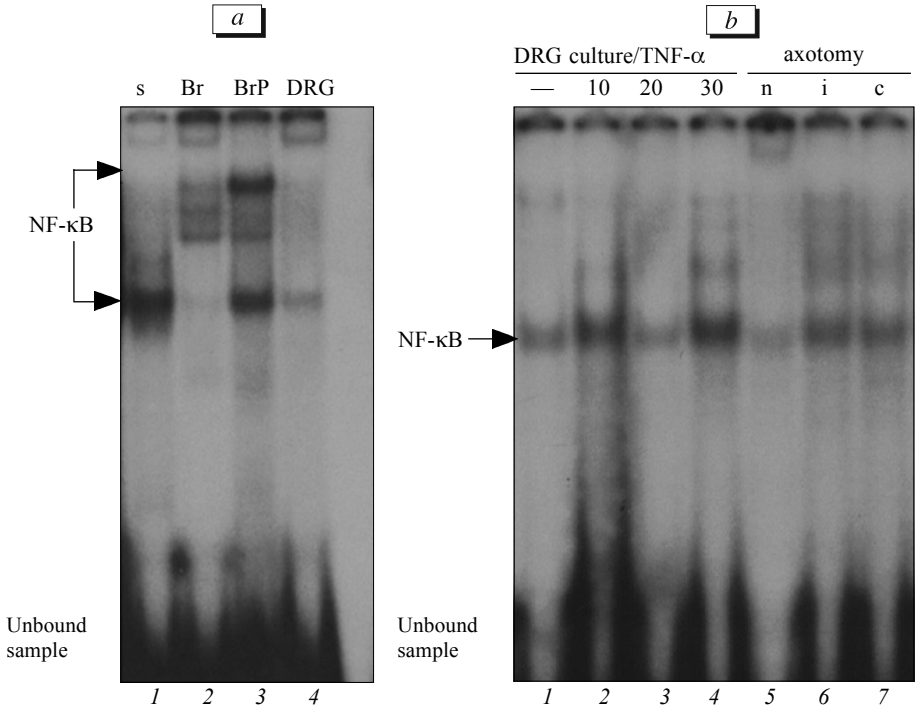

Fig. 2. EMSA of DNA-binding capacity of NF-κB. *a*) DNA-binding capacity of NF-κB in different rat tissues: *1*) splenic extract (S); *2*) brain cortex (Br); *3*) brain of 24-hour-old rats (BrP); *4*) intact rat spinal ganglia (DRG). *b*) DNA-binding capacity of NF-κB in culture of sensory neurons after incubation (during a certain period) with TNF-α (30 ng/ml; rows *1-4*) and 6 h after sciatic nerve crossing (rows *5-7*). n: nuclear extract from intact spinal ganglia; i: spinal ganglia on the side of injured nerve; c: spinal ganglia on the contralateral (intact) side. Here and in Fig. 3: position of NF-κB-DNA complex is shown by an arrow.

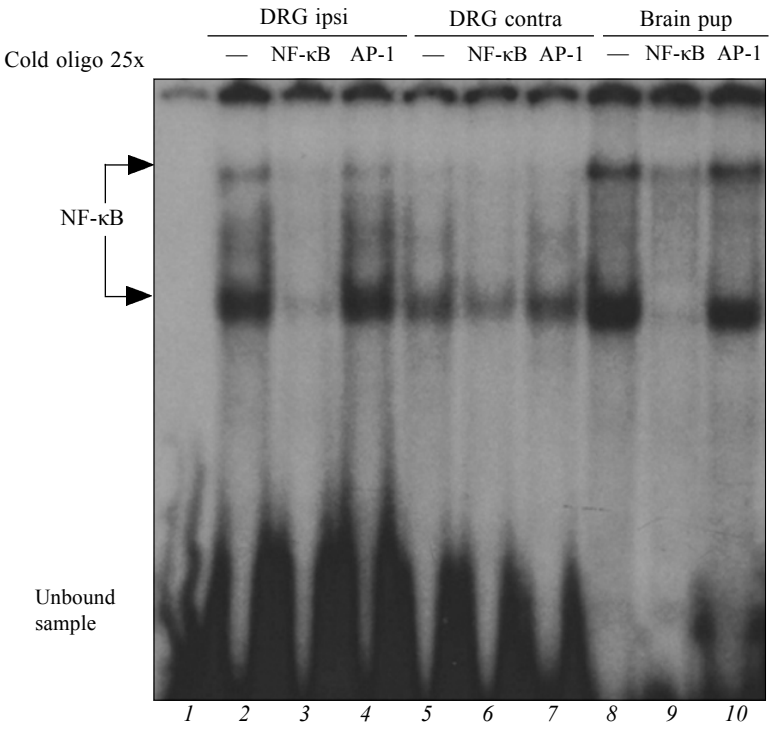

Fig. 3. Competitive analysis of NF-κB complexes in nuclear extracts isolated from rat spinal ganglia 6 h after axotomy and from the brain of newborn rats. Cold oligo 25×: 25-fold molar excess of unlabeled cold NF-κB or AP-1 oligonucleotides. Rows: *1* (probe): negative control without nuclear extract; *2-4* (DRG ipsi): nuclear extract of spinal ganglia on the side of damage; *5-7* (DRG contra): spinal ganglia on the contralateral (intact) side; *8-10* (brain pup): nuclear extract from newborn rat brain.

TNF-α stimulation *in vitro* and after peripheral nerve crossing *in vivo*. Activation of NF-κB in mature sensory neurons in response to TNF-α stimulation and after peripheral nerve crossing was studied in nuclear protein extract by the EMSA method on DNA fragments in gel. The location of the complex of radiolabeled DNA with the protein was visualized by autoradiography.

The EMSA of adult rat spleen and brain was carried out for control and identification of the NF-κB–DNA complexes. These organs are characterized by high constituitive NF-κB activity [6]. As was expected, EMSA of splenic nuclear extract showed a wide band of NF-κB–DNA complex (Fig. 2, *a*, row *1*). Higher NF-κB activity in the extract of brain cortex (Fig. 2, *a*, row *2*) compared to intact spinal ganglia extract (Fig. 2, *a*, row *4*) was detected. The EMSA profile for the brain of newborn rats differed from that of mature cortical tissue and constitutionally exhibited a potent band of the NF-κB–DNA complex (Fig. 2, *a*, row *3*). This NF-κB–DNA complex completely disappeared in a presence of 25-fold molar excess of unlabeled cold NF-κB–DNA oligonucleotides (Fig. 3, row *9*). Addition of oligonucleotides without AP-1 label under the same conditions had no effect on visualization of the NF-κB–DNA complex (Fig. 3, row *10*), which proves the formation of this complex by NF-κB–DNA oligonucleotides.

The next step of the study was EMSA evaluation of the impact of sciatic nerve crossing for the DNA-binding activity of NF-κB in neurons of the lumbar spinal ganglion 6 h after axotomy. Autoradiograms indicated sufficiently pronounced delay in gel of the NF-κB–DNA complex in the nuclear extracts from the ganglia in the crossed nerve and in the contralateral nerve (Fig. 2, *b*, rows *6*, *7*). A very pale band was visualized in the same position in extracts from intact animal ganglia (Fig. 2, *b*, row *5*).

The specificity of visualization of NF-κB–DNA complexes formed after axotomy (Fig. 3, rows *2*, *5*) was confirmed by displacement of oligonucleoides labeled with radionuclides and reduction of specific band of NF-κB–DNA complex delay in gel in the presence of 25-fold molar excess of unlabeled cold NF-κB oligonucleotides (Fig. 3, rows *3*, *6*). Addition of oligonucleotides without AP-1 label under the same conditions caused no effect of any kind on the presence of the NF-κB–DNA band (Fig. 3, rows *4*, *7*).

In order to evaluate the effect of TNF-α on DNA-binding capacity of NF-κB, sensory neurons of adult rats were cultured for 16 h and stimulated by adding TNF-α (30 ng/ml) to the nutrient media for 10, 20, and 30 min. EMSA of nuclear protein extract from sensory neurons showed a solitary band of delay in gel, similar to that observed in intact spinal ganglia *in vivo*, but

of higher intensity (Fig. 2, *b*, row *1*). Incubation with TNF-α significantly increased DNA-binding capacity of NF-κB as soon as after just 10 min. However, 20-min incubation with TNF-α led to a reduction of the band intensity, while 30-min incubation led to the maximum development of the NF-κB–DNA complex delay band in gel (Fig. 2, *b*, rows *2-4*).

Hence, the data indicate that NF-κB can be involved in posttraumatic regulation of gene transcription in cells of the spinal ganglions. The expression of NF-κB-dependent genes MCP-1 and IκBα increased in neurons of the spinal ganglion after axotomy *in vivo* and after TNF-α stimulation *in vitro*. The DNA-binding capacity of NF-κB also increased in neurons of the spinal ganglions *in vivo* after axotomy and *in vitro* after incubation with TNF-α.

After sciatic nerve injury, Schwann cells and macrophages migrate to the ganglionic zone and produce specific factors, including TNF-α. These factors are released from cells and bind to their receptors on neurons and glia, thus activating NF-κB in these cells. Hence, NF-κB transcription factor can serve as a messenger connecting the cytokine and neutrophine signal pathways for triggering, in turn, gene expression in sensory neurons. Presumably, this protects mature sensory neurons, as it is known that NF-κB prevents apoptosis caused by TNF-α in other cell types [2].

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