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Manganese Superoxide Dismutase Induced by TNF-α Is Regulated Transcriptionally by NF-κB after Spinal Cord Injury in Rats

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ABSTRACT

Antioxidant enzymes including superoxide dismutase (SOD) may play a role in the mechanism by which cells counteract the deleterious effects of reactive oxygen species (ROS) after spinal cord injury (SCI). Cu/Zn and MnSOD are especially potent scavengers of superoxide anion and likely serve important cytoprotective roles against cellular damage. We investigated expression of SOD after SCI to address its role during the early stages of injury. MnSOD activity was increased 4 h after SCI and persisted at elevated levels up to 24–48 h; by contrast, Cu/ZnSOD activity was not changed. RT-PCR and Western blot analyses showed increased levels of MnSOD mRNA and protein, respectively, by 4 h and reached maximum levels by 24–48 h. Double immunostaining revealed that MnSOD protein was localized within neurons and oligodendrocytes. Tumor necrosis factor–α (TNF-α) was administered locally into uninjured spinal cords to examine potential mechanisms for MnSOD induction after injury. TNF-α administered exogenously increased MnSOD expression in uninjured spinal cords. Western blot and immunostaining also revealed that a transcription factor, NF-κB, was activated and translocated into the nuclei of neurons and oligodendrocytes. By contrast, administration of neutralizing antibody against TNF-α into injured spinal cords attenuated the increase in MnSOD expression and activation of NF-κB. Double immunostaining revealed that MnSOD was co-localized with NF-κB in neurons and oligodendrocytes after SCI. These results suggest that TNF-α may be an inducer of NF-κB activation and MnSOD expression after SCI and that MnSOD expression induced by TNF-α is likely mediated through activation of NF-κB.

Key words: Cu/ZnSOD; MnSOD; NF-κB; spinal cord injury; TNF-α; TNF-α neutralizing antibody

INTRODUCTION

OXIDATIVE STRESS contributes to pathological processes after spinal cord injury (SCI) (Simonian and Coyle, 1996; Taoka et al., 1995). Accumulation of such free radicals as reactive oxygen species (ROS) and superoxide anion (O_2^-) (Swerdlow et al., 1996, 1997) may cause apoptotic cell death. Cells contain a variety of

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antioxidant enzymes like superoxide dismutase (SOD) which defend against free radical damage. SOD is a family of antioxidant enzymes converting superoxide radicals into H_2O_2; H_2O_2 then is catabolized to water and oxygen by catalase and glutathione peroxidase (Hassan, 1988). Two types of SOD are found in mammalian cells: mitochondrial manganese SOD (MnSOD) and cytoplasmic copper/zinc SOD (Cu/ZnSOD). Both have been implicated in neuronal recovery/degeneration in such neurodegenerative diseases as ALS and in such injury as axotomy (Ghadge et al., 1997; Rosenfeld et al., 1997).

Increased MnSOD has neuroprotective effects against oxidative stress or toxicity mediated by TNF-α (Manna et al., 1998; Siemankowski et al., 1999; Wong et al., 1989; Wong and Goeddel, 1988), IL-1β (Earnhardt et al., 2003; Hohmeier et al., 1998), or ischemia/reperfusion injury (Chen et al., 1998). Overexpression of Cu/ZnSOD is neuroprotective in transient focal ischemia-reperfusion and in traumatic brain injury (Chan, 1996; Kinouchi et al., 1991; Kondo et al., 1997; Saito et al., 2003). Overexpressing MnSOD in transgenic mice prevents neuronal apoptosis (Keller et al., 1998) reducing traumatic (Sullivan et al., 1999) and ischemic brain injury by suppressing production of peroxynitrite, lipid peroxides (Keller et al., 1998) and release of mitochondrial cytochrome c (Fujimura et al., 1999; Lewen et al., 2001). Overexpressing MnSOD exerts neuroprotective effects against MPTP (Klivenyi et al., 1998), NMDA and nitric oxide-mediated neurotoxicity (Gonzalez-Zulueta et al., 1998).

Inflammatory cytokines generate superoxide radicals and damage cells (Li et al., 1996; Skaleric et al., 2000). MnSOD is regulated transcriptionally and induced by a variety of cytokines including TNF-α, IL-1, IL-6, and IFN-γ (Dougall and Nick, 1991; Harris et al., 1991; Karube-Harada et al., 2001; Nelson et al., 1995; Nogae et al., 1995; Visner et al., 1990). We recently reported that TNF-α increased after SCI and may function as an inducer for apoptosis in neurons and glia (Yune et al., 2003). Furthermore, TNF-α and IL-1 cause rapid activation and nuclear translocation of the transcription factor, NF-κB (Beg et al., 1993; Osborn et al., 1989; Siebenlist et al., 1994) whose activation is required for the induction of MnSOD (Darville et al., 2000; Delhalle et al., 2002; Jones et al., 1997; Maehara et al., 1999; Sugino et al., 2002). We postulated that expression of MnSOD induced by TNF-α after SCI might be mediated through activation of NF-κB.

We examined the modulation of SOD after SCI. The roles of TNF-α and NF-κB in upstream induction of MnSOD were evaluated by local administration of either TNF-α or neutralizing antibodies against TNF-α into uninjured or injured spinal cord, respectively. We show that MnSOD expression was modulated at the transcriptional and posttranscriptional levels, whereas Cu/ZnSOD was not. Furthermore, induction of MnSOD by TNF-α after SCI may be mediated through activation of the transcription factor, NF-κB.

MATERIALS AND METHODS

Materials

A monoclonal antibody to NF-κB was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A neutralizing antibody against TNF-α and TNF-α were purchased from R & D (Minneapolis, MN). Polyclonal antibody against MnSOD was a kind gift from Dr. Kumagai, University of Tsukuba, Japan. Antibody to Cu/ZnSOD and SOD enzyme activity assay kits were purchased from Calbiochem (San Diego, CA). Trizol Reagent and MMLV reverse transcriptase were purchased from GibcoBRL (Gaithersburg, MD).

Spinal Cord Injury (SCI)

Traumatic injury was induced using the weight drop device developed at New York University (Gruner, 1992) using the injury protocol developed by a multicenter consortium (Multicenter Animal Spinal Cord Injury Study; Basso et al., 1995, 1996a,b) as reported previously (Lee et al., 2003; Liu et al., 1997; Xu et al., 1998). Briefly, adult rats [Sprague-Dawley; male; 250–300 g; Sam:TacN (SD) BR, Korea] were anesthetized with chloral hydrate (500 mg/kg, i.p.), and a laminectomy was performed at the T9-T10 level exposing the cord beneath without disrupting the dura. The spinous processes of T8 and T11 were then clamped to stabilize the spine, and the exposed dorsal surface of the cord was subjected to a weight drop impact using a 10-g rod (2.5 mm in diameter) dropped at a height of 12.5 mm. During surgery, temperature was maintained at 37°C by a heating regulated thermostatically pad governed by a rectal probe. After injury, the muscles and skin were closed in layers, and the rats were placed in a temperature- and humidity-controlled chamber overnight. Manual expression of the urinary bladder was performed three times per day until reflex bladder emptying was established. For the sham-operated controls, the animals underwent a T10 laminectomy without weight-drop injury. All surgical interventions and postoperative animal care were performed in accordance with the Guidelines and Polices for Rodent Survival Surgery provided by the Animal Care Committee of the Korea Institute of Science and Technology (KIST).

Intraspinal Microinjection of TNF-α or Neutralizing Antibody Against TNF-α

Intraspinal injections were performed as previously described (Lee et al., 2000; Yune et al., 2003). In brief,

TNF-α or TNF-α neutralizing antibody were injected into uninjured spinal cord or the epicenter of the injury lesion immediately after SCI, respectively. Rats were randomly assigned to one of three experimental groups according to which reagents were to be injected into the spinal cords. The groups were as follows: vehicle controls (2 or 4 μL of normal saline or normal serum; $n = 10$ for each group); 4 μL of TNF-α (10 ng/μL, $n = 15$); 2 or 4 μL of neutralizing antibody against TNF-α (10 ng/μL, $n = 15$ for each group).

Tissue Preparation

At specific time points after SCI, animals were anesthetized with chloral hydrate and perfused via cardiac puncture initially with 0.1 M PBS, pH 7.4 and subsequently with 4% paraformaldehyde in 0.1 M PBS, pH 7.4. A 20-mm section of the spinal cord, centered at the lesion site, was dissected out and post-fixed by immersion in 4% paraformaldehyde overnight. The segment was embedded in OCT for frozen sections as described previously (Du et al., 1999; Lee et al., 2000, 2003; Yune et al., 2003). Longitudinal frozen tissue sections were then cut at 10 μm.

Immunostaining

Frozen sections were processed for immunohistochemistry with polyclonal antibody against MnSOD (1:1,000 dilution) and a monoclonal antibody against NF-κB (Santa Cruz Biotechnology, 1:200 dilution). After three washes in PBS for 5 min, sections were incubated in 0.5% hydrogen peroxide in PBS for 30 min to inhibit endogenous peroxidase activity. The sections were blocked in 5% normal serum and 0.1% Triton X-100 in PBS for 1 h at RT and then incubated with primary antibodies overnight at 4°C, followed by HRP-conjugated secondary antibodies (Dako, Carpinteria, CA). The ABC method was used to detect cells labeled specifically using a Vectastain kit (Vector Laboratories, Burlingame, CA). DAB served as the substrate for peroxidase. Some sections stained for MnSOD were double-labeled using antibodies to either neuron specific protein (NeuN; 1:200 dilution; Chemicon, Temecula, CA), a monoclonal antibody to the oligodendrocyte-specific antigen, APC (1:200 dilution; Oncogene, Cambridge, MA), an antibody specific for microglia, OX-42 (1:200 dilution; Chemicon) or a monoclonal antibody against NF-κB (Santa Cruz Biotechnology). For double labeling, FITC or TRITC-conjugated secondary antibodies (Dako) were used. Also, nuclei were labeled with DAPI according to the protocol of the manufacturer (Molecular Probes, Eugene, OR). Images were collected using an Olympus microscope and SPOT™ (Diagnostic Instrument Inc). In all immunocytochemistry controls, reaction to the substrate was absent if the primary antibody was omitted or if the primary antibody was replaced by a non-immune, control antibody. Serial sections were also stained for histological analysis with Cresyl violet acetate.

Subcellular Fractionation and Western Blot

At appropriate times after treatment, segments of spinal cord (10 mm) were isolated using the lesion site as the epicenter. The experiments described were all performed at 4°C. The tissues were resuspended in a homogenizing buffer containing 10 mM PBS, pH 7.5, 250 mM sucrose, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL leupeptin and aprotinin and homogenized in a Dounce homogenizer. Tissue homogenate was centrifuged at 200 $\times$ *g* for 10 min at 4°C, and the supernatant was recentrifuged at 8,000 $\times$ *g* for 10 min at 4°C. The pellet was homogenized in a Dounce homogenizer and centrifuged at 200 $\times$ *g* for 10 min, and the supernatant was centrifuged at 8,000 $\times$ *g* for 10 min. The 200 $\times$ *g* pellets (nuclear fraction) and 8,000 $\times$ *g* pellets (mitochondrial fraction) were pooled and resuspended in PBS. The supernatant from 8,000 $\times$ *g* spins was centrifuged at 40,000 $\times$ *g* for 1 h at 4°C, and the pellet (plasma membrane fraction) was resuspended in PBS and briefly broken by sonication. The supernatant was saved and used as the cytosolic fraction. Protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL). For analyses of Western blot, 10–50 μg of protein was separated by 12.5% SDS-PAGE and transferred to polyvinylidene difluoride (Millipore, Bedford, MA) membranes by electrophoresis. The membranes were blocked with 5% nonfat skim milk in TBS for 1 h at room temperature and then incubated with antibodies against MnSOD (1:2,000), Cu/ZnSOD (Calbiochem, 1:500), and NF-κB (Santa Cruz Biotechnology, 1:200) overnight at 4°C. After washing three times with TBS-T (0.1% Tween 20), the membranes were processed with HRP-conjugated secondary antibody (Pierce) and immunoreactive bands were visualized by chemiluminescence using Supersignal (Pierce). Densitometry readings of bands were measured using a ChemiImager™4400 (Alpha Innotech Co, San Leandro, CA). β-actin (Sigma, 1:2,000; cytoplasmic fraction), cytochrome oxidase subunit IV (Molecular Probes, 1:500; mitochondrial fraction) and histone 3 (Cell Signaling, 1:500; nuclear fraction) were used as quantitative markers to confirm the purity of each fraction and to assess consistent protein loading in each lane. Experiments were repeated three times and the values obtained for the relative intensity were subjected to statistical analysis.

Enzyme Activity of MnSOD and Cu/ZnSOD

To determine the enzymatic activity of superoxide dismutase (SOD) after SCI, spinal cords were taken at predefined time intervals (sham, 0.5, 1, 4, 8, 24, and 48 h after injury; $n = 5$ for each time point) as described above. For SOD activity, the NBT-staining method was used as described by Beauchamp and Fridovich (1971). In brief, cytoplasmic (100 μg) and mitochondrial (10 μg) protein fractions obtained as described above were electrophoresed on native 12% PAGE, the gels were equilibrated with potassium-phosphate buffer (50 mM potassium-phosphate buffer, pH 7.8, 0.1 mM EDTA, pH 8.0) for 30 min at room temperature, and then stained with staining buffer solution (0.25 mM NBT, 0.33 mM riboflavin, 0.2% TEMED) for 30 min at room temperature (in the dark). After incubation, the gels were illuminated for 5–15 min under lighted conditions. During illumination, the gel appears to be stained blue/purple uniformly, except at the positions containing SOD. Illumination was stopped when maximum contrast between the achromatic zones and the blue/purple color had been achieved. The gels were then photographed. SOD activity was also analyzed by an SOD enzyme assay kit (Calbiochem) according to the manufacturer's instruction. The reaction was initiated by the addition of xanthine oxidase (19 mU) to a reaction mixture containing 80 μL of reaction buffer and 100 μL of cytoplasmic (100 μg) and mitochondrial (10 μg) protein. After incubation, the absorbance change at 560 nm was monitored at room temperature; the background reading of the blank was then subtracted from all values. SOD activity was expressed in terms of relative activity and experiments were repeated three times to ensure reproducibility.

Electrophoretic Mobility Gel-Shift Assay (EMSA) for NF-κB Activation

The spinal cord tissues were isolated and processed as described above. Three spinal cords per group were pooled, and nuclear extracts were prepared using a CelLytic nuclear extraction kit (Sigma) according to the manufacturer's instructions. After quantification of protein, the nuclear extracts were used in a mobility gel-shift assay similar to the methods described previously (Huang et al., 2001). In brief, oligonucleotide for NF-κB containing consensus κB-binding sequence (Santa Cruz Biotechnology) was radiolabeled with [^{32}P]-γATP (ICN, Costa Mesa, CA) by T4 polynucleotide kinase (New England Biolabs, Beverly, MA) to produce double-stranded DNA probes. Five micrograms of nuclear protein were added to 40 μL of binding buffer (1 mM $MgCl_2$, 4% glycerol, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 1 μg of poly (dI-dC) and 10 mM Tris-HCl, pH 7.5) containing ^{32}P-labeled double-stranded DNA (100,000 cpm) and incubated at room temperature for 1 h. After incubation, bound and free probes were separated by 6% non-denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. For competition experiments, radiolabeled DNA probes and nuclear proteins were incubated with a 100-fold molar excess of the unlabeled DNA oligonucleotide. Supershift assays were performed by preincubating nuclear extracts with polyclonal antibody (p65 subunits of NF-κB, Santa Cruz Biotechnology) for 1 h at 4°C before adding labeled probes.

RT-PCR Analysis

At 0.5, 1, 4, and 24 h after injury, rats ($n = 4$ for each time point) were anesthetized and perfused via cardiac puncture with 0.1 M PBS, pH 7.4. Total RNA was isolated using Trizol Reagent (GibcoBRL) according to the manufacturer's instructions. To ascertain that all RNA samples would be DNA-free, samples were treated with RNase-free DNase I (Sigma). After spectrophotometric quantification, the purified RNA was separated on a formaldehyde-agarose gel to check the extent of degradation. One microgram of total RNA was reverse-transcribed into first strand cDNA in each 20 μL of reaction mixture using MMLV reverse transcriptase (GibcoBRL) according to the manufacturer's instructions. A 20-μL PCR reaction contained 2 μL first strand cDNA, 0.6 U Amplitaq polymerase (Perkin-Elmer, Branchburg, NJ), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 250 μM dNTP, and 10 pmol of each specific primer. PCR samples were subjected to the following conditions: 25 cycles for β-actin (95°C; 1 min; 56°C; 1 min; and 72°C: 2 min), 25 cycles for Cu/ZnSOD (95°C: 1 min; 58°C: 1 min; and 72°C: 2 min) and 35 cycles for MnSOD (95°C: 1 min; 60°C: 1 min; and 72°C: 2 min) on a thermocycler (Perkin-Elmer). The primers used for this experiment were designed from sequences previously reported for MnSOD (Ho and Crapo, 1987; accession number 56690) and Cu/ZnSOD (Hass et al., 1989; accession number M25157). The β-actin primer used as an internal control, was designed according to the sequence previously reported (Nudel et al., 1983). The MnSOD primer sequences were 5′-GAGAAGTTTAAGGAGAAACTGACAG-3′ (forward) and 5′-GTCAGGTCTGACGTTTTTATACTGA-3′ (reverse). The Cu/ZnSOD primer sequences were as follows: forward primer was 5′-GGACCTCATTTTAATC-CTCACTCTA-3′ and reverse primer was 5′-CTTTG-TACTTTCTTCATTTCCACCT-3′. Primers for β-actin were as follow: forward primer was 5′-CTTTGATGT-CACGCACGATTTC-3′ and reverse primer was 5′-GGGCCGCTCTAGGCACCAA-3′. Negative controls in-

cluded PCR reactions lacking primers or without added reverse transcriptase. After amplification, RT-PCR products were separated on a 1.5–2% agarose gel containing 0.5 μg/mL ethidium bromide. The amplified cDNA fragments were visualized under ultraviolet light. Experiments were repeated three times for reproducibility.

Statistical Analysis

Data are presented as mean ± SD values. Data from Western analyses were evaluated for statistical significance using two-way ANOVA with a *post hoc* Tukey test. Differences were considered to be statistically significant at a level of $p < 0.05$.

RESULTS

Enzyme Activity of MnSOD after SCI

To determine the types of SOD modulated after SCI, we measured enzyme activity of SOD by both the NBT-staining method and the SOD enzyme assay kit using mitochondrial and cytoplasmic fractions (sham, 0.5, 1, 4, 8, 24, and 48 h after injury; $n = 5$ for each time point). Blue/purple gel bands representing SOD activity were observed using the NBT-staining method (Fig. 1A). MnSOD enzyme activity in the mitochondrial fraction was not observed in the sham control but began to appear at 4 h after injury and increased gradually up to 48 h after injury. By contrast, Cu/ZnSOD activity in the cytoplasmic fraction was observed in the sham control but was not changed after injury (Fig. 1A). Also, MnSOD activity determined by the SOD enzyme assay kit gradually increased after SCI, although Cu/ZnSOD activity was not changed up to 48 h. After inhibition of Cu/ZnSOD activity by addition of 3 mM KCN for 15 min in the assay buffer, total SOD activity was similar to SOD activity in the mitochondrial fraction. Also, SOD activity of the mitochondrial fraction was not inhibited by addition of 3 mM KCN. Therefore, we concluded that the fractionation procedure used in this experiment compartmentalized the two isoforms (data not shown).

Expression of MnSOD after SCI

We performed RT-PCR and Western blot analysis (sham, 0.5, 1, 4, and 24 h after injury; $n = 4$ for each time point) to examine the expression profiles of SOD after SCI. MnSOD mRNA was not detected in the sham control, was induced as early as 0.5 h after SCI, peaked at 4 h and persisted for up to 24 h (Fig. 2A). Cu/ZnSOD mRNA was detected in the sham control, but was not changed during the 24 h after SCI. Western blot analysis also showed that MnSOD protein in the mitochondrial fraction increased after SCI, but Cu/ZnSOD protein in the cytoplasmic fractions was not changed during the 24 h after SCI (Fig. 2B,C).

Immunohistochemical Detection of Cu/ZnSOD and MnSOD after SCI

Longitudinal sections containing the lesion's epicenter (20-mm) were examined for the immunocytochemical localization of SOD after SCI (sham, 1, 4, and 24 h; $n = 3$ for each time point). Weakly immunoreactive Mn-

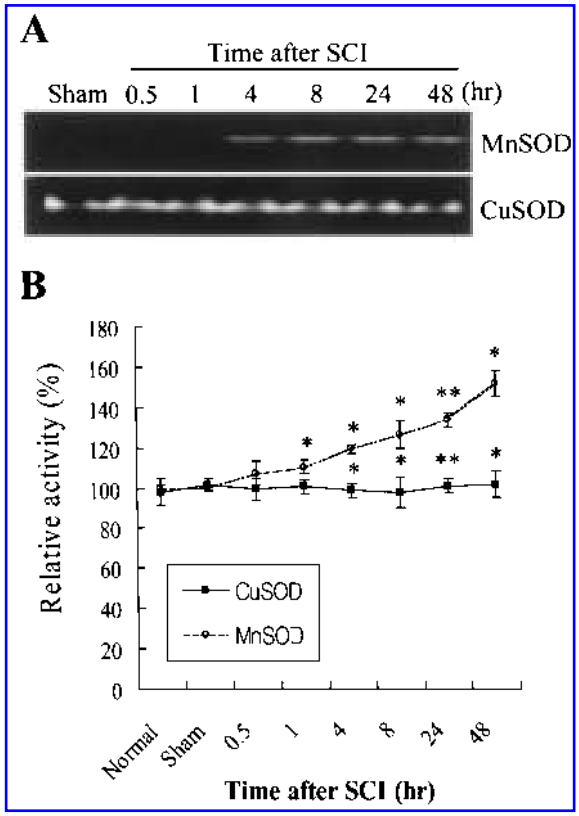

FIG. 1. Detection of enzyme activity of Mn- and Cu/ZnSOD after SCI. To examine the activity of SOD after SCI, spinal samples were taken at predefined time intervals (sham, 0.5, 1, 4, 8, 24, and 48 h after injury; $n = 5$ for each time point) as described in Materials and Methods. Cytoplasmic (100 μg) and mitochondrial (10 μg) protein was used for each assay. (**A**) NBT-staining of SOD activity. Note the achromatic (color-free) zones indicative of SOD activity that appear on the gels that have taken on a blue/purple color due to the assay processing, were detected at 4 h and persisted up to 48 h after SCI. There was no band at early times such as 0.5 and 1 h after injury and in the sham-operated spinal sample. These data were representative of three independent experiments. (**B**) SOD activity using SOD enzyme assay kit after SCI. Note that only MnSOD activity gradually increased up to 48 h after SCI, while Cu/ZnSOD activity was not changed. Values are mean ± SD of three separate experiments. $*p < 0.005$, $**p < 0.001$.

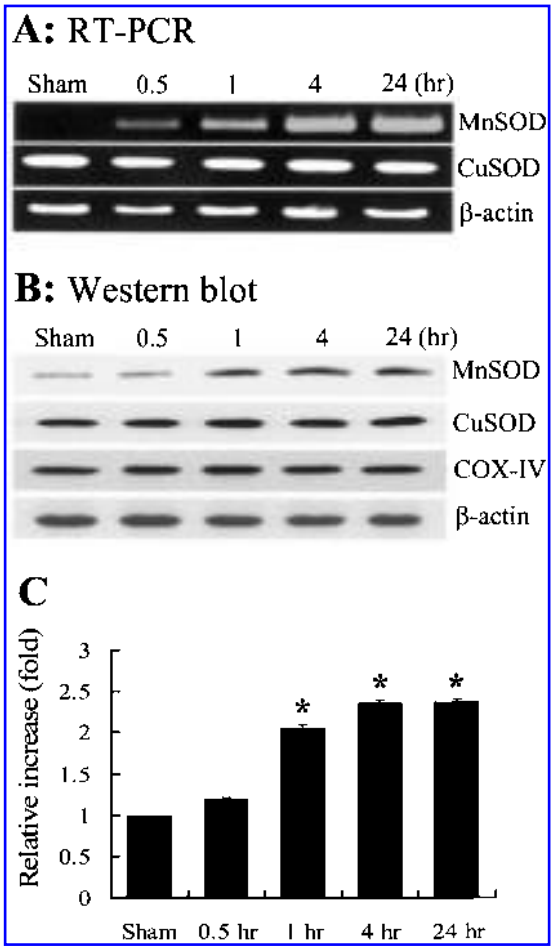

FIG. 2. Expression of MnSOD and Cu/ZnSOD after SCI. Tissues were harvested at 0.5, 1, 4, and 24 h after SCI ($n = 4$ for each time point). (**A**) RT-PCR; Isolation of total RNA and RT-PCR were performed as described in Materials and Methods. Note that the level of MnSOD mRNA was induced early (0.5 h), peaked at 4 h following injury and persisted for up to 24 h after injury. Note, by contrast, that Cu/ZnSOD mRNA was not changed during the 24 h after SCI. MnSOD mRNA was not detected in the sham operated control. The gels presented are representative of results from three separate experiments. β-Actin was used as an internal control. (**B**) Western blot; Preparation of mitochondrial, cytoplasmic protein and Western blot analysis were performed as described in Materials and Methods. Data show that MnSOD protein was increased and reached its peak at 24 h after SCI. Cu/ZnSOD protein was not changed during the 24 h after SCI. β-actin and COX-IV were used as internal controls of protein loading for cytoplasmic and mitochondrial fractions. The data shown are representative of results from three separate experiments. (**C**) Densitometry readings of the bands of MnSOD on Western blot were expressed as arbitrary units; these data were then converted to relative increase as compared to the sham control. $^*p < 0.01$.

SOD was detected in the gray matter (Fig. 3A) but not in the white matter of the sham control (Fig. 3B). This immunoreactivity increased with time after injury (Fig. 3C,D). Immunoreactivity of MnSOD was visible in both gray and white matter as early as 1 hr after injury, and increased further up to 24 h. At 4 h, a marked particulate cytosolic immunoreactivity was detected in the cells suggesting that MnSOD protein is localized in the mitochondria (Fig. 3C). Furthermore, cells positive for MnSOD were distributed ubiquitously in presumptive neuron cells in the gray matter (Fig. 3C) and in oligodendrocytes in the white matter (Fig. 3D), although these cells did not seem to exist at the lesion site throughout the entire period after injury (data not shown). These results show that MnSOD was induced and modulated after SCI. There were no differences in immunoreactivity of Cu/ZnSOD between injured and sham-operated spinal sections. Immunoreactivity of Cu/ZnSOD was distributed homogeneously in the cytoplasm of presumptive neuronal cells in the gray matter (Fig. 3E,G), while immunoreactivity of Cu/ZnSOD was observed in the cytoplasm and nucleus of glial cells in the white matter (Fig. 3F,H). Double immunostaining with cell type specific markers was performed to elucidate the cell types expressing MnSOD. As expected, double staining also revealed that MnSOD-positive cells in the gray matter were co-localized with a neuronal marker, NeuN (Fig. 4D). MnSOD-positive cells in the white matter were also co-localized with APC, a marker for oligodendrocytes (Fig. 4H).

Exogenous TNF-α Administered into the Uninjured Spinal Cord Induces MnSOD Expression

To examine the upstream molecules signaling the induction of MnSOD after SCI, we injected 4 μL of the pro-inflammatory cytokine, TNF-α (10 ng/μL), into the uninjured spinal cord (vehicle, 1, 4, and 8 h after injury, $n = 5$ for each time point). As shown in Figure 5A,B, Cu/ZnSOD expression was not changed after injection. However, MnSOD expression was induced and increased with time as compared to vehicle controls; this may mean that TNF-α serves as an upstream inducer of MnSOD expression after injury. At 8 h after treatment with TNF-α, the increase of MnSOD expression was 3.7 ± 0.6 fold compared to vehicle control (Fig. 5C). We also found that the induction of MnSOD was dependent on the concentration of TNF-α (data not shown).

Inhibition of MnSOD Expression by Treatment with Neutralizing Antibody to TNF-α

TNF-α was increased shortly after SCI (Lee et al., 2000; Yune et al., 2003). We injected 2 or 4 μL of neutralizing antibodies against TNF-α (10 ng/μL) into the

lesion site immediately after injury ($n = 5$ for each group) to examine whether the induction of MnSOD was mediated by TNF-α. At 4 h after injury, spinal cord samples were taken as described above. RT-PCR analysis showed that transcription of MnSOD mRNA was inhibited by treatment with neutralizing antibody against TNF-α, although Cu/ZnSOD mRNA was not affected (Fig. 6A). Western blot analysis of mitochondrial and cytoplasmic extracts also showed that the expression of MnSOD was significantly decreased by treatment with neutralizing antibodies against TNF-α after injury while Cu/ZnSOD expression was not changed (Fig. 6B,C).

Activation of NF-κB after Injury

Some reports have shown that activation of NF-κB is required for the induction of MnSOD (Darville et al., 2000; Delhalle et al., 2002; Jones et al., 1997; Maehara et al., 1999; Sugino et al., 2002). We tried to examine the temporal activation of NF-κB after SCI by Western blot and immunohistochemistry at pre-defined intervals (0.5, 1, 4, and 24 h after injury) in order to investigate whether this transcription factor might be involved in the induction of MnSOD. As shown in Figure 7A, Western blots of nuclear extracts with anti-p65 antibody showed that a single band with an apparent molecular weight of 65 kDa (p65) was detected. Translocated NF-κB increased markedly by 1 h after injury and remained at increased levels thereafter up to 24 h after injury; NF-κB in the cytoplasm decreased after injury. Nuclear extracts from the sham-operated spinal cords, however, showed only a low level of NF-κB (Fig. 7A,B). We used gel electrophoretic mobility shift assay (EMSA) to investigate the activation of NF-κB further after SCI. Activation of NF-κB is caused mainly by increased DNA binding activity after NF-κB release from I-κB. Multiple DNA–protein complexes were observed in the spinal cord (Fig. 7C). Furthermore, NF-κB DNA binding activity was increased after SCI and peaked at 4 h after injury (Fig. 7C, lane 2–6). The specificity of the DNA-protein complexes was verified using unlabeled competitor and by supershift assay using p65 antibody. These complexes were completely abolished by competition with a 100-fold molar excess of unlabeled NF-κB oligonucleotides (Fig. 7C, lane 1) and supershifted by antibodies against p65 (Fig. 7C, lane 8 and 9). We examined the immunocytochemical profiles of activated NF-κB to confirm its detection on Western blot. In sham-operated spinal cords, NF-κB immunoreactivity was detected in neurons in the gray matter and in presumptive glial cells in white matter throughout the extent of the spinal cord section as based on cell morphology and size (Fig. 7D,E). In addition, NF-κB immunoreactivity was localized primarily in the cytoplasm of neuronal and glial cells, particularly in the perinuclear region (Fig. 7D,E). By contrast, strong NF-κB immunoreactivity was detected mainly in the nucleus of neurons in the gray matter and in the nuclei of presumptive glial cells in the white matter throughout the extent of the spinal cord section after injury (Fig. 7F,G). Activated NF-κB–positive cells, likely to be neutrophils and/or macrophages (based upon their morphology and size), were observed in the lesion site as well.

Inhibition of NF-κB Expression by Neutralizing Antibody to TNF-α

As mentioned above, many reports have shown that activation of NF-κB was mediated by TNF-α under various conditions (Harris et al., 1991). As we found that the induction of MnSOD was influenced by TNF-α (as described above), we examined whether NF-κB activation might be affected by TNF-α after SCI by injecting neutralizing antibody to TNF-α. To investigate this, 4 μL of neutralizing antibody against TNF-α (10 ng/μL) was injected into the lesion site immediately after injury ($n = 5$ for each group) and spinal samples were harvested 1 and 4 h later. Western blot analyses of nuclear and cytoplasmic extracts showed that treatment with neutralizing antibodies against TNF-α significantly decreased the amount of activated NF-κB translocated into the nucleus as compared to the amount seen in the vehicle control (Fig. 8). NF-κB activation after SCI was decreased about twofold compared to vehicle controls. As demonstrated by EMSA studies, DNA-binding activity of NF-κB in nuclear extracts was also inhibited by treatment with neutralizing antibody against TNF-α (Fig. 8C).

MnSOD Expression Was Colocalized with Activated NF-κB–Positive Cells after SCI

We tried to observe the colocalization of NF-κB and MnSOD by double staining to investigate whether the activation of NF-κB might be directly involved in the induction of MnSOD expression after SCI. MnSOD expression was observed in the cytoplasm of MnSOD-positive cells (Fig. 9A). NF-κB immunoreactivity was also observed in the cytoplasm and nuclei in the same view, suggesting that NF-κB was activated and translocated into the nuclei after injury (Fig. 9B). Interestingly, MnSOD expression co-localized with activated NF-κB-positive cells in the gray matter 4 hr after SCI (Fig. 9D, merged image). These results show that activated NF-κB might be directly involved in the induction of MnSOD after SCI.

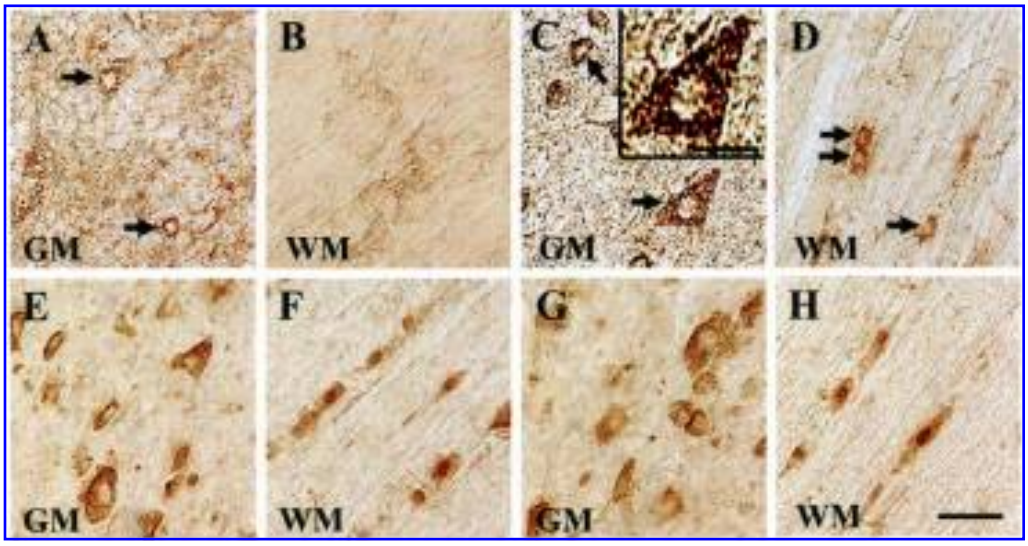

FIG. 3. Immunocytochemical detection of Mn- and Cu/ZnSOD protein after SCI. Spinal tissues were harvested at 0.5, 1, 4, and 24 h after SCI ($n = 4$ for each time point); the immunohistochemical processes were performed as described in Materials and Methods. Weak immunoreactivity of MnSOD was detected in the gray matter (**A**), but not in the white matter (**B**) of sham-operated controls. Immunoreactivity increased through time in gray matter (**C,** see arrows) and white matter (**D,** see arrows) at 4 h after injury. Note that a marked particulate cytosolic immunoreactivity was detected in cells suggesting that MnSOD protein is localized in the mitochondria (C). The insert (C) shows a higher magnification of a cell showing particulate cytosolic immunoreactivity to MnSOD. Cu/ZnSOD immunoreactivity was observed in presumptive neurons in the gray matter (**E,G**) and glial cells in the white matter (**F,H**) of sham-operated control (E,F) and injured spinal cord (G,H). Note that there were no differences of Cu/ZnSOD immunoreactivity between sham-control and injured spinal cord. Bar 30 μm, 20 μm (C insert).

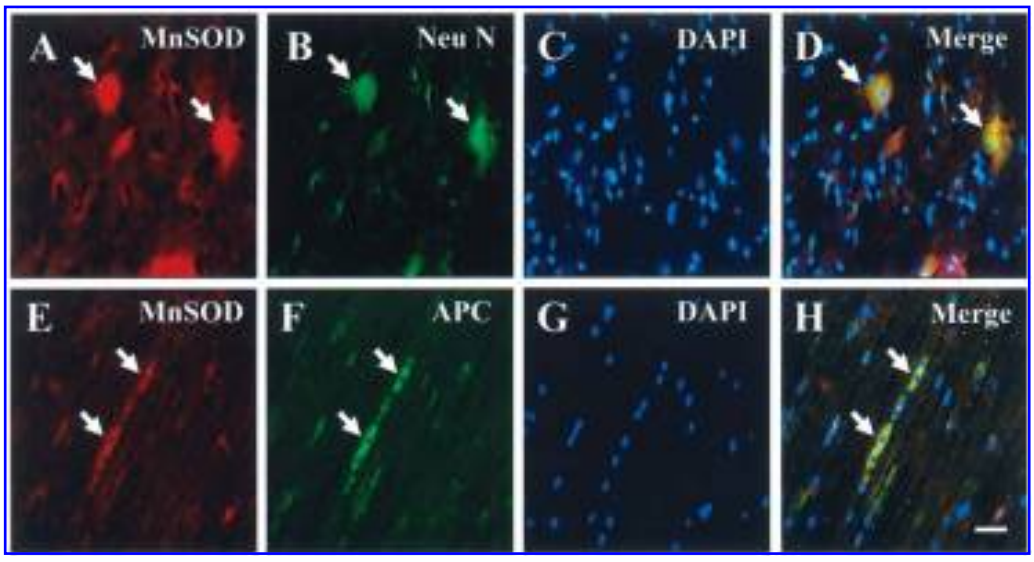

FIG. 4. MnSOD proteins were colocalized with NeuN and APC-1 after SCI. Some MnSOD stained sections were used for identification of cells expressing MnSOD protein with cell specific markers. Note that MnSOD-positive cells (**A,** see arrows) in the gray matter were co-localized with a neuronal marker, NeuN (**B,** see arrows) and its nucleus was labeled with DAPI (**C**) at 4 h after SCI (**D,** merged image, see arrows). MnSOD-positive cells (**E,** see arrows) in the white matter were also co-localized with APC (**F,** see arrows), a marker for oligodendrocytes and its nucleus was labeled with DAPI (**G**) at 4 h after SCI (**H,** merged image, see arrows). Bar = 30 μm.

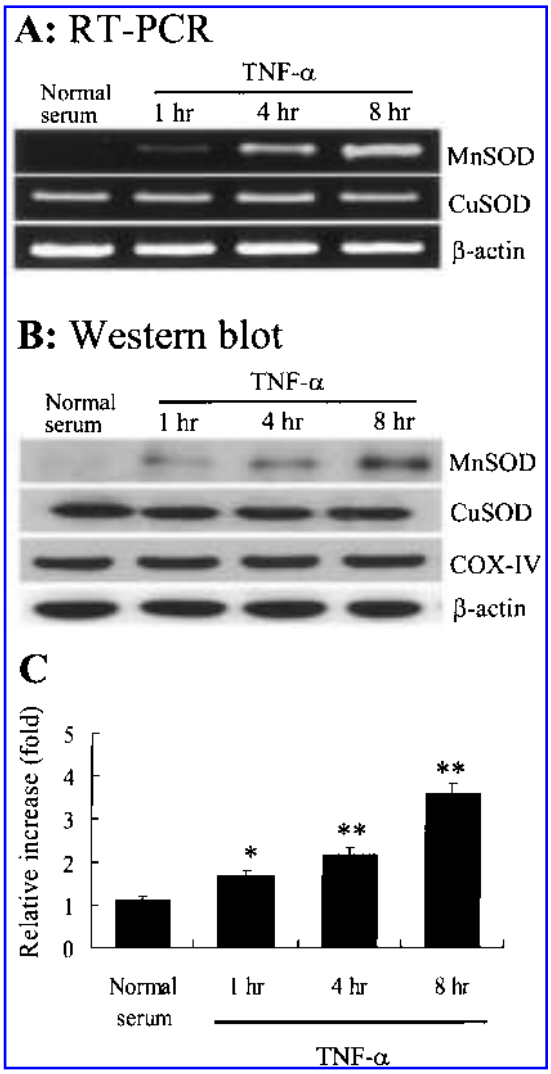

FIG. 5. Exogenous TNF-α administered into uninjured spinal cord induces MnSOD expression. TNF-α (4 μL, 10 ng/μL) was administered directly into the uninjured spinal cord ($n = 5$ for each time point). Note that MnSOD expression was significantly induced and increased through time as compared to vehicle control, although Cu/ZnSOD expression was not changed after injection, which examined by RT-PCR (**A**) and Western blot (**B**). Note, for example, at 8 h after treatment with TNF-α, the increase of MnSOD expression was 3.65 ± 0.6 ($p < 0.001$) fold compared to the vehicle control (**C**). β-actin and COX-IV were used as internal controls of protein loading for cytoplasmic and mitochondrial fractions. Values are mean $\pm$ SD of three separate experiments. $^*p < 0.005$, $^{**}p < 0.001$.

DISCUSSION

Here we report that of the two superoxide dismutases (SOD) known as antioxidant enzymes combating reactive oxygen species (ROS), only MnSOD was induced and modulated after spinal cord injury (SCI), while Cu/ZnSOD was not. The proinflammatory cytokine, TNF-α, which was increased after SCI, serves as an upstream signal triggering the induction of MnSOD through

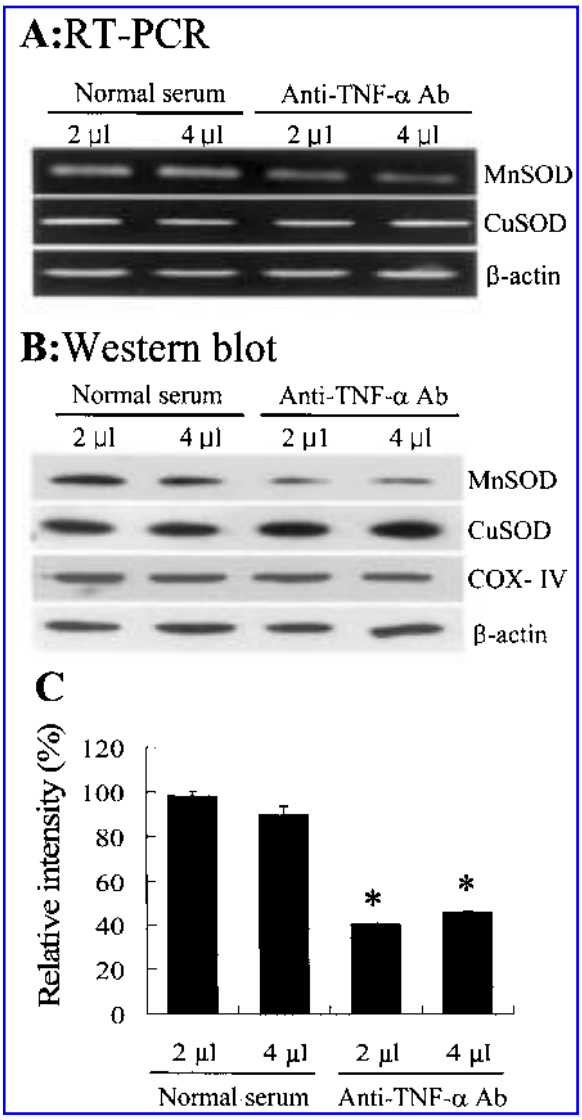

FIG. 6. Effect of neutralizing antibodies against TNF-α on expression of MnSOD after SCI. Normal serum or antibody against TNF-α (2 or 4 μL, 10 ng/μL) was injected into the lesion sites immediately after injury. Spinal cord tissues were then collected 4 h post-injury ($n = 5$ for each group). Note that mRNA (**A**) and protein of MnSOD (**B**) decreased following treatment with antibody against TNF-α, while Cu/ZnSOD was not affected suggesting TNF-α might act as an inducing molecule of MnSOD after SCI. β-actin and COX-IV were used as internal controls of protein loading for cytoplasmic and mitochondrial fractions. The gels shown are representative of results from three separate experiments. (**C**) Densitometry readings of bands of MnSOD on Western blot were expressed as arbitrary units; data were then converted to percentages as compared to the vehicle control. $^*p < 0.005$.

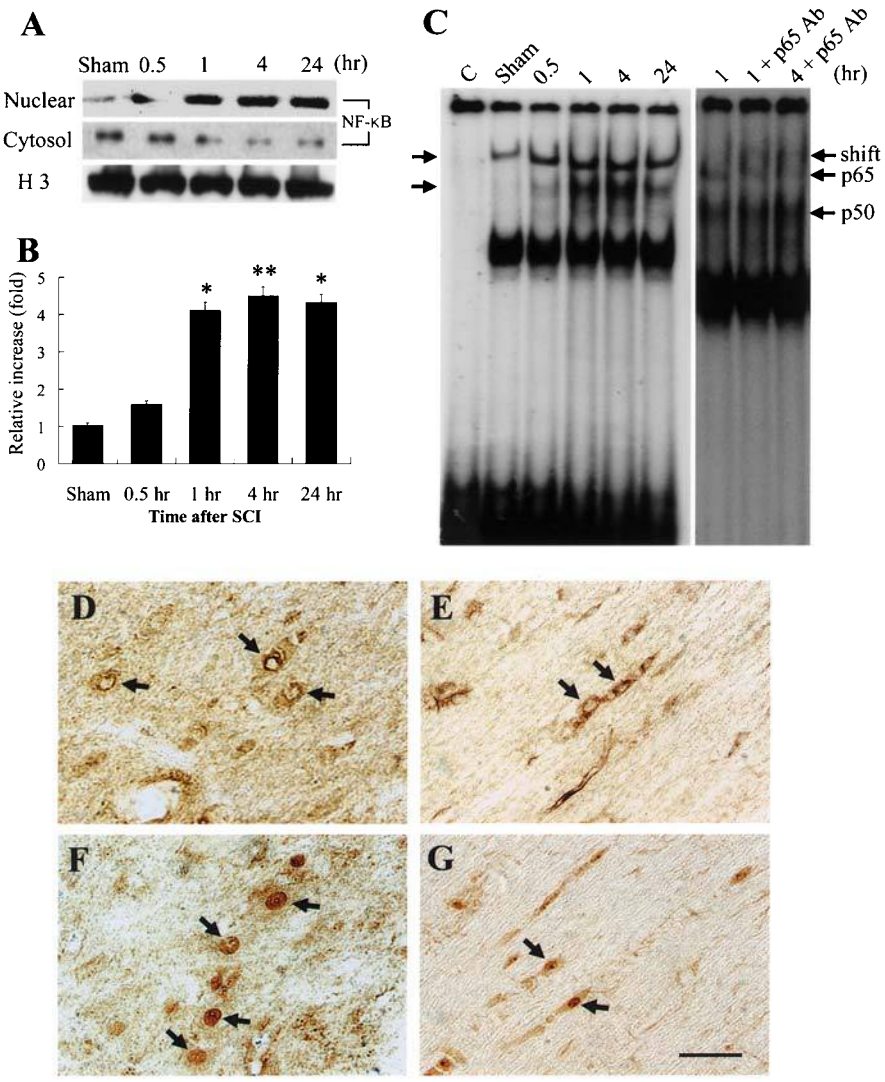

FIG. 7. Time-dependent changes of activated NF-κB in the spinal cord after injury. Samples from sham control and injured spinal cords were prepared for Western blot and immunohistochemistry at 0.5, 1, 4, and 24 h after SCI ($n = 4$ for each time point). (**A**) Western blot analysis of NF-κB in nuclear and cytoplasmic extracts using anti-p65 antibody. Note that activated NF-κB increased markedly by 1 h after injury and remained at increased levels thereafter for 24 h. Histone 3 was used as an internal control of protein loading for nuclear fraction. The gels presented are representative of results from three separate experiments. (**B**) Densitometry readings of gel bands were expressed as arbitrary units; data were converted to percentages as compared to the arbitrary densitometric units of the sham control. Values are mean ± SD of three separate experiments. *$p < 0.005$, ** $p < 0.001$. (**C**) EMSA analysis of NF-κB binding in nuclear extracts using a 5′-end labeled consensus oligonucleotide. Note that multiple bands interacted with radiolabeled (κB DNA and the protein complexes interacting with the NF-κB oligonuceotide prominently increased and peaked at 4 h after injury. The gels presented are representative of results from three separate experiments. (**D–G**) Immunocytochemical detection of NF-κB protein at 1 h after SCI. Tissues of sham and injured spinal cord were used as described in Materials and Methods. Note that NF-κB immunoreactivity was detected primarily in the cytoplasm of neurons (D, see arrows) and glial cells in the white matter (E, see arrows), which represents its inactive form. However, immunoreactivity of NF-κB was mainly observed in the nucleus of neurons in the gray matter (F, see arrows) and presumptive glial cells (G, see arrows) in the white matter, which represents its activation after SCI. Bar = 30 μm.

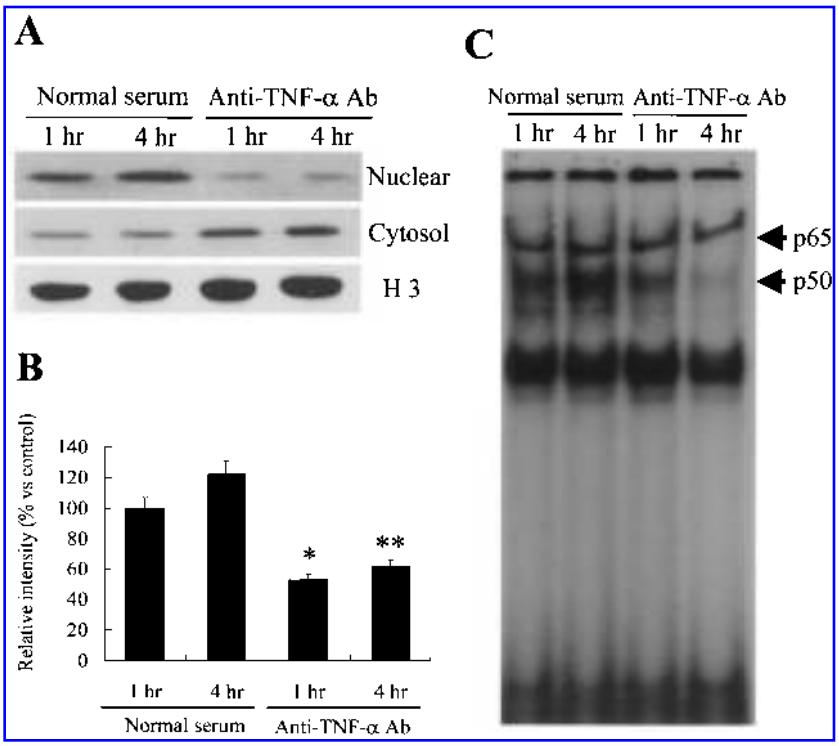

FIG. 8. Effect of neutralizing antibodies against TNF-α on the activation of NF-κB after SCI. Four microliter of normal serum or antibody to TNF-α (10 ng/μL) were injected into the lesion sites immediately after injury and spinal cord tissues were then collected at 1 and 4 h after injury ($n = 5$ for each group). (**A**) Inhibition of NF-κB activation by antibodies to TNF-α by Western blot analysis after SCI. Note that treatment with neutralizing antibody against TNF-α after injury significantly decreased the amount of activated NF-κB translocated into the nucleus. Histone 3 was used as an internal control of protein loading for nuclear fraction. The gels presented are representative of results from three separate experiments. (**B**) Densitometry readings of gel bands were expressed as arbitrary units; data were converted to percentages as compared to the vehicle control. Note that treatment with neutralizing antibody against TNF-α after injury significantly decreased the amount of activated NF-κB translocated into the nucleus. Values are mean $\pm$ SD of three separate experiments. *$p < 0.005$, **$p < 0.001$. (**C**) EMSA analysis of NF-κB binding in nuclear extracts after treatment of neutralizing antibody against TNF-α. Note that treatment with neutralizing antibody against TNF-α after injury significantly decreased the binding activity of NF-κB to its consensus oligonucleotides. The gels presented are representative of results from three separate experiments.

the activation of NF-κB. Our data suggest that activated NF-κB might be directly involved in the induction of MnSOD after SCI.

It has been reported that membrane disruption and the production of ROS are important factors causing immediate functional loss, progressive degeneration, and neuronal cell death after SCI (Liu et al., 1998, 1999; Luo et al., 2002; Mu et al., 2002). Under normal physiological conditions, there is a dynamic balance between ROS and their scavengers including SOD. However, when this balance between ROS and scavengers is disrupted, cells and tissues are damaged by oxidative stress. Oxidative damage to membranes may result in loss of membrane fluidity by lipid peroxidation. In addition, oxidative damage to proteins may progressively impair activity of the electron transport chain and, in a vicious cycle, cause further ROS production and oxidative damage (Symonyan and Nalbandyan, 1972). Progressive loss of ATP may ultimately lead to loss of Na^+/K^+-ATPase activity with a consequent plasma membrane depolarization, loss of cellular homeostasis, and activation of the apoptotic cascade (Bowling and Beal, 1995).

Based on the importance of antioxidant enzymes for cellular homeostasis after trauma, we examined the expression profiles of Cu/ZnSOD and MnSOD after SCI, which are known as antioxidant enzymes that destroy superoxide anion. Our data show that MnSOD activity was significantly increased after SCI, although Cu/ZnSOD activity was not (Fig. 1). In addition, as elucidated by RT-PCR, Western blot, and immunohistochemistry (Figs.

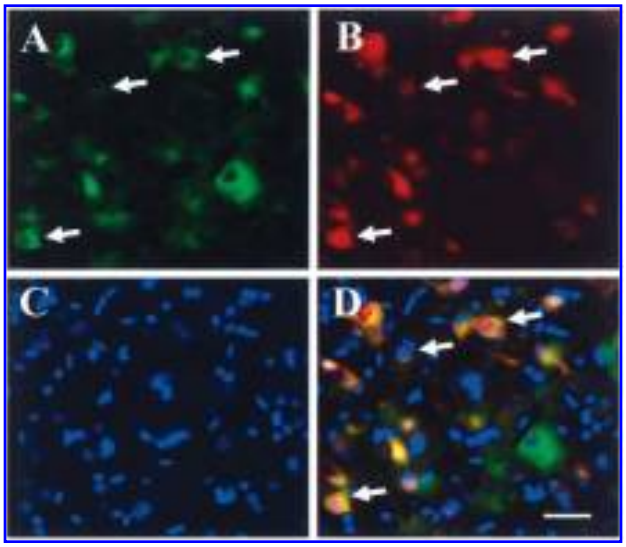

FIG. 9. Double immunofluorescent staining for MnSOD and NF-κB after SCI. Some MnSOD sections were used for double staining with NF-κB. (**A**) Expression of MnSOD was observed in the gray matter at 4 h after injury. (**B**) NF-κB immunoreactivity was observed in the cytoplasm and nuclei in the same view suggesting that NF-κB was activated and translocated into the nuclei after injury. (**C**) DAPI staining of nuclei in the same view. (**D**) Image overlap of A, B, and C demonstrates that MnSOD expression (A, see arrows) were co-localized with activated NF-κB positive cells (B, see arrows) in the gray matter at 4 h after SCI (D, merged image, see arrows). Bar = 30 μm.

2 and 3), we found that after SCI only MnSOD expression was induced and modulated, while Cu/ZnSOD expression was constitutive and not changed. This increased expression of MnSOD after SCI may be a marker of intracellular oxidative injury and play a cytoprotective function as it would serve to ameliorate the effects of oxidative injury. Our data correspond to the report by Earnhardt et al. (2003), which shows a rapid induction of MnSOD mRNA between 2 and 6 h after SCI. The report by Rosenfeld et al. (1997) also shows that MnSOD expression was increased in the motor and sensory neurons in dorsal root ganglion and spinal cord after complete axotomy of peripheral nerve.

Inflammatory reactions play an important role in the secondary injury cascade and contribute largely to the neuropathology associated with chronic SCI (Blight, 1992; Blight et al., 1997; Dusart and Schwab, 1994). Injury to the spinal cord also triggers a rapid and robust upregulation of proinflammatory cytokines that include TNF-α, IL-1β, and IL-6 (Hayashi et al., 2000; Pan et al., 2002; Streit et al., 1998; Wang et al., 1996; 2002; Xu et al., 1998). Studies have shown that TNF-α can elicit either a trophic or toxic effect, which is dependent on the target cell types and receptors. Two different TNFRs—TNFR1(p55) and TNFR2(p75)—have been identified (Beutler and Van Huffel, 1994a,b) and shown to mediate different cellular responses using distinct pathways (Kinouchi et al., 1991; Tartaglia et al., 1991). Some reports showed that TNF-α initiates an intracellular signaling pathway that leads to apoptosis through activation of caspase (Arch et al., 1998; Jarvis et al., 1994; Nagata, 1997). We recently reported that TNF-α, which was rapidly increased after SCI, plays an important role in neuronal and glial apoptosis; treatment with neutralizing antibody against TNF-α reduced apoptosis after SCI (Lee et al., 2000; Yune et al., 2003). Some reports showed that TNF-α initiates a pathway to cell survival through activation of the transcription factor, NF-κB (Barkett and Gilmore, 1999; Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996). Moreover, experiments using mice deficient in TNFR performed by Bruce et al. (1996) demonstrated that endogenous TNF-α protects neurons against ischemic and excitotoxic insults with induction of an antioxidative pathway, although a report by Xu et al. (1998) showed that methylprednisolone, the only known neuroprotective drug, inhibits TNF-α expression and NF-κB activation after SCI. MnSOD is induced by a variety of cytokines including TNF-α, IL-1, IL-6, and IFN-γ (Dougall and Nick, 1991; Harris et al., 1991; Karube-Harada et al., 2001; Nelson et al., 1995; Nogae et al., 1995; Visner et al., 1990). Some reports have shown that TNF-α induces MnSOD expression in various conditions (Guo et al., 2003; Karube-Harada et al., 2001; Lin et al., 1993; Nakata et al., 1993; Visner et al., 1990; Warner et al., 1991). The report by Rogers et al. (2001) is especially interesting in that it showed that TNF-α selectively induces MnSOD expression via mitochondria-to-nucleus signaling. As such, we injected TNF-α into the uninjured spinal cord to examine the role of TNF-α underlying the induction of MnSOD expression after SCI. Our results showed that exogenous TNF-α induced MnSOD expression in uninjured spinal cord (Fig. 5) and that treatment with neutralizing antibody against TNF-α after SCI inhibited the expression of MnSOD (Fig. 6). Therefore, these data suggest that TNF-α may play an important role in the induction of MnSOD as an inducing signal after SCI, although we have not examined which particular receptor of TNF-α is involved.

Although responses induced by NF-κB are associated most commonly with immunological and inflammatory processes (Chiarugi and Moskowitz; 2003; Makarov, 2000; Miagkov et al., 1998; Poynter et al., 2003), the role of NF-κB in normal or pathological conditions has not been established. Recent reports have shown that NF-κB

was activated in the spinal cord after injury or after traumatic brain injury (Bethea et al., 1998; Kim et al., 2001; Xu et al., 1998; Yang et al., 1995). In agreement with other reports, our data show that the amount of NF-κB translocated into nuclei of cells increased after SCI (Fig. 7). Also, NF-κB immunoreactivity was detected primarily in the nuclei of presumptive neurons in the gray matter and in glial cells in the white matter suggesting an activation of NF-κB after injury (Fig. 7E,F). To convincingly demonstrate activation of NF-κB by TNF-α after injury, we injected antibodies that neutralized TNF-α into the spinal cord after SCI. As shown in Figure 8, treatment with neutralizing antibody against TNF-α significantly inhibited the translocation of NF-κB, which means that activation of NF-κB was dependent on TNF-α after injury. Our results agree with other reports showing TNF-α–dependent/NF-κB activation (Beg et al., 1993; Beg and Baltimore, 1996; Osborn et al., 1989). Of special interest in this regard is the report by Kim et al. (2001) showing that deletion of tumor necrosis factor receptor reduces NF-κB activation and functional recovery after traumatic SCI. This suggests the activation of NF-κB is mediated by TNF-α after injury, although we have not elucidated whether TNF-α/NF-κB signaling might be neuroprotective or deleterious after SCI.

Despite the numerous reports related to regulation of MnSOD expression, little is known about the molecular mechanisms that govern transcription of the MnSOD gene. Three transcription factors—NF-κB, AP-1, and C/EBP-β—have been proposed as regulators of MnSOD expression in different cell types (Darville et al., 2000; Jones et al., 1997; Warner et al., 1996). Among these transcription factors, NF-κB has been proposed as a regulator for TNF-α–induced gene expression in a variety of cell types (Barnes and Karin, 1997). Recent studies have shown that activation of NF-κB may play an important role in the induction of MnSOD (Darville et al., 2000; Delhalle et al., 2002; Jones et al., 1997; Maehara et al., 1999; Sugino et al., 2002), although the report by Borrello and Demple (1997) shows NF-κB–independent transcriptional induction of the MnSOD gene. Our data also show that induction of MnSOD was mediated by activation of NF-κB after SCI (Fig. 9), although we freely admit that other transcription factors were not examined.

In conclusion, the results of these experiments show that after SCI, the transcription factor, NF-κB, was activated, and MnSOD was induced, while Cu/ZnSOD was not. In addition, this activation of NF-κB and induction of MnSOD might be dependent on TNF-α, which was increased after SCI. Our data show that after SCI, MnSOD expression induced by TNF-α is mediated through activation of NF-κB. This phenomenon could represent a self-defense mechanism combating oxidative stress by ROS that include superoxide anion.

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