Journal of Cellular Biochemistry 100:279–292 (2007)

ERK 1/2 Signaling Pathway Is Involved in Nicotine-Mediated Neuroprotection in Spinal Cord Neurons

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Abstract Evidence indicates that agonists of neuronal nicotinic receptors (nAChRs), including nicotine, can induce neuroprotective and anti-apoptotic effects in the CNS. To study these mechanisms, the present study focused on nicotine-mediated modulation of the extracellular regulated kinase 1 and 2 (ERK1/2) pathway in cultured spinal cord neurons. Exposure to nicotine (0.1–10 μM) for as short as 1 min markedly upregulated levels of phosphorylated ERK1/2 (pERK1/2) and increased total ERK1/2 activity. Inhibition studies with mecamylamine and α-bungarotoxin revealed that these effects were mediated by the α7 nicotinic receptor. In addition, pre-exposure to U0126, a specific inhibitor of the ERK1/2 signaling, prevented nicotine-mediated anti-apoptotic effects. To indicate if treatment with nicotine also can activate ERK1/2 in vivo, a moderate spinal cord injury (SCI) was induced in rats using a weight-drop device and nicotine was injected 2 h post-trauma. Consistent with in vitro data, nicotine increased levels of pERK1/2 in this animal model of spinal cord trauma. Results of the present study indicate that the ERK1/2 pathway is involved in anti-apoptotic effects of nicotine in spinal cord neurons and may be involved in therapeutic effects of nicotine in spinal cord trauma. J. Cell. Biochem. 100: 279–292, 2007.

Key words: spinal cord injury; nicotine; fatty acids; apoptosis; signal transduction

Spinal cord injury (SCI) is traditionally divided into primary and secondary trauma. Primary trauma indicates the initial injury of the spinal cord while secondary trauma refers to the cascade of events following the injury. Among a variety of secondary mechanisms of SCI [Dumont et al., 2001; Beattie et al., 2002b], apoptosis of neurons [Liu et al., 1997; Sugawara et al., 2002], and oligodendrocytes [Springer et al., 1999; Beattie et al., 2002a] appears to play one of the most important roles. Apoptotic cells in the lesion area can be detected within minutes following spinal trauma with the maximum presence of apoptotic neurons 8 h after the injury [Liu et al., 1997]. This process is associated with markedly upregulated caspase-3 around the injury site [Springer et al., 1999; Wingrave et al., 2003]. Apoptosis during spinal cord trauma appears to be related to the induction of oxidative stress and activation of microglial cells. For example, overexpression of superoxide dismutase can protect against neuronal death [Sugawara et al., 2002] and dying neurons are surrounded by activated microglial cells [Beattie et al., 2002b]. It has been hypothesized that the outcome of spinal cord trauma might depend on widespread apoptotic cell death in the lesion area [Liu et al., 1997] and also in regions distant to initial spinal injury [Crowe et al., 1997]. Apoptosis has also been

Grant sponsor: Philip Morris External Research Program; Grant sponsor: Kentucky Tobacco Research and Development Center.

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Received 10 January 2006; Accepted 2 May 2006
DOI 10.1002/jcb.21013

shown to occur in patients who died after spinal injury [Emery et al., 1998; Yamaura et al., 2002].

A growing line of evidence indicates that nicotine can exert neuromodulatory and neuroprotective effects. Administration of nicotine to the CNS can stimulate release of neurotransmitters [Okuda et al., 1994; Serova and Sabban, 2002] and neurotrophic factors, such as basic fibroblast growth factor (bFGF or FGF-2) and brain-derived neurotrophic factor (BDNF) [Belluardo et al., 2004]. In addition, nicotine exposure can lead to elevated cellular cAMP levels [Hsu et al., 1997]. Treatment with nicotine also can protect against apoptotic cell death. For example, we demonstrated that nicotine attenuated arachidonic acid-induced caspase activation and apoptosis of spinal cord neurons [Garrido et al., 2000, 2001]. The protective effects of nicotine also were demonstrated in other experimental systems [Kihara et al., 2001; Utsugisawa et al., 2002a; Guan et al., 2003; Hejmadi et al., 2003; Jonnala et al., 2003].

At the present time, nicotine appears to be a relatively safe medication and available over-the-counter. Nicotine gums or transdermal patches are commonly used for smoking cessation. Nicotine has also been used for treatment of Alzheimer's disease, Parkinson's disease, Tourette's syndrome, depression, and other diseases [Mihailescu and Drucker-Colin, 2000].

Nicotine exerts neuroprotective effects primarily through receptor-mediated mechanism(s). Specifically, nicotine binding to nAChRs induces conformational changes of the receptor, opens the calcium channel gate, and stimulates calcium influx into the cells [Dajas-Bailador and Wonnacott, 2004]. Increased intracellular calcium can subsequently activate a variety of interrelated signal transduction mechanisms involving activation of calcium-dependent kinases. The signaling pathways that can be implicated in nicotine-mediated neuroprotection include extracellular regulated kinase 1 and 2 (ERK1/2), which are components of the mitogen-activated protein kinase (MAPK). However, nicotine-induced signaling pathways have primarily been studied in PC12 cells [Qiu et al., 1998; Nakayama et al., 2001; Utsugisawa et al., 2002b], Xenopus oocytes expressing nAChRs [Hsu et al., 1997], or in other non-neuronal cell types [Hellstrom-Lindahl et al., 2000; Zhang et al., 2001; Sugano et al., 2005]. Only recently the effect of nicotine on MAPK was studied on neuronal cultures [Dajas-Bailador et al., 2002], hippocampal slices [Bell et al., 2004], and in mouse brains in vivo [Brunzell et al., 2003; Valjent et al., 2004]. No reports have focused on nicotine-induced signaling mechanisms in spinal cord neurons in relationship to neuroprotection in spinal cord trauma. Therefore, the present study focuses on the effects of nicotine on the ERK1/2 signaling pathway. We demonstrate that exposure to nicotine can specifically activate ERK1/2 in spinal cord neurons and that this pathway is involved in nicotine-mediated protection against arachidonic acid-induced apoptosis.

MATERIALS AND METHODS

Spinal Cord Neuron Cell Culture

Spinal cord neuron cultures were prepared and maintained according to the previously described protocol [Toborek et al., 1999; Garrido et al., 2000, 2001]. Briefly, spinal cords obtained from 13-day-old fetal mice were minced mechanically and digested during a 30 min incubation with 0.67 mg/ml of papain in D1SGH solution (135 mM NaCl, 5 mM KCl, 0.3 mM Na_2HPO_4, 0.2 mM $KHPO_4$, 15.5 mM glucose, 22 mM sucrose, and 9.86 mM HEPES) at 37°C. Enzymatic digestion was stopped by the addition of equal volume of 10% fetal bovine serum (FBS) in minimum essential medium (MEM) and tissue homogenate was centrifuged. The cell pellet was resuspended and triturated with a solution containing 40 μg/ml DNase in MEM supplemented with 10% FBS and 10% equine serum. The neurons were plated onto polyethyleneimine coated dishes at a density of 3×10^6 cells per 60-mm-diameter dish. The medium was completely removed 6 h after cell seeding and replaced with serum-free Neurobasal medium containing N2 supplement, 2 mM glutamine, 100 μg/ml gentamicin, and 2.5 μg amphotericin B. The cultures were maintained in atmosphere containing 10% CO_2 at 37°C. Three days after seeding, cultures were treated with 5.4×10^{-5} M fluorodeoxyuridine and 1.4×10^{-5} M uridine to inhibit proliferation of glial cell population. Twice per week, one third of the culture medium was replaced with fresh Neurobasal medium supplemented as described above. Spinal cord neuron cultures were used after 14–17 days in vitro when the cultures were considered mature and suitable for experiments.

Experimental Media

Arachidonic acid of high purity (>99%) was purchased from Nu-Chek-Prep (Elysian, MN). A stock solution of this fatty acid was prepared in absolute ethanol and diluted when added to the experimental media to achieve a 10 μM final concentration. The ethanol concentration did not exceed 0.1% in the culture dishes and did not show any effect on spinal cord neuron cultures. Stock solution of nicotine (RBI, Natick, MA) was prepared in sterile phosphate buffer saline (PBS). U0126, the specific inhibitor of MAPK kinase, was purchased from Promega (Madison, WI), stock solution was prepared in DMSO. U0126 was used at the final concentration of 10 μM.

Spinal Cord Trauma

Traumatic spinal injury was induced in adult male Long-Evans rats (Harlan, Indianapolis, IN) by the NYU weight drop device [Gruner, 1992]. All procedures and handling techniques were approved by the Institutional Animal Care and Use Committee. Briefly, rats (9–10 weeks old) were anaesthetized with pentobarbital (40 mg/kg) and the laminectomy was performed at vertebral level T-10, exposing the dorsal cord surface with the dura remaining intact. A 10-g weight was allowed to drop from 12.5 mm onto the T-10 segment, resulting in a moderate SCI. The impact rod was removed immediately following the injury and muscles, and the incision were closed in layers. Following surgery, animals were placed on a heating pad, which was maintained at 37°C. The rats were monitored until they recovered from anesthesia and then returned to their home cages.

Two hours following the trauma, the animals received either a single i.p. injection of saline or different doses (0.35, 3.5, or 7 mg/kg body weight) of freshly prepared nicotine solution (RBI, Natick, MA). The following experimental groups were used in the present study: Sham (only surgical procedures required for laminectomy were performed; i.p., injection with saline); Trauma (laminectomy, followed by the induction of spinal cord trauma and i.p. injection with saline); and Trauma plus nicotine (laminectomy, followed by the induction of spinal cord trauma and i.p. injection with nicotine at the dose of 0.35, 3.5, or 7 mg/kg). Rats were sacrificed 3 h after nicotine administration, that is, 5 h after the induction of SCI.

Immunostaining

Spinal cord neurons cultured on 60 mm dishes were fixed with ethanol for 30 min at 4°C. After washing three times with PBS and blocking with 3% BSA in PBS for 30 min, samples were incubated overnight at 4°C with anti-MAP2 antibody (Chemicon International, Temecula, CA). The excess of primary antibody was removed, slides were washed three times with PBS, and stained with anti-α7, anti-ERK1, or anti-pERK1/2 antibody for 2 h at room temperature (all antibodies from Santa Cruz Biotechnology, Santa Cruz, CA). Then, the slides were washed again with PBS and incubated with FITC- or rhodamine-conjugated secondary antibody for 2 h at room temperature. After washing with PBS, slides were mounted in aqueous mounting media and covered with coverslips. Specimens were evaluated under an epifluorescence Nikon Eclipse E600 microscope and the images were captured using a Spot CCD camera system.

Western Blot

Treated spinal cord neurons were washed with cold PBS and lysed in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 10% NP-40, 0.1% SDS, 1 mM EGTA, 100 μg/ml PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 100 μM of heat inactivated Na_3VO_3. Following 10 min incubation on ice, the lysates were cleared by centrifugation at 15,000*g* (4°C, 15 min). Protein concentration was determined according to Bradford assay with serum albumin as a standard. Aliquots of cell lysates containing 25 μg of total protein in gel loading buffer (50 mM Tris-HCl, 100 mM dithiotreitol, 2% SDS, 2% glycerol, 0.05% bromophenol blue, pH 6.8) were separated on 10% SDS–PAGE gel and transferred onto a nitrocellulose membrane. Non-specific binding was blocked overnight at 4°C with 5% non-fat dry milk in TBST (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20). The membranes were incubated with anti-phospho ERK1/2 antibody (1:1,000, Santa Cruz Biotechnology) for 1.5 h at room temperature. After removal of primary antibody, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000) for 1 h at room temperature. The immunoreactive bands were visualized by using ECL Western blotting detection system

(Amersham Biosciences, Piscataway, NJ) and Kodak XAR-5 film for autoradiography. Densitometric values of the bands corresponding to phosphorylated ERK1 (pERK1) and ERK2 (pERK2) were determined using UN-SCAN-IT gel^{TM} image analysis software (Silk Scientific, Inc., Orem, UT) and statistically analyzed.

A similar protocol was used to determine pERK1/2 in spinal cord tissue. However, tissue samples were prepared by homogenization in a lysis buffer of different composition (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.1 mg/ml phenylmethylsulfonyl fluoride, 0.5% NP-40, 1 mM EDTA, 2.5 μg/ml leupetin, 1 μg/ml pepstatin A, 1 μg/ml aprotonin, Na_3VO_4 1 mM).

ERK1/2 Activity Assay

Treated spinal cord neurons were lysed in 10 mM TRIS-HCl buffer, pH 7.4 containing 150 mM NaCl, 2 mM EGTA, 2 mM DTT, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM Na_3VO_3. Lysates were centrifuged at 25,000*g* for 20 min at 4°C and supernatants containing cytoplasmic MAPK were frozen at −80°C until analyses. ERK1/2 activity was determined using the commercially available kit (Amersham Biosciences). This assay system is based on a transfer of the (γ-^{32}phosphate) group of ATP to the threonine group of a highly selective peptide for ERK1/2. Results are expressed as pmoles of phosphate transferred per minute.

MTT Conversion Assay and LDH Activity

MTT conversion was performed as described earlier [Garrido et al., 2000]. This assay takes advantage of the conversion of the yellow MTT (3-[4,5-dimethylthiazol-2]-2,5 diphenyl tetrazolium bromide) to purple formazan crystals by mitochondrial succinate dehydrogenase in viable cells. Briefly, MTT was diluted in water and added to spinal cord neurons grown in 24-well plates at a final concentration of 0.25 mg/mL. Following a 2 h incubation to allow its conversion into formazan crystals, the media was removed and cells were lysed with DMSO to allow the crystals to dissolve. Absorbance was read at 595 nm using a microplate reader and the results were expressed as percentage of control.

Lactate dehydrogenase (LDH), located in the cytoplasm in viable cells, is released to the media by cells that have lost membrane integrity. Assessment of LDH activity in culture media was performed as described earlier [Garrido et al., 2000] by measuring the decrease in absorbance at 340 nm due to the conversion of enzyme cofactor NADH to NAD+ (one enzyme activity unit = 0.001ΔA/min). Briefly, 100 μl aliquots of culture media were collected and dissolved in pyruvate buffer (0.25 mg sodium pyruvate in PBS, 0.1 M, pH = 7.4). NADH (0.25 mg in phosphate buffer) solution was then added to the samples. Separate control cultures were lysed by repeated freezing and thawing and total released LDH was measured. Absorbance was measured immediately after the addition of NADH and 45 s after the initial reading. The data are presented as the percentage of the total releasable LDH.

Caspase-3 Activity

Caspase-3 activity was quantified using CaspACE Fluorimetric Assay System (Promega Corporation), according to the manufacturer's instructions. This assay is based on the release of the fluorochrome 7-amino-4-methyl coumarin (AMC) when the provided pseudosubstrate acetyl-Asp-Glu-Val-Asp-aldehyde-AMC is cleaved by caspase-3. Free AMC produces fluorescence that can be monitored and quantified to estimate caspase-3 activity. Briefly, cultured spinal cord neurons were lysed and cell extracts were centrifuged to eliminate cellular debris. Aliquots (20 μl) of the cell extracts were incubated for 2 h at 37°C in the presence of the substrate. Generation of free AMC was quantified using a fluorescent plate reader (360 nm excitation, 460 nm emission).

Hoechst Staining

Hoechst staining was employed to quantitatively assess apoptosis of spinal cord neurons. This technique allows the visualization of DNA condensation, a characteristic feature of apoptotic cells. Neurons were pre-exposed to 10 μM nicotine and treated with 10 μM arachidonic acid for 24 h. To inhibit ERK1/2, U0126 [5 μM, specific inhibitor of MAPK kinase (MEK)] was added into selected cultures 30 min before nicotine treatment. Following treatment exposure, neurons were washed with PBS (0.1 M, pH 7.4), fixed with 4% paraformaldehyde for 15 min and then washed again. The cells were stained with Hoechst 33342 (Molecular Probes; final concentration 1 μg/ml in PBS) for 15 min at room temperature. Cells were then washed

with PBS and the nuclei were visualized with a fluorescence microscope (340 nm excitation, 420 nm barrier filter) using a 40× objective. To quantify cellular apoptosis, neurons with fragmented or condensed DNA and apparently normal DNA were counted.

Statistical Analysis

Routine statistical analysis of data was completed using SYSTAT 7.0 (SPSS, Inc., Chicago, IL). One-way ANOVA was used to compare mean responses among the treatments. For each endpoint, the treatment means were compared using Bonferroni least significant difference procedure. Statistical probability of $P < 0.05$ was considered significant.

RESULTS

To visualize ERK1/2 expression, the cultures were double immunostained for the presence of pERK1/2 or total ERK and microtubule-associated protein 2 (MAP2), a specific marker for neuronal cells. Both pERK1/2 (Fig. 1A) and total ERK (Fig. 1B) immunoreactivity directly overlap with MAP2 staining, indicating localization in spinal cord neurons. While pERK1/2 immunoreactivity appears to be localized both to neuronal processes and cell bodies, the majority of total ERK1/2 immunoreactivity is distributed in cell bodies.

Nicotine Exposure Activates ERK1/2 in Spinal Cord Neurons

Time- and dose-dependent effects of nicotine on activation of ERK1/2 in cultured spinal cord neurons are shown in Figures 2 and 3, respectively. Levels of phosphorylated ERK1/2 (pERK1/2) reflect the activated form of ERK1/2, while the levels of total ERK1/2 indicate that the same amount of proteins were loaded into each well. As illustrated in Figure 2A, treatment with 10 μM nicotine for as short as 1–6 min markedly increased both pERK1 and pERK2 levels. Figure 2B depicts the effects of nicotine exposure on ERK1/2 activity as measured by radioactivity assay. These results confirm that a short treatment (i.e., 1 min) with 10 μM is sufficient to significantly elevate ERK1/2 activity. The levels of ERK1/2 activation remained at approximately the same level for up to 15 min of exposure to nicotine.

Figure 3 reflects the effects of exposure to different doses of nicotine on pERK1/2 levels. In

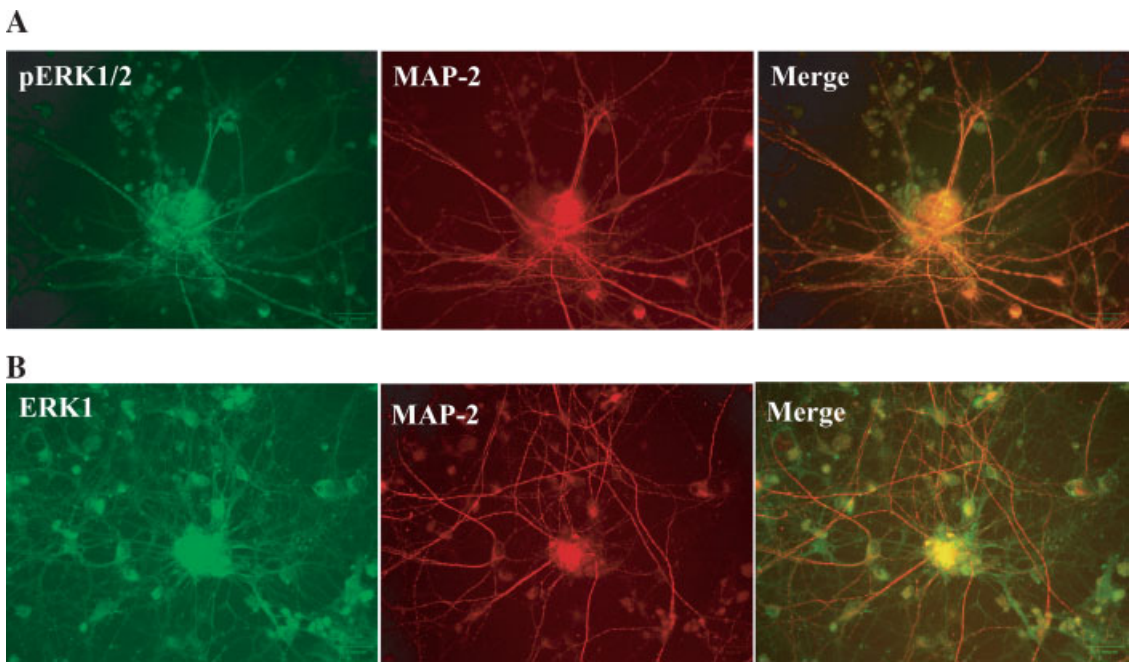

Fig. 1. Phosphorylated ERK1/2 (pERK1/2) and total ERK immunoreactivity in cultured spinal cord neurons. Neurons were stained for the expression of pERK1/2 (**A**) or ERK1 (**B**). In addition, the cells were stained for microtubule-associated protein 2 (MAP2), a specific marker for neuronal cells. As indicated, pERK1/2 immunoreactivity appears to be distributed in cell bodies and neuronal processes. In contrast, ERK1 immunoreactivity is localized primarily to cell bodies. Both pERK1/2 and ERK1 immunoreactivity directly overlap with MAP2 staining indicating localization in spinal cord neurons.

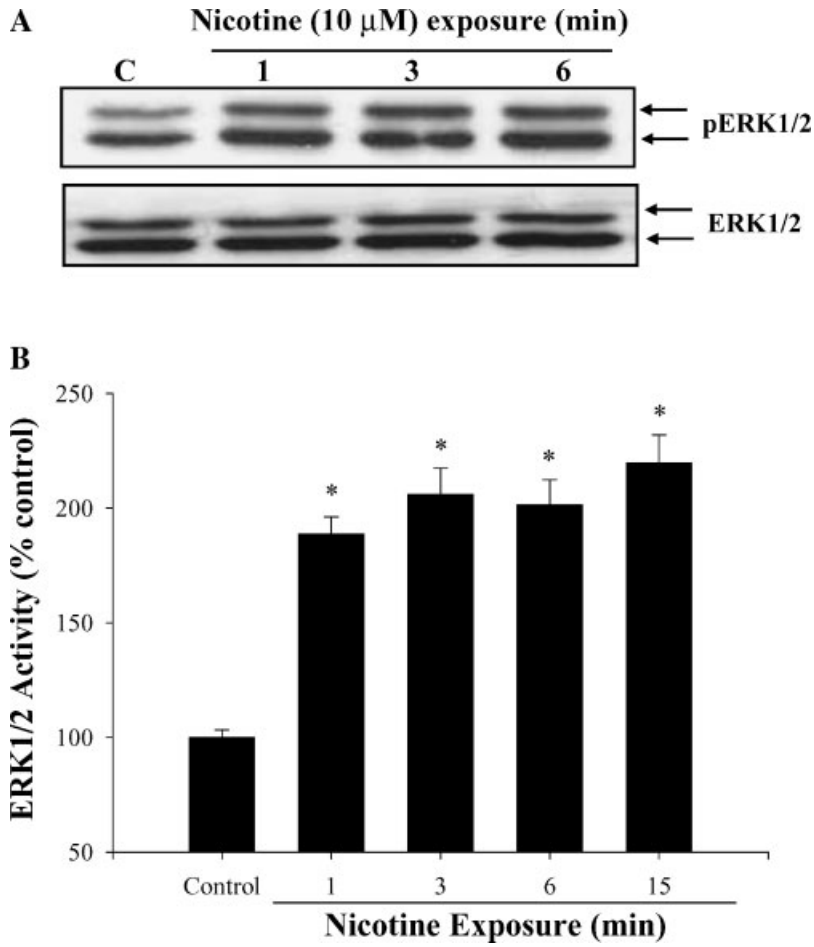

Fig. 2. Time-dependent effects of nicotine on ERK1/2 activation in spinal cord neurons. Neurons were treated with nicotine (10 μM) for the indicated time points and activation of ERK1/2 was evaluated either by Western blot (**A**) or total activity assay (**B**). The blot is the representative image from six independent experiments. The ERK1/2 activity assay was based on the transfer of the radiolabeled γ-phosphate group from ATP to a peptide that is highly specific for ERK1/2. Data shown are the mean ± SD of six determinations. *Statistically significant compared to the control group.

these experiments, spinal cord neurons were treated with nicotine for 5 min. The representative image of several Western blot analyses is shown in Figure 3A. These experiments were repeated four times, the bands were scanned and their intensity was statistically analyzed (Fig. 3B). As illustrated, pERK1/2 levels were significantly elevated by treatment with 0.1–10 μM nicotine. Phorbol myristoyl acetate (PMA, 50 ng/ml) was used as the positive control.

Nicotine-Mediated Activation of ERK1/2 Is Dependent on the α7 Neuronal-Nicotinic Receptor

Spinal cord neurons are characterized by a high level expression of nAChRs [Khan et al., 2003]. The present study is focused on the neuroprotective effects mediated by the α7 receptor. Figure 4A shows expression of this receptor in cultured spinal cord neurons. As demonstrated, the α7 immunoreactivity is present both in the cell bodies and neuronal processes. In addition, it directly overlaps with MAP2 positive staining, indicating neuronal localization.

To determine if nicotine-mediated activation of ERK1/2 is mediated by the nAChRs, neurons were pretreated with mecamylamine (10 μM for 30 min), followed by exposure to nicotine (10 μM) for 5 min. Mecamylamine is a general inhibitor of nAChRs. Figure 4B upper panel shows the representative image of several Western blot analyses that pretreatment with mecamylamine can abolish nicotine-mediated phosphorylation of ERK1/2. This effect was also observed in ERK1/2 activity assay (Fig. 4B lower panel).

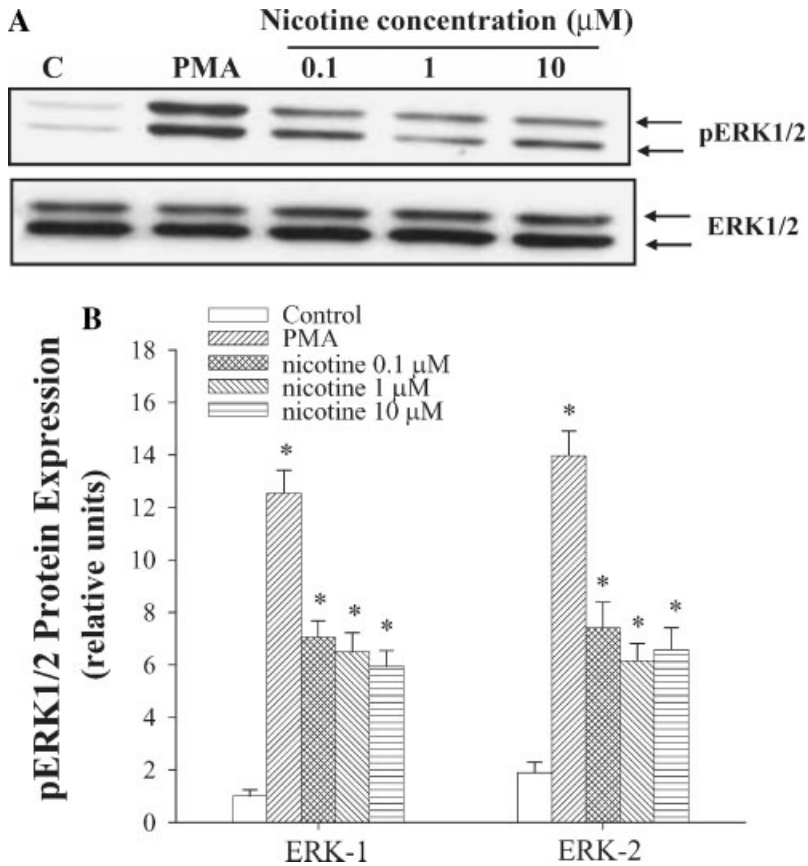

Fig. 3. Dose-dependent effects of nicotine on ERK1/2 activation in spinal cord neurons. Neurons were treated with nicotine (0.1–10 μM) for 5 min, and the levels of phosphorylated ERK1/2 (pERK1/2) and total ERK1/2 were evaluated by Western blot. The blot (**A**) is the representative image from six independent experiments and the bar graph (**B**) is the combined densitometry data from these experiments. Phorbol myristoyl acetate (PMA, 50 ng/ml) was used as the positive control. Data shown are the mean ± SD of six determinations. *Statistically significant compared to the control group.

To determine which specific type of nAChR is responsible for nicotine-mediated activation of ERK1/2, spinal cord neurons were pretreated with α-bungarotoxin, a specific inhibitor of the α7 nAChR (Fig. 4C) or with dihydro-β-erythroidine, an antagonist that blocks nAChR containing β2 subunits (Fig. 4D). Nicotine at the concentration of 10 μM was used in these experiments. Figure 4C indicates that nicotine-induced phosphorylation of ERK1/2 is effectively blocked by α-bugarotoxin. On the other hand, dihydro-β-erythroidine had no effects on pERK1/2 levels in nicotine-treated spinal cord neurons (Fig. 4D).

Nicotine Exposure Activates ERK1/2 in Experimental Spinal Cord Trauma

To indicate if nicotine can activate ERK1/2 in vivo, we employed a model of spinal cord trauma in which rats were treated with increasing doses of nicotine (0.35, 3.5, or 7 mg/kg). Levels of pERK1/2 were determined 3 h after nicotine administration and 5 h after the trauma. As shown in Figure 5, spinal cord trauma alone had no effects on pERK1/2 expression at the time point at which the determinations were performed. However, nicotine injections markedly, in a dose-dependent manner, increased ERK1/2 expression. The maximum activation of ERK1/2 was observed in rats injected with nicotine at the dose of 7 mg/kg.

ERK1/2 Is Involved in Anti-Apoptotic Effects of Nicotine

Nicotine can exert neuroprotective effects in various models of neurotoxicity, including injury to spinal cord neurons induced by arachidonic acid. To determine if ERK1/2 is involved in nicotine-mediated neuroprotective and anti-apoptotic effects, MTT conversion assay, LDH release, caspase-3 activity, and a

number of apoptotic cells were evaluated in spinal cord neurons treated with arachidonic acid and/or nicotine in the presence of U0126, a specific inhibitor of ERK1/2 signaling. Our previous studies indicated that arachidonic acid at the concentration of 10 μM causes injury to cultured spinal cord neurons [Toborek et al., 1999; Garrido et al., 2000, 2001]. Therefore, this concentration of arachidonic acid was used in the present study.

As indicated in Figure 6A, treatment with 10 μM arachidonic acid for 48 h markedly decreased MTT conversion in cultured spinal cord neurons. This effect was significantly attenuated in cultures pretreated with 10 μM nicotine. In addition, U0126 prevented nicotine-mediated protection against arachidonic acid-induced alterations in MTT conversion.

The effect of inhibition of the ERK1/2 pathways on nicotine-mediated protection against

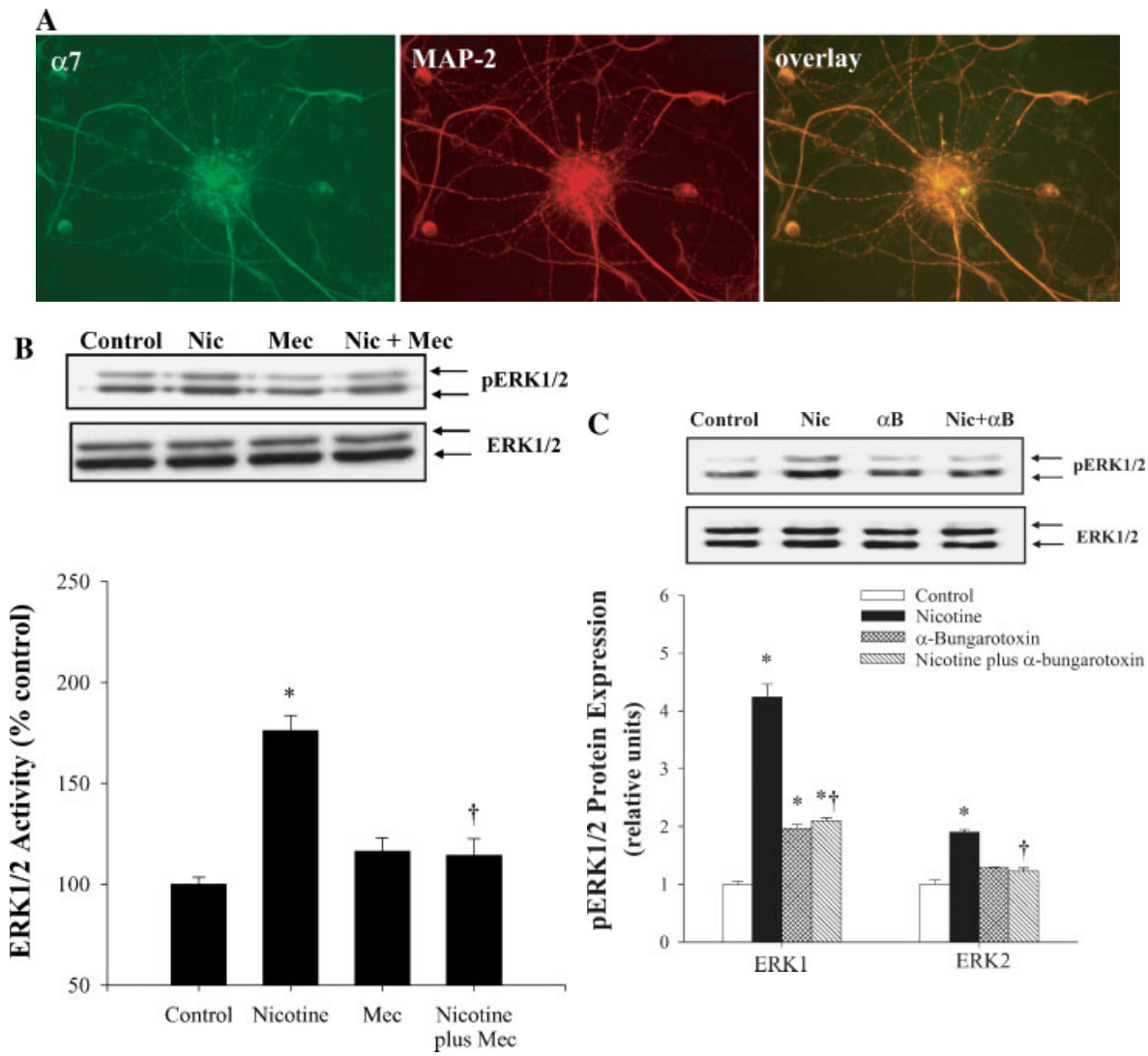

Fig. 4. Nicotine-mediated activation of ERK1/2 is mediated by the α7 receptor. Untreated cultured spinal cord neurons were immunostained for the presence of the α7 receptors and MAP2, a specific marker for neuronal cells. As indicated, α7 immunoreactivity directly overlaps with MAP2 positive staining and is distributed in both cell bodies and neuronal processes (**A**). In addition, neurons were pretreated with mecamylamine (Mec, 10 μM; **B**), α-bugarotoxin (αB, 1 nM; **C**), or dihydro-β-erythroidine (DHβE, 10 μM; **D**) for 30 min before co-exposure to nicotine (10 μM) for 5 min. Phosphorylated and total ERK1/2 were assessed by Western blots. The blots are the representative images from at least four independent experiments, and the bar graphs on C and D are the combined densitometry data from the respective experiments. In addition, ERK1/2 activation was assessed by the activity assay (B). Data shown are the mean ± SD SD of four determinations. *Statistically significant compared to the control group. †Data in the group Nicotine plus inhibitor (Mec or αB) are significantly different from those in the Nicotine group. #Data in the group Nicotine plus DHβE are significantly different from those in the DHβE group.

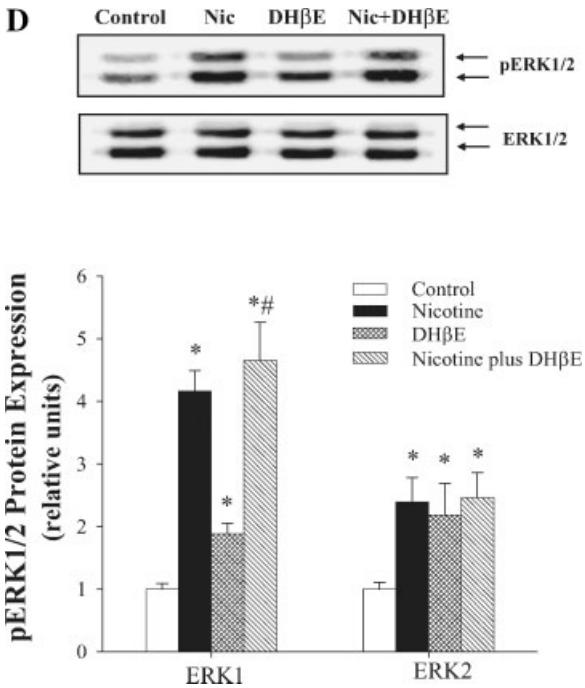

Fig. 4. (*Continued*)

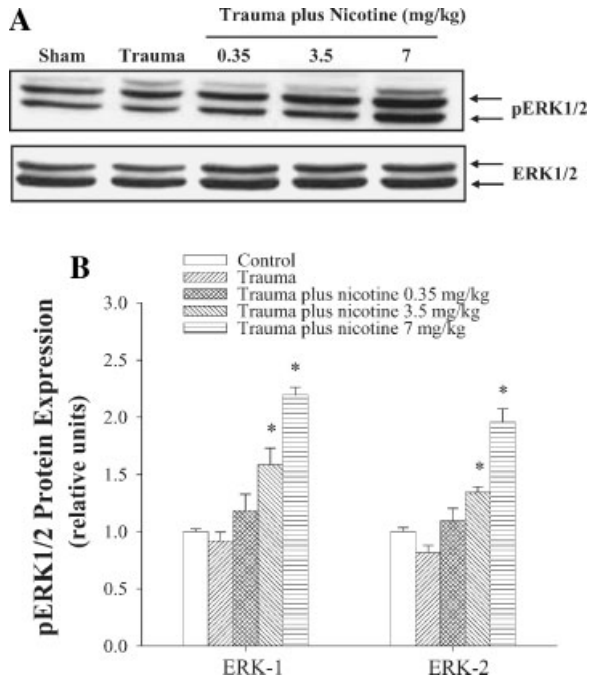

Fig. 5. Effects of nicotine administration on ERK1/2 activation in spinal cord trauma. Moderate compressive spinal cord trauma was induced by a 10-g weight drop from 12.5 mm onto the T-10 segment using the NYU impactor. Indicated doses of nicotine were injected i.p. 2 h after the trauma and animals were scarified 3 h following nicotine administration. Phosphorylation of ERK1/2 (pERK1/2) was determined by Western blot. The blot (**A**) is the representative image from four independent experiments and the bar graph (**B**) is the combined densitometry data from these experiments. Data shown are the mean ± SD of four determinations. *Statistically significant compared to the control (Sham) and Trauma groups.

arachidonic acid-induced LDH release in spinal cord neurons is illustrated in Figure 6B. Treatment with 10 μM arachidonic acid for 48 h increased LDH release by approximately 120% as compared to control. A 2 h pretreatment with nicotine reduced the LDH activity in cultures treated with arachidonic acid to control values. These protective effects were inhibited by a co-exposure to U0126.

Figure 6C indicates that nicotine can effectively inhibit arachidonic acid-induced activation of caspase-3. However, a co-treatment with U0126 markedly blocked this protective effect. In addition, exposure to U0126 alone was sufficient to significantly increase caspase-3 activity, a finding which is consistent with the hypothesis that ERK1/2 plays a critical role in neuronal survival [Hetman and Gozdz, 2004].

Figure 6D indicates the involvement of the ERK1/2 signaling in nicotine-mediated protection against arachidonic acid-induced apoptotic cell death. Apoptosis of spinal cord neurons was determined by Hoechst staining, which allows determination and quantification of cells with fragmented and condensed chromatin. Arachidonic acid treatment significantly increased the number of cells with fragmented and condensed chromatin. Nicotine treatment decreased the number of apoptotic cells to control levels and pretreatment with U0126 reversed this protection.

DISCUSSION

Activation of ERK1/2 is the primary pathway involved in protection against damage-induced cell injury and death [Hetman and Gozdz, 2004]. The ERK1/2 signaling cascade is initiated by stimulation of Ras, a low molecular weight (21 kDa) GTP-binding protein. Ras is anchored in cellular membranes and can be activated by a variety of stimuli, including receptor tyrosine kinases (RTK) and calcium influx [Mazzucchelli and Brambilla, 2000]. Activation of Ras results in subsequent activation of Raf (MAPK kinase kinase), MEK, and ERK1 and ERK2. Other members of the MAPK family include JNK/SAPK and p38 MAPK [Harper and LoGrasso, 2001]. In the present study, treatment with nicotine rapidly activated ERK1/2 (Figs. 2 and 3). These effects appeared to be specific because similar nicotine treatment did not affect p38 and/or JNK/SAPK

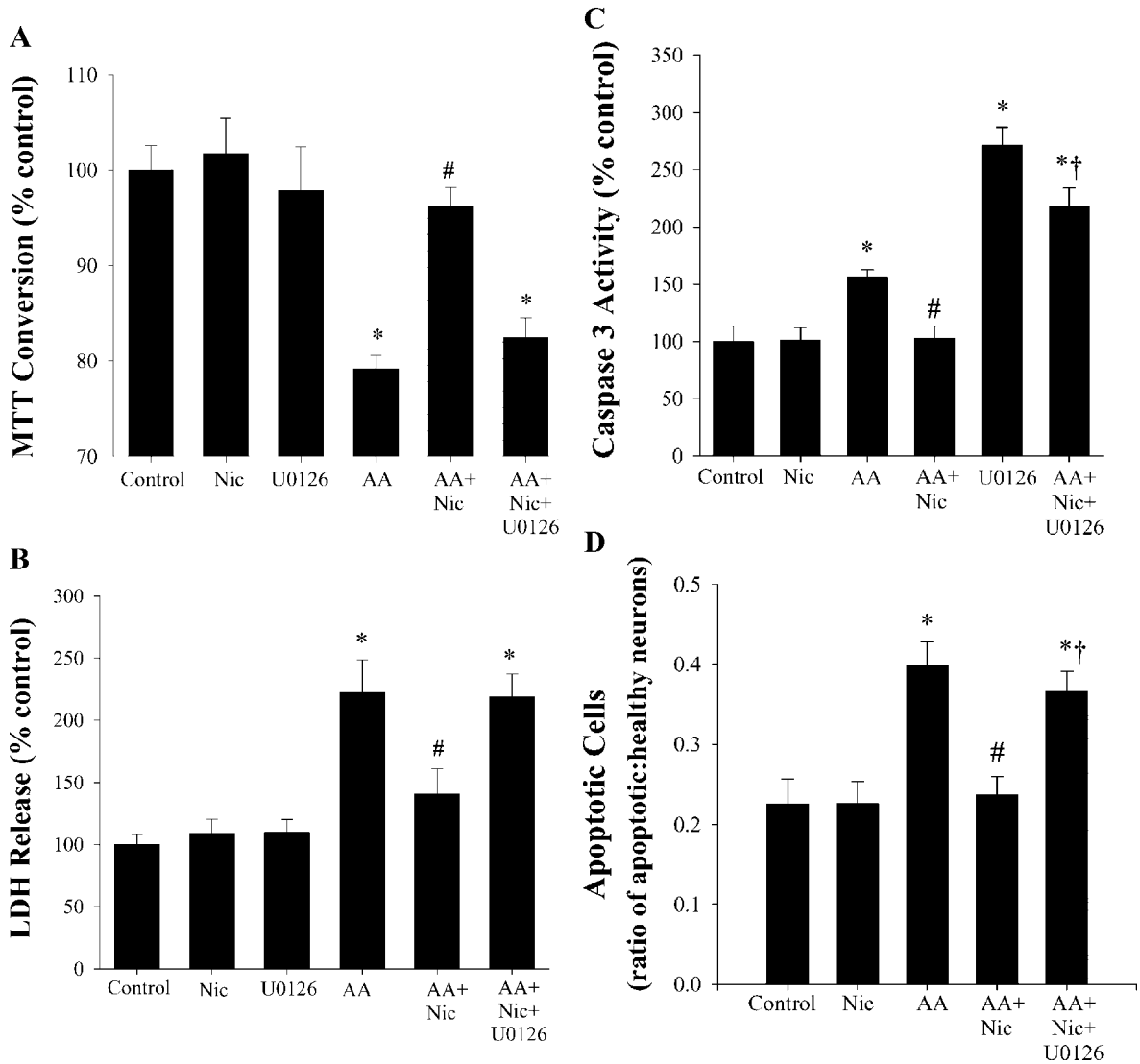

Fig. 6. Inhibition of ERK1/2 attenuates protective effects of nicotine on arachidonic acid-induced decreased viability and apoptosis of spinal cord neurons. Cultured spinal cord neurons were pretreated with 10 μM U0126 for 30 min before a 2 h nicotine treatment, followed by a 48 (**A** and **B**) or 24 h (**C** and **D**) exposure to 10 μM arachidonic acid. MTT conversion assay (A) and LDH release (B) were determined as general markers of cell viability. In addition, caspase 3 activity (C) was determined by a fluorescent method using acetyl-Asp-Glu-Val-Asp-aldehyde-AMC as a substrate. The nuclei (D) were stained with Hoechst 33342 and were visualized with a fluorescence microscope. Neurons with fragmented or condensed DNA and apparently normal DNA were counted. Data shown are the mean ± SD of six determinations. *Statistically significant compared to the control group. #Data in the group Arachidonic acid plus Nicotine are significantly different from those in the Arachidonic acid group. †Values in the Arachidonic acid plus Nicotine plus U0126 group are significantly different compared to the Arachidonic acid plus Nicotine group. N, nicotine; AA, arachidonic acid.

activation (data not shown). These are important findings because activation of ERK1/2 can stimulate cell survival, while activation of the JNK/SAPK and p38 MAPK are more related to induction of cell death [Harper and LoGrasso, 2001].

Nicotine-induced effects in the CNS are primarily mediated by specific nAChRs. nAChRs are formed from five subunits arranged around the central ion channel and are widespread in the brain and the spinal cord [McGehee, 1999]. Different combinations of these subunits form specific types of receptors. Comprehensive analysis of nAChR expression in the spinal cord revealed that immunoreactivity against specific nAChR subunits is the most intense in the dorsal horns, followed by the central and ventral horn regions [Khan et al.,

2003]. In the dorsal horns, the immunolabeling is particularly strong in the superficial layers comprising laminae II and III. In addition, the β4 expression is strong in the central canal region. Most of nAChR immunoreactivity shows presynaptic localization; however, nAChRs also can localize with post-synaptic sites in the dorsal and ventral horns, intermediolateral cell column, and the central canal spinal regions [Khan et al., 2003].

Among different nAChRs, it appears that the α7 and the α4β2 receptors may play the most significant role in the CNS [Nakayama et al., 1995; Hsu et al., 1997; Berg and Conroy, 2002]. Therefore, their influence on nicotine-mediated stimulation of ERK1/2 was examined in the present study. The obtained results indicated that phosphorylation of ERK1/2 in nicotine-treated spinal cord neurons can be specifically prevented by pre-exposure to α-bugarotoxin (a blocker of the α7 receptor) but not by dihydro-β-erythroidine (an antagonist of the β2 subunit and thus the α4β2 receptor) (Fig. 4B–D). These results are consistent with reports by ourselves [Garrido et al., 2001] and others [Kihara et al., 2001; Serova and Sabban, 2002], which indicate the important role of the α7 receptor in nicotine-mediated neuroprotection. As illustrated in Figure 4A, the α7 receptors are highly expressed in the cultured spinal cord neurons.

To study the role of ERK1/2 in the protective effects stimulated by nicotine, we employed an arachidonic acid-induced model of SCI that has been extensively developed in our laboratory [Toborek et al., 1999; Garrido et al., 2000, 2001]. The rationale of this model is based on the fact that trauma to the spinal cord is associated with calcium-dependent enzymatic hydrolysis of membrane lipids and the release of free fatty acids, primarily arachidonic acid [Murphy et al., 1994]. These reactions occur rapidly and the levels of free fatty acids are elevated already within the first few minutes after the experimental SCI [Demediuk et al., 1985; Bazan et al., 1995]. Arachidonic acid is freely diffusible in cell membranes and can affect activities of various ion channels, including both voltage- and ligand-gated ion channels as well as intracellular calcium release channels [Holmqvist et al., 2001; Liu et al., 2001]. In addition, exposure to elevated levels of arachidonic acid can induce neurotoxic effects. We indicated that arachidonic acid can enhance oxidative stress, elevate intracellular calcium levels, increase nitric oxide production, and stimulate caspase activities in cultured spinal cord neurons [Garrido et al., 2000, 2001]. Neurotoxic effects of this fatty acid also were observed in hippocampal neurons [Katsuki and Okuda, 1995] and in PC12 cells [Macdonald et al., 1999].

Due to the post-mitotic nature of adult neurons, apoptosis of neuronal tissue may have most significant pathological consequences. Thus, protection against neuronal death emerged as a priority for therapeutic strategies in spinal cord trauma. Evidence indicates that nicotine can exert anti-apoptotic effects [Garrido et al., 2000, 2001; Utsugisawa et al., 2002a; Hejmadi et al., 2003]; however, the mechanisms of these effects are poorly understood. In the present study, we provided evidence that the ERK1/2 pathways plays a critical role in the neuroprotective effects of nicotine. Indeed, we indicated that inhibition of ERK1/2 at the MEK level efficiently blocked nicotine-mediated protection against arachidonic acid-induced decreased spinal cord neurons viability, activation of caspase-3, and apoptotic cell death (Fig. 6A–D).

The ERK1/2 signaling can participate in neuroprotective effects of nicotine through a variety of different mechanisms. For example, it was shown that ERK2 can increase expression of bcl-2 and inhibit cellular apoptosis [Heusch and Maneckjee, 1998]. In addition, the neuroprotective effects of ERK1 and ERK2 may be related to activation of a variety of transcription factors, which, in turn, can regulate transcription rates of neurotrophic factors, leading to overexpression of "survival" genes and enhanced neuronal viability. Among the transcription factors that are involved in the ERK-mediated cellular survival are Elk1, nuclear factor-κB (NF-κB), and cAMP response element (CRE)-binding factor (CREB) [Mazzucchelli and Brambilla, 2000; Zhu et al., 2004]. The role of NF-κB in neuronal survival is well known [Mattson et al., 2000]. In addition, recent evidence strongly suggested the significance of CREB in regulation of cell viability [Finkbeiner, 2000; Mayr and Montminy, 2001]. To demonstrate the importance of CREB in neuronal survival, it was shown that this factor was required to induce transcription of BDNF [Zha et al., 2001]. Elk1 functions as a nuclear transcriptional activator through the interaction

with the serum response element (SRE) present in the promoter of many immediate early genes [Wasylyk et al., 1998]. Among others, Elk1 is involved in regulation of expression of FGF-2 [Schweppe et al., 1997; Shibata et al., 1991].

Our published results fully support the importance of neurotrophic factors, such as BDNF and FGF-2 in the neuroprotective effects of nicotine [Garrido et al., 2003]. Indeed, we reported that exposure to arachidonic acid markedly diminished expression of BDNF and FGF-2. These effects were fully prevented by pretreatment with 10 μM nicotine and blocked by inhibition of nicotinic receptors [Garrido et al., 2003]. BDNF plays a critical role in neuronal survival in different models of neurotoxicity, including injury to spinal cord neurons. For example, BDNF was shown to rescue approximately 50% of motoneurons from degeneration due to distal nerve axotomy [Yuan et al., 2000] BDNF-secreting cell grafts also stimulated growth of motor, sensory, and coerulospinal axons in injured spinal cords [Lu et al., 2001]. FGF-2 is another neurotrophic factor critical for survival of several neuron populations, such as cholinergic basal forebrain neurons [Minger et al., 1996], dopaminergic neurons [SiuYi et al., 2001], hippocampal neurons [Cheng et al., 2002], as well as spinal motor neurons [Teng et al., 1999]. To confirm its role in spinal cord trauma, it was demonstrated that FGF-2 infusions significantly enhanced functional recovery and tissue sparing in contusion models of SCI in rats [Rabchevsky et al., 2000; Romero et al., 2001]. It also was shown that expression of FGF-2 may be regulated by nicotine exposure. Specifically, acute, intermittent nicotine injections markedly upregulated expression of FGF-2 in different regions of the rat brain [Belluardo et al., 1998]. This effect was blocked by pretreatment with mecamylamine [Belluardo et al., 1998] but stimulated by epibatidine (a potent agonist of nAChRs) [Belluardo et al., 1999], indicating involvement of the nicotinic receptors in the regulation of FGF-2 levels.

In conclusion, the present study provides evidence that exposure to nicotine can activate ERK1/2 both in cultured spinal cord neurons and in a model of spinal cord trauma. This effect appears to have critical importance on survival of spinal cord neurons. Indeed, inhibition of the ERK1/2 pathway completely blocked nicotine-mediated protection against arachidonic acid-induced apoptosis. Based on these data, we conclude that the ERK1/2 signaling pathway plays an important role in nicotine-mediated neuroprotection.

ACKNOWLEDGMENTS

This work was supported in part by the Philip Morris External Research Program and the Kentucky Tobacco Research and Development Center.

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