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The Effects of Erdosteine, N-Acetylcysteine, and Vitamin E on Nicotine-Induced Apoptosis of Hippocampal Neural Cells

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Abstract This study investigated the frequency of apoptosis in rat hippocampal neural cells after intraperitoneal nicotine injection, examining the roles of the inflammatory markers myeloperoxidase (MPO) and tumor necrosis factor alpha (TNF-α) in nicotine-induced brain damage and the protective effects of three known antioxidant agents, N-acetylcysteine (NAC), erdosteine, and vitamin E. Female Wistar rats were divided into seven groups, each composed of nine rats: 2 negative control groups, 2 positive control groups, one erdosteine-treated group (500 mg/kg), one NAC-treated group (500 mg/kg), and one vitamin E-treated group (500 mg/kg). Nicotine was intraperitoneally injected at a dosage of 0.6 mg/kg for 21 days. Following nicotine injection, the antioxidants were administered orally; treatment was continued until the rats were killed. Apoptosis level in hippocampal neural cells was determined by using TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick endlabeling) method. Staining of cytoplasmic TNF-α in hippocampal neural cells and hippocampus MPO activity were evaluated by immunohistochemistry. Nicotine administration had no effect on local TNF-α production, or hippocampal MPO activity. The treatments with erdosteine, NAC and vitamin E significantly reduced the rate of nicotine-induced hippocampal neural cell apoptosis. This findings suggest that erdosteine and NAC can be as effective as vitamin E in protecting against nicotine-induced hippocampal neural cell apoptosis. J. Cell. Biochem. 104: 1740–1746, 2008.

Key words: hippocampal neural cells; apoptosis; nicotine; erdosteine; N-acetylcysteine; vitamin E

Nicotine is the neuroactive compound thought to be responsible for the development and maintenance of tobacco addiction. Despite the abuse potential of nicotine, the acute effects of this drug on the adult brain are considered primarily beneficial and, in particular, neuroprotective. However, in heavy smokers, abstinence from nicotine is accompanied by cognitive impairment, suggesting adverse effects of nicotine on brain plasticity [Abrous et al., 2002]. Nicotine is a neuroteratogen and alters the replication, differentiation, and apoptotic fate of neuronal cells in the developing brain, ultimately evoking permanent changes in synaptic activities and behavior [Abreu-Villaca et al., 2005]. Nicotine exposure has been shown to cause a number of changes that include persistent alterations in nicotinic receptor expression, nerve cell loss, reduced neurotrophic factor expression in astroglia, and reduced neurogenesis in the adult hippocampal formation [Belluardo et al., 2005]. Accordingly, in many neural precursor cells, nicotine inhibits cell proliferation and enhances apoptosis. Apoptosis, also known as programmed cell death, occurs in several pathological situations and constitutes part of a common mechanism for cell replacement, tissue remodeling, and the removal of damaged cells [Jang et al., 2002].

The adverse effects of nicotine are exerted by the disruption of the normal trophic functions of acetylcholine (ACh), mediated by nicotinic acetylcholine receptors (nAChRs), which appear developmentally as early as the neural tube stage and which increase as brain development progresses [Atluri et al., 2001; Abreu-Villaca et al., 2005]. Both human and animal studies

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have demonstrated that central stimulation of nAChRs can enhance cognitive ability. In addition to the direct stimulation of nAChRs, nicotine may provide cascading effects by stimulating the release of a variety of transmitters involved in cognitive function, including ACh, dopamine, norepinephrine, serotonin, and glutamate [Lee et al., 2001]. Neuronal nAChRs are distributed throughout the brain with a high concentration in the hippocampus, thalamus, and cerebral cortex. Functional studies have revealed that, in addition to their activity on synaptic transmission, nicotinic receptors may contribute to the control of several long-term processes in the brain, such as hippocampal-based learning, memory, and neuronal plasticity [Anagnostaras et al., 2001; Eidi et al., 2006].

Oxidative stress is a major mechanism for cellular damage and is associated with a wide variety of neurotoxicants. The brain is among the most vulnerable organs, because of its high oxygen consumption and because its membrane lipids are high in oxidizable polyunsaturated fatty acids. Certain regions of the central nervous system (CNS), such as the hippocampus and cerebellum, may be particularly sensitive to oxidative stress, with lower endogenous levels of biochemical antioxidants than other brain regions [Marino et al., 2004].

Increased oxidative stress can lead directly to nucleic acid, protein, and lipid damage, followed by a variety of secondary processes, such as impaired activity of membrane enzymes. In addition, an increase in cellular oxidation can also activate redox-responsive transcription factors, such as nuclear factor-kappa B (NF-κB), which is a critical step in tumor necrosis factor alpha (TNF-α) gene expression [Allen and Tresini, 2000]. The overexpression of such inflammatory mediators potentiate secondary injury to the brain via a variety of processes, including neutrophil infiltration, activation of microglia, and stimulation of astrocyte proliferation, which can in turn further increase the generation of neurotoxic reactive oxygen species (ROS) [Qiano et al., 2005].

Nicotine induces free radical generation both in vivo and in vitro and contributes a major portion of the net oxidative stress imposed by tobacco use, while at the same time depleting antioxidant defenses [Qiano et al., 2005]. Several studies have indicated that nicotine interferes with cellular protein synthesis and metabolism, inhibits thymidine incorporation into DNA, and reduces the transmembrane potential [Guan et al., 2003]. Therefore, antioxidants might be useful in preventing the neurodegeneration and functional impairments associated with nicotine exposure.

N-acetylcysteine (NAC) is a thiol compound which is the chemical formula $C_5H_9NO_3S$. NAC is a membrane-permeable precursor of glutathione, which interacts directly with intracellular oxidants. In addition to its antioxidant properties, NAC has the capacity to inhibit several inflammatory elements related to oxidant stress and is involved in the pathophysiology of inflammation [Blackwell et al., 1996; Blesa et al., 2003]. Erdosteine [*N*-(carboxymethylthioacetyl)-homocysteine thiolactone] is a novel mucoactive agent which has the chemical formula $C_8H_{11}NO_4S_2$. Erdosteine contains two blocked sulphydryl groups, which after hepatic metabolization, become available for free radical scavenging and antioxidant activity [Dechant and Noble, 1996; Braga et al., 2000]. Vitamin E (5,7,8-trimethyltocol), a lipid-soluble vitamin and potent free radical scavenging antioxidant, prevents lipid peroxidation and helps maintain the integrity of cellular organelles [Minko et al., 2002].

This study investigated the frequency of apoptosis in rat hippocampal neural cells after intraperitoneal nicotine injection, examining the roles of the inflammatory markers myeloperoxidase (MPO) and TNF-α in nicotine-induced brain damage and the protective effects of three known antioxidant agents, N-acetylcysteine, erdosteine, and vitamin E.

MATERIALS AND METHODS

Animals

This study was conducted at the experimental research center, University of Akdeniz (Antalya, Turkey). Sixty-three Wistar rats (200–250 g) were used in the study. The animals were fed a commercial balanced diet and tap water ad libitum. The rats were housed in cages and kept at a controlled temperature ($22 \pm 2^{\circ}C$) and humidty (55–60%) with a 12-h light/dark cycle. The investigation followed the National Research Council guidelines (NIH publication no. 85-23, revised l996) and was approved by the Animal Care and Use Committee of the University of Akdeniz.

Experimental Groups

The rats were divided into seven groups, each composed of nine rats: two negative control groups (intraperitoneal saline plus oral distilled water or sodium bicarbonate), two positive control groups (intraperitoneal nicotine plus oral distilled water or sodium bicarbonate), one erdosteine-treated group (nicotine plus erdosteine at a dose of 500 mg/kg), one NAC-treated group (nicotine plus NAC at a dose of 500 mg/kg), and one vitamin E-treated group (nicotine plus vitamin E at a dose of 500 mg/kg).

Experimental Procedure

Nicotine hydrogen bitartrate (Sigma, St. Louis, MO) was dissolved in 1 ml of sterile saline solution and injected intraperitoneally at a dosage of 0.6 mg/kg for 21 days, as previously described [Helen et al., 2003]. Erdosteine (Sandoz Drug Industries, Istanbul, Turkey) was dissolved with an equivalent molar quantity of sodium bicarbonate in distilled water, and NAC (Bilim Drug Industries, Istanbul, Turkey) and vitamin E (Bayer Drug Industries, Istanbul, Turkey) were each dissolved in distilled water. Following nicotine injection, the antioxidants were administered orally once a day via a syringe with a gavage needle; treatment was continued until the rats were killed. Control rats were intraperitoneally administered isotonic saline solution at a volume equal to that of the nicotine injection, and distilled water at a volume equal to that of the NAC and vitamin E treatments or a molar quantity of sodium bicarbonate equivalent to that of the erdosteine treatment dissolved in distilled water was given orally according to the drug administration protocol. The rats were killed with an overdose of urethan anesthesia at 21 days after the nicotine injection, all rats were decapitated and the brains were removed. The brain tissue was prepared for analysis of apoptosis, TNF-α, and MPO.

Analysis of Apoptosis

The apoptosis level in hippocampal neural cells was determined by using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, the sections were deparaffinized and rehydrated. Then the sections were incubated with proteinase K, rinsed, incubated in 3% H_2O_2, permeabilized with 0.1% Triton X-100, rinsed again, and incubated in the TUNEL reaction mixture. Following incubation, the sections were rinsed and visualized using Converter-POD with DAB. The sections were counterstained with hematoxylin and eosin (H&E). Apoptotic cells containing DNA fragmentation at a single cell level were identified by the TUNEL method. The neural cells per brain section were counted under a selected 400× microscopic field by two pathologists blind to the experimental protocol. The apoptosis index was expressed as a percentage of TUNEL-positive cells in 1,000 cells counted in the same section [D'Agostini et al., 2001].

Analysis of TNF-α

The local production of TNF-α was evaluated immunohistochemically using an anti-TNF-α kit (Histopathology Ltd., Akác, Hungary) according to the manufacturer's protocol. Briefly, the heart tissue samples on polylysine-coated slides were deparaffinized and rehydrated. Then, the microwave antigen retrieval procedure was performed, and the samples were incubated in a 3% H_2O_2 solution to inhibit endogenous peroxidase. To block nonspecific background staining, the sections were incubated with a blocking solution. Then the sections were incubated with primary anti-TNF-α antibody, followed by incubation with biotinylated goat anti-mouse antibody. After incubating with the chromogenic substrate (DAB), the sections were counterstained with hematoxylin and eosin (H&E). The slides were examined under a light microscope (Olympus BX51; Olympus Corp., Tokyo, Japan) at 400×, and all analyses were performed by two pathologists blind to the group assignments. The staining of cytoplasmic TNF-α in hippocampal neural cells was evaluated [Yang et al., 2004].

Analysis of MPO

Hippocampus MPO activity was evaluated immunohistochemically using an anti-MPO kit (NeoMarkers, Inc., Portsmouth, NH) according to the manufacturer's protocol. Briefly, the heart tissue samples on polylysine-coated slides were deparaffinized and rehydrated. Then, the microwave antigen retrieval procedure was performed, and the samples were incubated in a 3% H_2O_2 solution to inhibit endogenous

peroxidase. To block nonspecific background staining, the sections were incubated with a blocking solution. Then the sections were incubated with primary anti-MPO antibody, followed by incubation with biotinylated goat anti-mouse antibody. After incubating with the chromogenic substrate (DAB), the sections were counterstained with hematoxylin and eosin (H&E). The slides were examined under a light microscope (Olympus BX51; Olympus Corp.) at 400×, and all analyses were performed by two pathologists blind to the group assignments. The staining of cytoplasmic MPO in the neutrophils was evaluated [Genovese et al., 2005].

Statistical Analysis

Statistical analyses were conducted using the SPSS statistical package (SPSS 9. for Windows, Chicago, IL). The results were expressed as mean values ± standard deviation. Differences in quantitative variables between the groups were analyzed using one-way analysis of variance (ANOVA) followed by *post-hoc* multiple comparison tests and Student's *t*-test. All statistical tests were two-tailed, and a *P*-value less than 0.05 indicated significance.

RESULTS

Analysis of Apoptosis

There were no significant differences in apoptosis between the distilled water- and sodium bicarbonate-treated groups (data not shown). The effects of nicotine and antioxidants on apoptosis in hippocampal neural cells are given in Table I. The number of TUNEL-positive neural cells was significantly higher in the nicotine-treated group than in the control group ($P = 0$). Nicotine administration resulted in the induction of DNA fragmentation ladders, which are characteristic of apoptotic cell death (Fig. 1). Treatment with erdosteine ($P = 0$), NAC ($P = 0$), and vitamin E ($P = 0$) significantly reduced the rate of nicotine-induced neural cell apoptosis, and there were no significant differences in apoptosis among the three antioxidant-treated groups.

TABLE I. Effects of Treatment on Apoptosis in Hippocampal Neural Cells

Treated group	Apoptosis index (%) mean ± SD
Negative control	9.1 ± 1.2
Positive control	65.8 ± 2.0†
Erdosteine (500 mg/kg)	8.0 ± 6.0*
NAC (500 mg/kg)	11.0 ± 10.8*
Vitamin E (500 mg/kg)	18.6 ± 12.2*

†Statistical analysis: significantly higher compared with the negative control group ($P = 0$).
*Statistical analysis: significantly lower compared with the positive control group ($P = 0$).

Analysis of TNF-α

Nicotine had no effect on the local production of TNF in neural cells.

Analysis of MPO

Nicotine had no effect on hippocampal MPO activity.

DISCUSSION

Nicotine can disrupt mitochondrial functions, stimulate oxidative stress, inhibit neurogenesis, and enhance cell death. The mechanisms by which nicotine can induce changes in apoptotic expression include excitotoxicity, with increased calcium influx to neurons through the activation of nicotinic acetylcholine receptors. This calcium influx subsequently triggers the expression of p53, a cell cycle-related protein, and then cell death. This mechanism appears to be relevant only for undifferentiated cells, as nicotine does not trigger p53 expression in fully differentiated hippocampal progenitor cells [Machaalani et al., 2005].

Nicotine administration in the present study resulted in the induction of DNA fragmentation ladders in hippocampal neural cells, indicating apoptosis of these cells. These results suggest that nicotine abuse can have adverse consequences in the adult brain, raising an additional concern about the consequences of tobacco smoking. The effect of nicotine on hippocampal plasticity could contribute to the progressive development of cognitive deficits observed in smokers and consequently contribute to maintaining tobacco addiction [Foulds et al., 1996]. Along with the large amount of work demonstrating neuroprotective effects of nicotine, several papers have reported adverse effects of nicotine. To date, there are only a few studies examining the effects of nicotine on the expression of apoptotic markers in the brain. Studies have focused on prenatal nicotine exposure and its effects on the embryonic brain, postnatal nicotine exposure and its effects on the adoles-

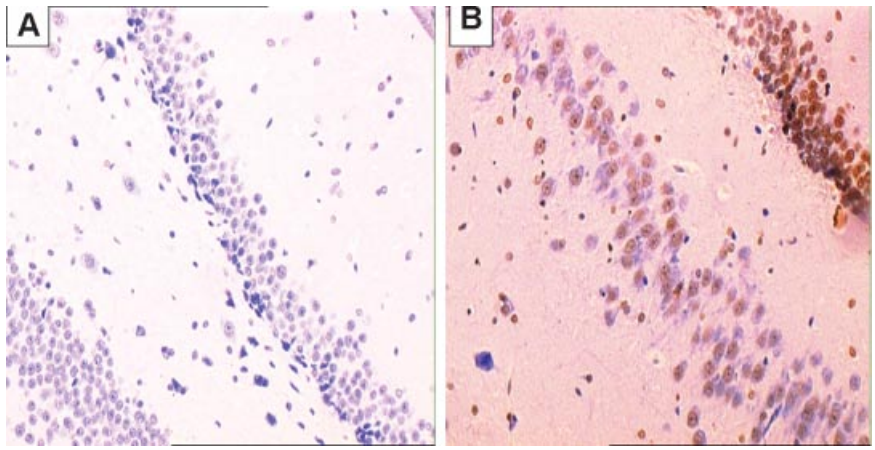

Fig. 1. Photomicrographs of apoptotic cells (brown-stained nuclei) in the hippocampal neural cells, as detected by the TUNEL method (original magnification, 200×). **A**: the negative control group; **B**: the nicotine-treated group. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cent brain, and direct effects of nicotine on differentiated and undifferentiated hippocampal cells in culture [Machaalani et al., 2005].

Treatment with erdosteine, NAC, or vitamin E significantly reduced the rate of nicotine-induced apoptosis in hippocampal neural cells. These results illustrate that nicotine toxicity may be mediated, at least in part, by oxidative stress and may be attenuated by preloading cells with antioxidants. It has been suggested that such effects on neural cell apoptosis might occur after supplementation with NAC and vitamin E, based on their abilities to down-regulate mitochondrial oxidant production and oxidative damage in vitro [Cuzzocrea et al., 2000; Chow, 2001]. However, the effects of erdosteine on the regulation of apoptosis have not been previously examined. NAC has been shown to reduce the generation of lipid peroxidation products, locomotor hyperactivity, and hippocampal neuron loss [Cuzzocrea et al., 2000]. Vitamin E has been shown to prevent the lowering of ACh levels in some parts of the brain involved in memory retention [Wortwein et al., 1994]. In addition to functioning as potent peroxyl radical scavengers, antioxidants are thought to have roles modulating cellular structure, metabolism, and signal transduction [Traber and Packer, 1995; Allen and Tresini, 2000].

The inflammatory response is the initial mechanism of self-defense by the innate immune system against endogenous and exogenous insults. Increasing evidence has highlighted the role of inflammation in most brain pathologies, including immune-mediated diseases and both acute and chronic neurodegenerative diseases. Among the mechanisms that regulate the inflammatory response, cross-talk between the immune and nervous systems plays an important role [Wang and Feuerstein, 2000; Wang et al., 2003].

TNF-α is a multifactorial cytokine mainly involved in inflammatory and other immune reactions. Several studies have indicated that TNF-α, together with other proinflammatory cytokines, may play a role in the development of CNS injury. Cytokines present in the CNS are produced not only by cells of the immune system but also by those of the brain, including astrocytes and neurons [Wang et al., 2003]. TNF-α appears to play a central role in the development of the acute-phase processes that occur in brain infarction as well as during CNS injury, participating in and modulating a complex neuroinflammatory network characterized by opposing neurotoxic and neuroprotective properties [Intiso et al., 2003].

In this study, the administration of nicotine did not modulate TNF-α expression, suggesting that the action of nicotine may not be linked to TNF-α transcription in neural cells. Nicotine and ACh effectively deactivate peripheral macrophages and inhibit TNF-α production in human macrophages as well as in mouse microglial cultures. The ACh-dependent macrophage deactivation is mediated by the α7 subunit of the nAChRs, which is expressed in peripheral

macrophages and has been described as essential for the so called "cholinergic anti-inflammatory pathway" [Wang et al., 2003; De Simone et al., 2005].

Many neurological insults and neurodegenerative disorders are accompanied by an acute inflammatory reaction that can contribute to neuronal damage. This inflammation involves the infiltration of blood-borne neutrophils into injured areas [Wang et al., 2003]. Since neutrophils rapidly infiltrate the injured brain parenchyma, it has been hypothesized that neutrophils can exacerbate post-insult injury by releasing noxious substances such as cytokines, oxygen radicals, and proteases. Neutrophils have the capacity to produce oxygen free radicals with two enzymes, the membrane-associated NADPH oxidase and MPO. However, the role of inflammation (harmful, beneficial, or non-relevant) in the pathogenesis of brain injury is controversial. For example, although neutrophils infiltrate injured CNS tissue at the time of cell death, studies have not indicated a clear cause and effect relationship between neutrophil recruitment and CNS pathogenesis [Beray-Berthat et al., 2003a; Dinkel et al., 2004].

In the present study, nicotine had no effect on hippocampal MPO activity, indicating that neutrophils may not play an important role in nicotine-induced hippocampal apoptosis and that anti-neutrophil strategies may not be neuroprotective in these conditions. Likewise, it has been reported that in severe ischemia, the suppression of neutrophil infiltration did not lead to a reduction of oxidative stress and nitric oxide production in brain lesions [Beray-Berthat et al., 2003b].

In conclusion, the results of this study suggest that long-term exposure to nicotine can lead to apoptotic damage in neural cells and induce structural change in the hippocampal formation, that antioxidant supplementation may be of therapeutic benefit in protecting the CNS from the damaging effects of nicotine in cigarette smoke, that erdosteine and NAC can be as effective as vitamin E in protecting against nicotine-induced neural apoptosis. Further studies are needed to elucidate the mechanisms of nicotine-induced apoptosis and to investigate the direct and specific effects of these antioxidants on apoptosis regulation. Apoptosis, as an active cellular process, represents a potentially preventable form of cell death. Understanding the molecular details of nicotine-CNS cell interactions may be the key to effective therapeutic intervention.

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