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Nicotine Suppresses TNF-α Expression in Human Fetal Astrocyte through the Modulation of Nuclear Factor-κB Activation

Il Hong Son[1,5,*], Yong Hoon Park[5,*], Hyun Duk Yang[1,5], Sung Ik Lee[1,5], Sun Jung Han[1,5], Jai Kyoo Lee[2,5], Dae Ho Ha[3,5], Hyung Won Kang[4,5], Joo Young Park[6] & Sung Soo Lee[7]

[1]Department of Neurology, Sanbon Medical Center, Wonkwang University, Gyeonggi-Do 435-040, Korea
[2]Department of Emergency Medicine, Sanbon Medical Center, Wonkwang University, Gyeonggi-Do 435-040, Korea
[3]Department of Orthopedics Sanbon Medical Center, Wonkwang University, Gyeonggi-Do 435-040, Korea
[4]Department of Oriental Neuropsychiatry, Sanbon Oriental Medical Center, Wonkwang University, Gyeonggi-Do 435-040, Korea
[5]Inam Neuroscience Research Center, Sanbon Medical Center, Wonkwang University, Gyeonggi-Do 435-040, Korea
[6]Department of Microbiology, Wonju College of Medicine, Yonsei University, Gangwon-Do 220-710, Korea
[7]Department of Neurology, Wonju College of Medicine, Yonsei University, Gangwon-Do, 220-710, Korea
Correspondence and requests for materials should be addressed to S. S. Lee (ssleeneuro@gmail.com)
*Il Hong Son and Yong Hoon Park contributed equally to this work.

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Abstract

Parkinson's disease (PD) progresses severely by a gradual loss of dopaminergic neurons in the substantia nigra (SN). Epidemiological studies showed that the incidences of PD were reduced by smoking of which the major component, nicotine might be neuroprotective. But the function of nicotine, which might suppress the incidences of PD, is still unknown. Fortunately, recently it was reported that a glial reaction and inflammatory processes might participate in a selective loss of dopaminergic neurons in the SN. The levels of tumour necrosis factor (TNF)-α synthesised by astrocytes and microglia are elevated in striatum and cerebrospinal fluid (CSF) in PD. TNF-α kills the cultured dopaminergic neurons through the apoptosis mechanism. TNF-α release from glial cells may mediate progression of nigral degeneration in PD. Nicotine pretreatment considerably decreases microglial activation with significant reduction of TNF-α mRNA expression and TNF-α release induced by lipopholysaccharide (LPS) stimulation. Thus, this study was intended to explore the role of nicotine pretreatment to inhibit the the expressions of TNF-α mRNA in human fetal astrocytes (HFA) stimulated with IL-1β. The results are as follows: HFA were pretreated with 0.1, 1, and 10 μg/mL of nicotine and then stimulated with IL-1β (100 pg/mL) for 2 h. The inhibitory effect of nicotine on expressions of TNF-α mRNA in HFA with pretreated 0.1 μg/mL of nicotine was first noted at 8 hr, and the inhibitory effect was maximal at 12 h. The inhibitory effect at 1 μg/mL of nicotine was inhibited maximal at 24 h. Cytotoxic effects of nicotine were noted above 10 μg/mL of nicotine. Moreover, Nicotine at 0.1, 1 and 10 μg/mL concentrations significantly inhibited IL-1β-induced NF-κB activation. Collectively, these results indicate that in activated HFA, nicotine may inhibit the expression of TNF-α mRNA through the pathway which suppresses the NF-κB activation. This study suggests that nicotine might be neuroprotective to dopaminergic neurons in the SN and reduce the incidences of PD.

Keywords: Nicotine, Tumor necrosis factor-α, Astrocyte, NF-κB

Parkinson's disease (PD) is a debilitating neurodegenerative movement disorder characterized by damage to the nigrostriatal dopaminergic system[1]. Epidemiological studies have identified a negative correlation between smoking and the development of Parkinson's disease[2]. The aetiology of sporadic PD, which accounts for the majority of the disease, is still unknown. Recently it was suggested that a glial reaction and inflammatory processes may also participate in the cascade of events leading to neuronal degeneration in PD. Human and animal studies support the role of neuro-inflammation in underlying pathogenesis of PD[3]. The levels of TNF-α expressed by astrocytes and microglia are elevated in striatum and cere-

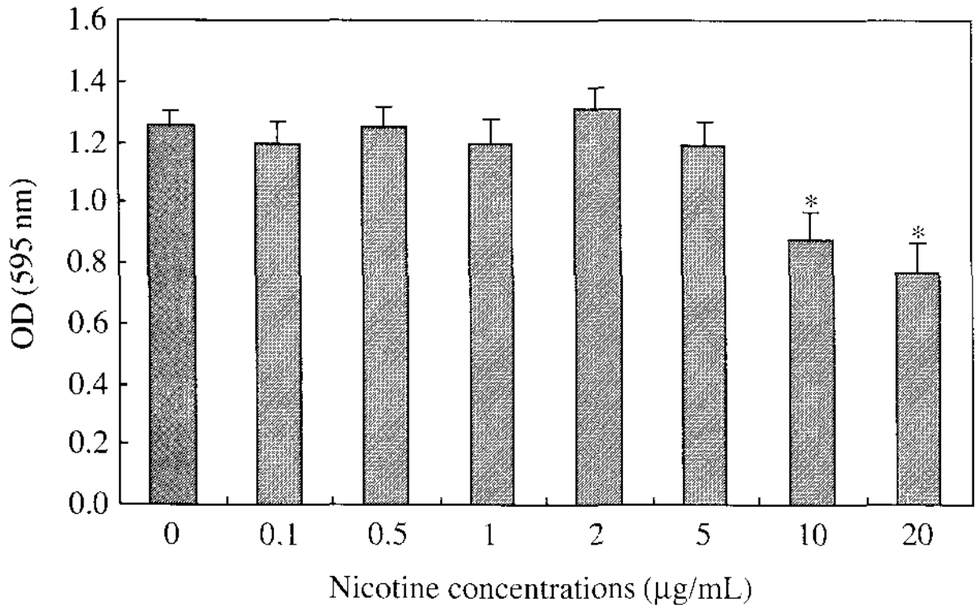

Figure 1. The effect of nicotine on human fetal astrocytes. Human fetal astrocytes are incubated with nicotine at various concentrations and the cellular viability is determined with MTT assay. The results were analyzed using the Mann-Whitney test and when compared with the control group, significant differences ($P<0.05$) are indicated by *.

brospinal fluid (CSF) in PD[4,5]. Previous studies have showed that activation of cultured astrocytes or microglia leads to increased synthesis and release of a number of cytokines including TNF-α[6]. TNF-α kills cultured dopaminergic neurons by an apoptotic mechanism involving mitochondrial dysfunction, free radical generation and activation of the transcription factor NF-κB[5]. Therefore, TNF-α release from glial cells may contribute to a cycle of events in dopaminergic neurons involving oxidative stress and mitochondrial dysfunction, and this may mediate progression of nigral degeneration in PD[6]. It was reported that nicotine pretreatment has considerably decreased microglial activation with significant reduction of tumor necrosis factor (TNF)-α mRNA expression and TNF-α release induced by LPS stimulation[3]. It is well known that NF-kappa B activates the transcription of genes encoding proteins involved with immune or inflammation responses[7]. Thus, this study was intended to explore the role of nicotine pretreatment to inhibit the expressions of TNF-α mRNA in higher proliferative human fetal astrocytes[18] stimulated with IL-1β through the pathway which suppresses the NF-κB activation.

Results

Cytotoxicity of Nicotine on Human Fetal Astrocytes

Cytotoxicity of nicotine on human fetal astrocytes showed that the concentrations of nicotine to reduce significantly the survivability of the cell were over 10 μg/mL (Figure 1).

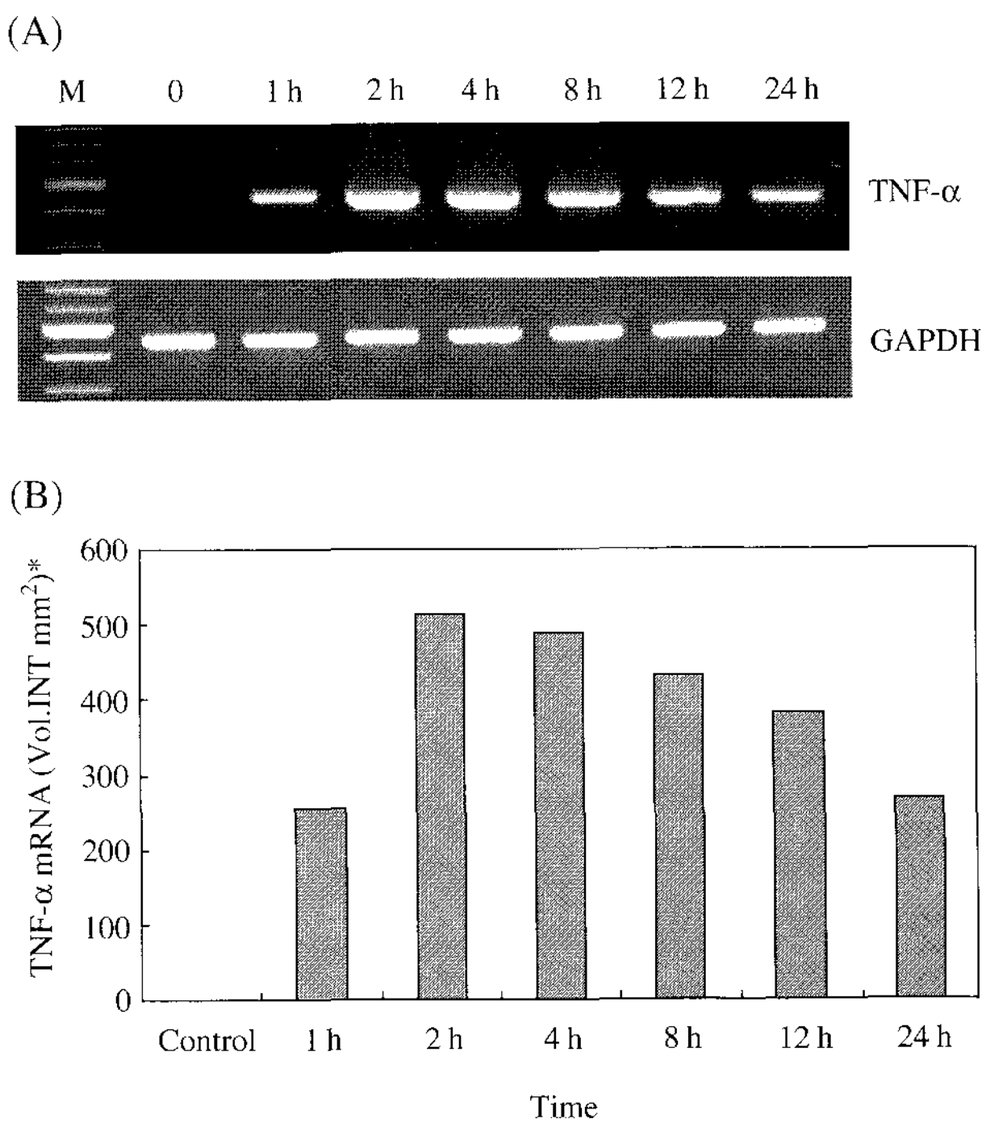

Figure 2. The effect of IL-1β on TNF-α mRNA expression in primary cultured human fetal astrocytes. The astrocytes are treated with IL-1β (100 pg/mL) for the indicated time periods. A, RT-PCRs using TNF-α and GAPDH primers are performed. B, The relative TNF-α mRNA expression levels are analysed using densitometer. *volume intensity count per mm^2.

Expression of TNF-α mRNA after Treatment with IL-1β in Human Fetal Astrocytes

To compare the level of TNF-α mRNA expressions at 1 h, 2 h, 4 h, 8 h, 12 h and 24 h after IL-1β (100 pg/mL) treatment, primary cultured human fetal astrocytes were stimulated by IL-1β for activation. In the cell treated only with IL-1β, TNF-α mRNA expression was induced and its level reached the most high at 2 h and afterwards decreased slowly (Figure 2) but in the IL-1β untreated cell, not induced (data not shown).

The Effect of Nicotine on TNF-α mRNA Expression in Human Fetal Astrocytes

To evaluate the effect of nicotine on the TNF-α mRNA expression, primary cultured human fetal astrocytes were pretreated with nicotine (0.1, 1 and 10 μg/mL) and then pre-incubated for 24 h, 12 h, 8 h, 4 h and 2 h. To induce the TNF-α mRNA expression, the cells were stimulated by IL-1β (100 pg/mL) for 2 h.

Analysis using RT-PCRs showed different TNF-α mRNA expressions in several concentrations of nicotine. In the group pretreated only with nicotine, TNF-α mRNA expression was not detected (data not sho-

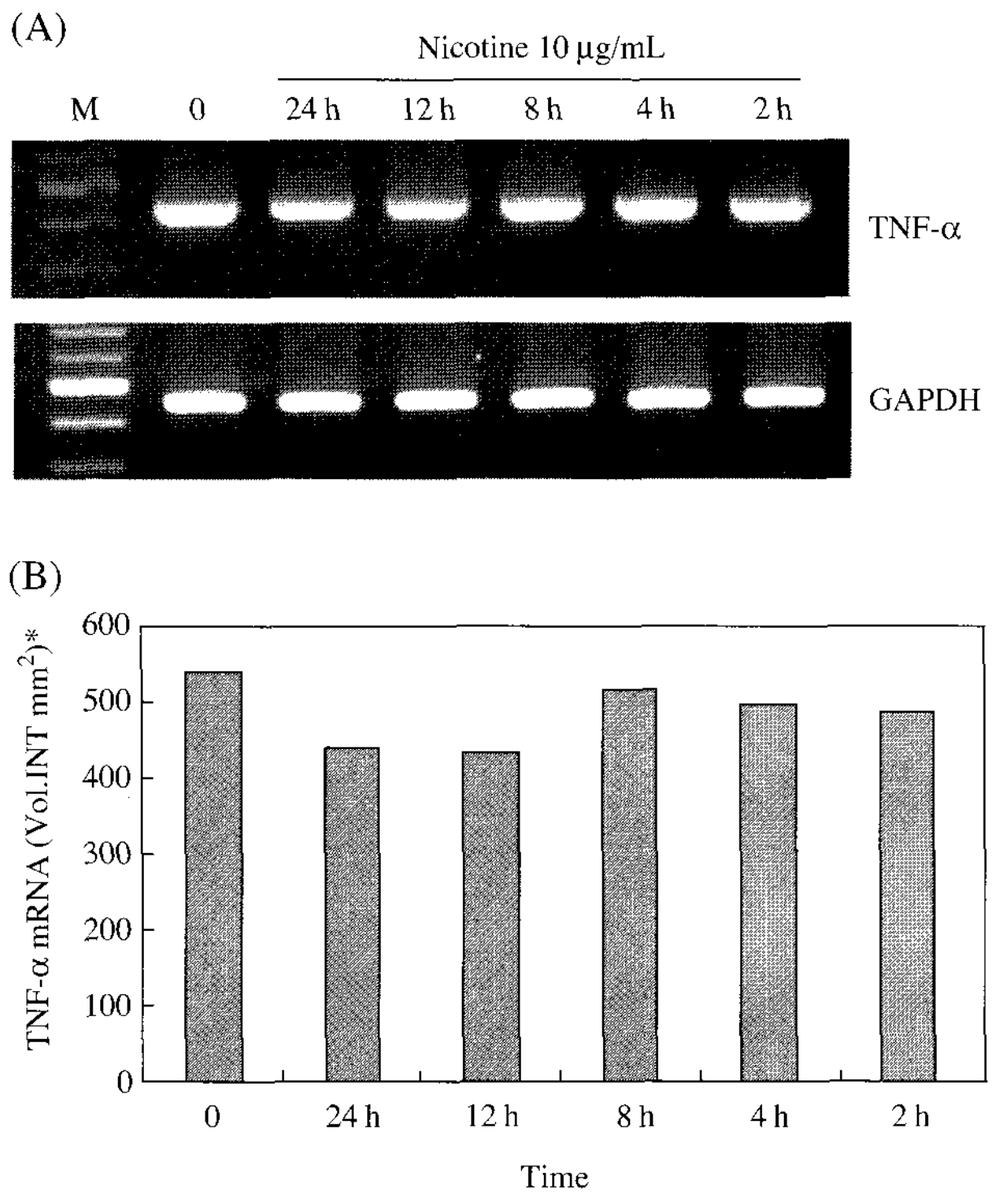

Figure 3. The effect of nicotine (10 μg/mL) on TNF-α mRNA expression in primary cultured human fetal astrocytes. Human fetal astrocytes are treated with 10 μg/mL nicotine for the indicated time periods and then stimulated with IL-1β (100 pg/mL) for 2 h. A, RT-PCRs using TNF-α and GAPDH primers are performed. B, The relative TNF-α mRNA expression levels are analysed using densitometer. *volume intensity count per mm^2.

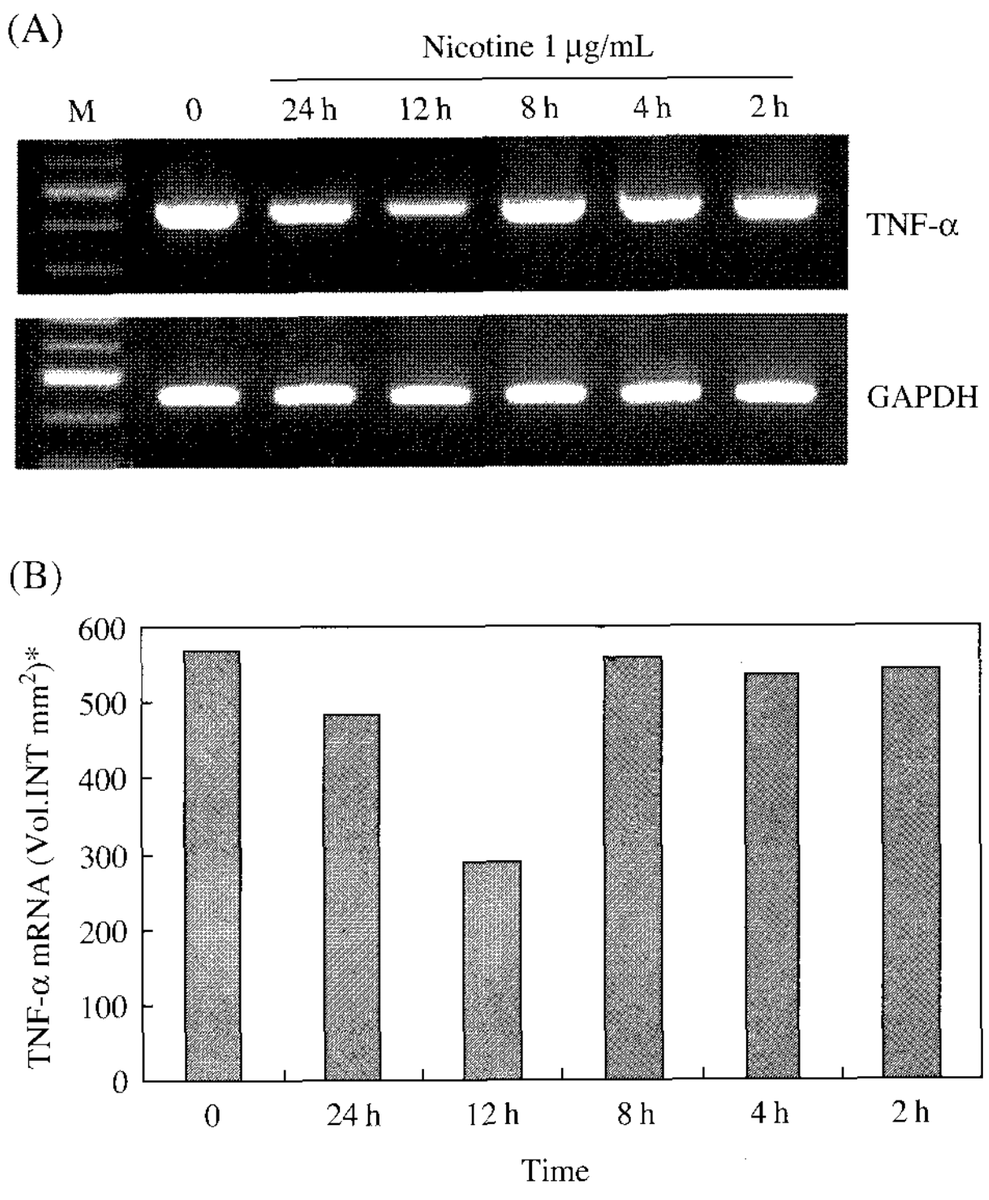

Figure 4. The effect of nicotine (1 μg/mL) on TNF-α mRNA expression in primary cultured human fetal astrocytes. Human fetal astrocytes are treated with 1 μg/mL nicotine for the indicated time periods and then stimulated with IL-1β (100 pg/mL) for 2 h. A, RT-PCRs using TNF-α and GAPDH primers are performed. B, The relative TNF-α mRNA expression levels are analysed using densitometer. *volume intensity count per mm^2.

wn). However, in the group treated only with IL-1β (100 pg/mL), TNF-α mRNA expression was induced and its level reached the most high at 2 h. Therefore, this was considered as the control group. When compared with the control group, groups pretreated with different concentrations of nicotine showed the different patterns of TNF-α mRNA expression according to their pretreatment times (Figures 3, 4 and 5).

In the group pretreated with nicotine (10 μg/mL), the changes of TNF-α mRNA expression were detected at 12 h and 24 h after nicotine pretreatment but not statistically significant (Figure 3). However, the groups pretreated with nicotine 1 μg/mL and 0.1 μg/mL showed the significant reduction of TNF-α mRNA expression at 12 h and 24 h after nicotine pretreatment, respectively, in these two groups (Figures 4 and 5).

Effects of Nicotine Treatment on IL-1β-induced NF-κB Activation

To examine the effects of nicotine treatment on IL-1β-induced NF-κB activation, primary cultured human fetal astrocytes were pretreated with nicotine (0.1, 1 and 10 μg/mL) for 30 min and stimulated by IL-1β (100 pg/mL) for 30 min. And then, NF-κB activity in nuclear protein extracts from the cells, were analyzed by electrophoretic mobility shift assay. In the astrocytes not treated or treated only with nicotine, NF-κB activity was not nearly detected (lane 2 and 3) but in the astrocytes treated only with IL-1β, strong NF-κB activity was shown (lane 7). In the astrocytes pretreated with nicotine and then stimulated by IL-1β, NF-κB activities decreased (lane 4-6) when compared with that of astrocytes treated only with IL-1β (lane 7) (Figure 6).

Discussion

Parkinson's disease (PD) is characterized clinically by rigidity, tremor and bradykinesia, which result from the progressive death of dopaminergic neurons

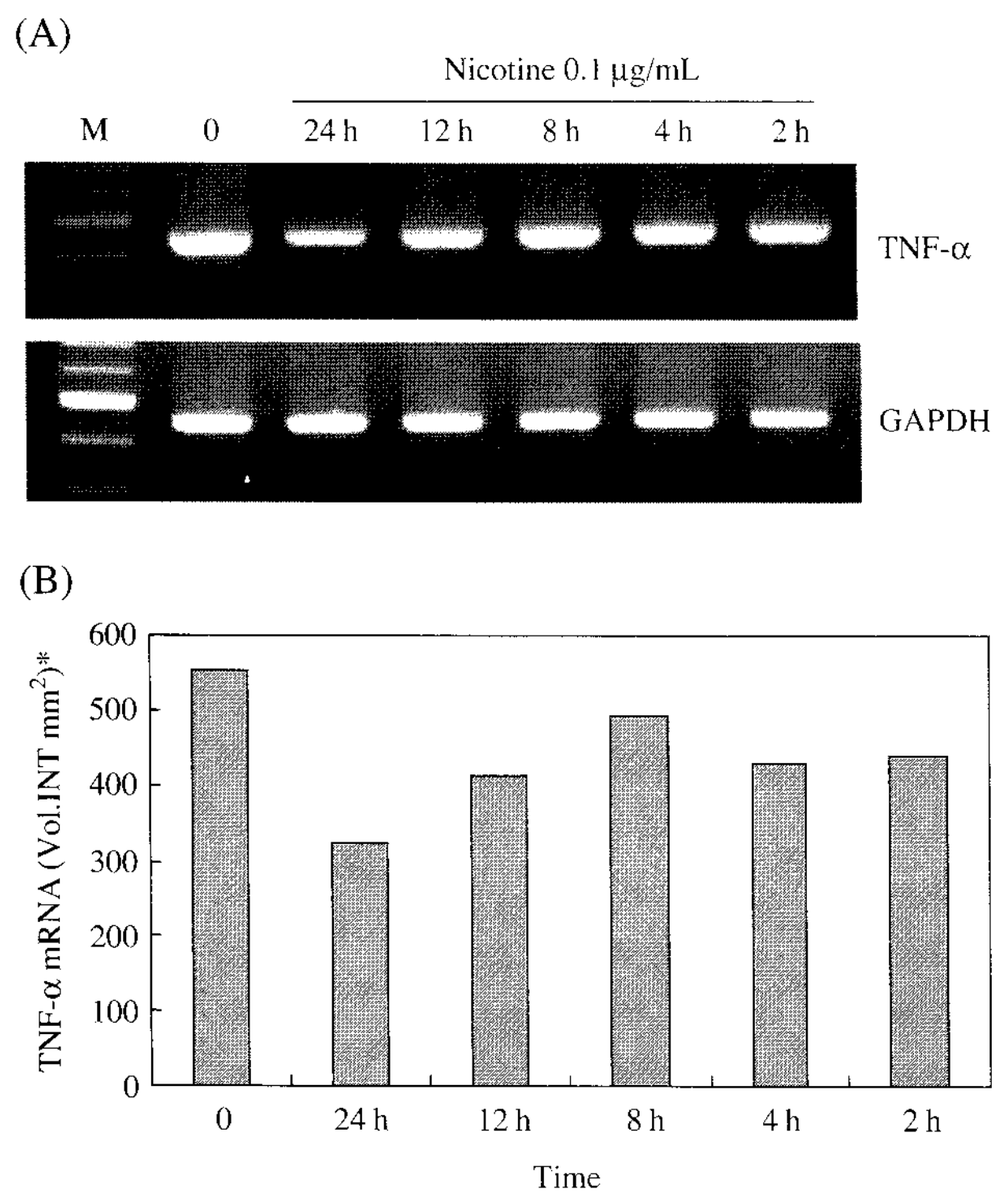

Figure 5. The effect of nicotine (0.1 μg/mL) on TNF-α mRNA expression in primary cultured human fetal astrocytes. Human fetal astrocytes are treated with 0.1 μg/mL nicotine for the indicated time periods and then stimulated with IL-1β (100 pg/mL) for 2 h. A, RT-PCRs using TNF-α and GAPDH primers are performed. B, The relative TNF-α mRNA expression levels are analysed using densitometer. *volume intensity count per mm^2.

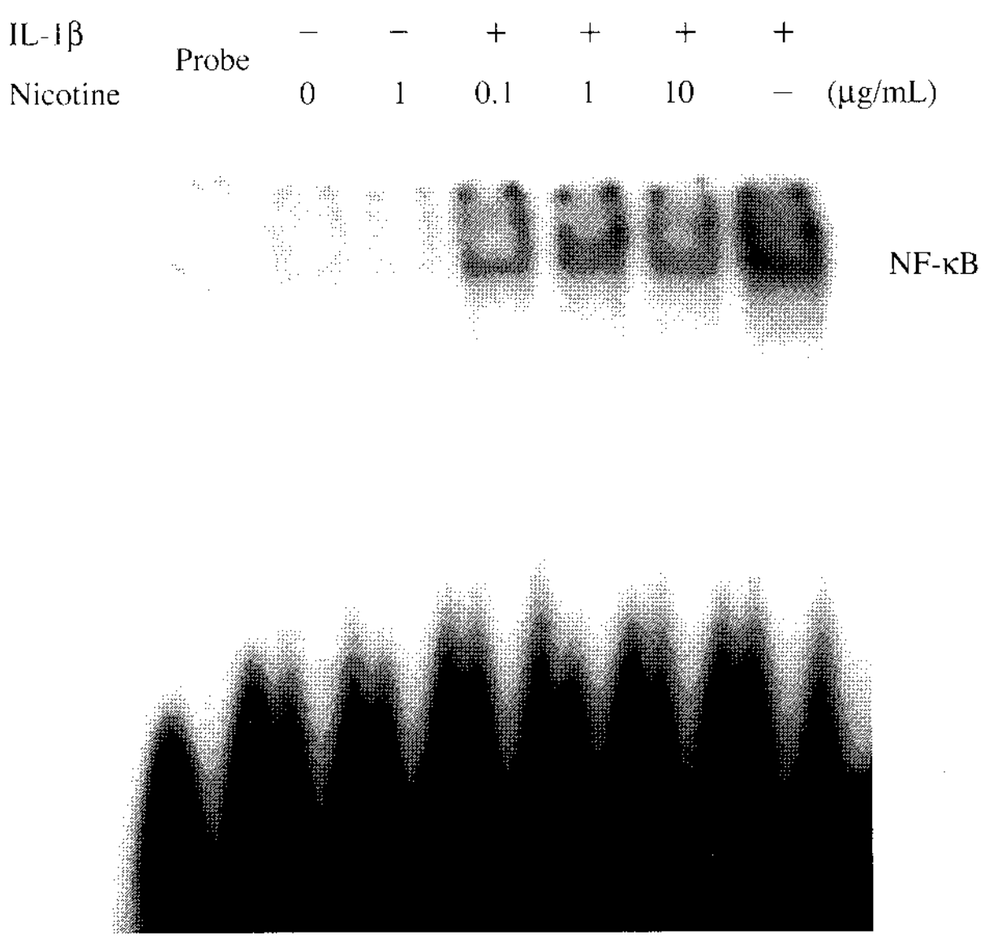

Figure 6. Effects of nicotine treatment on IL-1β-induced NF-κB activation. Electrophoretic mobility shift analysis of NF-κB binding activity in nuclear protein extracts from IL-1β activated, nicotine-pretreated human fetal astrocytes. Nuclear extracts were incubated with ^{32}P-end-labeled NF-κB consensus oligonucleotide in binding reactions. The binding complexes were analyzed on nondenaturing polyacrylamide gels in 0.25 × TBE and visualized by autoradiography.

in the substantia nigra. The etiology of PD is diverse and complex[15]. In PD, the levels of TNF-α expressed by astrocytes and microglia are elevated in striatum and CSF[4,5]. In the effects of TNF-α to neurons, it is known that TNF-α kills cultured dopaminergic neurons by an apoptotic mechanism involving mitochondrial dysfunction, free radical generation and activation of the transcription factor NF-κB[5]. On the basis of these reports, it was expected that if the suppression of TNF-α expressed by astrocytes and microglia might decrease the cell death of dopaminergic neuron. By the way, nicotine have been recently reported to inhibit tumor necrosis factor-α (TNF-α) production in human macrophages as well as in mouse microglial cultures[16]. Therefore, this study was intended to evaluate whether in human fetal astrocytes, nicotine pretreatment might suppress mRNA expression of IL-1β-induced TNF-α. To estimate the effects of nicotine on the TNF-α mRNA expression, primary cultured human fetal astrocytes were pretreated with nicotine (0.1, 1 and 10 μg/mL) and were preincubated for several hours and then for inducing the TNF-α mRNA expression, were stimulated by IL-1β. TNF-α mRNA expression levels of the groups pretreated with lower nicotine doses (0.1, 1 μg/mL) were reduced when compared with that of the control group which was not pretreated with nicotine. This meant that the nicotine pretreatment inhibited TNF-α mRNA expression. The inhibition times of nicotine on the IL-1β-induced TNF-α mRNA expression were the inverse correlation to the concentrations of nicotine. In higher dose of nicotine pretreatment (1 μg/mL), TNF-α mRNA expression was early inhibited significantly at 8 h but in lower dose of nicotine pretreatment (0.1 μg/mL), was late inhibited significantly at 24 h.

For elucidating the pathway of inhibitory effect of nicotine on TNF-α mRNA expression, a inflammatory cytokines-closely related signaling protein, nuclear factor-kappaB, NF-κB was analyzed. It is well known that one of several signaling proteins, NF-κB can be activated by exposure of cells to inflammatory cytokines such as TNF or IL-1 and activates the transcription of genes encoding proteins involved with immune or inflammation responses[7]. NF-κB activates the immune cells by upregulating the expression of many cytokines which are IL-1β, IL-2, IL-6, IL-8, GM-CSF, TNF-α, TNF-β, IFN-β[14]. On the other

hand, it is known that nicotine inhibits the production of inflammatory mediators in U937 cells through the modulation of NF-κB activation[17]. From the several reports, it was expected that TNF-α was regulated through the NF-κB signaling pathway in human fetal astrocytes. This study showed that in human fetal astrocytes, IL-1β stimulation induced NF-κB activation (Figure 6) and simultaneously increased TNF-α mRNA expression (Figure 2). Moreover, in nicotine pretreatment conditions, NF-κB activation and TNF-α mRNA expression stimulated by IL-1β were suppressed depending on the nicotine pretreatments (Figures 3 and 6).

In conclusion, in human fetal astrocytes, nicotine is capable of suppress the TNF-α mRNA expression and NF-κB is a possible signal mediator of TNF-α mRNA expression.

Methods

Cell Culture

Human fetal astrocytes were obtained anonymously as discarded tissue under a Wonju College of Medicine, Yonsei University Institutional Review Board (IRB).

Human fetal astrocytes were isolated from the brain tissue of human fetus which were aborted for therapeutic purposes between 20 and 25 weeks[8]. The purity of astrocytes were assessed by staining for glial fibrilary acidic protein (GFAP), an astrocyte-specific protein[9]; the cultured cells were >95% GFAP positive[10]. Cells were cultured in Dubecco's modified Eagle's medium (DMEM; Gibco BRL, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acid, 100 U/mL penicillin and 100 μg/mL streptomycin.

Measurement of Cell Viability MTT

Cell viability was measured by using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases[11]. Human fetal astrocytes were seeded in 96-well plates at a density of 4×10^4 cells per well and were treated with various concentrations of nicotine (Sigma, St. Louis, MO) (0.1, 0.5, 1, 2, 5, 10 or 20 μg/mL) at 37°C. After incubation for up to 24 h, the medium was incubated with 10 μL of 5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT; Sigma) solution for 4 h. Then the culture medium with MTT was removed and 200 μL dimethyl sulphoxide (DMSO) was added to each well to dissolve the formazan. Absorbance was measured with ELISA reader (read at 595 nm; Multiskan EX, Thermo Electron Corporation, France).

Induction of the Expression of TNF-α mRNA in Human Fetal Astrocytes with IL-1β

Cultured human fetal astrocytes were detached with 0.25% trypsin and were seeded in 6-well plates at a density of 2×10^5 cells per well and were cultured in DMEM supplemented with 10% fetal bovine serum for 24 h. Afterwards, the medium was changed with serum free DMEM. In the nicotine pretreatment group, several concentrations of nicotine were pretreated for 2, 4, 8, 12, and 24 hours and in control group, serum free DMEM was treated. At 1, 2, 4, 8, 12 and 24 hours after 100 pg/mL of IL-1β (R & D systems, Minneapolis, MN, USA) treatment for 2 hours, RNAs were isolated from the each group.

RNA Isolation

Total cellular RNAs were isolated by Trizol reagent. Briefly, cells were washed once in phosphate buffered saline (PBS) and lysed directly in culture dishes. RNAs were extracted using 1 mL of Trizol reagent per 1×10^6 cells and the amount of RNA was determined by spectrophotometery. RNAs of human fetal astrocytes were isolated at 0, 2, 4, 8, 12 and 24 hours.

cDNA Synthesis and PCR

Reverse transcriptions of total RNA 2 μg isolated from human fetal astrocytes were performed with 1 μg oligo-dT primer (Promega, Madison, WI) followed by the addition of a reaction mixture containing buffer, 40 U RNase inhibitor (Promega), 200 U MMLV reverse transcriptase (Promega), and 1 mM dNTP mix in a final volume of 25 μL. The mixture was incubated at 42°C for 1 hr and terminated at 95°C for 5 min: cDNAs were synthesized. To quantitate the concentration of TNF-α induced by IL-1β, TNF-α PCR product was amplified after 95°C for 5min for 30 cycles, at 94°C for 30 sec, at 60°C for 30 sec, and at 72°C for 1 min, followed by 7 min extension at 72°C and terminated at 4°C. The primer sets were TNF-α primers (sense 5′-GACTGACAAGCCTGTAGCCCATGTTGT AGCA-3′, antisense 5′-CAATGATCCCAAAGTAG-ACCTGCCCAGAC-3′) and GAPDH primers (sense 5′-ACCACAGTCCATGCCATCAC-3′, antisense 5′-TCCACCACCCTGTTGCTGTA-3′). All of the PCR products were viewed under ultraviolet light after 1 % agarose gel electrophoresis and staining in ethidium bromide. The semi-quantification results were analyzed using the Quantity One software (Bio-Rad, Hecules, CA, USA).

Preparation of Nuclear Extracts

Human fetal astrocytes were stimulated with IL-1β

(100 pg/mL) for 2 hours[8], and the nuclear extracs were prepared[12]. Briefly, cells were harvested by scraping and then pelleted. Next the cells were resuspended in 1 mL of buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothretol, 0.5 mM PMSF and 10 μg/mL of leupeptin and aprotinin), and incubated on ice for 10 min. The cells were then lysed using 0.6% Nonidet-P 40, and a nucleus pellet was obtained. The nucleus pellets were resuspended in 30 μL of hypertonic buffer C (20 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 μg/mL of leupeptin and aprotinin) and the solution was shaken at 4°C for 15 min. After centrifugation of the solution, the supernatant was collected and stored at −70°C until use, and protein concentrations were determined using a Bradford protein assay (Bio-Rad, Hecules, CA, USA).

Electrophoretic Mobility Shift Assay (EMSA)

NF-κB activation was determined by electrophoretic mobility shift assay using the method described by Chaturvedi *et al.*[8,13]. Briefly, 8 μg of nuclear extracts were incubated for 20 min at room temperature with or without a competitor in the binding buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 5 mM DTT and 1 μg of poly dI/ dC (Amersham, Little Chalfont, UK)). ^{32}P-end-labeled double stranded NF-κB binding oligonucleotide (5′-AGTTGAGGGGATTTCCCAGGC-3′), was added to the reaction mixture and incubated on ice for an additional 30 min. The reaction mixtures were then resolved by 6% nondenaturing polyacrylamide gel electrophoresis. After electrophoresis, dried gel was exposed to X-ray film (KODAK) at −70°C for over 12 hours, and the specificity of binding was examined by competition with the unlabeled oligonucleotide.

Statistical Analysis

Results of cellular viability in several concentrations of nicotine treatment were analyzed by the Mann-Whitney U-test.

Acknowledgements

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References

1. Quik, M., Bordia, T. & O'Leary, K. Nicotinic receptors as CNS targets for Parkinson's disease. *Biochem Pharmacol* **74**:1224-1234 (2007).
2. Picciotto, M. R. & Zoli, M. Neuroprotection via nAChRs: the role of nAChRs in neurodegenerative disorders such as Alzheimer's and Parkinson's disease. *Front Biosci* **13**:492-504 (2008).
3. Park, H. J. *et al.* Neuroprotective effect of nicotine on dopaminergic neurons by anti-inflammatory action. *Eur J Neuroscience* **26**:79-89 (2007).
4. CastanÄ, O. A., Herrera, A. J., Cano, J. & Machado, A. Lipopolysaccharide intranigral injection induces inflammatory reaction and damage in nigrostriatal dopaminergic system. *J Neurochem* **70**:1584-1592 (1998).
5. Hunot, S. *et al.* Nuclear translocation of NF-κB is increased in dopaminergic neurons of patients with Parkinson disease. *Proc Natl Acad Sci* **94**:7531-7536 (1997).
6. McNaught, K. S. & Jenner, P. Dysfunction of rat forebrain astrocytes in culture alters cytokine and neurotrophic factor release. *Neurosci Lett* **285**:61-65 (2000).
7. Baldwin, A. S. Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* **14**:649-683 (1996).
8. Lee, S. S. *et al.* Characterization of the two genes differentially expressed during development in human fetal astrocytes. *Yonsei Med J* **44**:1059-1068 (2003).
9. Winkler, M. K. & Beveniste, E. N. Transforming growth factor-beta inhibition of cytokine-induced vascular cell adhesion molecule-1 expression in human astrocytes. *Glia* **22**:171-179 (1998).
10. Park, J. Y. *et al.* Establishment of a culture method and characterization for human fetal astrocytes. *Kor J Virol* **31**:39-45 (2001).
11. Yamamoto, T. *et al.* Kinetic characterization of the nitric oxide toxicity for PC12 cells: effect of half-life time of NO release. *Eur J Pharmacol* **397**:25-33 (2000).
12. Duhamel-Clerin, E. *et al.* Cellular expression of an HMGCR promoter-CAT fusion gene in transgenic mouse brain: evidence for a developmental regulation in oligodendrocytes. *Glia* **11**:35-46 (1994).
13. Hanaka, S., Abe, T., Itakura, H. & Matsumoto, A. Gene expression related to cholesterol metabolism in mouse brain during development. *Brain Dev* **22**:321-326 (2000).
14. Ghosh, S., May, M. J. & Kopp, E. B. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* **16**:225-260 (1998).
15. Giasson, B. I. & Lee, V. M. A new link between pesticides and Parkinson's disease. *Nat Neurosci* **3**:1227-1228 (2000).
16. De Simone, R., Ajmone-Cat, M. A., Carnevale, D. & Minghetti, L. Activation of alpha7 nicotinic acetylcholine receptor by nicotine selectively up-regulates cyclooxygenase-2 and prostaglandin E2 in rat microglial cultures. *J Neuroinflammation* **2**:4 (2005).
17. Sugano, N., Shimada, K., Ito, K. & Murai, S. Nicotine

inhibits the production of inflammatory mediators in U937 cells through modulation of nuclear factor-kappaB activation. *Biochem Biophys Res Commun* **252**: 25-28 (1998).

18. Yong, V. W. & Antel J. P. Culture of glial cells from human brain biopsies. In *Biology and Pathology of Astrocyte-Neuron Interactions* (ed. Fedoroff S.), pp. 271-281, Humana, New York (1992).