BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **252,** 25–28 (1998)
ARTICLE NO. RC989599

Nicotine Inhibits the Production of Inflammatory Mediators in U937 Cells through Modulation of Nuclear Factor-*k*B Activation

Naoyuki Sugano,[1] Koichi Shimada, Koichi Ito, and Seidai Murai
Department of Periodontology, Nihon University School of Dentistry, 1-8-13 Kanda-Surugadai, Chiyoda-ku, Tokyo, 101-8310 Japan

Received September 22, 1998

Cigarette smoke is a major risk factor for lung cancer and respiratory infections. This increased susceptibility may result from cigarette smoke-induced impairment of the immune system. In this study, we evaluated the effect of nicotine on the production of inflammatory mediators by activated macrophages. Pretreatment with nicotine caused a significant inhibition of LPS-induced IL-1, IL-8, and PGE_2 expression at the transcriptional level in U937 cells. Nicotine inhibited the activation of a transcription factor, NF-kB, which in turn, binds to and mediates transcriptional activation of these genes. These inhibitory effects of nicotine may contribute to cigarette smoke-induced immunosuppression.

It is now well documented that cigarette smoke increases the incidence of diseases including lung cancer and respiratory infections. It has been speculated that the increased susceptibility of smokers to cancer and infections may reflect cigarette smoke-induced impairment of the immune system (1–4). Among the various immune cells, macrophages play a central role in conferring a first line of defense against invading pathogens. Inflammatory mediators secreted by activated macrophages play a crucial role in the protective function of macrophages against exogenous stimuli.

Cigarette smoke contains a range of compounds with pharmacological activity, of which nicotine is the most important component. There is increasing evidence that chronic nicotine treatment leads to inhibition of the cellular as well as the humoral immune system, indicating that nicotine is a major immunosuppresive component in cigarette smoke (5–7). However, the mechanisms underlying these inhibitory effects are unknown. It is clear that the transcription factor NF-*k*B plays a key role in the regulated expression of a large number of inflammatory mediators (8). In the present study we investigated the effect of nicotine on lipopolysaccharide (LPS)-induced IL-1β, IL-8, and PGE_2 expression and its regulation at the transcriptional level. Our results demonstrate for the first time that nicotine can impair macrophage function by interfering with the activation of NF-*k*B.

MATERIALS AND METHODS

Cell culture and nicotine treatment. The U937 cell line was maintained in RPMI1640 medium supplemented with heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (0.1 μg/ml), and L-glutamine (2 mM) in 75 cm^2 tissue culture flasks at 37°C in 5% CO_2 in air and high humidity. Nicotine (98% pure) was purchased from Sigma (St. Louis, MO). A 10% stock solution of nicotine was prepared in phosphate-buffered saline, neutralized to pH 7.2 with HCl, which was prepared freshly prior to each experiment. For differentiation, logarithmic phase cultures at $1x10^6$ cells/ml were preincubated with 10 nM phorbol myristate acetate (PMA, Sigma) for 48 h, after which they were made quiescent for 24 h by incubation in PMA-free fresh medium before the experiments (9). Cultures were pre-treated with nicotine for 30 min before the addition of 1 μg/ml LPS (*E. coli* 0111:B4, Sigma), and then cultured for various times.

Protein synthesis. Samples of the supernatants were collected 24 h later and assayed for IL-1β, IL-8, and PGE_2 with ELISA kits according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

Reverse transcriptase polymerase chain reaction (RT-PCR). Six hours after stimulation, 1 μg of total RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Promega, Madison WI) to evaluate IL-1β, IL-8, and cyclooxygenase-2 (COX-2) gene expression. Twenty percent of the resulting cDNA products were analyzed by 25 cycles of PCR with primers for β-actin, IL-1β, IL-8 (Stratagene, La Jolla, CA), and COX-2 (10).

Electrophoretic mobility shift assay (EMSA). NF-*k*B activation was assessed by EMSA, as described previously (11). Thirty minutes after stimulation, nuclear extracts were prepared, and 3 μg of each extract was incubated with a ^{32}P-end-labeled NF-*k*B consensus probe (GGGACTTTCC, Promega).

Western blot analysis. Western blotting was performed as described previously (13). Polyclonal antibodies against I*k*Bα and COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA) were used to assess I*k*B degradation and COX-2 expression.

[1] To whom correspondence should be addressed. Fax: 81-3-3219-8349. E-mail: sugano-n@dent.nihon-u.ac.jp.

0006-291X/98 $25.00

TABLE 1

The Effect of Nicotine on LPS-Induced IL-1β, IL-8, and PGE_2 Production

Treatment	(pg/ml) IL-1	IL-8	PGE_2
Control	34.2 ± 8.8	49.6 ± 5.8	76.8 ± 4.3
LPS	972.2 ± 145.8	295.2 ± 18.5	2345.3 ± 504.8
LPS + Nicotine (1 μg/ml)	2.8 ± 0.1	0.8 ± 0.1	101.2 ± 10.2
LPS + Nicotine (0.1 μg/ml)	975.7 ± 113.4	301.7 ± 12.4	2895.2 ± 334.1
Nicotine (1 μg/ml)	2.4 ± 0.1	0.8 ± 0.1	67.9 ± 9.0

Note. The U937 cells were induced to differentiate with PMA and allowed to become quiescent. A 30-min pretreatment with nicotine was followed by 24 h of incubation with 1 μg/ml LPS. Culture supernatants were assayed for IL-1β, IL-8 and PGE_2 using ELISA kits. The results are presented as means ± standard deviation of three experiments. Each experiment was done in triplicate.

RESULTS AND DISCUSSION

Effect of nicotine treatment on IL-1β, IL-8, PGE_2, and COX-2 synthesis. Initially, experiments were carried out to investigate the effect of nicotine treatment on inflammatory mediator synthesis. The cells were pretreated with nicotine (1 μg/ml or 0.1 μg/ml) for 30 min prior to 24-h stimulation with LPS (1 μg/ml). Culture supernatants were assayed for IL-1β, IL-8, and PGE_2 protein levels, as described in Methods. Pretreatment with 1 μg/ml nicotine inhibited LPS-induced IL-1β, IL-8, and PGE_2 protein production, but 0.1 μg/ml nicotine had no inhibitory effect (Table 1). Nicotine did not influence the viability of the cells at 1 μg/ml, as determined by their ability to exclude trypan blue dye (data not shown).

COX catalyzes the first committed step in the formation of prostaglandins. Arachidonic acid released from membrane phospholipids by phospholipase A2 is converted to prostaglandin H_2 (PGH_2) through the action of COX; PGH_2 is then converted to PGE_2 by cell-specific synthases. There are two COX enzymes, referred to as COX-1 and COX-2. COX-1 is thought to mediate the physiological responses to circulating hormones that require constant or rapid modulation, the so-called housekeeping functions. In contrast, COX-2 is an inducible enzyme that is normally absent from cells, but is expressed in response to cytokines, growth factors or mitogens (12). Therefore, we examined the effect of nicotine on COX-2 expression by western blotting using anti COX-2 antibodies. Whole cell lysates prepared from the cells pretreated for 30 min with nicotine and then activated with 1 μg/ml LPS for 6 h, were subjected to western blotting. As shown in Figure 1A, LPS stimulation resulted in increased amounts of COX-2. Pretreatment with 1 μg/ml nicotine inhibited LPS-induced COX-2 production, but 0.1 μg/ml nicotine had no inhibitory effect.

Effect of nicotine treatment on IL-1β, IL-8, and COX-2 mRNA production. To confirm that nicotine acted through inhibition of a transcription-related mechanism, RT-PCR was used to monitor the U937 message. LPS stimulated the production of IL-1β, IL-8, and COX-2 mRNA, which was inhibited by 1 μg/ml nicotine (Fig. 1B). However, 0.1 μg/ml nicotine had no significant inhibitory effect. The housekeeping gene β-actin was unaffected by nicotine.

Effects of nicotine treatment on NF-kB activation. Transcriptional activation of IL-1β, IL-8, and PGE_2 has been shown to involve the transcription factor NF-*k*B (8,13–15). Our next experiment was designed to investigate if nicotine pretreatment alters LPS-induced NF-*k*B activation. Nuclear extracts prepared from the cells pretreated for 30 min with nicotine and then activated with LPS for 30 min were incubated with ^{32}P-labeled NF-*k*B consensus oligonucleotide in binding reactions. The binding complexes were then analyzed on nondenaturing polyacrylamide gels and

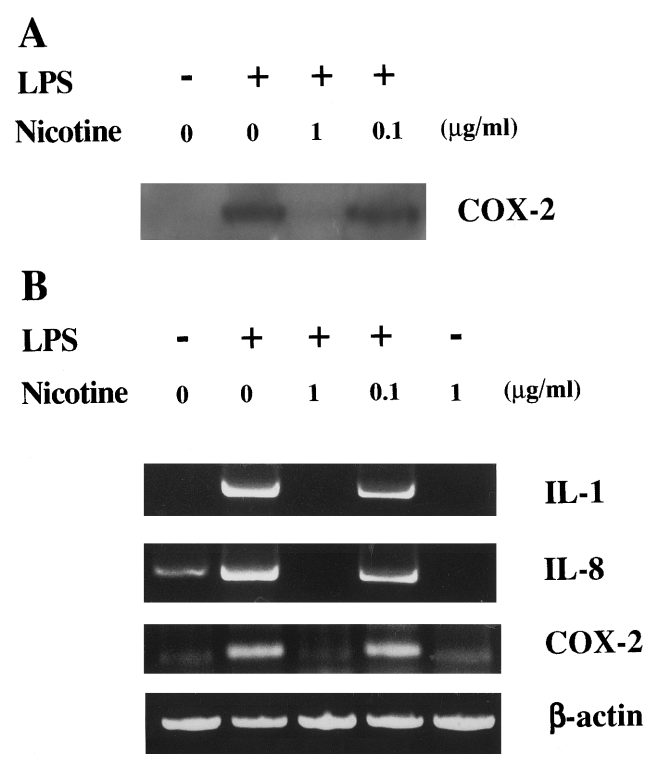

FIG. 1. (A) The effect of nicotine on LPS-induced COX-2 protein expression. A 30-min pretreatment with nicotine was followed 6 h of incubation with LPS. Whole cell lysates were subjected to western blot analysis using an antibody against COX-2. (B) The effect of nicotine on LPS-induced IL-1β, IL-8, and COX-2 mRNA expression. LPS treatment was carried out for 6 h. RT-PCR analysis of mRNA was performed as described in Materials and Methods. The results represent 3 independent experiments.

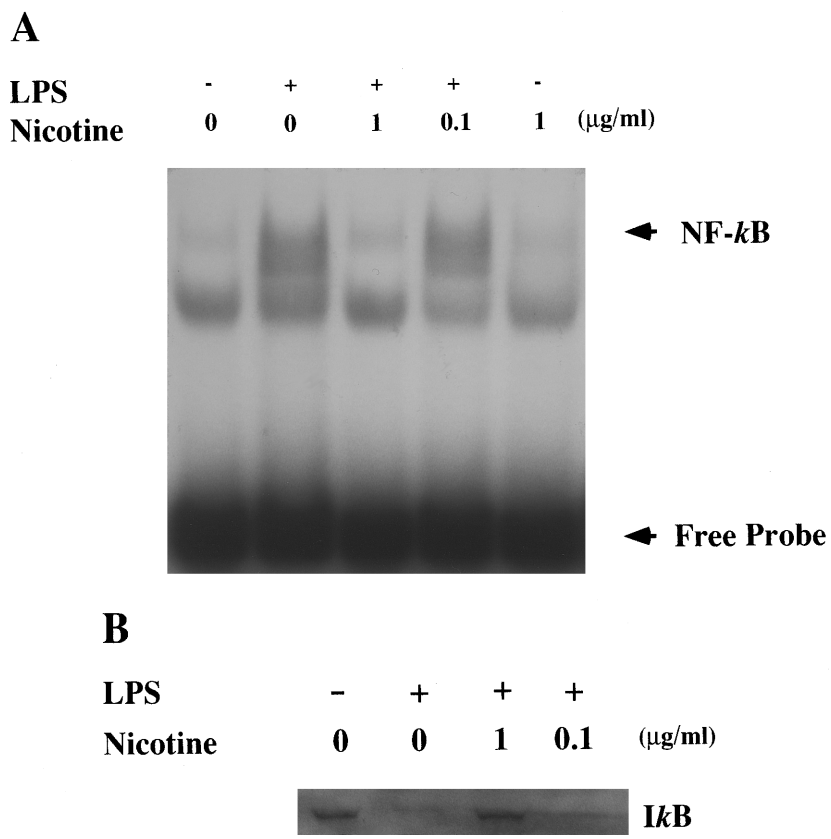

FIG. 2. (A) Electrophoretic mobility shift analysis of NF-*k*B binding activity in nuclear protein extracts from LPS-activated, nicotine-pretreated U937 cells. Nuclear extracts were incubated with ^{32}P-end-labeled NF-*k*B consensus oligonucleotide in binding reactions. The binding complexes were analyzed on nondenaturing polyacrylamide gels in 1xTAE and visualized by autoradiography. (B) Effect of nicotine treatment on cytosolic I*k*B protein levels in U937 cells. Cytosolic extracts were prepared 15 min after LPS stimulation and analyzed by western blotting using an antibody against I*k*Bα.

visualized by autoradiography. The NF-*k*B binding complexes are indicated in Figure 2A. Our results showed that 1 μg/ml nicotine significantly inhibited LPS-induced NF-*k*B activation, whereas 0.1 μg/ml nicotine had no significant inhibitory effect.

The active form of NF-*k*B is normally sequestered in the cytoplasm by the inhibitory protein, I*k*B. NF-*k*B activation is mediated by a process involving I*k*B phosphorylation on specific serine residues, ubiquitination, and degradation, which then allows the freed active form to translocate into the nucleus to initiate gene transcription by binding to its consensus element (8). To identify the mechanism by which translocation of the active form of NF-*k*B is modulated by nicotine, we investigated the effect of nicotine on I*k*B protein levels. Cytosolic extracts were prepared from the cells pretreated for 30 min with nicotine and then activated with LPS for 15 min. Samples were analyzed by western blotting using anti I*k*Bα antibodies. I*k*B protein was almost non-detectable in cells that had been treated with LPS alone (Fig.2B). However, in the nicotine (1 μg/ml) pretreated cells that had been activated with LPS there was significant accumulation of I*k*B protein in the cytosolic extract. These findings strongly suggest that the inhibitory effect of nicotine is associated with NF-*k*B activation and I*k*B degradation. It has been reported that nicotine inhibits receptor-induced Ca^{2+} mobilization and up-regulation of 1,4,5-triphosphate synthesis in T cells, although its site of action on the LPS-induced signal transduction pathway leading to I*k*B degradation remains unclear (16).

In conclusion, nicotine has inhibitory effects on IL-1β, IL-8, and PGE_2 production through modulation of NF-*k*B activation. These observations may account for the fact that smokers show increased susceptibility to infections, but a decreased susceptibility to some autoimmune diseases such as ulcerative colitis.

ACKNOWLEDGMENTS

This work was supported by Nihon University Research Grant for assistant and Sato fund Nihon University School of Dentistry.

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