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Nicotinic acetylcholine receptor activation mediates nicotine-induced enhancement of experimental periodontitis

T. Breivik[1,2], Y. Gundersen[2], P. Gjermo[1], S. von Hörsten[3], P. K. Opstad[2]

[1]Department of Periodontology, Faculty of Dentistry, University of Oslo, Oslo, Norway, [2]Norwegian Defence Research Establishment, Division of Protection, Kjeller, Norway and [3]Experimental Therapy, Franz-Penzoldt-Center, Friedrich-Alexander-University Erlangen-Nuremberg, Germany

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Background and Objective: Smokers have an increased risk of developing periodontitis as well as showing more rapid progression and resistance to treatment of the disease, but the biological mechanisms are poorly understood. Our objective was to investigate putative biological mechanisms by which nicotine may enhance the susceptibility and thus the course of periodontitis in an animal model.

Material and Methods: Ligature-induced periodontitis was applied in periodontitis-susceptible Fischer 344 rats. The animals were given daily intraperiotonal (i.p.) injections of the nicotinic acetylcholine receptor (nAChR) antagonist mecamylamine (1 mg/kg) 45 min before subcutaneous (s.c.) injections in the neck skin with nicotine (0.8 mg/kg), or treated with the same amount of saline i.p. and nicotine s.c., or with mecamylamine and saline. Control rats received i.p. and s.c. injections of saline only. Periodontal bone loss was assessed when the ligatures had been in place for 3 weeks. Two hours before decapitation, all rats received lipopolysaccharide (LPS; 100 μg/kg, i.p.) to induce a robust immune and stress response.

Results: Compared with saline/saline-treated control rats, saline/nicotine-treated rats developed significantly more periodontal bone loss, and LPS provoked a significantly smaller increase in circulating levels of the cytokines tumour necrosis factor α (TNF-α), transforming growth factor 1β (TGF-1β) and interleukin-10 (IL-10). Mecamylamine pretreatment of nicotine-treated rats abrogated the increased periodontal bone loss and the LPS-induced TNF-α decrease, but had no significant effects on the levels of TGF-1β and IL-10, or the stress hormone corticosterone.

Conclusion: The results indicate that nicotine enhances susceptibility to periodontitis via nAChRs, which may act via suppressing protective immune responses through the cholinergic anti-inflammatory pathway.

Torbjørn Breivik, Department of Periodontology, Faculty of Dentistry, University of Oslo, PO Box 1109, Blindern, Oslo, Norway
Tel: +47 70121221
Fax: +47 70124003
e-mail: tbreivik@odont.uio.no

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Cigarette smokers have an increased risk of developing periodontitis (1–3). The risk seems to be dose dependent and correlates with the duration of tobacco use (4,5). Augmented colonization of subgingival pathogenic bacteria (periodontopathogens), which include some gram-negative bacterial species, are currently regarded as initiating agents for periodontitis (6), and there is evidence of an increased colonization of subgingival perio-

dontopathogens in smokers (7). The periodontal tissue destruction seems mainly to be mediated by substances such as matrix metalloproteinases and reactive oxygen species (8,9). These tissue destructive components are most likely to be released from activated innate immune cells fighting and destroying the periodontopathogens that are infecting the gingival connective tissues (10). The pathophysiological mechanisms leading to this cascade of events are, however, poorly understood.

Recently, is has become clear that the brain is an important regulator of peripheral immune system responses via the autonomic nervous system and the neuro-endocrine (hormonal) systems, including the peptinergic/sensory system, the sympathetic nervous system (SNS) and the hypothalamic–pituitary–adrenal (HPA) axis (11–13). Our previous experiments in rats have revealed that the responsiveness of the SNS and HPA axis (which together comprise the stress response system) plays a significant role in the susceptibility and progression of periodontitis (14–17), and that the increased susceptibility to periodontitis, as found in patients with severe anxiety and major depression, may result from an over-responding stress response system (18,19).

Every inflammatory response, including but not limited to periodontitis, is associated with local and systemic cytokine responses, many of which are induced by lipopolysaccharides (LPS). Cytokines are proteins released from activated immune and tissue cells, which are key regulators of inflammatory and immune responses. Lipopolysaccharides are biologically active structural components of the outer cell membrane of all gram-negative bacteria, and a potent pathogen-associated molecular pattern (PAMP) as well as a cytokine inducer (20). Moreover, LPS is used experimentally to study how gram-negative bacterial infections and immune signals from the periphery activate the immunoregulatory systems within the brain (21). There is now accumulating evidence that the brain regulates immune system responses, including cytokine responses, not only via the peptinergic/sensory system and the stress response system, but also via the parasympathetic nervous system (PSNS). Cytokines released from activated immune cells may signal the brain through sensory afferent nerves of the PSNS. The brain network then activates efferent parasympathetic (cholinergic) nerves, leading to release of the neurotransmitter acetylcholine (ACh). Acetylcholine binds to ACh receptors (AChRs) on immune and tissue cells involved in immune and inflammatory processes (22,23). By its binding to AChRs, ACh can inhibit the activation of nuclear factor (NF)-κB in pathogen- and LPS-activated immune cells (24,25). Nuclear factor-κB is a transcription factor with a central role in the synthesis of pro-inflammatory cytokines, including tumour necrosis factor α (TNF-α). Thus, there exists an immunoregulatory system controlled by the brain, today called the 'the inflammatory reflex', and the efferent neural regulatory pathway of this bidirectional communication system is termed the 'cholinergic anti-inflammatory pathway' (22,23).

There are two types of AchRs, namely the muscarinic AChRs (mAChRs) and nicotinic AChRs (nAChRs), which include several subtypes, of which α7 nAChRs in particular have been found to be involved in the regulation of immune and inflammatory cells (26,27). Moreover, nicotine is a potent α7 nAChR agonist that activates the cholinergic anti-inflammatory pathway, leading to increased release of ACh, and nicotine alters immune system responses via this pathway (23,28). In addition, nicotine may bind directly to nAChRs on immune cells (27). Activation of α7 nAChRs on immune cells by ACh and/or nicotine inhibits the release of pro-inflammatory cytokines, including TNF-α, by transducing a cellular signal that inhibits the nuclear activity of NF-κB (23–25). Thus, nicotine can influence immune system responses, including cytokine responses, indirectly by binding to cells within the central nervous system that are involved in peripheral immune system regulation, and by its direct effect on immune cells. Until now, however, it has not been demonstrated in an animal model of periodontitis whether nicotine mediates the aggravation of the disease via activation of nAChRs.

Therefore, the present study was designed to determine whether pretreatment with mecamylamine, which is a centrally acting nAChR antagonist that blocks all subtypes of nAChRs (29), would influence the severity of periodontitis induced by chronic nicotine treatment, as well as associated changes of pre- and anti-inflammatory cytokines induced by gram-negative bacterial LPS. In addition, the role of nicotine in the responsiveness of the HPA axis to LPS stimulation, cytokine responses and susceptibility to periodontitis was explored.

Material and methods

Animals

Male Fischer 344 rats, weighing 270–280 g, were obtained from Charles River Laboratories (Sulzfeld, Germany), and used after 1 week of acclimation. Standard rat chow pellets and tap water were available *ad libitum*. The animals were housed in groups of four under a 12 h–12 h light–dark cycle (lights on from 07.00 to 19.00 h) with temperature and humidity at 22°C and 40–60%, respectively. The experiments were registered and approved by the Norwegian Experimental Animal Board.

Drugs

S(–)Nicotine di-D-tartrate and (±)-mecamylamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA), and were dissolved in physiological saline (0.9% sodium chloride) and adjusted to pH 7.4 with sodium hydroxide. Mecamylamine is a non-selective nAChR antagonist, i.e. it blocks the ability of nicotine to bind to all subclasses of nAChRs (30). The dosing was selected on the basis of available literature.

Experimental design

Experiment 1 — This experiment was designed to test whether administration of nicotine and pretreatment with

mecamylamine would affect ligature-induced periodontal breakdown, as well as immune and stress responses to the predominantly gram-negative bacterial cell wall constituent LPS. The rats were randomly assigned to three groups, each consisting of 10 rats. Periodontitis was induced in all rats (see subsection 'Experimental periodontitis'). Two days thereafter, group 1 (the mecamylamine/nicotine group) was injected intraperiotonally (i.p.) with mecamylamine (1 mg/kg) and 45 min thereafter subcutaneously (s.c.) in the neck with nicotine (0.8 mg/kg) for five consecutive days over a period of 3 weeks. Group 2 (the saline/nicotine group) received saline i.p. and 45 min thereafter nicotine s.c., while group 3 (the saline/saline group) had saline i.p. and 45 min thereafter saline s.c. Two hours before ending the experiment, all animals were injected i.p. with LPS to assess whether the treatment modified the cytokine and corticosterone responses to LPS.

Experiment 2 — This was a follow-up study to investigate whether mecamylamine by itself had any effect on the development of periodontitis, cytokine and stress responses. Twenty Fischer 344 rats were employed. The rats were divided into two groups with 10 rats in each group. They were of the same age and weight as in experiment 1 and were treated the same way over exactly the same time period. Group 1 (the mecamylamine/saline group) was injected i.p. with mecamylamine (1 mg/kg) and 45 min thereafter s.c. with saline at the same volume as the nicotine injection in experiment 1. The control group (the saline/saline group) was treated in the same way as the control group in experiment 1.

Experimental periodontitis

All animals were anaesthetized with a subcutaneous injection in the neck with Hypnorm andDormicum (fentanyl/fluanizone and midazolam 1:1), 0.2 mL/100 g body weight. A sterile silk ligature (Perma-hand® seide 3–0, Ethicon GmbH, Norderstedt, Germany) was tied around the neck of the maxillary right second molar tooth. The ligature was left in the same position during the entire experiment and served as a retention device for oral microorganisms. At the end of the experiment the maxillae were excised and fixed in 4% formaldehyde.

Lipopolysaccharide challenge

The animals were injected with LPS (*E. coli* serotype 0111:B4, Sigma; (St. Louis, MO, USA) 100 μg/kg; 100 μg/mL, i.p.) 2 h before ending the experiments to assess whether the treatment regimen influenced cytokine or corticosterone responses to this inflammatory agent. After decapitation of the rats, blood samples were collected (6–10 mL from each animal) in vacutainer tubes (10 mL without additives) and allowed to coagulate on ice for 1 h. Thereafter, the samples were centrifuged for 20 min at 2000 *g*, and the serum samples were removed, aliquoted and stored at −20°C prior to analysis of corticosterone and cytokines.

Assay of serum TNF-α, transforming growth factor (TGF)-β and interleukin (IL)-10

Serum TNF-α, TGF-β and IL-10 were measured by means of enzyme-linked immunosorbant assay (ELISA) kits from R&D Systems, Inc., Minneapolis, MN, USA, with catalogue numbers RAT00 for TNF-α, MB100 for TGF-1β and R1000 for IL-10. The minimal detectable concentration for TNF-α was less than 12.5 pg/mL, and less than 31.2 pg/mL for IL-10 and TGF-1β.

Assay of corticosterone

Corticosterone was measured with ^{125}I radioimmunoassay (RIA) coat-A-count kit from Diagnostic Products Corp., Los Angeles, CA, USA, catalogue number TKRC1. The detection limit was 5.7 ng/mL.

Radiographic examination

The specimens were stabilized with dental wax on a Sidexis digital X-ray sensor, orientated with the axis of the teeth parallel to the sensor surface by using ×4 magnification loupe glasses (Zeiss, Germany). The distance between the cemento-enamel junction and bone on mesial surfaces of the second molars were displayed digitally in the computer program and recorded as bone loss. The examiner was unaware whether the specimens came from experimental or control animals. Each X-ray was read three times, and the mean of the three readings calculated.

Statistical methods

Data are presented as means ± SD. The data were tested for normal distribution. Thereafter, differences between values were estimated with one-way repeated measurements ANOVA, or one-way repeated measurements ANOVA on ranks as appropriate, followed by Student–Newman–Keul's *post hoc* test. *p*-values less than 0.05 were considered statistically significant.

Results

Effect of nicotine and mecamylamine pretreatment on the weight of the animals

There was no significant weight difference between the groups, either at the start of the experiments or at the time of decapitation 21 days after ligature placement. Nicotine-treated rats, nicotine-treated rats pretreated with mecamylamine and saline-treated animals weighed 306.2 ± 11.2, 302.1 ± 10.8 and 302.5 ± 9.3 g, respectively, at the induction of the disease and 328.5 ± 11.1, 326.2 ± 12.1 and 327.7 ± 11.2 g, respectively, at the end of the experiments.

Effect of nicotine and mecamylamine pretreatment on periodontal tissue destruction

The saline/nicotine-treated animals had significantly more alveolar bone loss than the saline/saline-treated control animals (0.87 ± 0.05 vs. 0.81 ± 0.03 mm); (Fig. 1). Mecamylamine pretreatment abolished the enhanced periodontal breakdown induced by nicotine (0.76 ± 0.04 mm). Since the bone loss in the nicotine-treated rats pretreated with mecamyl-

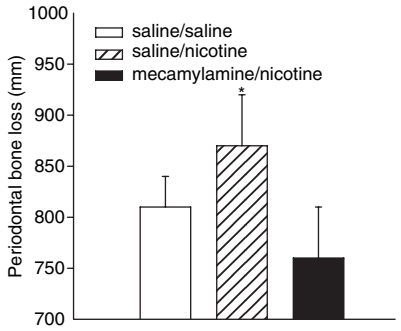

Fig. 1. The mean distance from the cemento-enamel junction to the alveolar bone crest in saline/saline-treated control rats, saline/nicotine-treated rats and mecamylamine/nicotine-treated rats as measured on digital radiographs. Nicotine-treated rats showed significantly more periodontal bone loss (*$p < 0.05$ vs. control rats), and mecamylamine pretreatment abolished this effect ($p < 0.001$).

amine tended to be even lower than in the saline/saline-treated control rats, we conducted a follow-up experiment (experiment 2) to test whether mecamylamine treatment by itself had any effect on alveolar bone loss. There was no significant difference between the two groups (0.82 ± 0.03 mm in the saline/saline-treated control rats vs. 0.81 ± 0.03 mm in the mecamylamine/saline-treated rats).

Effects of nicotine and mecamylamine pretreatment on selected serum cytokines after LPS challenge

After LPS challenge, saline/nicotine-treated rats reacted with significantly lower TNF-α serum levels than saline/saline-treated control rats (364 ± 251 vs. 1015 ± 338 pg/mL; Fig. 2A). Mecamylamine pretreatment abolished this reduction (1088 ± 598 pg/mL). In the follow-up experiment (experiment 2), treatment with mecamylamine alone did not significantly influence serum TNF-α levels after LPS challenge (1392 ± 227 vs. 1104.1 ± 605.9 pg/mL in the control rats).

The serum levels of TGF-1β were significantly lower in the saline/nicotine-treated rats compared with saline/saline-treated control animals (44.3 ± 3.8 vs. 50.0 ± 3.4 ng/mL). Mecamylamine pretreatment had no significant

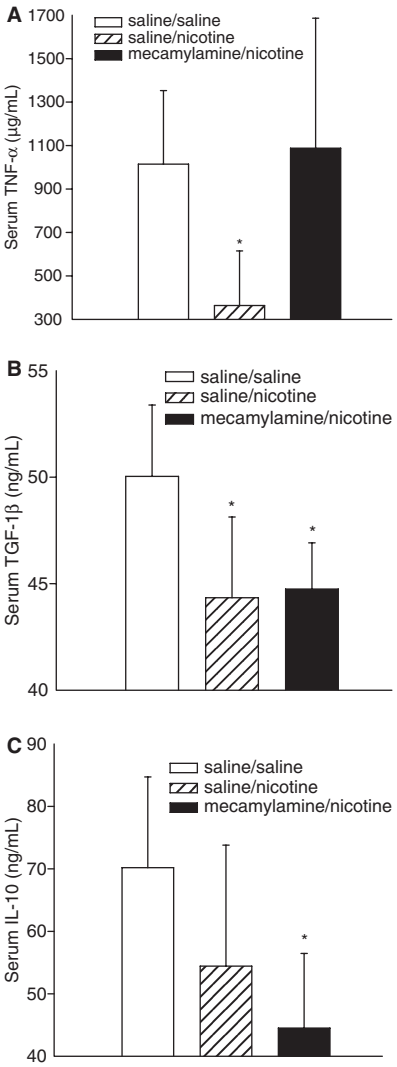

Fig. 2. Analysis of serum levels of the pro-inflammatory cytokine TNF-α (A) and the anti-inflammatory cytokines TGF-1β (B) and IL-10 (C) 2 h after intraperitoneal injection of LPS (100 μg/kg) in saline/saline-treated control rats, saline/nicotine-treated rats and mecamylamine/nicotine-treated rats. Compared to saline-treated controls, nicotine-treated rats demonstrated significantly lower serum levels of TNF-α ($p < 0.001$), TGF-1β ($p < 0.01$) and IL-10 ($p = 0.05$). Mecamylamine pretreatment abolished the nicotine-induced TNF-α reduction ($p < 0.01$), but had no significant effect on the serum levels of TGF-1β and IL-10.

effect on nicotine-induced TGF-1β levels (44.7 ± 2.2 ng/mL; Fig. 2B). The corresponding values for IL-10 were 54.4 ± 19.4 and 70.2 ± 14.5 pg/mL (Fig. 2C). In nicotine-treated rats pretreated with mecamylamine, the IL-10 levels tended to be even lower (44.5 ± 11.9 pg/mL; n.s. vs. saline/nicotine). The follow-up experiment (experiment 2) showed that the serum levels of TGF-1β were significantly higher in the mecamylamine/saline-treated rats compared with saline/saline-treated control animals (47.7 ± 6.2 vs. 53.2 ± 4.6 ng/mL). The same was true for IL-10 (48.2 ± 13.8 vs. 34.06 ± 5.7 pg/mL).

Effects of nicotine and mecamylamine pretreatment on plasma corticosterone levels after LPS challenge

The mean serum corticosterone levels following LPS challenge did not differ significantly between the groups. The values for saline/saline-treated control rats were 1447 ± 32 nmol/L, for saline/nicotine-treated rats1457 ± 54 nmol/L and for nicotine/mecamylamine-treated rats 1424 ± 45 nmol/L. In experiment 2, the corresponding concentrations in mecamylamine/saline-treated rats were 1612 ± 48 and 1595 ± 86 nmol/L in saline/saline-treated control animals (n.s. between groups).

Discussion

The scientific rationale for this study was clinical observations and epidemiological studies showing that smokers have a high incidence and more rapid development of periodontitis. The study demonstrates that chronic treatment with nicotine enhances the progression of ligature-induced periodontitis, and that pretreatment with the nAChR antagonist mecamylamine abolishes this effect in periodontal disease-susceptible Fischer 344 rats. The nicotine-treated animals also had significantly lower serum levels of the pro-inflammatory cytokine TNF-α and the anti-inflammatory cytokines TGF-1β and IL-10 subsequent to a LPS challenge. The reduction in TNF-α levels was reversed by pretreatment with mecamylamine, while no significant effect was observed in TGF-1β and IL-10 levels. Thus, the principal finding was that the nicotine-induced worsening of experimental periodontitis can

be blocked by pretreatment with the nAChR antagonist mecamylamine. Furthermore, nicotine alters TNF-α cytokine responses to a gram-negative bacterial LPS challenge via the same mechanisms. This suggests that nicotine-induced enhancement of periodontitis may be mediated via its effect on immune system responses controlling pathogenic dental plaque microorganisms.

The results are in line with recent investigations documenting that nicotine aggravates ligature-induced periodontitis (31) and inhibits TNF-α responses to LPS (27,32). The data also support our previous studies showing that animals responding with strong TNF-α responses to a LPS challenge are more resistant to experimental periodontitis than those responding with weaker TNF-α responses (19,33,34).

When LPS is recognized by specific recognition receptors (e.g. the Toll-like receptor 4; TLR4/CD14 complex) on macrophages and dendritic cells, the NF-κB pathway is activated (23). This leads to the release of a large number of immunoregulatory substances, including the pro-inflammatory cytokine TNF-α and the anti-inflammatory cytokines IL-10 and TGF-β. The anti-inflammatory cytokines counteract further release of pro-inflammatory cytokines (35,36). In addition, LPS-induced pro-inflammatory cytokines such as TNF-α activate the HPA axis and the SNS, leading to the release of glucocorticoid hormones and catecholamines (adrenaline and noradrenaline), respectively. The release of these 'stress hormones' counter-regulates a number of immune mediators, including the production and release of TNF-α, by altering the activation of NF-κB as well as other intra-cellular pathways (37). There is now evidence that the way in which the stress response system responds to LPS or other danger signals (whether they are of immunological, chemical, physical or psychological origin) plays a significant role in the final clinical outcome, i.e. the susceptibility/resistance to infectious and inflammatory diseases (38–40). Our studies have shown that this includes ligature-induced periodontitis (18,19, 33,34).

The attenuated increase of TNF-α levels following LPS challenge in the chronically nicotine-treated animals, and the reversion after pretreatment with mecamylamine, is in accordance with recent studies (24,32). Tumour necrosis factor-α is an important mediator of local and systemic immuno-inflammatory responses, primarily produced and released from activated tissue macrophages and dendritic cells in response to pathogens and other injurious stimuli, and it plays a significant role in the eradication of pathogens (41).

The results from the present study, showing that pretreatment with the nAChR antagonist mecamylamine of nicotine-treated rats alters systemic cytokine responses to an *in vivo* challenge with LPS and the susceptibility to ligature-induced periodontitis, suggest that nAChRs, and consequently the PSNS and the cholinergic anti-inflammatory pathway, are involved in the pathogenesis of ligature-induced periodontitis, as well as nicotine-induced worsening of experimental periodontitis. Nicotine binds to nAChRs within the central nervous system and activates the PSNS, including the cholinergic anti-inflammatory pathway. This pathway regulates immune system responses, including cytokine responses, by releasing ACh in the periphery (22–24,37,41). In addition, nicotine can bind to nAChRs in the periphery; including nAChRs on immune cells, such as macrophages and dendritic cells, which are expressing high levels of 7α nAChRs (24,37). In this way, nicotine may indirectly reduce TNF-α production subsequent to LPS stimulation by binding to nAChRs on brain and nerve cells, leading to increased release of ACh in the periphery, as well as directly by activating nAChRs on immune cells (24–27).

The present experiments also showed that nicotine-treated rats responded to LPS with significantly lower circulating levels of the anti-inflammatory and T regulatory cytokines TGF-1β and IL-10. Since nicotine treatment, and subsequent activation of nAChRs, represents an anti-inflammatory mechanism (23), this finding was unexpected. Furthermore, mecamylamine pretreatment failed to reverse this effect. This indicates that the reduction in TGF-1β and IL-10 is not mediated via the cholinergic anti-inflammatory pathway. Activation of a self-controlling immune regulating system offers an alternative explanation. When pathogenic components such as LPS are recognized by identification receptors on macrophages or dendritic cells, these immune cells are activated to release pro-inflammatory cytokines such as TNF-α, as well as anti-inflammatory cytokines such as TGF-1β and IL-10. These anti-inflammatory cytokines counteract further release of pro-inflammatory cytokines (36), and this they most likely do to prevent damage of the surrounding tissues induced by pro-inflammatory cytokine and the development of pro-inflammatory diseases (23). The reduced TGF-1β and IL-10 responses to LPS, as found in the nicotine-treated rats, may thus be a result of the lower pro-inflammatory TNF-α response of this self-controlling (autocrine) system, and not a brain-derived (endocrine) immunoregulatory output induced by nicotine.

Since nicotine administration or introduction of smoking to inexperienced individuals activates the HPA axis (42,43), we have earlier hypothesized that smoking may alter immune system responses and the susceptibility to periodontitis through activation of this non-specific regulatory system in the same way as emotional stressors may do (18). However, research has shown that chronic nicotine administration induces tolerance to the HPA axis, expressed by normal glucocorticoid concentrations (43). In addition, it has been shown that the weakened pro-inflammatory immune responses also persist after removal of the adrenal glands (44). This property of nicotine and HPA axis activation is supported by the present study. We did not find any statistically significant difference between corticosterone responses to LPS in nicotine-treated rats and saline-treated control animals, in spite of a reduced TNF-α response. Since the nicotine-induced drop in TNF-α levels

and the augmented periodontal breakdown were reversed by mecamylamine pretreatment, the results indicate that nicotine mediates most of its effect on TNF-α responses and periodontal breakdown via nAChRs and the subsequent activation of the cholinergic anti-inflammatory pathway.

In conclusion, we have described a potent inhibitory effect of the nAChR antagonist mecamylamine on nicotine-induced enhancement of experimental periodontal breakdown when given prior to chronic nicotine administration. Furthermore, mecamylamine pretreatment alters cytokine responses to a LPS challenge. These data, suggesting that a centrally acting nAChR antagonist, which blocks all subtypes of nAChRs (29), prevents nicotine-induced worsening of experimental periodontitis, are, to our knowledge, the first report demonstrating that cholinergic pathways are involved in the pathogenesis of periodontitis. This may constitute a novel pathway by which nicotine and other environmental factors activating the central nervous system may affect the growth of gram-negative bacteria and other pathogens in subgingival plaque. Nicotine may turn down the production of TNF-α and other pro-inflammatory cytokines through its binding to nAChRs on cells belonging to the central nervous system and/or the immune system. The reduced ability of the immune system to respond with an optimal immune response to gram-negative bacteria and/or other pathogenic microorganisms may render the patient more susceptible to the development of periodontitis. Thus, an inadequate cytokine response may, in part, represent an underlying mechanism explaining the increased colonization of periodontophatogens, and consequently one reason for smokers to be more prone to develop periodontitis. The practical implication is to motivate patients to quit smoking and other nicotine abuse. Furthermore, smoking may dysregulate/misguide brain neuro-endocrine regulatory mechanisms involved in peripheral and systemic immune regulation, which in turn may worsen periodontitis.

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