Anatabine Ameliorates Experimental Autoimmune Thyroiditis

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Tobacco smoking favorably influences the course of Hashimoto thyroiditis, possibly through the antiinflammatory proprieties of nicotine. In this study we tested anatabine, another tobacco alkaloid, in a model of experimental autoimmune thyroiditis. Experimental autoimmune thyroiditis was induced by different doses of thyroglobulin, to produce a disease of low, moderate, or high severity, in 88 CBA/J female mice: 43 drank anatabine supplemented water and 45 regular water. Mice were bled after immunization and killed to assess thyroid histopathology, thyroglobulin antibodies, T₄, and thyroid RNA expression of 84 inflammatory genes. We also stimulated in vitro a macrophage cell line with interferon-γ or lipopolysaccharide plus or minus anatabine to quantify inducible nitric oxide synthase and cyclooxygenase 2 protein expression. Anatabine reduced the incidence and severity of thyroiditis in the moderate disease category: only 13 of 21 mice (62%) developed thyroid infiltrates when drinking anatabine as compared with 22 of 23 (96%) controls (relative risk 0.59, \( P = 0.0174 \)). The median thyroiditis severity was 0.5 and 2.0 in anatabine and controls, respectively (\( P = 0.0007 \) by Wilcoxon rank sum test). Anatabine also reduced the antibody response to thyroglobulin on d 14 (\( P = 0.029 \)) and d 21 (\( P = 0.045 \)) after immunization and improved the recovery of thyroid function on d 21 (\( P = 0.049 \)). In the thyroid transcriptome, anatabine restored expression of IL-18 and IL-1 receptor type 2 to preimmunization levels. Finally, anatabine suppressed in a dose-dependent manner macrophage production of inducible nitric oxide synthase and cyclooxygenase 2. Anatabine ameliorates disease in a model of autoimmune thyroiditis, making the delineation of its mechanisms of action and potential clinical utility worthwhile.


Tobacco smoking has numerous detrimental effects on human health, but it has also been associated with a few apparent salutary actions, including the amelioration of autoimmune (Hashimoto) thyroiditis and ulcerative colitis. Smokers in the Third National Health and Nutrition Examination Survey were found to have lower prevalence of thyroperoxidase and/or thyroglobulin antibodies than nonsmokers (1). This protective effect of smoking was confirmed in two additional cross-sectional studies, one from the Amsterdam autoimmune thyroid disease cohort (2) and the other from the Danish population (3), as well as in a 5-yr prospective study also based on the Amsterdam autoimmune thyroid disease cohort (4). In the prospective study, cigarette smoking women who had one or more relative with documented thyroid autoimmunity but no thyroid dysfunction or autoantibodies at study entry showed lower odds of developing thyroperoxidase and/or thyroglobulin antibodies (4). Similarly in ulcerative colitis, smoking has been shown to decrease flares (5), hospitalizations (6), and a need for oral glucocorticoids (7) so that low-dose smoking resumption has been successfully used in ex-smokers with refractory disease (8).
The mechanisms underlying this influence of tobacco smoking on some autoimmune diseases have been related to the effects of tobacco components on the immune system (9). There are numerous (>4000) components in tobacco, including alkaloids (such as nicotine and anatabine), gases (e.g. carbon monoxide), and carcinogens (e.g. polycyclic aromatic hydrocarbons, aldehydes, free radicals, and solvents), and of them nicotine is known to possess antiinflammatory properties (10). Nicotine acts via binding to the nicotinic receptor, a pentameric ion channel (mainly for sodium and calcium) formed by the arrangement of 16 different subunits in hetero- or homomeric conformations (11). The receptor is classically expressed in the peripheral (all preganglionic fibers and neuromuscular synapses) and central nervous system, but more recently it has been described in cells of the immune system, including CD4 T lymphocytes, dendritic cells, and macrophages (12). Indeed, the α7-homopentameric nicotinic receptor has emerged as a novel therapeutic target for diseases with an inflammatory pathogenesis (13).

Nicotine has been used successfully in mice with experimental autoimmune encephalomyelitis in which it reduced disease severity, shifting the autoimmune profile from pathogenic Th1 and Th17 responses to protective Th2 responses (14). Nicotine, however, cannot be used in humans because it is addictive and toxic and has a short 3-h plasma half-life. Consequently, we reasoned that other alkaloids of tobacco could share similar antiinflammatory properties but have a more favorable pharmacological profile. The minor tobacco alkaloid anatabine is nonaddictive and nontoxic at therapeutic doses and has a longer 8-hr half-life. Furthermore, anatabine has been recently shown to inhibit nuclear factor-κB (NF-κB) activation and reduce neuroinflammation in a mouse model of Alzheimer disease (15). In the present study, we therefore tested the antiinflammatory properties of anatabine in a mouse model of experimental autoimmune thyroiditis.

Materials and Methods

Mice and induction of experimental autoimmune thyroiditis (EAT).

EAT was induced in 8-wk-old female CBA/J mice (The Jackson Laboratory, Bar Harbor, ME) as described (16). Briefly, mouse thyroglobulin was purified by S-300 gel chromatography and emulsified 1:1 in complete Freund’s adjuvant (which contains 5 mg/ml of Mycobacterium tuberculosis strain H37 Ra; Difco Laboratories, Detroit, MI). Mice were injected sc with the emulsion on d 0 and 7 and then killed on d 21 [see a video demonstration of the immunization procedure published elsewhere (17)]. Three different induction protocols were used to obtain a thyroiditis of low, moderate, or high severity, based on the amount of mouse thyroglobulin in the immunogen (50, 75, or 100 μg, respectively). Each experiment included two groups, both immunized in the same manner and coded for blinding. The control group drank regular water; the anatabine group drank water supplemented with anatabine. Eighty-eight mice were studied: 43 anatabine treated and 45 controls, immunized in eight separate experiments by three investigators (A.D.R., M.A.L.-S., and S.-C.T.). Experiments were conducted in accordance with the standards established by the U.S. Animal Welfare Act, set forth in the National Institutes of Health guidelines and approved by the Johns Hopkins University Animal Care and Use Committee.

Anatabine source and administration

Anatabine was provided by Rock Creek Pharmaceuticals (Washington, DC), its commercial producer. The racemic mixture was synthesized by Anthem Biosciences Pvt. Ltd. (Bangalore, India) and then purified and formulated by Emerson Resources Inc. (Norristown, PA). Anatabine was added to the water bottle at the start of the experiment (d 0) at a concentration of 0.05 mg/ml, a concentration derived from the first, and thus far only, study that used anatabine in mice (15). Based on a young adult CBA/J female mouse drinking approximately 5 ml of water per day and weighing approximately 20 g, we administered anatabine at a dose of 0.25 mg/d per mouse to approximate a dose of 12.5 mg/kg/d, which corresponds to a human adult dose of 1 mg/kg/d, based on the formula reported by Reagan-Shaw et al. (18). The same bottle was used for the 3-wk duration of the experiment, and the water was changed twice a week. The consumption of water in each cage was estimated by measuring the weight of the water bottle. There was no difference in water consumption between cages drinking anatabine water and cages drinking regular water.

Thyroid histopathology and macrophage staining

Thyroid glands were collected 21 d after the first immunization. For most mice (72 of 88), the entire gland still attached to the trachea was removed and fixed overnight in 10% buffered formalin. For 16 mice, eight anatabine treated and eight controls, one lobe was removed for gene expression analysis (see below), and the other lobe attached to the trachea was used for histopathology. After processing and embedding in paraffin, thyroid specimens were cut completely to obtain numerous nonsequential 5-μm sections, which were then stained with hematoxylin and eosin. Sections were scored blindly (by P.C.) to obtain a score expressed on a discrete scale from 0 (normal) to 5 (complete thyroid infiltration and destruction) that summarizes the severity of autoimmune thyroiditis. The presence of macrophages in the thyroid glands with ongoing EAT was assessed by immunohistochemistry using a rat antimouse antibody (F4/80; Serotec, Raleigh, NC; diluted 1:16) using the procedure previously described (19).

Thyroglobulin and purified protein derivative antibodies

Mice were bled on d 0, 10, 14, and 21 after the first immunization to collect serum for the measurement of thyroglobulin antibodies, as previously described (16). Briefly, Immunolon2 ELISA plates (Dynex Technologies, Chantilly, VA) were coated with 100 ng/well of mouse thyroglobulin, blocked, and then
incubated overnight with mouse sera diluted according to the day after immunization (1:100 for d 0, 1:400 for d 10 and 14, and 1:1600 for d 21). After washing, incubation with a secondary antibody recognizing mouse IgG and IgM conjugated with alkaline phosphatase, and color development by addition of the \( p \)-nitrophenylphosphate substrate, plates were read at 405 nm using Emax microplate reader (Molecular Devices, Sunnyvale, CA). Results were expressed as arbitrary units per milliliter using in each plate a homemade standard curve derived from serial dilutions of a serum sample with known thyroglobulin antibodies. To control for thyroid specificity, we also assessed the antibody response against purified protein derivative, a component of the \( M. \) tuberculosis contained in the complete Freund’s adjuvant. Purified protein derivative (Mycos Research, LLC, Loveland, CO) was used to coat (1 μg/well) ELISA plates (Immuno2; ISC BioExpress, Kaysville, UT), which were then incubated overnight with mouse sera (in triplicates) diluted 1:100 in Tris-buffered saline. After extensive washing, a secondary antibody conjugated to alkaline phosphatase recognizing mouse IgG and IgM was used to detect specific binding. After addition of the \( p \)-nitrophenylphosphate substrate, color development was assessed and expressed as OD.

**Assessment of thyroid function by serum total T4**

Total T4 was measured on d 0, 10, 14, and 21 using a commercial RIA (GammaCoat \(^{125}\)T4; Diasorin, Stillwater, MN) according to the manufacturer’s protocol.

**Analysis of thyroidal gene expression by real-time RT-PCR**

Thyroid lobes from d 21 mice of the moderate severity group, eight from anatabine treated and eight from controls, were pooled into separate microcentrifuge tubes and processed by RNeasy minikit (QIAGEN, Valencia, CA) to extract total RNA. We also used 10 lobes from unimmunized (d 0) wild-type CBA/J female mice as baseline control. Yield and quality of RNA were determined by spectrophotometry and denaturing agarose gel electrophoresis. Total RNA was reverse transcribed using the RT\(^2\) first-strand kit (QIAGEN) following the manufacturer’s in-
structions. cDNA products (175 ng per reaction) were mixed to the supplied quantitative PCR master mixture, added to a 96-well mouse inflammatory marker plate (PAMM-011; QIAGEN), and amplified using the Bio-Rad CFX96 modular thermal cycler platform (Bio-Rad Laboratories, Hercules, CA). This plate contains primers for the detection of 40 chemokines (27 ligands and 13 receptors), 37 cytokines (25 ligands and 12 receptors), and seven additional inflammatory molecules (the complete list of these 84 genes can be seen at the QIAGEN web site: http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-011A.html). Results normalized for the expression of housekeeping genes were analyzed using the QIAGEN web site software appropriate for the product. We considered significant those changes in gene expression that in pairwise comparisons yielded a 4-fold difference (i.e., values > 4 for increases and < 0.25 for decreases).

**In vitro effect of anatabine on inflammatory markers using a macrophage cell line**

Given the presence of macrophages infiltrating the thyroid gland in the EAT model and the availability of well-established mouse macrophage cell lines, we used the RAW 264.7 line (from American Type Culture Collection, Manassas, VA) to assess the effects of anatabine on key inducible inflammatory markers like cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS)-2. Macrophages were grown at 37 °C in a CO2 incubator using RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin. When confluent, cells were stimulated overnight with interferon-γ (10 ng/ml), a well-characterized activator of macrophages. Thirty minutes later, anatabine was added to the overnight culture at concentrations of 250, 350, 500, 1000, or 2000 μM. Protein extracts were then prepared by cell lysis, separated by SDS-PAGE on gradient gels (NuPAGE Novex 4–12%; Novex, San Diego, CA), and transferred onto polyvinyl difluoride membranes (Bio-Rad Laboratories). After blocking with 5% nonfat dry milk, membranes were incubated overnight with COX2 antibody (Cell Signaling Technology, Inc., Danvers, MA; diluted 1:5,000) or iNOS antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; also diluted 1:1,000). To adjust for loading in each well, membranes were probed with a tubulin antibody (Abcam, Cambridge, MA; diluted 1:5,000). Signal was detected by the addition of a secondary anti-IgG antibody conjugated to horseradish peroxidase and quantified using ImageJ (National Institutes of Health, Bethesda, MD), adjusting for the levels of α-tubulin in each lane. Parallel experiments were also performed with lipopolysaccharide as a stimulant, rather than interferon-γ, as indicated in the Supplementary Materials, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org.

**Statistical analysis**

The primary study outcome was the autoimmune response as assessed by thyroid histopathology. Its severity was compared between the two experimental groups (anatabine treated and controls) by the Mann-Whitney U rank-sum test and its incidence by risk ratio. Secondary outcomes of the study were serum thyroglobulin antibodies and total T4. They were analyzed using multiple linear regression with generalized estimating equations, which takes into account multiple values (at the various immunization days) from the same mouse, correcting for the possibility that characteristics of a single mouse over time are likely to be correlated with one another. Analyses were performed using Stata 12 (Stata Corp., College Station, TX).

**Results**

Anatabine decreases the incidence and severity of EAT

Anatabine significantly decreased the severity of EAT induced with our standard induction protocol. The median thyroiditis score decreased from 2.0 in the control group to 0.5 in the anatabine group (Fig. 1A, middle pair

**FIG. 2.** Antibodies to thyroglobulin (A), antibodies purified protein derivative (PPD) (B), and total T4 (C) during EAT in mice receiving anatabine or control water. Analytes were assessed before (d 0) and 10, 14, or 21 d after the first thyroglobulin immunization. The dashed lines (C) represent the mean (4.78 μg/dl) ± 1 sd (1.49 μg/dl) of total T4 in 70 unimmunized female CBA/J mice studied by our laboratory in previous publications (16, 30).
of box plots, and Fig. 1B, P = 0.0007). The incidence of EAT was also significantly decreased by anatabine (Fig. 1B): eight of 21 mice did not develop EAT when drinking anatabine compared with one of 23 of those drinking regular water (relative risk 0.59, 95% confidence interval 0.39–0.9, P = 0.0174). The microscopic appearance of the hematopoietic infiltrate was similar in both experimental groups, composed mainly of lymphocytes and macrophages (Fig. 1C, inset), but the infiltration was less extensive and destructive of the thyroid architecture in the anatabine-treated mice (Fig. 1D) than in controls (Fig. 1C). When EAT was induced to achieve complete disease penetrance and greatest severity, anatabine was not observed to ameliorate thyroiditis severity (Fig. 1A, right pair). Conversely, when EAT was induced with low thyroglobulin doses, thyroiditis was mild in both experimental groups (Fig. 1A, left pair), implying that anatabine per se is not harmful to the thyroid. Overall these results demonstrate that anatabine reduces the incidence and ameliorates the severity of this experimental form of autoimmune thyroiditis.

**Anatabine decreases specifically the antibody response to thyroglobulin and improves the recovery of thyroid function**

Serum thyroglobulin antibodies in EAT typically begin to increase on d 10 after the first immunization, peak at approximately d 60, and then gradually decrease by d 100 without disappearing entirely (16). Anatabine treated mice developed lower levels of thyroglobulin antibodies than controls on d 14 (P = 0.029) and d 21 (P = 0.045) (Fig. 2A), suggesting that anatabine attenuates the thyroid-specific autoimmune response induced by thyroglobulin immunization. This attenuation was specific to thyroglobulin antibodies because it was not observed for the antibody response against purified protein derivative (Fig. 2B), suggesting that anatabine administration is not associated with generalized immunosuppression.

Total T₄ is known to decrease markedly and rapidly after the injection of complete Freund’s adjuvant due to a nonthyroidal illness effect (20) and then to decrease at later time points due to the autoimmune destruction of the thyroid gland (16). Anatabine-treated mice showed higher serum T₄ concentrations than controls on d 21 after immunization (Fig. 2C, P = 0.049), suggesting a prompter recovery of euthyroidism. This effect tended to be present also on d 10 and 14 but did not reach statistical significance (Fig. 2C).

**Anatabine in vivo decreases specific proinflammatory signals in the thyroid gland with ongoing EAT**

To begin addressing the mechanism of action of anatabine, we compared the thyroidal expression of a panel of cytokines and chemokines between anatabine-treated and control EAT mice, each adjusted for the expression level of unimmunized thyroid genes. Of the total 84 genes present on the array, eight increased significantly (>4-fold) during EAT (Table 1). Anatabine suppressed the EAT-mediated increase of IL-1 receptor type 2 (IL-1R2, Figure 3A) and IL-18 (Fig. 3B), restoring their expression to levels similar to those seen in normal unimmunized thyroids. These results reveal a specific effect of anatabine on proinflammatory pathways that may explain the amelioration of thyroiditis incidence and severity observed histologically.

**Anatabine in vitro decreases the specific proinflammatory signals in a macrophage cell line**

We next assessed in vitro the effect of anatabine on iNOS and COX2, two enzymes that increase universally during inflammation. Macrophages were first activated with interferon-γ to induce the expression of iNOS and COX2 and then incubated with anatabine at concentrations of 250 or 350 μM. These concentrations were chosen based on a dose-response curve ranging from 250 to 2000 μM (Supplemental Fig. 1). Anatabine suppressed in a dose-dependent manner the increase of iNOS and COX2 in-

**TABLE 1.** Thyroidal expression levels of eight chemokine/cytokine genes that significantly increased (>4-fold) during EAT as compared with the expression in unimmunized thyroids

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Anatabine treated (fold increase over unimmunized)</th>
<th>Control (fold increase over unimmunized)</th>
<th>Net effect of anatabine</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1R2</td>
<td>3.80</td>
<td>15.74</td>
<td>Decrease 0.24</td>
</tr>
<tr>
<td>IL-18</td>
<td>1.24</td>
<td>5.34</td>
<td>Decrease 0.23</td>
</tr>
<tr>
<td>Spp1</td>
<td>32.65</td>
<td>20.85</td>
<td>No change 1.57</td>
</tr>
<tr>
<td>CCL8</td>
<td>20.37</td>
<td>16.72</td>
<td>No change 1.22</td>
</tr>
<tr>
<td>CCL6</td>
<td>6.74</td>
<td>7.32</td>
<td>No change 0.92</td>
</tr>
<tr>
<td>Itgam</td>
<td>7.05</td>
<td>6.34</td>
<td>No change 1.11</td>
</tr>
<tr>
<td>IL-1β</td>
<td>10.89</td>
<td>5.18</td>
<td>No change 2.10</td>
</tr>
<tr>
<td>CCR2</td>
<td>6.25</td>
<td>4.82</td>
<td>No change 1.30</td>
</tr>
</tbody>
</table>

The presence of anatabine suppressed the increase of IL-1R2 and IL-18.
duced by interferon-γ (Fig. 4), confirming in vitro its antiinflammatory properties. The effect seen with interferon-γ was also seen when macrophages were stimulated with lipopolysaccharide (Supplemental Fig. 1).

**Discussion**

Autoimmune (Hashimoto) thyroiditis, first reported in 1912 and considered a rarity until the mid-1950s, has now become the most common autoimmune disease. It has a genetic predisposition, such as the association with certain human leukocyte antigen haplotypes and polymorphisms in immunoregulatory genes (21), and a long preclinical phase of detectable autoimmunity without hypothyroidism (22). Consequently, there is the theoretical opportunity to intervene to maintain normal thyroid structure and function. However, despite the disorder’s long history and high prevalence, thyroid hormone replacement after gland failure remains the only established medical treatment for the condition.

It is now widely appreciated that autoimmune diseases have a complex and multifactorial pathogenesis in which several signaling pathways lead to overproduction of cytokines that damage the tissue and ultimately result in clinical disease (23). Drugs that specifically inhibit or neutralize these cytokines, such as the blockade of TNFα in rheumatoid arthritis or IL-1 in systemic-onset juvenile idiopathic arthritis (23), are now commonly used to treat severe autoimmune manifestations. For Hashimoto thyroiditis, however, the effectiveness of T4 therapy as well as the cost and potential for adverse events, make the cytokine-blocking agents inappropriate for prolonged use to maintain thyroid gland health.

Based on epidemiological observations that smoking is associated with lower prevalence and incidence of serological autoimmune thyroiditis, we hypothesized that a component of tobacco must be responsible for this effect. Anatabine is an alkaloid with a structure similar to nicotine, found in tobacco and other solanaceous plants, such
shown to increase during thyroid inflammation both in vitro (27) and but also on immune cells (12). Activation of the nicotinic receptor that has been clearly associated with antiinflammatory responses of inflammation (10, 24). The nicotinic receptor that has been found to initiate inflammation (25). Consistent with this mechanism, Paris and colleagues demonstrated that anatabine suppresses in a dose-dependent manner the transcription of NF-κB induced by tumor necrosis factor-α (15).

This study has elucidated another mechanistic pathway that could explain the antiinflammatory effects of anatabine. Using a panel of 84 cytokines and chemokines, we found that during experimental autoimmune thyroiditis, there is an up-regulation of thyroidal genes involved in attracting immune cells (e.g. CCL6 and CCL8), augmenting the function of lymphocytes and dendritic cells (e.g. CCR2 and integrin-αM), and tissue remodeling (e.g. the extracellular structural protein Spp1). These genes were not affected by anatabine administration. However, anatabine suppressed the thyroidal expression of IL-18 and IL-1R2. IL-18, a member of the IL-1 family, is produced by activated macrophages and stimulates production of interferon-γ from T cells and natural killer cells (26), overall acting as a proinflammatory stimulus. IL-18 has been shown to increase during thyroid inflammation both in vitro (27) and in vivo (28) and, it has been suggested, may contribute to tissue destruction in human autoimmune thyroiditis. The decreased IL-18 level with anatabine administration likely translates into decreased generation of pathogenic Th1-type lymphoid responses and lesser histopathological changes. The IL-1R2 is expressed principally on macrophages and B cells in which it increases in states of ongoing inflammation to dampen the inflammatory process. It binds IL-1α and IL-1β, like the type 1 IL-1 receptor, but does not convey an intracellular signal because of its very short cytoplasmic tail, serving in essence as a decoy receptor (29). The fact that the expression of IL-1R2 in thyroids of anatabine treated mice with experimental autoimmune thyroiditis is significantly lower than that of controls and almost identical with the levels observed in unimmunized thyroids suggests that anatabine administration is associated with an overall attenuation of the thyroid-specific autoimmune response.

Additional experiments will be needed to elucidate fully anatabine’s mechanism(s) of action, which could include effects not mediated by the nicotinic receptor and pathways not analyzed in the present study. Even if its antiinflammatory effects are mediated purely through the nicotinic receptor, it remains to be understood how the activation of a fast-switching, on/off type of ion channel leads to the prolonged effects on the control of inflammation (11).

In conclusion, these studies demonstrate that anatabine, a minor alkaloid originally derived from solanaceous plants, attenuates in a murine model histopathological, serological, and functional manifestations of thyroid autoimmunity. Further exploration of anatabine’s antiinflammatory mechanisms and potential clinical utility are worthwhile.

Acknowledgments

We express our gratitude to Drs. Michael Mullan and Fiona Crawford (Roskamp Institute, Sarasota, FL) for their guidance and advice.

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This work was supported by a grant from the Walton Family Foundation. S.I. was supported in part by a fellowship from the Uehara Memorial Foundation (Tokyo, Japan).

Disclosure Summary: P.C. and P.W.L. are consultants for Rock Creek Pharmaceuticals Inc. The other authors have nothing to declare.
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