Examination of antithyroid effects of smoking products in cultured thyroid follicles: only thiocyanate is a potent antithyroid agent

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We studied the antithyroid action of cigarette smoking products (nicotine, cotinine, and thiocyanate) in the physiological culture system of porcine thyroid follicles. Iodide uptake, iodine organification, de novo thyroid hormone formation, and iodide efflux were measured in the presence of 0–200 µmol/l nicotine, cotinine, or potassium thiocyanate. Nicotine and cotinine did not inhibit iodide transport or thyroid hormone formation. Thiocyanate concentrations equivalent to serum levels of smokers showed three independent antithyroid actions: (i) inhibition of iodide transport, (ii) inhibition of iodide organification, and (iii) increased iodide efflux. Inhibition of iodide transport by thiocyanate was competitive with iodide and independent of TSH concentration. Thiocyanate did not inhibit TSH mediated cAMP production or Na+K+ ATPase activity. a sodium pump for iodide transport. When 50 µmol/l thiocyanate was added 2 h after incubation with iodide or when 1 µmol/l thiocyanate was added from the beginning of incubation, iodine organification was inhibited without changing iodide transport. De novo thyroid hormone formation was clearly inhibited by 50 µmol/l thiocyanate. Thiocyanate increased iodide efflux although the degrees of iodide efflux by 10 µmol/l and 100 µmol/l thiocyanate did not differ significantly. In summary, thiocyanate, a product of smoking, has three independent antithyroid activities. The data of iodide transport kinetics suggest that thiocyanate can be an antithyroid agent particularly in iodine deficiency.

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Thyroid abnormalities caused by cigarette smoking include an increased incidence of nodular goiter (1–3) and a minor decrease or increase of serum thyroid hormone levels particularly in heavy smokers (3–5). Cigarette smoking can generate three major products: nicotine, cotinine (a product of nicotine), and thiocyanate (6). Whether nicotine or cotinine affects thyroid cell function has not been reported previously. Thiocyanate is generated from cigarette smoking as a detoxifying product of cyanide, which is derived from smoke. Thiocyanate has been considered as a possible cause of smoking-related thyroid disorders, because thiocyanate inhibits iodide transport (7–9). The action of thiocyanate, however, is complex: increased iodide efflux (10), inhibition of iodine organification (11, 12), inhibition of thyroid peroxidase activity in vitro (13), paradoxical increase in iodide uptake in iodine-deficient mice (14), and an increase in coupling of iodotyrosine in a cell-free system (15). We have established the physiological culture system of porcine thyroid follicles, which maintain a dome shape and form thyroid hormone in response to iodide (16–18). Using this system, we examined the effects of smoking products on iodide transport, iodide efflux, iodine organification, and de novo thyroid hormone formation. Particularly, we focused on the action of thiocyanate and discussed whether serum levels of thiocyanate in smokers can contribute to the development of hypothyroidism.

Materials and methods

Enzymes, hormones, and all reagents including culture medium were obtained from Sigma (St Louis, MO) unless otherwise specified.

Culturing porcine thyroid follicles

Fresh porcine thyroid glands were procured from Farmer John Co, Los Angeles, CA. Thyroid follicles were isolated with collagenase digestion and cultured in 6H medium that contained Coon’s modified Ham F-12, 1% calf serum, 1 U/l bovine TSH, 5 other hormones or peptides, 10 mg/l streptomycin, 100,000 U/l penicillin and 10 mg/l amphotericin-B as we described previously (15). On the fourth day, follicles (4000/well) were plated into 16 peripheral wells of 24-well culture dishes. In some experiments, follicles were exposed to 0 to 200 µmol/l
potassium thiocyanate, nicotine, and cotinine for 24 h in the medium that contained 0 to 1 U/l TSH, and the experiment for iodide uptake was started on the sixth day.

Effects of thiocyanate, nicotine, and cotinine on iodide uptake

Iodide uptake was started by adding the mixture of carrier-free 0.1 μCi Na^{125}I and 50 nmol/l NaI to each well. Incubations were done for 2 h at 37°C in a 95% air-5% CO_2 incubator for measurement of iodide uptake and iodine organification, since iodide uptake progressively increased up to 2 h. At the end of incubation, the medium was removed, and 1 ml water was added to remove follicles. The radioactivity of follicles in each well was counted in a gamma counter. The non-specific binding of ^{125}I to follicles was obtained from samples containing 1 mmol/l potassium perchlorate in the medium and subtracted from all samples. After counting the radioactivity of follicles, thyroid follicles in 1 ml water were sonicated for 20 sec and thyroid protein representing mainly thyroglobulin was precipitated by adding 1 ml of 20% trichloroacetic acid (TCA) at 4°C. After washing the pellet in 10% TCA, the amount of ^{125}I that was bound to protein was measured by counting the radioactivity of the pellet.

For the study of iodide transport kinetics, thyroid follicles were preincubated with 100 μmol/l methimazole for 10 h to block iodine organification. These follicles were incubated for 2 h in the presence of 50 μmol/l thiocyanate, 0.1 μCi Na^{125}I, and 1 to 10 μmol/l NaI.

Effect of thiocyanate on iodotyrosine and de novo thyroid hormone formation

This was to examine whether thiocyanate can actually decrease de novo formation of iodotyrosine and thyroid hormone. The mixture of 0.1 μCi Na^{125}I and 50 pmol/l NaI was added to the medium that contained 1 U/l TSH with or without 50 μmol/l thiocyanate, and incubation was done for 5 h at 37°C in a 5% CO_2-95% air incubator. At the end of incubation, follicles were washed in 2 ml of PBS (0.05 mol/l sodium phosphate buffer-0.9% saline, pH 7.2) and sonicated in 1 ml PBS. The sonicate was centrifuged at 12,500 × g for 30 min, and the supernatant containing thyroglobulin was digested with Pronase (Calbiochem, San Diego, CA) under nitrogen for 18 h at 37°C (16). The ^{125}I-labelled iodotyrosine and thyroid hormone were separated by Dowex-1 column chromatography (19). The radioactivity recovered in the fractions of iodotyrosine and thyroid hormone was expressed as percentage of total ^{125}I that was present in the 12,500 × g supernatant.

Measurement of cAMP content and Na^+K^+ATPase activity

TSH mediated cAMP production, an important signal for thyroid cell function, was measured in the thyroid follicles as described previously (18). Thyroid follicles were preincubated with 200 μmol/l potassium thiocyanate in TSH-free medium for 24 h followed by washing with hypotonic buffer. After addition of 200 μl of 3-isobutyl-1-methyl xanthine (0.5 mmol/l) containing hypotonic buffer to each well, the follicles were stimulated with 1 U/l TSH for 2 h. The supernatants were collected and stored at −20°C. The follicles were treated with 500 μl ice-cold absolute ethanol overnight at −20°C to extract intracellular cAMP. The ethanol extracts were collected in test tubes, evaporated, and then reconstituted with the original supernatant. The cAMP concentration was measured by RIA kit (Amerham Co., Arlington Height, IL).

Na^+K^+ATPase activity, a sodium pump necessary for iodide transport (7), was measured by the method we modified (20) from the original method of Brunberg and Halmi (21). After the follicles were preincubated with 200 μmol/l thiocyanate for 24 h, thyroid follicles were washed with 0.5 mmol/l TRIS-HCl-0.001 mmol/l EDTA buffer, pH 7.0 three times and homogenized as described (20). All samples were stored at −20°C until assay. The sample was centrifuged at 35,000 × g for 30 min at 4°C, and enzyme activity was measured in the pellet (10 to 20 μg protein) as described previously (20).

Iodide efflux study

Iodide efflux was studied by the method described previously (20). Thyroid follicles were preincubated with 100 μmol/l methimazole for 10 h to block iodine organification, and these follicles were incubated with the mixture of 0.1 μCi Na^{125}I and 50 pmol NaI for 3 h at 37°C. Iodide efflux was started by washing follicles with the washing medium that contained 6H medium, 50 nmol/l NaI and 100 μmol/l methimazole with or without potassium thiocyanate. Washing was done a total five times and the medium was collected at each time of washing for counting the radioactivity. The iodide efflux curve was constructed by calculating the percentage of radioactivity that remained in the follicle at each time of washing (7).

Protein measurement and statistical analysis

Protein content was measured by Bradford's method (22). Each experiment was performed at least three times with different batches of follicles. The results shown here were representative experiments with quadruplicate wells. The significance of the difference of the mean values was analyzed by unpaired Student’s t-test, and p<0.05 is considered as significant. Multiple compari-
Fig. 1. Iodide uptake by thyroid follicles in the presence of potassium thiocyanate (KSCN), nicotine (N), and cotinine (C) with three different TSH concentrations. The concentration of nicotine and cotinine was 200 \( \mu \text{mol/l} \). The results are the mean \( \pm \) so of quadruplicate wells. When so is very small it is not shown in the graph.

so sons employed the analysis of variance when it is indicated.

Results

Iodide uptake

We examined the effects of nicotine, cotinine, and thiocyanate on iodide transport in four different TSH concentrations. As shown in Fig. 1, thiocyanate concentration greater than 10 \( \mu \text{mol/l} \) inhibited iodide uptake in a dose response manner regardless of TSH concentration. Nicotine and cotinine even at the pharmacological concentration (200 \( \mu \text{mol/l} \)) did not inhibit iodide uptake. We also examined whether preincubation with thiocyanate is required to reduce iodide uptake. Follicles without preincubation and with 24 h preincubation showed identical inhibition (results are not shown).

Iodide transport kinetics

In the study of iodide transport kinetics, \( K_m \) values for iodide in the control group and the thiocyanate group were 27 \( \mu \text{mol/l} \) and 57 \( \mu \text{mol/l} \), respectively, by linear regression analysis. \( V_{\text{max}} \) values of iodide uptake in both groups were similar: 9.4 nmol I\(^-\) uptake/well (control) vs 7.5 nmol I\(^-\) uptake/well (Fig. 2). Thus, the result of kinetics suggests that thiocyanate is a competitive inhibitor with iodide.

Fig. 2. Iodide transport kinetics. Control group (\( \circ \longrightarrow \circ \)) and 50 \( \mu \text{mol/l} \) thiocyanate group (\( \Delta \longrightarrow \Delta \)). Iodine organification was blocked by 100 \( \mu \text{mol/l} \) methimazole in this experiment. The results are the mean \( \pm \) so of quadruplicate wells.
**Table 1.** Effects of low concentrations of thiocyanate on iodide uptake and iodine organification.

<table>
<thead>
<tr>
<th>Thiocyanate (µmol/l)</th>
<th>125I uptake (%/well)</th>
<th>125I organification (%/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>50.9 ± 2.6</td>
<td>26.3 ± 2.3</td>
</tr>
<tr>
<td>0.5</td>
<td>48.8 ± 1.6</td>
<td>24.1 ± 1.2</td>
</tr>
<tr>
<td>1.0</td>
<td>47.6 ± 2.3</td>
<td>18.5 ± 1.7</td>
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Follicles were incubated with the mixture of Na125I and NaI for 8 h in the presence of 0 to 1 µmol/l potassium thiocyanate. The results are the mean ± sd of quadruplicate wells. *p<0.01 when compared with that of the control sample.

**Fig. 3.** Effect of thiocyanate on iodine organification. Follicles were incubated with the mixture of Na125I and NaI for 2 h. Then 50 µmol/l potassium thiocyanate (KSCN) was added followed by incubation for another 2 h. Addition of thiocyanate did not change iodide uptake (O——O), whereas iodine organification (A———A) was decreased. When thiocyanate was not added at 2 h, iodide uptake at 4 h was 60 ± 5%. The results are the mean ± sd of quadruplicate wells. *p<0.05.

**Fig. 4.** Inhibition of de novo iodotyrosine and thyroid hormone formation by thiocyanate. Follicles were incubated with the mixture of Na125I and NaI in the presence of 50 µmol/l thiocyanate (O——O) or without thiocyanate (●——●) for 6 h. 125I-labeled iodotyrosine and thyroid hormone in the Pronase digest of follicles were separated by Dowex-1 column. B: addition of 30% butanol in the 0.1 mol/l HCl. A: addition of 60% acetic acid. Each fraction contained 50 drops.

**Iodine organification**

Thiocyanate decreased iodine organification in a dose response manner. The decrease in iodine organification occurred in parallel to the decrease in iodide uptake (results are not shown). Nicotine or cotinine at 200 µmol/l did not inhibit iodine organification. We also examined whether thiocyanate has an independent inhibitory action on iodine organification. Follicles were incubated with the mixture of Na125I and NaI for 2 h to load iodide; then 50 µmol/l thiocyanate was added. After the addition of thiocyanate, iodide uptake became plateau and iodine organification decreased significantly (Fig. 3), suggesting that thiocyanate inhibited iodine organification independent of iodide uptake. Additional evidence for independent inhibition of iodine organification was obtained by incubating follicles with 1 µmol/l potassium thiocyanate, which inhibited iodine organification without affecting iodide uptake (Table 1).

**Iodotyrosine and thyroid hormone formation**

Fig. 4 shows inhibition of iodotyrosine and thyroid hormone formation by 50 µmol/l thiocyanate, indicating that the action of thiocyanate extends to the reduction of thyroid hormone formation. Nicotine or cotinine at 200 µmol/l did not inhibit this process.

**Iodide efflux by thiocyanate**

Thiocyanate at 10 µmol/l or greater increased iodide efflux from the thyroid follicle (Fig. 5). Interestingly, the degrees of iodide efflux by 10 µmol/l and 100 µmol/l...
Discussion

This study was designed to examine whether products from cigarette smoking have a direct antithyroid activity in the thyroid cell. We examined nicotine, cotinine, and thiocyanate for antithyroid activity using cultured porcine thyroid follicles, which function in a more physiological fashion than do monolayered cells (16–18). Nicotine has been detected in smokers with its maximum serum level of 1 µmol/l (23). Cotinine, a product from nicotine, has a long serum half-life of 15 to 40 h with serum levels ranging from 0.1 to 1 µmol/l (24). None of the two agents showed antithyroid activity in our experiment. Serum levels of thiocyanate in smokers range from 75 to 200 µmol/l; serum half-life is greater than 6 days (25). Thus, concentrations of thiocyanate used in this experiment were physiological ranges of smokers. Thiocyanate is well known as an inhibitor of iodide transport (7, 8). We examined further whether TSH modifies the action of thiocyanate and whether thiocyanate affects cAMP generation and Na⁺K⁺ ATPase activity, important cellular functions for iodide transport (7). Our study indicates clearly that inhibition of iodide uptake by thiocyanate is independent of TSH concentration (Fig. 1) and that thiocyanate does not affect cAMP generation or Na⁺K⁺ ATPase activity. The question can be raised how thiocyanate inhibits iodide transport. Saito et al. have described thyroid iodide translocator, a Na-dependent iodide transport protein different from Na⁺K⁺ ATPase, in the phospholipid vesicle of plasma membrane (26) and speculated that thiocyanate inhibits this system (9). Their model is also convenient to explain competitive inhibition of iodide transport as thiocyanate and iodide are common substrates for this iodide transporting protein (9). Competitive inhibition of iodide transport is important to understand the action of thiocyanate in vivo because iodine deficiency may facilitate antithyroid action of thiocyanate or an excessive iodine intake may diminish the action of thiocyanate in the thyroid gland.

Thiocyanate has been reported to increase iodide efflux (10) and our present study confirmed it. Scranton et al. have reported in their in vivo model that thiocyanate is more potent for iodide efflux than for inhibition of iodide transport (10). In our culture system, the degree of iodide efflux by 10 µmol/l and 100 µmol/l thiocyanate did not differ significantly. Therefore, a profound decrease in ¹²⁵I content in the presence of 100 µmol/l thiocyanate cannot be explained by iodide efflux alone; inhibition of iodide uptake or iodide organification should be responsible.

In our study, iodine organification and de novo thyroid hormone formation were inhibited by thiocyanate (Figs. 3 and 4). This may be attributed to the decreased iodide transport or to independent inhibition of iodine organification, or both. Thiocyanate has been reported to inhibit iodide peroxidase in a cell-free system (11, 13) and iodine organification in rat thyroid lobe (12). Our present study also supports an independent inhibition of iodine organification by thiocyanate (Fig. 3 and Table 1) although our present study does not disclose its mechanism. Thiocyanate at 1 µmol/l has been reported to facilitate thyroid hormone formation in a cell-free system (15). We examined the effects of 0.5 µmol/l and 1 µmol/l thiocyanate on iodotyrosine and thyroid hormone formation by Dowex-1 column chromatography. We were unable to demonstrate such an increase in thyroid hormone formation in the culture system. We have not examined the effect of thiocyanate on thyroid hormone secretion. However, the decrease in hormone secretion can be expected by thiocyanate, since thyroid hormone secretion correlates with the amount of formed thyroid hormone in our culture system (16).

The potency of antithyroid activity of thiocyanate in vitro has to be interpreted carefully. For instance, 50 µmol/l thiocyanate has a potent antithyroid activity in vitro (Fig. 4), whereas most smokers have serum thiocyanate levels greater than 50 µmol/l without
developing hypothyroidism (5). The discrepancies of potency in vivo and in vitro appear to be attributed to the presence of iodide and pseudohologens in vivo because these agents are known to alter the effect of thiocyanate on the thyroid gland (27). Whether or not smoking contributes to the development of hypothyroidism in vivo needs more specific studies such as examination of thyroid function in smokers who live in iodine deficient areas or who have a marginal thyroid function such as Hashimoto’s thyroiditis.

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