Thyroid-stimulating hormone increases active transport of perchlorate into thyroid cells

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Perchlorate blocks thyroidal iodide transport in a dose-dependent manner. The human sodium/iodide symporter (NIS) has a 30-fold higher affinity for perchlorate than for iodide. However, active transport of perchlorate into thyroid cells has not previously been demonstrated by direct measurement techniques. To demonstrate intracellular perchlorate accumulation, we incubated NIS-expressing FRTL-5 rat thyroid cells in various concentrations of perchlorate, and we used a sensitive ion chromatography tandem mass spectrometry method to measure perchlorate accumulation in the cells. Perchlorate caused a dose-related inhibition of 125-iodide uptake at 1–10 μM. The perchlorate content from cell lysate was analyzed, showing a higher amount of perchlorate in cells that were incubated in medium with higher perchlorate concentration. Thyroid-stimulating hormone increased perchlorate uptake in a dose-related manner, thus supporting the hypothesis that perchlorate is actively transported into thyroid cells. Incubation with nonradioiodinated iodide led to a dose-related reduction of intracellular accumulation of perchlorate. To determine potential toxicity of perchlorate, the cells were incubated in 1 nM to 100 μM perchlorate and cell proliferation was measured. Even the highest concentration of perchlorate (100 μM) did not inhibit cell proliferation after 72 h of incubation. In conclusion, perchlorate is actively transported into thyroid cells and does not inhibit cell proliferation.

iodide; Na+/I− symporter

Although the mechanism by which iodide is concentrated in the thyroid has been well studied and characterized over the past 50 years, it was not until 1996 that the Na+/I− symporter (NIS) was cloned (12). NIS functions as the transport system for transferring iodide from the blood against an electrochemical gradient into thyroid follicular cells. This is the initial step in the biosynthesis of the iodine-containing hormones thyroxine and triiodothyronine, which are essential for growth, development, and many metabolic reactions (22). NIS has also been shown to have a high affinity for many ions of similar size and charge to iodide, such as perchlorate, thiocyanate, nitrate, bromide, perrhenate, and pertechnetate (31, 34). Many of these anions, including perchlorate, have been shown to be competitive inhibitors of iodide transport (34).

Perchlorate blocks iodide transport in human thyroid follicular cells in a dose-dependent manner (17, 27) and has been used pharmacologically to treat hyperthyroidism (33). In cells transfected with human NIS, NIS had a 30-fold higher affinity for perchlorate than for iodide (29). NIS is expressed in human placenta and lactating breast (20, 26) and thus may transport perchlorate across these tissues, resulting in exposure of the developing fetus and neonate to perchlorate (4, 19). This has led to public health concerns because low-level perchlorate exposure is common in the US population (5) and was associated with increased serum TSH and decreased thyroxine levels in women with low iodine status in one epidemiological study (3).

Perchlorate both inhibits iodide uptake by NIS and stimulates iodide efflux from thyroid follicular cells. These processes also are likely to occur in vivo on the basis of the historical use of the perchlorate discharge test to diagnose defects in iodine organization. Unlike iodide, however, perchlorate is not metabolized by the thyroid gland (1), and the mechanism of action of perchlorate on the NIS is not fully clear (31, 32). There are conflicting results from the electrophysiological research on NIS and anion transport studies in thyroid tissue (2, 16, 34, 35, 36) in regard to whether or not perchlorate is transported by NIS.

NIS transports two Na+ ions for every one I− ion, generating an inward current; this is the basis for electrophysiological studies of NIS using Xenopus oocytes (13, 16) and patch clamping in FRTL-5 cells (36). These methods define a selectivity series for anion transport as follows: I− > ClO4− > SCN− > NO3− > Br−. No measurable current was produced by the tetrahedral anions perchlorate and perrhenate in the two studies (16, 36), which led some researchers to the conclusion that these anions are not transported by NIS (13). This is in contradiction to previous studies showing that radiolabeled perchlorate was concentrated in thyroid tissue in vivo (9, 10, 32) and contrasts with the selectivity series based on physiologically relevant perchlorate transport in thyroid tissue: ClO4− > ReO4− > SCN− ≥ I− > NO3− > Br− (2, 34, 35). Recently, Dohan et al. (14) published additional evidence of NIS-mediated active transport of perchlorate both in vivo and in vitro. Their conclusions are based on indirect techniques for measuring perchlorate and thus need to be confirmed using direct perchlorate detection methods.

In the present work we used a very sensitive perchlorate measurement by ion chromatography-electrospray ionization-tandem mass spectrometry to directly measure perchlorate.
accumulation in FRTL-5 rat cells to test the hypotheses that perchlorate is actively transported into thyroid cells, that the process is TSH dependent, and that iodide can block perchlorate uptake by thyroid cells.

MATERIALS AND METHODS

Cell culture and reagents. FRTL-5 rat thyroid cells used in all experiments were generously provided by Dr. Leonard Kohn. FRTL-5 cells were used because no human thyroid cell line has functional NIS. The only human cell lines that can be maintained in cell culture are thyroid cancer cells that do not express NIS and do not transport iodide. Rat and human NIS cDNA share 93% similarity and are functionally interchangeable. FRTL-5 cells were cultured in Coon’s modified Ham’s F-12 medium supplemented with six hormones (bovine TSH, 2 U/l; insulin, 246 U/l; somatostatin, 10 µg/l; hydrocortisone, 10 nM; transferrin, 5 mg/l; glycy1-histidyl-lysine, 2.5 µg/l), 5% calf serum, and antibiotics (6H medium) as described previously (23, 25). Cells were maintained in a 5% CO2-95% air atmosphere at 37°C with a change of medium every 2nd or 3rd day and passed every 7 days. TSH is a pituitary hormone that induces NIS expression in thyrocytes. Cells to be studied in TSH-free (5H) medium were rinsed once with sterile saline, and the TSH-free medium was changed 3 days before and on the day of test substance addition. Three days was the minimum time required for TSH-stimulated effects on NIS expression to dissipate.

For experiments measuring uptake of 125I and perchlorate, FRTL-5 cells were transferred to 12-well plates and grown to confluence. FRTL-5 cells are contact inhibited and thus will not grow upon each other to physically block access to the medium. In experiments examining the effect of TSH on iodide and perchlorate uptake, FRTL-5 cells were grown in 12-well plates using 6H medium until ~50% confluence. They were then cultured in 5H medium for ≥3 days. When the confluence reached ~75%, TSH was reintroduced in concentrations designed for the particular experiment.

Chemicals, hormones, and reagents were purchased from Sigma. Iodide uptake assay. Na125I was purchased from Amersham and diluted for experiments in HBSS to 0.2 µCi/ml. During 1-h incubation, 0.5 ml of this assay buffer was placed in each well so that each well contained 0.1 µCi of Na125I. The concentrations of KClO4 and NaI were modified depending on the particular objective of each experiment.

The 6H medium was removed from each well in 12-well plates, and the cells were rinsed once with 1 ml of HBSS (previously heated to 37°C). After removal of HBSS rinse, 1 ml of the assay buffer containing 125I, perchlorate, and/or sodium iodide, as specified in each experiment, was added to each well. Cells were then incubated at 37°C water bath for 60 min.

The radioactive assay buffer was removed, and cells in each well were rinsed twice, each with 1 ml of ice-cold HBSS, as quickly as possible. The duration of cell contact to ice-cold HBSS was <30 s to avoid release of trapped iodide from cells. After the last rinse, 0.5 ml of 0.5 M NaOH was added to each well and cells were incubated at room temperature for 30 min. The NaOH acts as a cell lysate, breaking cell membranes and releasing trapped radioiodide into the NaOH solution. The entire volume (0.5 ml) was placed in a sample tube, and the 125I was counted in a γ-well counter. Total radioactivity of cells in each well was expressed as counts per minute per well. Each concentration was run in triplicate, and the uptake was calculated as an average of those three wells.

Perchlorate assay. For the purpose of analysis of perchlorate content in each well, an additional set of triplicate wells underwent exactly the same procedure as described above, but the Na125I was omitted. The cell pellets were lysed with NaOH and stored frozen at −20°C in plastic-stoppered tubes until shipment. Samples were shipped on dry ice by overnight courier to the Center for Disease Control’s National Center for Environmental Health in Atlanta, GA.

Perchlorate was analyzed using a modified version of the method of Valentin-Blasini et al. (30). Briefly, lysed cells (~100,000 cells) were spiked with 2 ng of 36Cl-perchlorate internal standard. Following a 30-min equilibration at room temperature, the solution was transferred to a microcentrifuge tube containing a 0.2-µm nylon filter. The sample was centrifuged at 16,060 g for 35 min. An aliquot of the filtrate was transferred to an autosampler vial and subsequently analyzed using ion chromatography-electrospray ionization-tandem mass spectrometry. Perchlorate was quantified on the basis of the peak area ratio of analyte to stable isotope-labeled internal standard. Two quality control pools were analyzed in each analytical batch with unknown samples. Reported results met the accuracy and precision specifications of the quality control/quality assurance program of the Division of Laboratory Sciences, National Center for Environmental Health, Center for Disease Control [similar to rules outlined by Westgard et al. (31a)]. We assessed perchlorate contamination by lot screening all reagents and analyzing blanks with each batch of unknowns and identified a background of trace levels of perchlorate in the medium and reagents. If background perchlorate levels exceeded the detection limit of 0.05 ng/ml, then the data were corrected for this small perchlorate background in the medium and NaOH (<0.17 ng/ml).

The results presented are representative of at least three replicate experiments. Iodide and perchlorate uptake data were fit to a curve using a single-site competitive ligand binding macro in Sigma Plot v10 (Systat Software, San Jose, CA). TSH response and washout data were curve fit using the Sigma Plot simple exponential rise to maximum curve fit.

Measurement of cell growth. FRTL-5 cells were grown to confluence in 6H medium and then maintained in 5H medium for 3 days. The medium was aspirated, and 200 µl of 6H medium containing various concentrations of ClO4− was added to quadruplicate wells of the 96-well plate. After 1–5 days, the perchlorate-containing medium was aspirated from each well, and growth inhibition due to possible perchlorate toxicity was tested using Promega’s MTS-CellTititer 96 AQueous One Solution Cell Proliferation Assay (Madison, WI).

RESULTS

Perchlorate is taken up by FRTL-5 cells. Figure 1A shows that, when cells were incubated with 1 µM iodide, the maximal uptake of radioactive iodide was 42,250 counts·min−1·well−1 when there was no perchlorate present. Addition of perchlorate caused a dose-related inhibition of iodide uptake. Figure 1B shows data on perchlorate uptake from a concurrent experiment with identical conditions, except that no 125I was used because this would contaminate the instrumentation for perchlorate measurement. The amount of perchlorate taken up by the cells increased with increasing perchlorate in the incubation medium. The percentage of perchlorate that was taken up by cells was calculated using the measured amount (in ng) divided into the expected amount present in the incubation medium (based on concentration of perchlorate used for each particular point). The percentage of perchlorate taken up by cells decreased as the concentration of perchlorate in the incubating medium increased. The perchlorate uptake was 8, 5, and 2% at perchlorate concentrations of 1, 2, and 10 µM, respectively.

Perchlorate uptake is TSH dependent. We investigated the effect of TSH on iodide and perchlorate uptake. FRTL-5 cells were grown in 6H medium at usual concentration of 2,000 µU/ml until ~95% confluent. They were then transferred to 12-well plates and grown in the absence of TSH (5H medium) for 7 days. The cells were then incubated in various concentrations of TSH ranging from 0 to 80 µU/ml for 96 h to stimulate various amounts of NIS expression. Subsequent in-
The results show that perchlorate is actively transported into FRTL-5 thyroid cells by a TSH-dependent process, that the amount of perchlorate taken up increases with the concentration of perchlorate in the medium and is saturated at high perchlorate concentration, and that the perchlorate uptake is blocked by iodide in a dose-related manner. These results support the hypothesis that perchlorate is actively transported into the thyroid cell; this active transport is most likely mediated by NIS. However, our data do not exclude the possibility that another transporter besides NIS may contribute to the transport of perchlorate. Based on the data shown in Fig. 2, a mean cell count of 5.0 \times 10^5/well and a mean thyroid cell volume of 3.73 \mu l (8), the calculated cell/medium ratio was 62 at the TSH-induced maximum uptake of perchlorate. Our results are consistent with studies of perchlorate uptake using ^{36}ClO_4^- in rats and guinea pigs showing that the isotope was concentrated in the thyroid tissue and that the concentration was TSH dependent (9, 21).

Electrophysiological studies showed that perchlorate did not elicit any current and that perrhenate elicited only a minor current in Xenopus laevis oocytes transfected with rat NIS, suggesting that these anions were not transported (16). In an
elegant study using both FRTL-5 cells and COS-7 cells transfected with human NIS, Van Sande et al. (31) showed that there was active transport of perrhenate. Their studies of the inhibition of transport by various anions suggested that perchlorate and perrhenate were similar in potency. They suggested that the "explanation for the discrepancy between the electrophysiological and tracer uptake measurement of tetrahedral oxyanion transport would be that the symporter would have equal stoichiometry for Na\(^+/\)H\(^+\) and these anions, i.e., that this transport would be electrically neutral" (31). NIS-mediated active vectorial transport of perchlorate was recently demonstrated by Dohan et al. (14) using polarized NIS-expressing Madin-Darby canine kidney cells in a bicameral setup. Studies of \(^{186}\text{ReO}_4\) in this elegant model showed that its transport stoichiometry was electoneutral, suggesting that Na\(^+/\)/ClO\(_4\) transport is also electoneutral (14). By directly measuring perchlorate accumulation in thyroid cells for the first time, we confirm physiological studies that inferred active perchlorate transport into thyroid cells rather than perchlorate’s being bound to NIS and functioning only as an inhibitor of iodide transport. A possible basis for the electrical neutrality is that each of the two sodium ions cotransported with iodide are bound by different regions of the symporter and that the symporter functions even when only one sodium ion is cotransported. Additionally, our findings agree with the conclusions of Clewell et al. (11), who believed that the weight of evidence favors active transport of perchlorate into the thyroid follicle against a concentration gradient.

Although perchlorate is an excellent oxidizer under some conditions, the activation energy required to initiate these chemical reactions is high. This high activation energy may explain why humans do not metabolize perchlorate; instead, humans rapidly excrete perchlorate in urine with a \(t_{1/2}\) of \(\sim 8\) h (17). Our data show that even a concentration of 100 \(\mu\)M, 50-fold greater than that which will markedly inhibit iodide uptake, is not cytotoxic in cultured FRTL-5 cells.

Although a rat cell line was used for the present study, we believe the results are applicable to humans. The cDNA that encodes human NIS exhibits an 84% identity and 93% similarity to rat NIS (26). The transcriptional regulation of human and rat NIS in thyroid cells is also very similar (20). Accordingly, our results are likely to be entirely applicable to human thyroid cells in regard to perchlorate uptake.

The principal concern regarding perchlorate exposure of humans is inhibition of thyroidal iodide uptake, possibly resulting in reduced synthesis of thyroid hormone. Because trace-level perchlorate exposure is common in the US population, there is considerable public and governmental interest in this issue (18). An epidemiological analysis of the National Health and Nutrition Examination Survey (NHANES) data has reported that background perchlorate exposure levels are associated with changes in thyroxine and TSH concentrations in women with low iodine status (3). Further analysis of the NHANES data found that the combination of exposure to perchlorate and cigarette smoke is associated with decreased thyroxine and increased TSH levels, consistent with inhibition of NIS-mediated iodide uptake (28). These published findings are consistent with our results; active transport of perchlorate is modulated by the presence of other anions, such as iodide. Additionally, our data suggest that the NIS expressed in pla-
central tissue could transport perchlorate across the placental barrier and lead to increased exposure to the developing fetus. Further research is needed to assess the toxicological implications of NIS expression in both placental and mammary tissue (11a) and the modulation of perchlorate transport by iodide. Active transport of perchlorate across membranes is also consistent with the active secretion of perchlorate in human and bovine milk (7, 15, 19, 24).

In conclusion, our data show that perchlorate is actively transported into FRTL-5 cells by a TSH-stimulated process and that iodide in high concentrations is a competitive inhibitor of perchlorate uptake.

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REFERENCES