Regulation of Thyroid Oxidative State by Thioredoxin Reductase Has a Crucial Role in Thyroid Responses to Iodide Excess

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The phenomenon that supraphysiological doses of iodide (I\(^{-}\)) temporarily inhibit thyroid hormone synthesis is known as thyroid iodide autoregulation. Recovery of thyroid function has been attributed to sodium-iodide symporter (NIS) inhibition, but the diversity of available data makes it difficult to reach definitive conclusions. Iodide excess induces reactive oxygen species production and cell toxicity. However, the roles of the oxidative state of the cell and antioxidant selenoproteins in I\(^{-}\) autoregulation have never been explored. Here we analyze the effects of high I\(^{-}\) doses in rat thyroids and in PCCl3 cells in the period comprising I\(^{-}\) autoregulation (i.e. 0–72 h after I\(^{-}\) administration), focusing on NIS expression, redox state, and the expression and activity of selenoproteins. Our results show that NIS mRNA inhibition by I\(^{-}\) does not occur at the transcriptional level, because neither NIS promoter activity nor Pax8 expression or its binding to DNA was modulated. Because I\(^{-}\) uptake was inhibited much earlier than NIS protein, and no effect was observed on its subcellular localization, we suggest that I\(^{-}\) is inhibiting NIS in the plasma membrane. The increased reactive oxygen species production leads to an increase in thioredoxin reductase mRNA levels and enzyme activity, which reduces the oxidative stress. Inhibition of thioredoxin reductase at either gene expression or activity levels prevented NIS recovery, thus illustrating a new role played by this selenoprotein in the regulation of cell homeostasis and consequently in I\(^{-}\) autoregulation. (Molecular Endocrinology 25: 1924–1935, 2011)

A part from its essential role in the synthesis of thyroid hormones T\(_3\) and T\(_4\), iodide (I\(^{-}\)) is one of the most important regulators of thyroid function, together with TSH (1, 2). Thyroid autoregulation by I\(^{-}\) has been extensively investigated in the last decades and was first described by Morton (3), who demonstrated the inhibitory effect of high doses of I\(^{-}\) on thyroid function. This effect, known as the Wolff-Chaikoff effect (4), is transient, and approximately 2 d after I\(^{-}\) administration, the thyroid escapes and hormone synthesis is restored (5).

After the cloning of the sodium-iodide symporter (NIS) (6, 7), a new field of investigation was opened to help in understanding the mechanisms of high I\(^{-}\) effects on the thyroid. The escape from the Wolff-Chaikoff effect has been associated with inhibition of NIS expression and I\(^{-}\) uptake, with a concomitant decrease of intracellular I\(^{-}\) concentration, which then becomes insufficient to sustain the effect (8, 9). However, the difficulty in clearly defining the boundaries that separate the changes in NIS expression during the Wolff-Chaikoff effect and the transition to its escape makes it difficult to delineate the mechanisms that are behind NIS modulation, and some discrepant results have been found depending on the approach used.

Abbreviations: ATG, Aurothioglucose; AUR, auranofin; Gpx, glutathione peroxidase; MMI, methylmercaptoimidazole; NIS, sodium-iodide symporter; NUE, NIS upstream enhancer; Pax8, paired-domain transcription factor 8; ROS, reactive oxygen species; siRNA, small interfering RNA; TPO, thyroperoxidase; Tnx, thioredoxin; TnxRd, thioredoxin reductase.
The thyroid gland has several mechanisms to protect itself against cell oxidation, given that thyroid hormone biosynthesis requires continuous synthesis of $H_2O_2$, produced by dual oxidase and used by TPO (thyroperoxidase) for $I^-$ organification. Therefore, control of thyroid oxidation levels is crucial for maintaining thyroid homeostasis. In this context, the importance of some selenium (Se)-containing antioxidative enzymes and proteins has been described; these proteins are expressed in thyrocytes and are partially secreted into the colloid lumen and mainly belong to the glutathione peroxidase (Gpx) and thioredoxin (Txn) reductase (TxnRd) families (10–12).

One effect of high doses of $I^-$ in the thyroid gland is the induction of toxicity, which has been attributed to an increased production of reactive oxygen species (ROS) that raise the intracellular oxidation levels to above the basal threshold (13–15). Previous work described the modulation of mRNA expression of some selenoproteins in thyroid cells treated with high $I^-$ doses (16), but it is not yet clear whether this modulation is associated to the changes in oxidative levels induced by $I^-$ and whether these changes are involved in thyroid autoregulation. The oxidative state may also be directly involved in NIS responses to $I^-$ excess, because a balanced cell oxidation level is critical for a normal functioning of gene expression machineries at the pre- and posttranscriptional levels (17).

In the present study, we have determined the effects of high $I^-$ doses on the oxidative state and on selenoprotein expression and activity in the thyroids of rats and in PCCl3 cells in a time-dependent manner. We show that the cell redox state and TxnRd activity are directly related to $I^-$ effects on NIS and thyroid hormone biosynthesis, giving new insights into understanding the molecular bases of thyroid autoregulation by $I^-$. In addition, we find that the blockage of $I^-$ uptake, which occurs faster than NIS mRNA and protein inhibition, is not due to internalization of NIS protein; instead, we suggest an inactivation of NIS in the plasma membrane by $I^-$ excess.

**Results**

**Iodide inhibits thyroid hormone secretion**

One of the difficulties in interpreting the studies on thyroid $I^-$ autoregulation arises from the discrepancies among the published data as a consequence of variations in parameters used by different authors, i.e. $I^-$ doses used, time of $I^-$ administration, and more importantly, the use of an *in vivo* or an *in vitro* system. Therefore, we decided to reevaluate the effects of high $I^-$ treatment on the thyroid both in rats and in PCCl3 cells between 0 and 72 h after administration.

A commonly agreed upon thyroid response to high $I^-$ treatment is the inhibition of thyroid hormone secretion, followed by the reestablishment of a normal intracellular $I^-$ concentration. This phenomenon has been observed in humans and in rats (18–20). Therefore, the first step of this study was to analyze whether the rat system we used was responsive to high $I^-$ treatment in agreement with these previous studies. Serum TSH and $T_3/T_4$ levels were determined in rats treated with 0.5 mg $I^-$ by ip injection. The inhibition of thyroid hormone production (Fig. 1) was as expected and similar to what was previously described by Eng et al. (21).

**NIS mRNA inhibition by iodide excess does not take place at the transcriptional level**

NIS inhibition at the mRNA level has been shown to be one of the main effects of high doses of $I^-$ in the thyroid (22, 23). In agreement with these data, we observed an 80% decrease in NIS mRNA in the thyroids of rats after 24 h $I^-$ treatment (Fig. 2A). In PCCl3 cells, we also observed decreased NIS mRNA levels after 24 and 48 h excess $I^-$ incubation, reaching a reduction of 20–30%, which was totally recovered at 72 h (Fig. 2B). In both rats and cells, NIS mRNA expression was not inhibited after treatment with $I^-$ and methylmercaptoimidazole (MMI) (Fig. 2, A and B, *hatched bars*).

The main transcription factor implicated in transcriptional regulation of NIS is paired-domain transcription factor 8 (Pax8) acting through its binding to the NIS upstream enhancer (NUE) region of the NIS promoter (24). Therefore, we analyzed whether $I^-$ treatment modulates Pax8 protein levels, but no effect was observed in
Iodide inhibits NIS protein expression and function but not its cellular localization

Having confirmed NIS mRNA inhibition by I\(^-\), the next step was to check whether this effect was accompanied by a decrease in NIS protein levels. Iodide excess markedly inhibits NIS protein expression in the thyroid of rats (Fig. 3A) from 6 h onward, reaching almost 90% inhibition at 24 h. In PCCl3 cells, we also observed a decrease in NIS protein expression after I\(^-\) excess treatment, starting at 6 h (Fig. 3B) and reaching approximately 40% inhibition at 24 h. After this period, NIS protein expression started to recover. In agreement with the data obtained on mRNA analysis, these effects were not observed in rats or cells treated with MMI (Fig. 3, A and B, hatched bars).

After confirmation of the inhibitory effect of I\(^-\) on both NIS mRNA and protein levels, our focus was to analyze its repercussion on I\(^-\) accumulation in PCCl3 thyroid cells. Using an \(^{125}\)I uptake assay, we observed that I\(^-\) treatment markedly blocked NIS activity, and surprisingly, this effect was much faster than that observed for mRNA and protein modulation, reaching almost 50% inhibition after just 1 h treatment and 80% inhibition at 24 h (Fig. 3C). The inhibitory effect was maintained during the 72 h treatment, although after 48 h, the activity started to recover.

Perhaps the most logical explanation to interpret the discrepancies observed between NIS mRNA and protein inhibition compared with the sharp blockage of I\(^-\) uptake is that the plasma membrane symporter is being internalized, thus becoming nonfunctional. Such an effect on NIS localization has been demonstrated for TSH in thyroid FRTL-5 cells (26). Thus, we quantified the amount of NIS protein present at the plasma membrane in PCCl3 cells (Fig. 3D) by a biotinylation assay (27), and we observed that the decrease of its expression was similar to that observed for total mRNA and protein (Figs. 2B and 3B). This reduction was about 30% after 24 h I\(^-\) treatment and was almost totally recovered after 72 h. These results demonstrate that I\(^-\) does not affect the trafficking and/or subcellular localization of NIS and suggest a direct inactivation of NIS protein present in the plasma membrane.
Iodide increases ROS levels

Thyroid cells preserve a basal level of ROS production necessary for maintaining their normal function (28). On the other hand, it has been described that I– excess can increase ROS production above these basal levels and induce cell toxicity (13). We decided to analyze the production of ROS species after excess I– treatment in the thyroid cell line PCC13 during the time period that spans I– autoregulation, i.e. from 0–72 h. We observed an increase in ROS production after 6 h (Fig. 4), reaching a maximum at 48 h (twice the normal levels). Interestingly, after 72 h I– treatment, we observed a reduction of 25% in ROS concentration with respect to the 48-h measurement. As a control of the effectiveness of the assay, we used treatment for 6 h with 5 μM H2O2, an oxidative stress inducer.

Iodide regulates TxnRd and Gpx mRNA expression

After the confirmation that I– excess increases ROS production in PCC13 cells in a time-dependent manner, we analyzed whether I– treatment modulates the expression of some of the most important regulators of cellular reduction-oxidation (redox) state, the selenoproteins. In a previous paper, Leoni et al. (16) showed that treatment of thyroid cells with I– modulates mRNA expression of Gpx2 and Txn1, selenoproteins, which have been implicated in the regulation of cellular oxidative stress. We therefore analyzed the two most important groups of selenoproteins: the Txn family, TxnRd1 (cytosolic) and TxnRd2 (mitochondrial), and the glutathione peroxidase family, Gpx1 (cytosolic), Gpx2 (gastrointestinal), Gpx3 (extracellular), and Gpx4 (phospholipid hydroperoxidase). Iodide excess significantly increased TxnRd1, TxnRd2, and Gpx2 mRNA expression in rats (approximately 2-fold, Fig. 5, A, C, and E) after 6 h treatment, followed by a notable decrease at 72 h. MMI addition blocked these effects of I– on the expression of TxnRd1, TxnRd2, and Gpx2 (Fig. 5, A, C, and E, hatched bars).

In PCC13 cells, TxnRd1 mRNA expression also increased (Fig. 5B), although with some delay when compared with the expression in rat thyroids, with a maximum after 48 h I– administration. TxnRd2 and Gpx2 mRNA levels in these cells were only slightly modulated (Fig. 5, D and F).

We also analyzed the mRNA levels of other selenoproteins (Gpx1, Gpx3, Gpx4, Sep15, Spp1, Selk1, Selm, and Sbp2) after I– excess treatment, but we did not observe any significant modulation in rats or cells (data not shown).

Iodide increases Txnrd activity but not Gpx activity

The most important role of selenoproteins controlling the redox state of the cell lies in their enzymatic activity. In thyroids of rats (Fig. 6A) and in PCC13 cells (Fig. 6B), I– increased TxnRd activity from 6–48 h after administration, and then activity started to decline. Also, TxnRd activity was incremented by MMI treatment, even though...
its administration had no significant effect on TxnRd mRNA expression. We did not observe any modulation of Gpx activity in rat thyroids (Fig. 6C) or in thyroid cells (Fig. 6D) after I− treatment, but an increased activity was observed after MMI administration (Fig. 6, C and D, hatched bars).

TxnRd activity regulates NIS responses to iodide

To correlate the regulation of NIS by I− excess with the increase of TxnRd activity, we analyzed NIS mRNA and protein expression in PCC13 cells treated with I−, but in the presence of a specific inhibitor of TxnRd activity, aurothioglucose (ATG). It has been described that 1 μM of this gold compound inhibits 25% of TxnRd activity, although having no effect on Gpx activity (29). Iodide, in the presence of 1 μM ATG, inhibits NIS mRNA and protein expression (Fig. 7, A and B, black bars) more dramatically than I− alone (compare with Figs. 2B and 3B). To confirm these results at the functional level, we analyzed I− uptake in PCC13 cells after high I− treatment in the presence of ATG and another inhibitor of TxnRd activity, auranofin (AUR) (30) (Fig. 7C). The administration of ATG with I− inhibits NIS activity from 70–90% from 6–72 h of treatment (Fig. 7C, black bars). The same was observed for AUR treatment (10 nM) plus I− (Fig. 7C, gray bars). Treatment with ATG and AUR without I− did not significantly inhibit NIS mRNA and protein expression (data not shown). The most important finding was that in the presence of the TxnRd activity inhibitors, NIS activity did not recover after 72 h, in contrast to what we observed when cells were treated with I− only (Fig. 3C).

To validate these findings conclusively, we used a small interfering RNA (siRNA) approach to inhibit TxnRd1 expression in PCC13 cells. The effectiveness of the siTxnRd1 approach was confirmed at the mRNA and protein levels (Supplemental Fig. 1, published on The Endocrine Society’s Journals Online web site at http://mend.endojournals.org). In these silenced cells, NIS expression and activity were inhibited from 6 h and did not show any sign of recovery after 72 h I− treatment (Fig. 7, A–C, white bars). Silencing of TxnRd1 in PCC13 cells without I− treatment had no effect on NIS expression or activity (data not shown). Together, these results effectively demonstrated that TxnRd activity is essential for recovery of NIS expression and function.

To determine whether these effects are specific for NIS, we analyzed the administration of I− excess and ATG on the expression of other thyroid genes involved in thyroid hormone biosynthesis. The results show that protein levels of thyroglobulin and TPO (Supplemental Fig. 2) and dual oxidase 2 and TSH receptor (data not shown) are not modulated by I− excess. Also, no modulation was observed in the presence of ATG (Supplemental Fig. 2), suggesting a specific effect of I− and TxnRd activity on NIS expression.

Discussion

Thyroid autoregulation by high iodide (I−) doses has been known since 1944 (3) and was characterized in 1948 (4). It is called the Wolff-Chaikoff effect and the escape from it. One of the features of this process, described in humans (18) and in rats (19, 31), is the inhibition of thyroid hormone secretion. Here we confirm the modification of rat thyroid status with a decrease in T3 levels at 24 h and an increase in TSH after 6 and 24 h of high I− administration, followed by a recovery between 48 and 72 h.

Although there have been many attempts to understand the mechanism of thyroid autoregulation by high doses of I−, it still remains uncertain. Eng et al. (21, 22) reported that high doses of I− decrease NIS mRNA and protein at posttranslational levels, affecting mainly the half-life of the protein in a dose-dependent manner. Our results support and extend these data by demonstrating that the decrease in NIS mRNA does not take place at the transcriptional level. This result is also in agreement with recent data showing I− effects on NIS mRNA stabilization (23) but is in contradiction to the findings of other authors who used higher I− doses (10 mM) (32). Furthermore, we found no regulation of Pax8 expression or its
specific binding to NIS promoter, confirming the lack of transcriptional regulation. A decrease of Pax8 mRNA levels by I− was described by other authors, but this was under different conditions (33).

The inhibition of iodide uptake by high doses of I− is extremely fast and was interpreted by Eng et al. (22) to be a consequence of NIS internalization or protein turnover. However, our results show that the amount of NIS protein at the plasma membrane was only slightly decreased, similar to total NIS protein, which does not match the sharp decrease in I− accumulation (compare Fig. 3, C and D). This result leads us to hypothesize that the rapid blockage of I− accumulation is possible only by inactivation of NIS protein in the plasma membrane.

There are many putative redox-sensitive sites in the NIS protein that could be involved in its functional inactivation. In silico analysis of Cys residues using three different programs (34–36) and based on topological analysis and protein modeling (De la Vieja, A., unpublished observations) predict only two intracellularly oriented and potentially redox-sensitive Cys residues (C272 and C440). Methionine residues also are important targets of oxidation within proteins, both in a reversible (methionine sulfoxide) and irreversible way (methionine sulfone). Other amino acids located in regions involved in I− and/or Na+ binding or translocation could also be direct targets of oxidation. Analysis of congenital iodide transport defects (revised in Refs. 7 and 37) reveals that single amino acid substitutions in the NIS protein can render an inactive protein without affecting synthesis or trafficking. Likewise, oxidation (in a reversible or irreversible way) of amino acids could generate an inactive protein. Moreover, there are indications that NIS may be a multimeric protein in its functional form (38, 39). Redistribution or alteration of these multimeric forms by direct oxidation may also result in a rapid inactivation of NIS protein.

The thyroid cell needs a minimal oxidative load to guarantee its normal function, and it has been described that when intracellular ROS levels drop below minimal concentrations, the expression of proteins involved in thyroid cell function is hampered (28). The activation of several pathways by ROS could be part of a defense mechanism against oxidative stress, an adaptive response serving to neutralize redox perturbations (40, 41). Increased expression and activity of antioxidant enzymes involved in H2O2 turnover, such as catalase, superoxide-dismutase, and peroxiredoxins have been described in the I−-deficient thyroid gland (42). However, available data on the induction of oxidation by I− excess, which besides H2O2 could involve other oxidized (e.g. O2•− or OH•) (43) or iodinated intermediates [e.g. 6-iodo-8-11,14-eicosatrienoic-∆-lactone (44) and 2-iodohexadecanal (45)], show that ROS production varies according to I− concentration and the species analyzed (46). In the present study, we observed that high I− treatment increased ROS production in PCC13 thyroid cells, but it was kept at a tolerable level suitable for the maintenance of normal cell functions: twice the normal levels and below cytotoxic concentrations that would not allow cell survival. Iodide cytotoxicity has been described in thyroid cells treated with 20 mM KI (13), a concentration much higher than the one used here. In our study, we
observed cytotoxicity and apoptotic signals only in control cells treated with 5 μM H₂O₂.

It has been documented that ROS at low levels are physiological mediators of cellular responses involved in processes such as mitogenic signal transduction (47, 48), gene expression, regulation of cell proliferation, replicative senescence, apoptosis, and cancer (49). In this context, it is plausible to conjecture that although only a small increase in ROS levels was observed, this was enough to trigger an oxidative stress response. Our study revealed that the increase in ROS levels by high I⁻ doses occurs progressively until 48 h and that this effect is accompanied by a modification of thyroid hormonal status.

Increased ROS levels induced not only TxnRd mRNA levels but, more importantly, also TxnRd activity. TxnRd, together with Txn and reduced nicotinamide adenine dinucleotide phosphate (NADPH) compose a highly conserved system (the Txn system) that regulates a variety of intracellular processes such as DNA synthesis, protein-DNA interactions, gene expression, and cellular growth (40, 50–52), and it has been described as one of the main effectors of ROS responses (53). In addition, high TxnRd1 expression and activity have been directly connected with cellular protection against oxidative stress induced by 4-hydroxynonenal, one of the end products of lipid peroxidation (54). Thus, this enzyme could be one of the main effectors responsible for restoration of normal oxidation levels in the thyroid cell after high I⁻ administration.

Although TxnRd has a general role as ROS scavenger (53), Gpx are mainly involved in regulation H₂O₂ levels in the colloid (42). This could explain not only the differences in Txnrd and Gpx activities that we have found but also why the alterations in Gpx mRNA levels were not observed in cells that lack follicular structure. In PCC13 cells, the effect is mainly due to the oxidized species produced in the cytoplasm. However, it is important to note that high I⁻ treatment significantly increased mRNA levels of Gpx2, which was described as a gastrointestinal enzyme (55) but is highly expressed in the thyroid. Our study also showed that the mRNA levels of other selenoproteins (Gpx1, Gpx4, Sep15, Sep1p, Selk1, Selm, and Sbp2) were not modulated by I⁻ treatment. Although it has been demonstrated that Se supply is directly associated with the control of selenoprotein expression (29, 56, 57), our results showed that Se supplementation (from 50–500 nm) of cells treated with I⁻ excess did not exert effects on selenoprotein modulation other than those reported here (data not shown).

In cells, although I⁻ uptake is still inhibited at 72 h, there is a tendency toward recovery in parallel with ROS decrease, which is in agreement with our hypothesis that the decrease in ROS levels is the first requirement for the thyroid to resume its normal function. The most striking observation was that this response was not observed in the presence of two inhibitors of TxnRd activity, ATG and AUR (29, 58–60). Reinforcing our affirmation that the Txn system is directly involved in the recuperation of thyroid function after oxidative stress, the same results were observed after silencing the TxnRd1 gene by siRNA. This effect is specific for NIS, because these treatments had no effect on other thyroid genes (such as Tg and Tpo). Besides its effects on NIS, the antioxidant role of TxnRd

FIG. 6. Effect of I⁻ treatment on TxnRd (A and B) and Gpx (C and D) activities. Enzyme activities were determined spectrophotometrically 6, 24, 48, and 72 h after I⁻ administration to rats (left panels) and PCC13 cells (right panels). Rats and cells were treated with MMI (c, hatched bars) or MMI plus I⁻ for 24 h (24, hatched bars). These data represent the mean ± se of at least three independent experiments performed in triplicate. Statistical significance was evaluated by a Student’s paired t test. Differences vs. control (c, black bars) for each time point normalized to 1 were considered significant: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
after excess $I^-$ administration seems to be crucial for re-establishment of thyroid homeostasis and the recovery of thyroid hormone biosynthesis.

In our study, all effects observed during $I^-$ autoregulation seem to occur faster and more pronounced in rats than in cells. The differences may be related to the follicular structures present in rat thyroid, which, besides allowing more ROS accumulation, seems to be a critical factor controlling NIS expression (61) and consequently $I^-$ uptake. Apart from the time shift and differences in intensity, we observed that the majority of the molecular events characterized present a similar behavior in both systems. These results make us more confident about the validation of this mechanism.

In summary, our results provide new information about the molecular events involved in thyroid autoregulation by high doses of $I^-$. First, we determined that the rapid blockade of $I^-$ uptake in thyroid cells is not occurring in parallel to NIS mRNA and protein modulation and also that it is not caused by NIS internalization. These observations suggest an inactivation of NIS localized at the plasma membrane. Second, the incorporation of large amounts of $I^-$ increases ROS species in the thyroid cell above basal levels. As a consequence, the expression and activity of TxnRd selenoproteins increase to compensate oxidation and avoid cell toxicity. Thus, these selenoproteins participate in thyroid $I^-$ autoregulation by allowing the restoration of a normal thyroid cell oxidation state and NIS reexpression.

Materials and Methods

Rats and treatment

Protocols for animal handling were approved by the local institutional Animal Care Committee, following the rules of the European Union. Male Wistar rats weighing 200–250 g were housed under controlled conditions of temperature (22 ± 2 C) and light (12-h light, 12-h dark cycle; lights on at 0700 h) and had free access to water and food. The iodine content of rat food was 0.4 mg/kg, and Se content was 100 μg/kg (Scientific Animal Food & Engineering). The Se concentration corresponds to a daily consumption of 0.12 parts per million, which is assumed to be sufficient to maintain its concentration at normal physiological levels (42, 62). Rats were divided into three groups, with three rats each: 1) control group, which received a single ip injection of 0.5 ml saline; 2) iodide-treated group, which received a single ip injection of 0.5 ml saline containing 0.03% MMI in drinking water for 6 d; on the seventh day, they received a saline or $I^-$ injection, as described for groups 1 and 2, respectively. Because MMI is a thionamine compound that blocks TPO activity (63), its use was intended to clarify the effects of $I^-$ itself (i.e., not of organified iodide or its oxidized intermediates) on thyroid responses to $I^-$ excess. After saline or $I^-$ injection, rats of groups 1 and 2 were killed at 6, 24, 48, and 72 h and rats of group 3 after 24 h.

Hormone measurements

Rat blood samples were collected for determination by RIA of total plasma T3 and T4 using T3 and T4 MAb kits (MP
Biomedicals, Solon, OH). TSH measurement was done using a Rat TSH RIA kit (IBL International GmbH, Hamburg, Germany).

Cell culture and treatment

Rat PCCl3 thyroid cells were cultured in Ham’s F12 medium supplemented with 5% donor calf serum and a six-hormone mixture: 1 mM TSH, 10 μg/ml insulin, 10 ng/ml somatostatin, 5 μg/ml transferrin, 10 mM hydrocortisone, and 10 ng/ml glycyl-l-histidyl-l-lysine acetate. Cells were maintained at 37 C and 5% CO² until reaching 30% confluence, when they received 1 mM (KI; Sigma) or not (controls). The medium does not contain I⁻ and Se, and when treatment was started, serum was reduced to 0.2%. Thus, the concentrations of both elements did not change significantly during the study. Cells were collected 6, 24, 48, and 72 h after I⁻ administration, with some variations depending on the assay as described in each experiment. One group of cells was pretreated with MMI (500 μM) for 12 h and then received I⁻ treatment for 24 h before being harvested.

TxnRd inhibition

To evaluate the involvement of TxnRd in I⁻ effects, we used two different approaches. First, PCCl3 cells were treated with two inhibitors of TxnRd activity (30, 60), 1 μM ATG or 10 nM AUR (Sigma), for 12 h before the addition of I⁻. Second, the TxnRd1 gene was silenced in PCCl3 cells using ON-TARGET plus SMARTpool siRNA molecules (Dharmacon, Lafayette, CO). We chose to silence TxnRd1, and not TxnRd2, because the increase of TxnRd1 mRNA expression by I⁻ was observed in both systems studied. After 12 h transfection, the silenced cells were treated with and without a high I⁻ dose, and all results were normalized against nontargeting siRNA (scrambled siRNA control).

RNA extraction and real-time quantitative RT-PCR analysis

Total RNA from rat thyroid tissue and PCCl3 cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Two micrograms of total RNA was reverse transcribed with 200 ng random primers, 10 U ribonuclease inhibitor, 0.1 mM dNTP, and 400 U Moloney murine leukemia virus reverse transcriptase. The obtained cDNA was used as a template for quantitative PCR using the SYBR Green Master Kit (Stratagene, La Jolla, CA) and the Stratagene Mx 3000P System. Cycling was done at 95 C for 10 min followed by 40 cycles of 95 C for 30 sec, 58–60 C for 30 sec, and 72 C for 30 sec. The primers used are described in Supplemental Table 1. Glucuronidase β (Gusb) was used as the standard.

Electrophoretic mobility shift assay

Nuclear extracts from PCCl3 cells were prepared following the procedure described by Andrews and Faller (64). An oligonucleotide probe corresponding to the Pax8-binding site in the rat NIS promoter (site PB within the NUE) (24) was labeled with [γ-32P]ATP by polynucleotide kinase. Nuclear extracts (7 μg) from untreated PCCl3 cells and from cells treated with I⁻ for 24 h were incubated with the labeled probe. Binding reactions were performed in a buffer containing 40 mM HEPES (pH 7.9), 200 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 150 ng/ml polydeoxyinosinic deoxyctydilic acid, and 5% Ficoll at room temperature for 30 min. Binding reactions were performed as described (65). Samples were electrophoresed on a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA. Gels were transferred to Whatman 3MM paper, dried, and autoradiographed.

Plasmids and transfection

A luciferase reporter construct containing a 2.854-bp DNA fragment of the rat NIS promoter (pNIS-2.8) (25) was used. pNIS-2.8 contains the NUE, a regulatory element necessary for full TSH response with two binding sites for the paired domain transcription factor Pax8, the main factor controlling NIS transcription. PCCl3 cells were transfected using jetPEI (Polyplus Transfection) and then treated with I⁻ for 24 and 48 h. pRL-TK, which contains a cDNA encoding renilla (Promega, Madison, WI), was used to monitor transfection efficiency. Cells were analyzed for luciferase and renilla activities by the Dual-Luciferase reporter assay system (Promega).

Iodide uptake

Cells were assayed for iodide transport as previously described (66). Briefly, control and I⁻-treated cells were incubated for 1 h at 37 C in Hank’s balanced salt solution containing 20 μM KI and carrier-free Na125I (specific activity, 100 μCi/mmol I⁻). Radioactivity was quantified in a γ-counter. Uptake was expressed as picomoles of I⁻ and normalized for the amount of DNA in each well. Perchlorate, a competitive inhibitor of NIS-mediated I⁻ transport, was used at 80 μM (not shown).

Protein extraction and Western blot analysis

Rat thyroid tissue and PCCl3 cells were homogenized in RIPA buffer containing PBS (1×), 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate and then centrifuged to obtain whole-cell extracts. Plasma membrane proteins were isolated from control and I⁻-treated PCCl3 cells using the membrane-impermeable biotinylation reagent Sulfo-NHS-SS-biotin (Pierce Biotechnology, Rockford, IL) as described (27). All samples were diluted in loading buffer and heated at 37 C for 30 min. Samples were then separated by SDS-PAGE and transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Immunoreactive bands were visualized with the Pierce ECL Western Blotting Substrate (Thermo Scientific, Pittsburgh, PA). Protein expression levels were quantified using ImageQuant software ( Molecular Dynamics, Sunnyvale, CA). The protein of interest was quantified and normalized in all cases to its loading control. Antibodies against pan-cadherin, tubulin, actin (Santa Cruz Biotechnology, Santa Cruz, CA), Na⁺/K⁺-ATPase-α (Affinity BioReagents, Golden, CO), TG (Dako, Carpinteria, CA), TPO (Santa Cruz), and Pax8 (BioPat) were used. rNIS antibody was a generous gift from Dr. N. Carrasco (Albert Einstein College of Medicine, Bronx, NY).

ROS measurement

Detection of ROS accumulation in PCCl3 cells was done by a carboxy-H₂DCFDA staining method (67). After incubation with I⁻ as described above, cells were incubated for 1 h at 37 C with 5 mM carboxy-H₂DCFDA. Then, cells were washed three times and incubated in PBS buffer without H₂DCFDA for 20 min. Fluorescence emission was read on an LS-50 fluorometer (PerkinElmer, Norwalk, CT) at 355 and 485 nm (excitation and
emission, respectively). Incubation with 5 μM H2O2 for 6 h was used as assay control. This concentration was chosen after analysis of a dose-response curve (0.5–100 μM). We observed that concentrations higher than 50 μM started to induce apoptosis (data not shown), as reported by other authors (68).

**TxnRd and Gpx enzymatic activity**

Measurement of TxnRd activity (69) was done using the Thioredoxin Reductase Assay Kit (Sigma), which uses 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) as the substrate for TxnRd. Fluorescence corresponding to DTNB reduction was measured in a spectrophotometer (Ultraspex 3000; Pharmacia Biotech, Piscataway, NJ) at 412 nm. Measurement of Gpx activity (70) was done using the Glutathione Peroxidase Assay Kit (Calbiochem, La Jolla, CA), which measures Gpx activity indirectly by measuring the NADPH oxidation produced to reduce the oxidized glutathione. NADPH oxidation is accompanied by a decrease in absorbance at 340 nm, which was measured in a spectrophotometer (Molecular Devices). For both assays, we started with 25–100 mg thyroid tissue and 10^6–10^8 cells.

**Statistical analysis**

The results are expressed as the mean ± SEM of at least three different experiments performed in triplicate. Data were analyzed with GraphPad Prism (Intuitive Software for Science). Statistical significance was determined by Student’s paired t test, and differences were considered significant at a P value of <0.05.

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