Down-regulation of the Sodium/Iodide Symporter Explains $^{131}$I-Induced Thyroid Stunning

Madeleine M. Nordén,¹ Fredrik Larsson,¹ Sofia Tedelind,¹ Therese Carlsson,¹ Charlotta Lundh,² Eva Forsell-Aronsson,² and Mikael Nilsson¹

¹Department of Medical Chemistry and Cell Biology, Institute of Biomedicine, The Sahlgrenska Academy at Göteborg University; ²Department of Radiation Physics, Göteborg University, Sahlgrenska University Hospital, Göteborg, Sweden

Abstract

$^{131}$I radiation therapy of differentiated thyroid cancer may be compromised by thyroid stunning (i.e., a paradoxical inhibition of radioiodine uptake caused by radiation from a pretherapeutic diagnostic examination). The stunning mechanism is yet uncharacterized at the molecular level. We therefore investigated whether the expression of the sodium/iodide symporter (NIS) gene is changed by irradiation using $^{131}$I. Confluent porcine thyroid cells on filter were stimulated with thyroid-stimulating hormone (TSH; 1 milliunit/mL) or insulin-like growth factor-I (IGF-I; 10 ng/mL) and simultaneously exposed to $^{131}$I in the culture medium for 48 h, porcine NIS mRNA was quantified by real-time reverse transcription-PCR using 18S as reference, and transepithelial iodide transport was monitored using $^{125}$I as tracer. TSH increased the NIS expression >100-fold after 48 h and 5- to 20-fold after prolonged stimulation. IGF-I enhanced the NIS transcription at most 15-fold but not until 5 to 7 days. $^{131}$I irradiation (7.5 Gy) decreased both TSH-stimulated and IGF-I-stimulated NIS transcription by 60% to 90% at all investigated time points. TSH and IGF-I stimulated NIS synergistically 15- to 60-fold after 5 days. NIS expression was reduced by $^{131}$I also in costimulated cells, but the transcription level remained higher than in nonirradiated cells stimulated with TSH alone. Changes in NIS mRNA always correlated with altered $^{125}$I transport in cultures with corresponding treatments. It is concluded that down-regulation of NIS is the likely explanation of $^{131}$I-induced thyroid stunning. Enhanced NIS expression by synergistically acting agents (TSH and IGF-I) partly prevents the loss of iodide transport expected from a given absorbed dose, suggesting that thyroid stunning might be pharmacologically treatable. [Cancer Res 2007;67(15):7512–7]

Introduction

Radioiodine therapy with $^{131}$I has two main indications: toxic diffuse goiter (i.e., Graves’ disease) and differentiated thyroid cancer. Successful outcome of treatment relies on the ability of the thyroid tissue to concentrate and retain $^{131}$I. In both normal and pathologic thyroid tissues, this is primarily achieved by the action of the sodium/iodide symporter (NIS) present at the thyroid cell surface (1). Loss of NIS expression leads to insufficient radioiodine uptake, which is the main reason why $^{131}$I therapy is not applicable to patients with poorly differentiated thyroid cancer.

Materials and Methods

Cell culture and hormone treatment. Pig thyroid glands obtained from the local abattoir were prepared for isolation and culture of thyroid epithelial cells as described (13). Briefly, purified thyroid follicle segments were plated on collagen type I–coated filters in Transwell bicameral chamber inserts (3413 or 93413, Corning Costar) at 37°C in a CO2 (5%) incubator. The cells were grown in Earl's MEM with 5% FCS, penicillin (200 units/mL), streptomycin (200 μg/mL), and fungizone (2.5 μg/mL); all reagents were purchased from PAA Laboratories GmbH) until forming a continuous monolayer resembling the native thyroid follicular epithelium.

Diagnostic scanning with low amounts of $^{131}$I is often used to estimate the mass of thyroid remnants after tumor surgery. Numerous studies have reported that diagnostic exposure to $^{131}$I may diminish subsequent uptake of the therapeutic dose, a phenomenon known as thyroid stunning, which potentially may compromise the therapeutic efficiency and outcome of radioiodine treatment (reviewed in refs. 2–5). The stunning mechanism has for long been obscure mainly because of lack of experimental data. However, recent studies in our laboratory, using an in vitro model of internal radiation of normal thyroid cells, show that $^{131}$I indeed reduces thyroid iodide transport in a dose-dependent manner (6, 7) at absorbed doses equivalent to those estimated to induce stunning in vivo (3). Importantly, as quiescent (Go) cells were irradiated and the cell number did not change, it could be excluded that the inhibited transport was related to compromised cell cycle progression or apoptosis otherwise readily induced when similar amounts of $^{131}$I were administered to proliferating thyrocytes (6, 7). Considering NIS as the key molecule responsible for the iodide-concentrating mechanism in the thyroid gland and in thyroid carcinoma cells with preserved iodide uptake, it can be hypothesized that the expression or function of NIS might be affected by ionizing radiation and that this could explain thyroid stunning. However, this possibility has not been experimentally investigated.

The NIS expression in thyroid cells is transcriptionally regulated by both positive and negative signals. Most importantly, thyroid-stimulating hormone (TSH) up-regulates the NIS mRNA level multifold via activation of a cyclic AMP (cAMP)-responsible element in the NIS promoter (8, 9). This favorable effect is likely responsible for the fundamental increase in tumor radioiodine uptake in thyroid cancer patients given recombinant human TSH before radioiodine therapy (10). Conversely, down-regulation of NIS mRNA expression can be experimentally induced by excess iodine (11, 12), which requires organization (i.e., iodide-protein binding) and thus explains the so-called escape of the Wolff-Chaikoff effect, a thyroid protective mechanism against toxicity of iodine overload. In this study, we investigated with real-time reverse transcription-PCR (RT-PCR) analysis whether $^{131}$I irradiation influences the NIS transcript levels in primary cultured normal thyrocytes stimulated with TSH and insulin-like growth factor-I (IGF-I).
after ~1 week. In the culture chamber, the basal and apical compartments facing either side of the epithelium correspond to the extracellular space and the follicular lumen, respectively, which makes the working model highly suitable for studies of transepithelial iodide transport normally taking place in vivo. Epithelial barrier function indicating tight junction integrity was assessed before experiments by monitoring the transepithelial electrical resistance with a Millicell-ERS ohmmeter (Millipore Corp.).

Confuent cells were stimulated alone or in combination with TSH (1 milliunit/mL; Sigma) and insulin-like growth factor 1 (IGF-I; 10 ng/mL; Roche Diagnostics GmbH). TSH and IGF-I were added to the medium at the start of 131I exposure, and hormonal stimulation (renewed every 2–3 days without irradiation. However, as IGF-I (but not TSH) is mitogenic also to confluent thyroid cells (14), we measured the total DNA content of individual filter cultures by a fluorimetric assay (15) to correlate data to cell number changes likely appearing in response to IGF-I stimulation with and without irradiation.

Irradiation with 131I. Both the apical (200 μL) and basal (400 μL) culture compartments were replaced with medium containing the same activity concentrations of 131I (Nycodem, Amersham). Calculations to obtain a mean absorbed dose of 7.5 Gy after irradiation for 48 h were carried out using Monte Carlo simulation as described (7), taking into account the fact that 131I gradually accumulated in the apical medium due to ongoing iodide transport during 131I exposure. The absorbed dose of 7.5 Gy was chosen to mimic a high diagnostic dose assumed to be received in thyroid remnants after whole-body scanning with 131I. Methimazole (1 mmol/L; Sigma) was present in the medium to prevent radiiodide-protein binding that might extend 131I exposure after removal of the free activity and thereby increase the absorbed dose in cells subjected to further culture. Culture plates were shielded with lead blocks to prevent external irradiation of control groups. Irradiation was stopped after 48 h by removing all 131I by repeated washings with activity-free medium.

Measurement of 125I transport. Transepithelial 125I transport was assessed 2, 5, or 7 days after the start of 131I irradiation. Triplicate cultures in each treated group were simultaneously replaced with basal medium (400 μL) containing 150 kBq/mL 125I– (Nycodem, Amersham; appreciated final iodide concentration, 10−7 mol/L) and apical medium (200 μL) that was free of radiotracer. After 30 min, 50 or 100 μL were taken from the apical side of each well, and the activities were measured in a gamma counter (Wallac 1480 WIZARD 3ª, Wallac Oy). The relative amount of 125I– being transported to the apical compartment was calculated after correction for detector background.

Real-time RT-PCR analysis of NIS mRNA expression. Total RNA was extracted from cultures at the indicated time points with the Qiagen RNeasy Micro kit (Qiagen GmbH), according to the manufacturer’s instructions, and quantified by UV spectrophotometry at 260 nm. For each sample, cDNA was synthesized from 0.5 μg RNA using random hexamers and Taqman reverse transcription reagents (Applied Biosystems) according to the manufacturer’s instructions. Oligonucleotide primers for the porcine NIS (pNIS) gene and for the reference gene (18S) were designed using the Primer Express software (Applied Biosystems) and purchased from TAG Copenhagen A/S. Primer sequences were as follows: pNIS, 5’-ctctctgctggagcaggtactct-3’ (forward) and 5’-gtgaggtggtcgcgctga-3’ (reverse); 18S, 5’-gtaaccgttgaaccctcctg-3’ (forward) and 5’-ctcctaatcggtagagc-3’ (reverse). The relative quantitative PCRs were done by using the Quantitect SYBR Green PCR kit (Promega) and the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Thermal cycling conditions consisted of an initial cycle of 2 min at 50°C, a cycle of 15 min at 95°C, and 40 cycles of 15 s of denaturation (94°C) followed by 1 min of annealing and extension (60°C). All amplification reactions were done in triplicates. The threshold cycle (Ct) values were used for calculation of the relative expression ratios between control and treated cells using the formula described by Pfaffl (16).

Statistical analysis. In total, two to six separate experiments with triplicates in each were carried out for each kind of assay and time point. The Student’s t test or analysis of confidence intervals was used to determine the statistical significance (P ≤ 0.05) of differences between data obtained in measurements of total DNA and 125I transport. The statistical significance of real-time RT-PCR data (P ≤ 0.001) obtained in a single analysis was calculated by using the Relative Expression Software Tool (REST; ref. 17). The Student’s t test (P ≤ 0.05) was used for comparing log2 values pooled together from several identical PCR experiments. Results presented in graphs show mean ± SE values.

Results

131I irradiation inhibits TSH-stimulated NIS mRNA expression and iodide transport. Before 131I irradiation experiments were carried out, it was necessary to characterize the magnitude and kinetics over time of NIS mRNA expression in the applied thyroid cell culture model. Primers against defined sequences of the pNIS gene were found to give reliable quantitative data on the transcript level as analyzed with real-time RT-PCR. As shown in Fig. 1A, 1 milliunit/mL TSH increased the NIS mRNA expression ~130-fold (log, 7.04) after 48 h. The stimulatory effect sustained after prolonged exposure to TSH for up to 7 days, although there was a significant reduction after the peak value at 2 days. This pattern correlated well with changes in the transepithelial transport of iodide from the basal to the apical medium (i.e., TSH enhanced the 125I transport at all time points, although most prominently 2 days after stimulation was started; data not shown). As previously reported (13, 18), no transport of 125I was measured in the opposite apical-to-basal direction (data not shown), illustrating the fact that NIS is solely expressed at the basolateral surface of fully polarized thyroid epithelial cells.

Next, cultures were stimulated with TSH and simultaneously exposed to 131I for 48 h to obtain the absorbed dose of 7.5 Gy, after which NIS mRNA was measured either directly or after culture for additional 3 days in activity-free medium. This showed that irradiation significantly reduced the NIS transcript levels measured at both time points (Fig. 1B). In average, 131I inhibited the NIS expression by 60%, but in two of five included experiments, the reduction was ~80%. Importantly, down-regulation of NIS was accompanied by a corresponding decrease of the iodide transport capacity in the irradiated cultures (Fig. 1C).

IGF-I-stimulated NIS transcription is also radiosensitive to 131I. NIS is up-regulated by TSH mainly via a cAMP-dependent mechanism (19). It was therefore of interest to investigate whether NIS gene transcription as stimulated by the activation of another intracellular signaling pathway also might be radiosensitive. Earlier studies from our laboratory have shown that the transepithelial transport of iodide in pig thyrocytes is stimulated by IGF-I, although this occurs with a delayed onset in comparison with the effect of TSH (14). We confirmed this in the present study (data not shown) and found in addition that 10 ng/mL IGF-I increased the NIS mRNA levels at the corresponding investigated time points (i.e., 5–7 days after IGF-I was added; Fig. 2A). The magnitude of enhanced NIS expression was lower than that monitored after TSH stimulation, and a significant stimulatory effect of IGF-I on NIS mRNA was not always measured. Nevertheless, in experiments showing a clear response (two of six), the IGF-I–induced transcription of NIS was nearly completely inhibited by the simultaneous exposure to 131I (Fig. 2B). Likewise, irradiation inhibited the IGF-I–stimulated iodide transport recorded in parallel cultures (Fig. 2C).

According to previous studies (14), IGF-I is mitogenic to confluent thyroid cells, which thus escape from contact inhibition, enter the cell cycle, and start to proliferate, resulting in a gradual
conversion of the cell monolayer into a multilayered epithelium. However, as the NIS mRNA quantification was normalized to 18S in all samples, it is conceivable to assume that the observed effects of IGF-I and 131I were not biased by altered cell numbers. To further corroborate this, we measured the total DNA content, which was found to be increased by 25% after stimulation with 10 ng/mL IGF-I, a submaximal mitogenic dose, after 5 days (Fig. 2D). Although simultaneous irradiation with 131I (7.5 Gy) expectedly blocked the IGF-I–stimulated DNA synthesis (Fig. 2D), the magnitude of altered NIS expression and iodide transport was much higher than can be explained by the relatively small changes in cell number.

IGF-I and TSH synergistically counteract the loss of NIS expression and iodide transport in 131I-irradiated cells. As TSH and IGF-I stimulate the expression of NIS by different modes of action, it was of interest to study the combined effect and determine whether NIS in costimulated cells were radiosensitive to 131I to the same extent as in single-stimulated cells. Incubation with TSH and IGF-I for 5 days increased the NIS transcript levels in average 3-fold (log2 1.48) in comparison with TSH alone (Fig. 3A). This correlated with a corresponding acceleration of iodide transport (Fig. 3B), indicating that the two stimuli acted synergistically. 131I irradiation at an absorbed dose of 7.5 Gy reduced both NIS transcription and iodide transport in the costimulated cells (Fig. 3A and B). However, the inhibitory effect was unexpectedly small and in fact both the expression and function of NIS remained at higher levels than those measured in unirradiated TSH-stimulated cells. Thus, TSH and IGF-I cooperatively counteracted the down-regulation of NIS appearing at a given absorbed dose of 131I.

Discussion
Thyroid stunning reflecting a reduced radioiodine uptake in thyroid remnants after whole-body diagnostic scanning with 131I has been an elusive phenomenon ever because it was first recognized more than 50 years ago (20). Although most experts in the field agree that it is a real side effect of the diagnostic use of 131I that needs to be considered in radioiodine therapy (2–5), the significance and even the existence of stunning have been questioned (21). Recently, we showed in cultured thyroid cells that 131I irradiation indeed inhibits iodide transport at absorbed doses equivalent to those estimated to be received in thyroid or tumor tissues after diagnostic administration of the same isotope (6, 7). In this article, we provide the first evidence of a molecular mechanism that may explain radiation-induced thyroid stunning (i.e., suppressed NIS gene transcription).

The experimental model was adopted to mimic in vitro a situation of internal radiation of the thyroid with 131I. For this purpose, primary cultured thyroid epithelial cells were continuously exposed to 131I and thereby allowed to take up and concentrate the isotope in the apical compartment (corresponding to the follicular lumen in vivo) for a period of 48 h. The received absorbed dose was calculated to be 7.5 Gy, which is comparable with a large diagnostic scanning dose clinically. Importantly, the irradiated cells were confluent and thus growth arrested already at start of the experiment, and no significant changes in cell number were noted in cultures in which TSH-stimulated NIS expression was markedly inhibited. In addition, NIS transcriptional activity

Figure 1. Effects of 131I irradiation on TSH-stimulated NIS mRNA expression and iodide transport. Thyroid cells were stimulated with 1 milliunit/mL TSH for 2 to 7 d and irradiated during the first 48 h. NIS mRNA quantified by real-time RT-PCR and 125I/C0 transport were investigated in triplicates of parallel cultures in the same experiments. Number of included experiments varied as indicated below. A, time-dependent stimulation of NIS transcription by TSH (n = 2). B, 131I-induced inhibition of NIS mRNA expression stimulated by TSH (n = 2 after 2 d and n = 5 after 5 d). C, 131I-induced inhibition of 125I transport stimulated by TSH after 5 d (n = 4). Columns, mean NIS mRNA data of obtained log2 values; bars, SE. **, P ≤ 0.001, REST analysis; *, P ≤ 0.05, Student’s t test.

Unpublished results.
was compared with 18S rRNA, which remained stable after irradiation, and the total RNA pool did not differ between irradiated and nonirradiated samples. Together, this indicates that stunning is not caused by a therapeutic effect due to compromised cell cycle machinery or loss of radiation-damaged cells by apoptosis, which would have been necessary to consider if the cells were proliferating. The distinction is not trivial, as stunning has been suggested to result from thyroid or tumor tissue destruction appearing early after administration of the ablation dose of 131I (22). In further support of stunning as a cell cycle–independent effect of 131I irradiation, we recently found that the dose response of growth arrest and inhibited iodide transport in proliferating and nonproliferating cells, respectively, are not entirely overlapping, the latter being more radiosensitive (7). Nevertheless, stunning might still be a consequence of nuclear events related to nonlethal DNA damage from ionizing radiation. Preliminary observations in our laboratory show that also nonproliferating G0 thyrocytes display definitive signs of DNA double-strand breaks [i.e., activation and recruitment of the histone variant H2AX to nuclear foci of DNA repair (23) after 131I exposure using the same experimental design as in this study].

IGF-I was found to partly counteract the 131I-induced decrease of the NIS mRNA and iodide transport levels in TSH-stimulated thyroid cells, although the weak stimulatory effect of IGF-I given alone was fully blocked by the same radiation dose. This strongly suggests that radiation affects NIS by interfering with its transcriptional regulation and argues against the unlikely scenario that the NIS gene would be, other than infrequently, damaged by a direct hit of randomly distributed ionizing events in the nucleus. Due to the complexity of NIS gene regulation, involving both protein kinase A–dependent and protein kinase A–independent signaling pathways (24, 25), there are several possible candidate mechanisms. Of particular interest may be the recently reported cooperative regulation of the NIS promoter activity by the thyroid transcription factor Pax8 and a redox factor, Ref-1 (26). Ref-1, synonymously called Ape1, is an endonuclease with multifunctional properties that can be triggered by genotoxic stress and participates in DNA repair (27). Speculatively, activated Ref-1/Ape1 might be recruited to sites of DNA damage whereby the transcriptional enhancer of NIS expression might suffer from relative lack of a necessary cofactor. Another possible mechanism leading to stunning might be that signals involved in physiologic or pathophysiologic down-regulation of NIS (e.g., as induced by growth factors) are activated by irradiation (28). In fact, both ligand-dependent and ligand-independent activation of growth factor receptor signaling pathways supposed to participate in the radioprotection response ultimately controlling cell survival are commonly observed in irradiated cells (29).

IGF-I alone stimulated NIS expression and iodide transport, although much less efficiently than TSH. Also contrasting the

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Effects of 131I irradiation on IGF-I–stimulated NIS mRNA expression, iodide transport, and cell proliferation. Thyroid cells were stimulated with 10 ng/mL IGF-I and irradiated identically to that indicated for TSH-treated cultures in Fig. 1. A, time-dependent stimulation of NIS transcription by IGF-I (n = 4 for 2 d, n = 6 for 5 d, and n = 3 for 7 d). B, representative graph of 131I-induced inhibition of NIS mRNA expression stimulated by IGF-I after 5 d (n = 2). C, 131I-induced inhibition of 125I/C0 transport stimulated by IGF-I after 5 d (n = 2). D, 131I-induced inhibition of IGF-I–stimulated increase in cell number estimated by quantification of total DNA content (n = 2). ***, P < 0.001, REST analysis; *, P < 0.05, Student’s t test.
stimulated by IGF-I and TSH after 5 d (identically to that indicated in Figs. 1 and 2. A, 131I-induced inhibition of accelerated NIS mRNA expression stimulated by IGF-I and TSH after 5 d (n = 4). Note that data are compared with the NIS transcript level in cultures stimulated with only TSH. B, 131I-induced inhibition of accelerated 125I transport stimulated by IGF-I and TSH after 5 d (n = 4). **, P ≤ 0.001, REST analysis; *, P ≤ 0.05, Student’s t test.

In conclusion, our findings indicate that 131I irradiation down-regulates the expression of NIS at the transcription level, leading to inhibited iodide transport in primary cultured thyroid cells. This effect most likely explains the phenomenon thyroid stunning observed after pretherapeutic diagnostic examination of 131I uptake in patients with thyroid cancer. The ability of IGF-I and TSH to concomitantly accelerate NIS expression and thereby antagonize the 131I-induced loss of NIS suggests that thyroid stunning might be treatable. However, in view of the possibility that IGF-I regulates NIS in a species-specific manner [i.e., IGF-I suppresses NIS transcription in a rat thyroid cell line (40)], it will be important to extend these studies to human thyroid cells and also carcinoma cells before a possible clinical protocol can be discussed. Likewise, referring to the currently debated application of 131I in future treatment modalities of mammmary adenocarcinomas endogenously overexpressing NIS (41) and of other cancers subjected to NIS gene transfer (42), it is pertinent to elucidate whether stunning involving down-regulation of NIS is thyroid specific or comprise a general response to radiation independently of cell type.

Acknowledgments

Received 2/28/2007; revised 5/7/2007; accepted 5/22/2007.

Grant support: Swedish Research Council grant 537 and Swedish Cancer Foundation grants 3427 and 5657.

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We thank the Department of Oncology and the Division of Medical Physics and Medical Engineering at the Sahlgrenska University Hospital (Gothenburg, Sweden) for help in supplying 131I.

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