Re-evaluation of the effect of iodine on thyroid cell survival and function using PCCL3 and Nthy-ori 3-1 cells

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Abbreviations: FBS, fetal bovine serum; IF, immunofluorescence; IB, immunoblotting; TSHR, TSH receptor; LC3, microtubule-associated protein 1 light chain 3; ROS, microtubule-associated protein 1 light chain 3; NIS, sodium/iodine symporter, U/L, units per liter.
Abstract

Appropriate amount of iodine is critical for normal function of thyroid cells synthesizing thyroid hormones. Although normal thyroid cell lines such as rat PCCL3 and FRTL5 and human Nthy-ori 3-1 have been widely used for in vitro studies on physiological and pathophysiological effects of iodine on thyroid cells, we have recently pointed out the critical differences between FRTL5/PCCL3 cells and Nthy-ori 3-1 cells. Therefore, we here directly compared some of the cellular characteristics - iodine uptake, differentiated status, iodine-induced cytotoxicity and iodine-regulation of autophagy - between PCCL3 and Nthy-ori 3-1 cells. PCCL3 cells express mRNAs for TSH receptor and sodium/iodine symporter and incorporate iodine in a TSH-dependent manner, while Nthy-ori 3-1 cells do not either. Nevertheless, both cells were comparably resistant to iodine cytotoxicity: only far excess iodine (5 x 10^{-2} M) killed 20 to 40% cells in 24 h with perchlorate exhibiting no effect, suggesting this cytotoxic effect is due to extracellular iodine. In contrast, a wide range of iodine (5 x 10^{-9} to 5 x 10^{-2} M) induced autophagy in PCCL3 cells, which was abolished by perchlorate, indicating intracellular iodine-induction of autophagy, but this effect was not observed in Nthy-ori 3-1 cells. In conclusion, it is critical to discriminate the effect of iodine incorporated into cells from that of extracellular iodine on thyroid cells. Iodine-uptake competent thyroid cells such as PCCL3 and FRTL5 cells, not Nthy-ori 3-1 cells, should be used for studies on iodine effect on thyroid cells.
Introduction

The thyroid follicular epithelial cells actively incorporate iodine from bloodstream to synthesize thyroid hormones, but excessive and deficient iodine both cause thyroid dysfunction. Normal thyroid cell lines are very useful to study in vitro physiological and pathophysiological effects of iodine on thyroid cell survival and function. They include rat normal thyroid cell lines, PCCL3 (1) and FRTL5 (2), and a human cell line, Nthy-ori 3-1 (3). Nthy-ori 3-1 cells have recently been used to show iodine-inductions of cell death (4) (5) (6) and of endoplasmic reticulum (ER) stress (7), and -inhibition of autophagy (8). However, we have recently pointed out the critical differences between FRTL5/PCCL3 cells and Nthy-ori 3-1 cells, and expressed our concern that the latter may not be suitable for studies on in vitro effect of iodine on thyroid cells (9). For example, we and others have previously shown that the formers take up iodine (10) (11), while the latter does not (12) (13). Therefore, we here directly compared some cellular characteristics such as the ability to take up iodine, differentiated status, the sensitivity to iodine-induced cytotoxicity and iodine-regulation of autophagy between PCCL3 and Nthy-ori 3-1 cells.

Materials and methods

Cell line used

A rat normal thyroid cell line PCCL3 (1) was previously described (14) and maintained in Coon’s modified F-12 medium supplemented with 5% fetal bovine serum (FBS), antibiotics and 3H (2 U/L bovine TSH, 5 U/L insulin and 5 mg/L transferrin) or 2H (insulin and transferrin) (all from Sigma-Aldrich, St Louis, MD). A human normal cell line Nthy-ori 3-1 (3), obtained from Health Protection Agency Culture Collections (Salisbury, UK), was cultured in RPMI 1640 with 10 % FBS and antibiotics (and 2 U/L TSH in Figure 1). The former was spontaneously immortalized and its growth is totally TSH-dependent, while the latter is derived from an HTori-3 cell line which was immortalized by transfection of simian virus 40 T-antigen and can grow without TSH (9). A human anaplastic thyroid cancer cell line 8505C and a human hepatocellular cancer cell line HepG2 were obtained from RIKEN Bioresource Center (Tsukuba, Japan), cultured in RPMI 1640 with 10 % FBS and antibiotics, and used as controls.
**131I-uptake**

PCCL3 cells were maintained in the presence of 3H or incubated with 2H for 3 days, and Nthy-ori 3-1 cells were maintained in the regular medium or incubated with 2 U/L TSH for 3 days. 5 x 10^4 cells in a 96-well plate were incubated with 10 μCi/ml (37 kBq/μCi) 131I (PerkinElmer, Waltham, MA, USA) for 30 min. Following extensive washing with PBS and resuspension by trypsinization, 131I incorporated into the cells was measured using the automatic gamma counter 2470 WIZARD2 (PerkinElmer). Perchlorate (NaClO4) was added to the culture medium at the final concentration of 5 μM (15) 1 h before 131I addition to block 131I uptake.

**Quantitative real-time PCR for TSHR and NIS expression**

Total RNA extraction and cDNA synthesis were performed with PCCL3 cells maintained with 3H and Nthy-ori 3-1 cells incubated with 2 U/L TSH for 3 days as previously described (16). cDNA synthesized from total RNA from a normal human thyroid tissue in the previous study (16) was also used. PCR was then carried out in a Thermal Cycler Dice Real-time system (Takara, Tokyo, Japan). The primer pairs used were previously described; human TSHR (length of a PCR product: 213 bp) (17), human NIS (420 bp) (18), rat TSHR (302 bp) (19), rat NIS (529 bp) (20), and β-actin (105 bp) (21). The PCR condition was 40 cycle of denature at 95 °C for 15 sec, annealing at 55 °C for 15 sec and extension at 72 °C for 30 sec. The cycle threshold values, which were determined using second derivative, were used to calculate the normalized expression of the indicated mRNAs using Q-Gene software using β-actin for normalization. The PCR product sizes were also confirmed with 1.5 % agarose gel electrophoresis.

**Cell viability assay**

1 x 10^4 cells in a 96-well plate were incubated for 24 to 48 h and then treated with up to 5 x 10^-2 M NaI for 24 h. In PCCL3 cells maintained with 3H, perchlorate was added to the culture medium (the final concentration of 5 μM) 1 h before NaI addition. Cell viability was then measured using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) in accordance with the manufacturer’s protocol.

**Monitoring of autophagy**

Autophagic activity was monitored in PCCL3 maintained with 3H and Nthy-ori 3-1 cells maintained in the regular medium by immunofluorescence (IF) and immunoblotting (IB), as previously described (22). The primary and secondary antibodies for p62, LC3 and anti-β-actin were also shown in refs. (22) (23) (24) (25)
(26) (27) (28). When autophagy is induced, LC3-I is converted to LC3-II by lipidation and recruited to autophagosome from the cytoplasm. Therefore, the amount of LC3-II increases in IB, and punctate staining of LC3 appears in IF. Furthermore, the amounts of p62, a substrate of autophagy, decrease by degradation in the autolysosome, which can be determined by IB and IF (see ref. (22) for more details).

**Statistical analyses**

Experiments were repeated twice with essentially same results. Statistical differences between different groups were examined with ANOVA test and Games-Howel test using SPSS. P < 0.05 was used to identify statistically significant differences.

**Results and Discussion**

We first confirmed the ability/ inability of 131I-uptake in PCCL3 and Nthy-ori 3-1 cells cultured in the presence or absence of TSH (10) (12) (13); an anaplastic thyroid cancer cell line 8505C and a hepatocellular carcinoma cell line HepG2 were used as negative controls. We have previously shown that PCCL3 cells incorporated 131I in a dose-dependent manner, reaching the peak at 30 min in the presence of TSH (10). Therefore, 131I-uptake was determined 30 min after addition of 131I into the culture medium. As shown in Figure 1A, PCCL3 cells maintained with 3H clearly incorporated 131I, which was completely abolished by 5 μM NaClO₄ (perchlorate), a competitive inhibitor of NIS, confirming NIS-mediated iodine uptake. Removal of TSH for 3 days reduced 131I uptake by approximately 50%, indicating TSH-dependent action of iodine uptake. On the other hand, Nthy-ori 3-1 cells, irrespective of the presence or absence of TSH, showed almost negligible 131I-uptake; these low levels of iodine uptake are likely non-specific, because the similar low levels of 131I-uptake were also observed in 8505C and HepG2 cells, both of which have been shown not to express NIS (29) (30).

We next examined mRNA expression of thyroid-specific molecules critical for TSH-dependent iodine uptake; namely TSHR and NIS. Thus, mRNAs for TSHR and NIS were readily detectable in PCCL3 cells and a human thyroid tissue (a positive control), but extremely low levels of their expression was barely observed in Nthy-ori 3-1 cells (Figure 1B), supporting the 131I-uptake results in Figure 1A.

These results clearly demonstrate the substantial differences in the differentiated status between PCCL3 and Nthy-ori 3-1 cells. PCCL3 cells are well differentiated, expressing TSHR and NIS mRNAs, and able to take up
iodine, while Nthy-ori 3-1 cells are conversely less differentiated, expressing little mRNAs for these two molecules, and unable to take up iodine. Nevertheless, both cell lines have long been used comparably as normal thyroid cell lines.

These results then caused doubts about the previous studies on the effects of iodine on survival and function of thyroid cells using Nthy-ori 3-1 cells (see the Introduction), and prompted us to re-evaluate and compare some of these results - iodine cytotoxicity and iodine modulation of autophagic activity - using PCCL3 and Nthy-ori 3-1 cells in parallel in our own hands. The concentrations of iodine we used are 5 x 10^{-9} M, the physiological concentration in human sera; 10^{-6} M, the concentration that suppresses iodine uptake in cultured thyroid cells; 10^{-3} M, excess iodine; and 1 and 5 x 10^{-2} M, far excess iodine (10). Figure 2 demonstrates the results of the study on iodine cytotoxicity. Up to 10^{-2} M iodine had no effect and only highest dose of iodine (5 x 10^{-2} M) induced 20 to 40 % cell death in 24 h in both cell lines as well as 8505C and HepG2 cells. Perchlorate had no effect on cell death in PCCL3 cells, indicating that this mild cytotoxic effect of iodine is attributed to extracellular iodine present in the culture medium, not intracellular iodine incorporated into the cells. Thus, both of thyroid cell lines as well as 8505C and HepG2 cells are comparably resistant against extracellular iodine cytotoxicity, and iodine incorporated into PCCL3 cells is likely harmless, even when the cells were exposed to far excess iodine (10^{-2} M). As far as we know, this is the first study on iodine cytotoxicity in PCCL3 cells. The similar results were reported in Nthy-ori 3-1 cells (4) (5) (6), human thyroid cells and FRTL5 cells (31) (32). Although the mechanism(s) for cytotoxic effect of far excess iodine (5 x 10^{-2} M) in the culture medium is at present unknown, some interaction between iodine and membranous proteins/lipids may explain this effect (33).

The results of autophagy experiments are shown in Figures 3 and 4. A wide range of iodine (5 x 10^{-9} M to 10^{-3} M) increased LC3 puncta and decreased p62 fluorescence intensity 0.5 to 2 h after addition of iodine in IF in PCCL3 cells (Figure 3A & B), clearly demonstrating induction of autophagy by iodine. The data are also confirmed by IB in the cells incubate for 1 hr with 10^{-3} M iodine (Figure 3C). Perchlorate abolished the effect of iodine (10^{-6} M) on autophagy almost completely, indicating that this effect is exerted by intracellular iodine (Figure 3D). Far excess iodine (5 x 10^{-2} M) induced the lower degree of autophagy as compared to the lower doses of iodine, which is likely attributed to cytotoxic effect of far excess iodine as shown in Figure 2. In contrast, 5 x 10^{-9} M to 10^{-3} M iodine had no effect, and 5 x 10^{-2} M iodine reduced LC3 puncta and increased p62 levels in Nthy-ori 3-1 cells (Figure 4), again suggesting that far excess iodine suppressed autophagy by its cytotoxicity. Although our data are somewhat different from the previous study, in which 10^{-4} to 5 x 10^{-3} M
iodine decreased LC3-II in western blotting and autophagosome and autolysosome formation with the LC3-RFP-GFP vector in Nthy-ori 3-1 cells (8), our results demonstrate that induction of autophagy by iodine ranging from $5 \times 10^{-9}$ M to $10^{-3}$ M in PCCL3 cells are due to iodine incorporated into cells, which cannot be seen in Nthy-ori 3-1 cells that are incapable of incorporating iodine.

The pathophysiological significance of iodine-induced autophagy is unknown. We first thought that iodine-increase in ROS might cause autophagy induction because it is well known that oxidative stress induce autophagy through the PERK-eLF2α-ATF4-CHOP pathway and MAPKs such as c-Jun N-terminal kinase 1 (34) (35). Indeed ROS elevation by iodine has previously been reported in FRTL5 and PCCL3 cells; a ~2-fold increase in intracellular ROS by $10^{-3}$ M iodine in 30 min and a huge increase in mitochondrial ROS by $10^{-2}$ M iodine in 2 h (36) and a biphasic increase (at 2 and 24, not 4, h) by $10^{-4}$ M iodine (37). However, we could not detect ROS increase in iodine-treated PCCL3 cells using 2',7'- dichlorofluorescin diacetate in our hands (data not shown).

We also found one report inconsistent with our data (38), in which low doses of iodine ($10^{-8}$ to $10^{-5}$ M) stimulated cell proliferation but higher dose ($10^{-3}$ M) induced apoptosis and autophagy in a thyroid cancer cell line BCPAP. BCPAP cells were established from a differentiated papillary thyroid cancer, but likely gained undifferentiated phenotype during long culture (39) (40). Indeed, BCPAP cells are reported not to express NIS nor take up iodine (12) (39). Therefore, the above data may indicate a possibility for high sensitivity of BCPAP cells to extracellular iodine.

In conclusion, our results indicate that the in vitro effects of high doses of iodine on thyroid cells so far reported using Nthy-ori 3-1 cells are likely due to iodine present in the culture medium not iodine incorporated into the cells. Although cultured cell lines are useful tools for in vitro studies on cell behavior and function, appropriate one(s) should be chosen for a certain experiment in general, and in particular, the effect of iodine, especially that incorporated into thyroid cells, should be studied with functional, iodine-uptake competent thyroid cells such as PCCL3 and FRTL5 cells, not Nthy-ori 3-1 cells.
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Author Disclosure Statement

The authors have no potential conflict of interest to declare.

Data Availability

Some or all data generated or analyzed during this study are included in this published article or in the data repositories listed in References.
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Figure legends

Figure 1.  131I-uptake in PCCL3, Nthy-ori 3-1, 8505C and HepG2 cells, and TSHR and NIS mRNA expression in PCCL3 and Nthy-ori 3-1 cells. A, the cells were cultured in the presence or absence of TSH and/or perchlorate and then incubated with 10 μCi/ml 131I for 30 min. 131I taken up into the cells were measured as described in the Materials and methods. The data are means ± S.D. (n=3). B, Total RNA was extracted from PCCL3 and Nthy-ori 3-1 cells, and subjected to RT-PCR for TSHR, NIS and β-actin mRNAs as described in the Materials and methods. The data are means ± ranges (n=2).

Figure 2. Cytotoxicity of iodine in PCCL3, Nthy-ori 3-1, 8505C and HepG2 cells. The cells, cultured in the presence or absence of perchlorate, were incubated with up to 5 x 10^{-2} M iodine for 24 h and the cell viability was determined as described in the Materials and methods. The data are means ± S.D. (n=3).

Figure 3. Control of autophagic activity by iodine in PCCL3 cells. A, the cells were incubated with up to 5 x 10^{-2} M iodine for up to 2 h, and LC3 puncta and P62 fluorescence intensities were quantified by IF as described in the Materials and methods. B, representative photographs for LC3 puncta and p62 fluorescence in the cells incubated with 10^{-3} M iodine for up to 1 h are shown. C, western blotting of LC3 and p62 in the cells incubated with 10^{-3} M iodine for 1 hr are shown. D, the cells, cultured in the presence or absence of perchlorate, were incubated with 10^{-3} M iodine for 1 h, followed by quantification of LC3 puncta and p62 fluorescence intensities by IF as described in the Materials and methods. *, p<0.05; **, p<0.01.

Figure 4. Control of autophagic activity by iodine in Nthy-ori 3-1 cells. The cells were incubated with up to 5 x 10^{-2} M iodine for up to 2 h, and LC3 puncta and P62 fluorescence intensities were quantified by IF as described in the Materials and methods. *, p<0.05; **, p<0.01.
**A**

131I uptake (cpm \( \times 10^4 \))

|       | PCCL3 | Nhry-ori 3-1 | 8505C | HepG2 |
|-------|-------|--------------|-------|-------|
| Perchlorate | + | + | + | - |
| TSH   | + | + | + | - |

**B**

Relative mRNA levels (fold increase vs each alone)

| Protein | PCCL3 | Nhry-ori 3-1 | Normal human thyroid tissue |
|---------|-------|--------------|-----------------------------|
| Promotor | + | + | + |
| TSP1    | + | + | + |
| TSP2    | + | + | + |

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A

Fluorescence intensity of p62 (10^5 / per cell)

Fluorescence intensity of p62 (10^5 / per cell)

Number of LC3 puncta (per cell)

Number of LC3 puncta (per cell)

B

LC3

p62

0 1 (h)

C

1 mM Na for 1 h

(-) (+)

LC3

p62

β-action

0 1 (h)

D

Fluorescence intensity of p62 (10^5 / per cell)

Number of LC3 puncta (per cell)

Nal

Perchlorate

- + - +
Number of LC3 puncta (per cell) vs. fluorescence intensity of p62 (10^6 per cell) under different NaI concentrations: 5 x 10^-9 M, 10^-6 M, 10^-3 M, and 5 x 10^-2 M.