Duox expression and related H₂O₂ measurement in mouse thyroid: onset in embryonic development and regulation by TSH in adult

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Abstract

In the thyroid, H₂O₂ is produced at the apical pole of thyrocytes by one or two NADPH oxidases (NOX), Duox1/2 proteins. The onset of Duox expression was analysed by immunohistochemistry in the developing mouse thyroid in parallel with thyroglobulin (Tg) iodination and the expression of other thyroid differentiation markers. Duox proteins were found at embryonic day (E) 15.5 and were mainly localised at the apical pole of thyrocytes. Tg was detected 1 day before (E14.5) and Tg iodination was concomitant with the expression of both Duox and Na⁺/I⁻ symporter (NIS; E15.5). The role of TSH in regulating Duox expression and H₂O₂ accumulation was evaluated in thyroids of adult mice with reduced (*Tshr^{hyt/hyt}* or mice treated with thyroxine) or increased (methimazole or perchlorate treatment) TSH/Tshr activity. In mice with suppressed TSH/Tshr activity, Duox

expression was only partially decreased when compared with wild-type, as observed by western blot. In *Tshr^{hyt/hyt}* strain, Duox was still expressed at the apical pole and H₂O₂ measurements were normal. On the other hand, chronic TSH stimulation of the gland led to a decrease of H₂O₂ measurements without affecting Duox expression. The onset of Duox protein expression is compatible with their proposed function in thyroid hormone synthesis and it can be considered as a functional marker of the developing thyroid. However, Duox expression in adult is much less regulated by TSH than NIS and thyroperoxidase. It is not always correlated with the overall thyroid H₂O₂ accumulation, highlighting the importance of additional regulatory mechanisms which control either the production or H₂O₂ degradation.

Journal of Endocrinology (2007) **192**, 615–626

Introduction

Thyroid hormones are synthesised within thyroglobulin (Tg) matrix. The iodination of tyrosyl residues of Tg and the oxidative coupling of the resulting iodotyrosines are catalysed by the thyroperoxidase (TPO) in the presence of hydrogen peroxide (H₂O₂), which is used as a terminal electron acceptor (Nunez & Pommier 1982, Dumont *et al.* 2005). H₂O₂ is generated at the apical membrane of thyrocytes by an NADPH-dependent oxidoreductase. Two cDNAs encoding thyroid NADPH oxidases (NOX) have been cloned: Duox1 and Duox2, formerly called p138^{Tox}, ThOX1/2 or LNOX1/2 (Dupuy *et al.* 1999, De Deken *et al.* 2000). Duox1 and Duox2 are closely related proteins which display 83% sequence similarity and belong to the NOX family (Geiszt & Leto 2004). Duox proteins are characterised by an extracellular N-terminal peroxidase-like domain, intracellular loop containing two EF-hands motifs and C-terminal NADPH oxidase core, common to all NOX family members (Dupuy *et al.* 1999, De Deken *et al.* 2000). Thyroid H₂O₂ generating activity is strongly stimulated by calcium and requires plasma membrane Duox, which is marked by the

N-terminal complex-type glycosylation (Bjorkman & Ekholm 1984, Deme *et al.* 1985, Raspe *et al.* 1991, Corvilain *et al.* 1994, De Deken *et al.* 2002, Morand *et al.* 2003).

During the thyroid embryonic development, proteins involved in the synthesis of thyroid hormones, like TPO, Na⁺/I⁻ symporter (NIS), Tg and the thyroid-stimulating hormone (TSH) receptor (Tshr), are expressed just after the thyroid precursor cells have completed their migration from the primitive pharynx and reached their final location around the trachea (Lazzaro *et al.* 1991, De Felice & Di Lauro 2004). In mouse, this terminal, morpho-functional maturation begins with the expression of Tg at embryonic day 14 (E14; Postiglione *et al.* 2002), followed 1 day later (E15) by the other markers of thyroid differentiation, Tshr, NIS and TPO (Postiglione *et al.* 2002, De Felice *et al.* 2004), and the apparition of active TSH (Lin *et al.* 1994, Japon *et al.* 1994), indicating that Tshr is not necessary for the Tg onset contrary to NIS and TPO. The first phases of thyroid development (in mouse from E8.5), which include thyroid bud growth, proliferation of primordial thyrocytes and their migration towards the trachea, depend at least in part on the simultaneous activity of several thyroid transcription factors:

Ttf1 (Nkx2.1, Ttf-1), Foxe1 (Ttf-2), Pax8 and Hhex. Their expression precedes by ~6 days the expression of markers of functional differentiation (Tg, Tshr, TPO and NIS; for review, see De Felice & Di Lauro 2004).

After birth, TSH through Tshr is the major regulator of growth, differentiation and function of the thyrocyte. It mediates the mRNA expression of several thyroid genes. NIS and TPO expressions are upregulated by the TSH/Tshr signalling pathway, but Tg demonstrates rather stable expression towards TSH (Chazenbalk *et al.* 1987, Gerard *et al.* 1989, Dohan *et al.* 2003). These differences in the TSH/Tshr regulation of NIS and TPO on one side and Tg on the other side were also demonstrated in mouse models without functional Tshr (*Tshr^{hyt/hyt}* and *Tshr*-knockout lines) or deprived of TSH (*pit^{duv}/pit^{duv}*; Postiglione *et al.* 2002, Marians *et al.* 2002).

Regulation of Duox expression has been studied on human thyroids presenting several pathologies (hot nodule, multinodular goitre) in which no dramatic change was observed (Caillou *et al.* 2001, Gerard *et al.* 2003), except in Graves' disease as described by Caillou *et al.* (2001). Hyperstimulated thyroids of rats treated with methimazole or perchlorate did not show any upregulation of Duox expression (Dupuy *et al.* 2000, Miot *et al.* 2002). This is in contrast with the strong upregulation of Duox expression observed in FRTL5 cells (Dupuy *et al.* 2000), dog (De Deken *et al.* 2000) and pig (Dupuy *et al.* 1999), but not in human (De Deken *et al.* 2000, Pachucki *et al.* 2004) thyrocytes in primary culture stimulated by TSH.

In this work, we have shown in developing mouse thyroid that Duox proteins start to be expressed when the thyroid becomes functional and when iodide organification is first observed (E15.5). We have used a mouse model of congenital hypothyroidism related to impaired TSH/Tshr regulatory axis and wild-type mice with increased or decreased TSH levels to determine the *in vivo* regulation of Duox expression. Finally, we have investigated the correlation between Duox expression and localisation with the *ex vivo* H₂O₂ measurements.

Materials and Methods

Mouse models

All mice were kept in the animal facility under 12 h light:12 h darkness cycle and were given water and AO3 mice breeding diet (Safe UAR). All procedures respected regulations and guidelines of the Belgian state and European Union and were approved by the local ethical committee.

Normal staged embryos were obtained by dissecting pregnant females. The day the vaginal plug was observed was designated as embryonic day 0.5 (E0.5). The following stages were analysed: E10.5, E12.5, E13.5, E14.5, E15.5, E17.5 and E18.5. Embryos were embedded in OCT and quick frozen. The moulds were kept frozen until they were cryosectioned.

Two- to five-month-old *Tshr^{hyt/hyt}* mice (genetic background BALB/c crossed and backcrossed with C57BL/6 for at least five

generations) were used. All *Tshr^{hyt/hyt}* mice were treated with 2 mg/l thyroxine (T₄; l-thyroxine, sodium salt pentahydrate; Sigma-Aldrich) in drinking water to avoid hypothyroidism. The mutation Pro556-Leu in the fourth transmembrane domain of the Tshr from *Tshr^{hyt/hyt}* mice (Stein *et al.* 1994) was verified by sequencing PCR products after amplification of genomic DNA. DNA was prepared from a tail biopsy incubated overnight at 55 °C in Tris 100 mM (pH 8), NaCl 200 mM, EDTA 5 mM, SDS 0.2% and proteinase K 132 µg/ml (Qiagen). DNA was isolated using phenol-chloroform-isoamyl alcohol extraction (PCI, USB Corporation) and ethanol precipitation and spectrophotometrically quantified (NanoDrop ND-1000). The primers used for PCR amplification were: forward 5'-ACC TGC TTC TCA TTG CCT C-3' and reverse: 5'-GAA GAT CAA CAC AGC CAT CC-3'. Cycling parameters: 94 °C, 3 min; 94 °C, 1 min; 57 °C, 1 min; 72 °C, 1 min; 30 cycles; 72 °C, 10 min. PCR product of 497 bp was purified with Qiaquick purification column (Qiagen) and sequenced with BigDye terminator cycle method on automated ABI Prism 7100 DNA Sequencer (Applied Biosystems).

In order to metabolically modulate TSH/Tshr signalling, adult wild-type (WT) C57BL/6 mice were given thyroxine (T₄) 2 mg/l in drinking water for 10 or 65 days (the daily intake of drugs calculated by measuring the uptake of water is indicated, 0.7 µg T₄/day per gram of body weight) or methimazole (MMI, 1-methyl-2-mercapto-imidazole) 0.25 g/l (71.1 µg/day per gram of body weight) for 10 or 65 days or 10 g/l sodium perchlorate (NaClO₄; 2.9 mg/day per gram of body weight) for 22 days (Sigma).

TSH measurement in the serum of treated mice

TSH concentration was measured using the TSH activity bioassay in the Chinese hamster ovary cells stably transfected with a human *TSHR* (JP26 cell line; Perret *et al.* 1990) as described by Moeller *et al.* (2003). Blanks contained the same volume of human serum depleted of TSH. cAMP was measured according to the method of Brooker *et al.* (1979). Standard curve TSH-cAMP was obtained with known concentrations of bovine TSH (0–1000 µU/ml; Sigma) and used to calculate sample TSH concentrations. Results are expressed as TSH µU/ml ± S.E.M. and the number of mice tested is indicated (*n*).

Western blot

Thyroids were removed and immediately frozen in liquid nitrogen. They were homogenised in denaturing Laemmli buffer (pH 6.8; 1.54% DTT, 2% SDS, 10% glycerol and 0.75% Tris) using a teflon-glass homogeniser. For *Tshr^{hyt/hyt}*, T₄ treated and WT controls, four or five thyroids per condition were pooled together to insure sufficient protein amount, while one or two thyroids from MMI- and perchlorate-treated mice were used. Protein concentrations were measured by paper dye-binding assay (Minamide & Bamburg 1990). For Duox and NIS expression analysis, 30 µg

total proteins were separated on a 6% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Duox proteins were detected using a polyclonal antibody raised against the first intracellular part of human Duox1 (dilution 1:8000; De Deken *et al.* 2000). Anti-NIS antibody (1:2000) is a gift from Dr N Carrasco, Albert Einstein College of Medicine (Levy *et al.* 1997). To immunodetect Tg with a polyclonal antibody (1:5000; Roger *et al.* 1985) or iodinated Tg with a monoclonal antibody (B2, 1:5000; given by Dr C Ris-Stalpers; Den Hartog *et al.* 1990), 1 µg thyroid proteins was separated on a 5% SDS-polyacrylamide gel. Immune complexes were detected with horseradish peroxidase-coupled anti-rabbit or anti-mouse antibodies (Amersham Pharmacia Biotech) according to the ECL method. β-Actin was immunodetected with a polyclonal antibody (Sigma; dilution 1:750) to verify that equal quantities of proteins were loaded on the gel.

The intensities of immunoreactive bands in western blot experiments were quantified using Bio-Rad S-800 scanner and QuantityOne software (Bio-Rad). Ratio between each band intensity and actin band intensity was compared with the ratio between WT staining intensity and actin band intensity. Such relative expressions in relation to WT were pooled and analysed using *t*-test. The results are expressed as means of relative intensities when compared with WT ± s.d.

Immunohistochemistry

Cryostat sections (8 µm) from frozen mouse embryos or adult thyroids were fixed in acetone for 10 min at room temperature. The slides were kept at -20 °C and before the experiment dried and fixed again for 10 min in acetone. To quench possible endogenous peroxidase activity, the sections were treated with 1% H₂O₂ in acetone for 10 min at room temperature and dried. Sections were rehydrated with PBS supplemented with 1% BSA (PAA Laboratories GmbH, Pasching, Austria) and thereafter incubated for 30 min in PBS - 1% BSA containing normal goat serum (dilution 1:50; DAKO, Heverlee, Belgium). Primary antibodies were then applied: anti-Duox antibody (De Deken *et al.* 2000) at the dilution 1:150, anti-rat NIS (Paire *et al.* 1997; 1:10 000) was a kind gift from B Rousset, anti-human Tg (1:2000; DAKO), B2 anti-human iodinated Tg (1:2000; Den Hartog *et al.* 1990) and anti-rat Titf1 (1:5000; Lazzaro *et al.* 1991) for 1 h at room temperature. Secondary antibody-binding and chromogen reactions were performed using EnVision, goat anti-rabbit, peroxidase-conjugated antibody and AEC chromogen according to the manufacturer's protocols (DAKO). Preimmune serum instead of the primary antibody was used as a negative control. The slides were finally counterstained with Mayer's haematoxylin (DAKO) and mounted in Faramount aqueous mounting medium (DAKO).

Measurements of H₂O₂ from mouse thyroid

Thyroids were dissected and preincubated 1 h in Krebs-Ringer Hepes medium (KRH; Corvilain *et al.* 1991). About

2 mg tissue were used for each experimental condition: this corresponds approximately to two lobes of WT mice, four lobes for *Tshr^{hyt/hyt}* and T₄ treated, and between one and two thyroid lobes from MMI- and perchlorate-treated mice. Equivalent quantities of tissue were incubated with or without 5 µM ionomycin (Sigma) for 3 h in KRH medium supplemented with 0.1 mg/ml horseradish peroxidase type II (Sigma) and 440 µM homovanillic acid (Sigma). One group of WT thyroids was also preincubated and incubated with 1 µM diphenylene iodonium (DPI; Sigma) in the presence of 5 µM ionomycin. The fluorescence intensity from the KRH medium was measured with Perkin-Elmer Luminescence Spectrometer LS50B (Perkin-Elmer, Wellesley, MA, USA) with excitation at 315 nm and emission at 415 nm. H₂O₂ concentrations were estimated from the fluorescence values when compared with the fluorescence from a standard curve obtained with known concentrations of H₂O₂ (Merck). Auto-fluorescence of the medium without the tissue (background) was subtracted from standard curve and experimental measurements. DNA was extracted from the incubated tissue and quantified in the same way as DNA from the tail biopsies. H₂O₂ content is expressed in nanograms of H₂O₂ per microgram DNA.

Statistical methods

The results from all H₂O₂ tests were pooled inside each experimental group (e.g. WT stimulated with ionomycin). Using Shapiro-Wilk normality test, it was observed that H₂O₂ measurements within each experimental group exhibited log-normal distribution. Therefore, the statistical significance of difference between WT and experimental groups was tested using Student's *t*-test with calculated log values of all measurements (Dumont 1964). All *P* values are two-tailed and the level of significance was fixed to 5%.

Results

Onset of Duox protein expression during mouse thyroid embryonic development

Cryosections of mouse embryonic thyroids from E10.5 to E18.5 were examined for Duox protein expression using a polyclonal antibody raised against human Duox1, which recognises both Duox1 and Duox2 in human and rat thyroid (De Deken *et al.* 2000, 2002, Wang *et al.* 2005). Duox expression appeared at E15.5 (Fig. 1A and B). The staining was observed at the apical pole of thyrocytes which begin to form small follicles at this stage. Preimmune serum of Duox antibody was used as a negative control and displayed no staining in the conditions used for detection of Duox (Fig. 1C).

As previously shown (Lazzaro *et al.* 1991), nuclear staining for Titf1 was already found at the earliest analysed stage (E10.5, data

Figure 1 Onset of Duox expression in the thyroid of mouse embryo when compared with the expressions of Titf1, NIS, Tg and iodinated Tg (Tg-I). (A) Transversal cryosections from E14.5 and E15.5 were immunostained with indicated antibodies: Duox, NIS, Tg, iodinated Tg (Tg-I) and Titf1. (B) Higher magnification of sections stained with anti-Duox and anti-Tg-I antibodies that are shown in (A). The insert shows Duox staining at the apical side of developing follicles. (C) Control for the specificity of anti-Duox antibody using the preimmune (Preim) serum on E17.5 thyroid sections. In all the sections, except those detected with Titf1 antibody, nuclei were counterstained with Mayer's haematoxylin. Scale bar, 200 (A), 10 (B) and (C) and 5 μ m insert. Presented images are a representative set from two independent and equivalent experiments. In each experiment, at least two mice were tested for every embryonic stage.

not shown) and was preserved throughout the development (i.e. E14.5; Fig. 1A). NIS was expressed concomitantly with Duox (E15.5), while Tg appeared 1 day before (E14.5; Fig. 1A). Iodinated Tg (Tg-I) was immunodetected in the lumen of the follicles at E15.5 (Fig. 1A and B).

Expression of Duox and H_2O_2 accumulation in mice with impaired TSH/Tshr signalling ($Tshr^{hyt/hyt}$ mice)

$Tshr^{hyt/hyt}$ mice bear a missense mutation in the fourth transmembrane domain of the Tshr, which makes the

receptor insensitive to TSH stimulation. Mice homozygous for the mutation have a normally located but hypoplastic and poorly functioning thyroid (Beamer *et al.* 1981, Stein *et al.* 1994, Gu *et al.* 1995). In western blot experiments, *Tshr^{hyt/hyt}* thyroids exhibited one wide immunoreactive band corresponding to protein(s) of approximately 150 kDa molecular mass (Fig. 2A). In *Tshr^{hyt/hyt}* thyroids, Duox proteins were still present but less expressed when compared with WT mouse thyroids (Fig. 2A). Densitometric quantification showed that, in *Tshr^{hyt/hyt}* thyroids, Duox expression was $42 \pm 15\%$ of the expression in WT thyroid (Fig. 2B). These results were confirmed with the second Duox antibody raised against human Duox2 (Morand *et al.* 2003), giving identical results as the first in all western blot experiments presented in this article (data not shown). In the same tissue, NIS expression was almost completely abolished ($2 \pm 2\%$ of WT), as previously described (Postiglione *et al.* 2002; Fig. 2A and B). Tg, immunodetected as two major bands, exhibited decreased expression and reached $40 \pm 10\%$ of the amount observed in WT mice. Iodinated Tg was dramatically decreased in *Tshr^{hyt/hyt}* mice.

Immunohistochemistry studies showed that Duox, expressed at the apical pole of thyrocytes in WT mouse thyroid, maintained its apical localisation in *Tshr^{hyt/hyt}* (Fig. 3A and B). NIS found at the baso-lateral membranes of WT thyrocytes was absent in *Tshr^{hyt/hyt}* (Fig. 3C and D), while Tg staining was localised in the lumen of the follicles of WT and *Tshr^{hyt/hyt}* thyroids (Fig. 3E and F).

H₂O₂ accumulation by thyroids of 2–5-month-old mice was measured in basal and calcium-stimulated conditions. A median value of 1.43 ng H₂O₂/μg DNA was measured in WT thyroids and 6.88 ng H₂O₂/μg DNA when stimulated with the calcium ionophore ionomycin (Fig. 4). H₂O₂ accumulation stimulated by ionomycin was totally inhibited by DPI (data not shown). *Tshr^{hyt/hyt}* thyroids released 3.00 ng H₂O₂/μg DNA in basal condition and 8.62 ng H₂O₂/μg DNA in ionomycin-stimulated condition. No statistically significant difference of H₂O₂ accumulation was observed between WT and *Tshr^{hyt/hyt}* thyroids, either for basal or for calcium-stimulated conditions as calculated after the logarithmic transformation (see table B under Fig. 4).

Figure 2 Duox expression in mice without functional Tshr (*Tshr^{hyt/hyt}*) when compared with the expression of NIS, Tg and iodinated Tg (Tg-I). Up to five thyroids per genotype were pooled and homogenised in Laemmli buffer and 30 μg proteins were loaded on the gel for the detection of Duox, NIS and β-actin. For total (Tg) and iodinated thyroglobulin (Tg-I), 1 μg protein was used. Duox proteins were immunodetected with anti-Duox antibody, raised against human Duox1 showing immunoreactive band(s) corresponding to a protein(s) of approximately 150 kDa. The arrows correspond to the indicated molecular mass markers. Asterisk: non-specific band. β-Actin was immunodetected as control for protein loading. Shown experiment is representative from four independent experiments. (B) Densitometric quantification of bands on the basis of all western blot experiments, where in total six *Tshr^{hyt/hyt}* and six WT thyroid pools were examined. Histogram shows the mean relative intensity of expression of each protein of interest as a fraction of its expression in WT, designated as 1. Error bars represent s.d. Significance of the difference between WT and *Tshr^{hyt/hyt}*: for Duox, NIS and Tg-I, $P < 0.001$ and for Tg, $P < 0.01$.

Figure 3 Immunohistochemical detection of Duox, NIS and Tg in WT and *Tshr^{hyt/hyt}* thyroids. (A and B) Cryosections of mouse thyroid were immunostained with anti-Duox antibody. Arrow points to the apical membrane where Duox is expressed. (C and D) NIS immunodetection predominantly found at the basal pole of WT thyrocytes. (E and F) Tg immunodetection in colloid of follicles. The images show a representative experiment of at least three pairs of WT and *Tshr^{hyt/hyt}* thyroids. Nuclei are counterstained with Mayer's haematoxylin. Scale bar, 20 μ m.

Expression of Duox and H₂O₂ measurement in mice with decreased TSH/Tshr signalling (mice treated with T₄)

Tshr activity can be reduced in adult mice by T₄ treatment. At 4 months of age, WT mice were given T₄ (2 mg/l) in drinking water for 10 or 65 days. TSH concentration was decreased under the limit of detection in the serum of a random sample of mice treated with T₄ for 10 and 65 days (table C and Fig. 4). TSH concentration in control mice was 0.32 ± 0.04 μ U/ml. After T₄ treatment, NIS expression was dramatically reduced in both treated groups (10 and 65 days) when compared with control mice, confirming the reduced thyroid stimulation by TSH (Fig. 5). In T₄-treated animals, Duox expression was only partially decreased as in *Tshr^{hyt/hyt}* mice. The extent of the decrease was independent of the length of T₄ treatment (Fig. 5). Tg content, as evaluated by western blot, was quite similar after 10- or 65-day treatment with T₄, but iodinated Tg progressively decreased after 10 and 65 days (Fig. 5). H₂O₂ accumulation by thyroids of mice treated with T₄ was also measured. As the TSH level (reduction) was similar in all animals, we pooled the H₂O₂ measurements for statistical analysis. Basal values of H₂O₂ measurements in T₄-treated mice thyroids were 2.79 vs 1.43 ng H₂O₂/μg DNA in untreated mice (Fig. 4). This accumulation was strongly increased by ionomycin and

reached 10.96 ng H₂O₂/μg DNA in T₄-treated mice and 6.88 ng H₂O₂/μg DNA in thyroids of untreated mice. Neither basal nor calcium-stimulated H₂O₂ detection was significantly different between untreated and T₄-treated mice.

Expression of Duox and H₂O₂ measurement in mice with increased TSH/Tshr signalling (mice treated with methimazole and sodium perchlorate)

Conversely to the previous models with decreased TSH–Tshr signalling, we have tested also the expression of Duox and H₂O₂ content in thyroid models with increased TSH concentrations. Mice were therefore treated with the anti-thyroid drug MMI (0.25 g/l) in the drinking water for 10 or 65 days. After this treatment, serum TSH concentrations were: control, 0.32 ± 0.04 μ U/ml; MMI 10 days, 1.56 ± 0.87 μ U/ml; and MMI 65 days, 1.38 ± 0.40 μ U/ml (table C and Fig. 4). The expression of NIS was strongly increased after MMI treatment (Fig. 6). Duox exhibited constant expression irrespective of MMI treatment and its duration. The amount of Tg in the thyroid was partially reduced by increased TSH stimulation. Iodination of Tg was strongly reduced in accordance with the mechanism by which MMI exerts its anti-thyroid effects (Fig. 6). Since the increased TSH/Tshr activity in this model is achieved through the inhibition of the TPO, which is known to interact with

Figure 4 H_2O_2 accumulated in the medium from thyroids of mice treated with T_4 , MMI or sodium perchlorate (PERC) and $\text{Tshr}^{\text{hyt/hyt}}$ mice, compared with WT mouse thyroid. Tissue was incubated with (+) or without 5 μM ionomycin. Each point on the graph represents one thyroid (or pool of two in the case of smaller thyroids from $\text{Tshr}^{\text{hyt/hyt}}$ and T_4 groups). Median value of each group is marked on the graph. Statistical significance (*P*) calculated using Student's *t*-test with data obtained after the logarithmic transformation of H_2O_2 (ng/ μg DNA) measurements is shown above graph A (see Materials and Methods). ns, Not statistically significant. (B) Table shows the number of measurements in each group (*n*), median H_2O_2 accumulation (ng H_2O_2 / μg DNA) and mean and S.E.M. of log-transformed values. (C) Serum TSH concentration in experimental groups shown as mean $\pm$ S.E.M. $\mu\text{U/ml}$.

Duox in human thyrocytes (Wang *et al.* 2005), it would be possible that MMI treatment exerts effects on Duox other than through TSH/Tshr. We therefore treated another group of mice for 22 days with 1% sodium perchlorate, which blocks iodide entry and leads to high serum TSH concentration without directly affecting TPO activity. The TSH concentration in the serum of perchlorate-treated mice was 29.40 ± 7.84 $\mu\text{U/ml}$,

which is around 80 times higher than in control mice and ~ 20 times higher than in mice treated with MMI (table C and Fig. 4). NIS expression was dramatically increased when compared with WT thyroid, while Duox expression again remained unchanged (Fig. 6A and C). The thyroid demonstrated, by western blot, a drastic decrease of both total and iodinated Tg after perchlorate treatment.

Figure 5 Western blot detection of Duox, NIS, Tg and Tg-I from thyroids of mice treated with 2 mg/l T₄ in the drinking water for 10 or 65 days. Pools of up to five thyroids from treated (T₄) or control mice were homogenised in Laemmli buffer. For Duox, NIS and β -actin, 30 μ g thyroid proteins were loaded on the gel, while 1 μ g protein extract was used for the detection of Tg and Tg-I. β -Actin was immunodetected in order to validate equal protein loading. The arrows mark the position of the molecular mass standard. Results are representative of at least four experiments. (B) Densitometric quantification of bands on the basis of all western blot experiments. Histogram shows the mean intensity of expression of each protein of interest as a fraction of its expression in WT, designated as 1. Error bars represent s.d. Significance of the difference between WT and treated groups: for Duox, NIS and Tg-I, $P < 0.002$; for Tg 10-day T₄ treatment, $P < 0.01$; and for Tg 65-day T₄ treatment, not significant.

Figure 6 Western blot detection of Duox proteins in thyroids from the untreated wild-type mice (WT) or treated with 0.25 g/l MMI in drinking water for 10 or 65 days (MMI) or 10 g/l sodium perchlorate for 22 days (NaClO₄). Pools of up to five thyroids for control and up to two thyroids in MMI and perchlorate conditions were homogenised in Laemmli buffer. Thirty micrograms of total proteins were loaded on gel except for the detection of Tg and Tg-I, where 1 µg protein was used. The arrows mark the position of the molecular mass standard. The same experiment was reproduced thrice with at least duplicate samples in each condition. (B and C) Densitometric quantification of bands on the basis of all western blot experiments with MMI- (B) and perchlorate (C)-treated mice. Histogram shows the mean intensity of expression of each protein of interest as a fraction of its expression in WT, designated as 1. Error bars represent s.d. Significance of the difference between WT and treated groups: for Duox, not significant; for NIS, $P < 0.0001$; for Tg MMI, $P < 0.01$; for Tg NaClO₄, $P < 0.001$; and for Tg-I, $P < 0.001$.

Thyroids of MMI-treated animals released 1.59 ng H₂O₂/µg DNA when measured in basal conditions and 4.26 ng H₂O₂/µg DNA when incubated with ionomycin, while thyroids of non-treated mice released 1.43 ng H₂O₂/µg DNA in basal and 6.88 ng H₂O₂/µg DNA when stimulated with ionomycin. In thyroids from perchlorate-treated mice, H₂O₂ measurements were 1.48 ng H₂O₂/µg DNA in basal conditions and 1.68 ng H₂O₂/µg DNA with ionomycin stimulation (Fig. 4). A statistically significant difference was observed between ionomycin-stimulated H₂O₂ accumulation in control thyroids and in thyroids of

MMI- and perchlorate-treated mice. This decrease of measured H₂O₂ was more pronounced in perchlorate-treated animals (76% decrease) than in animals treated with MMI (48% decrease).

The follicular structure of the thyroid was largely preserved in MMI-treated animals and Duox was still observed at the apical pole of many thyrocytes (Fig. 7). However, thyroids of perchlorate-treated mice had lost their follicular organisation and, therefore, even though Duox staining was still observed along the surface of compressed follicles, its apical cellular localisation was less obvious.

Figure 7 Immunohistochemical detection of Duox in thyroid cryosections from normal control mice (WT), and wild-type mice treated for 65 days with 0.25 g/l MMI in drinking water or 22 days with 10 g/l sodium perchlorate (NaClO₄). In the perchlorate group, note the presence of compressed follicles with Duox staining at the line of contact; four such follicles are marked with arrows. Scale bar, 20 µm. Nuclei were counterstained with Mayer's haematoxylin.

Discussion

Duox genes and corresponding proteins were identified by the purification of the NADPH oxidase (NOX) activity from thyroid plasma membranes and the screening of thyrocyte cDNA libraries for transcripts similar to neutrophil NOX2 (*gp91^{phox}*; Dupuy *et al.* 1999, De Deken *et al.* 2000). As previously suggested (De Deken *et al.* 2002, Wang *et al.* 2005), their full glycosylation, processing to the plasma membrane and activity, is dependent on an additional maturation factor shown to be an ER-resident transmembrane protein, named DuoxA1/2 (Grasberger & Refetoff 2006). In addition to *in vitro* demonstration of Duox activity, mutations in *Duox2* were detected in different patients suffering from hypothyroidism, attributed to a lack of iodide binding to proteins not caused by any other known genetic defect (Moreno *et al.* 2002, Vigone *et al.* 2005, Varela *et al.* 2006).

In this work, we first determined the onset of Duox protein expression in the thyroid of mouse embryos. The same approach using specific antibodies was used in previous studies to detect the onset of expression of thyroid transcription factors, such as *Titf1* (Lazzaro *et al.* 1991) and *Foxe1* (Dathan *et al.* 2002), and markers of thyroid functional differentiation, such as Tg (Kawaoi & Tsuneda 1985, Postiglione *et al.* 2002) and NIS (Postiglione *et al.* 2002). Duox was expressed at E15.5, precisely at the same time as NIS and 1 day after Tg (E14.5). The same temporal expression pattern of Duox, NIS and TPO suggests the existence of common upstream regulators in the mouse; however, the lack of similar promoter regulatory sequences between human Duox and other human thyroid-specific genes (Pachucki *et al.* 2004) and a recent study on thyroid-specific mRNA expression in human embryo (Szinnai *et al.* 2007) argue against common direct activators.

Iodinated Tg was previously detected in the rat embryo at E17, when TPO was located at the apical plasma membrane (Remy *et al.* 1980). In developing mouse thyroid, TPO mRNA was detected from E15 (De Felice *et al.* 2004). In the present study, we showed that iodide organification in mouse at E15.5 is concomitant with Duox, TPO (De Felice *et al.* 2004) and NIS expression and appears 1 day after Tg (E14.5). Obviously, the machinery for iodide transport (NIS) and iodide oxidation (Duox and TPO) must be present to iodinate the already present Tg.

In vivo regulation of Duox expression by TSH/Tshr/cAMP cascade has been until now characterised in hyperstimulated human thyroid (Caillou *et al.* 2001, Gerard *et al.* 2003) and in thyroid of rats treated with MMI (Dupuy *et al.* 2000) or perchlorate (Miot *et al.* 2002). The question of the 'requirement' of Tshr activity for the expression of Duox has not been directly addressed. In addition, the correlation between Duox expression and accumulation of its product, H₂O₂, has not been studied *in vivo*. In wild-type mouse thyroid, Duox expression levels were already maximal. Increased Tshr stimulation did not further enhance Duox

protein expression. In *Tshr^{hyt/hyt}* mice and in adult mice treated with T₄, two types of regulation by TSH were observed: TSH affected only partially the expression of Duox and Tg but strongly modulated NIS and TPO expression (Fig. 2B; Postiglione *et al.* 2002). Although all of these genes required Tshr signalling for their full expression, Duox and Tg preserved around 40% of the normal expression in its absence, while NIS, the paradigm of TSH-regulated/dependent thyroid gene, was almost completely inhibited in these conditions. It has been clearly established that the onset of Tg expression during mouse thyroid development precedes the onset of Tshr expression (De Felice *et al.* 2004) and we confirm in this study that it also takes place in mice deprived of functional Tshr (Postiglione *et al.* 2002, Marians *et al.* 2002). Even if TSH is not needed for the initiation of Tg expression during embryonic development, the decrease in Tg and Duox immunoreactivity observed by western blot in *Tshr^{hyt/hyt}* mice implies that TSH is necessary for their full expression.

Since Duox proteins were significantly expressed in *Tshr^{hyt/hyt}* mice, we examined whether the posttranslational maturation and activity of Duox were influenced by TSH/Tshr signalling. It is well documented that Duox proteins in human (Caillou *et al.* 2001, De Deken *et al.* 2002, Morand *et al.* 2004), pig (Duox2; Morand *et al.* 2003, 2004), dog (De Deken *et al.* 2002) and rat (our unpublished results) thyroids exist in two predominant glycosylation forms, detected as a doublet in western blot. The glycosylation status can predict the cellular localisation and activity of Duox (De Deken *et al.* 2002, El Hassani *et al.* 2005, Grasberger & Refetoff 2006). The characteristic doublet was hardly detected in mouse thyroid probably because of the low affinity of antibodies for mouse Duox. Nevertheless, the same pattern of Duox immunostaining in western blot experiments with all experimental groups suggests that the glycosylation and the localisation of the active form at the plasma membrane should be independent of TSH stimulation. This was confirmed in *Tshr^{hyt/hyt}* mouse thyroid where Duox was expressed at the plasma membrane and was active. This suggests that the component(s) of the H₂O₂ generating system, which are responsible for the maturation and glycosylation of Duox, like DuoxA1/2 (Grasberger & Refetoff 2006) should also be resistant to TSH deprivation.

It was surprising that thyroids with decreased expression of Duox proteins (in *Tshr^{hyt/hyt}* and T₄ group) accumulated as much H₂O₂ as WT thyroids, implying that either the relative Duox activity, in this condition, is higher than in normal thyroid or that the degradation of H₂O₂ is less efficient in hypostimulated thyroids. On the contrary, high TSH stimulation (MMI- and perchlorate-treated mice) was accompanied by dramatically decreased accumulation of H₂O₂ in spite of correctly expressed Duox. This strongly suggests that additional factors could modulate the activity of Duox or control H₂O₂ content. The existence of yet unidentified modulators of thyroid NADPH oxidase activity has been proposed before (Dupuy *et al.* 1988, Leseney *et al.* 1999).

TSH stimulation seems to be sufficient to explain reduced H_2O_2 accumulation, since both MMI and perchlorate treatments lead to a decreased H_2O_2 accumulation. This is in agreement with reduced H_2O_2 accumulation measured in autonomous adenomas when compared with normal tissue (Deleu *et al.* 2000). These constant high TSH levels also trigger antioxidant systems directed against an excess of H_2O_2 in the cell-like glutathione peroxidase, catalase and peroxiredoxin 1 and 5 (Kim *et al.* 2000, Gerard *et al.* 2005). Another explanation would be that highly expressed TPO exerts some catalase-like activity (Magnusson & Taurog 1983). It is also noteworthy that high TSH stimulation caused the decrease in thyroid follicular organisation which could be related to low content of H_2O_2 .

In conclusion, Duox proteins exhibit an expression onset that is compatible with their proposed function in the synthesis of thyroid hormones and can therefore be considered along with Tg, Tshr, NIS and TPO, as markers of functionally developing embryonic thyroid. However, the regulation of Duox expression by TSH is relatively limited and is not correlated with the regulation of thyroid H_2O_2 accumulation, suggesting that mechanisms other than the regulation of Duox expression would control thyroid H_2O_2 content through TSH/Tshr stimulation.

Acknowledgements

We thank Chantal Degraef for her excellent technical assistance and Bernadette Bournonville for her help in mice genotyping. We thank Jacqueline Van Sande for measuring TSH and Prathiba Mithbaokar for preparing sections of staged embryos.

Funding

This work was supported by the 'Fonds National pour la Recherche Médicale' (FRSM), 'Action de Recherches de la Communauté française de Belgique' (ARC), 'Fonds National de la Recherche Scientifique' (FNRS), the Fondation Van Buuren and the Fondation Tournay-Solvay. X D D: Chargé de Recherche FNRS. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

- Beamer WJ, Eicher EM, Maltais LJ & Southard JL 1981 Inherited primary hypothyroidism in mice. *Science* **212** 61–63.
- Bjorkman U & Ekholm R 1984 Generation of H_2O_2 in isolated porcine thyroid follicles. *Endocrinology* **115** 392–398.
- Brooker G, Harper JF, Terasaki WL & Moylan RD 1979 Radioimmunoassay of cyclic AMP and cyclic GMP. *Advances in Cyclic Nucleotide Research* **10** 1–33.
- Caillou B, Dupuy C, Lacroix L, Nocera M, Talbot M, Ohayon R, Deme D, Bidart JM, Schlumberger M & Virion A 2001 Expression of reduced nicotinamide adenine dinucleotide phosphate oxidase (ThOX, LNOX, Duox) genes and proteins in human thyroid tissues. *Journal of Clinical Endocrinology and Metabolism* **86** 3351–3358.
- Chazenbalk G, Magnusson RP & Rapoport B 1987 Thyrotropin stimulation of cultured thyroid cells increases steady state levels of the messenger ribonucleic acid for thyroid peroxidase. *Molecular Endocrinology* **1** 913–917.
- Corvilain B, Van Sande J, Laurent E & Dumont JE 1991 The H_2O_2 -generating system modulates protein iodination and the activity of the pentose phosphate pathway in dog thyroid. *Endocrinology* **128** 779–785.
- Corvilain B, Laurent E, Lecomte M, Van Sande J & Dumont JE 1994 Role of the cyclic adenosine 3',5'-monophosphate and the phosphatidylinositol- Ca^{2+} cascades in mediating the effects of thyrotropin and iodide on hormone synthesis and secretion in human thyroid slices. *Journal of Clinical Endocrinology and Metabolism* **79** 152–159.
- Dathan N, Parlato R, Rosica A, De Felice M & Di Lauro R 2002 Distribution of the *tif2/foxe1* gene product is consistent with an important role in the development of foregut endoderm, palate, and hair. *Developmental Dynamics* **224** 450–456.
- De Deken X, Wang D, Many MC, Costagliola S, Libert F, Vassart G, Dumont JE & Miot F 2000 Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family. *Journal of Biological Chemistry* **275** 23227–23233.
- De Deken X, Wang D, Dumont JE & Miot F 2002 Characterization of ThOX proteins as components of the thyroid H_2O_2 -generating system. *Experimental Cell Research* **273** 187–196.
- De Felice M & Di Lauro R 2004 Thyroid development and its disorders: genetics and molecular mechanisms. *Endocrine Reviews* **25** 722–746.
- De Felice M, Postiglione MP & Di Lauro R 2004 Minireview: thyrotropin receptor signaling in development and differentiation of the thyroid gland: insights from mouse models and human diseases. *Endocrinology* **145** 4062–4067.
- Deleu S, Allory Y, Radulescu A, Pirson I, Carrasco N, Corvilain B, Salmon I, Franc B, Dumont JE, Van Sande J *et al.* 2000 Characterization of autonomous thyroid adenoma: metabolism, gene expression, and pathology. *Thyroid* **10** 131–140.
- Deme D, Virion A, Hammou NA & Pommier J 1985 NADPH-dependent generation of H_2O_2 in a thyroid particulate fraction requires Ca^{2+} . *FEBS Letters* **186** 107–110.
- Den Hartog MT, De Boer M, Veenboer GJ & De Vijlder JJ 1990 Generation and characterization of monoclonal antibodies directed against noniodinated and iodinated thyroglobulin, among which are antibodies against hormonogenic sites. *Endocrinology* **127** 3160–3165.
- Dohan O, De la Vieja O, Paroder V, Riedel C, Artani M, Reed M, Ginter CS & Carrasco N 2003 The sodium/iodide symporter (NIS): characterization, regulation, and medical significance. *Endocrine Reviews* **24** 48–77.
- Dumont JE 1964 Action de l'hormone thyroïdienne sur le métabolisme énergétique du tissu thyroïdien: effets *in vitro* de l'hormone. *Bulletin de la Société de Chimie Biologique* **XLVI** 1131–1144.
- Dumont JE, Maenhaut C, Christophe D, Vassart G & Roger PP 2005 The phylogeny, ontogeny, anatomy and regulation of the iodine metabolizing thyroid. In *The Thyroid and its Diseases*, www.Thyroidmanager.org.
- Dupuy C, Deme D, Kaniewski J, Pommier J & Virion A 1988 Ca^{2+} regulation of thyroid NADPH-dependent H_2O_2 generation. *FEBS Letters* **233** 74–78.
- Dupuy C, Ohayon R, Valent A, Noel-Hudson MS, Deme D & Virion A 1999 Purification of a novel flavoprotein involved in the thyroid NADPH oxidase. Cloning of the porcine and human cDNAs. *Journal of Biological Chemistry* **274** 37265–37269.
- Dupuy C, Pomerance M, Ohayon R, Noel-Hudson MS, Deme D, Chaaraoui M, Francon J & Virion A 2000 Thyroid oxidase (THOX2) gene expression in the rat thyroid cell line FRTL-5. *Biochemical and Biophysical Research Communications* **277** 287–292.
- El Hassani RA, Benfares N, Caillou B, Talbot M, Sabourin JC, Belotte V, Morand S, Gnidehou S, Agnandji D, Ohayon R *et al.* 2005 Dual oxidase2 is expressed all along the digestive tract. *American Journal of Physiology Gastrointestinal and Liver Physiology* **288** G933–G942.
- Geiszt M & Leto TL 2004 The Nox family of NAD(P)H oxidases: host defense and beyond. *Journal of Biological Chemistry* **279** 51715–51718.

- Gerard CM, Lefort A, Christophe D, Libert F, Van Sande J, Dumont JE & Vassart G 1989 Control of thyroperoxidase and thyroglobulin transcription by cAMP: evidence for distinct regulatory mechanisms. *Molecular Endocrinology* **3** 2110–2118.
- Gerard AC, Daumerie C, Mestdagh C, Gohy S, De Burbure C, Costagliola S, Miot F, Nolleaux MC, Denef JF, Rahier J *et al.* 2003 Correlation between the loss of thyroglobulin iodination and the expression of thyroid-specific proteins involved in iodine metabolism in thyroid carcinomas. *Journal of Clinical Endocrinology and Metabolism* **88** 4977–4983.
- Gerard AC, Many MC, Daumerie C, Knoops B & Colin IM 2005 Peroxiredoxin 5 expression in the human thyroid gland. *Thyroid* **15** 205–209.
- Grasberger H & Refetoff S 2006 Identification of the maturation factor for dual oxidase: evolution of an eukaryotic operon equivalent. *Journal of Biological Chemistry* **281** 18269–18272.
- Gu WX, Du GG, Kopp P, Rentoumis A, Albanese C, Kohn LD, Madison LD & Jameson JL 1995 The thyrotropin (TSH) receptor transmembrane domain mutation (Pro556-Leu) in the hypothyroid hyt/hyt mouse results in plasma membrane targeting but defective TSH binding. *Endocrinology* **136** 3146–3153.
- Japon MA, Rubinstein M & Low MJ 1994 *In situ* hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. *Journal of Histochemistry and Cytochemistry* **42** 1117–1125.
- Kawaoi A & Tsuneda M 1985 Functional development and maturation of the rat thyroid gland in the foetal and newborn periods: an immunohistochemical study. *Acta Endocrinologica* **108** 518–524.
- Kim H, Lee TH, Park ES, Suh JM, Park SJ, Chung HK, Kwon OY, Kim YK, Ro HK & Shong M 2000 Role of peroxiredoxins in regulating intracellular hydrogen peroxide and hydrogen peroxide-induced apoptosis in thyroid cells. *Journal of Biological Chemistry* **275** 18266–18270.
- Lazzaro D, Price M, De Felice M & Di Lauro R 1991 The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* **113** 1093–1104.
- Leseny AM, Deme D, Legue O, Ohayon R, Chanson P, Sales JP, Pires de Carvalho D, Dupuy C & Virion A 1999 Biochemical characterization of a Ca²⁺/NAD(P)H-dependent H₂O₂ generator in human thyroid tissue. *Biochimie* **81** 373–380.
- Levy O, Dai G, Riedel C, Ginter CS, Paul EM, Lebowitz AN & Carrasco N 1997 Characterization of the thyroid Na⁺/I[−] symporter with an anti-COOH terminus antibody. *PNAS* **94** 5568–5573.
- Lin SC, Li S, Drolet DW & Rosenfeld MG 1994 Pituitary ontogeny of the Snell dwarf mouse reveals Pit-1-independent and Pit-1-dependent origins of the thyrotrope. *Development* **120** 515–522.
- Magnusson RP & Taurog A 1983 Iodide-dependent catalytic activity of thyroid peroxidase and lactoperoxidase. *Biochemical and Biophysical Research Communications* **112** 475–481.
- Marians RC, Ng L, Blair HC, Unger P, Graves PN & Davies TF 2002 Defining thyrotropin-dependent and -independent steps of thyroid hormone synthesis by using thyrotropin receptor-null mice. *PNAS* **99** 15776–15781.
- Minamide LS & Bamburg JR 1990 A filter paper dye-binding assay for quantitative determination of protein without interference from reducing agents or detergents. *Analytical Biochemistry* **190** 66–70.
- Miot F, Mahieu F & Corvilain B 2002 Regulation of ThOX protein expression is associated with the Wolff-Chaikoff effect and its escape. *Journal of Endocrinological Investigation* **25**(Suppl 7) 17.
- Moeller LC, Kimura S, Kusakabe T, Liao XH, Van Sande J & Refetoff S 2003 Hypothyroidism in thyroid transcription factor 1 haploinsufficiency is caused by reduced expression of the thyroid-stimulating hormone receptor. *Molecular Endocrinology* **17** 2295–2302.
- Morand S, Chaaraoui M, Kaniewski J, Deme D, Ohayon R, Noel-Hudson MS, Virion A & Dupuy C 2003 Effect of iodide on nicotinamide adenine dinucleotide phosphate oxidase activity and Duox2 protein expression in isolated porcine thyroid follicles. *Endocrinology* **144** 1241–1248.
- Morand S, Agnandji D, Noel-Hudson MS, Nicolas V, Buisson S, Macon-Lemaitre L, Gnidehou S, Kaniewski J, Ohayon R, Virion A *et al.* 2004 Targeting of the dual oxidase 2 N-terminal region to the plasma membrane. *Journal of Biological Chemistry* **279** 30244–30251.
- Moreno JC, Bikker H, Kempers MJ, van Trotsenburg AS, Baas F, De Vijlder JJ, Vulsma T & Ris-Stalpers C 2002 Inactivating mutations in the gene for thyroid oxidase 2 (THOX2) and congenital hypothyroidism. *New England Journal of Medicine* **347** 95–102.
- Nunez J & Pommier J 1982 Formation of thyroid hormones. *Vitamins and Hormones* **39** 175–229.
- Pachucki J, Wang D, Christophe D & Miot F 2004 Structural and functional characterization of the two human ThOX/Duox genes and their 5′-flanking regions. *Molecular and Cellular Endocrinology* **214** 53–62.
- Paire A, Bernier-Valentin F, Selmi-Ruby S & Rousset B 1997 Characterization of the rat thyroid iodide transporter using anti-peptide antibodies. Relationship between its expression and activity. *Journal of Biological Chemistry* **272** 18245–18249.
- Perret J, Ludgate M, Libert F, Gerard C, Dumont JE, Vassart G & Parmentier M 1990 Stable expression of the human TSH receptor in CHO cells and characterization of differentially expressing clones. *Biochemical and Biophysical Research Communications* **171** 1044–1050.
- Postiglione MP, Parlato R, Rodriguez-Mallon A, Rosica A, Mithbaokar P, Maresca M, Mariani RC, Davies TF, Zannini MS, De Felice M *et al.* 2002 Role of the thyroid-stimulating hormone receptor signaling in development and differentiation of the thyroid gland. *PNAS* **99** 15462–15467.
- Raspe E, Laurent E, Corvilain B, Verjans B, Erneux C & Dumont JE 1991 Control of the intracellular Ca(2+)-concentration and the inositol phosphate accumulation in dog thyrocyte primary culture: evidence for different kinetics of Ca(2+)-phosphatidylinositol cascade activation and for involvement in the regulation of H₂O₂ production. *Journal of Cellular Physiology* **146** 242–250.
- Remy L, Michel-Bechet M, Athouel-Haon AM, Magre S, Cataldo C & Jost A 1980 Development of the thyroid gland in the rat fetus *in vivo*. An ultrastructural and radioautographic study. *Archives D'Anatomie Microscopique et de Morphologie Experimentale* **69** 91–108.
- Roger PP, Van Heuverswyn B, Lambert C, Reuse S, Vassart G & Dumont JE 1985 Antagonistic effects of thyrotropin and epidermal growth factor on thyroglobulin mRNA level in cultured thyroid cells. *European Journal of Biochemistry* **152** 239–245.
- Stein SA, Oates EL, Hall CR, Grumbles RM, Fernandez LM, Taylor NA, Puett D & Jin S 1994 Identification of a point mutation in the thyrotropin receptor of the hyt/hyt hypothyroid mouse. *Molecular Endocrinology* **8** 129–138.
- Szinnai G, Lacroix L, Carre A, Guimiot F, Talbot M, Martinovic J, Delezoide AL, Vekemans M, Michiels S, Caillou B *et al.* 2007 Sodium/iodide symporter (NIS) gene expression is the limiting step for the onset of thyroid function in the human fetus. *Journal of Clinical Endocrinology and Metabolism* **92** 70–76.
- Varela V, Rivolta CM, Esperante SA, Gruneiro-Papendieck L, Chiesa A & Targovnik HM 2006 Three mutations (p.Q36H, p.G418fsX482, and g.IVS19-2A>C) in the dual oxidase 2 gene responsible for congenital goiter and iodide organification defect. *Clinical Chemistry* **52** 182–191.
- Vigone MC, Fugazzola L, Zamproni I, Passoni A, Di Candia S, Chiumello G, Persani L & Weber G 2005 Persistent mild hypothyroidism associated with novel sequence variants of the DUOX2 gene in two siblings. *Human Mutation* **26** 395.
- Wang D, De Deken X, Milenkovic M, Song Y, Pirson I, Dumont JE & Miot F 2005 Identification of a novel partner of duox: EFP1, a thioredoxin-related protein. *Journal of Biological Chemistry* **280** 3096–3103.

Received in final form 7 December 2006

Accepted 8 December 2006

Made available online as an Accepted Preprint
28 December 2006