Duox expression and related H$_2$O$_2$ measurement in mouse thyroid: onset in embryonic development and regulation by TSH in adult

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Abstract

In the thyroid, H$_2$O$_2$ 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 H$_2$O$_2$ accumulation was evaluated in thyroids of adult mice with reduced (Tshrhyt/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 Tshrhyt/hyt strain, Duox was still expressed at the apical pole and H$_2$O$_2$ measurements were normal. On the other hand, chronic TSH stimulation of the gland led to a decrease of H$_2$O$_2$ 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 H$_2$O$_2$ accumulation, highlighting the importance of additional regulatory mechanisms which control either the production or H$_2$O$_2$ degradation.

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Introduction

Thyroid hormones are synthesised within thyroglobulin (Tg) matrix. The iodination of tyrosyl residues of Tg is the oxidative coupling of the resulting iodotyrosines and is catalysed by the thyroperoxidase (TPO) in the presence of hydrogen peroxide (H$_2$O$_2$), which is used as a terminal electron acceptor (Nunez & Pommier 1982, Dumont et al. 2005). H$_2$O$_2$ 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$^{\text{TOX}}$, 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 H$_2$O$_2$ 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, Corvila 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:
Tft1 (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 and when iodide organification is first 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 (Caillou et al. 1997, Gerard et al. 2000; 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<sup>h<sub>yt/h</sub>yt</sup> mice (genetic background BALB/c crossed and backcrossed with C57BL/6 for at least five generations) were used. All Tshr<sup>h<sub>yt/h</sub>yt</sup> mice were treated with 2 mg/l thyroxine (T<sub>4</sub>, 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<sup>h<sub>yt/h</sub>yt</sup> 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 protease 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 Qiagen 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 (T<sub>4</sub>) 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 T<sub>4</sub>/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 (NaClO<sub>4</sub>; 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; 1·5% DTT, 2% SDS, 10% glycerol and 0·75% Tris) using a teflon–glass homogeniser. For Tshr<sup>h<sub>yt/h</sub>yt</sup>, T<sub>4</sub> 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 & Bamberg 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 Rouset, 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 DAB 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 Heps 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 Tshβ/pβ and Tγ 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 stain ing 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
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


thyroids exhibited one wide immunoreactive band corresponding to protein(s) of approximately 150 kDa molecular mass (Fig. 2A). In Tshr thyroids, Duox proteins were still present but less expressed when compared with WT mouse thyroids (Fig. 2A). 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 ± 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 ± 10% of the amount observed in WT mice. Iodinated Tg was dramatically decreased in Tshr thyroids.

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 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 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) 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 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: for Duox, NIS and Tg-I, P<0.001 and for Tg, P<0.01.
Expression of Duox and \( H_2O_2 \) measurement in mice with decreased TSH/Tshr signalling (mice treated with \( T_4 \))

Tshr activity can be reduced in adult mice by \( T_4 \) treatment. At 4 months of age, WT mice were given \( T_4 \) (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 \( T_4 \) for 10 and 65 days (table C and Fig. 4). TSH concentration in control mice was 0.32 ± 0.04 mU/ml. After \( T_4 \) 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 \( T_4 \)-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 \( T_4 \) treatment (Fig. 5). Tg content, as evaluated by western blot, was quite similar after 10- or 65-day treatment with \( T_4 \), but iodinated Tg progressively decreased after 10 and 65 days (Fig. 5). \( H_2O_2 \) accumulation by thyroids of mice treated with \( T_4 \) was also measured. As the TSH level (reduction) was similar in all animals, we pooled the \( H_2O_2 \) measurements for statistical analysis. Basal values of \( H_2O_2 \) measurements in \( T_4 \)-treated mice thyroids were 2.79 vs 1.43 ng \( H_2O_2/\mu g \) DNA in untreated mice. This accumulation was strongly increased by ionomycin and reached 10.96 ng \( H_2O_2/\mu g \) DNA in \( T_4 \)-treated mice and 6.88 ng \( H_2O_2/\mu g \) DNA in thyroids of untreated mice. Neither basal nor calcium-stimulated \( H_2O_2 \) detection was significantly different between untreated and \( T_4 \)-treated mice.

Expression of Duox and \( H_2O_2 \) 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 \( H_2O_2 \) 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 mU/ml; MMI 10 days, 1.56 ± 0.87 mU/ml; and MMI 65 days, 1.38 ± 0.40 mU/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...
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 µU/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.

![Graph showing H2O2 accumulation](image)

**Figure 4** H2O2 accumulated in the medium from thyroids of mice treated with T4, MMI or sodium perchlorate (PERC) and Tshr/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 Tshr/hyt/hyt and T4 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 H2O2 (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 H2O2 accumulation (ng H2O2/µg DNA) and mean and S.E.M. of log-transformed values. (C) Serum TSH concentration in experimental groups shown as mean ± S.E.M. µU/ml.
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 μg thyroid proteins were loaded on the gel, while 1 μg 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.
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 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.

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 (\textit{gp91phox}, 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\textit{vivo} 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.

\textit{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 (Mirot 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 \textit{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\textsuperscript{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\textsuperscript{hyt/hyt} mice implies that TSH is necessary for their full expression.

Since Duox proteins were significantly expressed in Tshr\textsuperscript{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) thyroid 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\textsuperscript{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 DuoX1/2 (Grasberger & Refetoff 2006) should also be resistant to TSH deprivation.

It was surprising that thyroids with decreased expression of Duox proteins (in Tshr\textsuperscript{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$_2$O$_2$ accumulation, since both MMI and perchlorate treatments lead to a decreased H$_2$O$_2$ accumulation. This is in agreement with reduced H$_2$O$_2$ 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$_2$O$_2$ in the cell-like glutathione peroxidase, catalase and peroxireroxidase 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 pentose phosphate pathway in dog thyroid. Endocrinology 128 779–785.

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$_2$O$_2$ accumulation, suggesting that mechanisms other than the regulation of Duox expression would control thyroid H$_2$O$_2$ content through TSH/Tshr stimulation.

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References


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.


Magnusson KP & Taurog A 1983 Iodide-dependent catalytic activity of thyroid peroxidase and lactoperoxidase. *Biochemical and Biophysical Research Communications* 122 475–481.


Moet F, Mahieu F & Corvilain B 2002 Regulation of TgOX gene expression is the limiting step for the onset of thyroid hormone synthesis in the human fetus. *PNAS* 99 15462–15467.


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