# The Expression of Dual Oxidase, Thyroid Peroxidase, and Caveolin-1 Differs According to the Type of Immune Response (TH1/TH2) Involved in Thyroid Autoimmune Disorders

Lancelot Marique,* Victoria Van Regemorter,* Anne-Catherine Gérard, Julie Craps, Maximin Senou, Etienne Marbaix, Jacques Rahier, Chantal Daumerie, Michel Mourad, Benoît Lengelé, Ides M. Colin, and Marie-Christine Many

Pôle de Morphologie (L.M., V.V.R., A.C.G., J.C., M.S., B.L., I.M.C., M.-C.M.), Institut de Recherche Expérimentale et Clinique, Départements d'Anatomo-Pathologie (E.M., J.R.), d'Endocrinologie (C.D.), and de Chirurgie Endocrinienne et de Transplantation rénale (M.M.), Secteur des Sciences de la Santé, Faculté de Médecine, Université catholique de Louvain, B-1200 Brussels, Belgium

**Context:** Hashimoto's thyroiditis (HT) and Graves' disease (GD) are thyroid autoimmune disorders driven by Th1 and Th2 immune responses, respectively. Caveolin-1 (Cav-1), thyroid peroxidase (TPO), and dual oxidase (DUOX) are thought to be part of the thyroxisome, which is essential to maintain thyroid hormone synthesis, at the apical membrane.

**Objectives:** To analyze the thyroxisome in HT and GD thyroids, we investigated Cav-1, DUOX, and TPO expression as well as markers of oxidative stress (OS), cell proliferation, apoptosis, and antioxidant defenses. The effects of cytokines on Cav-1 expression were analyzed in vitro.

**Results:** In HT, the decrease in Cav-1, DUOX, and TPO expression was marked in follicles having the morphological aspect of active follicles in normal glands and thus called active-like follicles. $T_4$ was not detected in the colloid but in the cytoplasm as well as DUOX and TPO. These abnormalities were associated with increased OS and cell damage. In the hypofunctioning follicles of HT and normal thyroids, Cav-1, DUOX, and TPO were not expressed. In GD, they were expressed at the apical pole of thyrocytes, and $T_4$ accumulated in the colloid of all follicles. Th1 cytokines IL-1$\alpha$/interferon$\gamma$ decreased Cav-1 expression in vitro, whereas the Th2 cytokine IL-4 had no effect.

**Conclusion:** Th1 cytokine-induced down-regulation of Cav-1 could be responsible for intracytoplasmic $T_4$ synthesis and mislocalization of DUOX and TPO, suggesting an important role for Cav-1 in the preservation of thyroxisome integrity. The thyroxisome's disruption, leading to uncontrolled OS and cell apoptosis, is a key, event in HT pathogenesis. (***J Clin Endocrinol Metab*** **99: 1722–1732, 2014)**

Hashimoto's thyroiditis (HT) and Graves' disease (GD) are the two main types of thyroid autoimmune disorders. HT is triggered by an autoimmune response against thyroglobulin (Tg) and thyroid peroxidase (TPO) autoantigens. It is characterized by the progressive destruction of most follicles, fibrosis, and inflammatory infiltration (1), eventually leading to glandular atrophy and hypothyroidism. In addition, Th1 cytokines, among which interferon (IFN)-$\gamma$, TNF-$\alpha$, TNF-$\beta$, IL1$\alpha$, IL1$\beta$, IL-2, platelet-derived growth factor (2) are predominant, play a key role in thyroid cell destruction by CD8+ cytotoxic cells. Th1 cytokines inhibit iodide uptake and thyroid hormone (TH) release from thyrocytes and down-regulate dual oxidase (DUOX), TPO, Tg, and $Na^+/I^-$

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

Received September 13, 2013. Accepted December 31, 2013.
First Published Online January 29, 2014

* L.M. and V.V.R. contributed equally to this work.

Abbreviations: Cav-1, caveolin-1; DUOX, dual oxidase; GD, Graves' disease; 4-HNE, 4-hydroxynonenal; HT, Hashimoto's thyroiditis; IFN, interferon; KO, knockout; NIS, $Na^+/I^-$ symporter; NOX, nicotinamide adenine dinucleotide phosphate oxidase; OS, oxidative stress; ROS, reactive oxygen species; Tg, thyroglobulin; Tg-I, iodinated-Tg; TH, thyroid hormone; TPO, thyroid peroxidase.

symporter (NIS) expression in thyrocytes without affecting cell viability (3, 4).

In GD, self-tolerance to autoantigens, mainly the TSH receptor, is also disrupted. Most autoantibodies produced in GD exert a TSH-like effect and induce thyroid overstimulation. Hypertrophy and hyperplasia of thyrocytes affect the whole gland and can sometimes cause the formation of small papillae. Diffuse lymphocytic infiltrates may be observed among the follicles and germinal centers are frequently found (5). GD is driven by the humoral immune response and Th2 cytokines (IL-4 and IL-10) (2).

HT and GD are therefore multifactorial diseases with opposite thyroid hormone levels (hypothyroidism vs hyperthyroidism) and cytokine profiles (Th1 vs Th2). In addition to cytokines, HT and GD thyroid follicles are exposed to opposite environmental factors: high TSH levels and low or normal $T_4$ levels in HT, thyroid-stimulating antibodies, thioamide drugs, and high $T_4$ levels in GD (5). From these factors that may influence the morphology and function of the thyrocytes, thyroid hormones are the best known to modulate immune responses at the cellular level (6, 7).

TH synthesis is a multistep process (8), during which iodide is actively taken up across the basolateral membrane of thyrocytes via NIS and then crosses the apical membrane. The organification of iodide into Tg by TPO requires $H_2O_2$. To avoid cytotoxicity, $H_2O_2$ is synthesized by DUOX in a restricted area located at the interface between the apical membrane and the colloid and is immediately consumed by TPO. This biochemical entity, referred to as the thyroxisome (9), comprises a multiprotein complex containing TPO and DUOX among other proteins, which must be located at the apical membrane to be active in terms of efficient hormone synthesis (9).

Caveolin-1 (Cav-1), a 22-kDa protein, is another member of the thyroxisome multiprotein complex required for TH synthesis and thyroid cell homeostasis (10). Cav-1, like the other two isoforms of caveolin, integrates into cell membranes and creates caveolae, which are nonclathrin-coated pits (11, 12). By creating these raft-like membrane structures, Cav-1 can compartmentalize different cell processes (13). In thyroid cells from Cav-1 knockout (KO) mice, the TPO/DUOX protein complex is mislocalized in the cytoplasm instead of at the apical membrane. This leads to intracytoplasmic TH synthesis but also to $H_2O_2$ and/or reactive oxygen species (ROS) production, thereby leading to excessive oxidative stress (OS) and cell apoptosis (10). Therefore, Cav-1 is likely required for the correct positioning of the TPO/DUOX protein complex at the apical membrane. It is also likely that, through binding to DUOX, Cav-1 inhibits DUOX until it is correctly localized in the apical membrane. By contrast, Cav-1 deficiency leads to insufficient inhibition of intracytoplasmic DUOX, which is associated with the production of intracytoplasmic $H_2O_2$ and thus increased OS (10). Similar effects were observed in the thyroid of a patient with Pendred syndrome (14). In addition, thyroid hormones are known as modulators of Cav-1 expression. In rat heart development, for instance, the level of Cav-1 is decreased by hypothyroidism (15).

The aim of this study was to investigate the role of Cav-1 in the expression of the TPO/DUOX protein complex or thyroxisome in HT vs GD. Because the effect of cytokines on Cav-1 expression is not known, the in vivo study was coupled to an in vitro study in which Th1 and Th2 cytokines were tested in primary cultures of human thyrocytes.

## Materials and Methods

### Thyroid samples

Paraffin-embedded tissue samples from five HT patients and 10 GD patients were selected from the anatomopathology collection of the Université catholique de Louvain (Brussels, Belgium). Paranodular tissues from multinodular goiters were used as control thyroid samples (n = 5). Other thyroid samples (three of paranodular tissues, four from GD patients, and two from HT patients) were frozen in liquid nitrogen and stored at −80°C until use for RT-PCR and Western blotting. All tissues were surgical specimens, obtained after patients gave informed consent.

HT patients had high levels of circulating anti-TPO and anti-Tg antibodies. At the time of surgery, TSH levels were low in two patients (0.03, 0.072 μU/mL), high in two patients (5.64, 6.15 μU/mL), or normal in three patients (0.35–2.75 μU/mL). The free $T_4$ levels were in the normal range in all patients (0.9–1.46 ng/dL).

All GD patients were treated with antithyroid drugs (propylthiouracil or strumazol) and Lugol solution for a few days before the surgical procedure. At the time of surgery, TSH levels were undetectable in two patients, low in three patients (0.01, 0.01, 0.09 μU/mL), and normal in five patients (0.7–3.15 μU/mL). Free $T_4$ levels were normal in nine patients (0.6–1.8 ng/dL) and low in one patient (0.3 ng/dL).

### Immunohistochemistry

All antigens, iodinated-Tg (Tg-I), $T_4$, DUOX, TPO, Cav-1, 4-hydroxynonenal (4-HNE), catalase, activated caspase-6, and cyclin D1 were detected in paraffin sections (see Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). To detect Cav-1, 4-HNE, catalase, cyclin D1, and activated caspase-6, sections in citrate buffer [0.01 mol/L (pH 6)] were heated in a microwave oven once for 3 minutes at 750 W, followed by four times for 3.5 minutes at 350 W. Negative controls were performed by omitting the primary antibody.

## Cell culture

Thyrocytes from paranodular tissues were isolated as previously described (16) and suspended in modified Earle's medium (BRL-Gibco) without phenol red, containing 5% newborn calf serum (Gibco), penicillin (100 U/mL; Gibco), streptomycin (100 U/mL; Gibco), glutamine (2.4 mM; Sigma), and Fungizone (2.5 μg/mL; Gibco). The cells were plated in 12-well plates and cultured in a humidified atmosphere (5% $CO_2$) with 1 mU/mL TSH (Sigma) for 1 week. Cells were then incubated for an additional 3 days with recombinant murine IL-1α (2 ng/mL) (R&D Systems) and recombinant murine IFNγ (10 ng/mL) (R&D Systems), with recombinant human IL-4 (2 ng/mL) (R&D Systems), or with all three of these cytokines in modified Earle's medium containing 0.5% newborn calf serum. Each experiment was repeated twice (one with the number of wells of three and one with the number of wells of five).

## RNA purification and reverse transcription

Cells and thyroid lysates were suspended in TriPure isolation reagent (Roche Diagnostics GmbH), and total RNA was purified according to the manufacturer's protocol. Reverse transcription was performed by incubating 2 μg RNA with 200 U Moloney murine leukemia virus reverse transcriptase (Promega), 1 μL RNasin (Promega), 0.625 mM of each deoxynucleotidetriphosphate (Promega), and 2 μM oligodeoxythymidine (Promega) in the recommended buffer containing 10 mM dithiothreitol overnight at 42°C (20 μL final volume). $H_2O$ (80 μL) was then added and the products were used for PCR amplifications.

## Quantitative PCR

cDNA samples (2 μL) were mixed with 500 nmol/L of each of the selected primers (Supplemental Table 2) and SYBR Green reaction mix (Perfecta; VWR) in a final volume of 15 μL. Reactions were performed using the iCycler apparatus (Bio-Rad Laboratories) as follows: 30 cycles of 95°C for 1 minute, followed by 40 cycles of 95°C for 15 seconds, the appropriate annealing temperature (see Supplemental Table 2) for 45 seconds, and 81°C for 15 seconds. The amplified level of the target gene was normalized against that of β-actin.

## Western blot analysis

Frozen thyroid samples were homogenized in Laemmli buffer containing a protease inhibitor cocktail (Roche). The protein concentration was determined using a bicinchoninic assay protein assay kit (Thermo Scientific). Proteins (50 μg/lane) were denatured by heating homogenates at 95°C for 5 minutes in the loading buffer containing dithiothreitol (100 mM) and bromophenol blue (0.1%), separated by SDS-PAGE (10%, 8%, or 6%) and then transferred onto nitrocellulose membranes (Hybond ECL; Amersham Biosciences). Membranes were blocked with PBS containing 5% nonfat dry milk and 0.1% Tween 20 for 1 hour at room temperature and were then incubated with the primary antibody (see Supplemental Table 3). Membranes were washed and incubated for 1 hour at room temperature with peroxidase-labeled secondary antirabbit monoclonal horseradish peroxidase antibody (1:5000; Thermo Scientific) or antimouse biotinylated antibody (1:200; Thermo Scientific) followed by avidin biotin complex detection (Vectastain avidin biotin complex kit; Vector Laboratories) according to the primary antibody. Finally, membranes were visualized using enhanced chemiluminescence (SuperSignalWestPico; Thermo

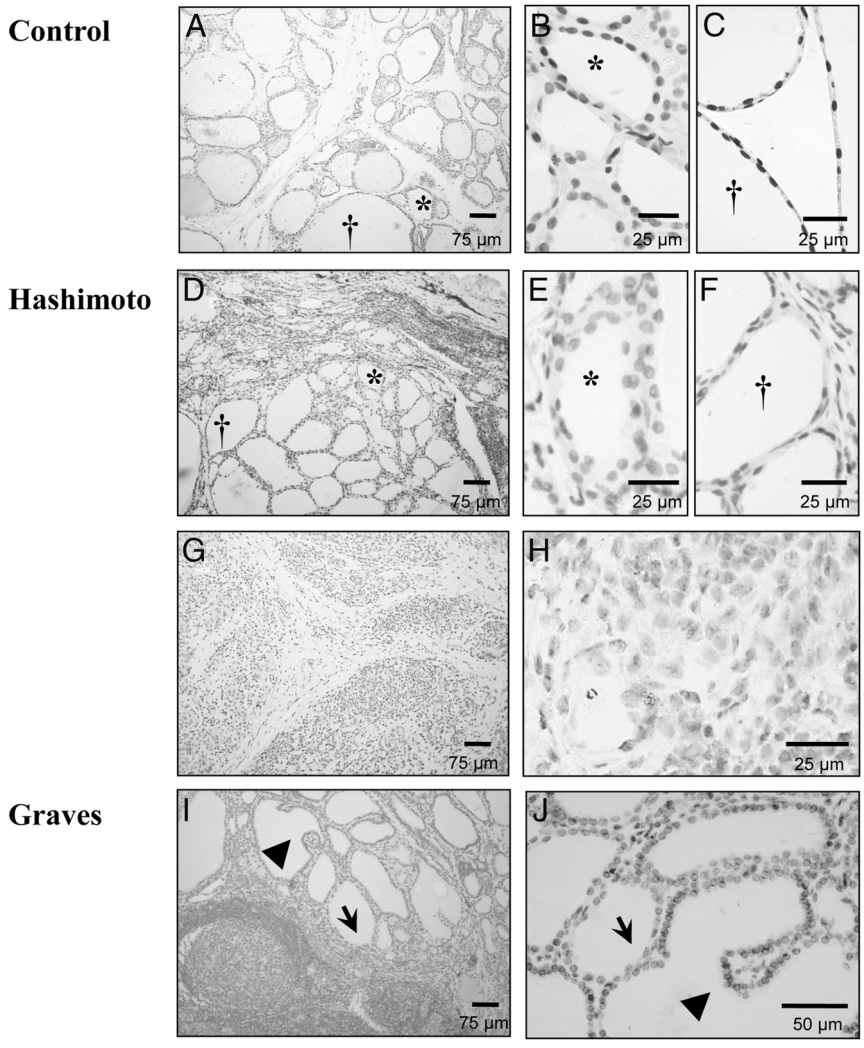


**Figure 1.** Thyroid morphology. In control thyroids, two types of follicles are observed (A): the majority are active follicles containing cuboidal cells and little colloid (B), and there are also hypofunctioning follicles surrounded by flat epithelium (C). In HT thyroids, two types of follicles are observed (D): a few so-called active-like follicles (E) with many cubic cells and little colloid, and numerous hypofunctioning follicles (F) lined with flat epithelium. These are both present within areas of cell damage (G) and inflammatory infiltrates (H). In GD thyroids (I and J), hyperactive follicles are present. Some of these follicles are small with a normal aspect (→), whereas others (▲) are larger with irregular lumina and sometimes papillae.

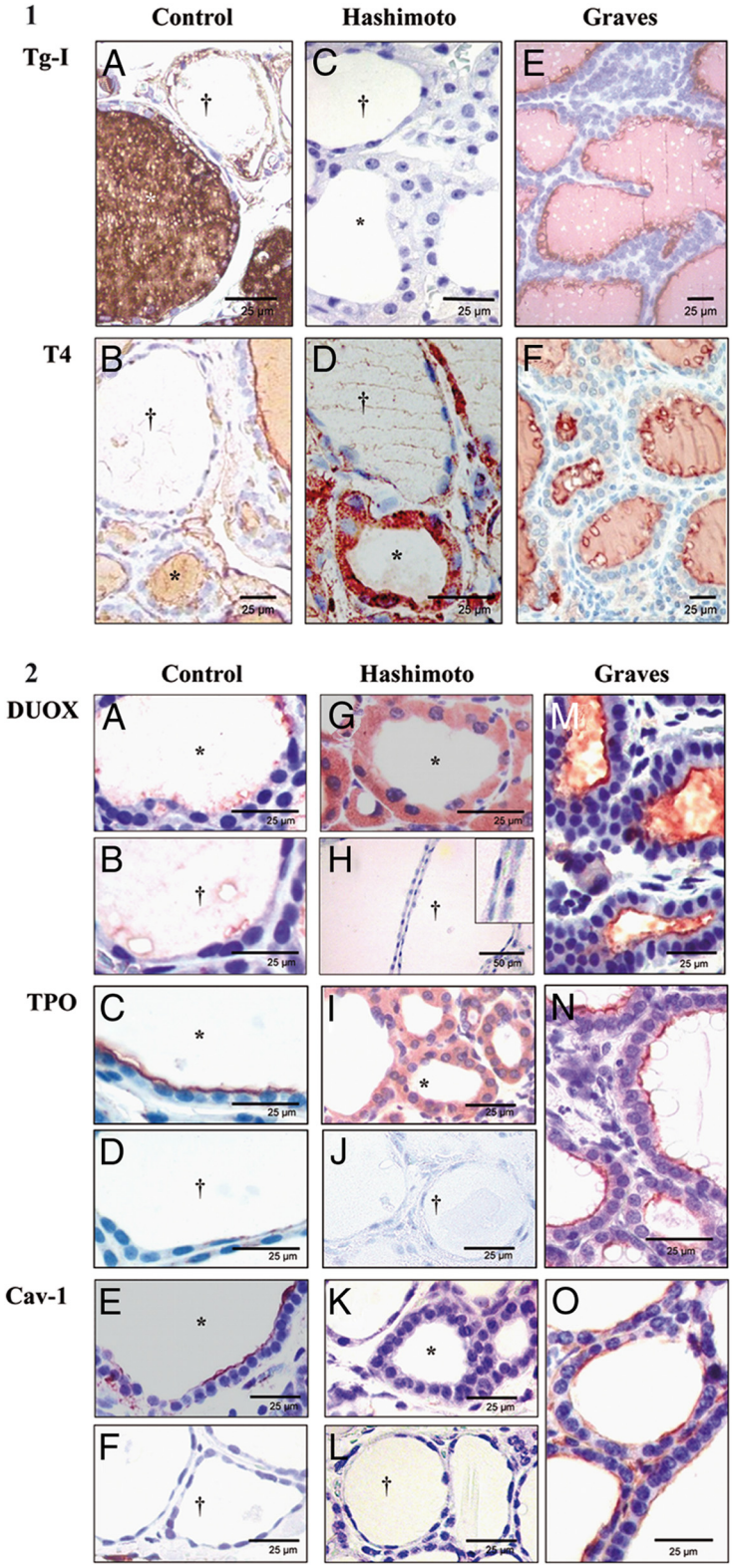


**Figure 2.** Immunohistochemical detection of Tg-I, $T_4$, DUOX, TPO, and Cav-1. Figure 2.1, In control thyroids, Tg-I (A) and $T_4$ (B) are detected in the follicular lumina of active follicles but not in hypofunctioning follicles. In HT thyroids, Tg-I (C) is not detected in the colloid of active-like or hypofunctioning follicles. $T_4$ (D) is detected in the cytoplasm of cells in active-like follicles but is not detected in most thyrocytes of hypofunctioning follicles. In GD thyroids, Tg-I (E) and $T_4$ (F) are highly expressed in the follicular lumina of all follicles. Figure 2.2, In control thyroids, DUOX (A and B), TPO (C and D), and Cav-1 (E and F) are expressed at the apical pole of cells in active follicles, whereas weak or no signals are detected in hypofunctioning follicles. In HT thyroids, DUOX (G and H) and TPO (I and J) are detected in the cytoplasm of cells in active-like follicles but not in hypofunctioning follicles. Cav-1 (K and L) is not detected in active-like follicles or hypofunctioning follicles. In GD thyroids, DUOX (M), TPO (N), and Cav-1 (O) are localized at the apical pole of all thyrocytes. *, Active or active-like follicle; †, hypofunctioning or hypofunctioning follicle.

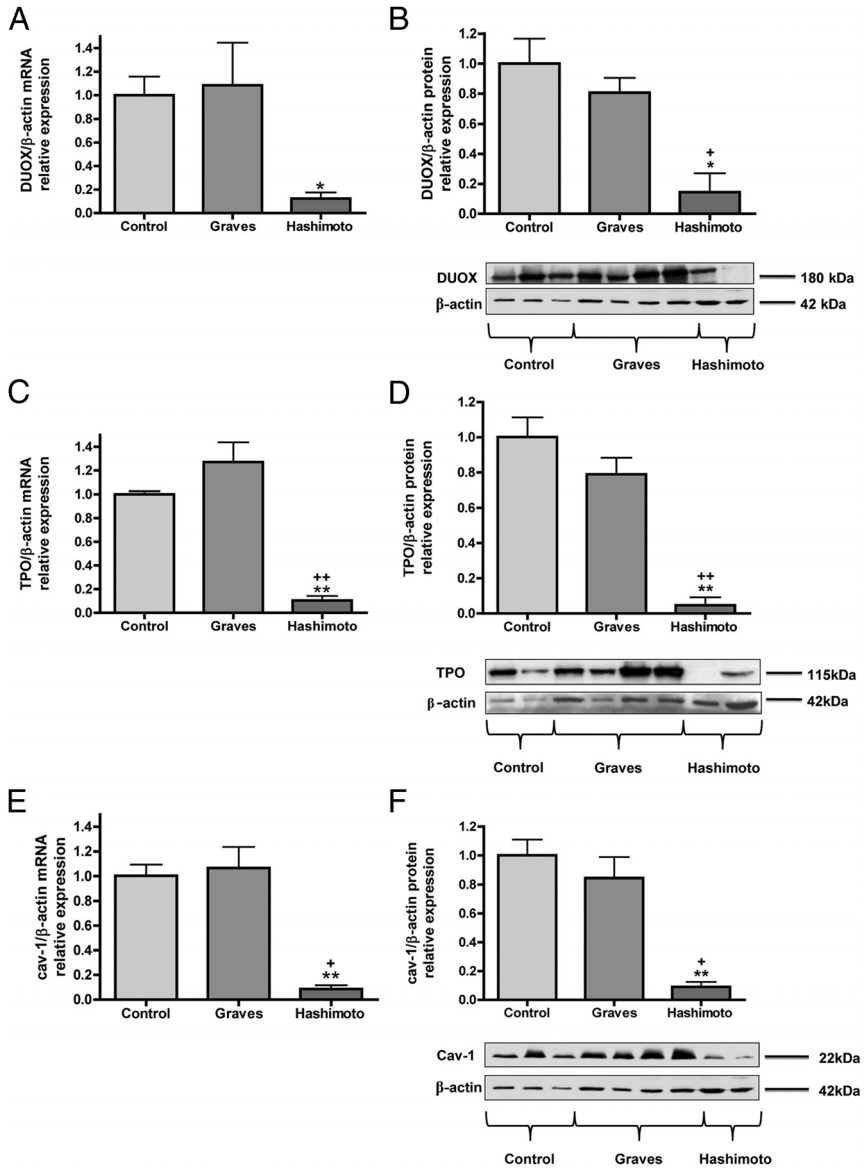


**Figure 3.** DUOX, TPO, and Cav-1 mRNA and protein expression. The expression of DUOX mRNA (A) and protein (B) relative to that of $\beta$-actin is expressed as mean ± SEM [control (n = 3); GD (n = 8); HT (n = 2)]. DUOX mRNA and protein expression is significantly lower in HT thyroids than in control and GD thyroids; however, there is no significant difference between GD and control thyroids. *, $P < .05$ vs control; +, $P < .01$ vs GD. The expression of TPO mRNA (C) and protein (D) relative to that of $\beta$-actin is expressed as mean ± SEM [control (n = 3); GD (n = 8); HT (n = 2)]. TPO mRNA and protein expression is significantly lower in HT thyroids than in control and GD thyroids; however, there is no significant difference between GD and control thyroids. **, $P < .01$ vs control; ++, $P < .01$ vs GD. The expression of Cav-1 mRNA (E) and protein (F) relative to that of $\beta$-actin is expressed as mean ± SEM [control (n = 3); GD (n = 8); HT (n = 2)]. Cav-1 mRNA and protein expression is significantly lower in HT thyroids than in control and GD thyroids; however, there is no significant difference between GD and control thyroids. **, $P < .01$ vs control; +, $P < .05$ vs GD.

Scientific) for 5–60 seconds on CL-Xposure films (Thermo Scientific) or using the Geliance Imaging =System 600 (PerkinElmer). Western blots were scanned and quantified by densitometry using NIH Scion image analysis software (National Institutes of Health, Bethesda, Maryland). All values are expressed as mean ± SEM.

### Statistical analysis

Quantitative PCR and Western blotting results were normalized against levels of $\beta$-actin. Statistical analysis was performed using an ANOVA2 followed by a Tukey-Kramer multiple comparison test or a *t* test (GraphPad). A value of $P < .05$ was considered statistically significant.

## Results

### HT, GD, and normal thyroid glands display marked morphological heterogeneity

In line with previous reports (17, 18), two types of follicles were observed in control thyroids (Figure 1A). The most abundant follicles, called active follicles (Figure 1B), were lined with cuboidal cells and contained little colloid. By contrast, the so-called hypofunctioning follicles were surrounded by flat epithelium (Figure 1C).

In HT thyroids (Figure 1D), these two types of follicles were also observed, but they were called active-like and hypofunctioning follicles. Even though there were fewer active-like follicles in HT thyroids (Figure 1E) than in control thyroids, these follicles were lined with high cubic cells and had little colloid, but as shown later, they lost their capacity of normal hormonal synthesis. Hypofunctioning follicles (Figure 1F) were most abundant and were morphologically and functionally similar to the hypofunctioning follicles in control thyroids. They were lined with flat epithelium and were close to areas of cell destruction (Figure 1G) and inflammatory infiltrates (Figure 1H).

In GD thyroids (Figure 1, I and J), some hyperactive follicles were small and had a feature similar to that in control thyroids, whereas others were larger with irregular lumina and sometimes papillae.

### Iodination is impaired in HT but not in GD

In control thyroids, Tg-I (Figure 2, 1A) and T4 (Figure 2, 1B) were detected in the colloid of active follicles but not in hypofunctioning follicles.

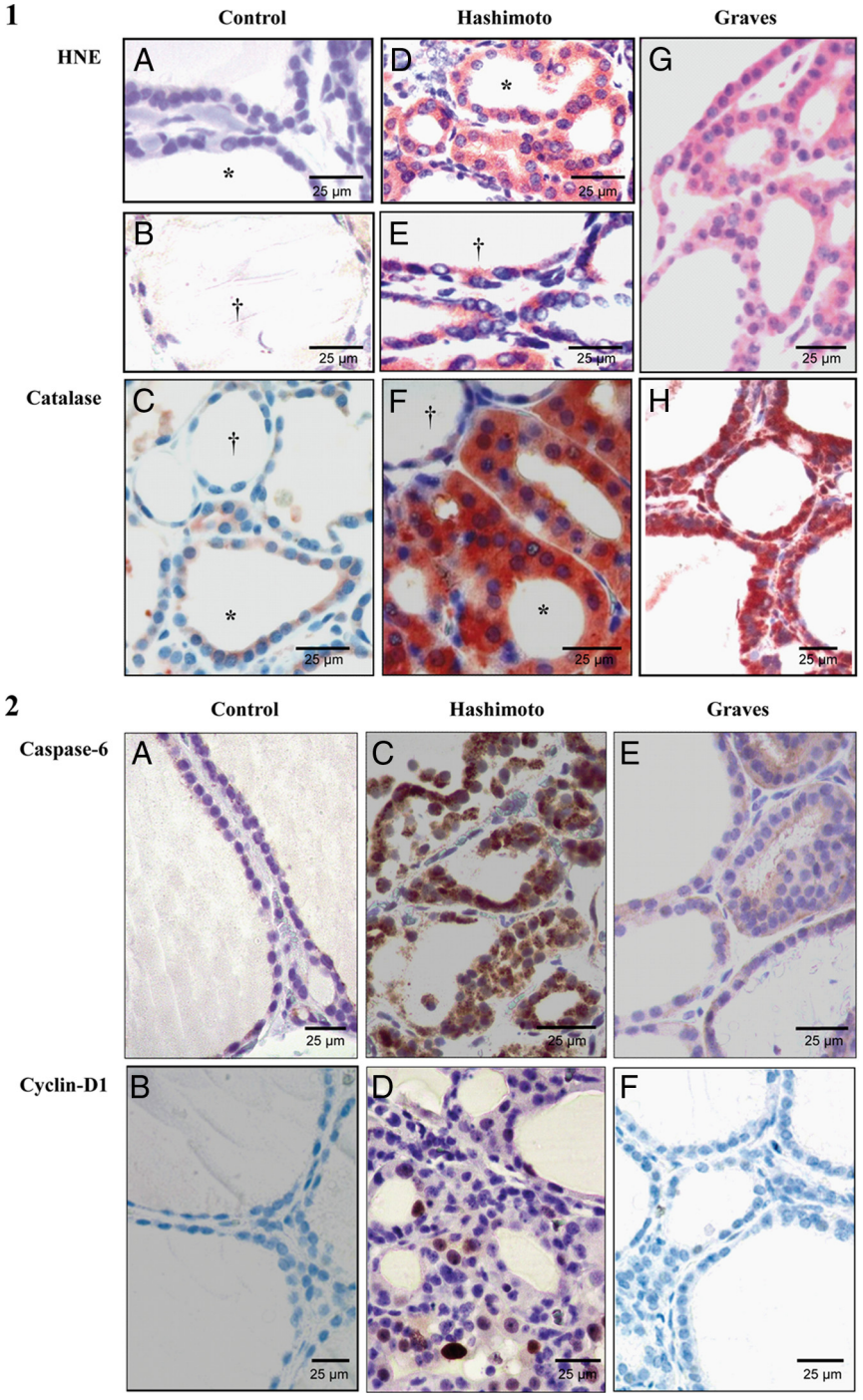


**Figure 4.** Immunohistochemical detection of 4-HNE, catalase, activated caspase-6, and cyclin-D1. Figure 4.1, In control thyroids (A and B), 4-HNE expression is low in active and hypofunctioning follicles. Catalase (C) is weakly expressed in active follicles and is not detected in hypofunctioning follicles. In HT thyroids (D and E), 4-HNE is highly expressed in thyrocytes of active-like follicles and hypofunctioning follicles. Expression of catalase (F) is high in active-like follicles but low in hypofunctioning follicles. In GD thyroids, 4-HNE (G) and catalase (H) are strongly expressed in all thyrocytes. Figure 4.2, In control thyroids, activated caspase-6 (A) is expressed in the nuclei of no or very few thyrocytes. Cyclin-D1 (B) is not detected in the nuclei of any thyrocytes. In HT thyroids, activated caspase-6 (C) is detected in numerous nuclei and cyclin-D1 (D) is detected in some nuclei. In GD thyroids, activated caspase-6 (E) is expressed in the nuclei of no or very few thyrocytes. Cyclin-D1 (F) is not detected in the nuclei of any thyrocytes. *, Active or active-like follicle; †, hypofunctioning or hypofunctioning follicle.

By contrast, in HT thyroids, Tg-I was not detected in the colloid of active-like follicles or hypofunctioning follicles (Figure 2, 1C). $T_4$ was not detected in lumina (Figure 2, 1D) but was detected in the cytoplasm of thyrocytes in active-like follicles. In hypofunctioning follicles, $T_4$ staining was weak or undetectable in most thyrocytes.

In GD thyroids, Tg-I (Figure 2, 1E) and T4 (Figure 2, 1F) were detected in the colloid of all follicles.

The observations are representative of HT and GD patients analyzed.

### The low level of Cav-1 expression in HT thyroid glands is associated with mislocalization and low expression of DUOX and TPO

In control thyroids, DUOX (Figure 2, 2A and 2B), TPO (Figure 2, 2C and 2D), and Cav-1 (Figure 2, 2E and 2F) proteins were detected at the apical pole of cells of active follicles, whereas weak or no signals were detected in hypofunctioning follicles, as previously reported (17–19).

In HT thyroids, DUOX (Figure 2, 2G and 2H) and TPO (Figure 2, 2I and 2J) were detected in the cytoplasm of cells of active-like follicles. The immunostaining was diffuse in the cytoplasm, rather than an intense linear labeling at the apical pole. DUOX and TPO were not detected in hypofunctioning follicles. Cav-1 was not detected in active-like or hypofunctioning follicles (Figure 2, 2K and 2L).

In GD thyroids, DUOX (Figure 2, 2M), TPO (Figure 2, 2N), and Cav-1 (Figure 2, 2O) were localized in the correct position at the apical membrane of all thyrocytes.

mRNA and protein levels of DUOX, TPO, and Cav-1 were significantly lower in HT thyroids than in control thyroids ($P < .05$) (Figure 3, A–F), whereas there were no significant differences between GD and control thyroids.

### The increased level of OS in HT is associated with cell apoptosis and a low level of cell proliferation

In control thyroids, 4-HNE, a marker of OS, was weakly expressed in active and hypofunctioning follicles (Figure 4, 1A and 1B). Catalase, which is involved in antioxidant defenses (20), was weakly expressed in active follicles and was not expressed in hypofunctioning follicles (Figure 4, 1C).

4-HNE expression was much higher in active-like follicles and hypofunctioning follicles (Figure 4, 1D and 1E) in HT thyroids than in control thyroids. In HT thyroids, catalase was strongly expressed in active-like follicles, whereas a weak signal was detected in hypofunctioning follicles (Figure 4, 1F).

4-HNE (Figure 4, 1G) and catalase (Figure 4, 1H) expressions were much higher in all thyrocytes in GD thyroids than in control thyroids.

In control thyroids, the levels of cell apoptosis and proliferation were low, and the nuclei of no or few thyrocytes were labeled with the antiactivated caspase-6 antibody (Figure 4, 2A), or with the anticyclin D1 antibody (Figure 4, 2B).

In HT thyroids, numerous nuclei were labeled with the antiactivated caspase-6 antibody (Figure 4, 2C). Some foci of cell proliferation were observed, as shown by the nucleus labeling with the anticyclin D1 antibody (Figure 4, 2D).

In GD thyroids, activated caspase-6 (Figure 4, 2E) was expressed in very few nuclei, whereas cyclin-D1 (Figure 4, 2F) was not expressed in the nuclei of any thyrocytes.

### Th1 cytokines down-regulate Cav-1 expression in primary cultures of human thyroid cells

Treatment of thyrocytes with IL-1$\alpha$/IFN$\gamma$ significantly decreased Cav-1 mRNA expression (10-fold decrease, $P <$ .01 compared with control) (Figure 5). IL-4, a Th2 cytokine that counteracts Th1 cytokine-induced effects on DUOX and TPO expression (3) and increases DUOX expression (21), did not significantly affect Cav-1 mRNA expression when added alone. By contrast, although Cav-1 mRNA expression was significantly higher in cells cotreated with IL-4 and IL-1$\alpha$/IFN$\gamma$ than in cells treated with IL-1$\alpha$/IFN$\gamma$ alone (2-fold increase, $P < .05$), Cav-1 mRNA expression was lower than in control cells ($P < .01$).

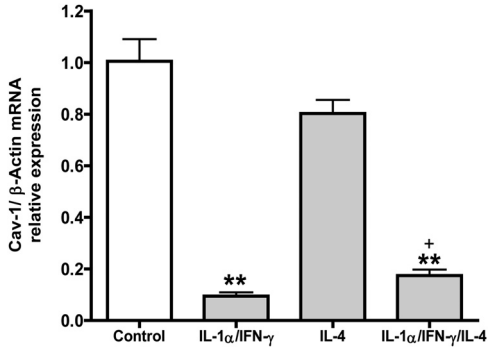


**Figure 5.** Cav-1 mRNA expression in human primary thyrocytes cultures. The mRNA expression of Cav-1 mRNA relative to that of $\beta$-actin is expressed as mean ± SEM of five independent wells in one representative experiment. IL-1$\alpha$ (2 ng/mL)/IFN$\gamma$ (100 U/mL) treatment decreases Cav-1 mRNA expression, whereas IL-4 (2 ng/mL) has no effect. Cav-1 mRNA expression is significantly higher in cells cotreated with IL-4- and IL-1$\alpha$/IFN$\gamma$ than in IL-1$\alpha$/IF$\gamma$-treated cells. However, Cav-1 mRNA expression is lower in cells cotreated with IL-4 and IL-1$\alpha$/IFN$\gamma$ than in control cells. **, $P < .01$ vs control cells; +, $P < .05$ vs IL-1-$\alpha$/IFN$\gamma$-treated cells.

## Discussion

Our data indicate that Cav-1 expression is down-regulated in HT thyroid glands along with an overall decrease in TPO and DUOX expression. Although decreased TPO expression has already been reported in HT (22), this is the first study to show that Cav-1 and DUOX mRNA and protein expressions are decreased in HT. Moreover, HT thyroids were characterized by marked tissue heterogeneity. Expression of differentiation-associated proteins was comparable between hypofunctioning follicles in HT thyroids and normal thyroids, whereas expression in active-like follicles markedly differed between normal and HT thyroids. This suggests that active-like follicles are affected by inflammatory cytokines. In HT, TPO, DUOX, and Cav-1 but also Tg-I and $T_4$ were not expressed in hypofunctioning follicles, as previously reported in normal thyroids (18). Of note, active-like follicles in HT thyroids also contained colloid that lacked Tg-I and $T_4$. This is in contrast to active follicles in normal thyroids, which contain Tg-I and T4 in the colloid and express DUOX, TPO, and Cav-1 at the apical pole of cells (17–19). The lack of Tg-I and $T_4$ in the colloid of active-like follicles of HT thyroids was associated with the intracytoplasmic localization of DUOX and TPO proteins along with abnormal intracellular TH biosynthesis. By contrast, in GD thyroids, TPO and DUOX were correctly located at the apical membrane to form the TH synthesis protein complex called the thyroxisome (23).

In HT, the ectopic location of DUOX and TPO associated with intracytoplasmic TH synthesis is reminiscent of that observed in Cav-1 KO mice (10) and in the thyroid of a patient with Pendred syndrome (14). These features could be the consequence of TPO/DUOX protein complex disruption. In this study, there was a correlation between the lack of Cav-1 expression and the mislocalization of the thyroxisome. This suggests that Cav-1 plays a major role in the correct apical positioning of TPO and DUOX. In HT, Th1 cytokines are at least in part responsible for Cav-1 down-regulation. Th1 cytokines inhibit many differentiation-associated proteins in thyrocytes, including

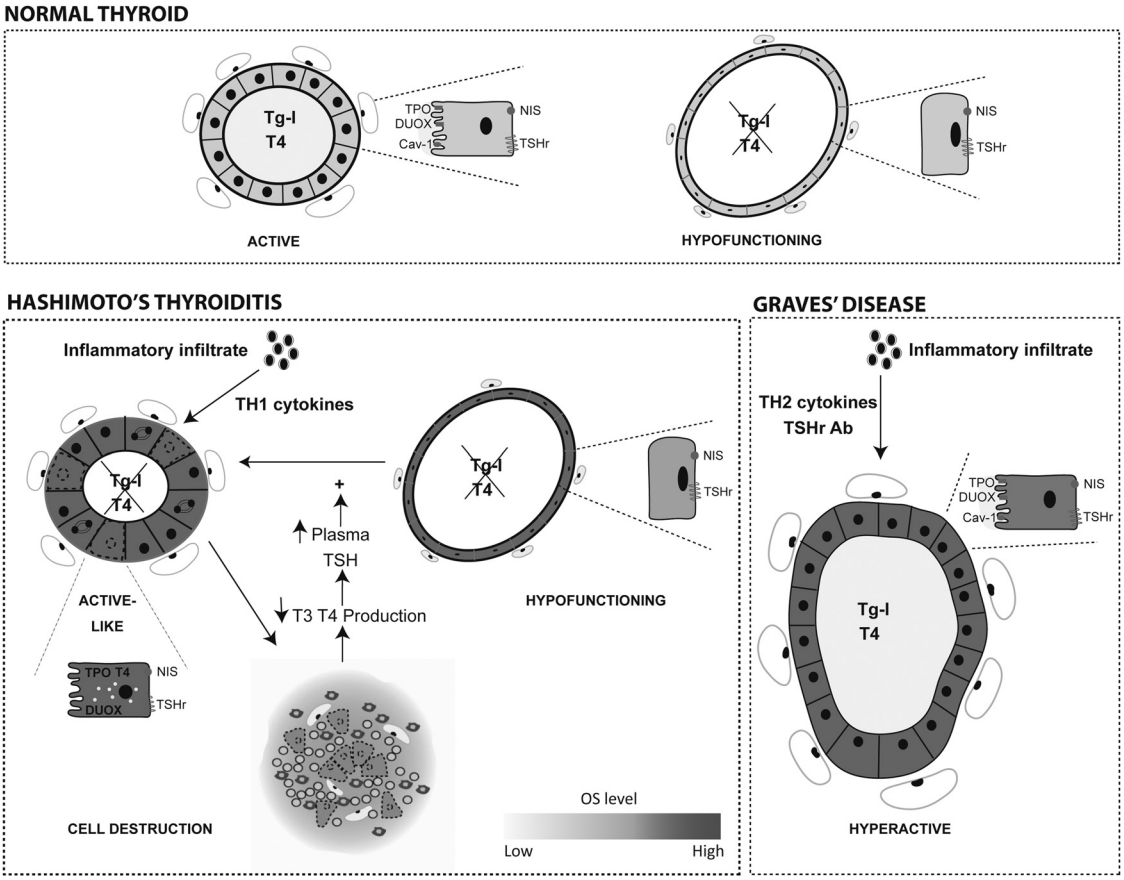


**Figure 6.** Schematic representation of hypothetical pathophysiological mechanisms in thyrocytes exposed to Th1 cytokine-driven inflammatory environment in HT. In control thyroids, as well as in multinodular goiters, there are two types of follicles, so-called active and hypofunctioning follicles. In active follicles, Cav-1 is associated with the TPO/DUOX protein complex to form the thyroxisome, which is located at the apical pole of thyrocytes. This complex is responsible for TH synthesis in the follicular lumina. In hypofunctioning follicles, the absence of DUOX and TPO expression is associated with the absence of both Cav-1 expression and TH synthesis. In both types of follicles, levels of OS, apoptosis, and proliferation are low. In GD thyroids, which are characterized by the production of Th2 cytokines and stimulating autoantibodies, DUOX, TPO, and Cav-1 are correctly located at the apical poles of cells in all follicles and TH synthesis occurs in the follicular lumen, as in control thyroids but at a higher rate. The hyperstimulation of cells is associated with increased OS, which is adequately managed by well-adapted antioxidant defenses. In HT thyroids, lack of Cav-1 expression in thyrocytes exposed to Th1 cytokines (IL-1$\alpha$/IFN$\gamma$) is associated with the cytoplasmic localization of DUOX and TPO. Consequently, TH synthesis occurs in the cytoplasm. This aberrant location of the thyroxisome is associated with increased OS and increased apoptosis. In this case, antioxidant defenses cannot adequately deal with the oxidative attack. In addition, cell proliferation cannot compensate for cell death and large zones of cell destruction arise. This leads to decreased TH synthesis and in turn to increased serum TSH levels. As long as they are not activated by TSH, the hypofunctioning follicles are unaffected by increased OS associated with the infiltrating inflammatory cells. We propose that upon TSH stimulation, hypofunctioning follicles are activated to compensate for impaired thyroid function. These hypofunctioning follicles express DUOX and TPO to increase TH synthesis. Similar to active-like follicles, the hypofunctioning follicles become sensitive to Th1 cytokines. Cav-1 is not expressed, causing destabilization of the thyroxisome and its mislocalization into the cytoplasm in which TH synthesis aberrantly occurs. The aforementioned pathological processes lead to cell damage and perpetuate the process of thyroid gland destruction.

TPO, DUOX, NIS, and Tg (3, 4), which may explain why hypothyroidism occurs independently of cellular destruction (21). In this study, Th1 cytokines induced a decrease in Cav-1 mRNA and protein expression in vitro. Because this effect was also observed in HT thyroids, Th1 cytokines released by the inflammatory infiltrate may be responsible for the observed changes in Cav-1 expression. The effect of Th1 cytokines on Cav-1 expression is of particular importance, considering that Cav-1 plays important roles in the regulation of multiple proteins in a wide range of cells (24, 25). In our model, the cytokine-induced effects on Cav-1 expression were IL-1$\alpha$/IFN$\gamma$ dependent, and IL-4, a Th2 cytokine, had no effect on Cav-1 expression or only a small effect on IL-1$\alpha$/IFN$\gamma$-induced Cav-1 down-regulation. It has also been shown in macrophages (26) that IFN$\gamma$ decreases Cav-1 expression and increases NO production by nitric oxide synthase-2. This is in agreement with the observation that a combination of IL-1$\alpha$ and IFN$\gamma$ induces nitric oxide production by nitric oxide synthase-2 in thyroid cells (27).

Cytokines likely affect Cav-1 and DUOX/TPO expression via different mechanisms. IL-4 and IL-13 increase

DUOX2 expression and $H_2O_2$ production (28), whereas IL-4 blocks IL-1$\alpha$/IFN$\gamma$-induced effects on TPO and DUOX expression (21). This suggests that the effects of cytokines on Cav-1 and on DUOX and TPO might involve different pathways. In addition, observations in active-like follicles of HT thyroid glands suggest that Th1 cytokines affect Cav-1 expression before they affect DUOX and TPO expression. Therefore, the loss of Cav-1 could be responsible for the mislocalization of DUOX and TPO and in turn for the disruption of the TPO/DUOX protein complex.

Our finding of high OS in HT thyrocytes is in agreement with a previous study of a mouse model of spontaneous thyroiditis in which thyroiditis was associated with increased OS and cell destruction (29). In HT, OS was observed in all follicles, regardless of whether DUOX and TPO were expressed. Therefore, the mediators of OS could come from multiple sources. DUOX and TPO are expressed but mislocalized in active-like HT follicles, thereby leading to aberrant intracytoplasmic TH synthesis. This may explain why high OS is observed in these apparently active follicles. Therefore, it is highly plausible that high OS is due to DUOX-associated intracytoplasmic $H_2O_2$ production, which is toxic because it does not occur at the apical pole of cells. This explanation is identical with the one given in the case of the patient with Pendred syndrome (14) and in Cav-1 KO mice in which $H_2O_2$ overproduction has been quantified (10).

In addition, Th1 cytokines induce intracellular ROS production by thyrocytes in vitro (29), which is associated with decreased DUOX expression. This suggests that Th1 cytokines stimulate other sources of ROS. ROS may come from the numerous inflammatory cells infiltrating the gland (29). They may also be produced in the thyrocytes by the family of nicotinamide adenine dinucleotide phosphate oxidases (NOX) (30). Thus, NOX4 and NOX2 are expressed in thyroid cells. NOX4 expression is increased by TSH and in carcinomas (31), whereas NOX2 expression is increased in HT and by Th1 (IL-1$\alpha$ and IFN$\gamma$) cytokines (32). Whatever the ROS origin, the antioxidant system is not sufficient to counteract their increased production, thereby leading to an increased rate of apoptosis, as occurs in Pendred syndrome (14), myxoedematous cretinism (23, 33, 34), and other autoimmune diseases such as osteoarthritis (35) and diabetes (36, 37).

In addition, as compared to controls and GD, the rate of cell proliferation was slightly increased in HT, as has been previously shown and quantified in KO Cav-1 mice (10). But this is likely insufficient to compensate for the increased rate of apoptosis. Because Cav-1 functions in the down-regulation of cell proliferation (38), possibly via direct repression of the *cyclin D1* gene (39), this might explain the increased rate of cell proliferation in HT thyroids. In this study, OS in GD was associated with increased expression of antioxidant proteins, as previously reported (40). This corresponds to accelerated cell metabolism, which is normally associated with increased ROS synthesis. In contrast to OS in HT, OS produced by the thyroxisome in GD is harmless because it occurs in cell compartments (as close to the apical pole) in which it is fully counteracted by competent antioxidant defenses.

In conclusion, our results show that Cav-1 expression is down-regulated and that DUOX and TPO are mislocalized in HT thyroid glands. This finding strongly suggests that Cav-1 governs the correct apical positioning of DUOX and TPO. Cav-1 expression is regulated by the Th1 cytokines IL-1$\alpha$ and IFN$\gamma$ but not by the Th2 cytokine IL-4. Our observations also suggest that active-like and hypofunctioning follicles are affected by cytokines differently. This is shown in Figure 6, which describes a putative mechanism for the outcome of HT. When Th1 cytokines act on active-like follicles, they decrease the expression of Cav-1, which leads to TPO/DUOX protein complex disruption and in turn to the mislocalization of TPO and DUOX from the apical membrane to the cytoplasm, an area that cannot cope with high OS. Because antioxidant defenses are not adapted to face such pathological levels of OS, cell damage ensues. This leads to hypothyroidism, which is responsible for increased TSH levels, which in turn stimulate hypofunctioning follicles. Then these hypofunctioning follicles are affected by Th1 cytokines, thereby entering the aforementioned pathological pathway. This process, which eventually leads to further cell destruction and functional impairment, may be considered as a key event in HT pathogenesis.

## Acknowledgments

We thank Professors J.-E. Dumont, F. Miot, and X. De Deken (Institut de Recherche Interdisciplinaire en Biologie Humaine et Moléculaire, Université Libre de Bruxelles) for their DUOX expertise; G. Behets-Wydemans [FATH, Institut de Recherche Expérimentale et Clinique (IREC), Université catholique de Louvain (UCL)] and V. Joris for their technical help; and L. Wallemme (Louvain Centre for Toxicology and Applied Pharmacology, IREC, UCL) for supplying cytokines. We also acknowledge C. de Ville de Goyet, M. de Bournonville, and V. Delacourt (Pôle de Morphologie, IREC, UCL) for their technical support.

Address all correspondence and requests for reprints to: Marie-Christine Many, PhD, Pôle de Morphologie, Institut de

Recherche Expérimentale et Clinique, Secteur des Sciences de la Santé, Université catholique de Louvain, UCL-5251, 52 Avenue E. Mounier, B-1200 Bruxelles, Belgium. E-mail: marie-christine.many@uclouvain.be.

This research was done thanks to Pôle de Morphologie, Institut de Recherche Expérimentale et Clinique, Université Catholique de Louvain, B-1200 Brussels, Belgium.

Disclosure Summary: The authors have nothing to disclose.

## References


1. **Mizukami Y, Michigishi T, Hashimoto T, Tonami N, Matsubara F, Takazakura E.** Silent thyroiditis: a histologic and immunohistochemical study. *Hum Pathol*. 1988;19:423–431.
2. **Phenekos C, Vryonidou A, Gritzapis AD, Baxevanis CN, Goula M, Papamichail M.** Th1 and Th2 serum cytokine profiles characterize patients with Hashimoto's thyroiditis (Th1) and Graves' disease (Th2). *Neuroimmunomodulation*. 2004;11:209–213.
3. **Gerard AC, Boucquey M, van den Hove MF, Colin IM.** Expression of TPO and ThOXs in human thyrocytes is downregulated by IL-1α/IFN-γ, an effect partially mediated by nitric oxide. *Am J Physiol Endocrinol Metab*. 2006;291:242–253.
4. **Ajjan RA, Watson PF, Findlay C, et al.** The sodium iodide symporter gene and its regulation by cytokines found in autoimmunity. *J Endocrinol*. 1998;158:351–358.
5. **Weetman AP, McGregor AM.** Autoimmune thyroid disease: further developments in our understanding. *Endocr Rev*. 1994;15:788–830.
6. **De Vito P, Incerpi S, Pedersen JZ, Luly P, Davis FB, Davis PJ.** Thyroid hormones as modulators of immune activities at the cellular level. *Thyroid*. 2011;21:879–891.
7. **De Vito P, Balducci V, Leone S, et al.** Nongenomic effects of thyroid hormones on the immune system cells: new targets, old players. *Steroid*. 2012;77:988–995.
8. **Colin IM, Denef JF, Lengele B, Many MC, Gerard AC.** Recent insights into the cell biology of thyroid angiofollicular units. *Endocr Rev*. 2013;34:209–238.
9. **Song Y, Driessens N, Costa M, et al.** Roles of hydrogen peroxide in thyroid physiology and disease. *J Clin Endocrinol Metab*. 2007;92:3764–3773.
10. **Senou M, Costa MJ, Massart C, et al.** Role of caveolin-1 in thyroid phenotype, cell homeostasis, and hormone synthesis: in vivo study of caveolin-1 knockout mice. *Am J Physiol Endocrinol Metab*. 2009;297:438–451.
11. **Rothberg KG, Heuser JE, Donzell WC, Ying YS, Glenney JR, Anderson RG.** Caveolin, a protein component of caveolae membrane coats. *Cell*. 1992;68:673–682.
12. **Glenney JR Jr, Soppet D.** Sequence and expression of caveolin, a protein component of caveolae plasma membrane domains phosphorylated on tyrosine in Rous sarcoma virus-transformed fibroblasts. *Proc Natl Acad Sci USA*. 1992;89:10517–10521.
13. **Harvey RD, Calaghan SC.** Caveolae create local signalling domains through their distinct protein content, lipid profile and morphology. *J Mol Cell Cardiol*. 2012;52(2):366–375.
14. **Senou M, Khalifa C, Thimmesch M, et al.** A coherent organization of differentiation proteins is required to maintain an appropriate thyroid function in the Pendred thyroid. *J Clin Endocrinol Metab*. 2010;95:4021–4030.
15. **Ratajcaak P, Oliviéro P, Marotte F, Kolar F, Ostadal B, Samuel JL.** Expression and localization of caveolins during postnatal development in rat heart : implication of thyroid hormone. *J Appl Physiol*. 2005;99:244–251.
16. **Nilsson M, Husmark J, Nilsson B, Tisell LE, Ericson LE.** Primary culture of human thyrocytes in Transwell bicameral chamber: thyrotropin promotes polarization and epithelial barrier function. *Eur J Endocrinol*. 1996;135:469–480.
17. **Gerard AC, Xhenseval V, Colin IM, Many MC, Denef JF.** Evidence for co-ordinated changes between vascular endothelial growth factor and nitric oxide synthase III immunoreactivity, the functional status of the thyroid follicles, and the microvascular bed during chronic stimulation by low iodine and propylthiouracyl in old mice. *Eur J Endocrinol*. 2000;142:651–660.
18. **Gerard AC, Many MC, Daumerie C, et al.** Structural changes in the angiofollicular units between active and hypofunctioning follicles align with differences in the epithelial expression of newly discovered proteins involved in iodine transport and organification. *J Clin Endocrinol Metab*. 2002;87:1291–1299.
19. **Costa MJ, Song Y, Macours P, et al.** Sphingolipid-cholesterol domains (lipid rafts) in normal human and dog thyroid follicular cells are not involved in thyrotropin receptor signaling. *Endocrinology*. 2004;145:1464–1472.
20. **Poncin S, Gerard AC, Boucquey M, et al.** Oxidative stress in the thyroid gland: from harmlessness to hazard depending on the iodine content. *Endocrinology*. 2008;149:424–433.
21. **Poncin S, Lengele B, Colin IM, Gerard AC.** Differential interactions between Th1/Th2, Th1/Th3, and Th2/Th3 cytokines in the regulation of thyroperoxidase and dual oxidase expression, and of thyroglobulin secretion in thyrocytes in vitro. *Endocrinology*. 2008;149:1534–1542.
22. **Yang D, Yuan Z, Li C.** [Gene expression of thyrotropin receptor, thyroid peroxidase and thyroglobulin in autoimmune thyroid disease]. *Zhonghua Nei Ke Za Zhi*. 1997;36:685–688.
23. **Song Y, Lothaire P, Dequanter D, et al.** Association of duoxes with thyroid peroxidase and its regulation in thyrocytes. *J Clin Endocrinol Metab*. 2010;95:375–382.
24. **Razani B, Lisanti MP.** Caveolins and caveolae: molecular and functional relationships. *Exp Cell Res*. 2001;271:36–44.
25. **Drab M, Verkade P, Elger M, et al.** Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science*. 2001;293:2449–2452.
26. **Lei MG, Tan X, Qureshi N, Morrison DC.** Regulation of cellular caveolin-1 protein expression in murine macrophages by microbial products. *Infect Immun*. 2005;73:8136–8143.
27. **van den Hove MF, Stoenoiu MS, Croizet K, et al.** Nitric oxide is involved in interleukin-1α-induced cytotoxicity in polarised human thyrocytes. *J Endocrinol*. 2002;173:177–185.
28. **Raad H, Eskalli Z, Corvilain B, Miot F, De Deken X.** Thyroid hydrogen peroxide production is enhanced by the Th2 cytokines, IL-4 and IL-13, through increased expression of the dual oxidase 2 and its maturation factor DUOXA2. *Free Radic Biol Med*. 2013;56:216–225.
29. **Poncin S, Colin IM, Decallonne B, et al.** N-acetylcysteine and 15 deoxy-δ12,14-prostaglandin J2 exert a protective effect against autoimmune thyroid destruction in vivo but not against interleukin-1α/interferon γ-induced inhibitory effects in thyrocytes in vitro. *Am J Pathol*. 2010;177:219–228.
30. **Bedard K, Krause KH.** The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev*. 2007;87:245–313.
31. **Weyemi U, Caillou B, Talbot M, et al.** Intracellular expression of reactive oxygen species-generating NADPH oxidase NOX4 in normal and cancer thyroid tissues. *Endocr Relat Cancer*. 2010;17:27–37.
32. **Colin IM, Poncin S, Levêque P, Gallez B, Gérard AC.** Differential regulation of the production of reactive oxygen species in Th1 cytokine-treated thyroid cells [published online December 13, 2013]. *Thyroid*. doi:10.1089/thy.2013.0142.
33. **Kohrle J, Jakob F, Contempre B, Dumont JE.** Selenium, the thyroid, and the endocrine system. *Endocr Rev*. 2005;26:944–984.

34. **Kohrle J, Gartner R.** Selenium and thyroid. *Best Pract Res Clin Endocrinol Metab*. 2009;23:815–827.
35. **Davies CM, Guilak F, Weinberg JB, Fermor B.** Reactive nitrogen and oxygen species in interleukin-1-mediated DNA damage associated with osteoarthritis. *Osteoarthritis Cartilage*. 2008;16:624–630.
36. **Suarez-Pinzon WL, Strynadka K, Schulz R, Rabinovitch A.** Mechanisms of cytokine-induced destruction of rat insulinoma cells: the role of nitric oxide. *Endocrinology*. 1994;134:1006–1010.
37. **Rabinovitch A, Suarez-Pinzon WL, Strynadka K, Lakey JR, Rajotte RV.** Human pancreatic islet β-cell destruction by cytokines involves oxygen free radicals and aldehyde production. *J Clin Endocrinol Metab*. 1996;81:3197–3202.
38. **Costa MJ, Senou M, Van Rode F, et al.** Reciprocal negative regulation between thyrotropin/3′,5′-cyclic adenosine monophosphate-mediated proliferation and caveolin-1 expression in human and murine thyrocytes. *Mol Endocrinol*. 2007;21:921–932.
39. **Hulit J, Bash T, Fu M, et al.** The cyclin D1 gene is transcriptionally repressed by caveolin-1. *J Biol Chem*. 2000;275:21203–21209.
40. **Gerard AC, Many MC, Daumerie C, Knoops B, Colin IM.** Peroxiredoxin 5 expression in the human thyroid gland. *Thyroid*. 2005;15:205–209.