Sexual dimorphism and thyroid dysfunction: a matter of oxidative stress?

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Abstract

Thyroid diseases, such as autoimmune disease and benign and malignant nodules, are more prevalent in women than in men, but the mechanisms involved in this sex difference is still poorly defined. H_2O_2 is produced at high levels in the thyroid gland and regulates parameters such as cell proliferation, migration, survival, and death; an imbalance in the cellular oxidant–antioxidant system in the thyroid may contribute to the greater incidence of thyroid disease among women. Recently, we demonstrated the existence of a sexual dimorphism in the thyrocyte redox balance, characterized by higher H_2O_2 production, due to higher NOX4 and Poldip2 expression, and weakened enzymatic antioxidant defense in the thyroid of adult female rats compared with male rats. In addition, 17β-estradiol administration increased *NOX4* mRNA expression and H_2O_2 production in thyroid PCCL3 cells. In this review, we discuss the possible involvement of oxidative stress in estrogen-related thyroid pathophysiology. Our current hypothesis suggests that a redox imbalance elicited by estrogen could be involved in the sex differences found in the prevalence of thyroid dysfunctions.

Key Words
► estrogen
► thyroid
► reactive oxygen species
► NADPH oxidases

Introduction

Thyroid diseases are more prevalent in women than in men. In the general population, the Framingham survey indicated that the prevalence of diffuse or nodular goiter is 6.4% in women and 1.5% in men (Vander *et al.* 1968), and the Wickham study reported a female-to-male thyroid disease prevalence ratio of 6.6:1 (Tunbridge *et al.* 1977). Additionally, the incidence of spontaneous hypothyroidism is higher in women than in men, with a mean incidence in women of 3.5/1000 survivors per year (2.8–4.5)

and in men of 0.6/1000 survivors per year (0.3–1.2) (Vanderpump *et al.* 1995). Moreover, the detection of thyroid autoantibodies is almost five times more common in women than in men (Hollowell *et al.* 2002). The mean incidence of Hashimoto's disease among women is ~3.5 cases/1000 people per year, whereas in men the incidence is 0.8 cases/1000 people per year (Brent & Davies 2011). The prevalence of hyperthyroidism in women is ~1%, and in men, it is approximately one-tenth of that,

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according to The National Health and Nutrition Examination Survey data from the USA, and it seems to be more prevalent after puberty (Mandel *et al.* 2011). Therefore, one can hypothesize that either sexual chromosomes and/or gonadal hormones are able to profoundly affect thyroid physiology and pathophysiology. In the present review, we focus on the known effects of estrogen on the thyroid gland and the possible involvement of reactive oxygen species (ROS) in the marked sexual dimorphism found in thyroid diseases, with women being most affected.

Review

Estrogen receptors in the thyroid gland

For decades, the thyroid gland has been known to be a target of estrogen (Molteni *et al.* 1979, Hampl *et al.* 1985, Schaefer *et al.* 1986). Nuclear estrogen receptors ERα and ERβ are ligand-regulated transcription factors and are classified as class I members of the superfamily of steroid/thyroid hormone nuclear receptors (Green *et al.* 1986, Mangelsdorf *et al.* 1995, Couse & Korach 1999, Cui *et al.* 2013). As such, binding of estrogen to ERs leads to the translocation of the hormone–receptor complex into the nucleus and interaction with DNA, specifically with the estrogen-responsive elements present in the promoter regions of target genes (Marino *et al.* 2006). In addition to nuclear ERs, which mediate the majority of the known effects of estrogens, some rapid effects of estrogens are transduced by ERs present at the cell membrane that activate intracellular signaling cascades (Pedram *et al.* 2006, Levin 2009, Cui *et al.* 2013). Membrane-localized ERα and ERβ activate Gαq and Gαs, leading to the activation of phospholipase C and adenyl cyclase respectively in addition to activating ERK (Razandi *et al.* 1999). Additionally, ERα directly binds to Gαi and Gβγ (Kumar *et al.* 2007). However, some rapid effects of estrogens are transduced by ER-independent pathways (Santen *et al.* 2009, Yue *et al.* 2010, Haas *et al.* 2012, Richardson *et al.* 2012). Another cell-surface receptor for estrogen has been identified as the orphan G protein-coupled receptor GPR30. Nevertheless, because GPR30 could also transduce the effects of compounds other than estrogens, such as chemokines (Catusse *et al.* 2010), the concept that GPR30 is an ER remains to be confirmed (Pedram *et al.* 2006, Levin 2009, Cui *et al.* 2013). Anyway, little is really known about the participation of GPR30 in effects of estrogens on the thyroid gland, although Vivacqua *et al.* (2006) have shown that GPR30 mediates the proliferative effect of estrogen in the thyroid cancer cell lines WRO and FRO because knockdown of GPR30 in these cells reduces their estrogen-induced proliferation (Vivacqua *et al.* 2006).

ER-X, another plasma membrane ER, seems to play a role during development (Toran-Allerand *et al.* 2002, Cui *et al.* 2013), but the function of this receptor in postnatal life is still elusive. An additional presumed cell membrane ER is Gq-mER, which binds Gq (Qiu *et al.* 2003) and seems to mediate estrogen's effects on the hypothalamic control of body temperature and energy homeostasis (Qiu *et al.* 2006, Roepke *et al.* 2010).

ERα and ERβ are expressed in the thyroid of both female and male rats, though female thyroid expresses higher ER levels than male thyroid (Stanley *et al.* 2010). Estrogen upregulates its own receptor in the thyroid of female and male rats, whereas gonadectomy reduces ER levels in the thyroid of both male and female rats (Banu *et al.* 2002). Vaiman *et al.* (2010) have shown that ERβ is detectable in benign and malignant lesions of human thyroid and also in normal thyroid. However, they did not detect ERα in 296 thyroid tissue samples (150 goiters, 90 papillary carcinomas, 19 follicular adenomas, 15 Hurthle cell adenomas, six Hashimoto's thyroiditis, five anaplastic carcinomas, four medullary carcinomas, four follicular carcinomas, two Hurthle cell carcinomas, and one squamous cell carcinoma of the thyroid) by immunohistochemical analysis (Vaiman *et al.* 2010). Furthermore, Ceresini *et al.* (2006) have shown that nuclear ERβ immunoreactivity is detectable not only in thyroid follicular cells but also in endothelial cells in both multinodular goiter and papillary thyroid carcinoma. Thus, in the human thyroid, ERβ seems to be the more relevant ER isoform under physiological conditions (Ceresini *et al.* 2006).

Thyroid cancer is the most common endocrine malignancy. Thyroid neoplasms can be classified as differentiated, which includes papillary and follicular carcinomas; undifferentiated (anaplastic carcinoma); tumors of parafollicular C cells (medullary carcinoma); and poorly differentiated thyroid carcinoma (Salvatore *et al.* 2011). Molteni *et al.* (1979) showed that thyroid adenocarcinomas have a high estradiol-binding capacity. The presence of ER was suggested to be higher in neoplastic lesions than in normal tissues (Jaklic *et al.* 1995), and it apparently decreases with the degree of malignancy (Hiasa *et al.* 1993, Tavangar *et al.* 2007), although many authors have not observed this difference (Métayé *et al.* 1993, Yane *et al.* 1994). The relationship between ERα and ERβ seems to differ among different thyroid samples, with lower ERβ-to-ERα mRNA ratios in

follicular adenomas compared with normal tissues (Egawa *et al.* 2001), indicating that the balance between the two ERs might influence thyroid pathogenesis. Zeng *et al.* (2007) have studied the effect of estradiol on proliferation of thyroid carcinoma cell lines (KAT5, papillary thyroid carcinoma cells and FRO, follicular thyroid carcinoma cells) (Zeng *et al.* 2007). These authors found that estradiol increased the cell proliferation rate and that this effect was positively correlated with the level of ERα but negatively with that of ERβ. Consistent with these results, Chen *et al.* (2008*b*) demonstrated that an ERα agonist stimulates thyroid cancer cell proliferation, whereas an ERβ agonist reduces it (Chen *et al.* 2008*b*). Thus, not only the levels of ER but also the balance between the expression of ERα and ERβ might affect the action of estrogen on the thyroid gland.

Thyroid function regulation by estrogen

Estrogen profoundly affects thyroid function, either directly or by regulating the hypothalamus–pituitary–thyroid axis. Estrogen increases the thyrotropin (TSH) response to thyrotropin-releasing hormone stimulation in ovariectomized rats (Chen & Walfish 1978). Additionally, the stimulatory effect of estradiol on thyroid radioiodine uptake in ovariectomized and hypophysectomized rats (Boccabella & Alger 1964) supports the hypothesis of a direct action of estrogen on the thyroid. Because ERs are expressed in both human and rat thyroid glands (Banu *et al.* 2002, Arain *et al.* 2003) and estradiol increases the proliferation rate of the FRTL-5 rat thyroid cell line independent of TSH (Furlanetto *et al.* 1999), it is clear that this hormone also regulates thyrocytes through a direct action.

Iodide transport is a fundamental step in thyroid hormone synthesis, which is catalyzed by the Na^+/I^- symporter (NIS; Dai *et al.* 1996, Smanik *et al.* 1996, Eskandari *et al.* 1997, Dohan *et al.* 2003). In FRTL-5 cells, estrogen reduces *Nis* (*Slc5a5*) gene expression in the presence of TSH (Furlanetto *et al.* 1999) and decreases cell iodide uptake in either the presence or the absence of TSH (Furlanetto *et al.* 2001). However, treatment of both ovariectomized adult and intact prepubertal rats with estrogen significantly increases thyroid iodide uptake (Lima *et al.* 2006), indicating that *in vivo* but not *in vitro* estrogen has a stimulatory effect on NIS. The difference between the two models might be related to the presence of thyroid stromal cells *in vivo* because these cells express ERα and Eβ and could play a role in the thyroid response to estradiol (Gantus *et al.* 2011).

Thyroperoxidase (TPO) plays a key role in the biosynthesis of thyroid hormones. TPO is a heme-containing glycoprotein bound to the apical membrane of thyroid cells, which in the presence of H_2O_2 catalyzes iodide oxidation, iodination of tyrosyl residues in the thyroglobulin (Tg) molecule, and coupling of iodo-tyrosines, leading to the formation of thyroid hormones (Ruf & Carayon 2006). In addition to the fundamental role in thyroid hormone biosynthesis, TPO is also the main thyroid autoantigen, and autoimmune thyroid disease is more frequent in women (Braverman & Utiger 2000, Bresson *et al.* 2005). The administration of estradiol to both intact and ovariectomized rats increases TPO activity (Lima *et al.* 2006), indicating that estrogen stimulates not only thyroid iodide uptake but also iodide organification.

In summary estrogen has a general stimulatory effect on thyroid function, at least in rats, increasing iodide uptake and TPO activity, which would be expected to increase thyroid hormone biosynthesis. However, the effect of estrogen and the consequences of ovariectomy on serum thyroid hormone and TSH concentrations are controversial and seem to depend on the model used (Böttner & Wuttke 2005, Seidlová-Wuttke *et al.* 2005, Sosić-Jurjević *et al.* 2005, Lima *et al.* 2006, Marassi *et al.* 2007, Abdel-Dayem & Elgendy 2009, Pantaleão *et al.* 2010). In addition, estrogen increases thyroxine-binding globulin (TBG) production both *in vivo* in women (Dowling *et al.* 1956, Robbins & Nelson 1958, Doe *et al.* 1967) and *in vitro* by hepatocytes of Rhesus monkeys (Glinoer *et al.* 1977). Thus, it is difficult to make a clear distinction between increases in serum thyroid hormone levels induced by estrogen directly, by increasing thyroid function, or indirectly, through an increase in TBG levels.

NADPH oxidases and thyroid physiology

Thyrocytes produce large amounts of H_2O_2 during their lives. At the apical membrane of thyrocytes, H_2O_2 acts as a TPO cosubstrate in thyroid hormone biosynthesis and is produced by calcium-dependent NADPH oxidases (NOX), namely dual oxidase (DUOX) (Dupuy *et al.* 1999, De Deken *et al.* 2000, Ameziane-El-Hassani *et al.* 2005). The NOX/DUOX family is composed of seven members, NOX1–NOX5 and DUOX1/2, which are differentially expressed among tissues (Weyemi & Dupuy 2012). The biological roles of NOXs are quite diverse, but the first physiological function described was related to the immune response, as NOX2 is activated during the neutrophil respiratory burst. However, other processes, such as cellular proliferation, apoptosis inhibition,

calcium release, and hormone biosynthesis, are well documented (Berdad & Krause 2007). Thyrocytes express both DUOX1 and DUOX2 at the apical membrane, but the source of H_2O_2 that sustains production of thyroid hormones (TH) seems to be DUOX2. The evidence that supports this idea is that mutations in *Duox2*, but not in *Duox1*, are associated with congenital hypothyroidism, and that mice deficient in *Duox2*, but not *Duox1*, are hypothyroid (Johnson *et al*. 2007, Donkó *et al*. 2010, Grasberger 2010). Both DUOX enzymes need the presence of their corresponding maturation factor, called DUOXA, to exit the endoplasmic reticulum and reach the apical plasma membrane (Grasberger & Refetoff 2006), where these proteins form stable complexes at the cell surface that are essential for DUOX activity (Morand *et al*. 2009). NOX4 is also present in thyrocytes, but it is localized intracellularly, specifically in endoplasmic reticulum and the nuclear membrane, and does not seem to be involved in TH biosynthesis (Weyemi *et al*. 2010). In contrast to DUOX enzymes, which need the presence of DUOXA for their activity, NOX4 seems to be more active in the presence of p22phox or possibly Poldip2 (Lyle *et al*. 2009), but this enzyme is constitutively active. NOX4 is detected in the nuclear or perinuclear regions of the cells and most probably acts as an intracellular signaling oxidase (Leto *et al*. 2009) (Fig. 1).

ROS and thyroid pathophysiology

As H_2O_2 is a ROS and might thus react with cellular components such as lipids, proteins, and DNA, some authors argue that H_2O_2 generated for TH biosynthesis could be toxic to thyrocytes. Maier *et al*. (2006) showed that the rat thyroid gland has a high level of DNA oxidative damage in comparison with other tissues, such as liver, spleen, and lung, indicating that the high frequency of somatic mutations and tumor initiation found in this organ is due to the oxidative environment that thyroid cells are subjected to during their long lives (Maier *et al*. 2006). In addition, carcinogenic effects of H_2O_2 on thyrocytes have been clearly demonstrated in two different *in vitro* studies. When a rat thyroid cell
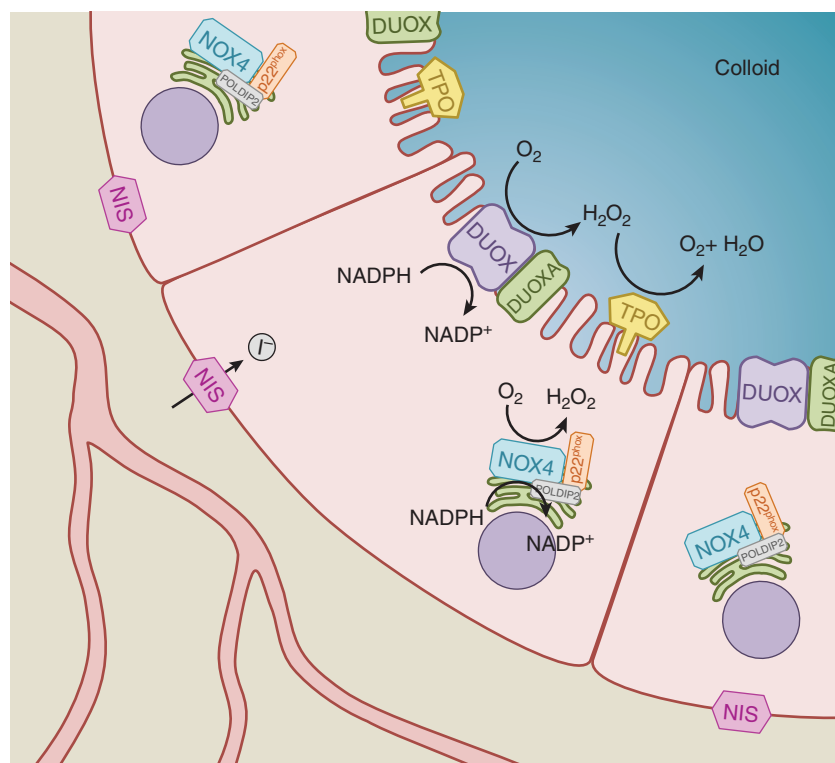

Figure 1

NADPH oxidase (NOX) enzymes expressed in the thyroid gland. DUOX-derived H_2O_2 is used as a cosubstrate by thyroperoxidase (TPO) for thyroid hormone biosynthesis at the apical membrane of thyrocytes. NOX4 produces ROS in intracellular compartments. NAPDH, NAD; NADP+, NADH; DUOX, dual oxidase; DUOXA, dual oxidase maturation factor; NIS, sodium/iodide symporter; POLDIP2, polymerase delta-interacting protein 2; ROS, reactive oxygen species.

line (PCCL3) was incubated with nonlethal H_2O_2 concentrations, the numbers of single- and double-strand breaks in DNA increased, as did the phosphorylation of histone H2AX, a marker of double-strand breaks (Driessens *et al.* 2009). Moreover, H_2O_2 exposure induced RET/PTC rearrangement formation in a human thyroid cell line, which was abolished when catalase was added to the incubation medium (Ameziane-El-Hassani *et al.* 2010).

In fact, any disturbance in ROS generation or consumption will promote ROS accumulation that can contribute to thyroid dysfunction. In this context, it is important to note that the site of ROS formation is closely linked to the type and extent of the damage. This can be explained by the short half-life of ROS molecules due to their high reactivity and the presence of antioxidant mechanisms in the whole cell (Block & Gorin 2012). DUOX enzymes are localized at the apical membrane of thyrocytes, physically and functionally interacting with TPO, and creating a producer–consumer unit that restricts the amount of H_2O_2 released into the lumen of follicular cells (Fortunato *et al.* 2010, Song *et al.* 2010). Furthermore, glutathione peroxidase 3 (GPx3), also located in the apical cell surface, and other intracellular antioxidant enzymes and molecules, such as GPx1, superoxide dismutase, catalase, and peroxiredoxins, most probably destroy H_2O_2 before it can react with DNA in the nucleus (Schweizer *et al.* 2008). On the other hand, NOX4 is an intracytoplasmatic ROS-generating enzyme, possibly located at the endoplasmic reticulum, and nuclear membrane where it was recently found in other cell types (Chen *et al.* 2008*a*,*b*, Weyemi *et al.* 2010, Spencer *et al.* 2011). Thus, H_2O_2 released by NOX4 can be linked to genomic instability.

We have previously compared DUOX and TPO activity in hypofunctioning thyroid lesions, and we found a negative correlation between these enzyme activities (Ginabreda *et al.* 2008). H_2O_2 can oxidize many proteins, changing their functions. Therefore, we proposed the hypothesis that H_2O_2 produced by DUOX could oxidize TPO, changing its activity, once both proteins are colocalized at the plasma membrane. Utilizing a heterologous system, we showed that indeed, H_2O_2 produced by DUOX reacts with TPO, decreasing its activity (Fortunato *et al.* 2010).

It has been suggested that ROS can contribute to autoimmune thyroid diseases, such as Hashimoto's thyroiditis and Graves' disease (Burek & Rose 2008). Tg and TPO are major autoantigens involved in autoimmune diseases (McIntosh & Weetman 1997). Tg fragmentation can occur during iodination and coupling of tyrosine residues in TH biosynthesis, forming immunoreactive peptides. Interestingly, it seems that Tg cleavage is a reaction that involves H_2O_2 produced in the apical membrane of thyrocytes (Duthoit *et al.* 2000, Raad *et al.* 2013). Raad *et al.* (2013) studied the effects of the main cytokines involved in Hashimoto's thyroiditis and Graves' disease, interferon γ (IFN-γ) and IL4/IL13 respectively, on DUOX expression and activity. IL4 and IL13 increase DUOX2 and DUOXA2 expression and calcium-dependent H_2O_2 generation, and IFN-γ treatment inhibits DUOX gene expression and blocks induction of Th2 by DUOX2/DUOXA2 (Raad *et al.* 2013). Taken together, the findings mentioned above indicate that DUOX enzymes could be involved in thyroid autoimmune pathophysiology.

Some studies have shown increases in peroxide content, lipid peroxidation, and the activity of the antioxidant enzyme catalase and GPx during goitrogenesis in rats (Poncin *et al.* 2008, Thomasz *et al.* 2010). The source of ROS during goitrogenesis has not been demonstrated, but NOX4 is a good candidate, according to recent findings. In the thyroid, NOX4 is present in activated, tall columnar cells and absent in quiescent, flat cells, indicating a role in thyrocyte functional activity. It is important to note that NOX4 and p22phox mRNA levels, as well as intracellular ROS generation, increase in a dose-dependent manner when human thyrocytes are incubated with TSH, indicating that ROS produced by NOX4 can act as second messengers in TSH signaling, as they do in other models (Weyemi *et al.* 2010).

A pro-oxidative environment can be involved in all steps related to carcinogenesis, such as initiation, promotion, and progression (Scandalios 2005). The main function of NOX is ROS production. Therefore, changes in NOX expression and/or activity could be related to carcinogenesis. ROS can directly interact with cellular macromolecules, such as nucleic acids leading to nucleotide oxidation and single- and double-strand breaks, thereby promoting genomic instability. However, a wide range of signaling pathways are redox-sensitive, so an increase in ROS can modulate autonomous cell growth and immortality (Block & Gorin 2012). Some previous studies evaluated DUOX expression and activity in samples of thyroid carcinomas, but no significant differences in the activity or expression of these enzymes were detected between normal and cancerous tissues (Lacroix *et al.* 2001, Ginabreda *et al.* 2008). In this context, NOX4 has gained attention in thyroid pathophysiology because its expression is higher in thyroid tumors in comparison with normal tissue. In fact, normal thyroid cells overexpressing an activated Ras oncogene

have increased NOX4 expression and activity, which is implicated in the stimulation of the DNA replication rate and DNA damage, leading to cellular senescence (Weyemi et al. 2012).

Estrogen effects on thyroid redox balance

A large body of evidence correlates different thyroid dysfunctions with redox imbalance, generally due to NOX activation. Our group proposed that the higher prevalence of thyroid diseases in women could be, at least

in part, due to sex-related differences in the thyroid redox environment. Utilizing rats as a model, we have shown higher H_2O_2 production and NOX4 expression in the thyroid of adult female rats in comparison with their male counterparts, but not in prepubertal animals, in which serum estradiol concentration is not significantly different between sexes (Fortunato et al. 2013). Weyemi et al. (2010) reported that in normal human thyroid tissue, NOX4 immunostaining is intracytoplasmic (Weyemi et al. 2010). However, although we confirmed that NOX4 immunostaining in the thyroids of both male
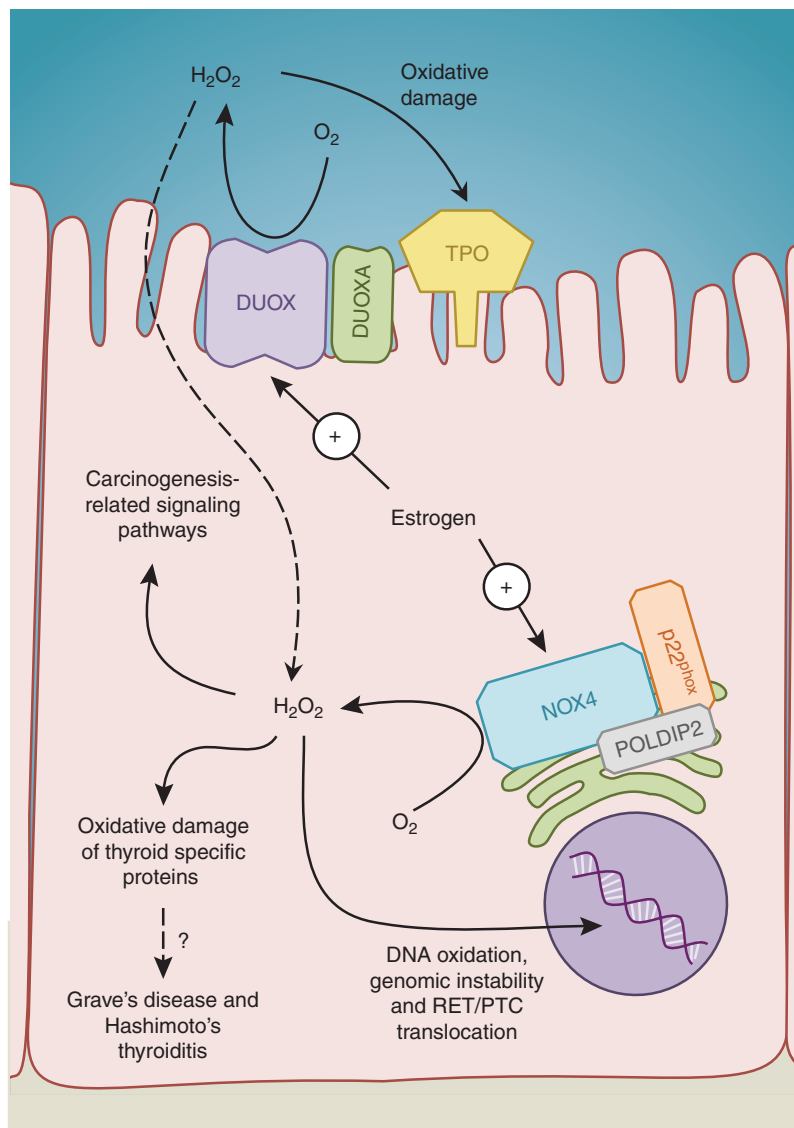
Figure 2

A hypothesis for thyroid dysfunctions related to estrogen exposure through NADPH oxidase activation. Estrogen signaling activates the production of ROS by NOX4 and DUOX in the thyroid tissue and may contribute to the establishment of an environment prone to the development of cancer and/or autoimmune disorders. DUOX, dual oxidase; DUOXA, dual oxidase maturation factor; NOX4, NAPDH oxidase 4; POLDIP2, polymerase delta-interacting protein 2; ROS, reactive oxygen species; TPO, thyroperoxidase.

and female rats is intracytoplasmic, we have also detected NOX4 immunostaining at the plasma membrane of thyrocytes. Furthermore, we have found higher NOX4 expression in the thyroid of female rats in the proestrus phase of the estrous cycle (characterized by an estrogen peak) and higher NOX4 expression and H_2O_2 production in PCCL3 cells treated with 17β-estradiol, indicating a role for estrogen in this process. DUOX2 was not different when we compared the thyroids from male and female rats, but estrogen also increased DUOX2 expression both *in vivo* and *in vitro* (unpublished data). In addition, catalase expression and activity, together with levels of thiol groups, were lower in adult female thyroid (Fortunato *et al.* 2013). Taken together, these results show sex-related differences in thyroid redox balance, with increased ROS production and decreased antioxidant defense in female thyroid (Fig. 2).

Although we have demonstrated the stimulatory effect of estrogen on NOX4 expression and activity, some questions remain to be elucidated. How could NOX4 be involved in estrogen-related thyroid dysfunction? First of all, because NOX4 is located intracellularly, the H_2O_2 produced by the enzyme could cause genomic instability through its reaction with cellular DNA. Cells overexpressing activated Ras oncogene have increased NOX4 expression and activity, which is responsible for the stimulation of DNA replication and DNA damage, leading to cellular senescence (Weyemi *et al.* 2012). In contrast, NOX4 and DUOX2 seem to regulate cell cycle entry via the p53-dependent pathway. PDGF-induced proliferation in HS68 fibroblast cells is abolished after NOX4 or DUOX2 knockdown, due to reduced ERK1 phosphorylation and increased levels of p53 and the cell cycle inhibitor protein p21 (Salmeen *et al.* 2010). It is important to note that estradiol is able to induce proliferation in normal and cancerous thyroid cells through ERK1/2 phosphorylation, so H_2O_2 produced by NOX4 could be involved in this signaling pathway (Manole *et al.* 2001, Kumar *et al.* 2010). Vascular endothelial growth factor (VEGF) is a proangiogenic factor with a central role in the function, development, and growth of blood vessels. Thyroid VEGF is upregulated by estrogen, and thyroid weight, vascular area, and VEGF protein expression are lower in ovariectomized rats in comparison with sham-operated rats and ovariectomized rats treated with 17β-estradiol (de Araujo *et al.* 2010). Interestingly, in PCCL3 cells, an increase in intracellular ROS, elicited by iodide deprivation, induces HIF-1α and VEGF protein expression, but concomitant treatment with the antioxidant *N*-acetyl-cysteine abolishes the effects of iodide deprivation (Gérard *et al.*

2009). Thus, it is tempting to speculate that NOX4 could also be involved in the regulation of thyroid VEGF expression induced by estradiol and iodide deprivation.

Summary and conclusions

In this review, we propose that ROS could be involved in the sexual dimorphism found in thyroid dysfunctions. Future studies are necessary to evaluate the involvement of NOX4-generated ROS in the estrogen-signaling pathway in thyrocytes. Elucidating this issue is crucial to improving our knowledge of the mechanisms involved in thyroid pathophysiology and will allow us to determine whether NOXs are potential therapeutic targets for thyroid dysfunctions.

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