Extracellular superoxide dismutase is a thyroid differentiation marker down-regulated in cancer

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Abstract

Reactive oxygen species, specifically hydrogen peroxide (H₂O₂), have a significant role in hormone production in thyroid tissue. Although recent studies have demonstrated that dual oxidases are responsible for the H₂O₂ synthesis needed in thyroid hormone production, our data suggest a pivotal role for superoxide dismutase 3 (SOD3) as a major H₂O₂-producing enzyme. According to our results, *Sod3* is highly expressed in normal thyroid, and becomes even more abundant in rat goiter models. We showed TSH-stimulated expression of *Sod3* via phospholipase C–Ca²⁺ and cAMP–protein kinase A, a pathway that might be disrupted in thyroid cancer. In line with this finding, we demonstrated an oncogene-dependent decrease in *Sod3* mRNA expression synthesis in thyroid cancer cell models that corresponded to a similar decrease in clinical patient samples, suggesting that SOD3 could be used as a differentiation marker in thyroid cancer. Finally, the functional analysis in thyroid models indicated a moderate role for SOD3 in regulating normal thyroid cell proliferation being in line with our previous observations.

Endocrine-Related Cancer (2010) 17 785–796

Introduction

The G protein-coupled receptor (GPCR) activating G α_s and G α_q proteins, such as TSH receptor (TSH-R), mediate the cellular responses to various extracellular stimuli via two main signal transduction pathways: the cAMP pathway and the phosphatidylinositol pathway that is involved in calcium signaling (Pierce *et al.* 2002). In thyroid, the TSH-R cAMP pathway is responsible for the thyroid cell proliferation, differentiation, and secretion, whereas the TSH-R phosphatidylinositol cascade controls thyroid hormone tri-iodothyronine (T₃) and thyroxine (T₄) synthesis. The TSH is crucially

important for normal thyroid function, and therefore, deregulation caused by, e.g. TSH-R mutations or external factors, affecting TSH signaling interferes with the cellular homeostasis in thyroid (Ludgate & Vassart 1995). A low iodide uptake leads to reduced thyroid hormone T₃ and T₄ production (hypothyroidism), which is compensated by increased TSH synthesis and subsequent activation of downstream signaling leading to cell proliferation and benign thyroid enlargement known as goiter (Dumont *et al.* 1989). Similarly, activating mutations of the TSH-R cause autonomous hyperfunctioning thyroid adenomas

and nonautoimmune toxic thyroid hyperplasia (Duprez et al. 1994).

TSH signaling has been shown to increase the synthesis of hydrogen peroxide (H_2O_2), which is used as an extracellular substrate by thyroperoxidase in thyroglobulin iodination and thyroid hormone synthesis (Corvilain et al. 1991). On the other hand, intracellular H_2O_2 contributes to mitogen signaling and consequent cellular proliferation in *in vitro* and *in vivo* models (Rao & Berk 1992, Guyton et al. 1996, Kuroki et al. 1996) by regulating the activation of protein tyrosine phosphatases and tyrosine kinase receptors (Rao 1997, Wang et al. 2000, Saito et al. 2002, Aslan & Ozben 2003, Konishi et al. 2004, Mehdi et al. 2005). We have previously demonstrated the ability of extracellular superoxide dismutase (SOD3), which is one of the main H_2O_2 -producing enzymes (Marklund 1984, Karlsson & Marklund 1987), to promote cell proliferation *in vivo* by activating Ras–Erk mitogen signaling, transcription factor upregulation and growth factor expression suggesting growth stimulatory role in tissues (Laurila et al. 2009).

In the current study, we investigated the signal transduction pathways and synthesis of *Sod3* in different experimental models to determine the function of SOD3-derived H_2O_2 in thyroid. We identified a novel TSH-R-mediated signaling pathway activating *Sod3* synthesis and decreased *SOD3* expression in thyroid malignancies.

Materials and methods

Animals

Male 4–5 weeks old Sprague–Dawley rats (University of Turku, Turku, Finland) were given 0.25% propylthiouracil (PTU; Sigma) *ad libitum* in drinking water for 2 weeks, killed, and tissues were collected for expression analysis. As controls for histological staining, we used nontreated rat thyroids, and for quantitative reverse transcription (qRT)-PCR analysis, a thyroid total RNA pool from four animals. Experimental procedures were done according to the European Union and the University of Turku guidelines (permission 2009/2502).

Histological analysis

Thyroid tissues were embedded in optimal cutting temperature compound, OCT (Tissue-Tek, Torrance, CA, USA). Ten-micrometer sections were stained with hematoxylin/eosin (Sigma) according to the standard protocols.

Cell lines and reagents

Rat PC Cl3 cells were grown in Ham's F-12 medium, Coon's modified (Sigma) supplemented with 5% calf serum (Life Technologies, Inc.) and 10 nM TSH, 10 nM hydrocortisone, 100 nM insulin, 5 µg/ml transferrin, 5 nM somatostatin, and 20 µg/ml glycyl-histidyl-lysine. PC RET/PTC1 and PC E1A stable cell lines, kindly provided by Prof. Alfredo Fusco, were grown in F-12 medium supplemented with 5% calf serum without hormones (Fusco et al. 1987, Santoro et al. 1993). PC-inducible RET/PTC1 cells were grown in the same medium as PC Cl3 cell administered with 1 µg/ml doxycyclin (Sigma). COS-7 cells were grown in DMEM (Sigma) supplemented with 10% fetal bovine serum (Life Technologies, Inc). Transfections (1 µg of plasmid) were done with Eugene6 (Roche Applied Science) for PC Cl3 cells, and with Polyfect (Sigma) for COS-7 cells. Human *SOD3* cDNA, kindly provided by Prof. Stefan L Marklund from the University of Umeå, Sweden, was cloned to pcDNA3 vector (Life Technologies). The plasmids pCEF- $G\alpha_s$ and pCEF- $G\alpha_q$, encoding constitutive active form (QL) of $G\alpha_s$ and $G\alpha_q$ and pCEF-protein kinase A (PKA), encoding the active catalytic subunit of PKA, were kindly provided by Dr Silvio Gutkind from NIH, MD, USA. PC Cl3 cells were incubated with 10 nM TSH (Sigma), 40 µM forskolin (Sigma), 1 µM *N*(2-((*p*-bromocinnamyl)amino)ethyl)-5-isoquinolinesulfonamide (H89; Calbiochem, San Diego, CA, USA), or 100 nM thapsigargin (Research Biochemicals International, Natick, MA, USA). SOD1 protein was purchased from Sigma.

PCRs

Total RNA was isolated from tissues pooled from four animals or cells using Tri-reagent (Sigma). The first-strand synthesis was done with QuantiTect RT (Qiagen) and qPCR with SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). Primers were rat *Sod1*-for GTC GTC TCC TTG CTT TTT GC and rat *Sod1*-rev TGC TCG CCT TCA GTT AAT CC; rat *Sod2*-for AAG GAG CAA GGT CGC TTA CA and rat *Sod2*-rev TGG CTA ACA TTC TCC CAG TTG; rat *Sod3*-for GAC CTG GAG ATC TGG ATG GA and rat *Sod3*-rev GTG GTT GGA GGT GTT CTG CT; human *SOD3*-for CTT CGC CTC TGC TGA AGT CT and human *SOD3*-rev GGG TGT TTC GGT ACA AAT GG; human β *ACTIN*-for TGC GTG ACA TTA AGG AGA AG and human β *ACTIN*-rev GCT CGT AGC TCT TCT CCA; rat β *Actin*-for TCG TGC GTG ACT TAA GGA G and rat β *Actin*-rev GTC AGG CAG CTC GTA GCT CT. Reactions were done in 60 °C annealing temperature.

Real-time PCR tissue samples

The qRT-PCR expression analyses from human tissues were done to normal thyroid ($n=7$), papilloma thyroid cancer (PTC, $n=9$), and anaplastic thyroid cancer (ATC, $n=6$) patient samples. The PTC patient samples had 30% genetic variation (six wild-type Braf, one Braf V600E, and two RET/PTC1 rearrangements). Five ATC patient samples had Braf V600E mutation and one had wild-type Braf. The analysis of mRNA expression was done from pooled samples.

DNA array tissue samples

The diagnoses of the hyperthyroidic patients were based on the long-standing hyperthyroidism, absence of thyroid-stimulating antibodies in the serum and in some cases, family history and confirmed by DNA sequencing of the TSH-R. The details of the patient material were described earlier (Hebrant *et al.* 2009). Shortly, thyroid samples were obtained from five familial nonautoimmune hyperthyroidism (FNAH) French patients (from Nancy (FNAH1 and FNAH4), Reims (FNAH2), Lyon (FNAH3), and Angers (FNAH5)), autonomous adenoma (AA) samples were obtained and pooled from four different patients as published previously (Hebrant *et al.* 2009).

In order to compare FNAH and AA, we used the reference pool of 23 normal thyroid tissues adjacent to different pathologies. The human HEEBO (human exonic evidence-based oligonucleotide) 70-mer oligonucleotide microarrays, containing ~48 500 probes (representing exonic sequences, alternatively spliced exons, expressed sequence tags (ESTs), and controls) were used for the array analysis. All hybridizations were performed in duplicates with dye swap. For the PTC and ATC microarray, 20 PTCs and 9 ATCs were hybridized on Affymetrix slides (HGU133 plus 2.0), according to Affymetrix protocol. The ethics committees of the institutions have approved protocols according to Declaration of Helsinki.

Calcium uptake assay

Intracellular Ca^{2+} concentrations were measured in PC Cl3 cells. Briefly, cells were detached from tissue culture plates with PBS containing 0.5 mmol/l EDTA, washed once with HEPES-buffered medium (HBM; 137 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l CaCl_2 , 0.44 mmol/l KH_2PO_4 , 4.2 mmol/l NaHCO_3 , 10 mmol/l glucose, 20 mmol/l HEPES, and 1.2 mmol/l MgCl_2 , pH 7.4), and then loaded with 2 $\mu\text{mol/l}$ fura-2 acetoxymethyl ester for 20 min at 37 °C. After loading, the cells were diluted with HBM without CaCl_2 to a

final concentration of 0.3 mmol/l CaCl_2 and stored at room temperature until use. For fluorescence recordings, an appropriate volume of the cell suspension was spun down, washed once in HBM, resuspended in HBM, and placed in a thermostated (37 °C) cuvette with magnetic stirring in a Hitachi F-2000 fluorescence spectrophotometer. The fluorescence was monitored at 340 nm (excitation) and 505 nm (emission). Experiments were calibrated with 60 μg digitonin/ml (F_{max}) and 10 mmol/l EGTA (F_{min}). The intracellular $[\text{Ca}^{2+}]$ was calculated from the fluorescence (F) using the equation $[\text{Ca}^{2+}] = (F - F_{\text{min}}) / (F_{\text{max}} - F) \times 224 \text{ nmol/l}$ (K_d for fura-2), in which the extracellular fura-2 fluorescence was subtracted from F values.

RNA interference

Short interfering *Sod3* or *Gapdh* OnTargetplus SMART pool oligos (Dharmacon, Lafayette, CO, USA) were transfected into PC Cl3 cells according to the manufacturer's protocol. Shortly, 20 $\mu\text{mol/l}$ of siSOD3 or siGAPDH control oligo were resuspended to total volume 100 μl transfection buffer, mixed with equal volume of Optimem (Life Technologies), and incubated 5 min at room temperature. The optimization of small interference RNA (siRNA) quantity used in the study was based on the siGAPDH silencing effect using KDAlert GAPDH assay kit (Ambion, Austin, TX, USA). To prepare the transfection solution, 5 μl Dharmafect 4 reagent was added to 190 μl of Optimem and incubated 5 min at room temperature. Oligo suspension and transfection reagent were combined, incubated 20 min at room temperature, and added to the cells for 48 h.

Bromodeoxyuridine analysis

Cells were serum starved for 16 h and TSH stimulated for 6 h before the addition of 10 mM bromodeoxyuridine (BrdU; Roche) for 2 h. Subsequently, cells were fixed in 3% paraformaldehyde (Sigma) and permeabilized with 0.2% Triton X-100 (Sigma). BrdU-positive cells were revealed with FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Cell nuclei were identified by Hoechst (Sigma) staining.

Chemiluminescence assay for O_2^- detection

Extracellular O_2^- release was detected by chemiluminescence using the SOD-inhibitable Diogenes reagent (National Diagnostics, Atlanta, GA, USA) on cells resuspended in Hanks' Balanced Salt Solution (HBSS)/10 mmol/l glucose. One $\mu\text{mol/l}$ ionomycin was added to the cells to stimulate the dual oxidase (DUOX)

activity. Measurements were taken with 22 s intervals for 10 min in 96-well white plates (1.5×10^5 cells/250- μ l well) at 37 °C using a Microplate Luminometer. Chemiluminescence curves were analyzed, and peak values of the curves were presented in Relative Light Units/s.

Fluorimetric assay for H₂O₂ detection

The H₂O₂ release was quantified 48 h after cell transfection by the homovanillic acid-based fluorimetric assay (Benard & Brault 1971) and by 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; Invitrogen). For homovanillic assay, the cells were incubated in Krebs–Ringer–HEPES medium pH 7.4 containing 0.1 μ g/ml HRP type II, 440 μ mol/l homovanillic acid, and 1 μ mol/l ionomycin during 2 h 30 min at 37 °C. Fluorescence intensity of oxidized homovanillic acid was measured at 425 nm after excitation at 315 nm. Results are reported as ng of H₂O₂/well representing the quantity of H₂O₂ accumulated during 2 h 30 min in each well (six-well plates). For the H₂DCFDA assay, the cells were grown in 96-well dishes, the reagent (1 mg/ml) was added to the cells for 15 min, and the fluorescence was measured with A₃₈₀/A₄₃₅.

Statistical analysis

Statistical analysis was done using *t*-test for means $\pm$ s.d.

Results

SOD3 is abundantly expressed in rat thyroid tissue and stimulated by TSH

SOD3 is an extracellular H₂O₂-producing enzyme expressed in various tissues (Marklund 1984, Laukkanen et al. 2000) protecting cell surface structures against deleterious effects of superoxide. The local administration of *Sod3*, causing twofold increased total SOD activity in tissues, or i.v. infusion of the native C-form of the isolated recombinant enzyme with affinity to cell surface structures results in significant attenuation of tissue damages and markedly increased tissue recovery (Hatori et al. 1992, Sjoquist & Marklund 1992, Laukkanen et al. 2002), suggesting that even minor changes in tissue SOD3 concentration have a significant impact on the redox balance.

Comparison of SOD isoenzyme mRNA expressions from normal rat tissues (Fig. 1A) demonstrated thyroid as one of the major expression sites for *Sod3*. Accordingly, mRNA extraction from normal rat tissues showed 2.5-fold higher expression levels in thyroid as compared with heart, corresponding to a previously

reported threefold and fivefold differences in thyroid versus heart protein level in rabbit and human respectively (Marklund 1984). The expression in the rat thyroid approached the levels detected in the aorta, which is one of the main expression sites for SOD3 (Laukkanen et al. 2002), indicating participation to ROS balance and signal transduction in thyroid tissue. The expression of *Sod1* and *Sod2* was in line with previously reported enzyme activities in the rat tissues (Marklund 1984).

Because SOD3 activation increases H₂O₂ synthesis that has been shown to be up-regulated in response to TSH, we used a rat *in vivo* model to investigate the expression status of SOD3 in benign thyroid enlargement caused by increased TSH secretion and to compare the animal data to human clinical samples. PTU treatment is known to cause decreased T₃ production with subsequent increase in TSH, thyroid enlargement, and activated thyroid function (Levey 1963, al-Alawi et al. 1995, Laezza et al. 2006); we therefore treated four rats with PTU *ad libitum* and killed the animals after 2 weeks to isolate the thyroids. As shown in Fig. 1, PTU-treated thyroids had on average fourfold increase in wet-weight (animals 4–7) as compared with normal untreated animals (rat 1–3; Fig. 1B), suggesting a typical goiter formation, which was further supported by hematoxylin–eosin staining demonstrating increased cellular division and loss of follicular lumen (Fig. 1C and D). The variation seen in thyroid growth is in line with previous reports (Levey 1963) due to *ad libitum* administration of PTU. To study the expression of *Sod3* mRNA, we pooled in two separate groups the normal and the treated rats, and showed tenfold increased ($P < 0.01$) mRNA synthesis in PTU-treated animals (Fig. 1E), thereby suggesting that the enzyme production is activated by TSH signaling. However, the DNA array analysis of human AA and FNAH samples failed to demonstrate significant differences between patient groups (Fig. 1F), suggesting that the human phenotype is milder than the phenotype of PTU-treated rats.

We then extended our analysis to clinical thyroid patient samples by comparing the SOD isoenzyme expression in normal human thyroid (Fig. 1G) and found that in humans, *SOD1* showed highest degree of expression differently from the rat thyroid samples (Fig. 1A). Finally, in order to confirm the significance of SOD3 in thyroid cell physiology, we compared the expression of *Sod1*, *Sod2*, and *Sod3* in a normal thyroid rat cell line (PC Cl3). According to qRT-PCR data, *Sod3* expression was significantly higher as compared with *Sod1* ($P < 0.01$) and *Sod2* ($P < 0.01$; Fig. 1H) validating the *in vivo* observations in the rat model (Fig. 1A).

Figure 1 Superoxide dismutase expression in rat and human tissues. (A) Quantitative RT-PCR analysis for *Sod1*, *Sod2*, and *Sod3* mRNA expression from normal untreated rat tissues. The analysis suggested high expression level for *Sod1* in liver, kidney, aorta, and in heart; for *Sod2* in kidney; and for *Sod3* in kidney, thyroid, and aorta. (B) Thyroid weight measurement from three normal and four PTU-treated rats. Rats 1–3: normal controls, rats 4–7: PTU-treated animals. The analysis showed approximately fourfold increase in wet weight of the PTU-treated tissues indicating goitrogenous development. The use of PTU *ad libitum* for 14 days in drinking water caused variation seen in thyroid weight development. (C and D) Hematoxylin–eosin staining for control rats (C) and for PTU-treated rats (D) showed hyperplasia and altered thyroid structure caused by the treatment. (E) *Sod3* mRNA expression upon PTU treatment. The *Sod3* levels in rat thyroid were significantly ($P < 0.01$) increased by PTU treatment. (F) Human DNA array analysis from normal thyroid, autonomous adenoma (AA), and from familial nonautoimmune hyperthyroidism (FNAH) showed insignificant differences for *SOD3* mRNA expression. (G) Real-time RT-PCR analysis for *SOD* mRNA expression from normal human thyroid samples. In human thyroid tissue, *SOD1* represents the major isoenzyme differently from rat thyroid tissue shown in panel 1A. (H) Comparison of *Sod1*, *Sod2*, and *Sod3* expression in rat thyroid PC Cl3 cells by real-time RT-PCR analysis suggested *Sod3* as a major isoenzyme in the cell line being in line with normal rat thyroid expression analysis shown in panel 1A. *Sod3* synthesis was significantly ($P < 0.01$) higher than *Sod1* and *Sod2* mRNA production.

TSH signal transduction increases *Sod3* mRNA expression

The upregulation of SOD3 in rat hyperfunctioning thyroids suggests that SOD3 could be under the control of TSH, and therefore, we investigated the effect of the different mediators of TSH signaling on regulating *Sod3* production in rat PC Cl3 cell line. As shown in Fig. 2A, the SOD3 mRNA synthesis was significantly induced by TSH stimulation (threefold increase, $P < 0.01$). Because TSH-R is coupled to both G proteins: to G_{α_s} , which causes accumulation of cAMP and activates PKA, and to G_{α_q} , which increases the intracellular Ca^{2+} level and activates protein kinase C (PKC), we studied the contribution of G_{α_s} -mediated pathway on *Sod3* mRNA production by transiently transfecting constitutively active G_{α_s} (pCEF- G_{α_s}) and PKA (pCEF-PKA) into PC Cl3 cells that resulted in 5.5-fold ($P < 0.01$) and sixfold increase ($P < 0.001$) in SOD3 mRNA synthesis respectively. Similarly, accumulation of cAMP after the activation of adenylyl cyclase with forskolin promoted *Sod3* mRNA transcription of sixfold ($P < 0.05$), supporting the involvement of TSH–cAMP pathway in the production of

SOD3. To further demonstrate the specificity of *Sod3* activation, we inhibited the PKA function in TSH-stimulated PC Cl3 cells by using a selective PKA inhibitor, H89. Interestingly, even though TSH–H89 incubation significantly decreased ($P < 0.05$) *Sod3* production, it was not able to completely abolish the mRNA induction, suggesting the contribution of other signaling routes in SOD3 regulation (Fig. 2A). Because in thyroid cells TSH-derived signaling is also mediated via G_{α_q} –PLC– Ca^{2+} signal transduction pathway (Song *et al.* 2007), we transfected PC Cl3 cells with an active form of G_{α_q} (pCEF- G_{α_q}) or incubated the cells with thapsigargin, which increases calcium influx. Transfection of pCEF- G_{α_q} as well as treatment with thapsigargin increased relative mRNA production by threefold ($P < 0.01$ and $P < 0.05$ respectively; Fig. 2B and C), suggesting that both G_{α_s} /cAMP/PKA and G_{α_q} /PKC γ / Ca^{2+} signal transduction pathways are involved in SOD3 regulation in PC Cl3 cells. The effect of thapsigargin on promoting the Ca^{2+} levels in PC Cl3 cells was determined by measuring the intracellular Ca^{2+} concentrations at different time points, upon treatment with the compound (Fig. 2C).

Figure 2 TSH activation increased *Sod3* mRNA production in PC Cl3 cells. (A) TSH-R cAMP pathway increased *Sod3* production. The expression was analyzed by qRT-PCR in cells treated with TSH and forskolin or transfected with *PKA* or $G\alpha_s$, which all induced the mRNA synthesis significantly ($P < 0.01$, $P < 0.05$, $P < 0.001$, and $P < 0.01$ respectively). Treatment with *PKA* inhibitor H89 reduced significantly ($P < 0.05$) the effect of TSH but was not able to completely inhibit the expression. (B) The effect of TSH-R phosphatidylinositol cascade on *Sod3* synthesis was studied by $G\alpha_q$ subunit transient transfection and by calcium uptake analysis using thapsigargin that blocks the calcium pumps in endoplasmic reticulum increasing the cytosolic calcium concentration. Both treatments, transfection and thapsigargin administration, increased the mRNA synthesis significantly ($P < 0.01$ and $P < 0.05$ respectively). (C) The intracellular calcium levels upon thapsigargin treatment were measured by fluorescent calcium uptake assay. The levels went promptly up after 100 nmol/l thapsigargin administration and stayed above the baseline values over the follow-up period.

SOD3 has a mitogenic effect in thyroid cells

We then aimed to investigate the physiological consequences of TSH-stimulated *Sod3* production in thyroid cells. In addition to antioxidative characteristics, we have recently shown that exogenously administered *Sod3* is able to stimulate Ras GTP loading *in vitro* and *in vivo* leading to the activation of mitogen

signaling pathway and consequent growth factor expression and cell proliferation (Laurila et al. 2009). This is further supported by numerous reports showing that physiological concentrations of H_2O_2 can activate cell membrane receptors and downstream signaling leading to cell proliferation *in vitro* and *in vivo* (Rao 1997, Wang et al. 2000, Saito et al. 2002, Aslan & Ozben 2003, Konishi et al. 2004, Mehdi et al. 2005).

Since *Sod3* is highly expressed in PC Cl3 cells (Fig. 1H), the overexpression of *SOD3* did not have any detectable effect on cell proliferation (data not shown). We therefore investigated the effect of RNAi on normal rat thyroid cell proliferation. The efficiency of *Sod3* siRNA transfection was checked by qRT-PCR analysis, showing 95% interference effect ($P < 0.001$) in *Sod3* mRNA production at 48-h time point (Fig. 3A). Importantly, by transfecting siRNA for *Sod3* (siSOD3) into TSH-stimulated PC Cl3 cells, we formally proved that SOD3 has a role in regulating the mitogenic effect of TSH on thyroid cells, as the transfection of siSOD3 significantly ($P < 0.05$) reduced the *in vitro* BrdU incorporation from 33 to 15% (Fig. 3B). This mitogenic effect of SOD3 is in line with our earlier observations (Laurila et al. 2009).

Because previous studies have shown that the production of H_2O_2 in thyroid cells correlates with the stimulation of T_3 and T_4 production (Corvilain et al. 1994), we determined the dismutase activity of SOD3 in Cos-7 heterologous system to predict the participation of SOD3 in hormone synthesis. For this, we used control cells expressing DUOX2 (D2) and the activator of DUOX1 (DUOXA1 or A1), which together produce $O_2^{\cdot -}$ rather than H_2O_2 (Zamproni et al. 2008, Morand et al. 2009). Transiently transfected *SOD3* showed significant ($P < 0.001$) reduction in extracellular $O_2^{\cdot -}$ concentration indicating functionality of SOD3 in our cellular model (Fig. 3C). Finally, because DUOXes have been indicated as the main sources for H_2O_2 production in thyroid, we compared H_2O_2 levels produced by DUOX and SOD3 (Fig. 3D), and found that indeed, the SOD3-derived H_2O_2 production was 28-fold lower than by DUOX2–DUOXA2 pair ($P < 0.05$), suggesting a role for SOD3-mediated H_2O_2 production in controlling TSH-dependent thyroid growth and differentiation rather than in hormone production.

SOD3 is down-regulated in thyroid cancer

In line with the observation that SOD3 could be involved in the TSH-dependent proliferation/dedifferentiation process, we decided to investigate the expression of *Sod3* in malignant thyroid proliferative disorders, firstly by determining the mRNA

levels from three rat PC Cl3 derived models of oncogene-dependent dedifferentiation, PC Braf, PC RET/PTC1, and PC E1A stable cell lines, which showed decreased *Sod3* synthesis in correlation with oncogene-mediated transformation and dedifferentiation as compared with normal PC Cl3 cells (Fig. 4A). PC Braf cells showed twofold decreased ($P < 0.05$) mRNA synthesis, PC RET/PTC1 cells showed threefold decreased ($P < 0.01$) *Sod3* mRNA amount where as PC E1A cell model lacked almost completely the expression ($P < 0.001$) as compared with control PC Cl3 cells.

To further study the effect of oncogene induced inhibition of SOD3 expression, we took advantage of an inducible oncogenic cellular model, doxycyclin *RET/PTC1*-inducible PC cells. As shown in Fig. 4B and C, the increased expression of the *RET/PTC1* oncogene upon doxycyclin treatment correlated with a significant reduction in *Sod3* expression at 24- and 72-h time points ($P < 0.05$ at 24 h and $P < 0.001$ at 72 h), indicating that the different levels of *Sod3* mRNA downregulation observed in Fig. 4A could also be due to the different expression levels of the specific oncogenes.

Since H₂O₂ represents the main product of *Sod3* activation, we confirmed the decreased *Sod3* expression in PC RET/PTC1 and PC E1A cells by measuring the H₂O₂ levels, which were significantly ($P < 0.001$ and $P < 0.01$ respectively) decreased in transformed cells as compared with wild-type cells (Fig. 4D).

To finally move our observation to human models, we then compared DNA array data obtained from normal human thyroid, PTC, and ATC tissues, and showed significantly ($P < 0.01$) decreased *SOD3* synthesis in papillary thyroid tumors and ATC tissue ($P < 0.01$) in line with *in vitro* cell models (Fig. 4A). These data were further confirmed by qRT-PCR analysis, which gave similar results (Fig. 4F).

Figure 3 Functional role of SOD3 in thyroid models. (A) RNAi knockdown of *Sod3* in PC Cl3 cells. Quantitative RT-PCR showed significant ($P < 0.001$) *Sod3* downregulation caused by siSOD3 interfering oligos. (B) BrdU incorporation analysis suggested significantly ($P < 0.05$) attenuated cell proliferation caused by siSOD3 in TSH-stimulated PC Cl3 cells. The TSH-stimulated proliferation was decreased by 44% in siSOD3-transfected cells as compared with siGAPDH-transfected cells. (C) The functionality of *SOD3* to dismutase DUOX2/DUOX1 (D2 + A1) derived superoxide was tested in Cos-7 heterologous system. The data showed that SOD3 was able to dismutase the DUOX2/DUOX1 produced superoxide significantly ($P < 0.001$) indicating functionality of the enzyme in this cell model. (D) SOD3-derived hydrogen peroxide production was tested in Cos-7 heterologous system. Properly folded DUOX2 produced significantly ($P < 0.05$) higher concentration of H₂O₂ than SOD3. Transfection of *SOD3* resulted in only minor increase in H₂O₂ synthesis suggesting that DUOX enzyme complex has the major role H₂O₂ synthesis in thyroid.

The expression of other isoenzymes, *SOD1* and *SOD2*, were more variable in patient samples; *SOD1* expression was mildly affected by tumorigenesis, whereas *SOD2* was decreased in PTC patients ($P < 0.01/P < 0.05$, DNA array/qRT-PCR) and significantly increased in ATC thyroid tumors ($P < 0.01/P < 0.05$, DNA array/qRT-PCR). The *in vitro* and *in vivo* data therefore showed that SOD3 is expressed in normal thyroid tissue, induced by TSH together with thyroid differentiation markers (Dumont *et al.* 1992, Kondo *et al.* 2006), and reduced in *in vitro* transformation models and thyroid cancer samples.

Discussion

Thyroid tissue physiology requires continuous high concentration of H₂O₂ for thyroid hormone T₃ and T₄ synthesis which is, according to recent publications, produced mainly by DUOX (Dupuy *et al.* 1991, 2000, De Deken *et al.* 2002, Rigutto *et al.* 2007). Inactivating mutations of DUOX2 completely abolish H₂O₂ generation and thyroid hormone synthesis in human thyroids, and constitute a major cause of congenital hypothyroidism (Moreno *et al.* 2002). SODs are H₂O₂-producing isoenzymes that are responsible for balancing the reduction–oxidation reactions in different cellular compartments. In thyroid tissue, H₂O₂ is utilized in thyroid hormone production by transmembrane glycoprotein thyroperoxidase that both

Figure 4 SOD3 expression decreases upon thyroid cell transformation. (A) Quantitative RT-PCR analysis for *Sod3* mRNA from wild-type rat PC Cl3 cells and PC Cl3 cells transformed with different oncogenes indicated decreased mRNA production correlating to the oncogene used for cell transformation. The *Sod3* synthesis decreased 50% ($P < 0.05$) in PC Braf cells, 60% ($P < 0.01$) in PC RET/PTC1 cell model, and was almost completely abrogated ($P < 0.001$) in PC E1A cells. (B and C) PC-inducible RET/PTC1 (PCindPTC1) cell model showed that the decreased *Sod3* mRNA synthesis depends on the level of oncogene expression. The doxycyclin-induced RET/PTC1 oncogene expression (B) correlates to the decreased *Sod3* production (C). (D) Determination of H₂O₂ production. The fluorimetric measurement of H₂O₂ synthesis showed decreased H₂O₂ production in PC RET/PTC1 ($P < 0.001$) and PC E1A ($P < 0.01$) cells as compared with PC Cl3 controls being in line with the reduced expression of the *Sod3* enzyme. (E) Analysis of DNA array data from normal thyroid samples derived from papillary thyroid (PTC) and anaplastic thyroid (ATC) cancers showed reduced *SOD3* expression in transformed tissues ($P < 0.01$). The differences of *SOD1* expression were statistically insignificant, whereas the expression of *SOD2* was significantly ($P < 0.05$) decreased in PTC samples and significantly ($P < 0.01$) increased in ATC patients. (F) Real-time RT-PCR analysis supported the array data, showing that only *SOD3* but not the other isoenzymes could be considered as a differentiation marker in cancer development. Both analyses, DNA array and qRT-PCR, showed that the expression of *SOD1* and especially *SOD2* was more variable.

oxidizes and incorporates iodine to thyroglobulin, which is then further metabolized to thyroid hormones T_3 and T_4 . In the present study, we therefore investigated the expression and role of SODs, more specifically extracellularly expressed SOD3, in thyroid cells *in vitro* models and further compared the results to patient samples data.

The expression analysis of different rat tissues suggested thyroid as a major expression site for the enzyme (Fig. 1). According to our previous studies, relatively low concentration of SOD3 *in vitro* or *in vivo* is able to induce a physiological response in the cells and in the surrounding extracellular tissue environment (Laukkanen *et al.* 2000, 2001b, 2002, Laurila *et al.* 2009), suggesting that even minor differences in the enzyme concentration can have an impact on cellular signaling and consequent biological effects.

We studied the *SOD3* expression in benign thyroid enlargement models to determine the expression of the enzyme in the proliferative environment. Even though previous clinical analysis has demonstrated decreased total SOD activity in the plasma of Graves' disease patients (Wilson *et al.* 1989, Abalovich *et al.* 2003, Rybus-Kalinowska *et al.* 2008), a recent paper suggested increased *Sod3* mRNA production in thyroid caused by long-term iodine deprivation in murine models (Maier *et al.* 2007). Since both the synthesis of autoantibodies against TSH-R in Graves' disease and the diet iodine deprivation lead to increased TSH-R signaling and consequent thyroid activation, we investigated the *Sod3* expression in experimental *in vivo* rat goiter model (Fig. 1). Increased *Sod3* production in rat goiter model was in line with previous murine models (Maier *et al.* 2007), suggesting the participation of TSH in its expression in mouse and rat thyroid cells. However, analysis of patient samples did not show significant increase in *SOD3* levels, which could be due to the existence of wide phenotypic variations in humans, ranging from mild to severe toxic hyperfunction of the gland. It is also noteworthy that unlike in rat normal thyroid and in rat PC Cl3 thyroid cells, in normal human thyroid tissue, *SOD1* expression was fivefold higher than *SOD3* mRNA production again indicating species-related differences (Fig. 1).

Because the TSH signaling is the main mediator of thyroid hyperfunction, we studied the effects of the different molecules of this pathway on *Sod3* mRNA synthesis. TSH signals to TSH-R, a GPCR that activates $G\alpha_s$ and $G\alpha_q$ heterotrimeric G-proteins in human and rodents (Laugwitz *et al.* 1996). Activation of $G\alpha_s$ leads to the stimulation of cAMP, a positive modulator of thyroid cell proliferation and the expression of differentiation markers such as sodium

iodide symporter and thyroperoxidase (Kogai *et al.* 1997, Pierce *et al.* 2002, van Staveren *et al.* 2006). The $G\alpha_q$ protein stimulation in thyroid activates DUOX-derived H_2O_2 production, which is the limiting factor in thyroglobulin iodination and in thyroid hormone T_3 and T_4 synthesis (Corvilain *et al.* 1994). To determine the role of *Sod3* in thyroid *in vitro* model, we utilized the PC Cl3 cell line that according to our present data contains higher concentration of SOD3 than other isoenzymes and is therefore responding to SOD3-mediated redox changes (Fig. 1H). By studying the effect of TSH signaling in these cells, we identified a novel GPCR-mediated induction of *Sod3* through both, a cAMP and phosphatidylinositol-mediated way, suggesting a role for the enzyme in thyroid physiology (Fig. 2). Since GPCR-mediated signal transduction plays a significant role also in other tissues, the current data could potentially further explain previous observations of SOD3-mediated effects in injury models (Laukkanen *et al.* 2002, Brasen *et al.* 2007).

In order to investigate the functional effect of the enzyme in thyroid environment, we used siRNA and overexpression of *SOD3* in heterologous Cos-7 model to study the proliferation and level of H_2O_2 production respectively (Fig. 3). According to the present data, knockdown of *Sod3* is able to attenuate TSH-stimulated proliferation proving a role for the

Figure 5 Scheme for activation of *Sod3* transcription in thyroid cells. TSH stimulation leads to signal transduction via cAMP–PKA and PLC– Ca^{2+} routes that both increase *Sod3* mRNA synthesis. In normal thyroid, the increased *Sod3* production has a role in thyrocyte proliferation whereas in thyroid cancer the signal transduction route leading to increased *Sod3* levels is silenced, as a result of the loss of differentiation markers.

enzyme in normal thyroid proliferation. In heterologous Cos-7 model, the transfected *SOD3* reduced extracellular superoxide generation by mismatched *DUOX1* and *DUOX2*, but the *SOD3* derived H_2O_2 was significantly lower than the H_2O_2 produced by matched *DUOX2* and *DUOX2* at equal levels of expression, suggesting that, differently from DUOXes, *SOD3* is not directly regulating thyroid hormone synthesis.

Finally, because the TSH signaling, as a differentiation marker, is down-regulated in cancer, we analyzed different PC Cl3 derived cancer cellular models and showed reduced expression of the *Sod3* mRNA, depending on both the specific oncogene used to transform the cells and the level of the oncogene expression (Fig. 4A–C).

To further analyze the SOD expression in thyroid tumorigenesis, we determined the mRNA expressions from human patient samples. Similarly to *in vitro* models, in both, papillary and anaplastic cancer tissue samples, we observed a reduced *SOD3* expression, suggesting that this gene behaves as a thyroid differentiation marker reduced in malignant transformation (Fig. 4E and F).

In conclusions, we have shown a novel TSH stimulation-dependent GPCR-mediated signal transduction pathway activating *Sod3* mRNA production in rat thyroid PC Cl3 cell line. We have further demonstrated differentiation-dependent *SOD3* expression both *in vitro* and *in vivo* in patient samples. The finding suggests that *SOD3* is involved in TSH mitogen effect in normal thyrocytes, a pathway that is disrupted in thyroid cancer (Fig. 5).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by Academy of Finland, Sigrid Juselius Foundation, Paolo Foundation, AIRC, and European Community Contract FP6-36495 (GenRisk-T).

Author contribution statement

L E Laatikainen, M D Castellone, A Hebrant, C Hoste, M C Cantisani, J P Laurila, G Salvatore, P Salerno, F Basolo, J Näsman, and M O Laukkanen contributed to the experimental part of the work; M D Castellone, J Näsman, J E Dumont, M Santoro, and M O Laukkanen contributed to the design of the experiments and editing of the manuscript; J E Dumont, M Santoro, and M O Laukkanen provided funding for the research.

Acknowledgements

The authors would like to thank Prof Stefan L Marklund, Prof Alfredo Fusco, and Dr Silvio Gutkind for providing materials.

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