Metabolic, Endocrine and Genitourinary Pathobiology

N-Acetylcysteine and 15 Deoxy- $\Delta^{12,14}$ -Prostaglandin J2 Exert a Protective Effect Against Autoimmune Thyroid Destruction *in Vivo* but Not Against Interleukin-1 α /Interferon γ -Induced Inhibitory Effects in Thyrocytes *in Vitro*

Sylvie Poncin,* Ides M. Colin,*
Brigitte Decallonne,[†] Isabelle Clinckspoor,[†]
Marie-Christine Many,* Jean-François Denef,*
and Anne-Catherine Gérard*

From the Unité de Morphologie Expérimentale,* Université catholique de Louvain, Brussels; and Legendo,[†] KULeuven, Belgium

Reactive oxygen species (ROS) are crucial for thyroid hormonogenesis, and their production is kept under tight control. Oxidative stress (OS) is toxic for thyrocytes in an inflammatory context. *In vitro*, Th1 pro-inflammatory cytokines have already been shown to decrease thyroid-specific protein expression. In the present study, OS level and its impact on thyroid function were analyzed *in vitro* in Th1 cytokine (interleukin [IL]-1 α /interferon [IFN] γ)-incubated thyrocytes (rat and human), as well as *in vivo* in thyroids from nonobese diabetic mice, a model of spontaneous autoimmune thyroiditis. N-acetylcysteine (NAC) and prostaglandin, 15 deoxy- $\Delta^{12,14}$ -prostaglandinJ2 (15dPGJ2), were used for their antioxidant and anti-inflammatory properties, respectively. ROS production and OS were increased in IL-1 α /IFN γ -incubated thyrocytes and in destructive thyroiditis. *In vitro*, NAC not only reduced ROS production below control levels, but further decreased the expression of thyroid-specific proteins in addition to IL-1 α /IFN γ -inhibitory effects. Thus, besides ROS, other intracellular intermediaries likely mediate Th1 cytokine effects. *In vivo*, NAC and 15dPGJ2 reduced OS and the immune infiltration, thereby leading to a restoration of thyroid morphology. It is therefore likely that NAC and 15dPGJ2 mainly exert their protective effects by acting on infiltrating inflammatory cells rather than directly on thyrocytes. (Am J Pathol 2010, 177:219–228; DOI: 10.2353/ajpath.2010.091253)

Thyrocytes continuously produce H₂O₂ and various reactive oxygen species (ROS) that are physiologically required for normal thyroid hormone synthesis. To control the toxicity resulting from ROS, thyrocytes possess several protective mechanisms. During thyroid hormone synthesis, H₂O₂ is produced in a limited area at the apical membrane and is immediately consumed in the peroxidation reaction catalyzed by thyroperoxidase (TPO). When ROS are produced in higher amounts, they are systematically eliminated by potent antioxidant systems such as peroxiredoxins, catalase, and glutathione peroxidases.^{1–5} Thus, a basal ROS production, which we define as oxidative load, is required to safeguard thyroid hormone synthesis, as recently demonstrated.⁶ Likewise, an harmless oxidative stress (OS) may also be important for cell division during goiter formation when thyrocytes are facing iodine deprivation.⁷

The context is quite different in the case of thyroid inflammation. Thus, in models of thyroiditis (transient or permanent), high amounts of ROS are produced and may become toxic.^{8–13} Using one of these models, we recently showed that increased OS associated with a strong inflammatory reaction can be controlled by 15 deoxy- $\Delta^{12,14}$ -prostaglandin J2 (15dPGJ2),² an anti-inflammatory prostaglandin¹⁴ that prevents OS-induced cytotoxicity.¹⁵ Iodine administration to goitrous thyrocytes produces an inflammatory reaction that is transient in most cases. However, in individuals genetically prone to develop autoimmune thyroiditis, this transient inflam-

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Address reprint requests to Anne-Catherine Gérard, Ph.D., Unité de Morphologie Expérimentale (MOEX), Université catholique de Louvain, UCL-5251, 52 Av. E.Mounier, B-1200, Brussels, Belgium. E-mail: anne-catherine.gerard@uclouvain.be.

mation may become permanent, thereby evolving toward destructive thyroiditis. A model of destructive thyroiditis can be obtained in nonobese diabetic (NOD) mice. In this model of Hashimoto's-like thyroiditis, the ongoing inflammatory reaction relies on pro-inflammatory Th1 cytokines^{16–18} that inhibit the expression of thyroid-specific proteins such as thyroglobulin, TPO, Na⁺/I[–] symporter (NIS), and dual oxidases (Duoxs).^{19–24} Mechanisms responsible for this inhibition are not yet known. In autoimmune processes targeting other cell types, such as pancreatic β cells, Th1 cytokine effects are mediated by nitric oxide (NO).^{25,26} In human, but not in rat thyrocytes, NO has also been identified as mediating the inhibitory actions of Th1 cytokines, but only partially.^{22,27–30} Intracellular factors other than NO should therefore mediate Th1 cytokine-induced inhibitory effects. ROS may represent, among others, alternative candidates, as suggested by previous studies. For instance, Th1 cytokines are known to increase ROS generation in the respiratory tract,³¹ in osteoarthritis,^{32,33} and in pancreatic islets.^{34,35} Up to now, nothing is known about the eventual involvement of ROS as intracellular mediators of Th1 cytokine-induced inhibitory actions in thyrocytes.

In the present study we aimed to evaluate the impact of Th1 cytokines (interleukin [IL]-1 α /interferon [IFN] γ) on ROS production and how they may influence thyroid cell function *in vitro*. Likewise, the role played by OS in thyroiditis was analyzed *in vivo* in the aforementioned NOD mouse model of spontaneous autoimmune thyroiditis. In both *in vitro* and *in vivo* models, the roles of ROS were evaluated by using *N*-acetylcysteine (NAC), a potent antioxidant, and 15dPGJ2 for its anti-inflammatory properties. We also investigated how antioxidant systems behave in these conditions.

Materials and Methods

Cell Cultures

PCCL3 cells, a continuous line of nontransformed rat thyroid follicular cells,³⁶ were a gift of Dr. F. Miot (Université Libre de Bruxelles, Institut de recherche interdisciplinaire en biologie humaine et moléculaire, Brussels, Belgium). They were grown to 80% to 90% confluence in Coon's modified Ham's F12 medium (BRL-Gibco, Paisley, Strathclyde, UK) supplemented with 5% newborn calf serum, penicillin (50 U/ml), streptomycin (50 μ g/ml), fungizone (2.5 μ g/ml; BRL-Gibco), 1 mU/ml thyroid stimulating hormone, 10 μ g/ml insulin, and 5 μ g/ml transferrin (Sigma, Bornem, Belgium), in a humidified atmosphere (5% CO₂). Recombinant rat IL-1 α (2 ng/ml, Chemicon International, Temecula, CA) and recombinant rat IFN γ (100 U/ml, Chemicon International) were added for three additional days, in combination or not with NAC (1 mmol/L, Sigma) or 15dPGJ2 (2.5 μ mol/L, Sigma) in the same medium containing 0.5% newborn calf serum and 1 mU/ml thyroid stimulating hormone. NAC, 15dPGJ2, or vehicle was added 2 hours before the cytokine cocktail. As a control, NAC or 15dPGJ2 were added on thyroid cells in the absence of cytokines.

Human thyroid tissues from patients who underwent thyroid surgery for benign multinodular goiter were obtained from the anatomopathology department after patients gave their informed consent. Thyrocytes were isolated according to Nilsson et al³⁷ and suspended in modified Earle's medium without phenol red containing 5% newborn calf serum, penicillin (50 U/ml), streptomycin (50 μ g/ml), and fungizone (2.5 μ g/ml; BRL-Gibco). They were plated in 6-well plates (50 μ g DNA/well) or in multichamber glass slide (Nunc International, Naperville, IL; 7 μ g DNA/chamber) and cultured in a humidified atmosphere (5% CO₂) with 1 mU/ml thyroid stimulating hormone. After 1 week, cells were incubated for three additional days with cytokines in combination or not with NAC (1 mmol/L) or 15dPGJ2 (2.5 μ mol/L), as described for PCCL3 cells. All experiments were repeated at least twice.

ROS Production

Thyrocytes were incubated in multichamber glass slides in appropriate medium. ROS production was measured by using a fluorescent dye, 2', 7' dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Paisley, UK). PBS-washed (pH 7.4) thyroid cells were incubated in Krebs-Ringer HEPES medium, pH 7.4, containing DCFH-DA (25 μ mol/L) at 37°C for 1 hour. The excess of dye was removed by two washes with PBS. Cells were stained with Hoechst for 20 minutes and rinsed in PBS. Cover slides were mounted in fluorescent mounting medium (DakoCytomation, Carpinteria, CA) for microscopic observation. ROS production was visualized on a fluorescent microscope equipped with a digital camera.

Viability Assay

Cell viability was assessed by using the Alamar blue assay (Biosource International, Camarillo, CA), as previously described.²²

Apoptosis Detection

Caspase activity was measured by using a CaspACE fluorescein isothiocyanate-VAD-fmk *in situ* marker (Promega, Madison, WI), which binds activated caspases, according to the manufacturer's instructions. Briefly, cells were incubated with 20 μ mol/L fluorescein isothiocyanate-VAD-fmk at 37°C for 20 minutes. Cells were then washed twice with PBS, fixed in 10% buffered formalin for 30 minutes, and rinsed with PBS. Coverslips were mounted in fluorescent mounting medium for microscopic observation. Cells treated with staurosporine (5 μ mol/L; Sigma) were used as positive control.

Nitrite Assay

Nitrite accumulation in the medium of human thyrocytes was measured by the Griess reaction by using a commercially available kit (Promega).

Table 1. Forward and Reverse Primers and Annealing Temperatures Used

Target	Primer forward	Primer reverse	Annealing temperature, °C
Actin	5'-CATCCTGCGTCTGGACCT-3'	5'-AGGAGGAGCAATGATCTTGAT-3'	62
rDuox	5'-GTGGCTGGAGGGAGCCAT-3'	5'-CCGTGAACAGACTCCTGT-3'	60
rDuox1	5'-CCTGCAAGCCAAAAGAAGAC-3'	5'-CCACTGAAGTTTCCCGTACA-3'	60
rDuox2	5'-AGAGGGAGCCATTACCCTGT-3'	5'-CGCATAGCTGAGATGGATGA-3'	60
rTPO	5'-CAGGTTTGGTGGGAGAA-3'	5'-CTGCACACTCATTAACATCTT-3'	58
rNIS	5'-GCGCTGCGACTCTCCCACTGAC-3'	5'-GGCGGTAGAAGATCGGCAAGAAGA-3'	60
hDuox	5'-GTGGCTGGCTGACATCAT-3'	5'-TGCAGGGAGTTGAAGAA-3'	58
h Duox1	5'-GGACCCCCAGGACCAGGAT-3'	5'-CTTACACTCACCGCCCCAACAC-3'	60
hDuox2	5'-AACCCTAACCTCCATCAACA-3'	5'-CCTTGTACCCCTTCCACTT-3'	58
hTPO	5'-CACGATGCAGAGAACTCAA-3'	5'-ATAGACTGGAGGGAGCCAT-3'	60
hNIS	5'-ACCGCGCCCCACCTCTTTCTTATT-3'	5'-CCCCCTCTGATTCTGGTTGTG-3'	62

Real-Time RT-PCR

Cells were suspended in TriPure isolation reagent (Roche Diagnostics GmbH, Mannheim, Germany), and total RNA was purified according to the manufacturer's protocol. Reverse transcription was performed by incubating 2 μ g RNA with 200 U Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, Merelbeke, Belgium) in the recommended buffer containing 1 μ l RNasin (Promega), 0.5 mmol/L dNTP (Promega), 2 μ mol/L oligodT (Sigma), and 10 mmol/L dithiothreitol (20 μ l final volume) overnight at 42°C. CDNA was diluted 1:5 in water for use in real time PCRs.

CDNAs (2 μ l) were mixed with 500 nmol/L of each selected primer (Table 1) and SYBR Green reaction mix (BioRad, Herts, UK) in a final volume of 25 μ l. Reactions were performed by using a iCycler apparatus (BioRad) as follows: 95°C for 1 minute, followed by 40 cycles of 95°C for 15 seconds, annealing temperature for 45 seconds (Table 1), and 81°C for 15 seconds. Amplification levels were normalized to that of β -actin.

Western Blottings

Thyrocytes were suspended in Laemmli buffer (50 mmol/L Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol), containing a protease inhibitor cocktail (Sigma), and were sonicated during 30 seconds. Protein concentration was determined by using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Duox (antibody provided by F. Miot, IRIBHN, Brussels), TPO (antibody provided by J. Ruf, Université de la Méditerranée, Marseille, France), catalase (Sigma), peroxiredoxin 3 and 5 (PRDX3, PRDX5; antibodies provided by B. Knoop, Université catholique de Louvain, Louvain La Neuve), and β -actin (Sigma) Western blottings were performed as previously described.²² Proteins (30 μ g/lane) were heated at 95°C for 5 minutes in the loading buffer (Laemmli buffer containing 100 mmol/L dithiothreitol and 0.1% bromophenol blue), separated by 8% SDS-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Rosenthal, The Netherlands). Membranes were blocked for 1 hour at room temperature in PBS (pH 7.4), 5% nonfat dry milk, 0.1% Tween, and incubated overnight at 4°C with the primary antibody at a dilution of 1:4000 (Duox, TPO), 1:10,000 (PRDX3, PRDX5), or 1:2000 (catalase, β -actin).

Membranes were incubated for 1 hour at room temperature with EnVision (1:200, DakoCytomation) peroxidase-labeled secondary antibody and visualized with enhanced chemiluminescence (SuperSignal West Pico, Pierce) on CLXposure TM films (Pierce). Western blots were scanned and quantified by densitometry using the NIH Scion Image Analysis Software (NIH, Bethesda, MD). Values were normalized by reporting the signal intensity to β -actin expression.

Immunofluorescence

Thyrocytes were cultured in multichamber glass slides in appropriate medium. Thyrocytes were fixed for 30 minutes in 4% paraformaldehyde, rinsed once with PBS, permeabilized for 15 minutes in a PBS-Triton 1% solution at room temperature, and washed with PBS supplemented with 1% bovine serum albumin. Cells were then incubated overnight with PRDX5 primary antibody (1:75) at room temperature. After being washed in PBS, fluorescein isothiocyanate-conjugated secondary antibody was added for 1 hour at room temperature at a dilution of 1:30 (anti-rabbit; DakoCytomation). Coverslips were mounted in fluorescent mounting medium for microscopic observation.

Animals and Treatments

Three-month-old female NOD mice, under a standard diet and kept under semibarrier conditions, were originally obtained from Professor Wu (Beijing, China) and inbred since 1989 (Proefdierencentrum, Leuven, Belgium). Animals were injected intraperitoneally with a saline solution of NAC (100 mg/kg/day) or with a saline solution of 15dPGJ2 (40 μ g/kg/day) for 4 days. NMRI mice were used as control. Mice were housed and handled according to Belgian Regulation of Laboratory Animal Welfare.

Preparation of Tissue Samples for Microscopy and Morphometric Analysis

Five animals of each group were anesthetized with pentothal, and thyroid lobes were dissected. One thyroid lobe was fixed in paraformaldehyde (4% in PBS)

Table 2. Experimental Conditions for Immunohistochemistry

Antibody	First antibody	Second antibody	Revelation substrate
4-HNE (Calbiochem, Darmstadt, Germany)	Rabbit polyclonal Dilution: 1:800 Incubation time: overnight	EnVision rabbit (DakoCytomation)	AEC (DakoCytomation)
PRDX5 (Polyclonal rabbit, B. Knoop, Université catholique de Louvain)	Rabbit polyclonal Dilution: 1:200 Incubation time: 1 hour	EnVision rabbit (DakoCytomation)	AEC (DakoCytomation)
PRDX3 (Polyclonal rabbit, B. Knoop, Université catholique de Louvain)	Rabbit polyclonal Dilution: 1:500 Incubation time: 1 hour	EnVision rabbit (DakoCytomation)	AEC (DakoCytomation)
Catalase (Sigma)	Monoclonal mouse Dilution: 1:200 Incubation time: 3 hours	EnVision mouse (DakoCytomation)	AEC (DakoCytomation)

for 24 hours and embedded in paraffin. The second lobe was frozen for cryostat sections. Thick sections (5 μ m) were used for morphology analysis and for immunohistochemistry. Morphometric measurements were performed by using the point-counting method described by Weibel et al.³⁸

For each thyroid, 1000 points were counted, and the relative volumes of epithelium, colloid, vessels, and interstitium were measured.

Immunohistochemistry

4-hydroxynonenal (4-HNE), PRDX5, and catalase immunostainings were performed on paraffin sections. Sections were dewaxed and rehydrated. Except for PRDX5 detection, paraffin sections were pretreated in a microwave oven in citrate buffer (pH 6.6) for one cycle of 3 minutes at 750 W, followed by four cycles of 3.5 minutes, each at 350 W. Endogenous peroxidases were quenched with 1% H₂O₂ for 30 minutes.

Then, paraffin sections were washed with PBS supplemented with 1% or 5% bovine serum albumin and thereafter incubated in PBS supplemented with 1% or 5% bovine serum albumin containing 2% or 5% normal goat serum at room temperature. Sections were incubated with the first antibody (4-HNE, PRDX5, and catalase) at room temperature (Table 2). The binding of antibodies was detected by using a second antibody conjugated to a peroxidase-labeled polymer (EnVision detection, DakoCytomation, Carpinteria, CA) or a biotinylated second antibody for 30 minutes followed by an avidin-biotin peroxidase complex for 30 minutes (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). The peroxidase activity was measured with 3-amino-9-ethylcarbazole substrate (DakoCytomation). Sections were counterstained with Mayer's hematoxylin, rinsed, and mounted in Faramount Aqueous mounting medium (DakoCytomation). To verify the binding specificity, some sections were incubated with the second antibody alone.

Data Analysis and Statistics

Data were expressed as mean $\pm$ SEM, $n = 6$ for all experiments. Each experiment was repeated at least twice. Statistical analyses were performed by using anal-

ysis of variance followed by Tukey-Kramer Multiple Comparison Test (GraphPad InStat, San Diego, CA), or by unpaired *t*-test. $P < 0.05$ was considered as statistically significant.

Results

IL-1 α /IFN γ Increase Intracellular ROS Production without Affecting Cell Viability In Vitro: Differential Effects of NAC and 15dPGJ2

Although Th1 cytokines are known to induce ROS production in various cell types,^{31,32,34,35} this has not been yet described in thyrocytes. IL-1 α /IFN γ -induced ROS, and nitrite production was therefore analyzed both in rat and human thyroid cells.

In rat PCCL3 control cells, ROS detected by DCFH-DA fluorescence were observed as granules within the cytoplasm (Figure 1A). The staining was greatly enhanced in Th1 cytokine-treated cells (Figure 1B), whereas ROS were detected both in the cytoplasm and in nuclei. In cells treated with NAC alone, and in accordance with NAC anti-oxidant properties, ROS fluorescence was strongly reduced compared with control cells (Figure 1C). In cells co-incubated with IL-1 α /IFN γ together with NAC, ROS fluorescence was below control levels (Figure 1D). By contrast, 15dPGJ2 influenced ROS production, neither in control cells (data not shown) nor in Th1 cytokine-treated cells (Figure 1E). Similar results were obtained in human primary cells (data not shown).

According to previous results, nitrite levels, the stable end-product of NO generation, were low in media from control human thyroid cells, but greatly enhanced in Th1 cytokine-treated cells (Figure 1F).^{22,30} When cells were co-incubated with IL-1 α /IFN γ and NAC or 15dPGJ2, nitrite levels were significantly reduced as compared with Th1-treated cells, but remained higher than in control cells (Figure 1F).

Cell viability was not affected, and no change in apoptosis was detected whatever the treatment used, indicating that the observed effects were not resulting from cell death (data not shown).

Figure 1. Detection of intracellular ROS in PCCL3 cells (A–E). ROS production was measured in control cells (A), in IL-1 α /IFN γ -treated cells (B), in NAC-treated cells (C), in IL-1 α /IFN γ -treated cells co-incubated with NAC (D), and with 15dPGJ2 (E). Scale bars = 200 μ m. Nitrite accumulation in the culture medium of human thyrocytes (F) is shown. Results are expressed as mean $\pm$ SEM of six individual wells of one representative experiment ($n = 6$). * $P < 0.05$ versus control cells; $^{\dagger}P < 0.05$ versus IL-1 α /IFN γ -treated cells; $^{\ddagger}P < 0.05$ versus NAC-treated cells; $^{\S}P < 0.05$ versus 15dPGJ2-treated cells.

IL-1 α /IFN γ Induce a Down-Regulation of Thyroid Cell Function In Vitro: Differential Effects of NAC and 15dPGJ2

As already reported,^{19,22} IL-1 α /IFN γ induced a down-regulation of the thyrocyte function, as indicated by decreased Duox (Duox1 and Duox2), TPO, and NIS expression, at mRNA or protein levels in rat PCCL3 and human cells (Figure 2; Supplemental Figure S1, see <http://ajp.amjpathol.org>).

Our data also confirm that Duox protein and mRNA expression was strongly decreased in PCCL3 cells incubated with NAC alone.⁶ When incubated together with IL-1 α /IFN γ , NAC further aggravated Th1-induced inhibitory effects (Figure 2, A and B). In human thyroid cells, NAC alone or in combination with Th1 cytokines significantly reduced Duox protein and mRNA expression without additional effect on Th1-induced down-regulation of Duox protein expression (Supplemental Figure S1, A and B, see <http://ajp.amjpathol.org>).

The distinct analysis of Duox1 and Duox2 expression in PCCL3 cells showed a different pattern. NAC influenced neither basal nor Th1-induced down-regulation of Duox1 mRNA expression (Figure 2C), but induced a decrease in Duox2 mRNA expression and further im-

Figure 2. Quantification of Duox protein expression in PCCL3 cells (A). Densitometric values of Western Blots are expressed as mean $\pm$ SEM of one representative experiment ($n = 6$). Relative expression of Duox (B), Duox 1 (C), Duox 2 (D), TPO (E), and NIS (F) mRNAs in PCCL3 cells, adjusted to the β -actin signal, are expressed as mean $\pm$ SEM of one representative experiment ($n = 6$). * $P < 0.05$ versus control cells; $^{\dagger}P < 0.05$ versus IL-1 α /IFN γ -treated cells; $^{\ddagger}P < 0.05$ versus NAC-treated cells; $^{\S}P < 0.05$ versus 15dPGJ2-treated-cells.

paired Th1 cytokine-induced down-regulating effects (Figure 2D).

As for Duox and already reported,⁶ NAC alone induced a down-regulation of TPO mRNA and protein expression both in PCCL3 and human thyroid cells (Figure 2E; Supplemental Figure S1, C and D, see <http://ajp.amjpathol.org>). In cells incubated together with NAC and IL-1 α /IFN γ , an additive effect of their respective inhibitory action was observed.

Although NAC alone had no effect on NIS mRNA, it further aggravated IL-1 α /IFN γ -induced NIS down-regulation, at least in human cells (Figure 2F; Supplemental Figure S1E, see <http://ajp.amjpathol.org>).

Both in PCCL3 and human cells, 15dPGJ2, when administered alone or together with Th1 cytokines, had no specific effect on Duox nor on NIS expression (Figure 2A–D and F; Supplemental Figure S1A, B, and E, see <http://ajp.amjpathol.org>). The only isolated modification we observed was on TPO mRNA expression in PCCL3 cells (Figure 2E).

The Expression and Intracellular Localization of PRDX3 and PRDX5 Antioxidant Enzymes Are Strongly Influenced by Th1 Cytokines, NAC, and 15dPGJ2

PRDX5 and PRDX3 protein expression in PCCL3 cells was significantly increased by IL-1 α /IFN γ (Figure 3, A

Figure 3. Expression of PRDX5, PRDX3, and catalase antioxidant enzymes. Quantification of PRDX5 (A), PRDX3 (B), and catalase (C) protein expression in PCCL3 cells. Densitometric values of Western blots are expressed as mean $\pm$ SEM of one representative experiment ($n = 6$). * $P < 0.05$ versus control cells; † $P < 0.05$ versus IL-1 α /IFN γ -treated cells; $\S P < 0.05$ versus 15dPGJ2-treated cells. Immunofluorescence of PRDX5 in PCCL3 cells in control (D), in IL-1 α /IFN γ -treated cells (E), in IL-1 α /IFN γ -treated cells co-incubated with NAC (F), and in IL-1 α /IFN γ -treated cells co-incubated with 15dPGJ2 (G). Scale bars = 200 μ m.

and B). The subcellular localization of PRDX5 also changed. In control cells, PRDX5 was expressed only as granules in the cytoplasm (Figure 3D). In Th1 cytokine-treated cells, PRDX5 expression not only increased in the cytoplasm, but also lighted up in the nuclei (Figure 3E). NAC treatment differentially regulated both PRDXs. NAC alone or together with Th1 cytokines reduced PRDX5 expression (Figure 3, A and F), but increased the expression of PRDX3 (Figure 3B). 15dPGJ2 significantly increased PRDX5 and PRDX3 expression in PCCL3 without affecting their expression induced by Th1 cytokines, or their localization (Figure 3A, B, and G). Other antioxidant enzymes reacted differently, as catalase protein expression remained stable in all experimental conditions (Figure 3C).

The Strong Increase of Both OS and Inflammatory Reaction in the NOD Mouse Model of Spontaneous Thyroiditis Is Down-Regulated by NAC and 15dPGJ2

To study the role of OS in thyroiditis *in vivo*, we used the NOD mouse model of spontaneous thyroiditis. At 3 months, 100% of mice exhibited an inflammatory infiltrate in the thyroid together with variable stages of tissue destruction. Instead of large and regular follicles filled with colloid and lined by cuboidal epithelial cells as observed in normal thyroids, the thyroids of NOD mice consisted of small follicles with narrowed follicular lumina lined by thicker cell walls. Signs of cell destruction were observed

in some areas: dead cells were observed in follicular lumina, some follicles were completely destroyed, and the interstitium was massively occupied by inflammatory cells (Figure 4A). These observations were confirmed by the morphometric analysis that showed a decrease in the relative volume of the colloid together with an increase in the relative volume of both epithelium and interstitium (Figure 4B).

4-HNE, a toxic product resulting from lipid peroxidation, was used as a OS marker.³⁹ Although 4-HNE immunostaining was fairly detected in thyroids from NMRI mice, it strongly increased in NOD mice, with localization in thyrocytes, as well as in some interstitial cells (Figure 5).

In NAC-treated NOD thyroid glands, OS was drastically reduced, as confirmed by the strong decrease in 4-HNE expression, although remaining higher than in NMRI mice (Figure 5). Follicles were larger and more regular in size. Follicular lumina were filled with colloid and lined by flattened epithelial cells. Although the inflammatory reaction was still observed in the interstitium, it was markedly less pronounced compared with thyroids of untreated NOD mice (Figure 4A). Hence, the colloid relative volume was significantly increased, but remained lower than in NMRI mice. The relative volume of the epithelium decreased to values similar to NMRI mice. The relative volume of the interstitium also decreased, but remained higher than in NMRI mice (Figure 4B).

As with NAC, the follicles of NOD mice treated with 15dPGJ2 were larger and more regular in size. Their

Figure 4. Thyroid morphology (A) and morphometric analysis (B) of thyroids from NMRI and NOD mice. Thyroiditis was observed in thyroids from NOD mice as shown by an interstitium massively infiltrated by inflammatory cells (arrows). In addition, follicles were small and narrowed compared with NMRI mice, and dead cells were observed. By contrast, when NOD mice were treated with NAC or with 15dPGJ2, the inflammatory reaction was markedly decreased; follicles were more regular, and lumina were filled with colloid and lined by flattened epithelial cells. Scale bars = 50 μ m. These observations were confirmed by morphometric analysis. * $P < 0.05$ versus NMRI mice; † $P < 0.05$ versus NOD mice.

lumina were filled with more colloid and lined by flattened epithelial cells (Figure 4A). The interstitium compartment was also significantly reduced. These morphological changes were associated with a decrease in 4-HNE expression, indicating a reduced OS (Figure 5).

Because of the strong OS induction in NOD thyroid glands, we analyzed the *in vivo* expression of two potent enzymatic antioxidant systems, namely PRDX5 and catalase (Figure 5). They were weakly expressed in the cytoplasm of control thyrocytes from NMRI mice. In thyroids from NOD mice, their expression was heterogeneously distributed, with a weak expression in follicles with flattened epithelium and a stronger expression in those with thicker epithelial cells. The total expression of PRDX5 and catalase was weaker in NOD mice treated either with NAC or with 15dPGJ2.

Discussion

In this study, we show for the first time that, *in vivo*, thyroiditis is associated with increased toxic OS and that, *in vitro*, Th1 pro-inflammatory cytokines induce intracellular ROS production in rat and human thyrocytes. This intracellular accumulation of ROS is quite different from that observed under physiological conditions. Hence, in a normal thyroid, H_2O_2 is produced by Duox in a limited area called thyroxisome that is located at the apical pole of the cell in microvilli.⁴⁰ H_2O_2 is either consumed during the hormone synthesis process or detoxified by potent antioxidant systems, thereby being harmless for cells.^{1,2,41,42} The outcome for thyrocytes is quite different when ROS are heavily produced in cells incubated with Th1 cytokines. ROS may then become toxic, being detected both in the cytoplasm and in nuclei. In previous studies, we have shown that Th1 cytokines induce a down-regulation of Duox,²² and a reduction in the production of extracellular H_2O_2 in the FRTL5 cell line (unpublished data). It is therefore likely that Th1 cytokine-induced ROS in thyrocytes are not generated from Duox enzymes, but instead from a source that remains to be discovered.

Another question that remains to be sorted out concerns the exact nature of ROS produced when thyrocytes are incubated with Th1 cytokines. The DCFH-DA probe used in our study is not sensitive enough to distinguish H_2O_2 from other ROS, likely more toxic, including peroxynitrite and hydroxyl radicals. It is, however, clear that the nature of ROS will be determinant in terms of cell survival, some of them being more deleterious than others.⁴¹ Among reactive oxygen species, reactive nitrogen species, especially NO, are known to be induced by IL-1 α /IFN γ in human thyrocytes^{27,30,43} and are partially responsible for the inhibitory effects of Th1 cytokines on thyrocytes.²² In addition, although NAC partially reduces the production of nitrite, ROS immunofluorescence detected by DCFH-DA fluorescence is completely abolished. Thus, ROS other than NO are likely produced in Th1 cytokine-incubated thyrocytes.

In vivo, the production of ROS was evaluated indirectly by measuring the induction of OS. In NOD mice, both toxic OS and inflammatory reaction affecting the whole thyroid gland were observed, as described in other models such as osteoarthritis, autoimmune encephalomyelitis, and lung diseases.^{33,44,45} Here, OS results from ROS produced by thyrocytes themselves facing Th1 cytokines (intrafollicular OS), but also from inflammatory cells colonizing the interstitium (extrafollicular OS). A way to explain the toxicity of OS in thyroiditis in NOD mice is the induction of intracellular adhesion molecule-1 by ROS.⁴⁶

In the presence of NAC, the intracellular ROS production and OS were significantly reduced both *in vitro* and *in vivo*. As precursor of glutathione, NAC may decrease cell OS directly or indirectly by restoring glutathione content.^{47,48} *In vitro*, NAC alone exerts an inhibitory effect on thyroid cell function.⁶ Combined with Th1 cytokines, the inhibitory effects were additive, suggesting that although ROS are produced in Th1 cytokine-treated cells, other intracellular mediators are involved in Th1 cytokine inhibitory effects. *In vivo*, the paradigm is not exactly the same

Figure 5. Immunohistochemical detection of 4-HNE, PRDX5, and catalase in thyroids from NMRI and NOD mice. The high expression of 4-HNE observed in NOD thyroid glands was strongly decreased after treatment with NAC or 15dPGJ2. PRDX5 and catalase were expressed in control thyroids from NMRI mice. In thyroids from NOD mice, their expression was heterogeneously distributed with a weak expression in follicles with flattened epithelium and a stronger expression in those with thicker epithelial cells (arrows). Scale bars = 50 μ m.

as NAC reduces the inflammatory reaction, thereby protecting indirectly thyrocytes against the cell destruction induced by the extrathyroidal autoimmune reaction. This could be due to the ability of NAC to decrease the inflammation by inhibiting various cytokines (tumor necrosis factor- α , IFN γ , IL-8, and IL-6)⁴⁵ and/or to restore the cellular redox-status and to modulate the activity of redox sensitive cell signaling pathways such as nuclear factor κ B that regulates pro-inflammatory genes.⁴⁴ The absence of NAC effects directly on thyrocytes *in vivo* compared with the *in vitro* experimental conditions could also be due to differences in terms of local concentration of NAC, probably reaching lower concentrations in thyroids when administered systemically *in vivo* than when added *in vitro* directly to cultured thyrocytes.

The effects of 15dPGJ2 were different from those of NAC, at least *in vitro*, as no reduction of ROS production was observed. By contrast, *in vivo*, as for NAC, OS was decreased, thereby allowing the recovery of a near normal thyroid morphology. It is not the first time that such protective effects are observed as they have been already reported in a nonautoimmune model of iodine-induced thyroid involution.² This is likely due to the ability of 15dPGJ2 to inhibit the expression of a variety of pro-inflammatory factors including cyclooxygenase-2, NOS2, and several cytokines (IL-6, IL-12, and tumor necrosis factor- α).^{49–51} 15dPGJ2 may also modulate or inhibit the nuclear factor κ B system^{51–53} and activate the mitogen activated protein kinase pathway through PPAR γ -dependent and independent mechanisms.⁵⁴ Because 15dPGJ2 has no direct effect on thyroid cells *in vitro*, but is able to

reduce inflammation *in vivo*, we suggest that 15dPGJ2 may favorably influence OS in the thyroid gland by acting directly on infiltrating inflammatory cells.

In this present study we report for the first time a differential regulation of Duox1 and Duox2 mRNA levels in PCCL3 rat thyroid cells. The respective roles of these two proteins encoded by two different genes remain unclear. Rigutto et al⁵⁵ have reported in PCCL3 cells that Duox1 alone is able to generate H₂O₂ and that the amount of Duox1 present is sufficient to generate enough H₂O₂. By contrast, other arguments suggest that in humans, thyroid Duox2 is the main H₂O₂ generator.⁵⁵ A study in the respiratory tract epithelium demonstrated that IL-4, a Th2 cytokine, increases Duox1 mRNA expression and that IFN γ , a Th1 cytokine, markedly induces Duox2 mRNA expression.³¹ In our study, the results were quite different. Here, Th1 cytokines inhibited both Duox genes. On the other hand, the antioxidant NAC negatively influenced only Duox2 mRNA. Thus, Duox2 expression seems to require a minimal oxidative load to be adequately expressed, whereas Duox1 expression does not depend on the thyroid cell ROS content. Obviously, further investigations are required to clarify the exact underlying mechanisms.

In conclusion, our results confirm that the maintenance of a minimal oxidative load, as in control cells, is essential to safeguard thyroid cell function. In addition, ROS are not the sole intracellular mediators of Th1 cytokine-induced inhibitory effects of thyroid cell function *in vitro*. *In vivo*, both the antioxidant NAC and the anti-inflammatory prostaglandin 15dPGJ2 protect the thyroid against toxic

OS mainly by acting on infiltrating inflammatory cells, thereby contributing to the reduction of the extrafollicular toxic OS. The intracellular OS remains under the control of efficient intracellular antioxidant systems, thereby allowing the thyroid cell function and morphology to recover.

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