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Metabolic, Endocrine and Genitourinary Pathobiology

Oxidative Stress

A Required Condition for Thyroid Cell Proliferation

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Goiter is associated with increased oxidative stress (OS). We studied the effects of an anti-inflammatory agent, 15 deoxy-$^{\Delta 12,14}$-prostaglandin J2 (15dPGJ2) and an antioxidant, N-acetylcysteine (NAC), on OS, thyroid function, and goiter expansion in a model of goiter induced by propylthiouracil (PTU) or perchlorate. OS was assessed by the immunodetection of 4-hydroxynonenal, thyroid function by measuring thyroxin (T4) and thyrotropin (TSH) plasma levels and detecting T4-rich thyroglobulin (Tg-I), and goiter expansion by weighing the thyroids and measuring cell proliferation (PCNA and cyclin D1 immunodetection). In both PTU and perchlorate-induced goiters, OS, TSH plasma levels, thyroid weight, and cell proliferation were strongly enhanced, whereas Tg-I expression was negative. All these parameters were reversed by NAC and 15dPGJ2 in PTU-goiters. In perchlorate-goiters, TSH plasma levels remained elevated and Tg-I–negative after NAC or 15dPGJ2 treatment. OS was reduced by NAC, but not by 15dPGJ2. In addition, NAC reduced PCNA and cyclin D1 immunostainings, as well as thyroid weight, whereas 15dPGJ2 influenced neither thyroid weight nor cell proliferation. In conclusion, NAC and 15dPGJ2 overcome PTU- but not perchlorate-induced effects. The retrieval of hormonal synthesis may result from direct chemical interactions between PTU and NAC/15dPGJ2. Although 15dPGJ2 has no effect in perchlorate-goiters, the reduction of OS by NAC is associated with altered goiter development, making OS a required condition for the growth of the thyroid gland. ***(Am J Pathol 2010, 176:1355–1363; DOI: 10.2353/ajpath.2010.090682)***

Thyrocytes produce constantly moderate amounts of reactive oxygen species (ROS), which are physiologically required for thyroid hormone synthesis.[1,2] To maintain cell integrity, several protective systems against ROS, such as antioxidant enzymes, peroxiredoxins, catalase, and glutathione peroxidases, are active in thyrocytes.[3,4] Nevertheless, when ROS are heavily produced, they may become toxic. Previous studies have shown that lipofuscins and 4-hydroxynonenal (4-HNE), a toxic product resulting from lipid peroxidation, are increased in goitrous and in involuting glands, indicating that the oxidative stress (OS) is greatly enhanced in these conditions.[5–7] Increased OS is not necessarily lethal for goitrous cells but is associated with large cellular destruction and inflammation in iodine-induced thyroid involution.[7,8] Moreover, peroxiredoxin 5 (PRDX5) and glutathione peroxidases are highly regulated in goitrous mouse thyroids and in thyroids from Graves disease patients, suggesting that they may play a role in regulating thyroid ROS levels.[3,7,9] We have previously shown that 15 deoxy-$^{\Delta 12,14}$-prostaglandin J2 (15dPGJ2), an anti-inflammatory prostaglandin, reduces OS-induced cell toxicity and inflammation in involuting glands.[7] In addition, Mutaku et al showed that vitamin E reduces goiter development by controlling thyrocyte growth, without interfering with the proliferation of endothelial cells and/or changing the thyroid hormone metabolism.[6]

To assess the role played by OS in goiter development and the influence of antioxidants on the thyroid function, we analyzed the effect of N-acetylcysteine (NAC), a potent antioxidant and of 15dPGJ2 in two different models of goitrogenesis (propylthiouracil [PTU] and perchlorate).

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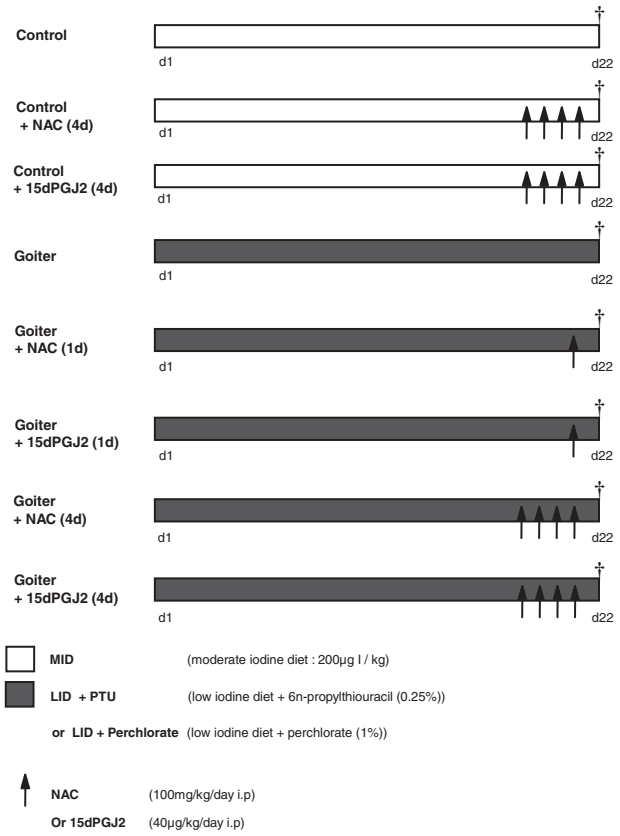

Figure 1. Design of the experimental procedure.

Materials and Methods

Animals and Treatments

Hyperplasic goiter was induced in six-week-old female Wistar rats or in six-week-old female NMRI mice (UCL, Brussels, Belgium) by feeding a low iodine diet (LID <20 μg iodine/kg, Animolabo, Brussels Belgium) supplemented with 0.25% 6-n-propyl-2-thiouracil (PTU, Sigma, St. Louis, MO), or with sodium perchlorate 1% in drinking water, for 21 days (Figure 1). The prostaglandin- and NAC-treated groups received, respectively, 40 μg/kg of 15dPGJ2 (Sigma) or 100 mg/kg of NAC (Sigma) for one or four days before sacrifice (i.p. in saline solution). Control groups received normal diet and tap water. Additional control groups were also performed by injection of 40 μg/kg of 15dPGJ2 or of 100 mg/kg of NAC for 4 days before sacrifice (i.p. in saline solution). Animals were sacrificed under thiopental anesthesia. Blood for thyrotropin (TSH) and thyroxin (T4) assays was collected and plasma stored at −20°C until use. Animals were maintained in accordance with the principles of Belgian laboratory animal welfare. Eight or five animals were used in control and goiter groups, respectively. Each experimental setting has been repeated twice and all analyses were realized both in rats and mice.

Preparation of Tissue Samples for Microscopy

Thyroids were dissected and weighed. Thyroids were fixed in formaldehyde and embedded in paraffin. Thick sections (5 μm) were used for immunohistochemistry.

TSH Assay

Plasma TSH levels were measured in duplicate by radioimmunoassay using a specific kit for detection of rat TSH (Biocode, Belgium or Amersham Biosciences, Little Chalfont, UK). All values were expressed as mean ± SEM. The statistical analysis was performed using analysis of variance followed by Tukey–Kramer multiple comparison test.

T4 Assay

Plasma T_4 levels were measured in duplicate by ELISA using a specific kit according to the manufacturer's instructions (Genway Biotech, San Diego, CA). All values were expressed as mean ± SEM. The statistical analysis was performed using analysis of variance followed by Tukey–Kramer multiple comparison test.

Immunohistochemistry

4-HNE, T4-rich thyroglobulin (Tg-I), PCNA, cyclin D1, and PRDX5 immunostainings were performed on paraffin sections. Sections were dewaxed, rehydrated and endogenous peroxidases were quenched with 1% H_2O_2 for 30 minutes. For 4-HNE and cyclin D1 detection, tissue sections were pretreated in microwave oven in citrate buffer (pH 6.6) for one cycle of three minutes at 750W, followed by four cycles of 3.5 minutes, each at 350W. All sections were washed with phosphate buffered saline (PBS) supplemented with 1% or 5% bovine serum albumin (PBS-BSA) and thereafter incubated in PBS-BSA containing 2% or 5% normal goat serum at room temperature. Sections were incubated with the first antibody (4-HNE, Tg-I, PCNA, cyclin D1, and PRDX5) at room temperature (see Table 1). The binding of antibodies was detected using a second antibody conjugated to a peroxidase-labeled polymer (EnVision detection, DakoCytomation, Carpinteria, CA). The peroxidase activity was revealed with AEC substrate (3-amino-9-ethylcarbazole, DakoCytomation). Sections were counterstained with Mayer hematoxylin, rinsed, and mounted in Faramount Aqueous mounting medium (DakoCytomation). To verify the binding specificity, some sections were incubated with the second antibody alone. Positive nuclei for PCNA and cyclin D1 were counted under light microscope, and results were expressed as a percentage of total nuclei. All values were expressed as mean ± SEM. The statistical analysis was performed using analysis of variance followed by Tukey–Kramer multiple comparison test.

Because similar results were obtained in rats and mice, only data coming from mice are presented in the result section.

Table 1. 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)
Tg-I (De Vijlder, Amsterdam, The Netherlands)	Mouse monoclonal, Dilution 1:3000, incubation time: overnight	EnVision mouse (DakoCytomation)	AEC (DakoCytomation)
PCNA (DakoCytomation)	Rabbit polyclonal, Dilution 1:75, incubation time: 1 hour	EnVision rabbit (DakoCytomation)	AEC (DakoCytomation)
Cyclin D1 (Abcam)	Rabbit polyclonal, Dilution 1:50 Incubation time: overnight	EnVision rabbit (DakoCytomation	AEC (DakoCytomation)
PRDX5 (B. Knoops, CL, Belgium)	Rabbit polyclonal, Dilution 1/200,incubation time: 1 hour	EnVision rabbit (DakoCytomation)	AEC (DakoCytomation)

Results

NAC and 15dPGJ2 Reduce OS in Goitrous Thyrocytes

4-HNE, the end-product of lipid peroxidation, was used as OS marker.[10] A weak 4-HNE immunostaining was detected in control thyrocytes with or without NAC or 15dPGJ2 (Figure 2, A–C). It was strongly enhanced in both types of goiters (Figure 2, D and I). In PTU-goiters, its expression was already decreased after one day of NAC or 15dPGJ2 treatment (Figure 2, E–H). In perchlorate-goiters, the expression of 4-HNE was reduced after four days of NAC treatment (Figure 2, J and L), but not after 15dPGJ2 treatment (Figure 2, K and M). Data presented in Figure 2 were obtained in mouse thyroids.

NAC and 15dPGJ2 Overcome PTU-Induced Effects

PTU treatment induced an increase in TSH and a decrease in T_4 plasma levels, confirming the functional hypothyroid status of goitrous animals (Figure 3G, Table 2). Unexpectedly, in goiters treated with NAC or 15dPGJ2 for 4 days, TSH plasma levels were significantly reduced and T4 plasma levels were increased. Similarly, Tg-I, used as a marker of T4-rich thyroglobulin, was absent in PTU-goiters (Figure 3, A and D), but became positive already after one day of NAC or 15dPGJ2 treatment (Figure 3, B, C, E, and F). Tg-I was mainly localized in cells or in the follicular lumen, but close to the apical pole of thyrocytes. After four days, the colloid was filled with iodinated thyroglobulin (Figure 3, H and I). These results indicate that thyroid hormone synthesis retrieves after NAC or 15dPGJ2 treatments.

Then, to evaluate the expansion of goiters, we analyzed the weight of thyroid. As expected, the thyroid weight was increased by PTU treatment (Figure 4A). By contrast, the weight of thyroids in goitrous mice treated with NAC for four days returned to control levels (Figure 4A). 15dPGJ2 also decreased the thyroid weight, but to a lesser extent (Figure 4A). This indicates that both NAC and 15dPGJ2 decrease the expansion of PTU-induced goiters. In fact, in thyroids of PTU-treated animals, the gland had the typical morphology of a goitrous thyroid (ie, blood vessels were enlarged, epithelial cells were hypertrophic, and follicular lumina were narrow compared with control groups; Figure 3, D and A). In PTU-goiters treated with NAC or 15dPGJ2 for four days, blood vessels were constricted; follicular lumina were widened and sometimes filled with colloid (Figure 3, H and I). Thus, treatment of PTU-induced goiters with NAC or 15dPGJ2 induces a return to a normal morphology of the gland after four days, which is in line with the decreased stimulation by TSH (Figure 3G).

To determine whether NAC- and 15dPGJ2-induced hindrance in PTU-goiters had consequences in terms of cell proliferation, we analyzed cell proliferation by using two markers, PCNA and cyclin D1. The number of PCNA-positive nuclei was very low in all control groups but was markedly increased in PTU-goiters (Figure 4B and supplemental Figure S1 at *http://ajp.amjpathol.org*). NAC induced a decrease in the number of PCNA-positive nuclei, already after one day of treatment. After four days of NAC treatment, the number of positive cells returned to control values. 15dPGJ2 decreased also the number of PCNA-positive nuclei, but only after four days of treatment. The number of cyclin D1–positive nuclei was very low in all control groups. The increased number of cyclin D1–positive nuclei in PTU-goiters was reduced after four days in animals treated with NAC or 15dPGJ2 (Figure 4C and supplemental Figure S2 at *http://ajp.amjpathol.org*).

Perchlorate-Induced Goitrogenic Effects Are Reduced by NAC, but not by 15dPGJ2

Perchlorate induced an increase in TSH plasma levels (Figure 3M), a decrease in T4 plasma levels (Table 2), and a loss in Tg-I immunostaining (Figure 3J). The treatment of perchlorate-goiters with NAC or 15dPGJ2, for one or four days, influenced neither TSH, nor T4 levels, nor Tg-I immunostaining (Figure 3, K, L, N, and O).

As for PTU-goiters, perchlorate induced an increase in the thyroid weight (Figure 4D). Whereas NAC treatment was not influencing the morphology of goiters, it de-

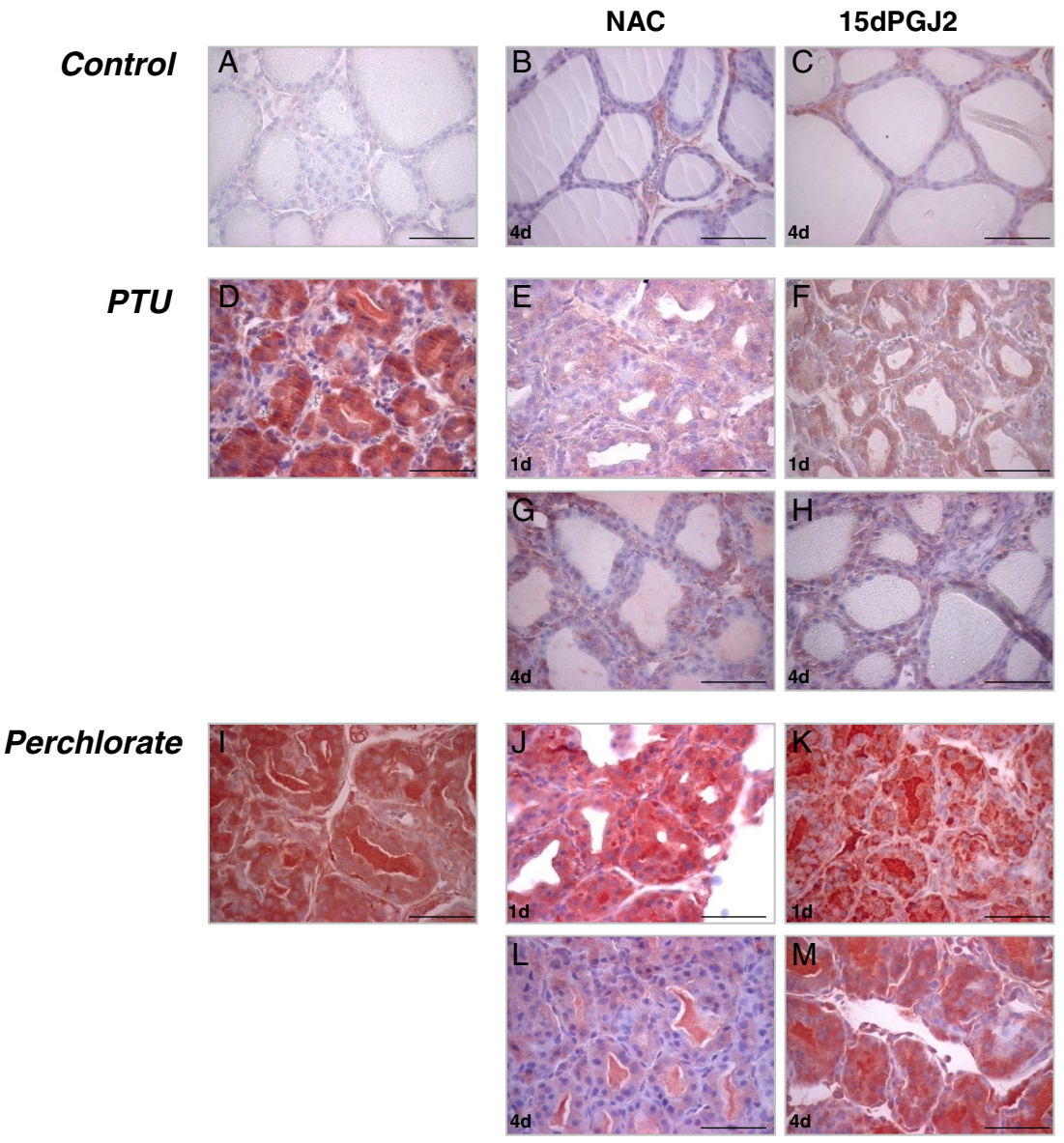

Figure 2. NAC and 15dPGJ2 reduce OS in goitrous mice. Detection by immunohistochemistry of 4-HNE, a marker of OS. 4-HNE was not detected in all control thyroids (**A**, **B**, **C**). It was highly expressed in thyrocytes from PTU and perchlorate goiters (**D**, **I**). In PTU-goiters treated with NAC or 15dPGJ2, 4-HNE staining was strongly decreased (**E**, **F**, **G**, **H**). In perchlorate-goiters, 4-HNE expression remained high after one day of NAC (**J**) or 15dPGJ2 (**K**, **M**) treatment, but decreased after four days of NAC treatment (**L**). Scale bar = 100 μm.

creased significantly the thyroid weight (Figure 4D). However, it remains higher than in control thyroids. By contrast, 15dPGJ2 treatment had influence neither on weight, nor on the morphology of goiters. NAC-induced effect on thyroid weight may be explained by alteration in cell proliferation. Hence, NAC decreased the number of nuclei positive both for PCNA and cyclin D1 (Figure 4, E and F, and supplemental Figures S1 and S2 at *http://ajp.amjpathol.org*), whereas 15dPGJ2 had no influence on these parameters. Results presented in Figures 3 and 4 were obtained in mouse thyroids.

PRDX5 Expression Is Strongly Enhanced in Goitrous Glands and Decreased by NAC and 15dPGJ2

PRDX5 was weakly expressed in the cytoplasm of thyrocytes in all control groups (Figure 5, A, B, and C). In goiters induced by PTU or perchlorate, PRDX5 expression was strongly increased (Figure 5, D and I). It was highly expressed in the cytoplasm of thyrocytes, but numerous nuclei were also labeled. In PTU-goiters, PRDX5

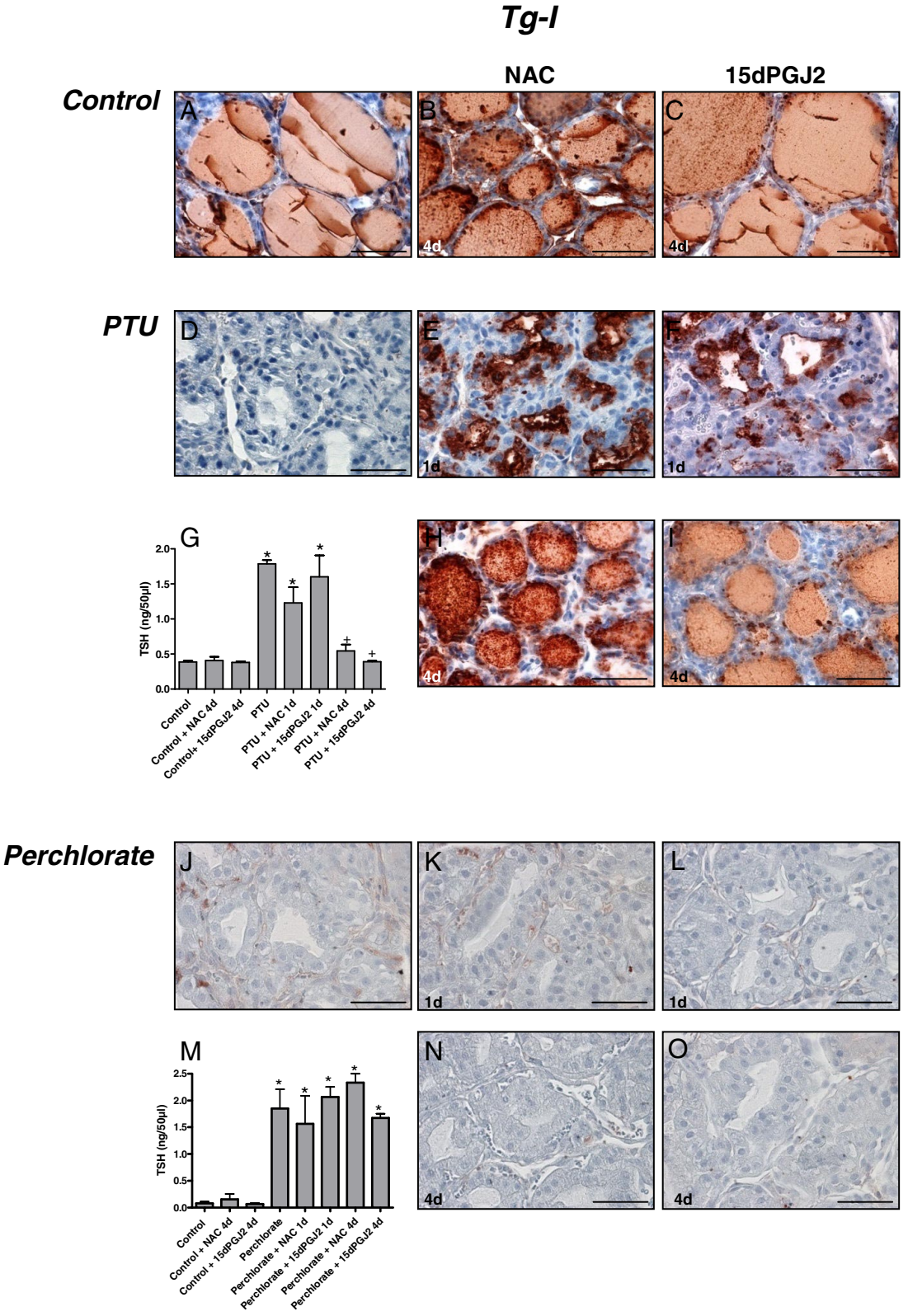

Figure 3. NAC and 15dPGJ2 restore thyroid hormone synthesis in PTU-goiters but not in perchlorate-goiters. Tg-I, a marker of iodinated-thyroglobulin, was detected in the colloid of all control mouse thyroids (**A**, **B**, **C**), but absent in both types of goiter (**D**, **J**). In PTU-goiters treated with NAC or 15dPGJ2, Tg-I immunostaining became positive (**E**, **F**, **H**, **I**), whereas it remained negative in perchlorate-goiters treated with NAC or with 15dPGJ2 (**K**, **L**, **N**, **O**). Mouse TSH plasma levels were measured by radioimmunoassay (**G**, **M**) and results expressed as mean ± SEM. TSH levels were in line with Tg-I expression, increasing when Tg-I was absent and decreasing when Tg-I was detected. $^{*}P < 0.05$ versus control, + $P < 0.05$ versus goiter. Scale bar = 100 μm.

Table 2. T4 Plasma Levels

Control	7.43 ± 1.33 μg/dl
Control + NAC 4 days	5.7 ± 1.16 μg/dl
Control + 15dPGJ2 4 days	3.57 ± 1.26 μg/dl
PTU	N.D.
PTU + NAC 1 day	1.55 ± 0.69 μg/dl
PTU + 15dPGJ2 1 day	3.03 ± 0.28 μg/dl
PTU + NAC 4 days	2.07 ± 0.51 μg/dl
PTU + 15dPGJ2 4 days	3.3 ± 0.87 μg/dl
Perchlorate	N.D.
Perchlorate + NAC 1 day	N.D.
Perchlorate + 15dPGJ2 1 day	N.D.
Perchlorate + NAC 4 days	N.D.
Perchlorate + 15dPGJ2 4 days	N.D.

T4 plasma levels were measured by ELISA. T4 plasma levels were detected in all control thyroids. In both types of goiters, T4 plasma levels remained below the detection levels. When PTU-goiters were treated with NAC or 15dPGJ2, T4 was detected, whereas it remained below the detection level in perchlorate-goiters treated with NAC or with 15dPGJ2. Results are expressed as mean ± SEM.

N.D. indicates not detected.

expression was decreased after four days of NAC and 15dPGJ2 treatment (Figure 5, E, F, G, and H). NAC had already an effect after one day of treatment (Figure 5E). The localization was mainly cytoplasmic after NAC treatment, but some nuclei remained positive after 15dPGJ2 treatment. In perchlorate-goiters, PRDX5 expression remained at a high level after one day of NAC or 15dPGJ2 treatment (Figure 5, J and K). After four days, NAC strongly decreased PRDX5 expression, whereas 15dPGJ2 decreased the staining only slightly (Figure 5, L and M). The cellular localization was mainly cytoplasmic. Results presented in Figure 5 were obtained in mouse thyroids.

Discussion

Our results show that OS is required for the growth of the thyroid gland when stimulated by goitrogen stimuli. As OS was known to increase during goiter formation and having demonstrated its key role in the maintenance of the thyroid function in basal conditions,[11] we looked at its potential role during goiter formation. We have chosen a pharmacological approach using two products (NAC and 15dPGJ2) that are known to reduce either OS in basal conditions (NAC),[11] or the inflammatory reaction in a model of iodine-induced thyroid involution (15dPGJ2).[7]

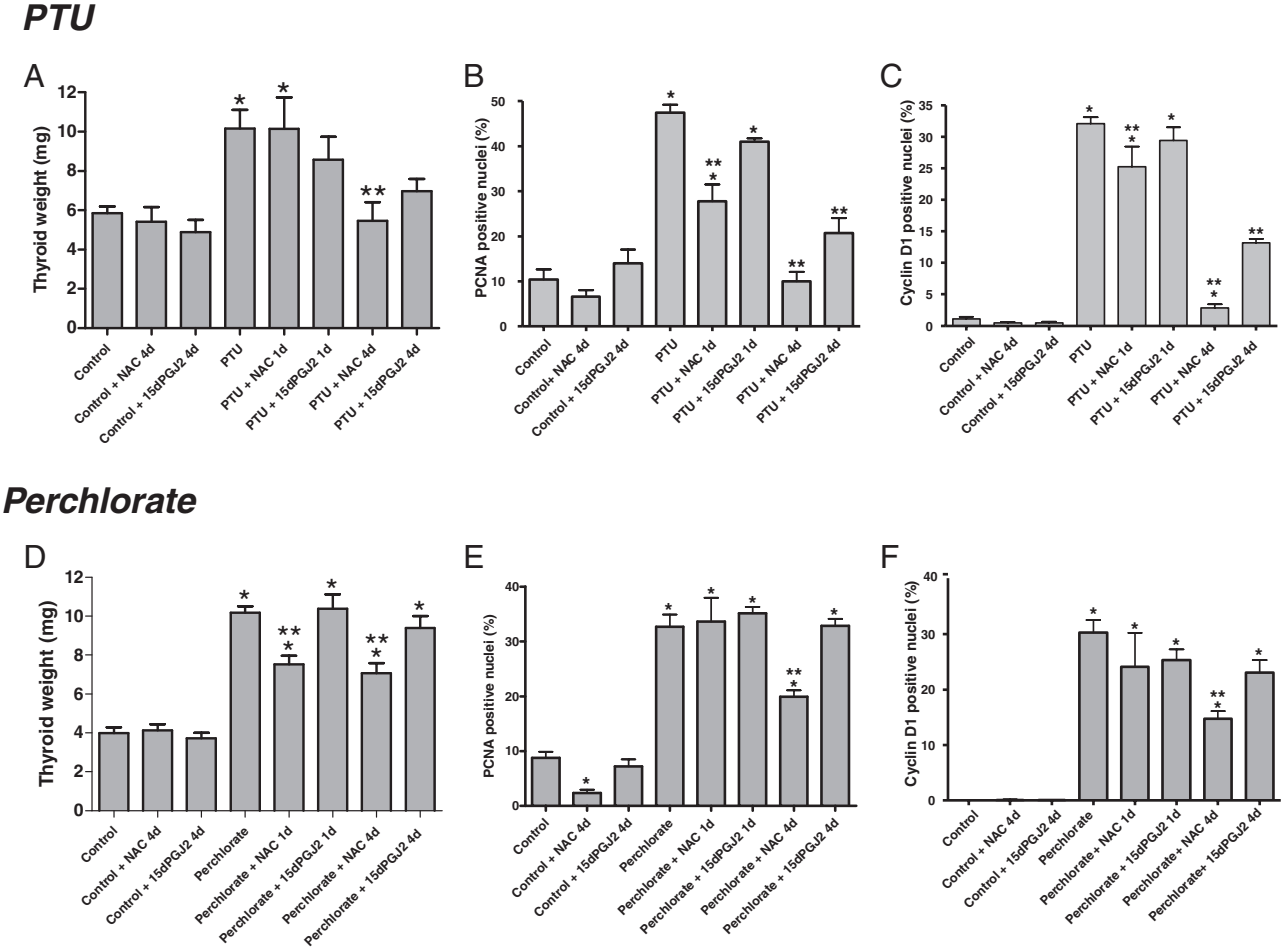

Figure 4. Mouse thyroid weight and number of PCNA- and cyclin D1–positive nuclei. The increased thyroid weight observed in both types of goiters was reduced by NAC and 15dPGJ2 in PTU-goiters (**A**), but only by NAC in perchlorate-goiters (**D**). Positive nuclei were counted and expressed as a pourcentage of nuclei. (mean ± SEM, $n = 6$; **B**, **C**, **E**, **F**). NAC induced a decrease in PCNA- and cyclin D1–positive nuclei in both types of goiters, whereas 15dPGJ2 decreased the number of PCNA- and cyclin D1–positive nuclei only in PTU-goiters. *$P < 0.05$ versus control; **$P < 0.05$ versus goiter.

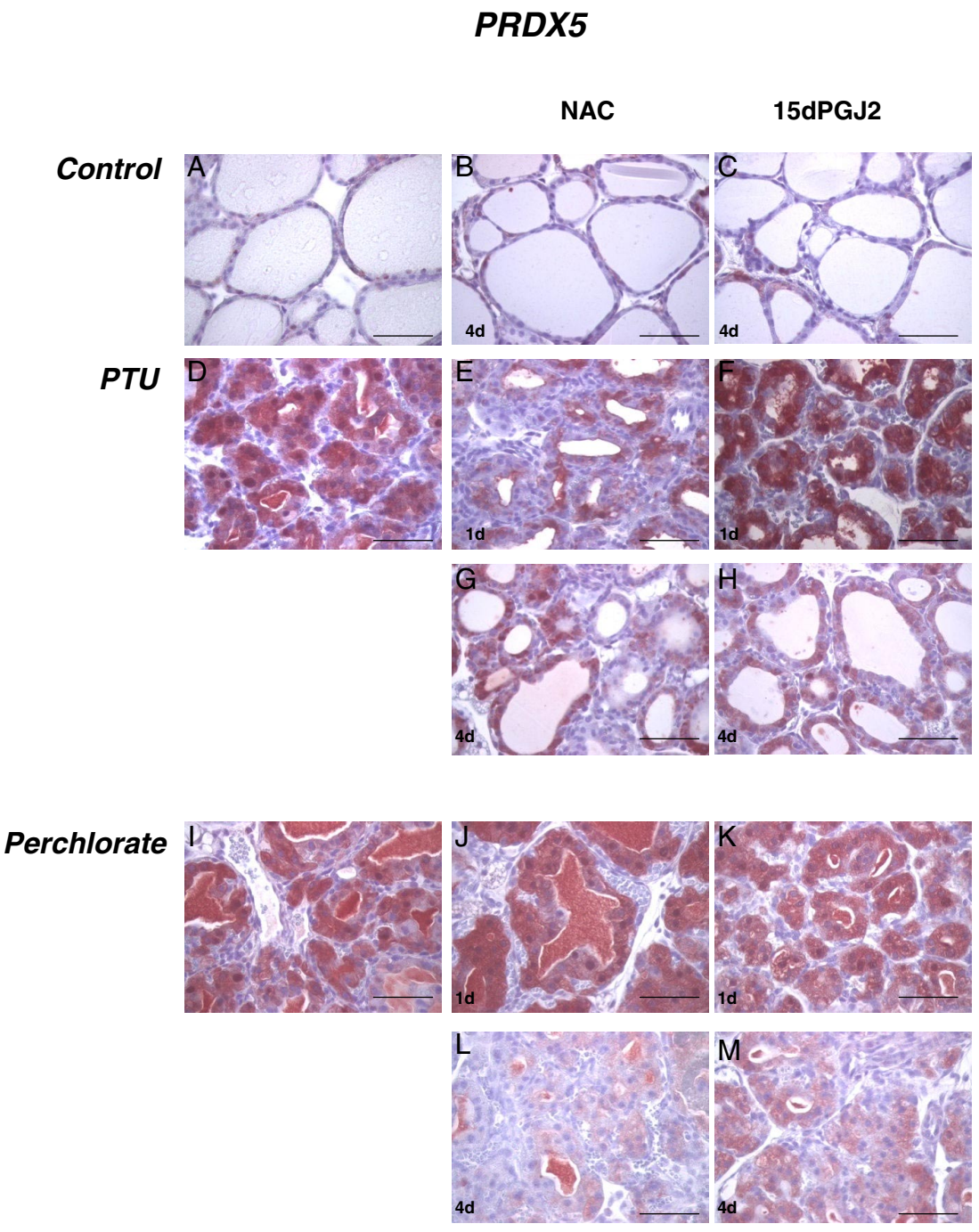

Figure 5. Expression of PRDX5. PRDX5 immunostaining was weak in control mouse thyroids (**A**, **B**, **C**), but highly expressed in thyrocytes from PTU- and perchlorate-goiters (**D**, **I**). In PTU-goiters treated with NAC or 15dPGJ2, PRDX5 expression decreased (**E**, **F**, **G**, and **H**). In perchlorate-goiters, its expression remained high after one day of NAC (**J**) or of 15dPGJ2 treatments (**K**) but decreased after four days of NAC or of 15dPGJ2 treatments (**L**, **M**).

We found that NAC and 15dPGJ2 blunt OS in PTU-induced goiters because they both overcome PTU-induced effects. In this model, NAC and 15dPGJ2 immediately restored the hormonal synthesis. These surprising results can be explained by the fact that chemical interactions may occur between PTU, 15dPGJ2, and NAC. Hence, the literature shows that 15dPGJ2 may covalently react with nucleophiles, such as the free sulfhydryls of glutathione and the cysteine thiol residues of cellular proteins.[12,13] It is therefore tempting to propose that PTU may be inactivated by chemical interactions between its thiol residues and 15dPGJ2, hindering in turn thyroperoxidase (TPO) inhibition. Similarly, NAC can also directly interact with the activity of some proteins through its reducing activity and might therefore also inactivate PTU.[14] To prove the involvement of OS in goiter formation, it was therefore mandatory to choose another approach that led us to use perchlorate to induce goiters. Now, the thyroid hormone synthesis was not restored, ruling out interferences between 15dPGJ2/NAC and perchlorate.

To evaluate the intensity of OS in our model, we used the labeling of 4-HNE, a marker of lipidic peroxidation. It was increased in both types of goiters according to previous results that showed that OS is greatly enhanced in hyperplastic glands.[6,7] The increased OS in goitrous thyrocytes is probably attributable to the accumulation of H_2O_2 after thyroperoxidase (TPO) blockade, or associated with the lack of iodine.[15–18] 15dPGJ2 reduced OS in PTU goiters, because of the inactivation of PTU, but not in perchlorate-induced goiters. The inability of 15dPGJ2 to suppress OS in perchlorate goiters indicates that 15dPGJ2 does not act directly on thyrocytes, as suggested in our previous study showing a reduction of OS in a model of involuting goiters where 15dPGJ2 was actually acting on infiltrating inflammatory cells.[7] By contrast, NAC, as an antioxidant, reduced OS in both PTU and perchlorate-induced goiters. In addition, it was able to decrease thyroid cell proliferation as indicated by the reduced cyclin D1 and PCNA immunostainings. The effect in PTU-goiters was attributable, as aforementioned for 15dPGJ2, to chemical interactions with PTU. It was not the case in perchlorate-goiters where both OS and cell proliferation were reduced, suggesting that both events are eventually linked. It is not the first time that such hindering effects of NAC on cell proliferation are described, as they were already reported in other models.[14,19–21] As far as we know, ROS have not been yet reported to have signaling roles in the thyroid, but this could be evoked based on the present results and in view of what is known in other models.[19,20]

By limiting cell proliferation, NAC decreases the thyroid weight and therefore limits goiter expansion. This observation is fully in line with a previous study that showed similar results with vitamin E, another antioxidant, which also reduces follicular cell proliferation.[6] Thus, NAC and vitamin E, like other antioxidants, might reduce cell proliferation because they inhibit ROS that are known to activate the cell cycle.[19]

OS in goiter was associated with a higher expression of the antioxidant enzyme PRDX5 that is located both in cytoplasm and nuclei. This up-regulation of antioxidant enzymes in goitrous rodent thyroids and in the thyroid of Graves disease patients has already been reported.[3,7,9] This suggests that PRDX5 plays a role in regulating ROS levels in the thyroid. In addition, changes in subcellular locations of PRDX5 suggest that it may act in different cellular compartments as a function of the localization, the nature, and the intensity of the oxidative attack. For instance, the nuclear localization of PRDX5 has been suggested to protect genome against oxidative attacks.[22–25] By contrast, in goitrous thyroids treated with NAC, PRDX5 was absent or very weakly expressed, suggesting that NAC acts as a substitution antioxidant in place of PRDX5.

In conclusion, NAC and 15dPGJ2 overcome PTU- but not perchlorate-induced effects. In the latter model, whereas 15dPGJ2 has no direct effect on goitrous thyroid cells, the antioxidant effect of NAC is associated with reduced goiter expansion, suggesting that increased OS is required for the growth of the thyroid gland.

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