Minimal oxidative load: a prerequisite for thyroid cell function

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Abstract

In addition to reactive oxygen species (ROS) produced by mitochondria during aerobic respiration, thyrocytes are continuously producing H₂O₂, a key element for hormonogenesis. Because nothing is known about ROS implication in normal non-stimulated cells, we studied their possible involvement in thyrocytes incubated with a potent antioxidant, N-acetylcysteine (NAC). NAC, which blocked the production of intracellular ROS, also decreased dual oxidases, thyreroxidase, pendrin, and thyroglobulin protein and/or gene expression. By contrast, Na⁺/I⁻ symporter mRNA expression was unaffected. Among antioxidant systems, peroxiredoxin (PRDX) five expression was reduced by NAC, whereas peroxiredoxin three increased and catalase remained unchanged. In vivo, the expression of both dual oxidases and peroxiredoxin five proteins was also decreased by NAC. In conclusion, when intracellular ROS levels drop below a basal threshold, the expression of proteins involved in thyroid cell function is hampered. This suggests that keeping ROS at a minimal level is required for safeguarding thyrocyte function.

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Introduction

Reactive species include both reactive oxygen species (ROS) and reactive nitrogen species (RNS). In all cell types, ATP generation by mitochondrial aerobic respiration generates ROS as by-products of oxidative phosphorylations. In physiological conditions, ROS in excess must be constantly detoxified by antioxidant systems to avoid cell damage. In thyrocytes, H₂O₂ is a ROS that is required for hormonogenesis. It is produced from dual oxidases (DUOX1/2) (Dupuy et al. 1999, De Deken et al. 2002) at the apical pole of the cell, where it oxidizes iodide (actively transported across the basal membrane by Na⁺/I⁻ symporter (NIS, product of SLC5A5) (Dai et al. 1996)) into iodine in a reaction catalyzed by thyreroxidase (TPO). Iodine is then incorporated into tyrosine residues of thyroglobulin (TG), again by TPO. Hence, H₂O₂ and free radicals are continuously produced in thyroid cells, even in physiological conditions (Denef et al. 1996, De Deken et al. 2002, Schweizer et al. 2008). Obviously, when ROS are over-produced in pathological situations, they may exert a wide range of actions, usually deleterious (Adler et al. 1999, Blokhina et al. 2003). To preserve cell integrity, several protective systems against ROS, such as peroxiredoxins (PRDX), catalase, and glutathione peroxidases are heavily expressed and active in thyrocytes. Thus, PRDX5 and glutathione peroxidase are increased in goitrous thyroids, both in human and rodents (Mano et al. 1997, Mutaku et al. 2002, Gerard et al. 2005, Poncin et al. 2008a). Cells can rely on various antioxidant tools to maintain the oxidative stress (OS) in strict limits to avoid OS becoming eventually harmful. Among them, selenium, which is required for glutathione peroxidase activity, has been shown to exert protective effects in various situations where OS is elevated, while its defect is deleterious (Contempre et al. 1993, 2004, Schomburg & Kohrle 2008, Schweizer et al. 2008).

Up to now, nothing has been reported about the physiological implication of ROS in normal resting thyrocytes. To answer this question, we analyzed the in vitro and in vivo expression of proteins involved in thyroid cell function in the presence of N-acetylcysteine (NAC), an agent supposed to deprive cells from intracellular ROS, because of potent antioxidant properties. We also investigated the regulation of antioxidant systems when the cell oxidative load is reduced.

Materials and Methods

Cell cultures

PCCL3 cells (a continuous line of non-transformed rat thyroid follicular cells, Fusco et al. 1987) were a gift from Dr F. Miot (Université Libre de Bruxelles, IRIBHN, Brussels, Belgium), and human thyroids from multinodular goiters were obtained at surgery after patients gave their informed consent. The study was performed after approval from the ethical committee had been received. Cells were cultured as previously reported (Poncin et al. 2008b). NAC (5, 2.5, 1, or 0.5 mM; Sigma–Aldrich) was added for 3 days in the cell medium containing 0.5% newborn calf serum and 1 mU/ml TSH. All experiments were repeated at least twice.
ROS production

Thyrotocytes were incubated in multichamber glass slides in appropriate medium. ROS production was measured using a fluorescent dye, 2′, 7′ dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Paisley, UK). Phosphate buffer saline (PBS, pH 7-4)-washed thyroid cells were incubated in Krebs-Ringer-HEPES (KRH) medium (pH 7-4) containing DCFH-DA (25 μM) at 37 °C for 1 h. The excess of dye was removed by two washes with PBS. Cells were stained with Hoechst for 20 min and rinsed in PBS. Cover slides were mounted in fluorescent mounting medium (DakoCytomation, Carpinteria, CA, USA) for microscopic observation. ROS production was visualized on a fluorescent microscope equipped with a digital camera (Zeiss, Zaventem, Belgium).

Viability assay

Cell viability was assessed using the Alamar blue assay (Biosource International, Camarillo, CA, USA), as previously described (Gerard et al. 2006).

Apoptosis detection

Caspase activity was measured by using a CaspACE FITC-VAD-fmk in situ marker (Promega) that binds activated caspasas, according to the manufacturer’s instructions. Briefly, cells were incubated with 20 μM FITC-VAD-fmk at 37 °C for 20 min. Cells were then washed twice with PBS, dried with Hoechst for 20 min, and rinsed in PBS. Cells were fixed in 10% buffered formalin for 30 min and rinsed with PBS. Cover slides were mounted in fluorescent mounting medium for microscopic observation. Cells treated with staurosporin (5 μM, Sigma) were used as positive controls.

Nitrite assay

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

Quantitative PCR

For each condition, cells from six individual wells were suspended in TriPure isolation reagent (Roche Diagnostics GmbH). Total RNA purification and reverse transcription were performed as previously described (Gerard et al. 2008). Quantitative PCR was performed in an iCycler apparatus (IQ5, Bio-Rad). cDNAs (2 μl) were mixed with 500 nM of each selected primers (Table 1) and SYBR green reaction mix (Bio-Rad) in a final volume of 25 μl. Reactions were performed as follows: 95 °C/1.5 min, followed by 40 cycles of 95°C/15 s, annealing temperature (Table 1)/45 s, and 81 °C/15 s. Amplification levels were normalized to those of β-actin. All melting curves were analyzed for each PCR to avoid genomic DNA amplification.

Western blotting

Thyrotocytes were suspended in Laemmli buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol), containing a protease inhibitor cocktail (Sigma), and were sonicated during 30 s. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockfort, IL, USA). DUOXs (antibody provided by F. Miot, IRIBHN), TPO (antibody provided by J. Ruf, Université de la Méditerranée, Marseille, France), PRDX5 (antibody provided by B. Knoops, UCL, Louvain La Neuve), and β-actin (Sigma) western blottings were performed as previously described (Gerard et al. 2006). Membranes were blocked for 1 h at room temperature (RT) in PBS (pH 7-4), 5% non-fat dry milk, 0.1% Tween, and incubated overnight at 4 °C with the primary antibody at a dilution of 1/4000 (DUOXs, TPO), 1/10 000 (PRDX5), or 1/200 (β-actin). Membranes were incubated for 1 h at RT 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 (National Institutes of Health, Bethesda, MA, USA). Densitometric values were normalized to β-actin expression.

Table 1 Forward and reverse primers and annealing temperatures

<table>
<thead>
<tr>
<th>Primer forward</th>
<th>Primer reverse</th>
<th>Annealing T (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>5′-CATCCTGGTCTGGACCT-3′</td>
<td>5′-AGGAGGAGCAATGTATCTTGAT-3′</td>
</tr>
<tr>
<td>r DUOX1/2</td>
<td>5′-GTGGCTGGAGGAGACCAT-3′</td>
<td>5′-CGTGAACAGACTCTGCTG-3′</td>
</tr>
<tr>
<td>r TPO</td>
<td>5′-CACCTTTCCTGTGGAAGA-3′</td>
<td>5′-CTGCACACTTAAACTCTT-3′</td>
</tr>
<tr>
<td>r Pendrin</td>
<td>5′-GTCGACAAACACTTCCCGGCT-3′</td>
<td>5′-GGTACTTCTGCTTACACTGGGC-3′</td>
</tr>
<tr>
<td>r TG</td>
<td>5′-GGACAAACACCCACCATCTGGAGC-3′</td>
<td>5′-TGCACTGGGGACGACTGTCAG-3′</td>
</tr>
<tr>
<td>h DUOX1/2</td>
<td>5′-GTGGCCTGGCTGACATAT-3′</td>
<td>5′-TGCAAGGGAGTGAAGA-3′</td>
</tr>
<tr>
<td>h TPO</td>
<td>5′-CACAGTTGGAGAAGAACCTC-3′</td>
<td>5′-ATAGACTGGAGGAGGACAT-3′</td>
</tr>
<tr>
<td>h NIS</td>
<td>5′-ACCGGCCGCCACCTCTTATT-3′</td>
<td>5′-CCCCCTCTGTATTGTTGTT-3′</td>
</tr>
<tr>
<td>h Pendrin</td>
<td>5′-TGGAACATCAAGACATATCTCAGTG-3′</td>
<td>5′-TGGCTCTGGATAGCAGAAATG-3′</td>
</tr>
<tr>
<td>h TG</td>
<td>5′-GCGGTCGGGCGGCTCAGTCTACCT-3′</td>
<td>5′-AGCAGTTTCTGCGTGGAG-3′</td>
</tr>
</tbody>
</table>
**PRDX5 immunofluorescence**

Thyrocytes were cultured in multichamber glass slides in appropriate medium. They were fixed for 30 min in 4% paraformaldehyde, rinsed once with PBS, permeabilized for 15 min in a PBS–Triton 1% solution at RT, and washed with PBS supplemented with 1% BSA (PBS–BSA). Cells were then incubated for 1 h with PRDX5 primary antibody (1/75) at RT. After being washed in PBS, a FITC-conjugated secondary antibody was added for 1 h at RT at a dilution of 1/30 (anti-rabbit; DakoCytomation). Cover slides were mounted in fluorescent-mounting medium for microscopic observation.

**Animals and treatments**

NMRI mice of 2-months-old received a standard diet. Animals were given i.p. injected of saline solution of NAC (100 mg/kg per day) for 4 days. Mice were housed and handled according to Belgian regulation of Laboratory Animal Welfare.

**Preparation of tissue samples for microscopy**

Five animals of each group (control and NAC) were anesthetized with pentothal and thyroid lobes were dissected. One thyroid lobe was fixed in paraformaldehyde (4% in PBS) for 24 h and embedded in paraffin. Thick sections (5 μm) were used for PRDX5 immunohistochemistry. The second lobe was frozen and cryostat sections (5 μm) were used for DUOXs immunohistochemistry.

**DUOXs and PRDX5 immunohistochemistry**

Tissue sections were washed with PBS–BSA and incubated at RT for 30 min with normal goat serum (1/50, Vector Labs, Burlingham, CA, USA) in PBS–BSA. Slides were then incubated at RT for 1 h with DUOXs (1/75) or PRDX5 (1/250) primary antibodies. The antibody was detected using Envision (DakoCytomation) or ABC kit (Vector labs) for DUOXs and PRDX5 detection respectively. The peroxidase activity was revealed using AEC (DakoCytomation) as substrate. Sections were counterstained with Mayer’s hematoxylin.

**Data analysis and statistics**

Data were expressed as mean ± s.e.m., n = 6 for all assays. All experiments were repeated at least twice. Statistical analyses were performed using ANOVA followed by Tukey–Kramer multiple comparison test (GraphPad InStat, San Diego, CA, USA), or by unpaired t-test. P < 0.05 was considered as statistically significant.

**Results**

**NAC reduces intracellular ROS production without inducing apoptosis or affecting cell viability**

In human thyrocytes, ROS, as detected by DCFH–DA fluorescence (Fig. 1A), were mainly observed in cytoplasm granules. NAC (1 mM)-treated cells showed a marked decrease in fluorescence. Identical results were obtained in PCCL3 cells (data not shown).

In human thyrocytes, nitrite levels (Fig. 1B, left panel), the stable end product of NO generation, were low in media from control cells and slightly increased in NAC (1 mM)-treated cells (1.6-fold, P < 0.05. Nos2 mRNA expression was not influenced by NAC (Fig. 1B, right panel).

In staurosporin-treated cells, used as positive control for apoptosis, all nuclei were labeled with CaspACE FITC–VAD-fmk marker (Fig. 1C). By contrast, apoptosis was detected neither in control, nor in NAC (1 mM)-treated cells. Cell viability was therefore unaffected by NAC (Fig. 1D).

**NAC reduces the expression of proteins involved in thyroid cell function**

In human thyrocytes, DUOXs and TPO proteins were detected by Western blot (Table 2). NAC at concentrations ranging from 0.5 to 5 mM strongly decreased the expression of both proteins. As no difference was observed from one concentration to another, 1 mM was selected as reference concentration in all experiments. NAC also decreased Dioxs and Tpo mRNA expressions, both in human and PCCL3 cells (Table 3). By contrast, NAC had no effect on Nis mRNA expression. Tg and pendrin mRNAs were also downregulated in PCCL3 cells, but not in human cells. Thus, except NIS, all thyroid differentiation genes studied in this work were downregulated in ROS-deprived cells.

**NAC differentially affects the expression of PRDX5 and PRDX3**

Both in human and PCCL3 cells, NAC decreased PRDX5 protein expression by 2.6-folds (P < 0.05, Fig. 2A). The analysis of PRDX5 protein expression by immunofluorescence (Fig. 2B) showed a cytoplasmic and granular pattern, suggesting a mitochondrial localization of the protein (Bannmeyer et al. 2005). By contrast, NAC induced a twofold increase in PRDX3 protein expression (P < 0.05, Fig. 2C). Catalase protein expression remained unchanged (Fig. 2D).

**NAC decreases DUOXs and PRDX5 expressions in mouse thyroids**

In mouse thyroids, DUOXs was detected at the apical pole of thyrocytes (Fig. 3A). In NAC–treated mice, DUOXs immunostaining was more faint and detected in few cells (Fig. 3B). A PRDX5 signal (Fig. 3C) was present in the cytosol and in nuclei, as previously reported (Poncin et al. 2008a). In NAC-treated mice (Fig. 3D), the signal was strongly reduced, which is in accordance with the above reported in vitro observations.
Discussion

This study shows that PCCL3 and human thyroid cells produce a given amount of intracellular ROS in basal conditions, likely as a result of physiological aerobic metabolism. But thyrocytes are somewhat unique in that they produce H$_2$O$_2$ that, along with iodine, is a key element for TH synthesis. Thus, ROS in the thyroid are definitively more than just by-products of aerobic respiration. They are in fact intrinsically involved in endocrine function. H$_2$O$_2$ is produced outside the cell by NADPH oxidases, DUOXs localized at the apical pole of the thyrocyte (De Deken et al. 2002). The hormone synthesis then occurs in the follicular lumen, close to the apical pole. Until now, H$_2$O$_2$ has always been detected in culture media (Bjorkman & Ekholm 1992, Fortemaison et al. 2005), never inside the cell. Here, we show for the first time that ROS are physiologically produced into cells without provoking significant cell damage, despite the fact that basal DNA damage in thyrocytes is higher than in other tissues (Krohn et al. 2007, Maier et al. 2007). H$_2$O$_2$ is likely one of these ROS as the DCFH-DA probe mainly reacts with H$_2$O$_2$. Besides DUOXs produced H$_2$O$_2$, other possible sources of intracellular ROS include mitochondria and peroxisomes (Bedard & Krause 2007). It is likely that ROS other than H$_2$O$_2$, such as hydroxyl radicals and anion superoxide, are produced in physiological conditions, but

Table 2 DUOXs and TPO protein expressions in human thyrocytes. DUOXs and TPO protein expressions in human thyrocytes were analyzed by western blot.

<table>
<thead>
<tr>
<th>Human</th>
<th>NAC 1 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DUOXs</td>
<td>2.39 ± 0.38</td>
</tr>
<tr>
<td>TPO</td>
<td>3.69 ± 0.72</td>
</tr>
</tbody>
</table>

Values, normalized to β-actin, are expressed as mean ±S.E.M. of one representative experiment (n=6). *P<0.05 versus control cells.
likely in very low amounts, as suggested by previous models (Denef et al. 1996). Further studies are required to find out the source and the type of intracellular ROS, and to sort out those resulting from aerobic metabolism as in every other organs, and those specifically involved in hormone synthesis.

NAC may decrease cell OS, either directly, as a source of sulphydryl groups that neutralize ROS, or indirectly as glutathione precursor by restoring glutathione levels (GSH) (Gillissen & Nowak 1998). The present study shows that NAC decreases intracellular ROS to very low levels without inducing apoptosis or affecting cell viability. This marked decrease in intracellular ROS levels is associated with duox1, tpo, tgs and pendrin expressions, but not nis mRNA expression. Such effects on duox1 expression were also observed in vivo. As NAC may also influence GSH intracellular levels, it is possible that some specific biochemical processes might be altered by glutathionylation, i.e., the reversible formation of disulfide bonds between protein cysteines and glutathione, as recently suggested (Cao et al. 2005, le-Donne et al. 2007). Although, ROS are sometimes known to act as transduction signals, for instance in cells stimulated by ligands (cytokines, growth factors), or in pathological situations (Lander 1997, Finkel 1998, Adler et al. 1999), it is the first time that the maintenance of a minimal intracellular oxidative load is shown to be required for safeguarding endocrine functions. This has already been suggested in pancreatic cells or in the lung (Lenzen 2008), but without any convincing demonstration. Thus, when the oxidative load in thyrocytes drops below a minimal threshold, various actors involved in the TH synthesis are also downregulated. This might eventually be relevant in terms of thyroid function in patients treated with NAC, but as far as we know, there is no robust data yet reporting such interactions in the literature.

In addition to ROS, thyrocytes also produce RNS in low amounts (basal nitrite release) associated with NOS2 and NOS3 expressions (Colin et al. 1995), suggesting a role for NO in normal thyroids. In contrast with ROS, NAC increased NO production without affecting nos2 mRNA expression. The interpretation of this result is somewhat difficult as NAC-induced effects on nos2 expression remains a matter of debate. It may either increase, or decrease, or have no effect on nos2 expression and NO release (Ramasamy et al. 1999, Vos et al. 1999, Chen et al. 2000, Zafarullah et al. 2003). Nevertheless, NO levels are particularly low when compared with those produced for instance in cells incubated with Th1 cytokines (Kasai et al. 1995, van den Hove et al. 2002, Gerard et al. 2006). Although this slight increase in NO production may appear odd in cells treated with NAC, one should keep in mind that superoxide anions, which are constantly produced

### Table 3

Relative mRNA expression of thyroid genes. Duox1, Tpo, Nis, Tg, and pendrin mRNA levels were measured by qRT-PCR in human thyrocytes and PCCL3 cells.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Human</th>
<th>NAC 1 mM</th>
<th>Control</th>
<th>NAC 1 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duox</td>
<td>1·61 ± 0·44</td>
<td>0·93 ± 0·27*</td>
<td>1·09 ± 0·13</td>
<td>0·62 ± 0·14*</td>
</tr>
<tr>
<td>Tpo</td>
<td>0·72 ± 0·11</td>
<td>0·15 ± 0·05*</td>
<td>0·81 ± 0·19</td>
<td>0·34 ± 0·08*</td>
</tr>
<tr>
<td>Nis</td>
<td>1·61 ± 0·55</td>
<td>1·59 ± 0·38</td>
<td>0·89 ± 0·17</td>
<td>0·79 ± 0·14</td>
</tr>
<tr>
<td>Tg</td>
<td>0·88 ± 0·26</td>
<td>0·85 ± 0·11</td>
<td>0·63 ± 0·16</td>
<td>0·33 ± 0·1*</td>
</tr>
<tr>
<td>Pendrin</td>
<td>0·83 ± 0·18</td>
<td>0·46 ± 0·08</td>
<td>0·74 ± 0·09</td>
<td>0·35 ± 0·05*</td>
</tr>
</tbody>
</table>

Values, normalized to β-actin, are expressed as mean ± S.E.M. of one representative experiment (n = 6). *P<0·05 versus control cells.

Figure 2 NAC differentially regulates the expression of antioxidant enzymes in human thyrocytes. PRDX5 (A), PRDX3 (C), and catalase (D) protein expressions were analyzed by western blot. Densitometric values normalized to β-actin are expressed as mean ± S.E.M. of one representative experiment (n = 6). *P<0·05 versus control cells. PRDX5 protein (B) localization was analyzed by immunofluorescence in control- and NAC-treated cells. Scale bars: 20 μm. Full colour version of this figure available via http://dx.doi.org/10.1677/JOE-08-0470.
In conclusion, our results showed for the first time that in physiological conditions, thyroid cells produce intracytoplasmic ROS. When their intracellular levels drop very low, as in NAC-treated cells, the expression of important proteins involved in TH synthesis is hampered. This strongly suggests that the maintenance of the oxidative load above a minimum threshold is required to safeguard the function of thyroid cells.

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.

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