**Regulation of H$_2$O$_2$ generation in thyroid cells does not involve Rac1 activation**

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**Abstract**

Objectives: The H$_2$O$_2$ generating system of the thyrocyte and the O$_2^-$ generating system of macrophages and leukocytes present numerous functional analogies. The main constituent enzymes belong to the NADPH oxidase (NOX) family (Duox/ThOX for the thyroid and NOX2 /gp91 phox for the leukocytes and macrophages), and in both cell types, H$_2$O$_2$ generation is activated by the intracellular generation of Ca$^{2+}$ and diacylglycerol signals. Nevertheless, although the controls involved in these two systems are similar, their mechanisms are different. The main factors controlling O$_2^-$ production by NOX2 are the cytosolic proteins p67 phox and p47 phox, and Rac, a small GTP-binding protein. We have previously reported that there is no expression of p67 phox and p47 phox in thyrocytes. Here, we investigated whether Rac1 is an actor in the thyroid H$_2$O$_2$-generating system.

Design and methods: Ionomycin- and carbamylcholine-stimulated H$_2$O$_2$ generation was measured in dog thyroid cells pretreated with the Clostridium difficile toxin B, which inhibits Rac proteins. Activation of Rac1 was measured in response to agents stimulating H$_2$O$_2$ production, using the CRIB domain of PAK1 as a probe in a glutathione S-transferase (GST) pull-down assay.

Results: Among the various agents inducing H$_2$O$_2$ generation in dog thyrocytes, carbamylcholine is the only one which activates Rac1, whereas phorbol ester and calcium increase alone have no effect, and cAMP inactivates it. Moreover, whereas toxin B inhibits the stimulation of O$_2^-$ generation by phorbol ester in leukocytes, it does not inhibit H$_2$O$_2$ generation induced by carbamylcholine and ionomycin in dog thyrocytes.

Conclusions: Unlike in leukocytes, Rac proteins do not play a role in H$_2$O$_2$ generation in thyroid cells. A different regulatory cascade for the control of H$_2$O$_2$ generation remains to be defined.

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**Introduction**

The thyroid cell shares with inflammatory cells a requirement for strong oxidizing machinery. In the thyroid cell, this machinery is formed by an H$_2$O$_2$-generating system, whose main enzymes ThOX1 and ThOX2 (thyroid oxidase, also called ‘Duox’ for dual oxidase) belonging to the NOX (NADPH oxidase) family have been recently cloned (1), and a thyroperoxidase, which uses this H$_2$O$_2$ to oxidize iodide and bind it to thyroglobulin. These steps are required for the synthesis of thyroid hormones (tri-iodothyronine (T$_3$) and thyroxine (T$_4$)) (2, 3).

In dog thyroid cells, H$_2$O$_2$ generation is strongly activated by carbamylcholine, acting through muscarinic receptors which activate the PLC/PKC cascade: by ionomycin, a calcium ionophore: by 12-O-tetradecanoylphorbol-13-acetate (TPA): activating PKC: and weakly by thyrotropin (TSH) and forskolin, through an increase of intracellular cyclic AMP. In leukocytes and macrophages, the H$_2$O$_2$-generating system is well characterized (4, 5). In these cells, the NOX2 NADPH oxidase (or gp91phox) generates O$_2^-$ and, after dismutation, H$_2$O$_2$. This H$_2$O$_2$ allows myeloperoxidase to oxidize chloride, as required for its bactericidal function (4, 6). NOX2 is associated with p22phox in the membrane, and its activation requires its association with the p47phox – p67phox complex, which is translocated from the cytosol with the Rac (Rac1 or Rac2) small GTPase in its active form (7). The leukocyte and thyroid H$_2$O$_2$-generating systems share several characteristics, including the fact that both are activated by intracellular Ca$^{2+}$ and diacylglycerol signals (8–10). However, although p22phox is expressed in the thyroid, p47phox and p67phox mRNAs were not detected (11). The absence of these proteins in thyroid cells does not exclude the possibility that other similar proteins could have a parallel role.

An important event required for oxidase activation in leukocytes is Rac (Rac1 or 2) activation (12, 13) and its translocation to the membrane (4). Rac1 is ubiquitous while Rac2 is only expressed in hematopoietic cells (14–16). Rac proteins are members of the Rho family of small GTPases, which are key regulators of
cells were still able to produce H\textsubscript{2}O\textsubscript{2} in response to stimulation by the use of toxin B and checked whether these cells were able to produce H\textsubscript{2}O\textsubscript{2} in response to stimulation. Toxins were kindly given by Dr M C Dinauer (Indiana University School of Medicine, Indianapolis, IN, USA), was cultured in RPMI 1640 medium with I-glutamine (Invitrogen) supplemented with 10% fetal calf serum (FCS), 1% penicillin, 1% streptomycin, and 1% Fungizone. These cells were differentiated into granulocytes by 6-day incubation with dimethyl formamide (DMF) (0.5%) (23).

\textbf{H\textsubscript{2}O\textsubscript{2} production assay}

H\textsubscript{2}O\textsubscript{2} generation was measured by the method of Bénard and Brault (24). Cells (1 \times 10^6) in 3 cm dishes were incubated in Krebs–Ringer Heps (KRH) medium containing 0.1 mg/ml horseradish peroxidase type II (Sigma) and 440 \mu M homovanillic acid (Sigma), and were stimulated with the various agents. After an incubation period of 1 h for PLB-985 cells and 2 h for thyroid cells, the medium was collected, and the fluorescence intensity was determined by excitation at 315 nm and emission at 425 nm with a luminescence spectrometer LS50B (Perkin-Elmer, Wellesley, MA, USA). In parallel, cells were scraped and lysed in CHAPS buffer (10 mM Tris–HCl pH 7.5, 1 mM MgCl\textsubscript{2}, 1 mM EGTA, 0.015 mg/ml benzamidine, 10% glycerol, 5 mM β-mercaptoethanol and 5 mg/ml CHAPS), and the protein amount was then estimated by the Bradford method (25). H\textsubscript{2}O\textsubscript{2} generation was then normalized by the protein amount.

\textbf{Staining of actin filaments}

Cells in Petri dishes were fixed with methanol for 10 min at −20 °C, permeabilized with 0.1% Triton X-100 in PBS, pH 7.5, at room temperature and blocked for 20 min with normal sheep serum (5% in PBS containing 0.05% bovine serum albumin (BSA). They were incubated for 2 h at room temperature with a rabbit antiactin antibody (1/50, Sigma) diluted in PBS/BSA and then for 2 h with Texas-red conjugated antirabbit immunoglobulin (Ig) G (1/50, Amersham). Between each incubation, cells were washed three times with PBS/BSA. Finally, cells were mounted, viewed and photographed with a 50 oil immersion lens mounted on a Zeiss Axiocover 135 microscope (Carl Zeiss, Thornwood, NY, USA).

\textbf{Rac activation assay}

Small G protein Rac1 activity assay was performed with GST protein fused to the Cdc42 and Rac1 interactive binding (CRIB) domain from human PAKIB. The vector for this GST-PAK-CRIB protein was a kind gift from Dr J G Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The recombinant protein was expressed as previously described (26).

Rac activation was determined as follows. Stimulated cells (from a 90 or 60 mm dish) were rapidly washed with ice-cold PBS, lysed and scraped in 800 µl GST-fish
buffer (10% glycerol, 50 mM Tris (pH 7.4), 100 mM NaCl, 1% NP-40 and 2 mM MgCl₂ supplemented with protease inhibitors: 1 μg/ml leupeptin, 60 μg/ml Pefabloc and 1 μg/ml aprotinin). The lysate was clarified by centrifugation. Supernatants (650 μl) were incubated for 30 min at 4°C with glutathione-agarose beads (Sigma) preloaded with the GST fusion protein. After incubation, beads were washed three times in GST-fish buffer and then resuspended in Laemmli buffer (60 mM Tris·HCl (pH 6.8). 2% SDS, 150 mM 2-β-mercaptoethanol and 10% glycerol).

Samples were analyzed by SDS-polyacrylamide gel electrophoresis (15%) with the MINI-Protean II dual slab cell from Bio-Rad, followed by transfer on PVDF membranes (Perkin-Elmer). Rac1 was immunodetected with an anti-Rac1 antibody (mouse monoclonal; BD Biosciences Pharmingen, San Jose, CA, USA). A secondary antibody coupled to horseradish peroxidase (Amer-sham) was used for detection by enhanced chemiluminescence (Western Lighting; Perkin-Elmer). For each sample, an aliquot (20 μl) of the initial total cell lysate, diluted two times in Laemmli buffer, was also resolved by Western blotting to ascertain that the same amount of material was assayed.

**Results**

**H₂O₂ generation in dog thyrocytes and PLB-985: effect of toxin B**

Bacterial toxin B is produced by *C. difficile* and monoglucosylate Rho family proteins, including Rho, Rac and Cdc42 at Thr³⁷/³⁵. Glucosylation renders the Rho GTPases inactive by inhibiting effector coupling (27, 28). Before using toxin B to inactivate Rac proteins and determine their role in H₂O₂ generation in dog thyroid cells, we verified its inhibitory effect on Rac1 activity, by GST pull-down assay, and on actin cytoskeleton assembly, by actin staining.

Dog thyroid cells were treated for 24 h with toxin B (500 pg/ml or 100 ng/ml). Treatment with toxin B at 100 ng/ml strongly disrupted the actin cytoskeleton, inducing rounding up and arborization (Fig. 1A). At this concentration, toxin B completely abolished the signal for the active Rac1 as well as the one for total Rac1 (Fig. 1B). The decrease of total Rac1 amount by toxin B has also been observed by other groups and is explained by the fact that the glucolysated protein could be more sensitive to degradation by the proteasome (29, 30). The actin cytoskeleton disruption is probably due to RhoA inactivation, as well demonstrated in many cell types (27, 28, 31). In dog thyrocytes, toxin B also inhibited RhoA activity (not shown). Conversely, treatment of the thyrocytes with a concentration of 500 pg toxin B/ml had no effect on the actin cytoskeleton (Fig. 1A), and did not inactivate Rac1 (Fig. 1B) and RhoA (not shown). Rac2 is known to be expressed only in hematopoietic cells. Its expression was not detected by Western blotting analysis in dog thyroid cells (not shown).

We then determined whether dog thyrocytes are still able to produce H₂O₂ in response to the PLCβ/PKC cascade and calcium pathway when they are pretreated for
24 h with toxin B (500 pg/ml or 100 ng/ml). An increase in H$_2$O$_2$ production was observed in cells stimulated for 2 h by carbamylcholine (carbachol, 10$^{-5}$ M), acting through muscarinic receptors and activating the PLCб/PKC cascade, or by ionomycin (5 μM), a calcium ionophore, compared with control nonstimulated cells. This H$_2$O$_2$ generation was not inhibited by toxin B (100 ng/ml), suggesting that Rac does not play a role in the thyroid H$_2$O$_2$-generating system (Fig. 2A).

As a control, we have investigated the effect of toxin B on a leukocyte cell line PLB-985 differentiated into granulocytes. In these cells, H$_2$O$_2$ production induced by TPA (100 ng/ml) for 1 h was inhibited by increasing concentrations of toxin B from 1 to 100 ng/ml, demonstrating that in this case Rac is well involved in H$_2$O$_2$ generation (Fig. 2B). Inhibition was not due to cell death, as confirmed by trypan blue staining (not shown).

**Rac1 activation in dog thyrocytes**

We investigated whether Rac1 was activated in dog thyrocytes when treated by various agents increasing H$_2$O$_2$ production. The activation of Rac1 was determined by pulling down the active GTP-bound Rac1 with the GST-PAK-CRIB fusion protein, and was detected by Western blotting with an anti-Rac1 antibody. Carbamylcholine activated Rac1 in dog thyrocytes after 1 min of treatment, and the effect persisted at least for 10 min (Fig. 3A). On the other hand, neither thapsigargin (1 μM), increasing intracellular Ca$^{2+}$ level, nor the PKC activator TPA (100 ng/ml), all of which are known to induce H$_2$O$_2$ generation (8) (M Milenkovic, unpublished results), had an effect on the amount of active Rac1 (Fig. 3B and C). In combination with thapsigargin, TPA activated Rac1 at the same level as carbamylcholine (Fig. 3D), indicating that activation of both PKC- and Ca$^{2+}$-dependent cascades is required for Rac1 activation. Furthermore, TSH (1 mU/ml) and forskolin (10$^{-5}$ M), which also weakly stimulate H$_2$O$_2$ generation through an increase of cyclic AMP level (8, 32), inactivated Rac1 (Fig. 3E). Taken together, these observations confirmed that Rac activation is not required for the H$_2$O$_2$ generation in dog thyrocytes.

**Discussion**

The H$_2$O$_2$-generating system of thyroid is poorly characterized, unlike that of leukocytes. Even though ThOXs share strong structural homologies with p91phox, their role in H$_2$O$_2$ formation has not been demonstrated. Their expression in PLB-XCGD cells, a human myeloid leukemia cell line possessing all the respiratory burst machinery except the gp91 phox gene, which was knocked out (33), did not reconstitute a functional H$_2$O$_2$-generating system (11).

Our results show that although thyrocytes and macrophages share a similar oxidative phenotype and regulation, the mechanisms involved are different in these cell types. Carbamylcholine, TSH, forskolin, Ca$^{2+}$ and phorbol esters all activate H$_2$O$_2$ generation in dog thyroid cells (8). We have shown that carbamylcholine stimulates Rac1: a phorbol ester used alone and Ca$^{2+}$ increase have no effect, and forskolin and TSH inactivate it. Toxin B, a large clostridial cytotoxin inhibiting Rac small G proteins, inhibits H$_2$O$_2$ generation in leukocytes, but not in thyroid cells. These results suggest therefore that there is no correlation between Rac activation and H$_2$O$_2$ production in dog thyrocytes. This is the first reported example of a Rac-independent NADPH oxidase. In leukocytes, Rac1 or Rac2, activation is essential for H$_2$O$_2$ generation. In its active form, Rac interacts with p67phox through its
tetratricopeptide repeat (TRP) motifs (34) and with gp91phox (35, 36), and it is presumed to support the translocation of the p47phox-p67phox complex from the cytosol to the membrane to form with gp91phox and p22phox an active oxidase complex (37). Rac1 activation is also required for H$_2$O$_2$ generation in nonphagocytic cells, which, like thyrocytes, do not express p47phox and p67phox. Indeed, overexpression of a dominant-negative mutant Rac1 inhibits production of H$_2$O$_2$ in the hepatoma cell line HepG2 stimulated by platelet-derived growth factor (PDGF) or nicotinamide adenine dinucleotide (NADH) (38, 39), and in NIH3T3 stimulated by growth factors, cytokines or overexpression of constitutively activated Ras (40, 41). Conversely, a constitutively activated form of Rac1 leads to an increase in intracellular H$_2$O$_2$ concentration in these cells (38, 40).

Recently, it was reported that Rho plays an important role in superoxide formation during phagocytosis of serum-(SOZ), C3bi-(COZ) and IgG-opsonized zymosan (IOZ) particles via phosphorylation of p47phox in macrophages (42). Nevertheless, in neutrophils, inactivation of Rho by the C 3 Clostridium botulinum toxin does not affect the respiratory burst (43). As toxin B inactivates Rac but also Rho and Cdc42 (44), our results suggest that these other small GTPases, in addition to Rac, do not take part in the thyroid H$_2$O$_2$-generating system.

The regulatory mechanisms mediating the control of H$_2$O$_2$ generation by Ca$^{2+}$, diacylglycerol and cAMP in the thyroid remain to be discovered as well as the exact composition of the NADPH oxidase complex. ThOXs, but not gp91phox, contain two EF-hand domains which are probably able to bind Ca$^{2+}$. The role of such domains in ThOXs function must be determined. Like thyrocytes, nonmyeloid tissues (except endothelial cells, vascular smooth muscle cells and adventitia) do not express p47phox and p67phox (45). Recently, two homologs of p47phox and p67phox,
called ‘NOXO1’ (NOX organizer 1) and ‘NOXA1’ (NOX activator 1) respectively, were identified in colon cells as activators of superoxide formation generated by NOX1 (46–49). The existence of such homologous proteins in thyrocytes is currently under investigation.

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