EXPERIMENTAL STUDY

Regulation of H₂O₂ generation in thyroid cells does not involve Rac1 activation

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Abstract

Objectives: The H₂O₂ generating system of the thyrocyte and the O₂[−] 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, H₂O₂ generation is activated by the intracellular generation of Ca²⁺ and diacylglycerol signals. Nevertheless, although the controls involved in these two systems are similar, their mechanisms are different. The main factors controlling O₂[−] 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 H₂O₂-generating system.

Design and methods: Ionomycin- and carbamylcholine-stimulated H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 O₂[−] generation by phorbol ester in leukocytes, it does not inhibit H₂O₂ generation induced by carbamylcholine and ionomycin in dog thyrocytes.

Conclusions: Unlike in leukocytes, Rac proteins do not play a role in H₂O₂ generation in thyroid cells. A different regulatory cascade for the control of H₂O₂ generation remains to be defined.

European Journal of Endocrinology 152 127–133

Introduction

The thyroid cell shares with inflammatory cells a requirement for strong oxidizing machinery. In the thyroid cell, this machinery is formed by an H₂O₂-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 H₂O₂ to oxidize iodide and bind it to thyroglobulin. These steps are required for the synthesis of thyroid hormones (tri-iodothyronine (T₃) and thyroxine (T₄)) (2, 3). In dog thyroid cells, H₂O₂ generation is strongly activated by carbamylcholine, acting through muscarinic receptors which activate the PLCβ/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 H₂O₂-generating system is well characterized (4, 5). In these cells, the NOX2 NADPH

oxidase (or gp91^{phox}) generates O₂[−] and, after dismutation, H₂O₂. This H₂O₂ allows myeloperoxidase to oxidize chloride, as required for its bactericidal function (4, 6). NOX2 is associated with p22^{phox} in the membrane, and its activation requires its association with the p47^{phox}–p67^{phox} complex, which is translocated from the cytosol with the Rac (Rac1 or Rac2) small GTPase in its active form (7). The leukocyte and thyroid H₂O₂-generating systems share several characteristics, including the fact that both are activated by intracellular Ca²⁺ and diacylglycerol signals (8–10). However, although p22^{phox} is expressed in the thyroid, p47^{phox} and p67^{phox} 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

cell function. They control diverse and essential functions in eukaryotes, linking extracellular signals received through the membranes to, depending on the cell type, cytoskeletal changes and activation of several signaling pathways, such as those leading to phagocytosis, mitogenesis, cell adhesion, gene expression, cell cycle progression and cell survival (17). Such GTPases function as molecular switches that are active when bound to GTP and inactive in the GDP-bound conformation (18). In their active GTP-bound form, they interact with and regulate the function of downstream effector molecules to mediate their cellular effects. Activation of Rho GTPases occurs when an upstream signal activates a guanine nucleotide exchange factor (GEF) that catalyzes the release of bound GDP to enable GTP loading. Conversely, inactivation of the Rho GTPase is mediated via GTPase-activating proteins (GAPs), which stimulate its intrinsic ability to hydrolyze GTP to GDP. In leukocytes, Rac2 is activated by P-Rex1, a leukocyte-specific Rac GEF (19), and Vav1 (7).

In this paper, we ask whether Rac1 could play a role in the thyroid H_2O_2 -generating system as in the leukocyte superoxide anion production machinery. To answer this question, we inactivated Rac in dog thyrocytes by the use of toxin B and checked whether these cells were still able to produce H_2O_2 in response to stimuli. We also investigated, by glutathione S-transferase (GST) pull-down assay, whether Rac1 was activated in dog thyrocytes treated by various agents that increase H_2O_2 production.

Materials and methods

Cell culture

Primary cell cultures of dog thyrocytes were obtained from follicles isolated by collagenase digestion and differential centrifugation from fresh dog thyroid, as described previously (20, 21). The thyrocytes were cultured in the following basal medium: DMEM plus Ham's F12 medium plus MCB 104 medium (2:1:1, by volume) (Life Technologies, Paisley, UK), supplemented with ascorbic acid (40 μ g/ml), sodium pyruvate (2 mM), 2% penicillin, 2% streptomycin and 1% fungizone. At day 4 or 5 of culture, the cells were quiescent and were stimulated for various periods of time with different agents: forskolin (Calbiochem, La Jolla, CA, USA); bovine thyroid-stimulating hormone (TSH); carbamylcholine; ionomycin, a calcium ionophore; thapsigargin, a SERCAs (sarcoendoplasmic reticulum Ca^{2+} -ATPases) inhibitor inducing Ca^{2+} liberation from intracellular stores (22); or 12-O-tetradecanoylphorbol-13-acetate (TPA), which were purchased from Sigma. For Rac inhibition, *Clostridium difficile* toxin B (Sigma) was added in the medium 24 h before the stimulation.

PLB-985 human myeloid leukemia cell line (23), kindly given by Dr M C Dinanuer (Indiana University

School of Medicine, Indianapolis, IN, USA), was cultured in RPMI 1640 medium with L-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).

H_2O_2 production assay

H_2O_2 generation was measured by the method of Bénard and Brault (24). Cells (1×10^6) in 3 cm dishes were incubated in Krebs–Ringer Hepes (KRH) medium containing 0.1 mg/ml horseradish peroxidase type II (Sigma) and 440 μ 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 $MgCl_2$, 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_2O_2 generation was then normalized by the protein amount.

Staining of actin filaments

Cells in Petri dishes were fixed with methanol for 10 min at $-20^\circ C$, 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 Axiovert 135 microscope (Carl Zeiss, Thornwood, NY, USA).

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 PAK1B. 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 μ l GST-fish

buffer (10% glycerol, 50 mM Tris (pH 7.4), 100 mM NaCl, 1% NP-40 and 2 mM $MgCl_2$ supplemented with protease inhibitors: 1 μ g/ml leupeptin, 60 μ g/ml Pefabloc and 1 μ g/ml aprotinin). The lysate was clarified by centrifugation. Supernatants (650 μ l) 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 (Amersham) was used for detection by enhanced chemiluminescence (Western Lighting; Perkin-Elmer). For each sample, an aliquot (20 μ l) 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

H_2O_2 generation in dog thyrocytes and PLB-985: effect of toxin B

Bacterial toxin B is produced by *C. difficile* and monoglucosylates Rho family proteins, including Rho, Rac and Cdc42 at Thr^{37/35}. 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 H_2O_2 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 glucosylated 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

Figure 1 Effects of toxin B on cell morphology and Rac1 activity. Quiescent cells cultured in basal medium for 4 days (ctr) were treated for 24 h with toxin B (TB) at 500 pg/ml or 100 ng/ml. (A) Antiactin immunofluorescence. (B) Determination of active Rac1 by GST pull-down assay with GST-PAK-CRIB protein. The arrow indicates the active form of Rac1. The lower panel shows the total amount of Rac1 contained in a fraction of the initial total lysate.

detected by Western blotting analysis in dog thyroid cells (not shown).

We then determined whether dog thyrocytes are still able to produce H_2O_2 in response to the PLC β /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_2O_2 production was observed in cells stimulated for 2 h by carbamylcholine (carbachol, 10^{-5} M), acting through muscarinic receptors and activating the PLC β /PKC cascade, or by ionomycin (5 μM), a calcium ionophore, compared with control nonstimulated cells. This H_2O_2 generation was not inhibited by toxin B (100 ng/ml), suggesting that Rac does not play a role in the thyroid H_2O_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_2O_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_2O_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_2O_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_2O_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_2O_2 generation through an increase of cyclic AMP level (8, 32), inactivated Rac1 (Fig. 3E). Taken together, these observations confirmed that Rac1 activation is not required for the H_2O_2 generation in dog thyrocytes.

Discussion

The H_2O_2 -generating system of thyroid is poorly characterized, unlike that of leukocytes. Even though ThOXs share strong structural homologies with p91^{phox} , their role in H_2O_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 $\text{gp91}^{\text{phox}}$ gene, which was knocked out (33), did not reconstitute a functional H_2O_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_2O_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_2O_2 generation in leukocytes, but not in thyroid cells. These results suggest therefore that there is no correlation between Rac activation and H_2O_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_2O_2 generation. In its active form, Rac interacts with p67^{phox} , through its

Figure 2 Effect of toxin B on H_2O_2 generation. (A) In dog thyrocytes, quiescent cells were treated for 24 h with 500 pg/ml or 100 ng/ml toxin B (TB) and then stimulated or not (ctr) for 2 h with carbamylcholine (cch, 10^{-5} M) or ionomycin (iono, 5 μM). (B) In PLB-985 cells, differentiated cells were treated with increasing concentrations of toxin B (TB) for 24 h and then stimulated or not (ctr) for 1 h with TPA (100 ng/ml). Each condition was done in duplicate, and the error bars represent the sample standard deviation. Similar results were obtained in two independent experiments.

Figure 3 Rac1 activation in dog thyrocytes in primary culture. Quiescent cells were stimulated for the indicated time with (A) carbamylcholine (cch, 10^{-5} M), (B) thapsigargin (tg, $1 \mu\text{M}$), (C) TPA (100 ng/ml), (D) a combination of thapsigargin ($1 \mu\text{M}$) and TPA (100 ng/ml), (E) forskolin (10^{-5} M) or TSH (1 mU/ml). Activation of Rac1 was determined by GST pull-down assay. Rac1-GTP was detected by Western blotting with a monoclonal antibody, anti-Rac1. (A) Upper panel shows the total amount of Rac1 contained in $20 \mu\text{l}$ total lysate.

tetratricopeptide repeat (TRP) motifs (34) and with gp91^{phox} (35, 36), and it is presumed to support the translocation of the p47^{phox}-p67^{phox} complex from the cytosol to the membrane to form with gp91^{phox} and p22^{phox} an active oxidase complex (37). Rac1 activation is also required for H₂O₂ generation in nonphagocytic cells, which, like thyrocytes, do not express p47^{phox} and p67^{phox}. Indeed, overexpression of a dominant-negative mutant Rac1 inhibits production of H₂O₂ 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 H₂O₂ 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 p47^{phox} in macrophages (42). Nevertheless, in neutrophils, inactivation of Rho by the C3 *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 H₂O₂-generating system.

The regulatory mechanisms mediating the control of H₂O₂ generation by Ca²⁺, diacylglycerol and cAMP in the thyroid remain to be discovered as well as the exact composition of the NADPH oxidase complex. ThOXs, but not gp91^{phox}, contain two EF-hand domains which are probably able to bind Ca²⁺. 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 p47^{phox} and p67^{phox} (45). Recently, two homologs of p47^{phox} and p67^{phox},

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.

Acknowledgements

We thank J G Collard and J P ten Klooster for providing the GST-PAK-CRIB construction; X De Deken, D Wang and M Milenkovic for their help with H₂O₂ measurement; and C Degraef for her technical assistance. This work was supported by the Fonds pour la formation à la Recherche dans l'Industrie et l'Agriculture (FRIA), Fondation Rose et Jean Hoguet, Fondation Van Buuren, and Pôle d'Action Interuniversitaire (PAI).

References

- De Deken X, Wang D, Many MC, Costagliola S, Libert F, Vassart G, Dumont JE & Miot F. Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family. *Journal of Biological Chemistry* 2000 **275** 23227–23233.
- Nunez J & Pommier J. Formation of thyroid hormones. *Vitamins and Hormones* 1982 **39** 175–229.
- Taurog A. In *Werner and Ingbar's The Thyroid*, 7th edn, pp 47–84. Eds LE Braverman & RD Utiger. Philadelphia: Lippincott, 1996.
- Babior BM. NADPH oxidase: an update. *Blood* 1999 **93** 1464–1476.
- Babior BM. NADPH oxidase. *Current Opinion in Immunology* 2004 **16** 42–47.
- Winterbourn CC, Vissers MC & Kettle AJ. Myeloperoxidase. *Current Opinion in Hematology* 2000 **7** 53–58.
- Price MO, Atkinson SJ, Knaus UG & Dinanuer MC. Rac activation induces NADPH oxidase activity in transgenic COS^{phox} cells, and the level of superoxide production is exchange factor-dependent. *Journal of Biological Chemistry* 2002 **277** 19220–19228.
- Raspé E, Laurent E, Corvilain B, Verjans B, Erneux C & Dumont JE. Control of the intracellular Ca(2+) concentration and the inositol phosphate accumulation in dog thyrocyte primary culture: evidence for different kinetics of Ca(2+)–phosphatidylinositol cascade activation and for involvement in the regulation of H₂O₂ production. *Journal of Cellular Physiology* 1991 **146** 242–250.
- Corvilain B, van Sande J, Laurent E & Dumont JE. The H₂O₂-generating system modulates protein iodination and the activity of the pentose phosphate pathway in dog thyroid. *Endocrinology* 1991 **128** 779–785.
- McPhail LC & Snyderman R. Activation of the respiratory burst enzyme in human polymorphonuclear leukocytes by chemoattractants and other soluble stimuli. Evidence that the same oxidase is activated by different transductional mechanisms. *Journal of Clinical Investigation* 1983 **72** 192–200.
- De Deken X, Wang D, Dumont JE & Miot F. Characterization of ThOX proteins as components of the thyroid H(2)O(2)-generating system. *Experimental Cell Research* 2002 **273** 187–196.
- Abo A, Pick E, Hall A, Totty N, Teahan CG & Segal AW. Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. *Nature* 1991 **353** 668–670.
- Knaus UG, Heyworth PG, Kinsella BT, Curnutte JT & Bokoch GM. Purification and characterization of Rac 2. A cytosolic GTP-binding protein that regulates human neutrophil NADPH oxidase. *Journal of Biological Chemistry* 1992 **267** 23575–23582.
- Shirsat NV, Pignolo RJ, Kreider BL & Rovera G. A member of the ras gene superfamily is expressed specifically in T, B and myeloid hemopoietic cells. *Oncogene* 1990 **5** 769–772.
- Didsbury J, Weber RF, Bokoch GM, Evans T & Snyderman R. Rac, a novel ras-related family of proteins that are botulinum toxin substrates. *Journal of Biological Chemistry* 1989 **264** 16378–16382.
- Haataja L, Groffen J & Heisterkamp N. Characterization of RAC3, a novel member of the Rho family. *Journal of Biological Chemistry* 1997 **272** 20384–20388.
- Etienne-Manneville S & Hall A. Rho GTPases in cell biology. *Nature* 2002 **420** 629–635.
- Scita G, Tenca P, Frittoli E, Tocchetti A, Innocenti M, Giardina G & Di Fiore PP. Signaling from Ras to Rac and beyond: not just a matter of GEFs. *EMBO Journal* 2000 **19** 2393–2398.
- Welch HC, Coadwell WJ, Ellison CD, Ferguson GJ, Andrews SR, Erdjument-Bromage H, Tempst P, Hawkins PT & Stephens LR. P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. *Cell* 2002 **108** 809–821.
- Roger PP, Hotimsky A, Moreau C & Dumont JE. Stimulation by thyrotropin, cholera toxin and dibutyryl cyclic AMP of the multiplication of differentiated thyroid cells in vitro. *Molecular and Cellular Endocrinology* 1982 **26** 165–176.
- Roger P, Taton M, Van Sande J & Dumont JE. Mitogenic effects of thyrotropin and adenosine 3',5'-monophosphate in differentiated normal human thyroid cells in vitro. *Journal of Clinical Endocrinology and Metabolism* 1988 **66** 1158–1165.
- Treiman M, Caspersen C & Christensen SB. A tool coming of age: thapsigargin as an inhibitor of sarco-endoplasmic reticulum Ca(2+)–ATPases. *Trends in Pharmacological Sciences* 1998 **19** 131–135.
- Tucker KA, Lilly MB, Heck L Jr & Rado TA. Characterization of a new human diploid myeloid leukemia cell line (PLB-985) with granulocytic and monocytic differentiating capacity. *Blood* 1987 **70** 372–378.
- Bénard B & Brault J. [Production of peroxide in the thyroid]. *Union Médicale du Canada* 1971 **100** 701–705.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 1976 **72** 248–254.
- Sander EE, Van Delft S, ten Klooster JP, Reid T, Van der Kammen RA, Michiels F & Collard JG. Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell–cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *Journal of Cell Biology* 1998 **143** 1385–1398.
- Just I, Fritz G, Aktories K, Giry M, Popoff MR, Boquet P, Hegenbarth S & Eichel-Streiber C. *Clostridium difficile* toxin B acts on the GTP-binding protein Rho. *Journal of Biological Chemistry* 1994 **269** 10706–10712.
- Dillon ST, Rubin EJ, Yakubovich M, Pothoulakis C, LaMont JT, Feig LA & Gilbert RJ. Involvement of Ras-related Rho proteins in the mechanisms of action of *Clostridium difficile* toxin A and toxin B. *Infection and Immunity* 1995 **63** 1421–1426.
- Clerk A, Pham FH, Fuller SJ, Sahai E, Aktories K, Marais R, Marshall C & Sugden PH. Regulation of mitogen-activated protein kinases in cardiac myocytes through the small G protein Rac1. *Molecular and Cellular Biology* 2001 **21** 1173–1184.
- Hahn A, Barth H, Kress M, Mertens PR & Goppelt-Strube M. Role of Rac and Cdc42 in lysophosphatidic acid-mediated cyclo-oxygenase-2 gene expression. *Biochemical Journal* 2002 **362** 33–40.
- Giry M, Popoff MR, Eichel-Streiber C & Boquet P. Transient expression of RhoA, -B, and -C GTPases in HeLa cells potentiates resistance to *Clostridium difficile* toxins A and B but not to *Clostridium sordellii* lethal toxin. *Infection and Immunity* 1995 **63** 4063–4071.
- Raspé E & Dumont JE. Tonic modulation of dog thyrocyte H₂O₂ generation and I[−] uptake by thyrotropin through the cyclic adenosine 3',5'-monophosphate cascade. *Endocrinology* 1995 **136** 965–973.
- Zhen L, King AA, Xiao Y, Chanock SJ, Orkin SH & Dinanuer MC. Gene targeting of X chromosome-linked chronic granulomatous disease locus in a human myeloid leukemia cell line and rescue

- by expression of recombinant gp91^{phox}. *PNAS* 1993 **90** 9832–9836.
- 34 Koga H, Terasawa H, Nunoi H, Takeshige K, Inagaki F & Sumimoto H. Tetratricopeptide repeat (TPR) motifs of p67(phox) participate in interaction with the small GTPase Rac and activation of the phagocyte NADPH oxidase. *Journal of Biological Chemistry* 1999 **274** 25051–25060.
 - 35 Bokoch GM & Knaus UG. NADPH oxidases: not just for leukocytes anymore! *Trends in Biochemical Sciences* 2003 **28** 502–508.
 - 36 Heyworth PG, Bohl BP, Bokoch GM & Curnutte JT. Rac translocates independently of the neutrophil NADPH oxidase components p47^{phox} and p67^{phox}. Evidence for its interaction with flavocytochrome b558. *Journal of Biological Chemistry* 1994 **269** 30749–30752.
 - 37 Diebold BA & Bokoch GM. Molecular basis for Rac2 regulation of phagocyte NADPH oxidase. *Nature Immunology* 2001 **2** 211–215.
 - 38 Cool RH, Merten E, Theiss C & Acker H. Rac1, and not Rac2, is involved in the regulation of the intracellular hydrogen peroxide level in HepG2 cells. *Biochemical Journal* 1998 **332** (Pt 1) 5–8.
 - 39 Bae YS, Sung JY, Kim OS, Kim YJ, Hur KC, Kazlauskas A & Rhee SG. Platelet-derived growth factor-induced H₂O₂ production requires the activation of phosphatidylinositol 3-kinase. *Journal of Biological Chemistry* 2000 **275** 10527–10531.
 - 40 Sundaresan M, Yu ZX, Ferrans VJ, Sulciner DJ, Gutkind JS, Irani K, Goldschmidt-Clermont PJ & Finkel T. Regulation of reactive-oxygen-species generation in fibroblasts by Rac1. *Biochemical Journal* 1996 **318** (Pt 2) 379–382.
 - 41 Irani K, Xia Y, Zweier JL, Sollott SJ, Der CJ, Fearon ER, Sundaresan M, Finkel T & Goldschmidt-Clermont PJ. Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. *Science* 1997 **275** 1649–1652.
 - 42 Kim JS, Diebold BA, Kim JI, Kim J, Lee JY & Park JB. Rho is involved in superoxide formation during phagocytosis of opsonized zymosans. *Journal of Biological Chemistry* 2004 **279** 21589–21597.
 - 43 Stasia MJ, Jouan A, Bourmeyster N, Boquet P & Vignais PV. ADP-ribosylation of a small size GTP-binding protein in bovine neutrophils by the C3 exoenzyme of *Clostridium botulinum* and effect on the cell motility. *Biochemical and Biophysical Research Communication* 1991 **180** 615–622.
 - 44 Just I, Hofmann F, Genth H & Gerhard R. Bacterial protein toxins inhibiting low-molecular-mass GTP-binding proteins. *International Journal of Medical Microbiology* 2001 **291** 243–250.
 - 45 Werner E. GTPases and reactive oxygen species: switches for killing and signaling. *Journal of Cell Science* 2004 **117** 143–153.
 - 46 Banfi B, Clark RA, Steger K & Krause KH. Two novel proteins activate superoxide generation by the NADPH oxidase NOX1. *Journal of Biological Chemistry* 2003 **278** 3510–3513.
 - 47 Geiszt M, Lekstrom K, Witta J & Leto TL. Proteins homologous to p47^{phox} and p67^{phox} support superoxide production by NAD(P)H oxidase 1 in colon epithelial cells. *Journal of Biological Chemistry* 2003 **278** 20006–20012.
 - 48 Takeya R, Ueno N, Kami K, Taura M, Kohjima M, Izaki T, Nunoi H & Sumimoto H. Novel human homologues of p47^{phox} and p67^{phox} participate in activation of superoxide-producing NADPH oxidases. *Journal of Biological Chemistry* 2003 **278** 25234–25246.
 - 49 Cheng G & Lambeth JD. NOXO1, regulation of lipid binding, localization, and activation of Nox1 by the Phox homology (PX) domain. *Journal of Biological Chemistry* 2004 **279** 4737–4742.

Received 21 June 2004

Accepted 30 September 2004