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Regulation of Phagocyte NADPH Oxidase by Hydrogen Peroxide through a Ca^{2+} /c-Abl Signaling Pathway

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Abstract

The importance of H_2O_2 as a cellular signaling molecule has been demonstrated in a number of cell types and pathways. Here we explore a positive feedback mechanism of H_2O_2 -mediated regulation of the phagocyte respiratory burst NADPH oxidase (NOX2). H_2O_2 induced a dose-dependent stimulation of superoxide production in human neutrophils, as well as in K562 leukemia cells over-expressing NOX2 system components. Stimulation was abrogated by addition of catalase, the extracellular Ca^{2+} chelator BAPTA, the T-type Ca^{2+} channel inhibitor mibefradil, the PKC δ inhibitor rottlerin, or the c-Abl non-receptor tyrosine kinase inhibitor imatinib mesylate, or by over-expression of a dominant-negative form of c-Abl. H_2O_2 induced phosphorylation of tyrosine³¹¹ on PKC δ and this activating phosphorylation was blocked by treatment with rottlerin, imatinib mesylate, or BAPTA. Rac GTPase activation in response to H_2O_2 was abrogated by BAPTA, imatinib mesylate, or rottlerin. In conclusion, H_2O_2 stimulates NOX2-mediated superoxide generation in neutrophils and K562/NOX2 cells via a signaling pathway involving Ca^{2+} influx and c-Abl tyrosine kinase acting upstream of PKC δ . This positive feedback regulatory pathway has important implications for amplifying the innate immune response and contributing to oxidative stress in inflammatory disorders.

Keywords

NADPH oxidase; NOX2; Neutrophil; Superoxide; Hydrogen peroxide; Signaling; c-Abl, Calcium; Free radicals

Introduction

Much of our understanding of the mechanisms of generation and functions of reactive oxygen species (ROS) is based on studies of peripheral blood neutrophils, wherein microbicidal oxidants released during the phagocytosis of microorganisms serve as a first line of host

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Author Disclosure Statement

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defense. In phagocytic cells, the major biochemical pathway for ROS formation proceeds through superoxide anion, which is generated through the single-electron reduction of molecular oxygen by an NADPH oxidase complex. Phagocyte NADPH oxidase is composed of the membrane-bound flavocytochrome b_{558} (a heterodimer of NOX2/gp91^{phox} and p22^{phox}), several modular cytosolic regulators (p47^{phox}, p67^{phox}, p40^{phox}), and a small GTPase, Rac1 or Rac2 [1,2]. The stimulus-dependent assembly of an active oxidase is tightly regulated, requiring translocation of the cytosolic components and their physical association with the membrane-bound flavocytochrome. Following cell stimulation by phagocytosis or soluble mediators, p47^{phox} undergoes extensive serine phosphorylation by a number of kinases and recruits p67^{phox} to the nascent membrane-associated complex. Rac, activated by GDP-GTP exchange, associates with the forming complex where, together with p67^{phox}, it regulates electron flow through the NOX2 catalytic subunit [3,4].

We recently demonstrated that NOX5, a Ca^{2+} -regulated NOX isoform, is activated by H_2O_2 through a novel signaling pathway requiring Ca^{2+} influx and c-Abl tyrosine kinase activation [5]. Whereas NOX5 activation ordinarily requires the direct binding of Ca^{2+} to the EF-hand domains present in its cytosolic N-terminal extension [6], the assembly of NOX2 features translocation of the cytosolic factors to the membrane-bound flavocytochrome, a process that involves Ca^{2+} only in proximal signaling events, rather than for direct binding to NOX [7,8]. In the present work, we investigated the hypothesis that NOX2, the index member of the NOX family, is regulated by H_2O_2 and explored the signaling mechanisms involved. In human blood neutrophils, as well as in K562 human leukemia cells over-expressing the NOX2 system, we found that H_2O_2 stimulates NOX2-catalyzed superoxide generation and that this regulation involves Ca^{2+} and c-Abl tyrosine kinase pathways upstream of the classical protein kinase C (PKC) pathway. Furthermore, H_2O_2 enhances and accelerates the oxidative burst in response to phorbol myristate acetate (PMA).

Experimental procedures

Isolation of neutrophils

Human neutrophils >90% pure were isolated from heparinized venous blood by dextran sedimentation, centrifugation on Hypaque-Ficoll, and hypotonic lysis of erythrocytes as described previously [9].

Expression of the NOX2 NADPH oxidase system in K562 cells

The clonal K562 human leukemia cell line stably expressing human NOX2 (gp91^{phox}) has been described previously [10]. The cells were maintained in complete RPMI medium (RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin). For the expression of p47^{phox} and p67^{phox}, cells in the logarithmic stage of growth were washed once, and resuspended in complete medium to 15×10^6 cells/ml. The cDNAs for human p67^{phox} and p47^{phox} (25 μg total) in the episomal vector pREP4 (Invitrogen, Carlsbad, CA), were added to 375 μl of the cells and 325 μl aliquots electroporated at 250 volts/960 μF d. Stable expressing cells were selected in medium containing 250 $\mu\text{g}/\text{ml}$ of hygromycin for 7 days, then subsequently maintained in medium with 150 $\mu\text{g}/\text{ml}$ of hygromycin. Expression of the p67^{phox}/p47^{phox} proteins in K562/NOX2 cells was confirmed by western blot analysis and by measuring superoxide generation (see below). These cells are referred to as K562/NOX2 cells. When the over-expression of a protein in addition to the *phox* proteins was required, we used transient transfection and pcDNA3.1 vectors containing the cDNAs of interest.

Stable expression of GFP-c-Abl fusion proteins

The expression plasmid pcDNA3.1/Zeo (+) containing cDNAs for either wild-type c-Abl (GFP-c-Abl) or kinase-dead c-Abl (GFP-KD-c-Abl) was linearized and transfected into K562 cells as described above. Stable expressing cells were selected in 250 µg/ml of zeocin for 14 days. Single-cell clones were established by limiting dilution in 96-well plates. The expression of GFP-c-Abl and GFP-KD-c-Abl in the selected clones was determined by fluorescence microscopy. These cells were then transiently transfected with pcDNA3.1 vectors encoding NOX2, p67^{phox}, and p47^{phox}.

Cell culture, inhibitors, and subcellular fractionation

Transfected K562 cells grown in complete RPMI medium, or freshly isolated neutrophils were treated as indicated in the text with inhibitors of PKC (staurosporine 1 µM, 30 min), PKCδ (rottlerin 20 µM, 3h), Src family kinases (genistein 10 µM, 30 min), c-Abl tyrosine kinase (imatinib mesylate 10 µM, overnight), SERCA (thapsigargin [TG] 100 nM, 30 min), T-type Ca²⁺ channels (mibefradil 15 µM, 1h), G-proteins (pertussis toxin [PTX] 10 µg/ml, 1h) and RhoGTPase (*Clostridium difficile* toxin B 1 nM, overnight). Cells were also treated, where indicated, with PMA 1 µg/ml or the extracellular Ca²⁺ chelator BAPTA (50 µM). Control cells were treated with vehicle dimethyl sulfoxide or phosphate-buffered saline (PBS) plus 10 mM glucose (PBS-G). At the end of the treatment the cells were washed in PBS-G and treated with H₂O₂ (100 µM) for 10 min at 37°C. Cell lysis was carried out in buffer A (20 mM HEPES, pH 7.9; 350 mM NaCl; 0.5 mM EDTA; 0.5 mM EGTA; 1 mM MgCl₂) plus 10% glycerol, 1% Nonidet P-40, 10 mM NaF, 0.1 mM NaVO₄, 8 mM β-glycerophosphate, phosphatase inhibitor cocktail I and II (Sigma), and a protease inhibitor cocktail (Roche, Mannheim, Germany; 1 tablet/50 ml buffer). Lysates were cleared by centrifugation, and when required, the total protein extracts were centrifuged at 100,000 g for 1 h to separate crude membranes from cytosolic proteins. Protein content was estimated as described [11].

Superoxide assay in whole cells

Superoxide generation was measured using a luminol-based chemiluminescence assay (Diogenes, National Diagnostics). Cells were collected by centrifugation, washed once in PBS, resuspended at 5×10⁶/ml in PBS-G, and kept on ice until assayed. For the assay, 100 µl of the luminol reagent were mixed with 0.25 to 0.5×10⁶ cells and incubated at 37°C for 2–4 min. Superoxide generation was stimulated by the addition of PMA 1 µg/ml in PBS-G, H₂O₂ 0–500 µM, or glucose oxidase (0.25 mU/ml) in the presence of glucose (5 mM) or the addition of formyl-methionyl-leucyl-phenylalanine (fMLF; 100 nM). Chemiluminescence was measured every 30–60 s using a Turner Designs 20/20 luminometer and a 5 s integration time.

Broken-cell NADPH oxidase assay

Neutrophils were disrupted by sonication in buffer B (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 1.25 mM EGTA, 10 mM PMSF, 10 mM NaF, 0.1 mM, NaVO₄, 8 mM β-glycerophosphate, phosphatase inhibitor cocktail I and II [Sigma], and a protease inhibitor cocktail [Roche, Mannheim, Germany; 1 tablet/50 ml buffer]). Lysates were cleared by centrifugation. Crude membranes were separated from cytosolic proteins by centrifugation at 100,000 g for 1 h. Protein content was estimated as described [11]. Mixtures containing PBS, pH 7.5, 2 mM MgCl₂, 20 mM GTPγS, 200 µM ATP, 10 nM arachidonic acid/µg of protein and 5 µg of membrane protein mixed with 45 µg of cytosolic proteins in a final volume of 100 µl were used to reconstitute NADPH oxidase activity. Superoxide production was initiated with the addition of 200 µM NADPH and determined by measuring the SOD-inhibitable reduction of cytochrome C (100 µM) and quantitated using $\Delta\epsilon_{550} = 21,000 \text{ M}^{-1} \text{ cm}^{-1}$ [12] blanked against identical wells containing 400 U/ml SOD. Average rates of superoxide

generation were calculated from the linear region of increase in absorbance at 550 nm and were expressed as nmol of $O_2^{\bullet}$ /min/mg of membrane protein.

Rac activation assay (GST-PAK-CRIB pull-down assay)

Cell lysates prepared as above in buffer A were pre-cleared with GST-bound GSH-Sepharose, then incubated at 4°C for 30 min with 10 µg of GST-Cdc42/Rac interactive binding (CRIB) region (amino acids 54–165) of PAK1 (p21-activated kinase 1) protein (GST-PAK-CRIB) immobilized on glutathione-Sepharose beads [13]. The bead pellet was thrice-washed with the lysis buffer and resuspended in 40 µl of SDS-PAGE sample buffer. The samples were separated on 12% SDS-PAGE gels, transferred to a nitrocellulose membrane, and blotted with a monoclonal antibody to Rac1/2 (Upstate Biotechnology). Parallel immunoblots were carried out on 20 µg of total cell lysate to quantitate the total amount of Rac1/2 protein. The antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL, Amersham Corp.).

Immunoblotting

Protein extracts (20–50 µg) were separated on 4–20% SDS polyacrylamide gradient gels and transferred to polyvinylidene difluoride membranes. The membranes were then incubated with antibodies against c-Abl (Sigma, cat # A5844), phosphotyrosine-245 c-Abl (Cell Signaling, cat # 2861), PKCδ (Santa Cruz Biotechnology, cat # sc937), phosphotyrosine-311 PKCδ (Cell Signaling, cat # 2055), p47^{phox} or p67^{phox} (William M. Nauseef, University of Iowa), or NOX2 [14]. Actin (Sigma) was used as a loading control. The antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL, Amersham Corp.).

Live cell imaging

Transfected K562 cells were washed twice in PBS-G and plated on poly-L-lysine-coated-glass coverslips for 60 min (3×10^5 cells/ml) in complete RPMI medium without phenol red. The cells were loaded with 6 µM fluo-4-AM and 300 nM dihydroethidium (DHE; Molecular Probes). Ca^{2+} influx and superoxide production were recorded using a confocal microscope (LSM 510; Carl Zeiss MicroImaging, Inc.) with a 63X/1.4 NA plan-apochromat objective. Fluo-4 and DHE fluorescence were excited with dual laser at 488/543 nm, attenuated to avoid photobleaching and saturation. Detection was through a 545 nm long-pass dichroic mirror and a band-pass filter at 500–530 nm for fluo-4 fluorescence and LP560 for DHE fluorescence. Image acquisition was performed with the Zeiss LSM510 Software 3.2 SP2.

Statistical analysis

Data are presented as the mean $\pm$ SEM of the values and were normalized to controls. Statistical analysis was performed using the Dunnett multiple comparisons test to adjust for multiple testing when comparing several means against the mean for a common control sample. A value of $P < 0.05$ was accepted as significant.

Results

H₂O₂ induces superoxide generation by NOX2 NADPH oxidase

Addition of 100 µM H₂O₂ to freshly isolated neutrophils induced a rapid and significant increase in chemiluminescence (via the Diogenes assay) that reached maximum values within 2 to 5 min and persisted at high levels for 20 to 40 min (Fig. 1A). Very little chemiluminescence was observed in the absence of H₂O₂ (Fig. 1A), cells, or Diogenes reagent (data not shown). Furthermore, the induction of chemiluminescence by H₂O₂ was completely abrogated by the prior addition of superoxide dismutase (400 U/ml) or diphenylene iodonium (10 µM), but not by azide (1 mM) (data not shown). Thus, H₂O₂ induced a marked and sustained production of

superoxide by neutrophils. The level of superoxide generated was directly dependent on the number of neutrophils (Fig. 1B) and the concentration of H_2O_2 (Fig. 1C) and was largely blocked by the addition of catalase (Fig. 1D). A combination of glucose oxidase 25 mU/ml and 5 mM glucose mimicked the effect observed with H_2O_2 (Fig. 1E), albeit with slower kinetics (Fig. 1F). Using this system, a period of about thirty min was required to reach the level of superoxide produced with 100 μM H_2O_2 in 5–10 min. Analogous to H_2O_2 , the effect was dependent on the concentration of glucose oxidase (Fig. 1F) and was inhibited by catalase (Fig. 1F). In comparison with fMLF, H_2O_2 was less potent in stimulating peak superoxide generation, but its effect was more sustained (data not shown). Furthermore, H_2O_2 pre-treatment increased superoxide production stimulated by PMA (Fig. 1G). These results show that not only does H_2O_2 serve as a primary activator of NOX2, but it also acts synergistically with PMA to enhance superoxide production.

Since neutrophils are short-lived cells and therefore only modestly amenable to molecular interventions *in vitro*, further studies of the mechanisms underlying the regulation of NOX2 by H_2O_2 were carried out using the human K562 cell line. Untransfected K562 cells express Rac and the p22^{phox} component of NADPH oxidase, but not NOX2, p67^{phox}, p47^{phox} or p40^{phox}. Therefore, to study the NOX2 system, the essential components NOX2, p67^{phox}, and p47^{phox} were transfected into K562 cells. H_2O_2 induced a substantial increase in superoxide production in these K562/NOX2 cells, with maximum activity observed 10–20 min after addition of 100 μM H_2O_2 (Fig. 2A). To confirm that the chemiluminescence detected was due to the activity of the NOX2 system, we compared superoxide generation in these cells with K562 cells expressing only the p67^{phox} and p47^{phox} cytosolic factors. Under these conditions there was little or no induction of superoxide generation by either H_2O_2 or PMA (Fig. 2A and 2D, respectively). The activation of NOX2 was dose-dependent from 10 to 500 μM H_2O_2 (Fig. 2B), over which range there was no effect on cell viability as determined by trypan blue exclusion (data not shown). The response to H_2O_2 was abrogated by the addition of catalase (Fig. 2C). Pre-incubation of K562/NOX2 cells with H_2O_2 enhanced their response to PMA (Fig. 2D and 2E). Relative to the effect of PMA alone, H_2O_2 pre-incubation resulted in increases in both the rate (6- to 10-fold) and total amount (3-fold) of superoxide produced. This effect gradually decreased and was undetectable after 1 h (Fig. 2D).

Role of Ca^{2+} influx in NOX2 activation by H_2O_2

Based on our previous work on H_2O_2 -induced NOX5-mediated superoxide generation [5], we investigated whether Ca^{2+} influx is involved in H_2O_2 -induced NOX2 activity. The induction of superoxide generation in K562/NOX2 cells by H_2O_2 was abrogated by treatment with either the Ca^{2+} chelator BAPTA or the T-type Ca^{2+} -channel blocker mibefradil (Fig. 3A). In contrast, depletion of intracellular Ca^{2+} stores by thapsigargin had no significant effect on H_2O_2 -induced superoxide generation, whereas it increased somewhat the basal level of superoxide production. The synergistic effect of H_2O_2 on PMA-stimulated superoxide generation was significantly reduced by pre-treatment with either BAPTA or mibefradil, but not thapsigargin (Fig. 3B). These results suggest that H_2O_2 activation of NOX2 as well as its synergistic effect on PMA stimulation requires an increase in cytosolic Ca^{2+} derived largely by influx from the extracellular pool.

The role of Ca^{2+} in the activation of NOX2 by H_2O_2 was also investigated by confocal imaging using the Ca^{2+} -sensitive probe fluo-4 and the superoxide-sensitive probe dihydroethidium (DHE) (Fig. 3C). Only K562 cells expressing the complete NOX2 system demonstrated superoxide formation (red fluorescence) following treatment with H_2O_2 , whereas Ca^{2+} influx (green fluorescence) was induced by H_2O_2 both in K562 cells expressing the complete NOX2 system and in those expressing p47^{phox} and p67^{phox} only. Notably, Ca^{2+} influx in response to H_2O_2 treatment was accentuated in cells expressing the complete NOX2 system, suggesting a

positive feedback effect of NOX2 products on H₂O₂ signaling, as we have described for NOX5 [5].

Role of c-Abl in NOX2 activation by H₂O₂

To investigate the role of c-Abl in H₂O₂-NOX2 regulation, we first treated K562/NOX2 cells with imatinib mesylate (10 μ M, overnight), an inhibitor of Abl tyrosine kinase. This agent completely blocked NOX2 stimulation by H₂O₂ (Fig. 4A) and significantly reduced the effect of H₂O₂ on PMA-stimulated superoxide production (Fig. 4B). Since imatinib is not totally specific for c-Abl tyrosine kinase, we also used stable K562 cell lines over-expressing either the GFP-tagged wild-type c-Abl (GFP-c-Abl), or the GFP-tagged kinase-dead c-Abl (GFP-KD-c-Abl), and transiently transfected with the NOX2 system components (gp91^{phox}/p67^{phox}/p47^{phox}). Over-expression of the enzymatically active GFP-c-Abl significantly enhanced both the basal and H₂O₂-induced activity of NOX2, whereas over-expression of the dominant-negative GFP-KD-c-Abl markedly reduced both basal superoxide production and the response to H₂O₂ (Fig. 4C). Similar effects were observed for the effect of H₂O₂ on PMA-stimulated NOX2 activity (Fig. 4D). These results show that c-Abl is a key intermediate in the NOX2-activating effects of H₂O₂.

Role of PKC δ in NOX2 activation by H₂O₂

PKC is an important mediator of neutrophil NOX2 activation. Since both Ca²⁺ influx and c-Abl can induce PKC activation, we analyzed the effect of PKC inhibitors, namely staurosporine, a broad inhibitor of all PKC subtypes, and rottlerin, which is specific for PKC δ . A member of the novel (n)PKC subfamily of isoforms requiring diacylglycerol (DAG), but not Ca²⁺, PKC δ participates in NOX2 activation in response to fMLF or zymosan by phosphorylating p47^{phox} [15,16]. PKC δ and c-Abl also cross-phosphorylate and activate each other [17]. We observed in K562/NOX2 cells that both H₂O₂ induction of NOX2 activity and its effect on PMA activation were abrogated by staurosporine (data not shown). When the cells were pre-treated with rottlerin, a significant reduction in stimulation of NOX2 activity by H₂O₂ was observed (Fig. 5A). In addition, rottlerin interfered with both the effect of H₂O₂ on PMA stimulation of NOX2 and the activation of NOX2 by PMA alone (Fig. 5B). Western blot analysis of PKC δ demonstrated that H₂O₂ induced phosphorylation of tyrosine³¹¹, a reaction that was blocked by rottlerin, BAPTA, or imatinib (Fig. 5C). PMA, used here as a positive control, predictably induced phosphorylation of PKC δ , but no additional phosphorylation was observed upon subsequent exposure to H₂O₂ (Fig. 5C). This observation correlates with marked superoxide production stimulated by PMA, but no further increment upon subsequent addition of H₂O₂. These results altogether suggest that PKC δ activation plays a major role in the regulation of NOX2 by H₂O₂ and that it lies downstream of Ca²⁺ entry and c-Abl activation.

Role of Rac in NOX2 activation by H₂O₂

Since Rac is required for agonist-stimulated NOX2 activation and a connection between c-Abl and Rac activation has been reported [18], we analyzed the potential role of Rac activation in NOX2 regulation by H₂O₂. In K562/NOX2 cells, H₂O₂ exposure resulted in activation of Rac, as assessed by the GST-PAK pull-down assay (Fig. 6A). Pre-treatment of the cells with the Rac inhibitor toxin B of *C. difficile*, BAPTA, imatinib, or rottlerin abrogated or reduced the effect of H₂O₂ on Rac activation. PMA-induced Rac activation was not affected by the subsequent addition of H₂O₂ (Fig. 6A). Pre-treatment with toxin B significantly reduced the production of superoxide stimulated by H₂O₂ (Fig. 6B). In cells pre-treated with the toxin, the effect of H₂O₂ on PMA-stimulated superoxide production was significantly reduced, and the stimulation by PMA itself was reduced by approximately 20% (Fig. 6C). These results suggest a common pathway for PMA and H₂O₂ in the activation of Rac through at least PKC δ and

demonstrate that the regulation of NOX2 by H₂O₂ requires activation of Rac downstream of Ca²⁺ influx and c-Abl activation.

Similar H₂O₂-NOX2 regulation pathways in neutrophils and K562/NOX2 cells

As for K562/NOX2 cells, H₂O₂-induced superoxide production by neutrophils was significantly reduced by BAPTA, mibefradil, or staurosporine, but not by thapsigargin (Fig. 7A). The synergistic effect of H₂O₂ pre-treatment for superoxide stimulation by PMA was also observed in these cells (see Fig. 1E).

Western blot analysis performed on neutrophil crude membranes showed that H₂O₂ induced the translocation to the membrane of the cytosolic factors p67^{phox} and p47^{phox} (Fig. 7B), a key feature of classical agonist-stimulated NOX2 activation. H₂O₂ also induced Rac activation in neutrophils (Fig. 7C). Furthermore, induction of superoxide production by H₂O₂ correlated with an increase in phosphorylation of both c-Abl (Fig. 7D) and PKCδ (Fig. 7E). These observations suggest that the signaling pathways involved in H₂O₂-NOX2 regulation in neutrophils are very similar to those demonstrated in K562/NOX2 cells.

Broken cell system analysis of effects of H₂O₂ pre-treatment

Reconstitution of NOX2 activity in the broken cell system was examined using different combinations of membranes and cytosol isolated from neutrophils pre-treated or not with 100 μM H₂O₂ (Fig. 7F). The rate of superoxide generation in the broken cell assay performed with membranes plus cytosol isolated from H₂O₂-treated cells, was much higher than in assays performed with membranes plus cytosol isolated from untreated control cells (*p*<0.05). Moreover, the level of superoxide produced by H₂O₂-treated cytosol plus untreated membranes tended to be increased relative to untreated cytosol plus untreated membranes and similar to the amount produced by untreated cytosol plus H₂O₂-treated membranes, although neither of these combinations generated as much activity as when both cytosol and membranes were derived from H₂O₂-treated cells (Fig. 7F). These observations may reflect both the presence of activated co-factors in H₂O₂-treated cytosol and translocated cytosolic factors in the plasma membranes isolated from H₂O₂-treated cells. The fact that superoxide generation was highest with the combination of treated membranes plus treated cytosol suggests that gp91^{phox} might itself be a final target of H₂O₂, particularly since the increased superoxide production was not attributable to an increase in NOX2 content of the membranes from H₂O₂-treated cells.

Lack of a priming effect of H₂O₂ on fMLF-stimulated superoxide production

Since H₂O₂ increases superoxide production induced by PMA, we investigated whether H₂O₂ could act as a priming factor for receptor-mediated superoxide production. We found that pre-incubation for 10 min with 10 nM to 100 μM H₂O₂ did not prime the cells for activation by 100 nM fMLF (Fig. 8A). On the contrary, a decrease in superoxide production was observed, suggesting that common signaling intermediates may be involved in H₂O₂ and fMLF-mediated superoxide production. Since signaling initiated by fMLF binding to its receptor proceeds through PTX-sensitive G proteins, which can be activated by H₂O₂ via cysteine oxidation [19], we investigated whether these G proteins might be involved in the effect of H₂O₂ on superoxide production. We observed a significant decrease in H₂O₂-induced superoxide production by PMNs pre-treated with PTX, an inhibitor of Gi/o proteins (Fig. 8B). A similar effect of PTX was observed in K562/NOX2 cells (data not shown). These observations point to at least one common proximal target of H₂O₂ and fMLF, namely a PTX-sensitive G protein.

Discussion

The work reported herein investigated whether NOX2 is regulated by H₂O₂ and examined the signaling pathways involved in this regulation (see Fig. 8C for a schema of proposed pathways).

We found that NOX2 is indeed positively regulated by H₂O₂ in both primary cells (i.e. human blood neutrophils) and K562 human leukemia cells over-expressing NOX2 and its cofactors. These results are in accord with our studies on NOX5 [5], as well as reports showing that H₂O₂ induces superoxide production by one or more non-phagocytic NADPH oxidases in vascular tissues [20]. We observed that Ca²⁺ influx is an essential proximal event required for the induction of NOX2-dependent superoxide production by H₂O₂. In accord with our findings, it has been shown recently that NOX2 regulates neutrophil membrane potential and Ca²⁺ influx not only via its electrogenic activity (i.e. catalytic transmembrane electron transfer), but also as a downstream consequence of the generation of ROS [21]. It is well known that Ca²⁺ mobilization plays an important role in classical receptor-mediated activation of superoxide production by phagocytic cells [22–24]. For example, similar to our observations in H₂O₂-treated cells, Ca²⁺ chelating agents block agonist/receptor-stimulated superoxide generation [22,25,26].

The receptor-mediated Ca²⁺ influx required for activation of NOX2-dependent superoxide production in phagocytic cells is predominantly a result of the activation of so-called capacitative Ca²⁺ entry via store-operated Ca²⁺ (SOC) channel(s), which are activated by the emptying of intracellular Ca²⁺ stores [27]. Although store-operated Ca²⁺ entry (SOCE) provides a mechanistic link between cytosolic [Ca²⁺] elevation and superoxide anion production, it cannot by itself account for the Ca²⁺ entry involved in H₂O₂-NOX2 regulation. For example, we observed that thapsigargin, an inhibitor of SERCA-mediated Ca²⁺ re-uptake that permits slow endoplasmic reticulum emptying followed by SOCE, did not significantly reduce superoxide production induced by H₂O₂ in either K562-NOX2 cells or neutrophils. The previous observation that Ca²⁺ ionophore and thapsigargin were unable to activate plasma membrane-associated NADPH oxidase in neutrophils [28], along with our finding that NOX2 activation by H₂O₂ is Ca²⁺-dependent but thapsigargin-resistant, supports the involvement of Ca²⁺ entry mechanisms other than SOC channels. Indeed, inhibitor experiments suggested that T-type voltage-gated channels were involved in H₂O₂-NOX2-regulation. However, given the long latency for NOX2 activation in neutrophils, it may be that after initial SOC channel-independent Ca²⁺ entry, SOCE is activated in a second phase. Such Ca²⁺ reinforcement/propagation has been observed in other cells [29].

Positive feedback regulation of NOX2 by H₂O₂ was blocked by imatinib mesylate, an inhibitor of Bcr-Abl as well as c-Abl [30], both of which are expressed in K562 leukemia cells. The essential role of native c-Abl in H₂O₂-NOX2 activation is suggested by the fact that H₂O₂ induced NOX2-dependent superoxide production in normal blood neutrophils, which do not express the Bcr-Abl fusion protein. Furthermore, in K562 cells stably over-expressing wild-type GFP-c-Abl, baseline NOX2 activity was enhanced and induction of activity by H₂O₂ was preserved, whereas over-expression of dominant-negative c-Abl abrogated the stimulatory effect of H₂O₂ on NOX2 activity. Overall, these results are in keeping with studies showing not only that c-Abl is activated by H₂O₂ [31], but that it induces an increase of ROS production when over-expressed in hematopoietic cells [32].

In our previous experiments, we showed that while Ca²⁺ was essential for the translocation of c-Abl to the membrane, it was not required for its activation by phosphorylation [5]. These data suggest that while Ca²⁺ is a key determinant of H₂O₂-NOX2 regulation, a Ca²⁺-independent pathway is also activated by H₂O₂. Several lines of evidence implicate Src as an upstream activator of c-Abl [33], although in H₂O₂-treated K562/NOX5 cells [5] and K562/NOX2 cells (data not shown), we were unable to demonstrate a role of c-Src upstream of c-Abl. Thus, the signaling intermediates directly responsible for c-Abl phosphorylation remain to be determined.

Activation of NOX2 is absolutely dependent on the presence of cytosolic co-factor proteins p67^{phox}, p47^{phox}, and Rac. PKC is a major signaling protein kinase required for assembly and activation of the NOX2 complex, acting at least in part through the phosphorylation of multiple serines on p47^{phox}. The PKC family, comprising eleven members, is categorized into three classes on the basis of structure and activation requirements. The classical PKC isoforms (α , β 1, β 2 and γ) are regulated by both Ca^{2+} and DAG, the novel PKC isoforms (δ , ϵ , θ and η) are regulated by DAG, but not Ca^{2+} , and the atypical PKC isoforms (ζ , τ and λ) require neither Ca^{2+} nor DAG for their activation. Several studies have shown that PKC δ is involved in NOX2 activation [15,16,34]. We found that H_2O_2 induced PKC δ tyrosine phosphorylation, an effect that was inhibited by BAPTA, imatinib, or rottlerin. Also, while PKC δ is known as a Ca^{2+} -independent PKC isoform, our results show that H_2O_2 induced Ca^{2+} /c-Abl-dependent regulation of PKC δ . In accord with these findings, Ca^{2+} ionophore [35] and c-Abl [17] were shown to induce PKC δ tyrosine phosphorylation. In addition, we found that inhibition of PKC δ by rottlerin reduced the effect of H_2O_2 on NOX2 activation. However, these results only partially correlate with the total abrogation of PKC δ tyrosine³¹¹ phosphorylation by rottlerin, suggesting that the activation of NOX2, while mediated in large part through PKC δ , may also involve a Ca^{2+} -dependent PKC [36]. Supporting this observation is the fact that staurosporine, a broad inhibitor of PKC, or Go6976, an inhibitor of classical PKC, either abrogated or reduced, respectively, superoxide production induced by H_2O_2 (data not shown). The potent effect of BAPTA on H_2O_2 -NOX2 regulation is probably related to the fact that classical PKC directly and PKC δ indirectly are regulated by Ca^{2+} . Furthermore, Ca^{2+} is implicated in the NOX2 assembly process through the regulation of cytoskeletal elements [37].

Since the activation of NOX2 is dependent upon activation of Rac through GTP/GDP exchange and c-Abl can activate small GTPases, including Rac1 [18,38,39], we investigated the role of Rac1/2 in H_2O_2 -NOX2 regulation. Furthermore, c-Abl tyrosine phosphorylates Sos-1, a guanine nucleotide-exchange factor (GEF) that can elicit Rac-GEF activity *in vitro* [40]. In accord with those observations, we found that the pre-treatment of K562/NOX2 cells with toxin B of *C. difficile* (Fig. 6) decreased significantly the effect of H_2O_2 on superoxide production. Furthermore, BAPTA, imatinib, or rottlerin counteracted the activation of Rac by H_2O_2 . These observations suggest that Rac is regulated by H_2O_2 downstream of the Ca^{2+} /c-Abl/PKC δ pathway. In accord with our results, both Ca^{2+} -dependent and Ca^{2+} -independent PKC have been implicated in the activation and translocation of Rac through the phosphorylation of Rho family GTPases [36,41,42].

Although a number of mechanisms for the priming of NOX2 activation have been proposed, current evidence appears to favor partial phosphorylation of p47^{phox} [43–45] and/or mobilization of the flavocytochrome from intracellular stores to the plasma membrane [44]. In these scenarios, Ca^{2+} signaling and tyrosine kinase pathways exhibit complementary roles [46–48]. In our study, while a synergistic effect of H_2O_2 was consistently observed with PMA (Fig. 1G, Fig. 2E), no priming was observed with 100 nM fMLF in PMNs (Fig. 8A). The absence of priming suggests that common signaling pathways are engaged by H_2O_2 and fMLF. Of note, fMLF involves a PTX-sensitive Gi protein and interestingly, PTX-sensitive Gi/Go proteins can be activated in a receptor-independent manner by H_2O_2 [19]. In PMNs, as well as K562/NOX2 cells, we found that pre-treatment with PTX significantly decreased the level of superoxide production induced by H_2O_2 . Also, it is possible that H_2O_2 inhibits fMLF signaling by competing for Gi proteins. The participation of Gi proteins is furthermore supported by the observation that they regulate Ca^{2+} channels [49,50]. Consistent with these mechanisms, pre-treatment with PMA induced extensive superoxide production that was not further enhanced by the subsequent addition of H_2O_2 , suggesting that maximal recruitment and NOX2 assembly by PMA sequestered or masked one or more targets of H_2O_2 .

Quite apart from the activation and translocation of the cytosolic cofactors (p67^{phox}, p47^{phox}, Rac) by H₂O₂, one cannot exclude that the catalytic core of the enzyme itself is modified by the treatment. If we assume that only the cytosolic factors are affected by H₂O₂, then reconstitution using any combination of membranes and/or cytosol isolated from treated cells should give the same result. The fact that superoxide produced by NOX2 reconstitution in the broken cell system using membranes plus cytosol obtained from treated cells is significantly higher than any other combination raises the possibility that the activity of the enzyme is increased. Since this increase was not correlated with any increase in NOX protein levels, it suggests that post-translational modification of NOX2 itself could contribute to superoxide production induced by H₂O₂. In accord with this hypothesis, it was recently reported that direct phosphorylation on serine/threonine in the C-terminal domain of NOX2 increased the diaphorase activity of a NOX2 fusion protein in the presence of p47^{phox}, p67^{phox}, and Rac2 [51]. Whether phosphorylation of NOX2 is involved in H₂O₂-NOX2 regulation remains to be determined, since our observations could also be explained by H₂O₂-induced activation events such that the membrane fractions harbor assembled complexes of flavocytochrome plus translocated cytosolic subunits.

Observations in many cell types suggest that Ca²⁺ and ROS signaling are interdependent [52–55]. Furthermore, sustained increases in either Ca²⁺ or ROS have been consistently associated with pathological processes. However, the mechanisms by which they mediate tissue injury remain poorly understood. Positive feedback redox regulation of NOX2, as well as other NOX family members [5], might represent a common mechanism for amplification of ROS generation. The potentially broad relevance of this mechanism is underscored by several facts: 1) NOX isoforms are expressed in all major organs and tissues [2]; 2) c-Abl is an ubiquitously expressed protein; and 3) H₂O₂ is a diffusible molecule that could act as either an autocrine or paracrine signaling factor. Furthermore, given the importance of neutrophils in tissue injury associated with immune reactions [56] and the enhancement of ROS production in many human diseases, such as acute respiratory distress syndrome [57], rheumatoid arthritis [58], atherosclerosis, ischemia/reperfusion-induced tissue injury, hypertension, and kidney disease [59–62], it appears worthwhile to consider NOX regulation by Ca²⁺ influx and c-Abl activation as both mechanisms and therapeutic targets in diseases associated with oxidative stress.

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Abbreviations

BAPTA	1,2-bis (2-aminophenoxy) ethane- <i>N,N,N,N</i> -tetraacetic acid
DAG	diacylglycerol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
fMLF	formyl-methionyl-leucyl-phenylalanine
GEF	guanine nucleotide-exchange factor

GFP	green fluorescent protein
GSH	glutathione
GST	glutathione S-transferase
DHE	dihydroethidium
H ₂ O ₂	hydrogen peroxide
NOX	NADPH oxidase
PBS	phosphate-buffered saline
PBS-G	PBS containing 10 mM glucose
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PTX	pertussis toxin
ROS	reactive oxygen species
SERCA	sarco-endoplasmic reticulum Ca ²⁺ -ATPase
SOC channels	store-operated Ca ²⁺ channels
SOCE	store-operated Ca ²⁺ entry
TG	thapsigargin

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Fig. 1. Effects of H₂O₂ on superoxide production by human neutrophils

Superoxide production by neutrophils was measured using a whole cell assay with the Diogenes reagent. (A) Chemiluminescence generated by 0.25×10^6 neutrophils incubated with $100 \mu\text{M}$ H₂O₂ (broken line) or PBS-G (solid line) was measured for the indicated times. (B) Chemiluminescence generated by increasing numbers of neutrophils incubated with $100 \mu\text{M}$ H₂O₂ (broken line) or PBS-G (solid line) was measured over 10 min, and after subtraction of the zero-time values the area under the curve was calculated (arbitrary units) as the measure of total superoxide production. (C) Superoxide production was determined in neutrophils stimulated with increasing concentrations of H₂O₂ and after subtraction of the zero-time values the area under the curve was calculated and expressed as a percent of the control cells without H₂O₂. (D) Neutrophils, either non-treated (-) or pre-treated with catalase (900 U/ml), were stimulated with $100 \mu\text{M}$ H₂O₂ and superoxide production was measured and expressed as in Fig. 1C. (E) Neutrophils were incubated with increasing concentrations of glucose oxidase and 5 mM glucose, or with $100 \mu\text{M}$ H₂O₂ and superoxide production was measured and expressed as in Fig. 1C. (G) Neutrophils were incubated with 25 mU/ml of glucose oxidase and 5 mM glucose in the presence or absence of catalase (900 U/ml), or with $100 \mu\text{M}$ H₂O₂ and superoxide production was measured for the indicated times. (G) Neutrophils were pre-incubated with or without $100 \mu\text{M}$ H₂O₂ for 10 min, then stimulated with PMA (1 $\mu\text{g/ml}$), and superoxide

production was measured and expressed as in Fig. 1C. The data are the means $\pm$ SEM of 3–6 independent experiments. Asterisks indicate statistical significance versus control cells without H₂O₂. *P<0.05, **P<0.01, ***P<0.001.

Fig. 2. Effect of H₂O₂ on superoxide production by K562/NOX2 cells

K562 cells over-expressing NOX2 plus p47^{phox}/p67^{phox} or only p47^{phox}/p67^{phox} were assayed for superoxide production as described in Fig. 1. (A) Chemiluminescence output was measured from 0.5×10^6 K562/NOX2 cells incubated with (broken line) or without (solid line) 100 μ M H₂O₂ and from K562 cells expressing only p47^{phox} and p67^{phox} (dotted line) incubated with H₂O₂ for the indicated times. (B) Superoxide production by K562/NOX2 cells incubated with increasing concentrations of H₂O₂ (0–500 μ M) was measured for 10 min and expressed as in Fig. 1C. (C) K562/NOX2 cells, either non-treated (–) or pre-treated with catalase (900 U/ml), were stimulated with 100 μ M H₂O₂ and superoxide production was measured and expressed as in Fig. 1C. (D) K562/NOX2 cells were pre-incubated with or without 100 μ M H₂O₂ for 10 min, then stimulated with PMA (1 μ g/ml), and chemiluminescence was measured and expressed as in Fig. 1A. Chemiluminescence was also measured in K562 cells expressing only p47^{phox} and p67^{phox} pre-incubated with H₂O₂ and then stimulated by PMA (dotted line). (E) K562/NOX2 cells were pre-incubated with or without 100 μ M H₂O₂ for 10 min, then stimulated with PMA (1 μ g/ml), and superoxide production was measured and expressed as in Fig. 1C. The data shown are the mean $\pm$ SEM of 3–9 independent experiments. Asterisks indicate statistical significance versus non-treated cells without H₂O₂. ** P < 0.01, *** P < 0.001.

Fig. 3. Role of Ca²⁺ in H₂O₂-NOX2 regulation

(A, B) K562/NOX2 cells were pre-treated with the indicated inhibitors: BAPTA (50 μM, 5 min), thapsigargin (TG; 100 nM, 30 min), mibefradil (15 μM, 1h). The cells were then tested for superoxide production with (hatched bars) or without (solid bars) 100 μM H₂O₂ for 10 min (A) or after further stimulation with PMA (1 μg/ml) for an additional 10 min (B). Superoxide production was expressed as in Fig. 1C as a percent of control cells (–) in the absence of H₂O₂. The data shown are the means ± SEM of 5–10 independent experiments. Asterisks indicate statistical significance versus non-treated cells without H₂O₂. **P* < 0.05; ****P* < 0.001. (C) Confocal microscopy of H₂O₂-induced superoxide generation and Ca²⁺ influx was performed using either K562 cells expressing NOX2, p67^{phox}, and p47^{phox} (right panels) or only p67^{phox} and p47^{phox} (left panels). The cells were loaded with fluo-4 (green fluorescence, Ca²⁺) and DHE (red fluorescence, superoxide) and stimulated with 100 μM H₂O₂. Confocal images using a Zeiss microscope were recorded for up to 10 min using dual excitation at 488/543 nm. Each set of four photomicrographic panels corresponds to superoxide (top left), Ca²⁺ (top right), the differential interference contrast image (bottom left), and a merge of the two fluorescence images (bottom right). The scale bar indicates 10 μm.

Fig. 4. Role of c-Abl tyrosine kinase in H_2O_2 -NOX2 regulation

Superoxide production was determined with (hatched bars) or without (solid bars) 100 μM H_2O_2 for 10 min (A, C), or after further stimulation with PMA (1 $\mu g/ml$) for an additional 10 min (B, D) using K562/NOX2 cells pre-treated or not (–) with imatinib mesylate (10 μM , overnight) (A, B) or using K562 cells over-expressing GFP-KD-c-Abl or GFP-WT-c-Abl and the NOX2 system (C, D). Superoxide production was expressed as in Fig. 1C as a percent of control cells without H_2O_2 (A, B) or versus K562/NOX2 cells without H_2O_2 (C, D). The data shown are the means $\pm$ SEM of 3–4 independent experiments. Asterisks indicate statistical significance versus nontreated cells without H_2O_2 (A, B) or versus K562/NOX2 cells without H_2O_2 (C, D). * $P < 0.05$; *** $P < 0.001$.

Fig. 5. Role of PKCδ in H₂O₂-NOX2 regulation downstream of Ca²⁺ and c-Abl

(A, B) K562/NOX2 cells were pre-treated with rottlerin 20 μM for 3h, then tested for superoxide production with (hatched bars) or without (solid bars) 100 μM H₂O₂ for 10 min (A) or after further stimulation with PMA (1 μg/ml) for an additional 10 min (B). Superoxide production was expressed as in Fig. 1C as a percent of control cells in the absence of H₂O₂. The data shown are the means ± SEM of 3–9 independent experiments. Asterisks indicate statistical significance versus non-treated cells without H₂O₂. **P* < 0.05, ****P* < 0.001. (C) A representative immunoblot experiment using antibodies against the phosphorylated tyrosine³¹¹-PKCδ and total PKCδ and cell lysates prepared from K562/NOX2 cells pre-treated with the indicated inhibitors [BAPTA (50 μM, 5 min), rottlerin (20 μM, 3 h), imatinib (10 μM, overnight)], or PMA (1 μg/ml, 5 min) and then stimulated (+) or not (–) with 100 μM H₂O₂ for 10 min.

Fig. 6. Role of Rac activation in H₂O₂-NOX2 regulation

(A) Total cell lysates (100 μ g of protein) were prepared from K562/NOX2 cells that had been pre-treated with the indicated inhibitors [BAPTA (50 μ M, 5 min), imatinib (10 μ M, overnight), rottlerin (20 μ M, 3 h), *C. difficile* toxin B (1 nM, overnight)], or PMA (1 μ g/ml, 5 min) and then stimulated (+) or not (-) with 100 μ M H₂O₂ for 10 min. The levels of active Rac (Rac-GTP) were determined using the GST-PAK-CRIB pull-down assay. (B, C) K562/NOX2 cells were pre-treated with toxin B of *C. difficile*, 1 nM overnight, then tested for superoxide production with (hatched bars) or without (solid bars) 100 μ M H₂O₂ for 10 min (B) or after further stimulation with PMA (1 μ g/ml) for an additional 10 min (C). Superoxide production was expressed as in Fig. 1C as a percent of control cells without H₂O₂. Data shown are the mean $\pm$ SEM of three independent experiments. Asterisks indicate statistical significance versus non-treated cells without H₂O₂. ** $P < 0.01$, *** $P < 0.001$.

Fig. 7. Assessment of H₂O₂-NOX2 signaling pathways in neutrophils

(A) Neutrophils were pre-treated with the indicated inhibitors: BAPTA (50 μ M, 5 min), thapsigargin (TG; 100 nM, 30 min), mibefradil (15 μ M, 1 h), staurosporine (1 μ M, 30 min). The cells were then tested for superoxide production with (hatched bars) or without (solid bars) 100 μ M H₂O₂ for 10 min. Superoxide production was expressed as in Fig. 1C as a percent of control cells without H₂O₂. Data shown are the mean $\pm$ SEM of 3-7 independent experiments. Asterisks indicate statistical significance versus control cells without H₂O₂. * P < 0.05, ** P < 0.01, *** P < 0.001. (B) NOX2 assembly was assessed by immunoblot analysis of membrane preparations from neutrophils stimulated (+) or not (-) with 100 μ M H₂O₂ using antibodies directed against NOX2, p47^{phox}, or p67^{phox}. (C) Total cell lysates (100 μ g of protein) were prepared from neutrophils that had been stimulated (+) or not (-) with 100 μ M H₂O₂. The levels of total Rac and active Rac were determined as in Fig 6A. (D, E) Activated (phosphorylated) c-Abl and PKC δ were assessed by immunoblot analysis with general and phosphospecific antibodies to c-Abl-Tyr²⁴⁵ (D) and PKC δ -Tyr³¹¹ (E), respectively, on whole cell lysates from neutrophils stimulated (+) or not (-) with 100 μ M H₂O₂. (F) Reconstitution of NADPH oxidase activity in the broken cell system was performed as described in experimental procedures using membrane and cytosol fractions from neutrophils pre-treated (+) or not (-) with 100 μ M H₂O₂ for 10 min. Superoxide production was measured as SOD-inhibitable cytochrome c reduction and expressed in nmol/min/mg of protein. Data shown are

the mean $\pm$ SEM of 3 independent experiments. Asterisks indicate statistical significance versus superoxide produced by the combination of membranes and cytosol isolated from untreated neutrophils. * $P < 0.05$.

Fig. 8. Effect of H₂O₂ on fMLF-stimulated superoxide production

(A) Neutrophils were pre-treated with various amounts of H₂O₂ and then stimulated with 100 nM fMLF. (B) Neutrophils were pre-treated with 10 μg/ml PTX for 1 h and then tested for superoxide production with (hatched bars) or without (solid bars) 100 μM H₂O₂ for 10 min. In A and B superoxide production was measured and expressed as in Fig. 1C as a percent of control cells without H₂O₂. Data shown are the mean $\pm$ SEM of three independent experiments. Asterisks indicate statistical significance versus non-treated cells without H₂O₂. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (C) The schema represents a model for H₂O₂-NOX2 regulatory pathways.