Ca\textsuperscript{2+}-dependent p47\textsuperscript{phox} translocation in hydroperoxide modulation of the alveolar macrophage respiratory burst

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Ca\textsuperscript{2+}-dependent p47\textsuperscript{phox} translocation in hydroperoxide modulation of the alveolar macrophage respiratory burst. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L1042–L1047, 1997.—Oxidative stress produces dual effects on the respiratory burst of rat alveolar macrophages. Preincubation with hydroperoxide concentrations [H\textsubscript{2}O\textsubscript{2} or tert-butyl hydroperoxide (t-BOOH); <50 µM] enhances stimulation of the respiratory burst, whereas higher concentrations inhibit stimulation. Both the enhancement and inhibition are markedly attenuated by buffering t-BOOH-induced changes in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i). Phosphorylation of the NADPH oxidase component p47\textsuperscript{phox} and its translocation from cytoplasm to plasma membrane are essential in respiratory burst activation. Phorbol 12-myristate 13-acetate (PMA)-stimulated p47\textsuperscript{phox} phosphorylation was negligibly affected by 25 or 100 µM t-BOOH. Nonetheless, 25 µM t-BOOH increased PMA-stimulated p47\textsuperscript{phox} translocation, whereas 100 µM t-BOOH decreased PMA-stimulated translocation. In unstimulated cells, however, neither phosphorylation nor translocation of p47\textsuperscript{phox} was affected by t-BOOH. Buffering of the t-BOOH-mediated changes of [Ca\textsuperscript{2+}]i (5, 16). Studies from our laboratory have shown that low concentrations of t-BOOH (<50 µM) enhance the respiratory burst stimulated by phorbol 12-myristate 13-acetate (PMA), whereas higher concentrations (but still sublethal; <100 µM) inhibit the burst (20). In this study, we examined the possibility that the alteration of the respiratory burst by t-BOOH could involve effects upon p47\textsuperscript{phox} phosphorylation and/or p47\textsuperscript{phox} translocation.

Although PMA does not stimulate changes in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]i), PMA stimulation of the respiratory burst can be modulated by changes in [Ca\textsuperscript{2+}]i (5, 16). Studies from our laboratory have shown that 25 µM t-BOOH caused a transient elevation in [Ca\textsuperscript{2+}]i in alveolar macrophages, whereas 100 µM t-BOOH caused a sustained elevation in [Ca\textsuperscript{2+}]i (19). This dual effect of t-BOOH on [Ca\textsuperscript{2+}]i, correlated with the effects on the alveolar macrophage respiratory burst (15). Furthermore, buffering of hydroperoxide-mediated changes in [Ca\textsuperscript{2+}]i, using the intracellular Ca\textsuperscript{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetate acid (BAPTA), suppressed the enhancement of the respiratory burst by 25 µM t-BOOH and attenuated the inhibition by 100 µM t-BOOH (16). These data suggest that changes in [Ca\textsuperscript{2+}]i, induced by t-BOOH are involved in the process that modulates the respiratory burst. Thus the effect of buffering [Ca\textsuperscript{2+}]i upon t-BOOH-mediated changes on p47\textsuperscript{phox} translocation was also investigated.

EXPERIMENTAL PROCEDURES

Materials. PMA, leupeptin, pepstatin A, aprotinin, phenylmethylsulfonyl fluoride (PMSF), sodium deoxycholate, and Nonidet P-40 (NP-40) were purchased from Sigma. Bio-lyte 3/10 ampholyte, piperazine diacrylamide, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), and twodimensional (2-D) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) standards were from Bio-Rad. Precast 10% tris(hydroxymethyl)aminomethane (Tris) buffers were from Bio-Rad. Dextran, bovine serum albumin (BSA), bicarbonate, and Tris were from Sigma. The N-acetyl-L-cysteine (NAC) solution was purchased from ICN. tert-Butyl hydroperoxide (t-BOOH) was purchased from BDH, and hydrogen peroxide [H2O2] was purchased from Mallinckrodt. PMA, leupeptin, and pepstatin A were dissolved in ethanol at a concentration of 1 mg/ml, and aprotinin was dissolved in 0.1% human serum albumin. Aminoethylisothiuronium (AET) was dissolved in Tris buffer (pH 7.5) and stored in aliquots at −20°C. All other reagents were from Sigma.

ALVEOLAR MACROPHAGES function as the first line of defense against bacteria and foreign particles in the lungs (11). Part of the mechanism by which phagocytic cells kill microbes depends on the generation of superoxide in a metabolic process known as the respiratory burst (22). The respiratory burst oxidase includes several cytosolic components, p47\textsuperscript{phox}, p67\textsuperscript{phox}, p40\textsuperscript{phox}, rac1, or rac2, and the transmembrane electron carrier flavocytochrome b\textsubscript{559}, a heterodimer of p22\textsuperscript{phox} and gp91\textsuperscript{phox}. During the activation of the respiratory burst oxidase of neutrophils, p47\textsuperscript{phox} is phosphorylated at multiple sites by protein kinases at the COOH-terminal domain (7, 8, 14, 26). The phosphorylated protein is then translocated from the cytoplasm to the plasma membrane for the assembly of the oxidase complex (4, 9). Failure to phosphorylate or translocate p47\textsuperscript{phox} results in failure to produce superoxide in stimulated cells (8).

Exposure of alveolar macrophages to air pollutants (e.g., nitrogen dioxide and ozone) or prolonged exposure to hyperoxia in vivo leads to significant inhibition of the respiratory burst (1, 12). Nevertheless, alveolar macrophages, after a brief exposure to hyperoxia in vivo, have an enhanced response to subsequent stimulation of the respiratory burst (28). We used tert-butyl hydroperoxide (t-BOOH), a relatively stable and water-soluble oxidant, to mimic both the enhancing and inhibitory effects of oxidant exposure on the rat alveolar macrophage respiratory burst. Because of their rapid metabolism, hydroperoxides cannot be precisely measured in vivo. Nonetheless, evidence for the generation of significant concentrations of hydroperoxides and their breakdown products in exposure to various oxidative stresses is well documented (27, 29). Previous studies from our laboratory have shown that low concentrations of t-BOOH (<50 µM) enhance the respiratory burst stimulated by phorbol 12-myristate 13-acetate (PMA), whereas higher concentrations (but still sublethal; <100 µM) inhibit the burst (20). In this study, we examined the possibility that the alteration of the respiratory burst by t-BOOH could involve effects upon p47\textsuperscript{phox} phosphorylation and/or p47\textsuperscript{phox} translocation.

This dual effect of t-BOOH on [Ca\textsuperscript{2+}]i, using the intracellular Ca\textsuperscript{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetate acid (BAPTA), suppressed the enhancement of the respiratory burst by 25 µM t-BOOH and attenuated the inhibition by 100 µM t-BOOH (16). These data suggest that changes in [Ca\textsuperscript{2+}]i, induced by t-BOOH are involved in the process that modulates the respiratory burst. Thus the effect of buffering [Ca\textsuperscript{2+}]i upon t-BOOH-mediated changes on p47\textsuperscript{phox} translocation was also investigated.

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EFFECT OF OXIDATIVE STRESS ON NADPH OXIDASE ACTIVITY

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Measurement of alveolar macrophage respiratory burst. Alveolar macrophages (1 x 10^6 cells) were treated (15 min, 37°C) with or without t-BOOH and then were activated by PMA (1 µg/ml) for 10 min. Cells were collected using a microcentrifuge for 10 s, resuspended in 0.5 ml of ice-cold lysis buffer [20 mM Tris·HCl, pH 7.4, 150 mM NaCl, 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacet-

acid, 5 mM EDTA, 50 µM leupeptin, 25 µM pepstatin A, 25 µM aprotinin, and 2 mM PMSF], sonicated 3 times for 10 s, and centrifuged at 2000 g, 10 min) to remove nuclei and unbroken cells. The supernatant was ultracentrifuged further (100,000 g, 60 min). The resulting supernatant was designated the cytosolic fraction. The membrane pellet was resuspended in 200 µl of the lysis buffer supplemented with sodium deoxycholate (1%) and NP-40 (1%). The protein concentration of each sample was measured using the bicinchoninic acid protein assay (Pierce). Cytosolic (5 µg protein/sample) and membrane (50 µg protein/sample) proteins were suspended in 20 µl of Laemml sample buffer, heated in boiling water for 5 min, separated by SDS-PAGE on a precast 10% polyacrylamide gel, and analyzed by immunoblotting.

Immunoblotting. Separated proteins were transferred to a nitrocellulose membrane and were incubated with the anti-p47phox antibody (diluted 1:5,000) overnight at 4°C. The p47phox-antibody complex was visualized using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) after a 1-h incubation with goat anti-rabbit immunoglobulin G (Kirkegaard and Perry, Gaithersburg, MD). The developed film was scanned using a Hewlett-Packard ScanJet 4c/T scanner and accompanying software. The images were then analyzed for relative density using SigmaScan software (Jandel Scientific Software, San Rafael, CA).

Statistical analysis. Data are expressed as means ± SE. The amounts of p47phox translocation from all groups were compared with the PMA stimulation group using one-way analysis of variance following Dunnett’s method. Differences in two groups were considered statistically significant at P < 0.05.

RESULTS

Effect of t-BOOH on p47phox phosphorylation. The relationship of p47phox phosphorylation to the activa-

Fig. 1. Superoxide production by alveolar macrophages stimulated with phorbol 12-myristate 13-acetate (PMA). Typical time course of superoxide production is shown. Respiratory burst was measured as described in EXPERIMENTAL PROCEDURES.

Fig. 2. Time course of p47phox phosphorylation stimulated with PMA. Alveolar macrophages were stimulated with PMA for 1, 5, 7, or 10 min. p47phox isoforms were analyzed by 2-dimensional gel electrophoresis as described in EXPERIMENTAL PROCEDURES. Data are representative of 2 experiments with different cell preparations.
tion of the respiratory burst oxidase in rat alveolar macrophages was examined. As a frame of reference, Fig. 1 shows the time course of superoxide production of alveolar macrophages stimulated by PMA. Superoxide production had a lag phase of \( \sim 1 \) min, reached a maximal rate at \( \sim 5 \) min, and then became progressively slower until \( \sim 30 \) min. Figure 2 shows \( p47^{phox} \) phosphorylation in alveolar macrophages stimulated by PMA for 0, 1, 5, 7, or 10 min, at which time point lysates were prepared for analysis by 2-D gel electrophoresis and immunoblotting. Resting alveolar macrophages contained one isoform of \( p47^{phox} \) predominantly. A single more acidic \( p47^{phox} \) isoform was distinctly apparent after 1 min of stimulation. Several additional \( p47^{phox} \) isoforms were apparent at 5 min. The more acidic \( p47^{phox} \) phosphoprotein forms reached a maximum at \( \sim 7 \) min.

The effect of \( t \)-BOOH treatment on the level of \( p47^{phox} \) phosphorylation was examined next. Two concentrations of \( t \)-BOOH were chosen [25 \( \mu \)M, which causes maximum enhancement (41 ± 5\%) of the PMA-stimulated respiratory burst, and 100 \( \mu \)M, which causes a 96 ± 2\% inhibition without cytotoxicity; see Ref. 16]. These effects were verified. Nonetheless, treatment of alveolar macrophages with 25 or 100 \( \mu \)M \( t \)-BOOH alone did not lead to the phosphorylation of \( p47^{phox} \) (Fig. 3). Furthermore, pretreatment with either 25 or 100 \( \mu \)M \( t \)-BOOH did not markedly change the pattern of \( p47^{phox} \) phosphorylation induced by PMA (Fig. 3). In other words, PMA induced the phosphorylation of \( p47^{phox} \), but \( t \)-BOOH did not alter this phosphorylation to any clear extent and certainly not in a manner consistent with its effect on the respiratory burst. Thus modulation of the phosphorylation of \( p47^{phox} \) does not appear to be the hydroperoxide-mediated event for either the enhancement or the inhibition of the burst.

Modulation of \( p47^{phox} \) translocation by \( t \)-BOOH. During the activation of the respiratory burst oxidase, phosphorylated \( p47^{phox} \) translocates from the cytosol to the plasma membrane. Such translocation of the cytosolic oxidase components is an essential step in the activation of the oxidase. Figure 4 shows that stimulation with PMA decreased the amount of \( p47^{phox} \) in the cytosolic fraction and increased the amount of \( p47^{phox} \) in the membrane fraction compared with the control as a function of time.

**Fig. 3.** Effect of tert-butyl hydroperoxide (\( t \)-BOOH) on \( p47^{phox} \) phosphorylation. Alveolar macrophages were treated with \( t \)-BOOH (0, 25, or 100 \( \mu \)M) for 15 min. Shown is phosphorylation of \( p47^{phox} \) from unstimulated alveolar macrophages and alveolar macrophages stimulated with PMA for 5 min. \( p47^{phox} \) phosphorylation was analyzed as in Fig. 2. Data are representative of 2 experiments with different cell preparations.
pretreatment with BAPTA-AM depresses the enhancement of the PMA-stimulated respiratory burst by 25 µM t-BOOH from 41 ± 5 to 8 ± 7% and reduces the inhibition of the burst by 100 µM t-BOOH from 96 ± 2 to 42 ± 10% (16). Thus there are Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent components to the inhibition of the respiratory burst by 100 µM t-BOOH.

To examine whether the modulation of p47\textsubscript{phox} translocation by t-BOOH resulted from t-BOOH-induced elevation of [Ca\(^{2+}\)], alveolar macrophages were incubated for 15 min with 5 µM BAPTA-AM before exposure to t-BOOH. Figure 6 shows that, after treatment of alveolar macrophages with BAPTA-AM, 25 µM t-BOOH did not enhance translocation after PMA stimulation. Furthermore, after treatment with BAPTA-AM, p47\textsubscript{phox} translocation was no longer decreased by 100 µM t-BOOH. Thus comparison of the results in the presence (Fig. 6) or absence (Fig. 5) of BAPTA suggests that hydroperoxide-induced changes in [Ca\(^{2+}\)], modulate the translocation of p47\textsubscript{phox}. The other component of inhibition of the respiratory burst, which is unaffected by BAPTA, apparently does not involve p47\textsubscript{phox} translocation.

**DISCUSSION**

The goal of this investigation was to determine whether the modulation of the respiratory burst by exposure to hydroperoxide is due to changes in the phosphorylation of p47\textsubscript{phox} and/or its translocation to the plasma membrane. Various protein kinases, including protein kinase C, guanosine 3',5'-cyclic monophosphate-dependent protein kinase, and p72\textsuperscript{syk} tyrosine kinase, can be activated by hydroperoxides (18, 23, 30). In addition, both serine-threonine and tyrosine protein phosphatases may be inhibited under similar conditions (30). Therefore, it seemed reasonable to investigate whether the phosphorylation of p47\textsubscript{phox}, an apparently obligatory step in the assembly of the respiratory burst, is affected by BAPTA, apparently does not involve p47\textsubscript{phox} translocation.
burst oxidase (8), was affected under conditions in which hydroperoxides modulate the respiratory burst. Although the phosphorylation of p47\textsuperscript{phox} appears to be a step required for the assembly of the NADPH oxidase in alveolar macrophages (Fig. 2), it is only marginally affected by t-BOOH (Fig. 3). Thus alteration of the pattern of p47\textsuperscript{phox} phosphorylation does not appear to play a role in the hydroperoxide modulation of the respiratory burst. This does not imply that protein phosphorylation is unaffected by oxidative stress in alveolar macrophages but only that one particularly important step in the activation of the respiratory burst was not the target of hydroperoxide modulation. Rather, many of the dual effects of sublethal concentrations of t-BOOH on the alveolar macrophage respiratory burst result from an increase in p47\textsuperscript{phox} translocation by 25 µM t-BOOH and the decrease in p47\textsuperscript{phox} translocation by 100 µM t-BOOH (Fig. 5). Buffering of the t-BOOH-mediated changes of \([Ca^{2+}]\) using BAPTA, which markedly attenuates the effects of t-BOOH on the respiratory burst (16), abolishes the dual effects of t-BOOH on p47\textsuperscript{phox} translocation (Fig. 6). The effect of BAPTA in eliminating the effect of 100 µM t-BOOH on translocation was more dramatic than its effect upon inhibition of the respiratory burst. This suggests that 100 µM t-BOOH had an additional \(Ca^{2+}\)-independent inhibitory effect on the respiratory burst. Taken together, these results suggest that oxidative stress induces 1) \(Ca^{2+}\)-dependent processes that modulate the assembly of the respiratory burst oxidase at a step that is separate from p47\textsuperscript{phox} phosphorylation and 2) a \(Ca^{2+}\)-independent process that inhibits the respiratory burst separately from both p47\textsuperscript{phox} phosphorylation or its translocation.

Recently, our laboratory demonstrated that sublethal concentrations of t-BOOH causes disassociation of annexin VI, a major \(Ca^{2+}\)-binding protein in macrophages, from the plasma membrane to the cytosol (17). This dissociation apparently results in the initial increase in \([Ca^{2+}]\), t-BOOH at 25 µM causes a transient elevation in \([Ca^{2+}]\), whereas 100 µM t-BOOH induces a sustained elevation in \([Ca^{2+}]\), because of reversible oxidation of the thiol group in \(Ca^{2+}\)-adenosinetriphosphate of the plasma membrane (17). Although the t-BOOH-induced elevation in \([Ca^{2+}]\) alone is not sufficient to activate the respiratory burst oxidase, this elevation in \([Ca^{2+}]\) can potentially modulate various components of signal pathways (e.g., phospholipases, protein kinases, and protein phosphatases).

Two possible mechanisms through which the elevation in \([Ca^{2+}]\) may then modulate the assembly of the respiratory burst oxidase may be hypothesized. First, an increase in \([Ca^{2+}]\) can result in the production of diacylglycerol, which augments both p47\textsuperscript{phox} translocation and superoxide anion production in a cell-free system (24). Diacylglycerol can be produced by the action of phospholipase C or by the sequential action of phospholipase D and phosphatidic acid phosphohydrolase (2). Phospholipase D can be activated by an increase in \([Ca^{2+}]\) or can be attenuated by the chelation of intracellular \(Ca^{2+}\) using BAPTA (21).

Results that have indicated the activation of phospholipase D by t-BOOH. Second, phospholipase A2 can also be activated by both an increase in \([Ca^{2+}]\) and dissociation of annexin VI from the membrane to the cytosol (3, 6). We have previously shown that t-BOOH releases arachidonic acid through the activation of phospholipase A2 in alveolar macrophages (25). Arachidonic acid can directly activate NADPH oxidase by causing p47\textsuperscript{phox} translocation without phosphorylation in a cell-free system (13); however, the relevance of this to physiological stimulation of the respiratory burst has been questioned (10).

In summary, our results demonstrated that the dual effects of t-BOOH on PMA-stimulated p47\textsuperscript{phox} translocation account for much of the dual effects on the alveolar macrophage respiratory burst. The regulation of p47\textsuperscript{phox} translocation results from the elevation of \([Ca^{2+}]\), caused by t-BOOH. This increase in \([Ca^{2+}]\) apparently results in an increase in p47\textsuperscript{phox} translocation with low oxidative stress but decreased translocation with greater oxidative stress. The remaining component of t-BOOH-induced inhibition is independent of changes in \([Ca^{2+}]\), and does not involve an effect upon translocation. Although the precise mechanism for the effect of the hydroperoxide-induced elevation in \([Ca^{2+}]\), on the translocation of p47\textsuperscript{phox} is unknown, it represents an example of the mimicry of physiological signaling that is becoming more apparent in low-level oxidative stress.

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