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

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Exposure of alveolar macrophages to air pollutants or prolonged exposure in failure to produce superoxide in stimulated cells (8). Failure to phosphorylate or translocate p47\(^{phox}\) results in failure to produce superoxide in stimulated cells (8).

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 inclusions several cytosolic components, p47\(^{phox}\), p67\(^{phox}\), p40\(^{phox}\), rac1 or rac2, and the transmembrane electron carrier flavocytochrome b\(_{553}\), a heterodimer of p22\(^{phox}\) and gp91\(^{phox}\). During the activation of the respiratory burst oxidase of neutrophils, p47\(^{phox}\) 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\(^{phox}\) 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 molecule, 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\(^{phox}\) phosphorylation and/or p47\(^{phox}\) translocation.

Although PMA does not stimulate changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]). PMA stimulation of the respiratory burst can be modulated by changes in [Ca\(^{2+}\)] (5, 16). Studies from our laboratory have shown that 25 µM t-BOOH caused a transient elevation in [Ca\(^{2+}\)] in alveolar macrophages, whereas 100 µM t-BOOH caused a sustained elevation in [Ca\(^{2+}\)] (19). This dual effect of t-BOOH on [Ca\(^{2+}\)], correlated with the effects on the alveolar macrophage respiratory burst (15). Furthermore, buffering of hydroperoxide-mediated changes in [Ca\(^{2+}\)], using the intracellular Ca\(^{2+}\) chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic 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\(^{2+}\)], induced by t-BOOH are involved in the process that modulates the respiratory burst. Thus the effect of buffering [Ca\(^{2+}\)] upon t-BOOH-mediated changes on p47\(^{phox}\) 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-(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and two-dimensional (2-D) sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) standards were from Bio-Rad. Precast 10% tris(hydroxymethyl)aminomethane

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(Tris)-glycine gels (15 well and 2-D) and SeeBlue prestained standard were from Novex. A rabbit polyclonal antibody against the COOH-terminal decapeptide of p47phox was generously provided by Dr. Bernard M. Babior of the Scripps Research Institute.

Preparation of alveolar macrophages. Alveolar macrophages were obtained by bronchoalveolar lavage of Sprague-Dawley rats (specific pathogen free, male, 250–350 g) with Dawley rats (specific pathogen free, male, 250–350 g) with sodium phosphate-buffered saline (pH 7.4, 0.01 M, 100 ml). Macrophages were resuspended and stored at 4°C in Krebs-Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM hydroxyethylpiperazine-2-ethanesulfonic acid, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM sodium phosphate, 125 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM Dawley rats (specific pathogen free, male, 250–350 g) with Dawley rats (specific pathogen free, male, 250–350 g) with sodium phosphate-buffered saline (pH 7.4, 0.01 M, 100 ml). Macrophages were resuspended and stored at 4°C in Krebs-Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM hydroxyethylpiperazine-2-ethanesulfonic acid, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM sodium phosphate, 125 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM hydroxyethylpiperazine-2-ethanesulfonic acid, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM sodium phosphate, 125 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM hydroxyethylpiperazine-2-ethanesulfonic acid, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM sodium phosphate, 125 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM hydroxyethylpiperazine-2-ethanesulfonic acid, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM sodium phosphate, 125 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM hydroxyethylpiperazine-2-ethanesulfonic acid, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM sodium phosphate, 125 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM hydroxyethylpiperazine-2-ethanesulfonic acid, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM sodium phosphate, 125 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM hydroxyethylpiperazine-2-ethanesulfonic acid, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM sodium phosphate, 125 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM hydroxyethylpiperazine-2-ethanesulfonic acid, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM sodium phosphate, 125 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM hydroxyethylpiperazine-2-ethanesulfonic acid, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM sodium phosphate, 125 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM hydroxyethylpiperazine-2-ethanesulfonic acid, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM sodium phosphate, 125 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM hydroxyethylpiperazine-2-ethanesulfonic acid, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM sodium phosphate, 125 mM N8Ringer-phosphate buffer (KRPH, pH 7.4; 10 mM hydroxyethylpiperazine-2-ethanesulfonic acid, 125 mM NaCl, 5 mM KCl, 5 mM glucose, 10 mM sodium phosphate, 125 mM N8

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.

Measurement of alveolar macrophage respiratory burst. Alveolar macrophages (1 x 10^6 cells) were incubated (37°C, 15 min) in 1 ml of KRPH buffer. The respiratory burst was stimulated by the addition of PMA (100 ng/ml) in the presence of 80 µM ferricytochrome c. Superoxide production was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome c, which was monitored continuously at 550–540 nm in a dual-wavelength spectrophotometer.

Analysis of p47phox phosphorylation by 2-D gel electrophoresis. Alveolar macrophages (2 x 10^6 cells/ml) were incubated (37°C, 15 min) in 1 ml of KRPH buffer with or without t-BOOH (25 or 100 µM t-BOOH) and, subsequently, were stimulated with or without PMA (100 ng/ml) for 5 min, at which time the rate of superoxide anion production is maximal. Cells were isolated using a microcentrifuge for 10 s and were dissolved in 0.1 ml of first-dimension sample buffer (0.1 g dithiothreitol, 0.4 g CHAPS, 5.4 g urea, 0.5 ml Bio-lyte 3/10 ampholyte, and 6 ml H2O). Aliquots (40 µl of each sample) were subjected to nonequilibrium pH gradient isoelectric focusing in a minicapillary gel (monomer solution: 9:2 M urea, 4% acrylamide, 4% Bio-lyte 3/10 ampholyte, 1.5% CHAPS, and 0.5% NP-40). The top and bottom chambers of the mini-Protean II electrophoresis apparatus contained 10 mM Na2HP04 and 100 mM NaOH, respectively. The samples were electrophoresed for 4 h (100 V, 10 min; 200 V, 10 min; 400 V, 3.5 h; and 600 V, 10 min), and the tube gels were extruded and transferred to the top of the minislab 2-D gel for the second run. p47phox (nonphosphorylated and phosphorylated isoforms) was detected by immunoblotting (see Immunoblotting).

Translocation of p47phox from cytosol to plasma membrane. Alveolar macrophages (1 x 10^6 cells/ml) were treated (15 min, 37°C) with or without t-BOOH and then were activated by PMA (1 µg/ml) for 5 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'-tetraace-

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

RESULTS

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

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 ~1 min, reached a maximal rate at ~5 min, and then became progressively slower until ~30 min. Figure 2 shows p47phox 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 p47phox predominantly. A single more acidic p47phox isoform was distinctly apparent after 1 min of stimulation. Several additional p47phox isoforms were apparent at 5 min. The more acidic p47phox phosphoprotein forms reached a maximum at ~7 min.

The effect of t-BOOH treatment on the level of p47phox phosphorylation was examined next. Two concentrations of t-BOOH were chosen [25 µM, which causes maximum enhancement (41 ± 5%) of the PMA-stimulated respiratory burst, and 100 µM, which causes a 96 ± 2% inhibition without cytotoxicity; see Ref. 16]. These effects were verified. Nonetheless, treatment of alveolar macrophages with 25 or 100 µM t-BOOH alone did not lead to the phosphorylation of p47phox (Fig. 3). Furthermore, pretreatment with either 25 or 100 µM t-BOOH did not markedly change the pattern of p47phox phosphorylation induced by PMA (Fig. 3). In other words, PMA induced the phosphorylation of p47phox, 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 p47phox does not appear to be the hydroperoxide-mediated event for either the enhancement or the inhibition of the burst.

Modulation of p47phox translocation by t-BOOH. During the activation of the respiratory burst oxidase, phosphorylated p47phox 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 p47phox in the cytosolic fraction and increased the amount of p47phox in the membrane fraction compared with the control as a function of time.
increased the translocation of p47phox from the cytosol with t-BOOH. The translocation of p47phox at a step(s) separate from the respiratory burst involves, in part, the alteration of the results suggest that hydroperoxide modulation of the 100 µM t-BOOH to the membrane upon stimulation with PMA, whereas 25 µM t-BOOH affected p47phox distribution. However, 25 µM t-BOOH alone affected p47phox localization after stimulation with PMA, whereas 100 µM t-BOOH decreased the translocation. These results suggest that hydroperoxide modulation of the respiratory burst involves, in part, the alteration of the translocation of p47phox at a step(s) separate from the phosphorylation of p47phox.

Ca2+-dependent p47phox translocation during exposure to t-BOOH. Incubation of alveolar macrophages with t-BOOH causes reversible elevation of [Ca2+] (17, 19). Buffering of this t-BOOH-induced elevation of [Ca2+], with the Ca2+ chelator BAPTA attenuates the dual effects of t-BOOH on the burst (16). Although incubation with BAPTA-acetoxymethyl ester (AM) alone does not affect the PMA-stimulated respiratory burst, 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 Ca2+-dependent and Ca2+-independent components to the inhibition of the respiratory burst by 100 µM t-BOOH.

To examine whether the modulation of p47phox translocation by t-BOOH resulted from t-BOOH-induced elevation of [Ca2+], 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, p47phox 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 [Ca2+], modulate the translocation of p47phox. The other component of inhibition of the respiratory burst, which is unaffected by BAPTA, apparently does not involve p47phox 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 p47phox and/or its translocation to the plasma membrane. Various protein kinases, including protein kinase C, guanosine 3',5'-cyclic monophosphate-dependent protein kinase, and p72syk tyrosine kinase, can be activated by hydroperoxides (18, 23, 30). In addition, both serine-threonine and tyrosine protein phosphatases may be inactivated under similar conditions (30). Therefore, it seemed reasonable to investigate whether the phosphorylation of p47phox, an apparently obligatory step in the assembly of the respiratory

![Fig. 4. Time course of p47phox translocation stimulated with PMA. Alveolar macrophages were stimulated with PMA for 1, 5, or 10 min before sonication and preparation of cytosolic and membrane fractions. Cytosolic (5 µg protein/sample) and membrane (50 µg protein/sample) proteins were analyzed by immunoblotting as described in EXPERIMENTAL PROCEDURES. 0, Control; 1, 1 min; 5, 5 min; and 10, 10 min. Results are representative of 3 experiments using different cell preparations.](http://ajplung.physiology.org/)

![Fig. 5. Effect of t-BOOH on p47phox translocation. Alveolar macrophages were treated with or without t-BOOH (0, 25, or 100 µM) for 15 min. Then macrophages were treated with or without PMA for 5 min. Shown is p47phox in the membrane fractions (50 µg protein/sample) analyzed as in Fig. 4. Lane 1, control (no t-BOOH or PMA); lane 2, 25 µM t-BOOH only; lane 3, 100 µM t-BOOH only; lane 4, PMA only; lane 5, 25 µM t-BOOH then PMA; and lane 6, 100 µM t-BOOH then PMA. Figure is representative of 4 experiments from different cell preparations. Mean and SE compared with density of PMA-stimulated alveolar macrophages are shown in bottom. *P < 0.05 vs. PMA stimulation by analysis of variance (ANOVA).](http://ajplung.physiology.org/)

![Fig. 6. Effect of buffering changes of intracellular Ca2+ concentration ([Ca2+]i) mediated by t-BOOH on p47phox translocation. Alveolar macrophages were treated with PMA for 15 min before treatment with t-BOOH (25 or 100 µM) for 15 min. Amounts of p47phox in membrane fractions (50 µg protein/sample) were analyzed as in Fig. 4. Lane 1, control (no BAPTA, no t-BOOH, and no PMA); lane 2, BAPTA only; lane 3, PMA only; lane 4, BAPTA plus PMA; lane 5, BAPTA, 25 µM t-BOOH, and PMA; and lane 6, BAPTA, 100 µM t-BOOH, and PMA. Figure is representative result of 3 experiments from different cell preparations. Mean and SE compared with PMA-stimulated alveolar macrophages from all 3 experiments are shown. *P < 0.05 vs. PMA stimulation by ANOVA.](http://ajplung.physiology.org/)
burst oxidase (8), was affected under conditions in which hydroperoxides modulate the respiratory burst. Although the phosphorylation of p47phox 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 p47phox 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 p47phox translocation by 25 µM t-BOOH and the decrease in p47phox translocation by 100 µM t-BOOH (Fig. 5). Buffering of the t-BOOH-mediated changes of [Ca2+]i using BAPTA, which markedly attenuates the effects of t-BOOH on the respiratory burst (16), abolishes the dual effects of t-BOOH on p47phox 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 Ca2+-independent inhibitory effect on the respiratory burst. Taken together, these results suggest that oxidative stress induces 1) Ca2+-dependent processes that modulate the assembly of the respiratory burst oxidase at a step that is separate from p47phox phosphorylation and 2) a Ca2+-independent process that inhibits the respiratory burst separately from both p47phox phosphorylation or its translocation. Recently, our laboratory demonstrated that sublethal concentrations of t-BOOH causes dissociation of annexin VI, a major Ca2+-binding protein in macrophages, from the plasma membrane to the cytosol (17). This dissociation apparently results in the initial increase in [Ca2+]i. t-BOOH at 25 µM causes a transient elevation in [Ca2+]i, whereas 100 µM t-BOOH induces a sustained elevation in [Ca2+]i, because of reversible oxidation of the thiol group in Ca2+-adenosinetriphosphatase of the plasma membrane (17). Although the t-BOOH-induced elevation in [Ca2+]i alone is not sufficient to activate the respiratory burst oxidase, this elevation in [Ca2+]i can potentially modulate various components of signal pathways (e.g., phospholipases, protein kinases, and protein phosphatases). Two possible mechanisms through which the elevation in [Ca2+]i may then modulate the assembly of the respiratory burst oxidase may be hypothesized. First, an increase in [Ca2+]i can result in the production of diacylglycerol, which augments both p47phox 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 [Ca2+]i or can be attenuated by the chelation of intracellular Ca2+ using BAPTA (21). Preliminary results have indicated the activation of phospholipase D by t-BOOH. Second, phospholipase A2 can also be activated by both an increase in [Ca2+]i 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 p47phox 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 p47phox translocation account for much of the dual effects on the alveolar macrophage respiratory burst. The regulation of p47phox translocation results from the elevation of [Ca2+]i caused by t-BOOH. This increase in [Ca2+]i, apparently results in an increase in p47phox 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 [Ca2+]i and does not involve an effect upon translocation. Although the precise mechanism for the effect of the hydroperoxide-induced elevation in [Ca2+]i on the translocation of p47phox 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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REFERENCES


