Priming of Alveolar Macrophage Respiratory Burst by H\textsubscript{2}O\textsubscript{2} Is Prevented by Phosphatidylcholine-Specific Phospholipase C Inhibitor Tricyclodecan-9-yl-xanthate (D609)

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ABSTRACT
The respiratory burst in alveolar macrophages is enhanced in vitro by pre-exposure to nontoxic concentrations of hydroperoxides before stimulation by an agonist, which may represent a feed-forward regulatory mechanism. Tricyclodecan-9-yl-xanthate (D609), an inhibitor of phosphatidylcholine-specific phospholipase C (PC-PLC), suppresses this priming of the respiratory burst by pre-exposure to H\textsubscript{2}O\textsubscript{2} (Fig. 1) in NR8383 alveolar macrophages (up to 100 μM D609, 400 nmol of H\textsubscript{2}O\textsubscript{2} added to 5 × 10\textsuperscript{6} cells 15 min before stimulation with ADP). D609 has potential as an antioxidant due to its dithiocarbonate functional group that allows it to slowly react with H\textsubscript{2}O\textsubscript{2} and rapidly reduce cytochrome c, which interferes with a common assay for the respiratory burst. Nonetheless, the antioxidant properties of D609 do not account for its inhibition of priming of the respiratory burst by H\textsubscript{2}O\textsubscript{2}. Reduction of nitro blue tetrazolium is the basis for an assay for superoxide production with which D609 does not interfere. With this assay, it was found that D609 does not inhibit the respiratory burst per se, but prevents its enhancement by pre-exposure to H\textsubscript{2}O\textsubscript{2}. Consistent with a role of diacylglycerol generation by phospholipase C, this enhancement was mimicked by pre-exposure to phorbol ester. In contrast with priming, receptor-mediated stimulation of the respiratory burst depends on the better characterized phosphatidylinositol-specific phospholipase C. Priming of the respiratory burst by H\textsubscript{2}O\textsubscript{2} joins the list of inflammatory responses that are inhibited by D609. Nevertheless, the results herein indicate that caution should be exercised in the interpretation of the effects of D609 to consider both antioxidant effects and inhibition of PC-PLC.

The xanthogenate compound tricyclodecan-9-yl-xanthate (D609; Fig. 1) has antiviral and antitumor properties (Amtmann et al., 1985; Waldeck, 1990; Villanueva et al., 1991; Walro and Rosenthal, 1997), and also inhibits events related to the activation of leukocytes during inflammation (Schutze et al., 1991; Bauldry et al., 1996; Spitsin et al., 1997; Carter et al., 1998; Tschaikowsky et al., 1998; Monick et al., 1999; Wooten et al., 1999; Zhang et al., 2001). D609 is a well characterized competitive inhibitor of phosphatidylcholine-specific phospholipase C (PC-PLC) with a K\textsubscript{i} of 6.4 μM, but shows no inhibitory activity toward phospholipase A\textsubscript{2} and D (Amtmann, 1996). Thus, D609 is frequently used in signal transduction research as a specific inhibitor of PC-PLC. Phospholipase D and sphingomyelinase, which are downstream effectors of PC-PLC, are indirectly inhibited by D609.

The respiratory burst in alveolar macrophages can be primed in vitro by pre-exposure to nontoxic concentrations of hydroperoxides (H\textsubscript{2}O\textsubscript{2} or tert-butylhydroperoxide) (Murphy et al., 1995; Hoyal et al., 1998; Giron-Calle and Forman, 2000). In primary isolates the respiratory burst is enhanced by up to 40% when a population of suspended cells is exposed to about 25 μM H\textsubscript{2}O\textsubscript{2} for 15 min before stimulation (Murphy et al., 1995). In the NR8383 alveolar macrophage cell line the enhancement is maximal, 100% above the basal stimulated level, when cells are exposed to 50 to 100 μM H\textsubscript{2}O\textsubscript{2} before stimulation with the agonist ADP (Giron-Calle and Forman, 2000). Using primary isolates of alveolar macrophages, Hoyal et al. (1996a) discovered that this effect depends on a transient increase of the cytosolic concentration of Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{c}), and correlates with increased translocation of p47\textsuperscript{phox} to the plasma membrane (Zhou et al., 1997). Higher, but still nontoxic, concentrations of H\textsubscript{2}O\textsubscript{2} cause inhibition of the respiratory burst, which correlates with a more sustained increase in the [Ca\textsuperscript{2+}]\textsubscript{c} and decreased translocation of p47\textsuperscript{phox} to the plasma membrane. Phosphorylation of p47\textsuperscript{phox}, which increased upon stimulation of the respiratory

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ABBREVIATIONS: D609, tricyclodecan-9-yl-xanthate; PC-PLC, phosphatidylcholine-specific phospholipase C; [Ca\textsuperscript{2+}]\textsubscript{c}, cytosolic concentration of Ca\textsuperscript{2+}; U73122, 1-(6-(17β-3-methoxyestra-1,3,5(10)-tri-en-17-yl)aminohexyl)-1H-pyrole-2,5-dione; PI-PLC, phosphatidylinositol-specific phospholipase; PBS, phosphate-buffered saline; BSA, bovine serum albumin; KRP, Krebs-Ringer phosphate buffer; NBT, nitro blue tetrazolium; PMA, phorbol-12-myristate-13-acetate.
The possible involvement in this phenomenon of H$_2$O$_2$ has also been investigated. Activation of the respiratory burst by receptor-mediated agonists such as ADP involves the activation of phosphatidylinositol-specific phospholipase C (PI-PLC) to produce inositol-1,4,5-trisphosphate. Nonetheless, priming of the respiratory burst is not mediated by phospholipase D. A view emerges from these studies in which PC-PLC has a function very different from that of the better characterized PI-PLC in the stimulation of the respiratory burst; whereas PI-PLC is essential for receptor-mediated stimulation of the respiratory burst, PC-PLC is involved only in the priming by H$_2$O$_2$.

**Experimental Procedures**

**Reagents and Materials.** Chemicals and enzymes were purchased from the following suppliers: H$_2$O$_2$, nitro blue tetrazolium (NBT), superoxide dismutase, fatty acid-free bovine serum albumin, superoxide dismutase, cytochrome c, and ADP (Sigma-Aldrich, St. Louis, MO); catalase (Worthington Biochemicals, Lakewood, NJ); and D609 and U73122 (BIOMOL Research Laboratories, Plymouth Meeting, PA). D609 was kept as lyophilized aliquots and reconstituted in phosphate-buffered saline (PBS). U73122 was dissolved in chloroform, aliquoted, kept dried under nitrogen gas, and reconstituted in PBS containing 1% bovine serum albumin (BSA). Cell culture medium was purchased from Invitrogen (Carlsbad, CA).

**Cell Culture and Preparation for Incubations.** NR8383 rat alveolar macrophages were obtained by Helmke et al. (1989), who kindly provided the cell line to us. They were cultured in F-12K nutrient mixture containing 15% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells in suspension were collected and resuspended in fresh medium twice a week when concentration was about 2 × 10$^6$ cells/ml (Helmke et al., 1989).

In preparation for the experiments, cell suspensions in culture medium were cooled down on ice, pelleted by centrifugation at low speed, and washed once with ice-cold Krebs-Ringer phosphate buffer (KRP). Viability was routinely assessed by using the vital stain trypan blue. Incubation of cells was carried out in KRP at 37°C.

**Spectrophotometric Analysis of Reaction of D609 with Cytochrome c or H$_2$O$_2$.** The time course of the reaction of D609 with cytochrome c or H$_2$O$_2$ was followed by UV/VIS spectrophotometry. Stock solutions of the reagents were prepared in phosphate-buffered saline (D609, cytochrome c, dithiothreitol) or water (H$_2$O$_2$). Reactions were carried out in KRP pH 7.4 in a cuvette kept at 37°C, by using a Spectromax Plus spectrophotometer (Molecular Dynamics, Sunnyvale, CA).

**Respiratory Burst Determination.** Analysis of superoxide production by determination of cytochrome c reduction was carried out essentially as described (Babior et al., 1973) by using a 20 μM concentration of cytochrome c, and cells were suspended in KRP at a concentration of 1 × 10$^6$ cells/ml. Reduced cytochrome c was measured spectrophotometrically as the difference between the absorbance at 550 nm (reduced cytochrome c) and 540 nm (background given by the isosbestic point between the absorbance peaks of oxidized and reduced cytochrome c). Superoxide production was given by the difference between samples incubated in the absence or presence of superoxide dismutase (120 U/ml).

For analysis of superoxide production by reduction of NBT (Giron-Calle and Forman, 2000), 5 × 10$^6$ cells in 2.5 ml of KRP were seeded into BSA-coated six-well plates (35-mm-diameter wells), and incubated without shaking at 37°C. After allowing the cells to attach for 15 min, the buffer was replaced by 1 ml of fresh, prewarmed KRP, and treatments were carried out as described in figures before stimulation of the respiratory burst. Superoxide production was visualized by addition of 0.25 mM NBT together with the agonist. NBT turns blue and precipitates when reduced by superoxide (Elferink, 1984). Superoxide production was allowed to proceed for 10 min, after which cells were fixed in the dishes by incubating in 3.7% formaldehyde for 5 min. Dishes were rinsed with phosphate buffer before scanning for image recording.

Tissue culture-treated plastic plates were coated with BSA by briefly rinsing with 5% BSA in phosphate-buffered saline and letting it dry at room temperature. For analysis of superoxide production by reduction of NBT, tissue culture-treated 60-mm-diameter plates were coated with BSA. Tissue culture-treated 60-mm-diameter plates were coated with BSA by briefly rinsing with 5% BSA in phosphate-buffered saline and letting it dry at room temperature.
them dry at room temperature. Before being used, BSA-coated dishes were rinsed gently twice with water.

Data as shown are representative of at least three repetitions.

**Results**

**D609 Reacts with Cytochrome c and H$_2$O$_2$.** Superoxide dismutase-inhibitable reduction of cytochrome c is the basis for the most widely used assay for determination of extracellular superoxide production by phagocytes (Babior et al., 1973). Nevertheless, this assay has been found to be inappropriate when incubations include the PC-PLC inhibitor D609, because D609 reacts with cytochrome c. As shown in Fig. 2A, addition of D609 at a concentration normally used for the purpose of PC-PLC inhibition caused increased absorbance at 550 nm, characteristic of reduced cytochrome c. Concomitantly, the absorbance at 302 nm, maximum of absorbance for D609, decreased (Fig. 2B).

In addition, D609 is able to react slowly with H$_2$O$_2$ at neutral pH, yielding a product with a maximum absorbance at 348 nm. In Fig. 3A the reaction of D609 with a high concentration of H$_2$O$_2$ is shown to illustrate the formation of this product. The presence of an isosbestic point between the peak absorbance of D609 at 302 nm and the new product absorbance peak at 348 nm indicates that reaction with H$_2$O$_2$ yields a single product. At lower H$_2$O$_2$ concentrations, as illustrated in Fig. 3B, the reaction of D609 with H$_2$O$_2$ proceeds more slowly. The reaction is slowed down in the presence of the sulfhydryl-reducing agent dithiothreitol (data not shown), and is completely reversed by dithiothreitol if excess H$_2$O$_2$ is previously eliminated by addition of catalase (Fig. 3B). Ethyl xanthate (sodium-O-ethyl dithiocarbonate) (Fig. 1) is a simpler molecule than D609 that has a dithiocarbonate group, presumably responsible for the chemical properties of D609. Ethyl xanthate reacted with H$_2$O$_2$ to the same extent as D609 (data not shown), showing a kinetics identical to that shown in Fig. 3A for the reaction between H$_2$O$_2$ and D609.

**Effect of D609 on Modulation of Respiratory Burst by H$_2$O$_2$.** NBT is another chemical that can be used to

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**Fig. 2.** Reduction of cytochrome c by D609. D609 (100 µM) was added to 20 µM cytochrome c in KRP buffer, pH 7.4, 37°C. A, spectra were taken before (--) and 15 (○) and 30 (□) min after addition of D609. B, time course of the same reaction followed at 302 nm.

**Fig. 3.** Reaction of H$_2$O$_2$ with D609. H$_2$O$_2$ (2 mM) was added to 50 µM D609 in KRP buffer pH 7.4, 37°C. A, spectra were taken before and 1, 5, and 15 min after addition of D609. B, time course of the reaction of a lower H$_2$O$_2$ concentration (100 µM) with 50 µM D609, followed at 302 and 348 nm; catalase (CAT, 23 units/ml) and dithiothreitol (DTT, 1 mM) were added 10 and 20 min after addition of H$_2$O$_2$, respectively.

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measure superoxide production by phagocytes (Elferink, 1984). NBT is a yellowish, water-soluble tetrazolium salt, which upon reduction by superoxide, forms a purple insoluble formazan. Incubation of NBT with D609 yielded no formazan product (data not shown). Thus, although cytochrome c is readily reduced by D609, NBT is not. Consequently, reduction of NBT has been chosen to study the effect of PC-PLC inhibition by D609 on the enhancement of the respiratory burst by H$_2$O$_2$.

Rather than incubating the cells with different concentrations of H$_2$O$_2$, a population of cells was exposed to a gradient of the peroxide as previously described (Giron-Calle and Forman, 2000). Briefly, cells were allowed to attach to BSA-coated dishes, and a solution of H$_2$O$_2$ was added to the center of the dishes. Dishes were not shaken, so that diffusion of the peroxide toward the periphery of the dishes along with consumption by the cells created a concentration gradient. After this pre-exposure to an H$_2$O$_2$ gradient for 15 min, the respiratory burst was stimulated by addition of an agonist together with NBT to cover the entire surface. This experimental system readily allows visualization of the enhancing effect of H$_2$O$_2$ on the respiratory burst that occurs in a markedly narrow range of H$_2$O$_2$ concentrations. In addition, attached cells more closely resemble the situation of alveolar macrophages in vivo. In the following figures, a dark ring at the center of the dishes revealed where exposure of the cells to H$_2$O$_2$ concentrations capable of enhancing stimulation of the respiratory burst occurred. The basal ADP-stimulated burst is short-lived (Giron-Calle and Forman, 2000), and without enhancement by pre-exposure to H$_2$O$_2$, only causes a small reduction of NBT, barely visible as a faint darkening of the entire plate compared with plates of unstimulated cells.

Treatment with D609 before exposure to H$_2$O$_2$ caused a concentration-dependent inhibition of the enhancement of the ADP-stimulated respiratory burst by H$_2$O$_2$ (Fig. 4). D609 also inhibited the enhancement of the respiratory burst triggered by zymosan A (data not shown) and phorbol-12-myristate-13-acetate (Fig. 7A) in a similar manner, indicating that this phenomenon was not specific to ADP stimulation, and occurred when the respiratory burst was stimulated by either soluble (ADP) or particulate (zymosan A) agonists. Addition of D609 after pre-exposure to H$_2$O$_2$ but just before addition of the agonist did not inhibit the enhancement of the respiratory burst by H$_2$O$_2$ at any concentration of D609 (Fig. 4). This indicated that the effect of D609 was not on the stimulation of the cells by the agonist, but on the priming by H$_2$O$_2$. Furthermore, the basal ADP-stimulated burst that was demonstrated by reduction of NBT throughout the whole plate was not inhibited by D609, although as mentioned above, this was too faint to be readily imaged for publication. Therefore, PMA stimulation, which produces a much greater basal respiratory burst than ADP, was used to illustrate this phenomenon. Figure 7A shows that D609 inhibited priming of the respiratory burst by H$_2$O$_2$ as observed by the great diminution of the ring of NBT reduction. Nonetheless, the surrounding basal respiratory burst was not inhibited by D609.

**D609 Inhibition of Priming of Respiratory Burst by H$_2$O$_2$ Is Not Due to Its Antioxidant Properties.** As shown above, D609 acts as a reductant in its reaction with cytochrome c, and is also able to react with H$_2$O$_2$. This points to the possibility that the mechanism of action of D609 may not only involve inhibition of PC-PLC as commonly argued but also the reducing power of the dithiocarbonate group. Therefore, the potential effects on priming by H$_2$O$_2$ of several related and unrelated compounds with reductive capacity were examined for comparison. The effect of the D609 analog ethyl xanthate (Fig. 1) and the sulphydryl agents glutathione, glutathione ester, N-acetyl cysteine, and dithiothreitol, and the lipid antioxidant butylhydroxytoluene were studied. The effect of pyrrolidinedithiocarbamate was studied as well. Pyrrolidinedithiocarbamate contains a moiety that resembles the chemical structure of the dithiocarbonate group, and is frequently used as an antioxidant, although its effect may be more related to metal chelating activity. As reported by Zhou et al. (2001), pyrrolidinedithiocarbamate reproduced the protective effect of D609 against ionizing radiation, supporting an antioxidant effect of the latter. If the antioxidant properties of D609 caused the inhibition of the enhancement of the respiratory burst by H$_2$O$_2$, at least some other antioxidants should mimic this effect; however, none of them were able to mimic the effect of D609 in a wide range of concentrations tested. Despite being able to react with H$_2$O$_2$ as well as D609, ethyl xanthate also failed to reproduce the effect of D609 (Table 1). Some of the treatments are also shown in Fig. 5.
Inhibition of PI-PLC, but not PC-PLC, Blocks Activation of Respiratory Burst. The effect of D609 is suggestive of an involvement of PC-PLC in the priming of the respiratory burst by H$_2$O$_2$, but not in the signal transduction pathway leading to stimulation of the respiratory burst by ADP directly. Another isoform of PLC, PI-PLC, is involved in the stimulation of the respiratory burst in phagocytes. Production of inositol-1,4,5-trisphosphate by PI-PLC leads to phosphorylation of certain cytosolic components of the NADPH oxidase, most prominently p47$^{phox}$, which allows for assembly of the functional NADPH oxidase enzymatic complex (Dusi et al., 1993; Lopes et al., 1999). The effect of the generic PLC inhibitor U73122 corroborated the involvement of PLC in the respiratory burst (Fig. 6). In contrast to D609, U73122 inhibited the respiratory burst not only when added before H$_2$O$_2$ but also when added after preincubation with H$_2$O$_2$ but before addition of ADP, which is consistent with its inhibition of PI-PLC.

Priming of Respiratory Burst by Pre-exposure to H$_2$O$_2$ Is Mimicked by Pre-exposure to Substimulatory Concentrations of Phorbol-12-myristate-13-acetate. PMA is a tumor promoter frequently used in signal transduction research as an activator of protein kinase C. PMA acts as an analog of the second messenger diacylglycerol, although activation of protein kinase C by PMA lasts longer (Castagna et al., 1981). If the priming effect of H$_2$O$_2$ depends on activation of PC-PLC, leading to release of diacylglycerol, PMA could mimic the effect of H$_2$O$_2$. We examined whether pre-exposure to PMA before activation of the respiratory burst has an effect similar to exposure to H$_2$O$_2$. PMA is able to stimulate a respiratory burst by itself (Giron-Calle and Forman, 2000), which can be enhanced by pre-exposure to H$_2$O$_2$ in a D609-inhibitable manner (Fig. 7A). Thus, to test the hypothesis that pre-exposure to PMA mimics the effect of pre-exposure to H$_2$O$_2$, PMA concentrations below the threshold for activation of the respiratory burst were used. Figure 7B shows the result of adding substimulatory concentrations of PMA to the cells instead of H$_2$O$_2$, 15 min before stimulation by ADP. A concentration of PMA that was not sufficient to activate a respiratory burst by itself was able to prime the respiratory burst subsequently activated by ADP.

Discussion

Reactive oxygen species were considered for a long time only as toxic by-products of xenobiotics or cell metabolism with deleterious effects on cells; however, considerable evidence has accumulated for their participation in the regulation of cell growth and function (Suzuki et al., 1997). The modulation of the respiratory burst by pre-exposure to H$_2$O$_2$ in alveolar macrophages constitutes an example of a signaling role (Hoyal et al., 1998). Generation of secondary messengers by phospholipases plays a major role in the signal transduction pathways leading to activation of the respiratory burst (Bauldry et al., 1988; Burnham et al., 1989; Smith et al., 1990; Cockcroft, 1992). Stimulation of macrophages by adenine nucleotides leads to assembly of the respiratory burst oxidase in a Ca$^{2+}$-dependent manner (Murphy et al., 1993; Hoyal et al., 1996a). Because the modulation of the respiratory burst in alveolar macrophages also depends on increases in the [Ca$^{2+}$]$_c$, Ca$^{2+}$-dependent phospholipases have been suggested as possible mediators of the modulatory effect of H$_2$O$_2$.

As a well documented inhibitor of PC-PLC, D609 was chosen to ascertain whether this enzyme might be involved in the response to H$_2$O$_2$. We found however that D609 is able to reduce cytochrome c and react with H$_2$O$_2$, although the reaction with H$_2$O$_2$ proceeds slowly at physiologically relevant concentrations. Oxidation of the dithiocarbonate group

![Fig. 5. Effect of analog ethyl xanthate and antioxidants on the enhancement of the respiratory burst by H$_2$O$_2$. Cells were allowed to attach to BSA-coated plates for 15 min. Cells were then exposed to a gradient of H$_2$O$_2$ formed by diffusion of peroxide added in the center of the wells (400 nM, 10 μl of 40 mM). D609 (100 μM), 100 μM ethyl xanthate, 100 μM glutathione ethyl ester, or 100 μM pyrrolidinedithiocarbamate was added 5 min earlier. The respiratory burst was stimulated 15 min after addition of H$_2$O$_2$ by replacing the buffer for prewarmed KRP containing 400 μM ADP and 25 mM NBT. Superoxide production was allowed to proceed for 10 min before fixation.](image)
present in the D609 molecule is responsible for these chemical properties, which indicate that D609 may be considered as a reducing agent with potential antioxidant properties. Reaction with H$_2$O$_2$ affords a single product that can be reduced by dithiothreitol, probably reflecting the formation of an oxidized D609 dimer. During the course of our studies, Zhou et al. (2001) demonstrated that D609 inhibits lipid peroxidation, glutathione consumption, and protein oxidation in lymphocytes exposed in vitro to ionizing radiation. They also showed that the antioxidant effect of pyrrolidinedithiocarbamate on the oxidation of dihydrorhodamine by a Fenton reaction system (FeSO$_4$/H$_2$O$_2$) was mimicked by D609. Our observations are consistent with theirs, and point to the need for considering the potential antioxidant effects of D609. With this in mind, the D609 analog ethyl xanthate can be used as a control because it possesses a reactive dithiocarbonate group, but does not inhibit PC-PLC.

Ethyl xanthate and a variety of cell-permeable as well as -impermeable antioxidants did not reproduce the inhibitory effect of D609 on the priming of the respiratory burst by H$_2$O$_2$. Although D609 or ethyl xanthate react with H$_2$O$_2$, at the H$_2$O$_2$ concentrations used in this study, the reaction rate is too slow to be of any consequence. The total lack of effect of ethyl xanthate on priming is consistent with the conclusion that, in this case, the antioxidant properties of D609 are not a factor.

D609 inhibited priming of the respiratory burst by H$_2$O$_2$ if present during incubation with H$_2$O$_2$, but not if added just before stimulation, suggesting that D609 affected priming but not the respiratory burst per se. In contrast, the generic PLC inhibitor U73122 inhibited the stimulation of the respiratory burst when added before or after preincubation with H$_2$O$_2$. This is consistent with the established involvement of
PI-PLC in signaling for the receptor-mediated stimulation of the respiratory burst (Burnham et al., 1989; Edwards, 1994).

It is interesting to note that although D609 is generally considered a specific inhibitor of PC-PLC, some reports claim that D609 can inhibit phospholipase D. This inhibition was observed at concentrations similar to those that inhibit PC-PLC (Kiss and Tomono, 1995) or higher (Gratas and Powis, 1993). Nevertheless, it was previously shown that activation of phospholipase D by \( \text{H}_2\text{O}_2 \) is not responsible for the enhancement of the respiratory burst by \( \text{H}_2\text{O}_2 \) in NR8383 macrophages (Giron-Calle and Forman, 2000), so that the hypothetical inhibition of phospholipase D cannot explain the effects of D609 described in this article.

An elegant regulatory mechanism involving two isoforms of PLC emerges from our results, in which activation of PC-PLC has a priming effect, whereas PI-PLC participates in the signaling for basal stimulation of the respiratory burst. There are two main differences in the signaling triggered by phosphatidylcholine versus hydrolysis of phosphatidylinositol. These are production of diacylglycerol without causing changes in the \( [\text{Ca}^{2+}]_i \), and a longer duration of diacylglycerol release with prolonged activation of protein kinase C. Some data also indicate that diacylglycerol derived from phosphatidylcholine may activate different protein kinase C isoforms than those activated by phosphatidylinositol-derived diacylglycerol (Exton, 1994). The modulation of the respiratory burst by peroxides is mediated by an increase in \( [\text{Ca}^{2+}]_i \) (Hoyal et al., 1996a), but the source of this \( \text{Ca}^{2+} \) and the characteristics of the increase are different from agonist (ADP)-triggered \( \text{Ca}^{2+} \) release, which depends on release from the endoplasmic reticulum due to phosphatidylinositol hydrolysis (Robison et al., 1995; Hoyal et al., 1996b).

Interestingly, pretreatment with substimulating doses of PMA, a phorbol ester that mimics prolonged release of diacylglycerol, activates different protein kinase C isoforms than those activated by phosphatidylcholine (Exton, 1994). Thus, the \( \text{Ca}^{2+} \) release that is necessary for the modulation by \( \text{H}_2\text{O}_2 \) most likely occurs upstream of PC-PLC activation. A likely downstream target of the PC-PLC-mediated diacylglycerol release is activation of protein kinase C. A relatively prolonged activation of protein kinase C may conceivably put the cell into a primed state that would allow an enhanced burst upon stimulation by a receptor-mediated agonist. Another possible downstream target of diacylglycerol production is phospholipase A2, which has been linked with priming by diacylglycerol in neutrophils (Bauldry et al., 1988).

Using D609 as a specific inhibitor of PC-PLC, recent reports claim the involvement of this enzyme in various events related with the activation of leukocytes. Thus, D609 has been shown to inhibit mitogen-activated protein kinases activation by lipopolysaccharide (Monick et al., 1999), protein kinase C activation by tumor necrosis factor (Schutze et al., 1991), nitric-oxide synthase activation (Spitsin et al., 1997; Zhang et al., 2001), outside-in signaling for spreading of lymphocytes (Wooten et al., 1999), nuclear factor-\( \kappa \)B activation (Yamamoto et al., 1997; Carter et al., 1998; Zhou et al., 2001), and cytokine release (Carter et al., 1998). Using radiolabeled phospholipids, Grove et al. (1990) determined that a phosphatidylcholine-specific phospholipase is involved in the activation of macrophages by lipopolysaccharide. D609 also inhibited endotoxin shock in whole animals, and has been proposed as an anti-inflammatory agent (Tschaikowsky et al., 1998). In this context, D609 inhibition of priming by \( \text{H}_2\text{O}_2 \) of the respiratory burst adds another dimension to its anti-inflammatory action.

Although priming of the respiratory burst by \( \text{H}_2\text{O}_2 \) has only been demonstrated in vitro, it may constitute a mechanism of regulation of the respiratory burst in the lung. Potentially, generation of hydrogen peroxide derived from the superoxide generated by macrophages and neutrophils during their respiratory burst could prime neighboring macrophages for greater subsequent superoxide production. Considering the concentrations of \( \text{H}_2\text{O}_2 \) that are effective for priming the respiratory burst (25–100 \( \mu \)M, or 25–100 nmol/10\(^6\) cells, for maximal enhancement of the respiratory burst), this phenomenon would probably be of relevance in sites of inflammation in which vigorous production of superoxide by phagocytes is taking place. At the same time, it would be advantageous if this priming were self-limiting to avoid excessive superoxide production. Previously published studies show that concentrations of \( \text{H}_2\text{O}_2 \) higher than those causing priming can inhibit the respiratory burst without being toxic to alveolar macrophages (Murphy et al., 1995).

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References


References


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