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Activation of phospholipase D involved in both injury and survival in A549 alveolar epithelial cells exposed to H_2O_2

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ABSTRACT

To determine the role of the phospholipase D (PLD) pathway in injury and survival of alveolar epithelial cells, A549 cells were exposed to H_2O_2 (500 μM) which resulted in time-dependent injury and bi-phasic increase of PLD activity at 5 min and at 3 h, respectively. *n*-Butanol (0.5%) inhibited PLD activation, attenuated cell injury at 5 min of H_2O_2 exposure, but enhanced injury at 3 h of exposure. This activation was inhibited by treatment with catalase (500 units/ml). Exogenous phosphatidic acid mimicked the effects of PLD activation, and diphenyliodonium (NADPH oxidase inhibitor) reversed the decline in cell viability induced by H_2O_2 exposure. Propranolol (phosphatidic acid phospholydrolase inhibitor) and quinacrine (phospholipase A2 inhibitor) had weak effects on H_2O_2-induced PLD activation but reversed H_2O_2-induced injury. We speculate that PLD activation at the initiation of H_2O_2 exposure predominantly results in NAPDH oxidase activation, which mediates A549 cell injury, but turns to mediating cell survival as the H_2O_2 attack continues, which might be mainly due to the accumulation of intracellular phosphatidic acid.

1. Introduction

Acute lung injury and acute respiratory distress syndrome are characterized by alveolar epithelial and alveolar-capillary damage which result in nonhydrostatic pulmonary edema and severe hypoxemia (Lucas et al., 2009; Manicone, 2009). Oxidative damage plays an important role in the loss of integrity of the epithelial barrier which leads to the influx of protein-rich edema fluid and accumulation of neutrophils in the alveolar space (Lucas et al., 2009; Manicone, 2009). In particular, a mass of reactive oxygen species (ROS) released by neutrophils in the alveolar space contribute greatly to alveolar tissue injury (Mendez and Hubmayr, 2005; Tsushima et al., 2009). However, a new paradigm of redox signaling has emerged recently, whereby some oxidants are considered to function as intracellular signaling molecules which can contribute to either cell death or survival (Giorgio et al., 2007; Trachootham et al., 2008).

Phospholipase D (E.C.3.1.4.4; PLD) is ubiquitous in mammalian cells and is activated by various extracellular stimuli, including H_2O_2, in different cell types. H_2O_2 stimulates PLD activity by a poorly understood, probably direct or indirect signaling pathway (Min et al., 2001; Natarajan et al., 1993; Oh et al., 2000; Xiao et al., 2005). Two mammalian PLD isozymes, PLD1 and PLD2, have been identified, characterized and cloned (Cockcroft, 2001; Jenkins and Frohman, 2005). PLD catalyzes the hydrolysis of phosphatidylcholine and other membrane phospholipids to phosphatidic acid (PA) and choline. PA can be subsequently converted to lyso-PA by phospholipase A2 (PLA2) or to diacylglycerol (DAG) by PA phospholydrolase (PAP), where PA is considered to be the main effector of the functions of PLD in cells (Cockcroft, 2001; Cockcroft and Frohman, 2009; Exton, 2002; Jenkins and Frohman, 2005). In the presence of primary alcohols, PLD catalyzes a transphosphatidylation reaction producing phosphtidylalcohols at the expense of PA; this feature provides a tool to implicate PLD in cellular responses (Exton, 2002).

PLD and its metabolites are involved in various cellular functions, such as activation of NADPH oxidase (oxidative burst), membrane trafficking, exocytosis (Cockcroft et al., 2002), phagocytosis (Corrotte et al., 2006), cell adhesion and chemotaxis (Gomez-Cambronero et al., 2007), cytoskeletal reorganization, cell proliferation, apoptosis, and survival (Cockcroft and Frohman, 2009). In inflammatory cells or phagocytic cells, PLD plays a role in stimulation of NADPH during the respiratory oxidative burst. PLD functions both directly, by generating PA, which binds to and

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stimulates the p47 (phox) component of the NADPH oxidase complex (Usatyuk et al., 2009), and indirectly, by converting of some of the PA into DAG, which is required for NADPH activation (Palicz et al., 2001). Once NADPH oxidase is activated, it generates H_2O_2 which leads to further PLD activation (Xiao et al., 2005). These features constitute a positive feedback cycle: exogenous H_2O_2 activates PLD and PLD activation promotes NADPH oxidase activation which leads to H_2O_2 production.

However, it is unclear that whether this positive feedback cycle exists in non-phagocytic cells, e.g., in the alveolar epithelial cell. Moreover, the roles of PLD and its metabolites play in alveolar epithelial cells during acute lung injury are unclear. Here, we report the involvement of the PLD pathway in H_2O_2-induced A549 alveolar epithelial cell injury and survival.

2. Materials and methods

2.1. Drugs

Phosphatidylcholine (C10:0), 4-aminoantipyrine, sodium oleate, 1-butanol, propranolol, phosphatidic acid (C10:0), catalase (from bovine liver) (Sigma, St. Louis, MO, USA), phenol (ShengGong Biotechnology Co. Ltd., Shanghai, China), and horseradish peroxidase (XiTang Biotechnology Co. Ltd., Shanghai, China), were dissolved in Millipore water. Quinacrine, diphenyliodonium (DPI), and 2,7-dichlorofluorescein diacetate (Sigma) were dissolved in dimethyl sulfoxide (DMSO, Sigma) and diluted in Millipore water before use (the concentration of DMSO was less than 0.001% after dilution). H_2O_2 (Sigma) was diluted in Earle's solution (concentrations in mM: NaCl, 117; KCl, 5.3; $CaCl_2$, 1.8; $NaHCO_3$, 26; $MgSO_4$, 0.8; NaH_2PO_4, 1.0; glucose, 5.6; pH 7.4). Choline oxidase (Sigma) was dissolved in a solution containing (in mM) Tris–HCl, 10; EDTA, 2.0; KCl, 134; pH 8.0.

2.2. Cell culture

A549 alveolar epithelial cells (Institute of Cell Biology, Chinese Academy of Science, Shanghai, China) were cultured in F-12 Kaighn's nutrient mixture (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Invitrogen) in a culture flask with 1×10^6 cells/75 cm^2 and in 96-well plates with 4000 cells/well under air and 5% CO_2 at 37 °C. Before exposure to H_2O_2, the culture medium was switched to F-12 Kaighn's nutrient mixture containing 1% fetal bovine serum for 48 h and then the cells were rinsed twice with Earle's solution.

2.3. Agent treatments

Catalase (500 units/ml), phosphatidic acid (0.1 μM), the PLD inhibitor 1-butanol (0.5%), the NADPH oxidase inhibitor diphenyliodonium (1 nM), the PAP inhibitor propranolol (100 μM), and the PLA2 inhibitor quinacrine (1 μM) were used. The agents were continuously applied from 30 min before H_2O_2 exposure to the end of exposure. The control group received the same treatment except for H_2O_2 exposure.

2.4. Assessment of cell viability and cell injury

At the end of H_2O_2 exposure, 3-(4,5-dimehythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well to a final concentration 0.5 mg/ml. After incubation for 4 h at 37 °C, the medium was removed and 100 μl DMSO was added to each well for 10 min at 37 °C. The plate was read at 570 nm using a microplate reader (Elx800, Bio-Tek Instruments, Vermont, USA). Results are reported as percentages of control. In another series, the cytotoxicity of H_2O_2 on A549 cells was quantified using lactate dehydrogenase (LDH) activity assays in the medium and the cell layer. The LDH activity assay was carried out by a colorimetric method using an LDH assay kit (JianCheng Biotechnology Co. Ltd., Nanjing, China). The net percentage of LDH release was calculated as follows: net percentage of LDH release = 100% × (stimulated release − spontaneous release)/(total release − spontaneous release).

2.5. Phospholipase D-catalyzed reaction

After treatment, the cells were trypsinized and rinsed with PBS (phosphate buffered saline, pH 7.4) 3 times at 4 °C. The cells were resuspend in lysis solution (KangCheng Biotechnology Co. Ltd., Shanghai, China), lysed by ultrasound in an ice bath, and the lysate was centrifuged to eliminate nuclei and unbroken cells. All lysates were tested for protein quantity. A PLD-catalyzed reaction was carried out in a 37 °C water bath for 90 min. The 360 μl reaction system (Vinggaard et al., 1996) was composed of 25 mM HEPES (pH 7.4), 2 mM phosphatidylcholine, 6 mM oleic acid, 1.6 M $(NH_4)_2SO_4$, 1 mM $CaCl_2$ and 5 mM $MgCl_2$. A549 cell lysate containing 100–300 μg protein (in 95 μl) was added at the onset of the reaction. To terminate the reaction, the tubes were placed in boiling water for 10 min. When cooled to room temperature, each sample was mixed with 360 μl chloroform and vortexed for 1 min before centrifugation (4000 × *g*, 10 min). After centrifugation, the supernatant was used for PLD activity assay.

2.6. PLD activity assay

As described previously (Lucas et al., 1995; Pedruzzi et al., 1998; Wu et al., 2001), the PLD activity assay was based on a reaction system containing 200 μl supernatant and 800 μl color reagent (Tris–HCl 45 mM, pH 8.0, peroxidase 5 units, choline oxidase 1 unit, 4-aminoantipyrine 0.3 mg, phenol 0.2 mg). The tube containing this reaction was incubated in a 37 °C water bath for 90 min. The reaction was terminated by adding 0.5 ml ice-cold 50 mM Tris–HCl (pH 8.0). A standard curve was constructed each time with a fresh choline standard (10, 20, 40, 80, and 160 nmol). The PLD activity was quantified by calculation of produced choline using a standard curve. One unit of PLD activity was defined as 1 nmol choline produced by 1 mg cell lysate protein during 1 min at 37 °C.

2.7. Measurement of intracellular H_2O_2

The amount of intracellular H_2O_2 was assessed using the fluorescent probe 2,7-dichlorofluorescein diacetate (DCF-DA). After diffusion into cells, the acetate group of DCF-DA is cleaved by esterases, trapping it inside; subsequent oxidation yields a fluorescent adduct. A549 cells cultured on 96-well plates were rinsed with Earle's solution, and incubated with 25 μM DCF-DA in Earle's solution for 30 min at 37 °C. Then the cells were exposed to H_2O_2 at indicated concentrations and durations. At the end of H_2O_2 exposure, the cells were rinsed twice to remove excess probe, and fluorescence was measured with a multi-well plate fluorescence reader (FLUOStar, BMG LABTECH GmbH, Offenburg, Germany) at excitation 485 nm and emission 520 nm. Results are expressed as percentage of control.

2.8. Statistical analysis

All data are expressed as mean ± SD. For comparison, unpaired *t*-test, one-way ANOVA and Dunnett's test or Dunn's test were performed using GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA). A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. H_2O_2-induced A549 cell viability changes and cell injury

We determined the effects of exposure to various concentrations of H_2O_2 at different durations on A549 cell viability by assay of MTT reduction, and on cell injury by assay of LDH release. The results showed that exposure to H_2O_2 induced a decline of cell viability and increment of LDH release in a concentration- and time-dependent manner (Fig. 1). After exposure to 500 μM H_2O_2, the cell viability gradually decreased by 8.6%, 9.2%, 9.7%, 26% and 40% of control at 5, 30, 60, 180 and 360 min, respectively (Fig. 1A). When the H_2O_2 exposure was longer than 3 h, the LDH release increased dramatically (Fig. 1B).

3.2. H_2O_2-induced changes of PLD activity in A549 cells

PLD activation was induced by H_2O_2 in a concentration-dependent and a time-dependent manner (Fig. 2). The basal activity (control level) of PLD in A549 cells was 0.16 units. When cells were exposed to 0–1000 μM H_2O_2 for 30 min, the highest PLD activity appeared when H_2O_2 concentration was 500 μM (Fig. 2A). Thus, in another series of experiments, cells were treated with 500 μM H_2O_2 for 5–360 min (Fig. 2B). We found the highest PLD activity appeared at an exposure of 5 min, when the value was 0.41 ± 0.06 units (2.56-fold control; $^{**}P < 0.01$, $n = 5$), and then the value decreased, but remained higher than control at 60 min. At 180 min, the value of PLD activity increased to 0.32 ± 0.04 units but was lower than the peak value. The PLD activity decreased to control at 6 h.

On the basis of these results, we assessed the responses of A549 cells to H_2O_2 exposure at 500 μM for 5 min and 180 min in the subsequent experiments.