THE EFFECT OF EXTRACELLULAR CARDIOLIPIN IN BOVINE PULMONARY ARTERY ENDOTHELIAL CELLS

by

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Abstract:

Group B Streptococcus is the most common cause of bacterial infection in the newborns. Infection by Group B Streptococcus often results in pulmonary hypertension. The bacterial components causing pulmonary hypertension had been identified as cardiolipin and phosphatidylglycerol. Cardiolipin not only induces pulmonary hypertension in human, but also in lambs. Due to very little research on cardiolipin and other phospholipids in lung injury, it is important to understand how cardiolipin and other phospholipids play a role in lung pathophysiology.

In our study, we are the first to demonstrate that no significant difference in cell viability is shown in the treatment with phosphatidylcholine alone (300 uM) nor with a combination of cardiolipin (30uM) and phosphatidylcholine (70uM) in bovine pulmonary artery endothelial cells at 4 hr and 24 hr. However, cells treated with 30uM cardiolipin and 70 uM phosphatidylcholine for 24 hr have a significant increase in caspase-3 and caspase-7 activity. Furthermore, caspase-3 and caspase-7 activity was elevated by treatment with CL alone at 10uM and 30uM.
**Conclusions:**

Cardiolipin and co-treatment with phosphatidylcholine induces apoptosis pathways in caspase-3 and caspase-7 cascades in bovine pulmonary artery endothelial cells.

**Public health significance:**

This study provides insight into the potential role of cardiolipin in the pathophysiology of lung injury. This may further open new approaches for the development of therapeutic intervention for pulmonary diseases.
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1.0 Background

1.1 Oxidized phospholipids and lung:

Oxygenated fatty acids are well-known signaling molecules: numerous oxygen-containing eicosanoids - prostaglandins, thromboxanes, leukotrienes, lipoxenes, resolvins and protectins - participate in regulation and coordination of cell and body metabolism (Bannenberg, Arita et al. 2007; Schwab, Chiang et al. 2007). Their important roles in cell proliferation, modulation of apoptosis, angiogenesis, inflammation and immune surveillance have been demonstrated (Ariel and Serhan 2007; Bannenberg, Arita et al. 2007). Several decades ago, activation of lipid peroxidation in the lung under hyperoxic conditions was evidenced by accumulation of malonyldialdehyde - one of the secondary products of lipid peroxidation (Jamieson, Chance et al. 1986). Moreover, elevation of oxygenated products formed from arachidonic acid - isoprostanes and isofuranes - has been documented in hyperoxic mouse lung (Fessel and Jackson Roberts 2005). The selective oxidation of the other major phospholipid, phosphatidylcholine (PC), is the key elements of apoptosis, inflammation and clearance of apoptotic cells (Wright, Howe et al. 2004).

1.2 Pulmonary endothelium:

Pulmonary endothelium is the locus of earliest structural and functional changes in acute lung injury (ALI) (Crapo 1986), and apoptosis of pulmonary endothelium is an important part of genesis and maintenance of ALI in experimental animals (Ray, Devaux et al. 2003;
Bhandari, Choo-Wing et al. 2006; Mokres, Parai et al. 2010) and human subjects (Abadie, Bregeon et al. 2005). Some studies have reported on LPS- and hyperoxia mediated apoptosis in cultured pulmonary endothelium although the mechanism remains unclear (Hoyt, Mannix et al. 1995; Tang, Wasserloos et al. 2002).

1.3 Cardiolipin (CL) and lung injury:

CL was originally isolated from beef heart in early 1940s. CL, the mitochondrial-specific phospholipid, helps maintain mitochondrial function, membrane potential and structural support for the inner mitochondrial membrane and the proteins in it, indicating that CL is a mitochondrial-specific lipid essential for bioenergetics in metabolism and catabolism and is often linked to the apoptotic program (Chicco and Sparagna 2007). Concentration and composition changes of CL have been implicated in pathological conditions including ischemia, hypothyroidism, aging, heart failure, cardioskeletal myopathy and pulmonary diseases (Curtis, Kim et al. 2003; Chicco and Sparagna 2007). CL has also been shown as a signaling molecule in apoptosis (Kagan, Tyurina et al. 2006; Ritov, Menshikova et al. 2006). Almost all functional information is in the context of its intracellular mitochondrial location, for example, oxidized CL triggers apoptosis.
1.4 **Extracellular CL in pulmonary epithelium**

Elevated CL levels are seen in chronic obstructive pulmonary disease (COPD) patients and in lung fluids of chemical alveolar injury model in dogs and in a sepsis model in rats.

In addition, in unpublished data (personal communication, Rama Mallampalli, University of Pittsburgh). His study shows that CL is elevated in the lung fluid of patients with pneumonia and in mice infected with H. influenza and E coli. His study also demonstrated that CL appears to play a role in pulmonary epithelial cell dysfunction (aberrant surfactant synthesis) in influenza infection in humans and mice.

Fig 1. Mouse Lung Epithelial Cell Viability.
This figure in Dr. Rama Mallampalli’s data shows the cell viability of mouse lung epithelial cells after treatment with different concentrations of CL. From the figure, it is shown that CL produced a dose-dependent decrease in epithelial cell viability.

1.5 Extracellular CL in pulmonary endothelium

Group B Streptococcus is the most common cause of bacterial infection in the human newborns (Schuchat 1999). Despite prompt treatment with antibiotics, mortality rate from Group B Streptococcus is significant at 5% (Schrag, Zywicki et al. 2000). Group B Streptococcus infection often causes severe pulmonary diseases in newborns, especially pulmonary hypertension, which causes an increase in pulmonary vascular resistance, impairing gas exchange of oxygen and carbon dioxide (Rojas, Green et al. 1981; Rojas and Stahlman 1984). In 2003, the bacterial components causing pulmonary hypertension had been identified as CL and phosphatidylglycerol (Curtis, Kim et al. 2003). Phosphatidylglycerol is a glycerophospholipid found in pulmonary surfactant. Two phosphatidylglycerols form CL, the constituent molecule of the mitochondrial inner membrane. Cardiolipin not only induces pulmonary hypertension in human, but also in lambs. Due to limited research on CL and other phospholipids in lung injury, it is crucial to understand how CL and other phospholipids play the role in lung pathophysiology.

In this study, we aimed to examine the potential toxic effects of exogenous CL on pulmonary endothelium in a cell model using bovine pulmonary artery endothelial cells.
Fig 2. Outline of experimental design.
2.0 Methods

2.1 Cell culture of bovine pulmonary artery endothelial cells (BPAECs)

BPAECs were isolated from neonatal calves as previously described (Stiebellehner, Frid et al. 2003), (Frid, Kale et al. 2002). BPAECs were maintained in MCDB 131 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 1% L-glutamine (Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (Invitrogen; Carlsbad, CA). Once BPAECs reach 90% confluency in T-75 flasks, the cells were trypsinized by trypsin (Invitrogen; Carlsbad, CA), washed twice with HBSS, centrifuged at 900x rpm for 5 minutes, re-suspended in MCDB 131 cultured medium, and then determine cell numbers by Countess® Automated Cell Counter (Invitrogen; Carlsbad, CA) before seeding cells to plates. Cells were serum-starved for 24 hours before exposed to phospholipids.

2.2 Preparation of liposomes

The dioleoyl forms of phosphatidylcholine (PC) and cardiolipin (CL) were obtained from Avanti Polar Lipids Inc., Alabaster, AL. Liposomes were formed by mixing with chloroform and methanol (ratio 2:1), dried and evaporated from nitrogen, and then rehydrated with HBSS buffer (Hanks' balanced salt solution with calcium and magnesium, bubbled with nitrogen before use) with vigorous vortexing and sonication. Liposomes in HBSS buffer can be ready to use or stored in freezer at -20 °C.
2.3 Exposure of BPAEC to phospholipids

Cultured BPAECs were exposed to various concentrations of PC and/or CL for 4 hr and 24 hr. PC was used at 0, 10, 30, 100, 300 uM; CL was used at 0, 1, 3, 10, 30 uM; The mixture of PC and CL was used at 0 uM PC+CL, 100 uM PC, 1 uM CL+99 uM PC, 3 uM CL+97 uM PC, 10 uM CL+90 uM PC, 20 uM CL+80 uM PC, 30 uM CL+70 uM PC. Therefore CL was studied from range 1~30 Mol% in PC liposomes. After each treatment, cells were collected and used to analyze cell viability and cell apoptosis (caspase-3 and caspase-7) activity.

2.4 Cell viability assay

Cell viability in BPAECs was measured by Alamar blue assay (Invitrogen, Carlsbad, CA). Alamar blue reagent was added to the cells, protected from light, and incubated for 3 hrs at 37 °C. The florescence was read by fluorescence spectrophotometer using 560nm excitation/590nm emission filter settings. The cell viability was expressed as fluorescence intensity (or absorbance).
2.5 Caspase-3 and caspase-7 activity

Caspase-3 and caspase-7 activity in BPAECs was measured using a luminescence Caspase-Glo assay kit purchased from Promega (Madison, WI). Luminescence was determined after 1hr of incubation at room temperature using a ML1000 luminescence plate reader (Dynatech). Caspase-3 and caspase-7 activity was expressed as the luminescence produced within 1hr of incubation per microgram of protein.

2.6 Statistical analysis

The data were expressed as mean ± S.E.M. Statistical significance was performed with analysis of variance followed by post-hoc analysis. A value of P<0.05 was considered to denote statistical significance.

3.0 Results

3.1 Cell viability

Fig 1 shows cell viability of BPAECs after 4hr treatment with PC at different concentrations. Fig 2 shows cell viability of BPAECs after 24hr treatment with PC at different concentrations. From Fig1 and 2, we can see that 4hr and 24hr treatment with PC alone show no significant changes in cell viability in BPAECs. Fig 3 and Fig show cell viability of BPAECs after 4hr and 24hr co-treatment with CL+PC. These two graphs
indicate that combination of CL and PC shows no significant changes in cell viability in BPAECs. The concentration here (in Fig 3 and 4) also represents that CL Mol% is 1%, 3%, 10%, 30% in PC liposomes.

3.2 Caspase 3/7 activity

Fig 5 shows caspase 3/7 activity of BPAECs after 4hr co-treatment with CL+PC. It is shown that no significant difference in caspase 3/7 activity after 4hr treatment with CL+PC. Fig 6 shows caspase 3/7 activity of BPAECs after 24hr co-treatment with CL+PC. This figure demonstrates that caspase 3/7 activity begins to increase after 24hr treatment with 30 Mol% in PC liposomes by 1.7-fold increase over the control. Next we would like to compare the toxic effect between CL in PC liposomes and CL alone. Fig 7 shows caspase 3/7 activity of BPAECs after 24hr treatment with CL at different concentrations. This figure clearly demonstrates that treatment with CL alone show great significance in caspase 3/7 activity at 10uM (by 1.7-fold increase over the control) and at 30uM (by 2.8-fold increase over the control).

4.0 Conclusion & Discussion

In this study, we conclude that 1) no significant differences in cell viability were detected in 4 hr and 24hr treatment with CL alone or CL+PC; 2) 30 Mol% of CL in PC liposomes induces apoptosis activity in caspase-3 and caspase-7 cascades in BPAECs; 3) Non-liposomal CL appears to be more toxic than the liposomal CL+PC in BPAECs.
The strengths of the study are that we are the first to demonstrate the toxic effects of extracellular CL in BPAECs. We are also the first to show that CL triggers apoptosis at 24hr in cultured BPAECs.

For the first time, our study demonstrated that PC, within concentration of 300 uM, has no significant toxic effects on cell viability at 4hr and 24 hr in cultured BPAECs. Similarly, co-treatment with CL (30uM) and PC (70uM), for 4 hr and 24 hr also shows no significant changes in cell viability in BPAECs. The range of Mol% of CL selected in this study was based on Dr. Rama Mallampalli’s manuscripts. Our study is also the first to show that cardiolipin activates apoptosis at 24hr in cultured BAPCSs. In the caspase3/7 study, it is found that 4 hr treatment with PC and CL does not make any differences in caspase-3 and caspase-7 activity. At 24hr, the activity of caspase-3 and caspase-7 begins to increase gradually and the significance shows at the co-treatment with CL (30uM) and PC (70uM) by 1.7-fold over the control. The results were confirmed by 4 separate individual experiments. Moreover, cells treated with CL alone even shows greater significance in caspase-3 and caspase-7 activity starting at 10uM CL (by 1.7-fold over control) and 30uM CL (by 2.8-fold over control). These results were further confirmed by 6 individual experiments per group.

The limitations of the study are some of the cell viability experiments were only repeated twice, it will be good to have at least 3-4 individual experiments as the future work. Also we could not know whether CL molecules were fully absorbed and uptake by BAPCSs. It is possible that free CL outside the cells affects the caspase 3/7 activity. Future work, such as microscopy imaging or CL tracing using radioactive labeling or
fluorescence labeling, needs to be done to confirm the actual distribution of CL inside and outside of the cells. It will also be beneficial to use other cell lines such as murine pulmonary artery endothelial cells and human pulmonary artery endothelial cells to compare the differences of the results.

In conclusion, single treatment with cardiolipin and/or co-treatment with phosphatidylcholine induce apoptosis pathways in caspase-3 and caspase-7 cascades at 24 hr in bovine pulmonary artery endothelial cells. This study provides insight into the potential role of cardiolipin and other major phospholipids in the pathophysiology of pulmonary hypertension and lung injuries. This may further open new approaches for the development of therapeutic intervention for pulmonary diseases.
Fig 3. Cell viability of BPAECs after 4h treatment of phosphatidylcholine (PC) at different concentrations.
Fig 4. Cell viability of BPAECs after 24h treatment of phosphatidylcholine (PC) at different concentrations.
Fig 5. Cell viability of BPAECs after 4h co-treatment with cardiolipin (CL) and phosphatidylcholine (PC) at different concentrations.
Fig 6. Cell viability of BPAECs after 24h co-treatment with cardiolipin (CL) and phosphatidylcholine (PC) at different concentrations.
Fig 7. Caspase 3/7 activity of BPAECs after 4h co-treatment with cardiolipin (CL) and phosphatidylcholine (PC) at different concentrations.
Fig 8. Caspase 3/7 activity of BPAECs after 24h co-treatment with cardiolipin (CL) and phosphatidylcholine (PC) at different concentrations.

(n=4 per group) * p<0.01 vs. control
Fig 9. Caspase 3/7 activity of BPAECs after 24h treatment with cardiolipin (CL) at different concentrations.

(n=6 per group) * p<0.05 vs. control; **p<0.01 vs. control


