Pulmonary surfactant protein A inhibits the lipid peroxidation stimulated by linoleic acid hydroperoxide of rat lung mitochondria and microsomes

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Abstract

Reactive oxygen species play an important role in several acute lung injuries. The lung tissue contains polyunsaturated fatty acids (PUFAs) that are substrates of lipid peroxidation that may lead to loss of the functional integrity of the cell membranes. In this study, we compare the in vitro protective effect of pulmonary surfactant protein A (SP-A), purified from porcine surfactant, against ascorbate–Fe\textsuperscript{2+} lipid peroxidation stimulated by linoleic acid hydroperoxide (LHP) of the mitochondria and microsomes isolated from rat lung; deprived organelles of ascorbate and LHP were utilized as control. The process was measured simultaneously by chemiluminescence as well as by PUFAs degradation of the total lipids isolated from these organelles. The addition of LHP to rat lung mitochondria or microsomes produces a marked increase in light emission; the highest value of activation was produced in microsomes (total chemiluminescence: 20.015 ± 1.735 \times 10^5 cpm). The inhibition of lipid peroxidation (decrease of chemiluminescence) was observed with the addition of increasing amounts (2.5 to 5.0 \mu g) of SP-A in rat lung mitochondria and 2.5 to 7.5 \mu g of SP-A in rat lung microsomes. The inhibitory effect reaches the highest values in the mitochondria, thus, 5.0 \mu g of SP-A produces a 100\% inhibition in this membranes whereas 7.5 \mu g of SP-A produces a 51.25 ± 3.48\% inhibition in microsomes. The major difference in the fatty acid composition of total lipids isolated from native and peroxidized membranes was found in the arachidonic acid content; this decreased from 9.68 ± 1.60\% in the native group to 5.72 ± 1.64\% in peroxidized mitochondria and from 7.39 ± 1.14\% to 3.21 ± 0.77\% in microsomes. These changes were less pronounced in SP-A treated membranes; as an example, in the presence of 5.0 \mu g of SP-A, we observed a total protection of 20:4 n-6 (9.41 ± 3.29\%) in mitochondria, whereas 7.5 \mu g of SP-A produced a 65\% protection in microsomes (5.95 ± 0.73\%). Under these experimental conditions, BSA was unable to inhibit lipid peroxidation stimulated by linoleic acid hydroperoxide of rat lung mitochondria or microsomes, thus indicating that this effect is specific to SP-A.

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Keywords: Rat lung; Lipid peroxidation; Linoleic acid hydroperoxide; Pulmonary surfactant protein A

Abbreviations: ARDS, acute respiratory distress syndrome; LHP, linoleic acid hydroperoxide; LOOH, lipid hydroperoxide; PUFAs, polyunsaturated fatty acid; SP-A, pulmonary surfactant protein A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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1. Introduction

The effects of oxidative stress on lung tissue are multiple; reactive oxygen and nitrogen species finally produced acute lung injury. In particular, they can initiate lipid peroxidation of intracellular membranes, and lipid peroxides are detrimental to cellular function. Lipid peroxidation could then play a role in the pathogenesis of acute respiratory distress syndrome (ARDS) [1,2]. The ARDS is a disease process that is characterized by diffuse inflammation in the lung...
Pulmonary surfactant is a mixture of lipids and proteins synthesized and secreted by alveolar type II cells. Its principal property is to reduce the surface tension by lining the alveolar surface although certain surfactant components have also been shown to participate in host defence. The pulmonary surfactant consists of 80–90% phospholipids, 3–8% neutral lipids, mainly cholesterol, and 5–10% surfactant proteins. The major phospholipid components are dipalmitoylphosphatidylcholine (30–45%), unsaturated phosphatidylcholine (25–35%) and small amounts of phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine and sphingomyelin. The surfactant proteins consist of SP-A and SP-D, which are members of the C-type lectin or collectin (collagen-like lectin) super family of proteins and SP-B and SP-C, which are small hydrophobic proteins that play a role in pulmonary surfactant homeostasis by regulating both surfactant secretion and clearance. It has also been shown that SP-A inhibits phospholipase A2 activity in the lung and may play a role in the regulation of the degradation of surfactant phospholipids in intracellular and extracellular compartments [10–11].

The exposure of surfactant to ambient oxygen and potent environmental oxidants such as ozone results in the peroxidation of unsaturated phospholipids, surfactant inactivation, airspace collapse and impaired gas exchange. Surfactant dysfunction is an important factor contributing to the pathophysiology of ARDS, as a result of free radical damage to lipid and protein components. In this disease, plasma proteins inactivate surfactant, and SP-A blocks foreign protein-induced inhibition and augments the adsorption of phospholipids to an air–liquid interface. SP-A has been measured in the systemic circulation and may be a useful biomarker of lung disease [12]. Previous reports involving the organic extract of surfactant and whole surfactant have shown that there exist potential differences with respect to susceptibility to inactivation [13].

Gillard et al. [14] have demonstrated that the in vitro lipid peroxidation of hydrophobic components of lung surfactant results in the loss of surface tension lowering function and loss of polyunsaturated fatty acids. Matalon et al. [15] have attributed the antioxidant activities of pulmonary surfactant to the presence of antioxidant enzymes such as catalase and superoxide dismutase.

Lipid peroxidation is one of the main events induced by oxidative stress and is particularly active in biomembranes like mitochondria, which are rich in PUFAs. Some lipid peroxidation products are light-emitting species, and their spontaneous chemiluminescence can be used as an internal marker of oxidative stress [16]. Once initiated, lipid peroxidation proceeds as a free-radical chain reaction, resulting in the formation of fatty acid hydroperoxides (LOOH). These hydroperoxides are ubiquitous components of biological systems; hence, their contribution to lipid peroxidation initiation processes is likely to be of a great significance in vivo [17]. In vitro, fatty acid hydroperoxides enhance the ascorbic–Fe2+ induced lipid peroxidation. Fe2+ will reductively cleave LOOH to highly reactive alkoxyl radicals (LO•) formed at the membrane surface. These radicals may penetrate into the hydrophobic region and trigger the initiation reaction by abstracting hydrogen from lipids to form new lipid alkyl radicals [18,19].

Kornbrust et al. [20] showed that highly oxygenated tissues such as lung and heart microsomes peroxidized at a 25- to 50-fold lower rate than liver, kidney, testes and brain microsomes. The ratio of peroxidable PUFAs to vitamin E was several-fold lower in lung and heart microsomes than in microsomes from the other tissues, which accounted for the unique resistance of lung and heart to lipid peroxidation. Moreover, Leedle and Aust [21] have demonstrated that rat lung microsomes were found to be much more resistant to lipid peroxidation than those from liver in both enzymatic and nonenzymatic systems.

Taken together, these observations have analyzed the effect of SP-A on the non-enzymatic (ascorbate–Fe2+ dependent) lipid peroxidation stimulated by linoleic acid hydroperoxide of the rat lung mitochondria and microsomes. The aim of this study was to determine if the SP-A contribute to the prevention of lipid peroxidation in the lung. The process was followed simultaneously by the determination of chemiluminescence and fatty acid composition of total lipids.

2. Materials and methods

2.1. Materials

Sepharose 4B was from Pharmacia Fine Chemicals Inc. Piscataway N.J. Butylated hydroxytoluene (BHT), phenyl
methylsulfonyl fluoride (PMFS), Lipoxidase (EC 1.13. 11.12) Type I S from soybean were from Sigma. Bovine serum albumin (BSA) (fraction V, fatty acid free) was obtained from Wako Pure Chemical Industries Ltd. (Osaka) Japan. Fatty acids and standards of fatty acid methyl esters were from Nu Chek Prep. Inc. Elysian, MN, USA. L-(-)-Ascorbic acid and boron trifluoride–methanol complex were from Merck; Micro-spin filter, 10,000 MW sterile, was from Cole Parmer Instrument Company, IL, USA. All other reagents and chemicals were of analytical grade.

2.2. Isolation of rat lung mitochondria and microsomes

Male Wistar rats were fed with commercial rat chow and water ad libitum. Rats weighing 180–220 g were sacrificed by cervical dislocation. Lungs were collected on ice immediately after sacrifice and were minced with scissors to small pieces and washed extensively with 0.15 M NaCl. The minced lungs were homogenized with ice-cold solution, 0.25 M sucrose, 10 mM Tris–HCl, pH 7.4, and 0.1 mM PMFS (3 ml/g of tissue) using the Potter-Elvehjem homogenizer for 20 s and transferred to a centrifuge tube. After homogenization, mitochondrial preparations were isolated by differential centrifugation. The homogenate was spun at 3000×g for 10 min to remove nuclei and cell debris, the pellet was discarded and the supernatant was centrifuged at 20,000×g for 10 min and the mitochondrial pellet was collected and resuspended in buffer, 10 mM Tris–HCl, pH 7.4, 0.1 mM PMFS. The post-mitochondrial supernatant (5 ml) was applied to a Sepharose 4B column containing 5 mM EGTA, 1 mM MgCl₂. Purified SP-A was obtained in the supernatant and was dialyzed against 150 mM NaCl for the removal of EGTA by 48 h according to [34] or filtrated for the removal of EGTA, and the free protein of EGTA was tested to demonstrate that low molecular weight contaminants are not responsible for the antioxidant activities.

2.2. Preparation of linoleic acid hydroperoxide

The hydroperoxide of linoleic acid was prepared by a modification of the method described by Armstrong [23] using the enzyme lipoygenase. About 125 mg of linoleic acid was converted to sodium salt and dissolved in 50 ml of 0.1 M borate buffer, pH 9. Next, 5 mg of soybean lipoygenase was added and the mixture was incubated in an oxygen-saturated atmosphere at 4 °C for 2 h. A second aliquot (5 mg) of enzyme was added and the incubation was continued for another 2 h. After incubation, NaCl (2.5 g) was dissolved in the solution and the hydroperoxides were extracted three times with 30 ml of anhydrous diethyl ether. The ether extract was dried with 12.5 g of sodium sulfate anhydrous to remove any water and filtered through paper to trap crystals. Aliquots were evaporated to dryness under nitrogen, resuspended in methanol and stored at −80 °C. Thin layer silica-gel chromatography was used to compare unoxidized fatty acids against corresponding hydroperoxides. Linoleic acid hydroperoxide and their fatty acid precursor linoleic acid were resuspended in methanol and applied to the plate, developed in heptane/ethyl acetate/acetic acid (95:5:1, v/v/v) as mobile phase, dried, sprayed with 0.1 M sulfuric acid and heated at 250 °C for 20–30 min. Under the conditions described, conversion was >95%. The maximal absorption at 234 nm, corresponding to the conjugate-diene system of hydroperoxides, was used to calculate final concentration of LHP applying Beer’s law using ε=23,000 as molar extinction coefficient.

2.4. Isolation of purified porcine surfactant protein A

SP-A was isolated from porcine lung surfactant as described previously [24]. Briefly, porcine lung lavage was collected and cells and debris were removed by low-speed centrifugation (300×g for 10 min at 4 °C); the cell-free supernatant was centrifuged at 7100×g for 60 min. The crude surfactant pellet was suspended in 0.15 M NaCl and it was layered over 0.75 M sucrose in 0.15 M NaCl and centrifuged at 40,000×g for 60 min. The pellet containing purified surfactant was aspirated and washed with Ca²⁺-buffer (1 mM Ca²⁺, 133 mM NaCl, 5 mM KCl, 10 mM Hepes, 2.6 mM phosphate, pH 7.4) to eliminate contaminant proteins, followed by washing of SP-A with Ca²⁺-buffer containing 5 mM EGTA, 1 mM MgCl₂. Purified SP-A was obtained in the supernatant and was dialyzed against 150 mM NaCl for the removal of EGTA by 48 h according to [34] or filtrated for the removal of EGTA, and the free protein of EGTA was tested to demonstrate that low molecular weight contaminants are not responsible for the antioxidant activities.

2.5. SDS -polyacrylamide gel electrophoresis (PAGE)

SP-A purity was demonstrated by 15% SDS-PAGE according to Laemmli [25].

2.6. Protein determination

Proteins were quantified by the method of Lowry et al. [26] using BSA as standard.

2.7. Lipid peroxidation and chemiluminescence assay. Effect of SP-A

Each determination was made using 1 mg of mitochondrial or microsomal protein. Lipid peroxidation was assayed by incubating rat lung mitochondria or microsomes in 0.05 M phosphate buffer, pH 7.4, at 37 °C, in final volumes of 1 ml and 2 ml, respectively. The reaction was started by the addition of ascorbate, final concentration 0.4 mM, and LHP
(50 nmol/mg of protein). Although no additional iron was added to the reaction mixture, there always remained sufficient iron in the phosphate buffer to provide the necessary ferrous or ferric iron for lipid peroxidation (the final concentration in the incubation mixture was 2.15 μM). Simultaneously, treated membranes containing increasing amounts (0 to 5.0 μg of SP-A in mitochondria and 0 to 7.5 μg of SP-A in microsomes) were assayed. In all the cases, controls without ascorbate were carried out. Lipid peroxidation was measured by monitoring light emission [27] with a liquid scintillation analyzer Packard 1900 TR. Chemiluminescence was determined over a 180-min period and recorded as cpm every 10 min. The sum of the total cpm was used to compare the inhibitory effect produced by SP-A.

2.8. Lipid extraction and fatty acid analysis

Total lipids were extracted from native and peroxidized with and without hydroperoxides rat lung mitochondria or microsomes with chloroform/methanol (2:1 v/v) containing 0.01% butylated hydroxyltoluene as antioxidant [28]. Lipids were transmethylated using BF₃/methanol (20% w/v) at 60 °C for 180 min under nitrogen atmosphere. After esterification, the fatty acid methyl esters were extracted with hexane/water (2:1 v/v) and then analyzed using a GC-14 A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a packed column (1.80 m × 4 mm id) GP 10% DEGS-PS on 80/100 Supelcoport. Nitrogen was used as a carrier gas. The injector and detector temperatures were maintained at 250 °C. The column temperature was held at 200 °C during 60 min. Individual fatty acid methyl esters were identified by comparing retention times with known standards. The content of each individual fatty acid was expressed as % by area of total fatty acids.

2.9. Statistical analysis

Results are expressed as the mean±S.D. of three independent determinations. Data were evaluated statistically by one-way analysis of variance (ANOVA) and by the Tukey test. The statistical criterion for significance was selected at different P values, which were indicated in each case.

3. Results

3.1. Effects of SP-A on lipid peroxidation of lung mitochondria and microsomes induced by linoleic acid hydroperoxide

We measured the chemiluminescence as a marker of lipid peroxidation. The addition of ascorbate–Fe²⁺ plus linoleic acid hydroperoxide (LHP) to the mitochondria and microsomes isolated from rat lung resulted in lipid peroxidation as evidenced by the emission of light. The data for the time-course of light emission are given in Figs. 1 and 2. Chemiluminescence as a function of time was measured every 10 min during 180 min at 37 °C in rat lung mitochondria or microsomes with and without 0.4 mM ascorbate plus LHP 50 nmol/mg protein. The same procedure was applied to rat lung mitochondria previously treated with 2.5, 3.75 and 5.0 μg of SP-A and rat lung microsomes previously treated with 2.5, 3.75, 5.0 and 7.5 μg of SP-A. In both cases, in the absence of ascorbic acid plus LHP, the light emission was very low (control groups). Ascorbic acid alone added to this system caused a small increase of chemiluminescence (data no shown). The co-addition of LHP to mitochondrial or microsomal membranes caused an increase in the light emission (lipid peroxidation). Light emission gradually increased and reached a maximal value at about 60 min of incubation. A much greater production of chemiluminescence occurred in microsomes (maximum peak 148,986 at 60 min).

Figs. 3 and 4 show the total chemiluminescence during incubation of rat lung mitochondria and microsomes, respectively, with different amounts of SP-A. In the control groups, the total cpm was 4.726±0.133×10⁵ and 4.219±0.582×10⁵, respectively. When those membranes were peroxidized with ascorbic acid plus LHP, the total chemiluminescence was increased to 15.997±1.615×10⁵ and 20.015±1.734×10⁵, respectively. When an increasing amount of SP-A was added, the total chemiluminescence decreased proportionally to the SP-A concentration. In mitochondria with 3.75 μg of SP-A, the total cpm decreased to 7.998±2.55×10⁵ instead; with 5.0 μg of SP-A, the total cpm was similar to the control (4.910±0.541×10⁵), obtaining an antioxidant protection of 100%. In microsomes with 7.5 μg of SP-A, the total cpm was 11.947±1.612×10⁵.

![Fig. 1. Chemiluminescence as a function of time during the Fe²⁺–ascorbate lipid peroxidation induced by LHP of rat lung mitochondria (1 mg of protein).](image-url)
If we compare the difference in the total cpm during peroxidation with and without ascorbic acid plus LHP, the induction produced in microsomes is greater than the one produced in mitochondria and the inhibitory effect of SP-A in mitochondria is greater than in microsomes. In both cases, samples without SP-A and treated with 2.5 μg of SP-A show differences which are highly significant \((P<0.001)\) with respect to the light emission of the control samples. On the other hand, mitochondria show highly significant differences between the peroxidized group in the absence of SP-A and SP-A treated groups with 3.75 and 5.0 μg of SP-A.

Fig. 5 shows the inhibition by SP-A on the lipid peroxidation of rat lung mitochondria and microsomes. The percent of inhibition was calculated as a decrease of total chemiluminescence measured every 10 min during 180 min. An increase of inhibition of lipid peroxidation was observed when increasing concentrations of SP-A were added to rat lung mitochondria or microsomes. The inhibition was concentration dependent. The antioxidant effect reaches the highest values in mitochondria with 5.0 μg of SP-A (the inhibition produced was 100%). In microsomes, the effectiveness of SP-A as an antioxidant was lesser, thus, with 7.5 μg of SP-A, the inhibition of lipid peroxidation reaches only 51.25±3.48%. Additional studies of lipid peroxidation of rat lung mitochondria and microsomes using
equal amounts of albumin and even higher compared to SPA were carried out. Our results indicate that under our experimental conditions, BSA was unable to inhibit lipid peroxidation stimulated by linoleic acid hydroperoxide of rat lung mitochondria or microsomes (Table 1), thus indicating that this effect is specific to SP-A. Purified SP-A (free of EGTA) was tested to demonstrate that low molecular weight contaminants are not responsible for the antioxidant activities. Our results clearly indicate that SPA free of EGTA has similar antioxidant activities to those reported in Figs. 3 and 4.

SP-A was submitted to 15% SDS-PAGE in order to determine the absence of contaminant proteins (Fig. 6).

3.2. Fatty acid composition of total lipids from rat lung mitochondria and microsomes. Effect of SP-A on lipid peroxidation stimulated by linoleic acid hydroperoxide

Simultaneously, we measured the loss of PUFAs to assess the oxidative damage to the cell membranes in the lungs. The content and composition of total lipids recovered from the mitochondria and microsomes have been analyzed, 16:0 was the prevalent fatty acid (30–34%), followed by 18:1n-9 (19%), 18:2 n-6 (14–15%), 18:0 (11–12%), 20:4 n-6 (7–9%) and 22:6 n-3 (2–2.5%).

Table 2 shows the fatty acid composition of total lipids from rat lung mitochondria: native, peroxidized and peroxidized in the presence of 2.5, 3.75 and 5.0 µg of SP-A. Statistically significant differences (P<0.05) in the content of docosahexaenoic acid were observed when native and peroxidized groups with or without the addition of 2.5 µg of SP-A were compared. Docosahexaenoic acid decreased markedly in the peroxidized rat lung mitochondria from 2.50±0.24% to 0.73±0.57%. However, the decreases of the other PUFAs were not statistically significant.

Table 3 shows the fatty acid composition of total lipids from rat lung microsomes: native, peroxidized and peroxidized in the presence of 2.5, 3.75, 5.0 and 7.5 µg of SP-A.

Table 1

<table>
<thead>
<tr>
<th>Ascorbic acid-LHP</th>
<th>BSA µg</th>
<th>Total chemiluminescence (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mitochondria Microsomes</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>582,912</td>
</tr>
<tr>
<td>Peroxidized</td>
<td>5</td>
<td>864,014</td>
</tr>
<tr>
<td>10</td>
<td>1,144,640</td>
<td>1,357,792</td>
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<td>20</td>
<td>798,150</td>
<td>1,322,080</td>
</tr>
<tr>
<td>50</td>
<td>1,047,610</td>
<td>1,445,712</td>
</tr>
</tbody>
</table>

Chemiluminescence during the Fe²⁺–ascorbate lipid peroxidation induced by LHP of rat lung microsomes or mitochondria (1 mg of protein). Control without ascorbic acid and without LHP. Peroxidized (0.4 mM ascorbic acid+50 nmol LHP) plus BSA as indicated. Chemiluminescence was recorded as cpm every 10 min during 180 min of incubation. The sum of the total cpm was used to compare the inhibitory effect produced by BSA.

Changes in the fatty acid profile of the rat lung microsomal lipids were observed when the native and peroxidized with the addition of ascorbic acid plus LHP groups were compared. The long-chain fatty acids mainly affected during the lipid peroxidation process were 18:2 n-6, 20:4 n-6 and 22:6 n-3, which decreased considerably. As a consequence, they brought about a relative increase in the percentage of saturated and monoenoic fatty acids. Statistically significant differences in the content of these long-chain peroxidable fatty acids were observed between native and peroxidized or peroxidized + 2.5 µg of SP-A, respectively.

In order to compare the oxidative damage, values of unsaturation index (UI) has been analyzed. The UI was calculated as the sum of the percentage of each fatty acid divided by the number of olefinic bonds. The UI of native mitochondria was 110.62±10.10; this value was reduced to 94.07±15.36 when these organelles were peroxidized. The addition of 2.5 µg of SP-A produced a marked decrease of this value (72.97±1.14%) in the control group to 3.21±0.94% in the peroxidized or peroxidized + 2.5 µg of SP-A, respectively.

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4. Discussion

Reactive oxygen species play an important role in several models of acute lung injury. Various enzymes such as...
superoxide dismutase, catalase and glutathione peroxidase as well as antioxidants such as glutathione and vitamins A, C and E present in the lung tissue decreased the concentration of these toxic species. When the production of reactive oxygen species exceeds the ability of the antioxidant system to eliminate them, oxidative stress can occur leading to the accumulation of lipid peroxidation products. A possible mechanism of oxidative lung injury is that superoxide anion is converted to an active oxidant-hydroxyl radical through the Fenton reaction and these radical may cause direct tissue injuries by lipid peroxidation. Peroxidative destruction of internal cell membranes such as mitochondria may lead to loss of their functional integrity [29].

An increased production of oxygen radicals in lung cells has been postulated to be a major factor in the etiology of lung damage during hypoxia. Freeman and Crapo [30] provide evidence for an association between hypoxia and the increased production of partially reduced species of oxygen by the lung and lung mitochondria. In rat lung, CuZn superoxide dismutase that resides in both the cytoplasm and mitochondria is induced by hypoxia as an adaptative response.

The most abundant surfactant protein (approximately 50% of total protein in isolated surfactant), SP-A, is a glycoprotein that is secreted into the airspace of the lung by the pulmonary epithelial cells, including alveolar type II cells and non-ciliated bronchiolar cells of the terminal

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Native (control)</th>
<th>Peroxidized + Asc + LHP</th>
<th>Peroxidized + 2.5 µg SPA</th>
<th>Peroxidized + 3.75 µg SPA</th>
<th>Peroxidized + 5.0 µg SPA</th>
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</thead>
<tbody>
<tr>
<td>16:0</td>
<td>30.53 ± 1.04</td>
<td>29.74 ± 6.07</td>
<td>30.64 ± 3.64</td>
<td>34.96 ± 2.85</td>
<td>35.95 ± 5.32</td>
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<tr>
<td>16:1</td>
<td>3.38 ± 0.24</td>
<td>1.89 ± 1.42</td>
<td>4.75 ± 0.41</td>
<td>2.94 ± 1.89</td>
<td>3.50 ± 0.96</td>
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<tr>
<td>18:0</td>
<td>10.93 ± 0.09</td>
<td>13.51 ± 2.47</td>
<td>14.12 ± 2.03</td>
<td>13.68 ± 1.38</td>
<td>12.30 ± 0.78</td>
</tr>
<tr>
<td>18:sn-9</td>
<td>19.74 ± 1.86</td>
<td>28.74 ± 8.23</td>
<td>25.39 ± 2.42</td>
<td>24.63 ± 0.34</td>
<td>19.94 ± 2.61</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>15.44 ± 2.49</td>
<td>17.83 ± 5.04</td>
<td>11.60 ± 2.01</td>
<td>13.57 ± 0.45</td>
<td>13.96 ± 3.67</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>0.96 ± 0.77</td>
<td>0.17 ± 0.23</td>
<td>0.03 ± 0.04</td>
<td>0.09 ± 0.13</td>
<td>0.06 ± 0.08</td>
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<tr>
<td>20:4n-6</td>
<td>9.68 ± 1.60</td>
<td>5.72 ± 1.64</td>
<td>4.55 ± 0.83</td>
<td>5.89 ± 1.30</td>
<td>9.41 ± 3.29</td>
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<tr>
<td>22:6n-3</td>
<td>2.50 ± 0.24</td>
<td>0.73 ± 0.57</td>
<td>0.22 ± 0.31</td>
<td>1.39 ± 0.89</td>
<td>1.73 ± 0.29</td>
</tr>
<tr>
<td>Saturated</td>
<td>41.46 ± 0.99</td>
<td>43.25 ± 4.76</td>
<td>44.77 ± 5.49</td>
<td>48.64 ± 4.21</td>
<td>48.26 ± 4.75</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>23.12 ± 1.84</td>
<td>30.52 ± 6.77</td>
<td>30.13 ± 2.06</td>
<td>27.57 ± 2.18</td>
<td>23.45 ± 3.54</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>28.59 ± 2.87</td>
<td>24.45 ± 7.43</td>
<td>16.41 ± 0.99</td>
<td>20.95 ± 1.56</td>
<td>25.16 ± 1.75</td>
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<td>Total unsaturated</td>
<td>51.70 ± 4.48</td>
<td>54.98 ± 4.27</td>
<td>46.54 ± 2.89</td>
<td>48.53 ± 2.29</td>
<td>48.60 ± 3.01</td>
</tr>
<tr>
<td>Saturated/Unsaturated</td>
<td>0.81 ± 0.07</td>
<td>0.79 ± 0.15</td>
<td>0.97 ± 0.18</td>
<td>1.01 ± 0.12</td>
<td>1.00 ± 0.16</td>
</tr>
<tr>
<td>UI</td>
<td>110.62 ± 10.10</td>
<td>94.07 ± 15.36</td>
<td>72.97 ± 1.30</td>
<td>93.45 ± 5.25</td>
<td>99.55 ± 5.13</td>
</tr>
</tbody>
</table>

Data shown are given in percentages of total fatty acids content (area %) and are ± S.D. of three separate experiments. Native (control): without ascorbate and without linoleic acid hydroperoxide (LHP). Peroxidized groups: with 0.4 mM ascorbate + 50 nmol of LHP. Statistically significant differences between the native and peroxidized groups are indicated by *P < 0.05 using analysis of variance (ANOVA).
bronchioles and conducting airways. Over 99% of SP-A in lavage fluid is bound to phospholipid. SP-A binds avidly to dipalmitoilphosphatidylcholine. Sphingomyelin is also preferred ligand but SP-A does not bind to the other major phospholipid components of surfactant including phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine or phosphatidylserine. The two adjacent glycerol coupled fatty acid chains are required for the interaction. The lipid binding site of SP-A was located in the neck region of the protein, a concept that was consistent with the hydrophobic nature of this domain but C-terminal carbohydrate recognition domain rather than the neck as the major lipid interaction site [10]. Moreover Bridges et al. [9] found that the antioxidant activity of SP-A is located in this region.

Rodriguez Capote et al. [31] have demonstrated that SP-A can reverse the detrimental effects of surfactant oxidation on the biophysical properties of surfactant by a mechanism that is dependent on interchain disulfide bond formation and the phospholipid binding domain.

Mark and Ingenito [32] have compared purified aqueous SP-A and whole surfactant after exposure to radical species generated with different transition metals, in vitro. They demonstrated that surfactant lipid and protein components, specifically SP-A, are chemically altered and observed that the lipid binding does not influence the susceptibility of SP-A to free radical attack.

Previous data from other group showed that the hydrophilic surfactant proteins SP-A and SP-D show protection from in vitro lipid peroxidation. These proteins directly protect surfactant phospholipids and macrophages from oxidative damage. Both proteins block the accumulation of thiobarbituric acid-reactive substances and conjugated dienes during copper-induced oxidation of surfactant lipids by a mechanism that does not involve metal chelating or substrate sequestration or accessibility of lipids to free radicals. Low-density lipoprotein oxidation is instantaneously arrested upon SP-A or SP-D addition, suggesting direct interference with lipid radical’s formation or by action as free radical chain terminators [9].

Katsura et al. [33] suggested that superoxide production of alveolar macrophages can be inhibited by SP-A and speculated that SP-A may have a protective role for oxidant injury in the lung.

Recently, Kuzmenko et al. [34] have analyzed the ability of human SP-A to inhibit the CuSO₄-induced oxidation of a mixture of surfactant lipids and concluded that SP-A inhibits the oxidation of lipid in a dose-dependent fashion by a mechanism that includes an oxidative modification of SP-A. The antioxidant effect of SP-A was slightly greater than that of albumin.

In previous reports, we have analyzed the peroxidation stimulated by lipid hydroperoxides on bovine rod outer segments and retinal pigment epithelium mitochondria [35,36].

A recent definitive study clearly shows that SP-A increases the production of nitric oxide by alveolar macrophages isolated from the lungs of patients with lung transplant [37]. These data further support the hypothesis that the ability of SP-A to modulate cell responses is dependent on the activation state of the cell and that the cellular environment is of extreme importance when attempting to understand cellular responses to external stimuli (e.g., bacteria). Furthermore, a recent paper shows that C57BL/6 mice lacking SP-A have lower levels of nitric oxide under control (basal) conditions but elevated levels after infection with mycoplasmas [38]. These data indicate that SP-A may help regulate NO production in response to a specific stimulus, i.e., suppression of NO in the absence of bacteria and increased NO in the presence of bacteria. The data also indicate that the contribution of SP-A to mycoplasma killing may be limited to lower doses of pathogens. In this paper, we have demonstrated that the Fe²⁺-ascorbate-dependent peroxidation induced by linoleic acid hydroperoxide of rat lung microsomal and mitochondrial lipids was inhibited by SP-A. The levels of SP-A used in our studies correspond to those found in vivo. This protein was more effective in inhibiting the lipid peroxidation on the mitochondrial than microsomal lipids, and LHP was more active to produce chemiluminescence in rat lung microsomes in the presence of a metal catalyzed oxidation system. SP-A isolated from porcine lung inhibited oxidation of membranes in a dose-dependent fashion that was half-maximal at a concentration of 3.25 μg and complete at 5.0 μg in mitochondria, whereas in microsomes supplemented with 7.5 μg of SP-A, the inhibition reaches the $51.25 \pm 3.48\%$.

In order to examine the effects of lipid peroxidation on the fatty acid composition of the lung mitochondria and microsomes exposed to LHP, the lipids were extracted, and the fatty acid composition of membranes, which have been under oxidative stress, was analyzed using gas chromatography. These results are compared with the control group.

The fatty acid profile of rat lung mitochondria do not differ substantially from that of rat lung microsomes; both are rich in polyunsaturated fatty acids, particularly of the n-6 family such as 18:2n-6. Microsomes have relatively less 20:4n-6 and 22:6 n-3 than mitochondria. Here, we demonstrated that the changes of the lipid composition of the mitochondria and microsomes under oxidative stress conditions are manifested by a decrease of the PUFAs and increase of the saturated fatty acid levels. These changes were less pronounced in the presence of increasing amounts of SP-A. The alterations that take place in the fatty acid composition of the lung mitochondria and microsomes can play an essential role in the mechanism of cell damage development. In a previous study from our laboratory [24], the porcine SP-A-induced inhibition of lipid peroxidation of isolated porcine surfactant mediated by ascorbate–Fe⁺² was reported using chemiluminescence. In addition, the fatty acid 20:4 n-6 was almost completely protected from lipid peroxidation by SP-A. However, it is important to note that the fatty acid composition of lung surfactant differs from...
that of rat lung microsomes or mitochondria. Thus lung surfactant contains 69.67 ± 1.50% of saturated fatty acids, mainly palmitic acid, and only 7.25 ± 0.37% of polyunsaturated, whereas rat lung microsomes and mitochondria contain 24.95 ± 4.04 and 28.59 ± 2.87 of PUFA, respectively. This difference in polyunsaturated fatty acid composition may influence the SP-A-mediated protection of lipid peroxidation.

Although the mechanism by which SP-A reduces lipid peroxidation remains unknown, a plausible hypothesis is that SP-A may limit the propagation of free radical chain reaction by scavenging lipid peroxyl radicals and thereby blocking the reaction of lipid peroxidation protecting PUFAs against oxidative damage. To clarify the antioxidant mechanism of SP-A, investigations involving interactions of the protein with iron, antioxidants or free radical intermediaries such as fatty acid hydroperoxides are necessary. In our assay, the SP-A minimized the mitochondrial and microsomal damage caused by fatty acid hydroperoxides in proportion to the protein concentration; in lung cells, SP-A may have a similar effect and thus protects against the potential cytotoxic effects produced during oxidative stress of lung membranes, where fatty acid hydroperoxides may be present. In conclusion, our results suggest that one function of SP-A is to act as a free radical scavenger, protecting membranes exposed to lipid peroxidation from deleterious effect, and, thus, may play a significant physiological role as an antioxidant protecting the lung during the oxidative stress due to atmospheric or supplemental oxygen, air pollutants or lung inflammation.

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