Free Radical Research, April 2006; 40(4): 419–425

Taylor & Francis
Taylor & Francis Group

Oxidative damage to surfactant protein D in pulmonary diseases

VITALITY STAROSTA, & MATTHIAS GRIESE

Lung Research Group, Children's Hospital of Ludwig Maximilians University, Munich, Germany

Accepted by Professor FJ Kelly

(Received 2 March 2005; in revised form 18 May 2005)

Abstract
Surfactant protein D is an important innate host defence molecule that has been shown to interact with a variety of pathogens and to play a role in surfactant homeostasis. The aim of this study was to examine the influence of oxidation on surfactant protein D in different lung diseases. Bronchoalveolar lavage fluids (BALFs) from patients with different grade of protein oxidation were examined for changes in the primary chain and the quaternary structure of surfactant protein D. Significant changes of quaternary surfactant protein-D (SP-D) structure were detected under oxidative conditions *in vitro* and *in vivo*. The functional capacity of surfactant protein D to agglutinate bacteria was impaired by oxidation. We conclude that surfactant protein D is an important target of free radicals generated in the lungs. Host defence may be impaired due to the oxidation of surfactant protein D and may contribute to the suppurative lung diseases like cystic fibrosis (CF).

Keywords: *Cystic fibrosis, children, SP-D, oxidation*

Introduction

In patients with chronic suppurative lung diseases, such as cystic fibrosis (CF) or chronic bronchiectasis, the high influx of neutrophil granulocytes into the airspaces is regularly accompanied by high levels of elastase and other proteolytic enzymes and large amounts of free radical species [2,35]. Reactive oxygen species (ROS) can damage all types of biomolecules [13] and although loss of function is very likely to occur, not much is known about the oxidation of specific proteins in CF or chronic bronchitis. The goal of this study was to investigate in detail the effect of oxidative stress on one specific protein in these disease conditions. Pulmonary surfactant protein-D (SP-D) is a member of the collectin family of C-type lectins that is produced and secreted by alveolar type II cells and nonciliated bronchial epithelial cells of the airways. SP-D is assembled predominantly as dodecamers (12-mers) consisting of four homotrimeric (3-mers) subunits each [37]. SP-D is involved in the innate immune system through its ability to recognize a broad spectrum of pathogens and to modulate immune functions [1,11]. Several studies have shown that SP-D interacts with a number of viruses, bacteria and fungi [11,30], including CF relevant organisms such as *Pseudomonas aeruginosa* [8,9,31]. It is known that SP-D can undergo structural and functional changes after exposure to neutrophil elastase *in vitro* and *in vivo* [1,19,36], but the role of oxidation in the damage of SP-D has not yet been determined. The damage of SP-D by oxidative stress may lead to a loss of many important functions of this molecule. Here, we investigated the changes of the primary chain, the quaternary structure and functional capacities of the SP-D by oxidation *in vitro* and *in vivo*.

Material and methods

Subjects, bronchoalveolar lavage and sample preparation

The cell free supernatant of bronchoalveolar lavage fluid (BALF), was analysed from 6 patients with chronic bronchitis (median age 5.8 years, range 2.5–15.2) and 9 patients with CF (median age was 14.0, range 7.7–20). The patients were lavaged during clinical diagnostic work up. Aliquots of the lavages

Correspondence: M. Griese, Children's Hospital, Ludwig-Maximilians-University, Lindwurmstr 4, 80337 Munich, Germany. Tel: 49 89 5160 7870. Fax: 49 89 5160 7872. E-mail: matthias.griese@med.uni-muenchen.de

ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Taylor & Francis
DOI: 10.1080/10715760600571248

were stored frozen immediately at −80°C until assay. Bronchoalveolar lavage was performed in the right middle lobe or most affected lobes with a flexible bronchoscope using 4 ml/kg 0.9% NaCl in four aliquots. Written informed consent was obtained from parents and/or older children before the study. The usage of BALF for these analytical purposes was approved by the institutional review board.

Biochemical methods

The content of protein carbonyls was assessed as a measure of oxidative stress in the BAL proteins by sensitive dot-blot assay [32]. The lower limit of the detection was 2 pmol carbonyls/μg protein.

2-D-SDS-PAGE was performed on the NuPage 2-D gels (Invitrogen, Carlsbad, CA) with modification for protein carbonyl identification [10]. Briefly, following sample (80 μg) in-gel rehydration and isoelectric focussing (IEF) on the IPG strips pH 3–10 L, 7 cm for 2000 V-h strips were incubated for 15 min in 2 N HCl with 10 mM 2,4-dinitrophenylhydrazin (DNPH, Sigma, Taufkirchen, Germany). Afterwards, the strips were washed with 2 M Tris-Base/30% glycerol (Plusone, Uppsala, Sweden) for 15 min. The second dimension was run on 4–12% Bis-Tris gels (Invitrogen). Proteins were transferred to a PVDF membrane (Millipore, Bedford, MA), and probed with anti-2, 4-dinitrophenyl (DNPH) antibodies (Sigma, Munich, Germany) for protein carbonyls, and anti-SP-D-antibodies (Davids Biotechnologie, Regensburg, Germany) for surfactant protein D. After development all X-ray films were generally exposured for 30 s, 1 min, 5 and 30 min. After 30 min no change or further enhancement of the signal was observed.

Preparation of human SP-D

Native SP-D was isolated by affinity chromatography from BAL fluid of pulmonary alveolar proteinosis (PAP) patients undergoing therapeutic BAL as described previously [33]. The purity of the isolated SP-D was confirmed by Coomassie-stained sodium dodecyl sulfate (SDS) -polyacrylamide gel electrophoresis and Western immunoblotting. *In vitro* oxidation was performed using the synthetic oxidizing agent AAPH (2,2-Azobis (2-amidinopropane)-dihydrochloride, Sigma, Taufkirchen, Germany). AAPH has temperature-dependent properties and generates a continuous flow of free radicals, thus allowing to study well-defined dose-dependent effects of oxidation as shown previously [17,20,26].

Gel filtration to determine the macromolecular organization of native SP-D in BAL

The separation of SP-D isoforms was done as described previously, using an Äkta apparatus and Superose-6-gel-chromatography column (Pharmacia, Uppsala, Sweden) [18,21].

Agglutination assay

A clinical isolate of *P. aeruginosa* (Ps 12, nonmucoid, from a patient with CF), previously selected for Ca^{2+} and carbohydrate-dependent agglutination, was washed three times in 50 mM Tris–HCl, 150 mM NaCl, pH 7.4, resuspended in the same buffer with 2 mM $CaCl_2$ ($OD_{623\,nm} = 2.0$) and incubated on glass cover slides with the different SP-D preparations (5 μg/ml SP-D incubated with or without oxidizing agent AAPH (12.5 mmol/l)). Control experiments were performed in the presence of maltose (100 mM). After 4 min, the slides were viewed under microscope and photographs were taken [9,19].

Statistical analysis

Statistical analysis was performed with Prism 4.02 (Graph Pad Software, San Diego, CA). Differences among experimental groups were determined by performing the Kruskal Wallis test with Dunn's *post hoc* test as a multiple comparison procedure. Spearman correlation analysis and linear regression were performed. A P value of <0.05 was considered significant. Values are expressed as mean ± SEM.

Results

Protein carbonylation was used as a measure of oxidation of proteins. In addition to total carbonyl content of the lavages, carbonylation of SP-D after separation from the other proteins by 1- and 2-D-electrophoresis was assessed on Western blots. Interestingly, in a number of patients who had various degrees of overall oxidation of their proteins in their lavages, assessed as total carbonyl content (Figure 1, left column), no signs of carbonyl formation in the area of SP-D were detected (Figure 1, column anti-DNPH-immunostaining). All these patients had SP-D in their lavages (Figure 1, column anti-SP-D immunostaining) and other neighbouring proteins were heavily oxidized at the same time, depending on the overall degree of oxidation (Figure 1, column anti-DNPH-immunostaining). This suggested that the primary structure of SP-D was very resistant to oxidation. No proteolytic fragments of SP-D were found on Western blots in any sample investigated.

These results prompted us to investigate the carbonylation of SP-D *in vitro*. Native purified SP-D could be oxidized *in vitro* dose-dependently, and carbonyl formation was clearly confirmed (Figure 2). This oxidation could be diminished by the presence of 4 mM dithiothreitol (DTT) (Figure 3). Taken together the data showed that the primary structure of SP-D could be oxidized, albeit at high oxidative stress *in vitro*.

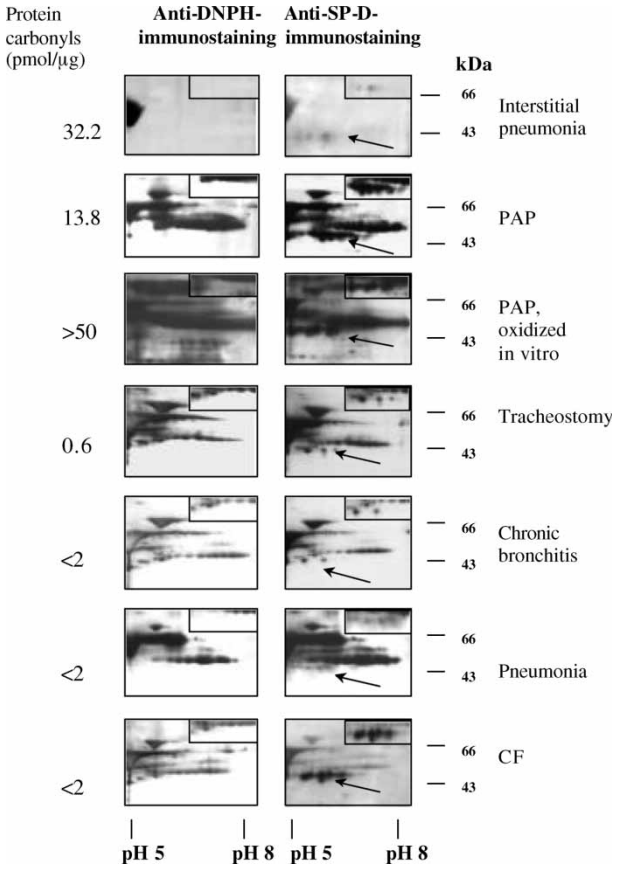

Figure 1. Oxidation of surfactant protein D in different lung diseases. BALF protein samples of patients with different lung diseases were separated by 2-D-electrophoresis (post-IEF-DNPH-derivatisation) followed by Western blotting and immunostaining. First carbonylated proteins were detected by anti-DNPH antibodies (left side). Next SP-D was detected on the same blots with anti-SP-D-antibodies (right side). Note remaining stain from DNPH detection. SP-D spots observed were indicated by an arrow. The insert in the right upper corner depicts the corresponding area where SP-D was detected in detail. Carbonyl content determined in total protein is indicated on the left side.

We next evaluated the susceptibility to oxidation of the quaternary structure of SP-D. The oligomeric organisation of SP-D can be resolved by gel filtration. After *in vitro* exposure of purified SP-D to different concentrations of oxidizing agent these SP-D oligomers were separated according to size and molecular weight (Figure 4). With increasing oxidation, a progressive change of the native macromolecular structure of SP-D (fractions 7–9, with an apparent molecular weight of >1000 kDa) was observed. The large SP-D polymers were depolymerised (Figure 4) towards smaller sized molecules. Mild oxidation led to a decrease of the peak in fractions 7–9 (containing 12-mers and higher forms) and to an increase of the low molecular weight forms (fractions 13–14, containing mainly 6-mers and 15–16, containing mainly 3-mers) [18]. Further oxidation resulted in a complete absence of the higher molecular forms and the accumulation of depolymerised and/or oxidatively damaged parts of molecules (fractions 18–22, containing proteins with a molecular weight lower than 45 kDa).

To investigate whether the quaternary structure of SP-D was damaged by oxidation *in vivo* as easily as *in vitro*, we assessed BAL fluid samples from patients with high (>10 pmol/μg protein) and with low (<2 pmol/μg protein) content of total carbonyls in their BALFs. In 9 subjects (6 chronic bronchitis, 3 CF) with little carbonyls present in their BAL fluids, the typical pattern of SP-D with an intact macromolecular organisation was observed (Figure 5, top and middle panel), whereas, in samples from patients with a high degree of oxidative stress, significant lower ($p < 0.05$) amounts of multimeric SP-D (fractions 7–9, Figure 5 bottom panel) were found. The total protein carbonyl level negatively correlated to the content of high molecular forms of SP-D (fraction 9) ($n = 15$,

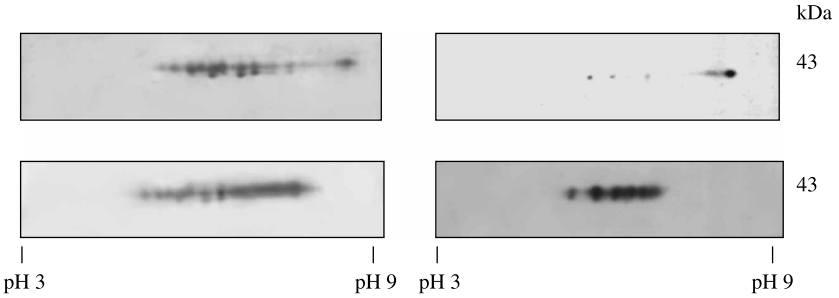

Figure 2. *In vitro* oxidation of purified SP-D. 2-D-electrophoresis (post-IEF-DNPH-derivatisation) followed by Western blotting and immunostaining was performed on SP-D purified from a patient with pulmonary alveolar proteinosis (PAP). Blots were stained with anti-DNPH-antibodies and anti-SP-D-antibodies. Relevant areas from the 2-D gel between pH 3–10 and a molecular weight 38–48 kDa are displayed. Upper left panel: Silverstaining. Upper right panel: Western blotting and anti-DNPH-immunostaining. Lower left panel: Western blotting and anti-SP-D-immunostaining. Lower right panel: Western blotting after *in vitro* oxidation with 37 mM AAPH for 2 h 37°C and anti-DNPH-immunostaining.

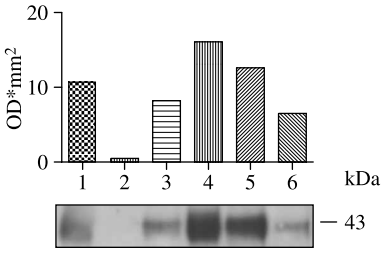

Figure 3. Dose-dependent *in vitro* oxidation of SP-D. Upper panel (diagram): semi-quantitative estimation of the degree of oxidation by densitometry. OD-optical density. Representative of two experiments. Purified SP-D (1 μg, lane 1) after pretreatment with reduced with $NaBH_4$ (lane 2), 10 mM AAPH 2 h (lane 3), 37 mM AAPH 2 h (lane 4), 74 mM AAPH 2 h (lane 5), 37 mM AAPH 2 h in the presence of 4 mM DTT (lane 6) were separated by1-D-SDS-PAGE after pre-electrophoresis DNPH-derivatisation. Western blotting (lower panel).

$r = -0.78$, $p = 0.0005$). *In vitro* oxidized SP-D had lost its capacity to agglutinate bacteria (Figure 6).

Discussion

Here, we showed that under circumstances of increased oxidative burden in the lungs, the quaternary structure of SP-D was altered, causing its depolymerisation. The extent of degradation was dependent on the overall degree of protein oxidation, used as a measure of oxidative stress. *In vitro* mild oxidation of SP-D also led to a depolymerisation of the macromolecular structure of SP-D. This loss of the oligomeric organisation of SP-D was also proportional to the extent of overall oxidation. Importantly, both *in vivo* and *in vitro* depolymerisation of SP-D was associated with a loss of its functional properties. In contrast, the evaluation of the primary chain of SP-D with electrophoretic techniques did not reveal any oxidative damage as assessed by carbonyl formation of bronchoalveolar lavage samples from patients with a broad range of oxidative burden. This may indicate a resistance of SP-D to oxidation and is in line with its antioxidative properties previously reported by Bridges et al. [4]. Nevertheless, *in vitro* the primary chain of SP-D could be oxidized in a dose-dependent manner with the free-radical generator AAPH.

Proteins constitute one of the major targets of ROS and it has previously been shown that oxidation can alter protein structure and that oxidation of proteins can lead to a loss of protein function as well as conversion of proteins to forms that are more susceptible to degradation by proteinases [14]. ROS elicit a variety of modifications in amino acid residues [14] and the formation of carbonyls is a result of the oxidation of arginine, lysine, threonine or proline amino acids. However, very little information is yet available about the exact identity of lung specific proteins targeted by ROS. It has been shown that free radical species may contribute to surfactant protein damage [15,28]. For radiolabelled surfactant protein A (SP-A) it has been shown that with storage time and concomitant exposure to oxidizing species, its hexameric quaternary structure was destroyed. The SP-A polypeptides generated had decreased binding activity [34]. Such changes of SP-A structure under the influence of free radicals of oxygen and nitrogen have been reported in a number of publications [23,25,38], suggesting an impairment of the physiological role of SP-A in acute lung injury. The finding reported here extend the role of oxidative damage to an additional specific protein, i.e. SP-D, and to other chronic type of lung diseases like CF.

Very similar changes of the macromolecular organisation of SP-D with loss of its function to those observed here, were described previously

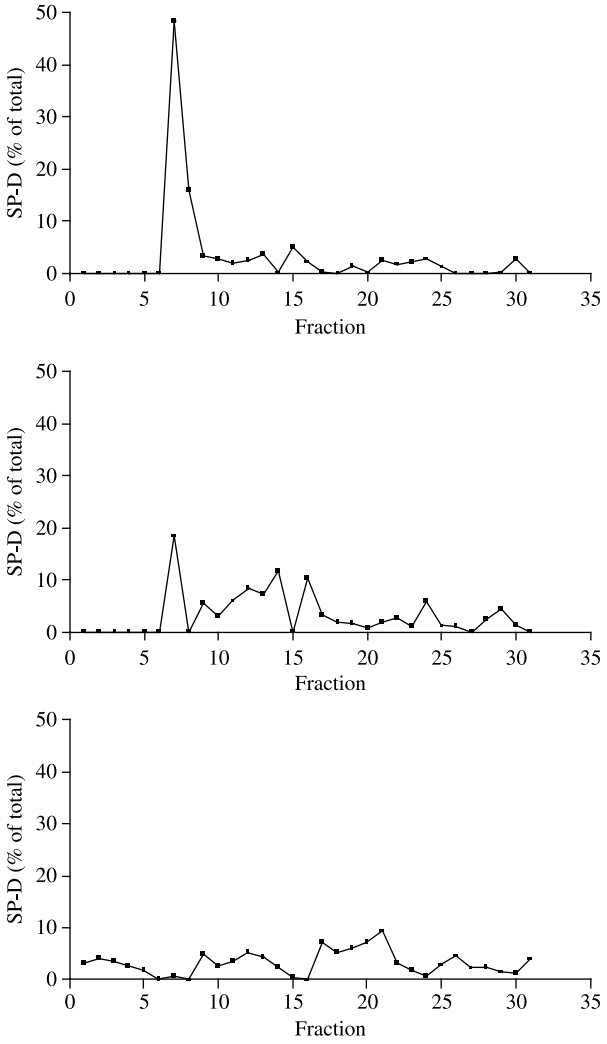

Figure 4. Separation of purified SP-D under native, non reducing conditions by gel filtration. Five hundred nanogram of purified SP-D were injected onto the column, 1 ml fractions were collected and SP-D concentration was determined in each fraction by dot-blotting. Upper panel: untreated SP-D, middle panel: *in vitro* treatment with 12.5 mM AAPH for 2 h by 37°C and lower panel: after *in vitro* treatment by 36 mM AAPH for 2 h by 37°C. Representative of two experiments is shown.

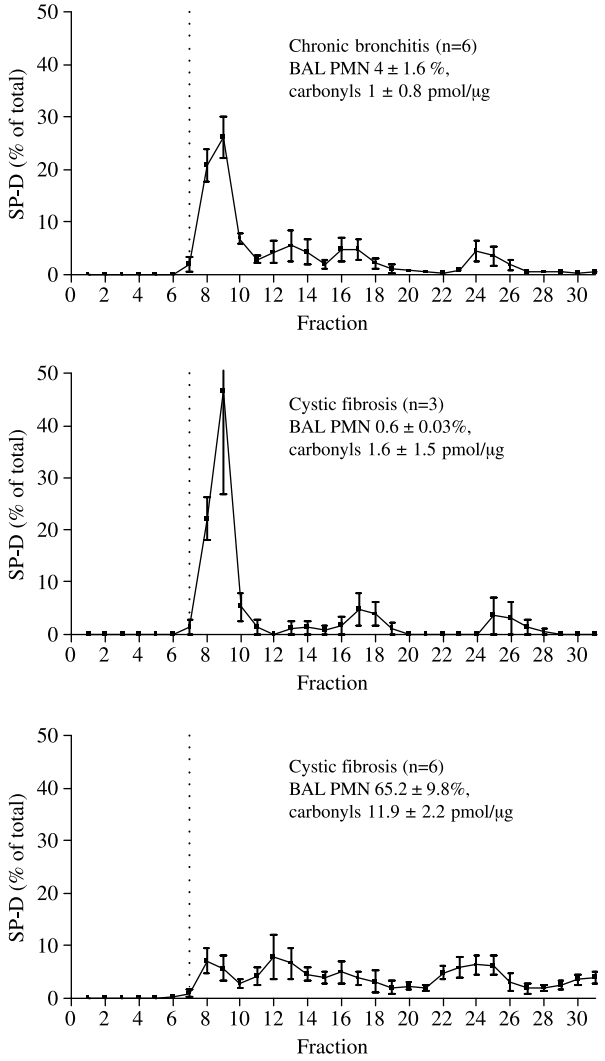

Figure 5. Separation of SP-D in BAL samples from patients with chronic bronchitis (upper panel) or CF with low (middle panel) or high (lower panel) neutrophil count and protein carbonyls by gel filtration under native and non reducing condition. Data are means ± SEM of the indicated subjects.

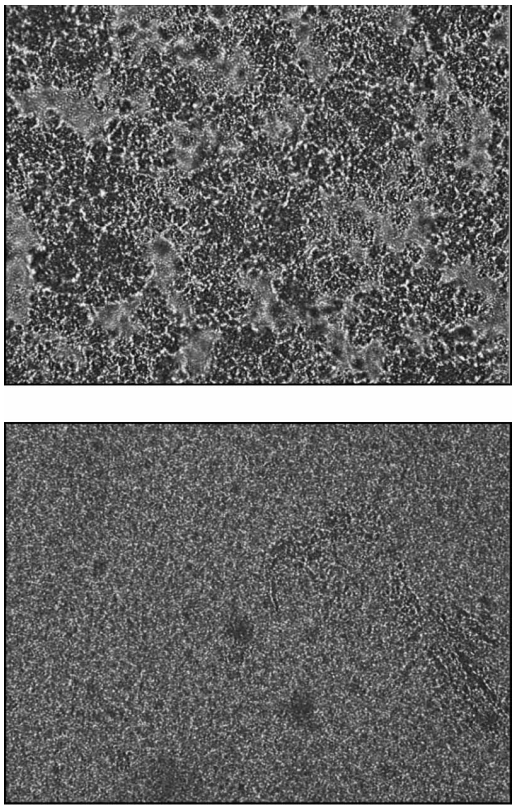

Figure 6. Agglutination of *Pseudomonas aeruginosa* (strain 12) by native SP-D (5 μg/ml, upper panel) and lack of agglutination by SP-D treated *in vitro* for 3 h with 12.5 mM AAPH (lower panel).

in children with gastro-esophageal reflux disease [18]. In that report a distinct relation between SP-D depolymerisation and oxidative status was not established, however, major effects of proteolytic breakdown were excluded. The results presented here extend those findings and are consistent with the hypothesis that oxidative damage may be a major cause for specific protein damage in a variety of lung diseases. In line with this, CF patients with high levels of total BALF protein oxidation had significantly less intact multimeric forms of SP-D in their lungs than CF patients with lower levels of BALF protein oxidation. It must be noted that our *in vivo* observed changes of the macromolecular structure of SP-D may not be caused exclusively by protein oxidation, but possibly also by increased proteolysis that may accompany states with increased neutrophil accumulation. However, there were no direct evidence for the generation of proteolytic SP-D fragments in these CF patients, in agreement with a previous study where we only observed immunoreactive lower molecular weight band [36].

Our initial observation that the primary chain of SP-D was very resistant to oxidative stress was unexpected. Even *in vitro* oxidation of a sample from BALF of a patient with PAP did not lead to significant carbonylation of SP-D, although, the lead majority of other visible proteins were heavily oxidized. Potential explanations include the fact that in the epithelial lining fluid, which contains a complex mixture of proteins, phospholipids and other biologically active substances, free radical reactions can progress differently as *in vitro*. It is known that SP-D arrests low density lipoprotein (LDL) oxidation almost instantaneously when added during the propagation phase [4]. This suggests that the collectins may directly interfere with lipid oxidation by inhibiting the formation of lipid radicals, by acting as free radical chain terminators or perform a chain termination function through their capacity to recognize, approximate and extinguish lipid radicals [4]. Also, having the possibility to quench lipid free radical formation and progression in its environment *in vivo*, SP-D may become a target of radical species itself. The AAPH-derived free radicals generated *in vitro* produce

a constant flow of radicals that are not recognized or intercepted by SP-D and thus may produce persistent oxidative damage to the protein.

Trimerisation of the collectin lectin domains leads to an increased binding affinity for ligands, such as complex carbohydrates, e.g. on the surfaces of microorganism [24]. Further increase of affinity is obtained by multimerisation of the trimeric units to tetramers of SP-D [11]. Similarly, mere trimers of SP-D failed to agglutinate bacteria and viruses [7,16], whereas, multimerised SP-D was very active [6]. Thus, the functional activity of the collectins at physiological concentrations is dependent on its multimeric organisation. We and others showed previously that proteolytic cleavage of the primary chain of SP-D destroyed the carbohydrate recognition domain (CRD) of SP-D and was associated with a reduction or loss of its functions [19,22]. This was demonstrated by a complete loss of the binding capacity of SP-D to maltose-coated surfaces after exposure to human leukocyte elastase (HLE). In addition, the ability to agglutinate *P. aeruginosa* by native SP-D was lost after exposure to the protease HLE.

Here, we demonstrated a loss of the biological activity of the oxidized SP-D caused by the alteration and disruption of its quaternary structure. In addition to the impaired agglutination of microorganisms shown here, it is likely that other functions of the protein that depend on its macromolecular organisation will be similarly altered, i.e. participation in processes of chemotaxis [12], phagocytosis [29], secretion of cytokines [27], function of lymphocytes [3] and antigen presentation [5]. We also demonstrated that alterations of functional SP-D properties by oxidation might be relevant for chronic pulmonary disease conditions, associated with an increased risk for pulmonary infections.

Acknowledgements

This study was supported by a DAAD grant to V.S.

References

[1] Alcorn JF, Wright JR. Degradation of pulmonary surfactant protein D by *Pseudomonas aeruginosa* elastase abrogates innate immune function. J Biol Chem 2004;279:30871–30879.

[2] Birrer P, McElvaney NG, Rudeberg A, Sommer CW, Liechti-Gallati S, Kraemer R, Hubbard R, Crystal RG. Protease-antiprotease imbalance in the lungs of children with cystic fibrosis. Am J Respir Crit Care Med 1994;150:207–213.

[3] Borron PJ, Mostaghel EA, Doyle C, Walsh ES, McHeyzer-Williams MG, Wright JR. Pulmonary surfactant proteins A and D directly suppress CD3+/CD4+ cell function: Evidence for two shared mechanisms. J Immunol 2002;169:5844–5850.

[4] Bridges JP, Davis HW, Damodarasamy M, Kuroki Y, Howles G, Hui DY, McCormack FX. Pulmonary surfactant proteins A and D are potent endogenous inhibitors of lipid peroxidation and oxidative cellular injury. J Biol Chem 2000;275: 38848–38855.

[5] Brinker KG, Martin E, Borron P, Mostaghel E, Doyle C, Harding CV, Wright JR. Surfactant protein D enhances bacterial antigen presentation by bone marrow-derived dendritic cells. Am J Physiol Lung Cell Mol Physiol 2001; 281:L1453–L1463.

[6] Brown-Augsburger P, Chang D, Rust K, Crouch EC. Biosynthesis of surfactant protein D. Contributions of conserved NH2-terminal cysteine residues and collagen helix formation to assembly and secretion. J Biol Chem 1996; 271:18912–18919.

[7] Brown-Augsburger P, Hartshorn K, Chang D, Rust K, Fliszar C, Welgus HG, Crouch EC. Site-directed mutagenesis of Cys-15 and Cys-20 of pulmonary surfactant protein D. Expression of a trimeric protein with altered anti-viral properties. J Biol Chem 1996;271:13724–13730.

[8] Bufler P, Schikor D, Schmidt B, Griese M. Cytokine stimulation by *Pseudomonas aeruginosa*–strain variation and modulation by pulmonary surfactant. Exp Lung Res 2004; 30:163–179.

[9] Bufler P, Schmidt B, Schikor D, Bauernfeind A, Crouch EC, Griese M. Surfactant protein A and D differently regulate the immune response to nonmucoid *Pseudomonas aeruginosa* and its lipopolysaccharide. Am J Respir Cell Mol Biol 2003; 28:249–256.

[10] Conrad CC, Choi JG, Malakowsky CA, Talent JM, Dai R, Marshall P, Gracy RW. Identification of protein carbonyls after two-dimensional electrophoresis. Proteomics 2001;1: 829–834.

[11] Crouch E, Wright JR. Surfactant proteins A and D and pulmonary host defense. Annu Rev Physiol 2001;63(521/54): 521–554.

[12] Crouch EC, Persson A, Griffin GL, Chang D, Senior RM. Interactions of pulmonary surfactant protein D (SP-D) with human blood leukocytes. Am J Respir Cell Mol Biol 1995; 12:410–415.

[13] Dalle-Donne I, Giustarini D, Colombo R, Rossi R, Milzani A. Protein carbonylation in human diseases. Trends Mol Med 2003;9:169–176.

[14] Davies KJ, Delsignore ME, Lin SW. Protein damage and degradation by oxygen radicals. II. Modification of amino acids. J Biol Chem 1987;262:9902–9907.

[15] Davis IC, Zhu S, Sampson JB, Crow JP, Matalon S. Inhibition of human surfactant protein A function by oxidation intermediates of nitrite. Free Radic Biol Med 2002; 33:1703–1713.

[16] Eda S, Suzuki Y, Kawai T, Ohtani K, Kase T, Fujinaga Y, Sakamoto T, Kurimura T, Wakamiya N. Structure of a truncated human surfactant protein D is less effective in agglutinating bacteria than the native structure and fails to inhibit haemagglutination by influenza A virus. Biochem J 1997;323:393–399.

[17] Gieseg SP, Pearson J, Firth CA. Protein hydroperoxides are a major product of low density lipoprotein oxidation during copper, peroxyl radical and macrophage-mediated oxidation. Free Radic Res 2003;37:983–991.

[18] Griese M, Maderlechner N, Ahrens P, Kitz R. Surfactant proteins A and D in children with pulmonary disease due to gastroesophageal reflux. Am J Respir Crit Care Med 2002; 165:1546–1550.

[19] Griese M, Wiesener A, Lottspeich F, von Bredow C. Limited proteolysis of surfactant protein D causes a loss of its calcium-dependent lectin functions. Biochim Biophys Acta 2003;1638:157–163.

[20] Headlam HA, Davies MJ. Markers of protein oxidation: Different oxidants give rise to variable yields of bound and released carbonyl products. Free Radic Biol Med 2004; 36:1175–1184.

[21] Hickling TP, Malhotra R, Sim RB. Human lung surfactant protein A exists in several different oligomeric states: Oligomer

size distribution varies between patient groups. Mol Med 1998;4:266–275.

[22] Hirche TO, Crouch EC, Espinola M, Brokelman TJ, Mecham RP, DeSilva N, Cooley J, Remold-O'Donnell E, Belaaouaj A. Neutrophil serine proteinases inactivate surfactant protein D by cleaving within a conserved subregion of the carbohydrate recognition domain. J Biol Chem 2004;279:27688–27698.

[23] Huang W, Wang G, Phelps DS, Al Mondhiry H, Floros J. Human SP-A genetic variants and bleomycin-induced cytokine production by THP-1 cells: Effect of ozone-induced SP-A oxidation. Am J Physiol Lung Cell Mol Physiol 2004; 286:L546–L553.

[24] Kishore U, Wang JY, Hoppe HJ, Reid KB. The alpha-helical neck region of human lung surfactant protein D is essential for the binding of the carbohydrate recognition domains to lipopolysaccharides and phospholipids. Biochem J 1996;318: 505–511.

[25] Kuzmenko AI, Wu H, Bridges JP, McCormack FX. Surfactant lipid peroxidation damages surfactant protein A and inhibits interactions with phospholipid vesicles. J Lipid Res 2004; 45:1061–1068.

[26] Kwon HY, Choi SY, Won MH, Kang T, Kang JH. Oxidative modification and inactivation of Cu,Zn-superoxide dismutase by 2,2′-azobis(2-amidinopropane) dihydrochloride. Biochim Biophys Acta 2000;1543:69–76.

[27] Limper AH, Crouch EC, O'Riordan DM, Chang D, Vuk-Pavlovic Z, Standing JE, Kwon KY, Adlakha A. Surfactant protein-D modulates interaction of *Pneumocystis carinii* with alveolar macrophages. J Lab Clin Med 1995; 126:416–422.

[28] Mark L, Ingenito EP. Surfactant function and composition after free radical exposure generated by transition metals. Am J Physiol 1999;276:L491–L500.

[29] O'Riordan DM, Standing JE, Kwon KY, Chang D, Crouch EC, Limper AH. Surfactant protein D interacts with *Pneumocystis carinii* and mediates organism adherence to alveolar macrophages. J Clin Investig 1995;95:2699–2710.

[30] Reid KB. Interactions of surfactant protein D with pathogens, allergens and phagocytes. Biochim Biophys Acta 1998; 19(1408):290–295.

[31] Restrepo CI, Dong Q, Savov J, Mariencheck WI, Wright JR. Surfactant protein D stimulates phagocytosis of *Pseudomonas aeruginosa* by alveolar macrophages. Am J Respir Cell Mol Biol 1999;21:576–585.

[32] Robinson CE, Keshavarzian A, Pasco DS, Frommel TO, Winship DH, Holmes EW. Determination of protein carbonyl groups by immunoblotting. Anal Biochem 1999;266:48–57.

[33] Strong P, Kishore U, Morgan C, Lopez BA, Singh M, Reid KB. A novel method of purifying lung surfactant proteins A and D from the lung lavage of alveolar proteinosis patients and from pooled amniotic fluid. J Immunol Methods 1998; 220:139–149.

[34] Stuart GR, Sim RB, Malhotra R. Characterization of radioiodinated lung surfactant protein A (SP-A) and the effects of oxidation on SP-A quaternary structure and activity. Exp Lung Res 1996;22:467–487.

[35] Van der Vliet A, Nguyen MN, Shigenaga MK, Eiserich JP, Marelich GP, Cross CE. Myeloperoxidase and protein oxidation in cystic fibrosis. Am J Physiol Lung Cell Mol Physiol 2000;279:L537–L546.

[36] Von Bredow C, Wiesener A, Griese M. Proteolysis of surfactant protein D by cystic fibrosis relevant proteases. Lung 2003;181:79–88.

[37] Zhang L, Ikegami M, Crouch EC, Korfhagen TR, Whitsett JA. Activity of pulmonary surfactant protein-D (SP-D) *in vivo* is dependent on oligomeric structure. J Biol Chem 2001;276:19214–19219.

[38] Zhu S, Ware LB, Geiser T, Matthay MA, Matalon S. Increased levels of nitrate and surfactant protein a nitration in the pulmonary edema fluid of patients with acute lung injury. Am J Respir Crit Care Med 2001;163:166–172.