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Effect of canine surfactant protein (SP-A) on the respiratory burst of phagocytic cells

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Cells obtained from bronchoalveolar lavage, or neutrophils of peripheral blood of dog, were incubated with the canine surfactant-associated protein A (SP-A). A significant decrease of the production of superoxide anion was observed after subsequent stimulation with phorbol-12-myristate-13-acetate (PMA) as measured by the lucigenin-dependent chemiluminescence (CL). Several other proteins used for control experiments did not decrease lucigenin-dependent CL, indicating a specific effect of SP-A on phagocytes. Treatment of SP-A with collagenase prior to incubation with neutrophils destroyed the depleting effect on oxygen radical production of PMA-stimulated cells. We propose that SP-A acts as a regulatory factor of the respitatory burst of alveolar macrophages and neutrophils in the lungs. The inhibitory effect of SP-A is down-regulated by collagenase released from stimulated alveolar macrophages.

Surfactant; Surfactant associated protein A; Respiratory burst; Chemiluminescence; Neutrophil; Alveolar macrophage; Beagle dog

1. INTRODUCTION

The major surfactant-associated protein, SP-A, a glycoprotein with M_r = 28,000–36,000 Da, is assumed to be involved in metabolism of lung surfactant compounds, as it has been shown to accelerate uptake [1] and to inhibit secretion [2,3] of lipids by isolated alveolar type II cells. SP-A can bind lipids [4] and mannose in a lectin-like manner [5]. Amino acid sequences from human [6,7] and dog SP-A [8] revealed a collagen-like region within the NH_2-terminal half and a globular region within the COOH-terminal half of the polypeptide chain. Analysis of the macromolecular organization proved that SP-A aggregates to a complex of 18 polypeptides with 6 collagen-triple helices [9,10], and that this structure is highly homologous to the hexameric structure of C1q [9], a subcomponent of the first component of the classical complement pathway. Tenner et al. [11] have shown, that SP-A can subsitute for C1q in enhancing the FcR and CR1 mediated phagocytosis of IgG- and C4b-opsonized targets by monocytes and macrophages. It is the collagenous region of C1q, which is responsible for binding to mononuclear phagocytes [12]. However, as recently reported [13], binding of SP-A to macrophages is impaired by mannose coupled to albumin, leading to the conclusion that at least part of the recognition process is based on the mannose binding property of SP-A.

The striking homology between SP-A and C1q in quaternary structure and function in phagocytic processes prompted us to study whether SP-A modulates the oxidative metabolism of phagocytes. We found that SP-A from dog depletes the PMA-induced production of superoxide anion by phagocytes from canine lung and neutrophils from dog blood in our in vitro system. The inhibitory effect of SP-A developed during preincubation with the phagocytes. The effect was destroyed by collagenase treatment.

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Abbreviations: SP-A, surfactant associated protein A (28,000–36,000 Da); CL, chemiluminescence; PMA, phorbol-12-myristate-13-acetate; BAL, bronchoalveolar lavage

2. MATERIALS AND METHODS

2.1. *Chemicals*

Collagenase (Chlostridiopeptidase A from *Chlostridium histolyticum*), Hepes (4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid), sodium bromide, *n*-octyl-β-D-glucopyranoside, lucigenin (*bis*-*N*-methylacridinium nitrate) and PMA were from Sigma (Deisenhofen). Dulbecco's buffer solution (phosphate-buffered saline) and Dulbecco's buffer solution without Ca^{2+} and Mg^{2+} (PM 16) were from Serva (Heidelberg). Polyprep medium for the isolation of blood neutrophils was from Nycomed (Oslo).

Proteins for CL assay: dog albumin, bovine RNase-A, human α_1-antitrypsin, dog alkaline phosphatase, dog IgG and bovine β-*N*-acetyl-glucosaminidase were from Sigma. Yeast glucose-6-phosphate dehydrogenase and porcine lactate dehydrogenase were from Boehringer (Mannheim). Bovine serum albumin was from Serva. All proteins were dialyzed for 12 h against 5 mM Hepes (pH 7.4). Chemicals for polyacrylamide gel electrophoresis were from Pharmacia-LKB (Freiburg). All other chemicals were from Merck (Darmstadt).

2.2. *Isolation of bronchoalveolar lavage (BAL) cells*

Sequential lung lavage of anesthesized 2–4-year-old beagle dogs was performed by washing 3 lobes through a fiberoptic bronchoscope, using 3 × 15 ml body-warmed PM 16 buffer for each lobe.

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The fluid recovered by suction was filtered through gauze and centrifuged at 400 × g for 10 min. Cells were washed 1 × and resuspended in PM 16 buffer. Viability (Trypan blue exclusion) was always higher than 95%. The cell suspension contained at least 80% alveolar macrophages and less than 3% neutrophils. The cell-free supernatant was stored at −80°C for isolation of surfactant.

2.3. *Isolation of neutrophils*

Polyprep medium (4 ml) as mixed with 300 μl 1.5% NaCl and overlayered with 5 ml citrate-anticoagulated fresh dog blood. After centrifugation at 450 × g for 30 min the neutrophils containing band was collected and the cells were washed in PM 16 buffer (Beck-Speier et al., personal communication). Contaminating erythrocytes were lyzed by treating the cell pellet with distilled water for 30 s. Isotonicity was restored by adding the same volume of 1.8% (w/v) NaCl. The purified neutrophils (purity > 90%) were resuspended in 0.5 ml PM 16 buffer, 0.1% glucose and kept at room temperature.

2.4. *Isolation of canine lung surfactant and SP-A*

Surfactant was isolated from cell-free BAL fluid in a discontinuous gradient of NaBr according to Liau et al. [14]. The surfactant material, collected from the gradient was diluted 1:1 with distilled water and pelleted at 48,000 × g for 60 min at 4°C. SP-A was isolated from surfactant as described by Haagsman et al. [5]. Collagenase digestion of SP-A was performed by incubating 50 μg SP-A with 1.5 μg collagenase in 50 mM Tris-Cl (pH 7.4), 2 mM $CaCl_2$, (total volume 300 μl) at 37°C for 17 h. Protein content of SP-A preparations was determined by the method of Bradford [15] with IgG as standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of SP-A was performed as described by Lenz et al. [16].

2.5. *Chemiluminescence assay*

Production of superoxide anion by phagocytes was measured by lucigenin-dependent CL (Beck-Speier, personal communication). Cells (1×10^5 BAL cells or 3×10^4 neutrophils) were mixed with assay buffer phosphate-buffered saline, 0.1% glucose, pH 7.0) in chemiluminescence cuvettes (Tecator, Rodgau) and preincubated, in the absence (control) or presence of SP-A (3 μg) or test proteins (3 μg), for 20 min at 37°C. Lucigenin was added to a final concentration of 0.8 mM and the cuvettes were transferred into a 6-channel Biolumat LB 9505 (Berthold, Wildbad). After a further 10 min incubation at 37°C, measurement was started and PMA was added to a final concentration of 0.32 μM (total assay volume 250 μl). Measurement was carried out at 37°C for 20 min. Maximal CL counts and integrals of CL counts over 20 min were monitored. Changes of superoxide anion production in different cell incubations were calculated as percentages of the control at the maximal CL response.

3. RESULTS

3.1. *Effect of SP-A on the superoxide anion production of BAL cells and neutrophils*

Relative superoxide anion production of phagocytes was measured by lucigenin-dependent CL. In the standard assay, phagocytes were preincubated with SP-A (12 μg/ml assay) for 30 min prior to CL measurement. During subsequent stimulation with PMA, BAL cells (containing at least 80% alveolar macrophages and less than 3% neutrophils) as well as neutrophils from blood showed a significant decrease of the CL response as compared to the cells, preincubated in absence of SP-A (control (Table I)). In contrast, lucigenin-dependent CL of resting phagocytes was not influenced by SP-A (data not shown). A dose-response curve established for BAL cells showed maximal reduction of PMA-stimulated CL with 40 μg SP-A/ml assay (residual CL 50% of control) and half-maximal reduction with a 4 μg SP-A/ml assay (data not shown). Depletion of lucigenin-dependent CL of PMA-stimulated phagocytes was dependent on the time of preincubation of the cells with SP-A. When SP-A was added to the cells just before stimulation with

Table I

Effect of SP-A on the lucigenin-dependent chemiluminescence of PMA-stimulated phagocytes

Preincubation of cells with SP-A (12 μg/ml)	Chemiluminescence in % of control[a] (mean ± SD)	
	BAL cells	Neutrophils
0 min	105.8 ± 2.7 (n = 3)	81.0 ± 19.7 (n = 8)
30 min	61.2 ± 15.4 (n = 21)	41.9 ± 23.7 (n = 25)

[a]30 min preincubation of cells (3×10^4 neutrophils/250 μl; 1×10^5 BAL cells/250 μl) at 37°C in absence of SP-A and subsequent stimulation with PMA.

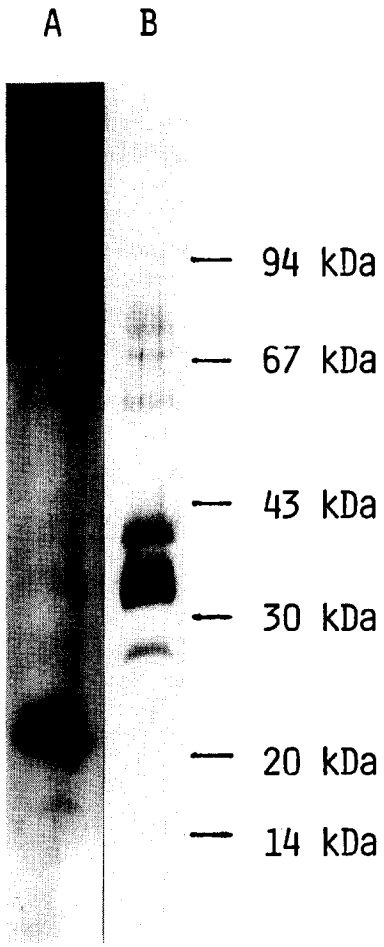

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified SP-A and collagenase-digested SP-A. Samples containing approximately 1.5 μg protein were reduced with dithiothreitol prior to electrophoresis. Protines were visualized by silver staining according to Blum et al. [17]. Lane A: collagenase treated SP-A (17 h/37°C); lane B: purified SP-A. Molecular weights on the right refer to standards.

Table II

Effect of collagenase-treated SP-A on the lucigenin-dependent chemiluminescence of PMA-stimulated neutrophils

Additions for preincubation of neutrophils[a]	Chemiluminescence in % of control[b] (mean ± SD)	
SP-A[c]	38.8 ± 13.2	(n = 5)
SP-A + collagenase[d] (17 h/37°C)	180.3 ± 42.1	(n = 5)
SP-A + collagenase[e] (0 h)	40.6	(n = 2)
SP-A[f] (17 h/37°C)	66.8 ± 25.9	(n = 4)
Collagenase[g] (17 h/37°C)	116.5 ± 30.1	(n = 3)

[a]Neutrophils (3×10^4 cells/250 μl) were preincubated after addition of proteins for 30 min at 37°C (see Materials and Methods) followed by stimulation with PMA.
[b]30 min preincubation of cells in absence of SP-A and collagenase and subsequent stimulation with PMA.
[c]Native SP-A (3 μg/250 μl).
[d]SP-A (3 μg/250 μl) digested with 0.1 μg collagenase for 17 h at 37°C.
[e]Collagenase (0.1 μg) pretreated for 17 h/37°C and SP-A (3 μg/ml) were mixed and immediately given to the cells for preincubation.
[f]SP-A (3 μg/250 μl) incubated as in (d), but without collagenase.
[g]Collagenase (0.1 μg) incubated as in (d), but without SP-A.

PMA, BAL cells showed no reduction and neutrophils only a slight reduction of the CL response compared with the controls (Table I).

3.2. *Effect of collagenase-treated SP-A on the superoxide anion production of neutrophils.*

Treatment of SP-A with collagenase (50 μg SP-A with 1.5 μg collagenase) for 17 h at 37°C resulted in a near complete conversion to the 'collagenase resistant fragment' [8] (22,000 Da molecular weight), as shown by polyacrylamide gel electrophoresis (Fig. 1). In contrast to the native SP-A, the collagenase-digested SP-A markedly enhanced the CL response of neutrophils after PMA stimulation to 180.3% as compared with the control under standard conditions (Table II). The following control measurements were performed to exclude artifacts: (1) Collagenase (incubated for 17 h at 37°C) and native SP-A were mixed at the same ratio as used in the digestion-mixture and immediately added to the cells (0 h digestion time). The CL response of PMA-stimulated neutrophils was identical to that obtained with native SP-A in the standard assay. (2) SP-A was pretreated for 17 h at 37°C in absence of collagenase and then added to the neutrophils for the standard assay. The CL response of the PMA-stimulated cells was increased from 38.8% with native SP-A to 66.8% with pretreated SP-A. Electrophoresis of the pretreated SP-A showed a faint band in the position of the collagenase resistant peptide, which was absent in the native SP-A (data not shown). (3) Collagenase alone, pretreated for 17 h at 37°C, did not markedly influence the CL response of PMA-stimulated neutrophils.

3.3. *Effect of various proteins on the lucigenin-dependent CL of PMA-stimulated BAL cells and neutrophils*

The effect of various proteins on the CL response of phagocytes was investigated to exclude an unspecific effect of SP-A (Table III). The CL response of PMA-stimulated BAL cells after preincubation for 30 min at 37°C with the different proteins ranged between 102.6% (lactate dehydrogenase) and 137.9% (alkaline phosphatase) showing an average CL response of 120% compared with the control. Surprisingly, the CL

Table III

Effect of various proteins on the lucigenin-dependent chemiluminescence of PMA-stimulated BAL cells and neutrophils

Proteins (12 μg/ml) used for preincubation	Chemiluminescence in % of control[a] (Mean ± SD)	
	BAL cells	Neutrophils
Glucose-6-phosphate dehydrogenase (yeast)	117.8 ± 36.5 (n = 4)	n.d.[b]
Lactate dehydrogenase (porcine)	102.6 ± 24.4 (n = 4)	198.9 (n = 2)
α_1-proteinase inhibitor (human)	112.9 ± 16.5 (n = 4)	248.4 (n = 2)
β-*N*-Acetyl-glucosaminidase (bovine)	104.6 ± 8.6 (n = 4)	130.6 ± 24.6 (n = 4)
RNase (bovine)	121.7 ± 12.6 (n = 4)	104.4 ± 8.8 (n = 4)
Serum albumin (bovine)	131.4 ± 18.8 (n = 4)	498.7 (n = 2)
Serum albumin (dog)	n.d.[b]	216.0 (n = 2)
Alkaline phosphatase (dog)	137.9 ± 5.0 (n = 4)	283.0 ± 54.5 (n = 4)
IgG (dog)	131.1 ± 39.8 (n = 6)	173.2 (n = 2)

[a]Preincubation of cells under standard conditions (see Materials and Methods) without addition of proteins followed by stimulation with PMA.
[b]Not determined.

response of PMA-stimulated neutrophils was more drastically increased by these test proteins with an average CL response of 231.7% compared with the control. The stimulating effect of the 3 dog proteins ranged between 173.2% (IgG) and 283% (alkaline phosphatase).

4. DISCUSSION

In recent years data are accumulating that surfactant has distinct effects in the host defence system of the lung, beside the well-known major function of preventing lung collapse. The surfactant-associated protein SP-A was recently described to bind to alveolar macrophages [18]. Since the structurally related C1q fragment of the complement system influences phagocytosis by binding to a receptor protein of alveolar macrophages, this is good evidence for the assumption that SP-A might modulate phagocytosis-associated processes like the respiratory burst of alveolar macrophages and neutrophils. Investigations of the oxidative metabolism of those cells were performed by monitoring lucigenin-dependent CL as a very sensitive and specific assay for the superoxide anion [19]. Instead of a homogeneous population of alveolar macrophages we used cells from BAL fluid, containing at least 80% alveolar macrophages and less than 3% neutrophils. The results presented here show that SP-A has no marked effect on the CL response of resting BAL cells and neutrophils, but significantly suppresses superoxide anion production of these cells after stimulation with PMA. This effect of SP-A was strongly dependent on preincubation with the phagocytes suggesting the necessity of an interaction of the protein with the cells. Thus, quenching of CL by SP-A can be excluded. The possibility that the depletion of the CL response by SP-A is an unspecific protein effect is ruled out, since various proteins tested did not deplete CL of PMA-stimulated BAL cells and neutrophils. All of these test proteins had very slightly stimulating effects on the lucigenin-dependent CL of BAL cells. However, a strong stimulation of the lucigenin-dependent CL of neutrophils was seen with albumin, the main protein component of the alveolar lining layer, α_1-PI and alkaline phosphatase, whereas no marked stimulation was observed with β-*N*-acetyl-glucosaminidase and RNase. An inhibitory effect on the oxidative response of alveolar macrophages after stimulation with PMA or opsonized zymosan was recently found for artificial and natural surfactant mediated by the phospholipids [20]. Since natural surfactant was more active, surfactant apoproteins were also believed to be involved in this process. In contrast, Webb and Jeska [21] found enhanced luminol-dependent CL of rat alveolar macrophages by pretreatment with the lipid fraction from the alveolar lining material and subsequent stimulation with latex beads. Van Iwaarden et al. [22] recently published data, indicating an increase of lucigenin-dependent CL of rat alveolar macrophages by human SP-A and rat surfactant without further stimuli. The CL of peritoneal macrophages, polymorphonuclear leukocytes and monocytes was not influenced by SP-A. We cannot confirm these data, since canine SP-A exhibited no stimulating effect on the lucigenin-dependent CL response of resting dog alveolar macrophages in our experiments. Further studies should clarify, whether these differences are due to different properties of dog and rat alveolar macrophages.

The concentration of SP-A necessary for depletion of the superoxide anion production in our in vitro system by 40–60% is very low (12 μg/ml). In contrast, other proteinaceous inhibitors of the respiratory burst of phagocytes like α_1-acid glycoprotein [23] and the elastase-generated IgG fragments Fc and Fabc [24] have to be present in much higher concentrations (α_1-acid glycoprotein, 100 μg/ml; Fc, 160 μg/ml; Fabc, 214 μg/ml). There exists less information on the SP-A concentration in the alveolar lining layer. Ryan et al. [25] estimated that the total SP-A concentration in this compartment is 360 μg/ml. However, the amount of SP-A which is bound to the surfactant and how much exists in free pools, is not known.

Treatment of SP-A by collagenase results in a collagenase resistant fragment (22,000 Da), which is also bound to alveolar macrophages [18]. Since both alveolar macrophages and neutrophils can release collagenases (reviewed in [26]) into the alveoli especially in inflammatory lung diseases, we tested the effect of collagenase-digested SP-A on neutrophils. In contrast to the suppressive effect of native SP-A, the digested protein markedly increased the superoxide anion production of PMA-stimualted neutrophils, and this increase was comparable to that found after treatment of the cells with IgG from dog.

We conclude from our in vitro studies that SP-A might have an important function in the control of the generation of reactive oxygen species by phagocytes in the lungs. This could prevent unnecessary oxygen radical production, which would be dangerous for the lung. Oxygen metabolism of phagocytes might only be activated, when free or membrane bound SP-A is split by a stimuli-induced release of collagenase. Further work is in progress to characterize the interaction of SP-A with phagocytes and to study the relevance of the effect on the respiratory burst in vivo.

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