Role of clathrin-mediated endocytosis of surfactant protein A by alveolar macrophages in intracellular signaling

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Moulakakis C, Stamme C. Role of clathrin-mediated endocytosis of surfactant protein A by alveolar macrophages in intracellular signaling. Am J Physiol Lung Cell Mol Physiol 296: L430–L441, 2009. First published January 9, 2009; doi:10.1152/ajplung.90458.2008.—We recently provided evidence that anti-inflammatory macrophage activation, i.e., the inhibition of constitutive and signal-induced NF-κB activity by the pulmonary collectin surfactant protein (SP)-A, critically involves a promoted stabilization of IkB-α, the predominant inhibitor of NF-κB, via posttranscriptional mechanisms comprising the activation of atypical (a)PKCζ, SP-A uptake and degradation by alveolar macrophages (AMφ) occur in a receptor-mediated, clathrin-dependent manner. However, a mutual link between endocytosis of and signaling by SP-A remains elusive. The aim of this study was to investigate whether clathrin-mediated endocytosis (CME) of SP-A by AMφ is a prerequisite for its modulation of the IkB-α/NF-κB pathway. The inhibition of clathrin-coated pit (CCP) formation and clathrin-coated vesicle (CCV) formation/budding abrogates SP-A-mediated inhibition of LPS-induced NF-κB activation in freshly isolated rat AMφ, as determined by Western analysis, fluorescence-activated cell sorting, confocal microscopy, and EMSA. Actin depolymerization and inhibition of CCP formation further abolished SP-A-mediated inhibition of LPS-induced TNF-α release, as determined by ELISA. In addition, SP-A-induced atypical PKCζ activation was abolished by pretreatment of AMφ with CCP inhibitors as determined by in vitro immunocomplex kinase assay. Although CME is classically considered as a means to terminate signaling, our results demonstrate that SP-A uptake via CME by AMφ has to precede the initiation of SP-A signaling.

constitutively, alveolar macrophages (AMφ) are the most abundant immunocompetent cells in the alveolar space with high phagocytic and antimicrobial properties when challenged with inhaled pathogens or invasive agents. Because of their unique localization within the alveolar surfactant film, which consists of phospholipids and proteins that are produced by type II alveolar lining cells (43), AMφ are the only macrophage to be exposed to air, thus having the most prevalent contact with inhaled pathogens and particles (43, 16) forming the first line of cellular defense (48).

Components of the surfactant lining layer support the resident AMφ in their immune response (44). Pulmonary surfactant is composed of lipids, mainly phospholipids (80–90%), and proteins (~10%). So far, four surfactant-associated proteins have been identified: the hydrophilic surfactant proteins A (SP-A) and D (SP-D), of which SP-A is most abundantly expressed in the lung, and the hydrophobic surfactant proteins B (SP-B) and C (SP-C). Whereas SP-B and SP-C are essential to reduce surface tension at the air-liquid interface of the lung, SP-A and SP-D have been shown to mediate host defense functions by recognizing, targeting, and enhancing the clearance of a variety of pathogens by AMφ (24, 40, 41, 62, 67, 68, 72). SP-A not only enhances macrophage phagocytosis (68), but also can affect other macrophage functions, including chemotaxis (74), release of cytokines, and production of reactive oxygen species (64, 68, 72).

SP-A belongs to the collectin family of proteins, along with SP-D, mannose-binding lectin, and bovine conglutinin (72). Collectins contain NH2-terminal collagen-like regions and C-type carbohydrate recognition domains (CRD) that form trimeric structures with high affinity for clustered oligosaccharides (72).

Clearance and degradation of SP-A by AMφ constitute the major route of removal of SP-A (3, 5, 6, 11, 74). The association of SP-A with human macrophages is very rapid, reaching a steady-state maximum by 10 min with ~90% of cell-bound SP-A being resistant to removal from the cell surface (11).

Recent studies showed that the uptake and degradation of SP-A by AMφ involves a clathrin-dependent endocytic pathway (3, 11, 32). Inhibition of clathrin-coated structures and actin significantly reduces the uptake of SP-A by rat and rabbit AMφ (3, 32). It was further reported that SP-A trafficking via the endolysosomal pathway occurs in a receptor-mediated, clathrin-dependent pathway in human macrophages (11). In these cells, SP-A was shown to colocalize sequentially with the early endosome marker EEA1, the late endosome marker lamp-1, and the lysosome marker cathepsin D.

Clathrin-mediated endocytosis (CME), responsible for the internalization of nutrients, pathogens, antigens, growth factors, and receptors, is the most well-characterized mechanism for the entry of molecules into cells. The central feature of CME is the recruitment of soluble clathrin from the cytoplasm to the plasma membrane. Clathrin triskelia assemble into a polygonal lattice at the plasma membrane to form clathrin-coated pits (CCP) that bud and pinch off from the membrane in a dynamin-dependent manner, yielding a clathrin-coated vesicle (CCV) (7, 37). The most abundant proteins in a coated vesicle, after clathrin, are those of the adaptor protein (AP)2 complex, consisting of two large subunits, α- and β2-adaptins, and two smaller ones, μ2 and σ2. AP-2 plays a central role in CCP formation and function, being responsible for the assembly of clathrin triskelia, recruitment of accessory proteins (27,
49, 56), and cargo recognition (28). So far, more than 20 clathrin adaptor proteins have been identified (53). Besides AP-2, the recruitment of specific cargo into CCVs involves clathrin-associated sorting proteins as well as the arrestins (14).

β-Arrestins were found to function in the desensitization of most seven-transmembrane receptors and as adaptors for AP-2 and clathrin (12). They escort activated receptors to CCPs for endocytosis and bind to other proteins involved in receptor internalization (12). In addition, β-arrestins have been implicated to play a role in cellular signaling and transcriptional regulation (12). A transcription factor family regulated by β-arrestins is NF-κB, a central regulator of genes involved in inflammation, cell proliferation, and apoptosis (12). The most prominent NF-κB heterodimer, p50/p65, is sequestered in the cytoplasm by a family of inhibitory proteins, among which IkB-α is assumed to function as the primary regulator of NF-κB in both stimulated and resting cells (54, 66). In response to many stimuli, including LPS, IkB-α is degraded via ubiquitination in an IκK-dependent manner (8, 26, 54). The removal of IkB-α allows the nuclear translocation of NF-κB and, subsequently, the transcription of downstream target genes, including IκB-α itself (9). The direct interaction of β-arrestins with IkB-α prevents IkB-α phosphorylation and degradation, thus attenuating NF-κB activation and the transcription of NF-κB-dependent target genes (71).

Endocytosis has been considered as a means to control the activity of signaling cascades and to terminate signaling of activated receptor complexes after their internalization (39, 47). The functional consequences of inhibiting endocytosis have been thoroughly investigated, revealing the functional importance of endosomes as a nucleation site of unique signaling complexes (29, 47, 70).

The current study investigated the role of CME in SP-A modulation of AMΦ function. It was hypothesized that SP-A endocytosed via CME by AMΦ is a prerequisite for SP-A anti-inflammatory immunomodulation in AMΦ. In this report we provide evidence that, indeed, SP-A has to be internalized via CME to mediate IkB-α stabilization, to inhibit LPS-induced NF-κB activity, and to induce atypical (α)PKCζ activation.

MATERIALS AND METHODS

Animals and reagents. Primary cells were obtained from pathogen-free male Sprague-Dawley rats (Charles River, Sulzfeld, Germany). Animal care and experiments were conducted according to protocols approved by the Schleswig-Holstein Ministry of Environment, Nature and Forestation. Rats were maintained at the Research Center Borstel animal facility under specific pathogen-free conditions.

The smooth LPS from Salmonella fredenau strain H909 was extracted using the phenol-water method, purified, lyophilized, and transformed into the triethylamine salt form (18). RPMI 1640 medium was purchased from GIBCO BRL (Karlsruhe, Germany). FCS was obtained from Bio- and Dulbecco’s phosphate-buffered saline were obtained from Transformed into the triethylamine salt form (18). RPMI 1640 medium was purchased from GIBCO BRL (Karlsruhe, Germany). FCS was obtained from Bio- and Dulbecco’s phosphate-buffered saline were obtained from Transformed into the triethylamine salt form (18). RPMI 1640 medium was purchased from GIBCO BRL (Karlsruhe, Germany). FCS was obtained from Bio-

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SP-A purification. Human SP-A was purified from bronchoalveolar lavage of patients with alveolar proteinosis as described in detail previously (73). Briefly, the lavage fluid was treated with butanol to extract SP-A, and the resulting pellet was sequentially solubilized in octylglucoside and 5 mM Tris, pH 7.4. SP-A was treated with polymyxin B agarose beads to reduce endotoxin contamination. SP-A preparations were tested for the presence of bacterial endotoxin using a Limulus amebocyte lysate assay (Cambrex, East Rutherford, NJ); all SP-A preparations used contained <0.2 pg endotoxin/μg SP-A. We express the possibility that in our SP-A preparations an effect of SP-A on the production of free radicals and cytokines may promote SP-A anti-inflammatory properties.

SP-A labeling. Purified SP-A was labeled with fluorescein-5-isothiocyanate isomer I (FITC) for 1 h at room temperature. The reaction was stopped by adding hydroxyamine hydrochloride, pH 8.5 (300 mM), and was dialyzed overnight in a QuisSep Micro Dialyzer (Roth, Karlsruhe, Germany) against 1× Tris-buffered water. Labeled proteins were analyzed by SDS-PAGE with subsequent Western analysis and Coomassie staining and were compared with unlabeled protein. Labeled and unlabeled SP-A exhibited identical bands in both approaches (Fig. 1).

Stimulation of rat alveolar macrophages. Rat AMΦ were isolated as described previously (64). The cells were plated at 1×10⁶ cells/ml in 24-well plates (Nunc, Wiesbaden, Germany) and allowed to attach for 90 min at 37°C in a 5% CO₂ atmosphere. The medium was then changed, and the cells were left untreated or treated with EDTA (10 mM, 30 min), mannose (1%, 30 min), or mannann (2.5 mg/ml, 30 min) before the addition of labeled or unlabeled SP-A (40 μg/ml, 1 h) at 37°C in the presence of 0.2% heat-inactivated (HI) FCS. In separate experiments, AMΦ in suspension at 1×10⁶ cells/0.5 ml per tube (Protein LoBind 1.5-m1l microfuge tubes; Eppendorf, Hamburg, Germany) were treated with the CME inhibitors Aman (2.5 mM, 30 min), PAO (2 μM, 30 min), CPZ (50 μM, 1 h), CytoD (10 μM, 30 min), an α-adaptin blocking peptide (10 μg, 10 min), or a β-arrestin blocking peptide (10 μg, 10 min) before the addition of labeled or unlabeled SP-A (40 μg/ml, 1 h) and/or LPS (100 ng/ml, 1 h) at 37°C in the presence of 0.2% HI-FCS to determine the possible role of the endocytic machinery on IkB-α protein expression. Cytosolic cell fractions (30–40 μg of protein/lane) were immunoblotted for IκB-α. Nuclear extracts (2 μg of protein/lane) of the cells were analyzed by EMSA for NF-κB DNA binding activity. For TNF-α determination, AMΦ in suspension at 1×10⁶ cells/tube were treated with CytoD (10 μM, 30 min), an α-adaptin blocking peptide (10 μg, 10 min), or a β-arrestin blocking peptide (10 μg, 10 min) before the addition of SP-A (40 μg/ml, 1 h) and/or stimulation with LPS (100 ng/ml) for 4 h at 37°C in the presence of 0.2% HI-FCS. After stimulation, cells were centrifuged at 200 g, and cell-free supernatants were collected for TNF-α determination.

TNF-α ELISA. TNF-α was determined in cell-free supernatants of stimulated rat AMΦ by BD OptEIA sandwich ELISA using anti-rat TNF-α antibody and biotinylated anti-rat TNF-α antibody (BD Biosciences, San Diego, CA) according to the manufacturer’s protocol.

Immunoprecipitation and in vitro αPKCζ kinase assay. Rat AMΦ (2×10⁶ cells/ml per well) were stimulated as described above. After culture, cells were lysed on ice for 30 min in 500 μl of lysis buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, and 1% Nonidet P-40). The lysates were spun at 9,300 g for 15 min, the supernatants were incubated with anti-αPKCζ antibody for 2 h or overnight at 4°C, after
which 50 μl of protein A-agarose were added for 2 h at 4°C with gentle rotation. The immune complexes were collected by centrifugation at 9,300 × g for 1 h, FITC-labeled SP-A (40 μg/ml, 1 h), smooth LPS (100 ng/ml, 1 h), or both (SP-A + LPS and FITC-SP-A + LPS). Functional activity was assessed based on the ability of SP-A to enhance IκB-α protein expression, constitutively and in the presence of LPS, and on its inhibitory effect on LPS-induced NF-κB activity by Western analysis and EMSA, respectively. Cytosolic cell fractions were immunoblotted for IκB-α (C). Nuclear extracts of the cells were analyzed by EMSA for NF-κB DNA binding activity (D). Data are means ± SE expressed as arbitrary units (a.u.) and are representative of 3–5 independent experiments. **P < 0.01 vs. control; ##P < 0.01 vs. LPS. Data were analyzed using a 1-way ANOVA with a post hoc Newman-Keuls test.

Western analysis. Western analysis was performed on cytosolic extracts. Experiments employing the inhibitors described above were performed under four different conditions: 1) in the absence of SP-A and LPS, 2) in the presence of SP-A (40 μg/ml, 1 h), 3) in the presence of LPS (100 ng/ml, 1 h), and 4) in the presence of SP-A (40 μg/ml, 1 h) before LPS (100 ng/ml, 1 h). After treatment, cytosolic fractions were assayed for protein content by using the bichinchoninic acid reagent (Pierce Biotechnology, Rockford, IL), separated on SDS-PAGE, and transferred to nitrocellulose membrane. The membranes were then incubated with anti-IκB-α or anti-actin (mouse monoclonal) at a 1:700 or 1:1,000 dilution, respectively. Goat anti-rabbit IgG-HRP or rabbit anti-mouse IgG-peroxidase conjugate served as secondary antibodies. Immunoreactive proteins were visualized using the ECL Western blotting detection system (Amersham, Munich, Germany).

Nuclear protein extraction and NF-κB activation assay. After the cells were exposed to the experimental conditions, nuclear extracts were prepared and analyzed as described previously (50). The activity of NF-κB in the nuclear extracts was determined by EMSA. NF-κB oligonucleotides were end-labeled with [γ-32P]ATP using T4 kinase. Crude nuclear extract (2 μg) was incubated for 20 min in binding buffer containing 50 μg of poly(di-cytosine)/ml with 7.5 fmol of the 32P-labeled oligonucleotides encoding the consensus NF-κB site 5'-AGTTGAGGGGACCTTCCAGGC-3'. Samples were separated by electrophoresis in 5% polyacrylamide gels, after which gels were analyzed using a PhosphorImager (Molecular Dynamics).

Uptake assay and fluorescence-activated cell sorting. Freshly isolated rat AMs were cultured at 0.5 × 10⁵ cells/0.5 ml per tube (Protein LoBind 1.5-ml microfuge tubes; Eppendorf) were incubated in the absence or presence of CME and actin inhibitors before the addition of FITC-labeled SP-A with gentle rotation at 37°C. After stimulation, cells were centrifuged at 200 g for 5 min at 4°C. The supernatant was discarded, and the cells were washed three times by centrifugation (200 g, 5 min, 4°C) with PBS without CaCl₂ and MgCl₂. The pellet was then resuspended in 100 μl of fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% formaldehyde) and transferred to a FACS tube. To exclude apoptotic cells, we added 1 ml of propidium iodide (1 μg/ml) and immediately centrifuged at 290 g for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in FACS buffer. Samples (20,000 cells/treatment) were analyzed for relative fluorescence per cell at 514 nm after excitation at 488 nm.
secondary antibody. FITC-SP-A and FITC-BSA were detected at 493 nm. Samples were analyzed using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Bensheim, Germany). All images were acquired under identical settings with Leica TCSNT software and assembled using Adobe Photoshop 6.0.

Statistical analysis. Data were statistically analyzed using one-way ANOVA with a Dunnett’s posttest or by one-way ANOVA with GraphPad Prism (version 4.0; GraphPad Software, San Diego, CA). Values were considered significant when P < 0.05. Data are means ± SE.

RESULTS

Labeled SP-A preparations are active. SP-A was isolated from human bronchoalveolar lavage and labeled with FITC. SP-A preparations were subjected to SDS-PAGE and subsequently stained with Coomassie (Fig. 1A) or blotted for SP-A (Fig. 1B). In both approaches, unlabeled SP-A and FITC-labeled SP-A exhibited identical protein bands with apparent molecular masses of ~30 and ~60 kDa. The functionality of labeled and unlabeled SP-A was assessed using a standard assay to determine SP-A activity, i.e., its ability to increase binding and/or uptake of FITC-SP-A was analyzed by FACS. Untreated or treated cells with EDTA or mannose, followed by treatment of FITC-SP-A with CytoD alone resulted in a significant decrease SP-A uptake and degradation by AMΦ(3, 33). SP-A increased IκB-α protein expression significantly, both constitutively (P < 0.001) and in the presence of LPS (P < 0.01). In the present study, CytoD did not change baseline IκB-α but abolished the SP-A effect on IκB-α both constitutively (P < 0.01) and in the presence of LPS (P < 0.05) (Fig. 3A), suggesting that intact actin filaments are required for SP-A-mediated IκB-α stabilization in primary AM.

SP-A-mediated inhibition of LPS-induced NF-κB DNA binding activity and TNF-α release requires intact actin filaments. Treatment of the cells with CytoD alone resulted in a significant enhancement of NF-κB activity (P < 0.001) in resting AMΦ compared with control (Fig. 3B). In parallel, CytoD increased TNF-α release by ~260% (Fig. 3C, right) in resting cells compared with control conditions (Fig. 3C, left). LPS-induced NF-κB DNA binding activity and TNF-α release were significantly reduced (P < 0.01) by SP-A (Fig. 3, B and C, left). However, the basal effect of CytoD on NF-κB activity and TNF-α release was not affected by SP-A, LPS, or both (Fig. 3, B and C, right).

SP-A-mediated IκB-α stabilization requires CCV formation and budding. We next asked whether SP-A internalization via CME by AMΦ is a prerequisite for IκB-α/NF-κB modulation by the collectin. It has been shown that SP-A is cleared by AMΦ in a clathrin-dependent manner (11, 33). Therefore, we...
used a panel of the most common pharmacological inhibitors of CCV formation or budding to prevent SP-A internalization and to investigate the effect on SP-A-mediated IkB-α stabilization: Aman, a specific clathrin inhibitor that prevents budding of CCV; PAO, which prevents formation of CCV; and the cationic amphiphilic agent CPZ, which causes a loss of the AP-2/clathrin assembly from the cell membrane (31).

SP-A enhanced IkB-α protein expression significantly, both constitutively and in the presence of LPS. Compared with control, only CPZ significantly decreased baseline IkB-α protein expression ($P < 0.05$). Pretreatment of AMφ with Aman, PAO, or CPZ significantly inhibited SP-A enhancing effect on IkB-α protein expression ($P < 0.001$) as well as SP-A-mediated inhibition of LPS-induced IkB-α degradation ($P < 0.001$ for Aman and CPZ, $P < 0.01$ for PAO) (Fig. 4A).

The inhibitors significantly enhanced NF-κB activity in resting cells compared with control ($P < 0.05–0.01$) (Fig. 4B). However, treatment of the cells with Aman, PAO, and CPZ before the addition of SP-A, LPS, or both did not affect the basal effect of the inhibitors, suggesting that CCV formation and budding are required for SP-A modulation of the IkB-α/NF-κB pathway.

Cell association of FITC-SP-A to AMφ is significantly reduced by inhibition of CCV formation and budding. To evaluate the impact of CCV formation and budding and the actin cytoskeleton on cell association of SP-A with rat AMφ, we left cells untreated or treated them with Aman, PAO, CPZ, or CytoD, followed by exposure to FITC-labeled SP-A. FACS analysis revealed that cell association of FITC-SP-A with AMφ was significantly ($P < 0.05–0.01$) inhibited in the presence of each inhibitor (Fig. 5).

**SP-A-mediated IkB-α/NF-κB modulation requires CCP formation.** To identify clathrin adaptors involved in SP-A’s effects, we targeted the most prominent clathrin-associated adaptors, AP-2 and β-arrestin. The α-subunit of the AP-2 complex binds to phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P$_2$] and recruits AP-2 to the plasma membrane, where clathrin is connected to AP-2 via β-arrestins (53). AMφ were treated with or without the respective β-arrestin blocking peptide and α-adaptin blocking peptide, followed by exposure to SP-A, LPS, or both.

The inhibition of β-arrestin or α-adaptin did not affect basal IkB-α protein expression. However, SP-A-mediated IkB-α stabilization was significantly reduced when either β-arrestin or α-adaptin was inhibited ($P < 0.001$). Likewise, SP-A-mediated increase of IkB-α in the presence of LPS was significantly ($P < 0.05$) reduced by β-arrestin or α-adaptin inhibition (Fig. 6A), suggesting that both β-arrestin and α-adaptin are involved in SP-A-mediated IkB-α stabilization.

In parallel, nuclear extracts were analyzed for NF-κB activity by EMSA. The inhibition of β-arrestin or α-adaptin alone significantly ($P < 0.01$ and $P < 0.001$, respectively) increased
Densitometric results are expressed as a.u. 

Data are means ± SE and are representative of 4–6 independent experiments. Densitometric results are expressed as a.u. *P < 0.05; **P < 0.01; ***P < 0.001 vs. control. ###P < 0.001 vs. SP-A. SP < 0.05; $$$P < 0.001 vs. LPS. §§P < 0.01; §§§P < 0.001 vs. SP-A+LPS. Data were analyzed using 1-way ANOVA with a post hoc Newman-Keuls test.

Fig. 4. Clathrin-coated vesicle (CCV) formation and budding are required for SP-A-mediated IκB-α stabilization and inhibition of LPS-induced NF-κB activity. Freshly isolated primary rat AMβ cells were left untreated or treated with 2.5 mM amantadine (Aman; 30 min), 2 μM phenylarsine oxide (PAO; 30 min), or 50 μM chlorpromazine (CPZ; 1 h) and then exposed to medium, 40 μg/ml SP-A (1 h), and/or 100 ng/ml LPS (1 h). A: cytosolic fractions were subjected to SDS-PAGE and immunoblotted for IκB-α protein expression. B: nuclear extracts of the cells were analyzed by EMSA for NF-κB DNA binding activity. Data are means ± SE and are representative of 4–6 independent experiments. Densitometric results are expressed as a.u. *P < 0.05; **P < 0.01; ***P < 0.001 vs. control. ###P < 0.001 vs. SP-A. SP < 0.05; $$$P < 0.001 vs. LPS. §§P < 0.01; §§§P < 0.001 vs. SP-A+LPS. Data were analyzed using 1-way ANOVA with a post hoc Newman-Keuls test.

basal NF-κB activity compared with control conditions. LPS-induced NF-κB activity was further enhanced when cells had been pretreated with the β-arrestin blocking peptide (P < 0.01) (Fig. 6D). SP-A-mediated inhibition of LPS-induced NF-κB activity could not be restored when cells had been pretreated with the β-arrestin (P < 0.01) or the α-adaptin blocking peptide (P < 0.001). Instead, the basal effect observed for β-arrestin or α-adaptin inhibition was further augmented in the presence of SP-A and LPS (P < 0.05). These results strongly suggest that both clathrin adaptors are required for SP-A-mediated inhibition of LPS-induced NF-κB activity.

We next examined the effect of β-arrestin or α-adaptin inhibition on TNF-α release. Treatment of AMβ with the β-arrestin or α-adaptin blocking peptide did not significantly enhance basal TNF-α release compared with resting cells, whereas SP-A-mediated inhibition of LPS-induced TNF-α release was significantly abrogated (P < 0.05) by treatment of the cells with the β-arrestin blocking peptide (Fig. 6C). Together, the data indicate that the clathrin adaptors β-arrestin and α-adaptin are involved in SP-A-mediated IκB-α stabilization, inhibition of LPS-induced NF-κB activity, and inhibition of TNF-α release.

We next performed confocal microscopy to visualize the effects of the above mentioned inhibitors on SP-A cell-association with or uptake by AMβ. Cells were seeded into eight-well chamber slides and either left untreated (Fig. 6Dg) or treated with the inhibitors (Fig. 6Db–f and h), followed by the addition of FITC-labeled SP-A (Fig. 6D). In a control condition, cells were treated with FITC-BSA (Fig. 6Di), EDTA (Fig. 6Dk), mannose (Fig. 6Dk), mannan (Fig. 6D), or normal mouse IgG (data not shown). Compared with cells that had been treated with mannose or mannan before the addition of

Fig. 5. CCV formation and budding are required for cell association of SP-A with AMβ. Freshly isolated primary rat AMβ in suspension at 0.5 × 10⁶ cells/tube were incubated in duplicate tubes with 2.5 mM Aman (30 min), 2 μM PAO (30 min), 50 μM CPZ (1 h), or 10 μM CytoD (30 min), followed by the addition of 40 μg/ml FITC-SP-A (1 h). Cells were washed, and cell association of FITC-SP-A was measured by flow cytometry. Data are means ± SE expressed as a percentage of total cell-associated SP-A. The overlay of FACS histograms is representative of 3 independent experiments. *P < 0.05; **P < 0.01 vs. cell association of FITC-SP-A. Data were analyzed using 1-way ANOVA with a post hoc Newman-Keuls test.

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FITC-SP-A or those treated with FITC-SP-A alone (Fig. 6A), the presence of all inhibitors tested prevented FITC-SP-A internalization by AMs. FITC-SP-A remained outside the cell, attached to the cell membrane.

SP-A-induced \( \alpha \)-PKC\( \zeta \) kinase activity requires CCV formation. SP-A-enhanced \( \alpha \)-PKC\( \zeta \) kinase activity in AMs has been shown previously to be critically involved in SP-A-mediated IκB-\( \alpha \) stabilization (50). We next investigated SP-A-induced \( \alpha \)-PKC\( \zeta \) kinase activity in the presence of Aman, PAO, CPZ, and CytoD in an in vitro immunocomplex kinase assay as described in MATERIALS AND METHODS (Fig. 7).

Confirming our previous data, \( \alpha \)-PKC\( \zeta \) kinase activity in AMs was significantly enhanced by SP-A, CPZ, and LPS (C), and LPS (P < 0.001) compared with control. Treatment of AMs with CytoD, Aman, PAO, and CPZ alone did not alter baseline \( \alpha \)-PKC\( \zeta \) activity. However, treatment of the cells with the inhibitors before the addition of SP-A or LPS significantly reduced SP-A- and LPS-induced \( \alpha \)-PKC\( \zeta \) activity. These results strongly suggest that the inhibition of the actin cytoskeleton and CCV formation and budding counteracts \( \alpha \)-PKC\( \zeta \) activation induced by both SP-A and LPS. Impaired SP-A activation of \( \alpha \)-PKC\( \zeta \) by treatment of the cells with CytoD suggests that SP-A-induced signaling events do not precede actin polymerization.

DISCUSSION

The pulmonary collectins are critical components of the immune host defense of the lung, mediating inflammatory responses of innate and adaptive immune cells to protect the host from lung damage or impaired gas exchange (55). The mechanisms of SP-A-mediated immunomodulation have been thoroughly investigated over the past several years both in vitro and in vivo. SP-A upregulates the expression of cell surface receptors involved in microbial recognition and modulates the specificity of gene transcription by primary lung cells to maintain lung homeostasis. SP-A modulation of the NF-κB pathway has been investigated by several groups with controversial results (1, 21, 30, 50, 59–61, 63, 76). Inhibition of the actin cytoskeleton and CCV formation and budding counteracts \( \alpha \)-PKC\( \zeta \) activation induced by both SP-A and LPS. Impaired SP-A activation of \( \alpha \)-PKC\( \zeta \) by treatment of the cells with CytoD suggests that SP-A-induced signaling events do not precede actin polymerization.

bition of constitutive and signal-induced NF-κB activity by SP-A, critically involves a promoted stabilization of IκB-\( \alpha \), the predominant inhibitor of NF-κB, via posttranscriptional mechanisms comprising a SP-A-enhanced activity of atypical PKC\( \zeta \) (50, 75).

Resident AMs substantially contribute to SP-A uptake and degradation (3, 5, 25, 74). SP-A uptake by rabbit AMs (3) and rat AMs (32) is clathrin dependent, and SP-A-containing vesicles traffic through the endolysosomal pathway, where they mature into lysosomes in human macrophages (11). The possibility that the uptake of SP-A by macrophages and its trafficking through the endolysosomal pathway contribute to

**Fig. 6.** Clathrin-coated pit (CCP) formation is required for SP-A-mediated IκB-\( \alpha \)/NF-κB modulation. Freshly isolated primary rat AMs were left untreated or treated with 10 μg of \( \alpha \)-arrestin blocking peptide (10 min) or 10 μg of \( \alpha \)-adapin blocking peptide (10 min) and then exposed to medium, 40 μg/ml SP-A (1 h), or/and 100 ng/ml LPS (1 h). A: cytosolic fractions were subjected to SDS-PAGE and immunoblotted for IκB-\( \alpha \) protein expression. B: nuclear extracts of the cells were analyzed by EMSA for NF-κB DNA binding activity. Data are means ± SE and are representative of 4–6 independent experiments. Densitometric results are expressed as a.u. ***P < 0.001 vs. control. §§§P < 0.001 vs. SP-A, §P < 0.05; $P < 0.01; $§§P < 0.001 vs. SP-A + LPS, §§§P < 0.001 vs. \( \alpha \)-arrestin block. +P < 0.05 vs. \( \alpha \)-adapin block. Data were analyzed using 1-way ANOVA with a post hoc Newman-Keuls test. C: cell-free supernatants were harvested after 4 h for the determination of TNF-\( \alpha \) by ELISA. Data are means ± SE expressed as a percentage of TNF-\( \alpha \) release in resting cells or in the presence of SP-A and LPS (dotted line, 100%) and are representative of 3–5 independent experiments. §P < 0.05 vs. TNF-\( \alpha \) release in the presence of SP-A and LPS. Data were analyzed using 1-way ANOVA with Dunnett’s posttest. D: freshly isolated rat AMs adhered to chamber slides at 1 × 10⁴ cells/well and were left untreated (control, g) or incubated with 2.5 mM Aman (30 min, h), 2 μM PAO (30 min, c), 50 μM CPZ (60 min, d), 10 μM CytoD (30 min, e), 10 μg of \( \alpha \)-adapin blocking peptide (10 min, f), 10 μg of \( \alpha \)-arrestin blocking peptide (10 min, h), 10 nM EDTA (30 min, i), 1% mannose (30 min, j), 2.5 mg/ml mannan (30 min, k), and 10 μg/ml FITC-BSA (30 min, l) and then exposed to medium or treated with 40 μg/ml FITC-SP-A (60 min, e; green). Slides were blocked and incubated with an anti-human IgG conjugated to NorthernLights 557 (red). Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (blue). Cells were visualized by confocal microscopy. Images in a–l are shown as differential interference contrast (DIC; left) and overlays of single staining (right) and are representative of 3–5 independent experiments with similar results.
SP-A biological activities was initially raised by Crowther and Schlesinger (11). In support of this notion, the aim of the present study was to investigate whether CME of SP-A by AMβδ is a prerequisite for its modulation of the IkB-α/NF-κB pathway.

The initial steps in CME involve the recruitment of clathrin to the plasma membrane and the formation of CCP that grow into CCV and finally pinch off the membrane (37). In the current study, we applied the most commonly used pharmacological inhibitors that are available to prevent CCV formation and budding and specific blocking peptides for the most prominent clathrin adaptors, α-adaptin and β-arrestin (31). In addition, actin polymerization, which has been linked to preformed clathrin-coated structures and vesicle scission (35, 45), was disrupted to investigate the effect on SP-A modulation of the IkB-α/NF-κB pathway in primary AMβδ. The actin-depolymerization agent CytoD, as well as the CCV formation/budding inhibitors, altered SP-A-mediated IkB-α profiles, i.e., decreased IkB-α protein expression constitutively and in the presence of LPS, which corresponded well with NF-κB DNA binding activities determined in parallel.

The significant enhancement of basal NF-κB activity after treatment of AMβδ with CytoD, Aman, PAO, and CPZ could not be restored by SP-A, either constitutively or in the presence of LPS. These agents have been successfully used to inhibit CME by a number of groups (4, 23, 31, 32, 36, 58), including studies investigating SP-A uptake by type II pneumocytes, the primary cells to synthesize, secrete, and recycle SP-A. In these studies, CytoD, as well as Aman, PAO, and potassium depletion, prevented SP-A uptake and degradation (32, 65). However, one should keep in mind that these pharmacological inhibitors also have other effects on cellular function, especially PAO (22, 23, 31, 57, 65). These side effects may have contributed to the release of proinflammatory mediators by AMβδ after PAO treatment and cannot be attributed solely to the inhibition of CME.

The treatment of AMβδ with CytoD alone led to a significant increase of TNF-α release by resting cells. The enhancing effect of CytoD on NF-κB activity and IkB-α degradation is in line with previously observed findings in intestinal epithelial cells, linking the actin cytoskeleton with the regulation of NF-κB activation and inflammatory mediator release (51). The mechanism(s) involved may include a direct interaction between p65 and actin (2). This is supported by several recent reports that actin assembly has a critical role in clathrin-coated vesicle formation, receptor-mediated endocytosis, and coated pit-dependent uptake processes (34, 35, 46, 52). Together, the data imply that, indeed, CCV formation and budding and an intact actin cytoskeleton are required for SP-A-modulated IkB-α stabilization and SP-A-mediated inhibition of LPS-induced NF-κB activity.

The clathrin assembly machinery constitutes a number of adaptor proteins and accessory proteins involved in CCP and CCV formation. Clathrin coat formation was thought to be initiated by the AP-2 complex, but recent data suggest that its major function lies in cargo recruitment (10). β-Arrestin, on the other hand, is an adaptor for both clathrin and AP-2. Both clathrin adaptors differ by means of cargo recognition. Whereas α-adaptin is a cargo-motif adaptor that binds to PtdIns(4,5)P2 (17), bridging clathrin and the membrane, β-arrestin is a cargo-specific adaptor (53). A role for the β-arrestins has been implicated in cellular signaling and regulation of transcription factors such as NF-κB (12). Independent studies by Witherow et al. (71) and Gao et al. (19) identified β-arrestin 1 and 2 as binding partners of IkB-α. They found that the interaction of the β-arrestins with IkB-α prevented NF-κB activation and expression of NF-κB target genes (19, 71).

In support of these findings, we have shown in the present study that the inhibition of β-arrestin 1 or α-adaptin significantly increased basal NF-κB activity in AMβδ compared with resting cells and abolished SP-A’s inhibiting effect on LPS-induced NF-κB activity. In addition, blocking β-arrestin abrogated SP-A’s inhibiting effect on LPS-induced TNF-α release, and SP-A-mediated IkB-α stabilization, both constitutively and in the presence of LPS, was abolished by both β-arrestin and α-adaptin inhibition. Together, these data suggest that the inhibition of constitutive CCP formation, i.e., the inhibition of the constitutive availability of clathrin and its recruitment to the membrane in resting cells, increases basal NF-κB activity in an IkB-α-independent way. Thus the data also imply that SP-A immunomodulation of AMβδ function depends on both clathrin adaptors, α-adaptin and β-arrestin, to mediate CCP formation.

The central role of AP-2 adaptors in CCP formation and recruitment of accessory proteins has been demonstrated by reduced numbers of coated pits in AP-2-depleted HeLa cells (49). However, virtually nothing is known about the signaling events following AP-2 depletion. In the current study, we inhibited the α-subunit of AP-2 that would abrogate the connection to the plasma membrane and the recruitment of accessory proteins for vesicle formation. Blocking α-adaptin prevented SP-A uptake and led to a significant increase in NF-κB activity, strongly suggesting that AP-2 plays an important role in SP-A internalization.

Compared with the induction of basal NF-κB activity by the general CME inhibitors, the overriding effect observed after treatment with the blocking peptides in the presence of SP-A and LPS on basal NF-κB activity may be due to the specificity of the blocking peptides and emphasizes the essential role of these adaptors in SP-A and LPS uptake via CME in immunocompetent cells.

When β-arrestin was inhibited, LPS-induced NF-κB activity in primary AMβδ was strongly enhanced, supporting the data obtained by Husebye et al. (29). They found that LPS is endocytosed by a receptor-mediated mechanism dependent on clathrin and colocalizes with TLR4 on early endosomes. This endosomal trafficking was not associated with signaling, but this sorting pathway had important functional consequences inasmuch as the inhibition of proteins involved in endosomal trafficking and maturation significantly increased LPS-induced NF-κB activity in HEK-293 cells. Thus the endosomal uptake of LPS appeared to participate in the termination of signaling by LPS initiated by receptors of the cytoplasmic membrane (29).

Although LPS uptake by CME terminates LPS signaling, the data presented in this report suggest that SP-A has to enter the AMβδ via clathrin- and actin-dependent endocytosis to initiate signaling. In the present study, we visualized the effect of preventing SP-A uptake by AMβδ by confocal microscopy. Actin depolymerization and the inhibition of CCP initiation or CCV formation and budding abrogated the internalization of
FITC-labeled SP-A by AMφ. These images add emphasis to and support our presented data.

With respect to our previous findings on SP-A induced aPKCζ kinase activity in primary AMφ (50), in the present study we investigated the effect of actin cytoskeleton disruption and inhibition of CCV formation and budding on SP-A-induced aPKCζ kinase activity. Whereas baseline aPKCζ activation was not altered in the presence of the inhibitors, SP-A as well as LPS-induced aPKCζ activity was prevented in the presence of all inhibitors tested. The role of CME in aPKCζ activation is unknown. Activation of aPKCζ induced by SP-A or LPS leads to an anti-inflammatory (50) or proinflammatory response, respectively, in immune cells (38). Although diverse, both activation mechanisms seem to occur through internalization of the distinct ligands via CME in an actin-dependent manner. Several studies have shown that PKCζ affects remodeling of the cytoskeleton through direct or indirect interaction with actin (15, 42). The evaluation of this dual activation mechanism of aPKCζ and the induced immune response triggered by SP-A and LPS requires future investigation in our laboratory.

Endocytosis has been considered as a means to terminate signals and prevent autotaxic mediator release by the effector cell. Recent data suggest that endosomes have functional importance by providing compartment-specific molecular interactions leading to the assembly of unique signaling complexes (47). A variety of cell surface receptors, including receptor tyrosine kinases, G protein-coupled receptors (GPCRs), and serine-threonine receptor kinases, use endosomes as a compartment for the activation of highly specialized signaling complexes (69, 70). The impairment of CME has been shown to inhibit the uptake of activated receptors and reduce the propagation of downstream signaling events (47). Thus endocytosis seems to have a dual role: signal termination and propagation, and receptor activation. Alterations in the endocytic machinery, where the balance between stimulation and attenuation of signaling is subverted, suggest an important role for endocytic proteins in pathological conditions, e.g., cancer (13).

In summary, we have provided evidence that actin-dependent CME of SP-A, in particular CCP initiation and CCV formation and budding, is required for SP-A-mediated IκB-α stabilization, inhibition of both LPS-induced NF-κB activity and TNF-α release, and SP-A-induced activation of aPKCζ. Still, virtually nothing is known about the signaling capacity of SP-A once endocytosed, as well as the subsequent signaling events involving that modulate AMφ immune functions. Given that SP-A is a central immunomodulatory protein in pulmonary innate host defense, the understanding of its physiological role and its interaction with immune cells in the lung is essential for the development of potential therapeutic measures and strategies. The identification and characterization of CME-associated scaffold proteins and kinases involved in SP-A signaling are therefore of major interest for future studies.

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REFERENCES


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