Recombinant human activated protein C reduces human endotoxin–induced pulmonary inflammation via inhibition of neutrophil chemotaxis

Jerry A. Nick, Christopher D. Coldren, Mark W. Geraci, Katie R. Poch, Brian W. Fouty, James O’Brien, Michael Gruber, Simona Zarini, Robert C. Murphy, Katherine Kuhn, Don Richter, Kelly R. Kast, and Edward Abraham

Recombinant human activated protein C (rhAPC) is a natural anticoagulant with potentially important anti-inflammatory properties. In humans with severe sepsis, rhAPC treatment reduces mortality, but mechanisms responsible have not been well characterized. Accumulation of activated neutrophils in the lungs and other organs during severe infection contributes to sepsis-induced organ dysfunction, including acute inflammatory lung injury. Because neutrophils express an APC receptor, we hypothesized that immunomodulatory effects of rhAPC occur, in part, via modulation of neutrophil responses. To examine this issue, we performed a double-blinded, placebo-controlled study of rhAPC in a human model of endotoxin-induced pulmonary inflammation. Administration of rhAPC significantly reduced leukocyte accumulation to the airspaces, independent of pulmonary cytokine or chemokine release. Neutrophils recovered from bronchoalveolar lavage fluid of volunteers receiving rhAPC demonstrated decreased chemotaxis ex vivo. Decreased neutrophil chemotaxis following exposure to rhAPC was confirmed in vitro. No differences were detected in gene expression, kinase activation, cytokine release, cell survival, or apoptosis of neutrophils recovered in the presence or absence of rhAPC. These studies demonstrate that rhAPC reduces both endotoxin-induced accumulation of leukocytes in the airspaces and neutrophil chemotaxis. These rhAPC-induced effects on neutrophil function may represent a mechanism by which rhAPC improves survival in patients with sepsis. (Blood. 2004;104:3878-3885)

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Introduction

Activated protein C (APC) is a natural anticoagulant that plays an important role in coagulation homeostasis by inactivating the procoagulant factors Va and VIIIa. Recently, recombinant human APC (rhAPC) has been demonstrated to have potentially important anti-inflammatory properties. rhAPC is able to inhibit leukocyte adhesion to vascular endothelial cells and to reduce activated neutrophil accumulation in rat lungs. In monocytes, which express the endothelial protein C receptor (EPCR), exposure to rhAPC inhibits lipopolysaccharide (LPS)–induced release of tumor necrosis factor α (TNFα) and nuclear translocation of nuclear factor (NF)–κB. In a lethal baboon model of E coli–induced sepsis, infusion of rhAPC was demonstrated to be protective, and in humans with multiple organ system dysfunction due to severe infection, treatment with rhAPC reduces mortality.

Despite the ability of rhAPC to improve survival in primate models of severe infection and in humans with sepsis, the mechanisms responsible for such effects have not been fully characterized. Although initial hypotheses focused on interruption of the coagulopathic and fibrinolytic cascades activated in sepsis, other agents that also have potent effects on such pathways, such as tissue factor pathway inhibitor and antithrombin, did not demonstrate the same clinical benefit in severe sepsis as was seen with rhAPC. In human models of endotoxemia, infusion of rhAPC did not affect proinflammatory responses, including elevations in circulating levels of proinflammatory cytokines, nor did it limit thrombin generation.

Recent data have shown that neutrophils express receptors for APC and also that neutrophil chemotaxis is inhibited by exposure to protein C, APC, or rhAPC. Activated neutrophils accumulate in the lungs and other organs during severe infection, and contribute to organ system dysfunction and mortality in this setting. Thus, the ability of rhAPC to affect neutrophil functions, particularly those associated with neutrophil migration, provides an additional potential mechanism for its beneficial effects in sepsis. In order to test this hypothesis, we used a human model of endotoxin-induced pulmonary inflammation to examine the effects of rhAPC in vivo on inflammatory responses in which neutrophils play a major role.

Patients, materials, and methods

Materials

Endotoxin-free reagents and plastics were used in all experiments. LPS isolated from E coli (EC-LPS) strain 0111:B4 (National Institutes of Health, 1400 Jackson St, Denver, CO 80206; e-mail: nickj@njc.org).

E.A. has served as a paid consultant for Eli Lilly and Company. An Inside Blood analysis of this article appears in the front of this issue.

Reprints: Jerry A. Nick, National Jewish Medical and Research Center, D202, 1400 Jackson St, Denver, CO 80206; e-mail: nickj@njc.org.

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Model of endotoxin-induced pulmonary inflammation

Subjects eligible for this study had to meet the following criteria: (1) 18 to 40 years of age, male or female; (2) nonsmoker; (3) no active medical problems; and (4) no concurrent medications, including aspirin or nonsteroidal anti-inflammatory drugs. Women taking oral contraceptives were not excluded. Exclusions included pregnancy; lactation; history of recent clinically significant asthma; allergies to both trimethoprim/sulfamethoxazole and penicillin (or amoxicillin); allergy to lidocaine or related compounds; allergy to opiates or benzodiazepines, used for sedation during bronchoscopy; history of asthma or history of exercise-induced wheezing; signs of any acute illness on the day of endotoxin instillation; abnormalities on screening laboratory tests, electrocardiogram, chest radiograph, or pulmonary function tests; and any personal or family history of bleeding disorders.

All subjects were admitted to the General Clinical Research Center (GCRC) at the University of Colorado Hospital. Approval for this study was obtained from the Colorado Multiple Institutional Review Board, and informed consent was provided according to the Declaration of Helsinki. The timeline for the study is shown in Figure 1. Using a double-blinded placebo-controlled design, subjects (n = 16) were randomized to receive either rhAPC (drotrecogin alfa [activated], 24 mcg/kg per hour) or normal saline (the solution for drotrecogin alfa [activated]) starting 2 hours before the initial bronchoscopy and continuing for 16 hours. The infusion of rhAPC or placebo was discontinued 2 hours prior to the second bronchoscopy to lessen the risk of hemorrhage resulting from anticoagulant therapy. Five hours before the second bronchoscopy, rhAPC AND LPS-INDUCED PULMONARY INFLAMMATION 3879

Figure 1. Experimental design of the endotoxin-induced pulmonary inflammation study.

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Microarray analysis of BALF neutrophil gene expression

Total RNA was stabilized in freshly isolated neutrophils by resuspension of 2 × 10⁶ cells in 1 mL RNAalater (Ambion, Austin TX), then stored at −20°C. Subsequent isolation with Trizol (Life Technologies, CA) and purification with RNeasy MinElute columns (Qiagen, CA) was performed following the manufacturer’s protocol. From 1 μg to 5 μg total RNA was used for microarray target labeling using standard methods for reverse transcription and one round of in vitro transcription. HG-U133A microarrays were hybridized with 20 μg cRNA and processed per the manufacturer’s protocol (Affymetrix, Foster City, CA).

Hybridization signals were quantified using the statistical algorithms implemented in the Affymetrix Gene Chip Operating System. Individual arrays were determined to be of high quality by visual inspection, comparison of the overall fluorescence intensity (scaling factor) to other arrays in the group, and low 3’/5’ ratios for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and B-actin (ratio < 3). This insures that all of the arrays in the group can be directly compared, and that the input mRNA was intact.

Microarray data were analyzed using BRB ArrayTools v3.1. Tabular intensity data were imported, log₂ transformed, and each array was normalized (mean centered) to the median intensity array. Class comparison using the univariate 2-sample t test was performed using the set of 11 853 genes that were reliably detected on 2 or more arrays. Exact multivariate permutation testing was conducted using all 126 possible permutations.

Neutrophil functional assays

Human neutrophils were isolated from blood by the plasma Percoll method and suspended in RPMI 1640 culture medium (Bio-Whittaker, Walkersville, MD). Neutrophils isolated from peripheral blood or BALF were resuspended in RPMI 1640 and human HIPP (2%) containing 10 × 10⁶ cells/mL. One mL of the cell suspension combined with LPS and left unstimulated was placed in a 1.5 mL microcentrifuge tube (Eppendorf, Germany) and rotated continuously for up to 4 hours at 37°C. Release of cytokines and various growth factors was screened for by enzyme-linked immunosorbent assay (ELISA) and by an antibody-based protein microarray (RayBiotech, Norcross, GA). Selected cytokines (TNF-α, interleukin 8 [IL-8], macrophage inflammatory protein 1 β [MIP-1β], monocyte chemoattractant protein 1 [MCP-1]) were quantified by immunoassay (ELISA Tech, Denver, CO). Quantification of neutrophil apoptosis was performed by immunoassay for cytoplasmic histone-associated DNA fragments (Roche, Mannheim, Germany). Cell death via necrosis was assessed by percent of total lactate dehydrogenase (LDH) release (Cytotoxicity Detection Kit; Roche).

Neutrophil chemotaxis assay

Migration of neutrophils through a microporous polyethylene terephthalate membrane to gradients of IL-8 was measured using a 96-Multiwell Insert System (HTS FluoroBlok; BD Falcon, Bedford, MA) in which a 3-μm pore
filter separated the upper from the lower chamber as a modified Boyden system. Cells were labeled in situ with 10 μM Calcein AM in Hanks balanced salt solution (HBSS) for 15 minutes at 37°C and washed once with HBSS, then resuspended in Krebs-Ringer phosphate buffer with 2% dextrose (KRPD) with 2.5% HIPPP. For in vitro studies of rhAPC deactivation, neutrophils were incubated following labeling for 20 minutes at a range of concentrations of rhAPC (10⁻⁴ g/mL to 10⁻¹⁰ g/mL) as previously described. In all experiments neutrophil chemotaxis was tested toward IL-8 (1 nM) in the lower chamber, and nondirectional migration was tested toward KRPD with 2.5% HIPPP. Migration was assayed by fluorescence of cells passing through the microporous membrane on a FLX 800 Microplate Fluorescence Reader with KC Junior Software (Bio-Tek Instruments, Winooski, VT) using excitation/emission wavelengths of 485 nm/530 nm from 2 minutes to 120 minutes at 5-minute intervals at 37°C with constant rotation of the plate. Migration was reported as a chemotaxis index, which is the ratio between the fluorescence intensity of directed and undirected migration through the filters for each sample.

**Protein expression and kinase phosphorylation**

Whole cell extracts from neutrophils were collected from cells denatured in ice-cold lysis buffer (50 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA [ethylene glycol-bis(beta-aminoethyl)ether)-N,N,N',N'-tetraacetic acid], 1 mM Na₃ vanadate, 10 mM Na pyrophosphate, 10 mM NaF, 300 μM p-nitrophenyl phosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, pH 7.3) for 15 minutes. The protein concentration of each sample was assayed using the BCA protein assay kit (Pierce, Rockford, IL) standardized to bovine serum albumin.

For Western blots, 70 μg protein from whole cell extracts was loaded on a 10% Tris-HCl sodium dodecyl sulfate (SDS) polyacrylamide gel. Protein was electrophoresed to a nitrocellulose membrane and then blocked with 5% nonfat dry milk, 20 mM Tris-buffered saline, with 0.1% Tween. After blocking, the membrane was incubated overnight at 4°C with antibodies to phos-p38 MAPK, total p38 MAPK, phos-p42/44 extracellular signal-regulated kinase (ERK) MAPK (1/2), total ERK 1/2 MAPK, phos-Akt, total Akt, or total 5-lipoxygenase (5-LO) using a dilution of 1:1000, followed by horseradish peroxidase–coupled secondary antibody at a dilution of 1:2000. After washing 5 times, bands were detected using chemiluminescence Western blotting detection reagents (ECL Detection System, Amersham-Pharmacia, NJ). Densitometry was performed using chemiluminescence system and analysis software (BioRad, Hercules, CA). Kinase phosphorylation was quantified as a normalized ratio of phosphorylated to total kinase, while total 5-LO was quantified as a normalized ratio to total p38 MAPK, and expressed in arbitrary units.

**Statistical analysis**

Data were analyzed using JMP statistical software (SAS Institute, Cary, NC). Studies of neutrophil chemotaxis were expressed as mean index value plus or minus standard error of the mean (SEM). Significance of the effect of rhAPC administration on neutrophil chemotaxis over time (Figure 7) was determined by 2-way analysis of variance (ANOVA). Log values of leukocyte recovery by BAL (Figure 2) were determined to have a normal distribution, and the significance of the effect of rhAPC administration was determined by Student’s t test. Significance of the effect of rhAPC administration on cytokine and receptor recovery (Figure 4) in BALF, and functional responses of BALF and blood-derived neutrophils (Figures 5 and 6), where a normal distribution was not present, were determined by Wilcoxon unpaired exact test. For all tests, P < .05 was considered significant.

**Results**

There were no differences in baseline parameters, including age or sex, between the groups randomized to receive rhAPC or placebo. No severe or unexpected adverse events occurred in either group, and there were no episodes of bleeding complications associated with rhAPC administration.

**Effects of rhAPC on bronchoalveolar lavage cell and neutrophil counts**

Accumulation of inflammatory cell populations was found in BALF from the pulmonary subsegment where endotoxin was instilled, but not BALF from the contralateral lung. Significantly fewer leukocytes were found in BALF from volunteers treated with rhAPC, as compared with those randomized to placebo (Figure 2A, P = .037). The majority of leukocytes recovered by BALF were neutrophils, thus fewer neutrophils were present in BALF from rhAPC-treated volunteers compared with those receiving placebo (Figure 2B, P = .048). As expected, fewer leukocytes were recovered from the contralateral lobe receiving saline instillation. For the saline-exposed control lobe, 178 146 ± 95 598 WBCs (mean ± SEM) and 18 470 ± 9 710 neutrophils per mL were recovered in subjects administered placebo, while 113 375 ± 45 437 WBCs and 23 150 ± 13 960 neutrophils per mL were recovered from subjects receiving APC (not significantly different). There were no differences in peripheral total white blood cell or neutrophil counts between rhAPC- and placebo-treated subjects (data not shown).

**Lack of effect of rhAPC on pulmonary indices of inflammation**

To assess inflammatory responses, we measured protein concentration in the BALF and screened for the presence of major cytokines, chemokines, and growth factors with an antibody-based protein array. There were no differences in BALF protein concentrations from the endotoxin-exposed lungs between rhAPC- and placebo-treated subjects: 234 ± 40 μg/mL in those given rhAPC and 238 ± 54 μg/mL in the placebo group. We screened BALF samples for the presence of 79 cytokines, chemokines, and growth factors by protein array. Although a number of peptides were present in an equivalent manner in both the saline- and the LPS-treated lobes, only IL-6, IL-8, and MCP-1 were consistently elevated in response to LPS 16 hours following its administration (Figure 3). The presence of rhAPC did not consistently modify the pattern of cytokines and chemokines released into either the LPS- or the saline-treated lung lobes.

**Figure 2. Administration of rhAPC reduces endotoxin-induced leukocyte accumulation to the airspaces.** Leukocytes present in BALF recovered 16 hours after endobronchial LPS-instillation from volunteers who received rhAPC (●) or placebo (○). (A) Total WBCs recovered per mL of lavage fluid. Administration of rhAPC significantly reduced recovery of all leukocytes, P = .037 by Student’s t test. (B) Total neutrophils recovered per mL of lavage fluid. Administration of rhAPC reduced recovery of neutrophils, P = .048 by Student’s t test.
To quantify the effects of rhAPC administration on pulmonary inflammatory responses, levels of the cytokines IL-1β, IL-6, IL-8, IL-10, TNF-α, and MCP-1, as well as of the soluble cytokine receptors and receptor-blocking agents TNF receptor type 1 (TNF RI), TNF receptor type 2 (TNF RII), and the interleukin-1 receptor antagonist (IL-1ra), were assayed in BALF from the pulmonary subsegment into which LPS had been placed and from the contralateral lung of the volunteers treated with rhAPC or saline. No detectible quantity of any of these inflammatory mediators was found in the BALF from the unexposed lung, and no levels of IL-1β or IL-10 were detectible in the endotoxin-exposed lung.

Increased concentrations of IL-6, IL-8, TNF-α, and MCP-1 were found in BALF from pulmonary subsegments exposed to LPS, but no significant differences were present when rhAPC- and saline-treated volunteer groups were compared (Figure 4A). Simi-
Gene expression in pulmonary neutrophils after rhAPC or placebo treatment

High-quality gene expression microarray data were obtained from 9 BAL neutrophil samples in which adequate numbers of cells were obtained for such analysis: 4 samples were from individuals treated with rhAPC, and 5 were from individuals treated with placebo. Comparison of these 2 groups did not reveal a statistically significant gene expression signature attributable to rhAPC administration. Some individual genes did attain a nominal significance in this class comparison: 2 genes are significant at the α = 0.001 level, 32 are significant at the α = 0.01 level (Table 1). However, both sets of nominally significant genes failed to sustain significance upon permutation testing, resulting in an 80% probability that there is no difference between the rhAPC- and placebo-treated groups.

Activated protein C blocks neutrophil migration in vitro

Previous studies had shown that rhAPC inhibited in vitro neutrophil chemotaxis. However, in those experiments, neutrophil migration was measured at a single time point, 30 minutes after establishment of the chemotactic gradient. To more completely characterize the time course for the effects of rhAPC on neutrophil chemotaxis, IL-8–induced migration was monitored over a 2-hour period using neutrophils from healthy humans that had been pretreated with a range of concentrations of rhAPC.

Pretreatment of neutrophils with rhAPC resulted in a significant reduction in IL-8–induced migration in a concentration-dependent manner, while simultaneous analysis of nondirectional migration (NDM) of the neutrophils demonstrated no effect of APC (data not shown). IL-8–induced migration relative to NDM was also reduced by APC in a concentration-dependent manner (Figure 7A). Although decreased chemotaxis was noted in rhAPC–exposed neutrophils 30 minutes after the initiation of the chemotactic gradient, the greatest effect of rhAPC on neutrophil migration was observed from 60 minutes to 120 minutes.

Effect of in vivo rhAPC administration on pulmonary neutrophil migration

BALF neutrophils recovered from individuals treated with rhAPC demonstrated a significant decrease in IL-8–induced chemotaxis relative to NDM when assayed ex vivo (Figure 7B). As with the in vitro studies (Figure 7A), the greatest differences in relative migration among pulmonary neutrophils from the rhAPC- and placebo-treated groups was found at later time points following the initiation of chemotaxis.

Discussion

RhAPC administered in vivo reduced neutrophil accumulation into the airways in response to endobronchial endotoxin administration,
which rhAPC administration reduced leukocyte accumulation in BALF or the recovery of cytokines, chemokines, and intracellular signaling, cytokine release, survival, or apoptosis were detected in circulating neutrophils exposed to rhAPC in vitro.

Chemotaxis toward other agents, but confirmation of the effect of rhAPC does not reduce responses such as bacterial phagocytosis, respiratory burst, or apoptosis.11

The mechanism by which rhAPC reduces neutrophil migration is unknown. However, 30 minutes after establishment of a chemotactic gradient, migration induced by formyl-Met-Leu-Phe (fMLP), C5a, antithrombin (AT), as well as IL-8, was significantly reduced in neutrophils pretreated with rhAPC.11 It was speculated that since rhAPC inhibits migration toward a variety of receptor ligands, apoptosis may be involved.11

The mechanism by which rhAPC blocks neutrophil migration to a number of chemokines in vitro, but does not reduce responses such as bacterial phagocytosis, respiratory burst, or apoptosis.11

Table 1. Gene expression of pulmonary neutrophils from rhAPC- and placebo-treated volunteers

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<th>Gene symbol</th>
<th>Gene title</th>
<th>Gene title</th>
<th>Mean signal</th>
<th>Fold increase, APC relative to placebo</th>
<th>Probeset ID</th>
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*All genes shown are transcripts with a parametric P < .001.

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analyses (Table 1) does not support a consistent effect of rhAPC on neutrophil gene expression. In particular, none of the approximately 80 chemotaxis-related genes (as assigned by Gene Ontology categorization) exhibit differential expression between these groups. However, the relatively small list of significant genes found in these analyses (Table 1) contains at least one gene implicated in lung inflammation. The gene encoding 5-LO had the greatest magnitude of IL-8–induced migration (1 nM gradient) over a 2-hour period in a modified Boyden chamber system. Pretreatment with rhAPC resulted in a significant reduction in IL-8–induced migration over time for all concentrations tested when compared with untreated cells (10^{-6} g/mL, P < .0001; 10^{-5} g/mL, P < .0001; 10^{-6} g/mL, P < .0001; 10^{-5} g/mL, P = .01; by 2-way ANOVA). (B) Effect of rhAPC on neutrophil chemotaxis in vivo. Neutrophils isolated from blood of healthy volunteers were exposed to rhAPC (●, 10^{-4} g/mL; ○, 10^{-5} g/mL; △, 10^{-6} g/mL; ○, left untreated (☐) for 20 minutes. Plot depicts mean chemotaxis index (n = 5) of IL-8–induced migration (1 nM gradient) over a 2-hour period in a modified Boyden chamber system. Pretreatment with rhAPC resulted in a significant reduction in IL-8–induced migration over time for all concentrations tested when compared with untreated cells (10^{-6} g/mL, P < .0001; 10^{-5} g/mL, P < .0001; 10^{-6} g/mL, P < .0001; 10^{-5} g/mL, P = .01; by 2-way ANOVA).

The present results, showing that rhAPC decreases neutrophil chemotaxis and accumulation in a relevant in vivo human model of acute pulmonary inflammation, provide important insights into a potential mechanism by which rhAPC decreases organ injury and mortality in sepsis. Although inflammation induced by endobronchial instillation of LPS is less severe, of shorter duration, and less “complex” than the proinflammatory stimuli encountered in the setting of sepsis or pneumonia, no anti-inflammatory effects of rhAPC were found in this study. A similar lack of anti-inflammatory properties was demonstrated when rhAPC was administered to endotoxemic humans. Minimal effects of rhAPC on circulating cytokine levels were found in the PROWESS (Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis) study, in which rhAPC reduced mortality from severe sepsis. Such findings suggest that beneficial effects of rhAPC in severe infection may result from directly inhibiting neutrophil migration, thereby reducing the injurious effects associated with excessive accumulation of activated neutrophils into the lungs and other organs. The specificity of this action, independent of detectable modulation of other neutrophil functions or inflammatory responses, has the potential to be a very selective intervention to reduce overly exuberant neutrophil recruitment without inducing immunosuppression.

References


Recombinant human activated protein C reduces human endotoxin-induced pulmonary inflammation via inhibition of neutrophil chemotaxis

Jerry A. Nick, Christopher D. Coldren, Mark W. Geraci, Katie R. Poch, Brian W. Fouty, James O’Brien, Michael Gruber, Simona Zarini, Robert C. Murphy, Katherine Kuhn, Don Richter, Kelly R. Kast and Edward Abraham