α₁-Antitrypsin, Old Dog, New Tricks

α₁-ANTITRYPSIN EXERTS IN VITRO ANTI-INFLAMMATORY ACTIVITY IN HUMAN MONOCYTES BY ELEVATING cAMP

Regulation of serine protease activity is considered to be the sole mechanism for the function of α₁-antitrypsin (AAT). However, recent reports of the anti-inflammatory effects of AAT are hard to reconcile with this classical mechanism. We discovered that two key activities of AAT in vitro, namely inhibition of endotoxin-stimulated tumor necrosis factor-α and enhancement of interleukin-10 in human monocytes, are mediated by an elevation of cAMP and activation of cAMP-dependent protein kinase A. As expected with this type of mechanism, the AAT-mediated rise in cAMP and the impact on endotoxin-stimulated kinase A. Elevation of cAMP and activation of cAMP-dependent protein kinase A.}

Regulation of proteolytic activity by endogenous anti-proteases represents a major mechanism limiting host tissue destruction at the sites of inflammation. α₁-Antitrypsin (AAT), the major circulating serine protease inhibitor, was first isolated in 1955 and was so named because of its ability to inhibit trypsin. It is now recognized that AAT, also known as α₁-protease inhibitor, is a potent inhibitor of multiple serine proteases with particularly high activity toward the neutrophil serine proteases, neutrophil elastase, and proteinase-3. Most of the circulating AAT is synthesized by the liver and is released rapidly during the acute phase response to inflammation or infection. Alterations of the AAT molecule that compromise its structure and/or secretion and thereby lead to AAT deficiency are known to predispose the individual to diseases. Clinical expressions of AAT deficiency can be seen in the lung, liver, and the skin, with considerable variability in the severity of disease. In fact, AAT deficiency is the only known genetic risk factor for the development of chronic obstructive pulmonary disease, a chronic inflammatory lung disease characterized by progressive proteolytic destruction of the lung.

Although it is generally assumed that the anti-inflammatory effects of AAT are mediated by its anti-protease activity, recent data suggest that other mechanisms may be involved. Our own in vitro studies, using monocytes stimulated with lipopolysaccharide (LPS), have demonstrated inhibition of TNFα production by native AAT and by AAT chemically modified to abolish its protease inhibitor activity. Furthermore, we have shown that both native and modified forms of AAT enhance LPS-stimulated IL-10 generation. The data generated with IL-10 was important because it inferred a specific mechanism for the effects of AAT rather than a general depressive effect of AAT on cell function.

The aforementioned studies raised the important question as to how AAT might exhibit anti-inflammatory activity independent of protease inhibitor activity. Because the effects of AAT on LPS-stimulated monocytes TNFα and IL-10 were similar to those reported for PDE4 inhibitors and receptor agonists such as PGF2, we hypothesized that AAT may mediate its anti-inflammatory activity through elevation of cAMP. cAMP is ubiquitously found in all mammalian cells and plays a key role in the regulation of many cellular functions. Classically, cAMP is thought to exert the majority of its intracellular effects by binding and activating cAMP-dependent PKA, thereby controlling the phosphorylation status and activity of multiple intracellular substrates. In inflammatory cells, elevation of cellular cAMP either through activation of multiple membrane receptors or inhibition of cAMP catabolism results in inhibition of LPS-stimulated cytokine and chemokine release and leukocyte recruitment and proliferation. This activity forms the mechanistic basis for the action of a number of new generation anti-inflammatory drugs.

Here we report that AAT, independently of its proteinase inhibitor activity, increases cAMP levels in monocytes and thereby exerts its anti-inflammatory effects in a model of LPS-mediated inflammation in vitro.

**EXPERIMENTAL PROCEDURES**

**AAT Preparations**—The α₁-antitrypsin (human) Prolastin® (lot 26N3PT2) was donated by Bayer Corp. (Elkhart, IN). The vial of Prolastin® contained 1059 mg of functionally active AAT, as determined by its capacity to inhibit porcine pancreatic elastase. Prolastin® was dissolved in sterile water provided by the manufacturer for injections and stored at 4 °C. Purified human AAT were obtained from Sigma and Calbiochem.

© 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
mutant), 10–50 μM calcium and magnesium. Fresh medium was added, and cells removed by washing three times with PBS supplemented with HEPE (Invitrogen). After 1 h, 15 min, nonadherent cells were collected and counted with a hemocytometer. The purity of the AAT preparations was determined according to the Bradford method (31). Various AAT preparations were tested in all the experimental models, with the objective of demonstrating that our results are not dependent on the specific properties of one AAT preparation.

Noninhibitory Forms of AAT—Temperature-inactivated AAT was produced by incubation at 60 °C for 10 h. The AAT was oxidized by N-chlorosuccinimide (Sigma) in a 25 M excess in a 0.1 M Tris-HCl buffer, pH 8. The buffer was changed to PBS using a centrifugal micro-concentrator (Centrifuge YM30, Millipore, MA). The temperature-inactivated and -oxidized AATs were tested for their ability to form complexes with pancreatic elastase (EC 3.4.21.36) (Sigma) and to inhibit elastase activity. Samples of inactivated or native AAT were incubated with pancreatic elastase at a 1:2:1 molar ratio for 15 and 30 min, respectively. The reaction was stopped by adding SDS sample buffer, and the mixtures were analyzed using 7.5% SDS-PAGE and stained with Coomassie Blue. Temperature-inactivated and oxidized AAT did not form any complexes with elastase.

Elastase inhibitory activity was assessed spectrophotometrically (spectrophotometer DU 600; Beckman Instruments). In brief, native, oxidized, or polymerized AAT was incubated with pancreatic elastase at a molar ratio of 1:2:1 for 5 min at room temperature in 0.1 M Tris buffer, pH 8. After addition of 25 μl of chromogenic elastase substrate (succinyl-(Ala)3-p-nitroanilide, 1 mg/ml stock solution) to give a total sample volume 300 μl, absorbance was measured at 405 nm for 280 s. The absorbance values used for the calculation of elastase inhibition by AATs were corrected with blanks for buffer plus substrate. Temperature-inactivated and -oxidized AAT had no inhibitory activity.

Monocyte Isolation—Human blood monocytes were isolated fromuffy coats (total blood was obtained from 65 donors in this study) using Ficoll-Paque PLUS (Amersham Biosciences). Briefly,uffy coats were diluted 1:2 in PBS with addition of 10 mM EDTA and layered on Ficoll. After centrifugation at 400 × g for 35 min at room temperature, the cells in the interface were collected and washed three times in PBS/EDTA. Cell purity and amounts were determined in an Autocounter AC900EO cell counter (Swelabs Instruments AB, Sweden). The granulocyte fractions were less than 5%. Cells were seeded at a concentration of 5 × 10⁶ cells/ml in RPMI 1640 medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 1 × non-essential amino acids, 2 mM sodium pyruvate, and 20 mM HEPES (Invitrogen). After 1 h, 15 min, nonadherent cells were removed by washing three times with PBS supplemented with calcium and magnesium. Fresh medium was added, and cells were stimulated with lipopolysaccharide (LPS, 10 ng/ml, J5 Rc mutant), 10–50 μM rolipram, 10–50 μM forskolin (Sigma) or predetermined concentrations of AAT, separately and in combination for 30 min and 1 and 18 h at 37 °C, 5% CO₂.

Neutrophil Isolation—Human neutrophils were isolated from the peripheral blood of healthy volunteers using Polymorph Prep™ (Axis-Shield PoC AS, Oslo, Norway) as recommended by the manufacturer. In brief, 25 ml of heparin anti-coagulated blood was gently layered over the 12.5 ml of Polymorph Prep™ and centrifuged at 1600 rpm for 35 min. Neutrophils were harvested as a low band of the sample/medium interface and washed with PBS, and residual erythrocytes were subjected to hypotonic lysis. The neutrophil purity was more than 95% as determined on an AutoCounter AC900EO.

Cytokine Assays—Cell culture supernatants from monocytes treated with LPS alone or in combination with AAT were analyzed to determine TNFα and IL-10 levels by using DuploSet enzyme-linked immunosorbent assay sets (R&D Systems; detection levels 15.6 and 31.2 pg/ml, respectively).

Quantitative Real Time Reverse Transcription-PCR Analysis—500 ng of total RNA was used for cDNA synthesis with the high capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Real time PCR was performed on cDNA corresponding to 25 ng of total RNA in a 7900HT fast real time PCR system (Applied Biosystems) in a total volume of 25 μl. Samples were diluted 25 times for 18 S RNA analysis. IL-10 primers were designed to span an intron, and melting curve analysis was performed on the 7900 HT instrument after the run to make sure that the signals were generated from cDNA and not genomic DNA. IL-10 and 18 S RNA transcript levels were analyzed using the SYBR® Green PCR master mix (Applied Biosystems) with the following primers: IL-10 forward, GGAGAACCCTG-AAGACCTCATA and reverse, TGCTCTTGTTTTCACAGGGA; 18 S RNA forward, CCGTACCACATCACAAGGAA, and reverse, GCTGGAATTACCGCGGCTTA. Taqman® gene expression assay HS00174128_m1 was used to quantify TNFα mRNA levels according to the standard protocol (Applied Biosystems, Foster City, CA). Fold expression values were calculated versus the sample with the lowest levels of each transcript using the ΔΔCt method after normalization to the internal control 18 S RNA.

Total Cellular cAMP Assay—Total cAMP levels in monocytes (1 × 10⁷ cells/ml) alone, treated with various concentrations of rolipram, forskolin, AAT (0.1–4 mg/ml), or LPS (10 ng/ml) alone, or pretreated with LPS, 10 μM rolipram, or 30 μM forskolin for 1 h following exposure to AAT were determined by the cAMP Direct Biotrak scintillation proximity assay system according to the manufacturer’s recommendations (Amersham Biosciences). Briefly, cells alone or with stimulating agents were incubated for the pre-determined time periods and lysed for 5 min. The antiserum, tracer, and scintillation proximity assay were reconstituted with lysis reagent and mixed with the analyzed sample. The antibody-bound cAMP reacts with the scintillation proximity assay reagent that contains anti-rabbit second antibody bound to fluoromicrospheres. Any 125I cAMP that is bound to the primary rabbit antibody will be immobilized on the fluorosphere, which will produce light. Measurement in a β-scintillation counter enables the amount of fluorosphere-bound labeled cAMP to be calculated.
α₁-Antitrypsin Induces cAMP in Monocytes

Concentration of unlabeled cAMP in a sample is then determined from a standard curve.

Electrophoresis and Western Blot Analysis—Monocytes were incubated alone, with 30 μM forskolin, or 0.5 mg/ml AAT separately or in combination with 10 μM H89 for different time periods and lysed, and protein was determined using the Bradford method. Equal amounts of analyzed protein were subjected to 10% SDS-polyacrylamide gel. Proteins were transferred to a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA) using a semi-dry blot electrophoretic transfer system. Western blot analysis was performed using rabbit polyclonal anti-PKA catalytic α/β (pT197) phosphospecific antibodies (BioSource International, Inc.) and polyclonal goat anti-actin (I-19) antibodies (Santa Cruz Biotechnology). The immunocomplexes were visualized with secondary horseradish peroxidase-conjugated rabbit anti-mouse antibodies (1:10,000) (DAKO, A/S, Denmark) and developed using the ECL Western blot analysis system (Amersham Biosciences).

Phosphodiesterase Type 4 (PDE4)-specific Enzymatic Activity Determination—PDE4 activity in the absence and in the presence of 10 μM rolipram (positive control) or 0.5, 1, and 2 mg/ml AAT was determined using the PDE4 enzymatic assay kit (Fab-Gennix Inc). Ten μl (7.5 μg of protein) of PDE4 enzyme supplied with the kit was incubated with rolipram or various concentrations of AAT at 30 °C for 5 min. The enzyme was incubated in buffer only for total PDE4 activity measure. Freshly prepared PDE-buffered substrate containing 15 μl of [2,8-3H]cAMP ammonium salt (25–40 Ci/mmol, PerkinElmer Life Sciences) was added to the reaction tube and incubated for 10 min at 30 °C with shaking. The reaction was terminated by transfer of the tubes to a 100 °C water bath for 3 min. According to the manufacturer’s protocol, the cAMP is hydrolyzed by PDE activity into the noncyclic form. The PDE4 enzymatic activity is directly proportional to the adenosine formed. Separation of adenosine from AMP and cAMP was performed according to kit recommendations. The PDE4 activity was calculated by subtracting non-PDE4 activity from total PDE activity.

PKA Activity Assay—Monocytes (1 × 10⁷ cells/treatment) alone and treated for 1 h with 0.5 mg/ml AAT or 50 μM forskolin, an activator of PKA (32), were harvested, washed with PBS, and suspended in 0.5 ml of cold extraction buffer (25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Cells were homogenized using cold homogenizer, centrifuged for 5 min at 14,000 × g at 4 °C, and assayed for PKA using the protein kinase A assay kit (Calbiochem) according to the manufacturer’s recommendations. [32P]ATP (specific activity 25–40 Ci/mmol) was obtained from PerkinElmer Life Sciences. The PKA reaction mixture contained 0.2 μCi/μl of [32P]ATP. One unit of activity is defined as the amount of PKA that catalyzed the incorporation of 1 pmol of phosphate from ATP into Kemptide/min/mg protein at 30 °C and pH 7.2.

To confirm our findings we also used a nonradioactive PKA assay kit based on enzyme-linked immunosorbent assay that utilizes a synthetic pseudosubstrate and a monoclonal antibody that recognizes the phosphorylated form of the peptide (Calbiochem).

Statistical Analysis—The Statistical Package (SPSS for Windows, release 13.0) was used for the statistical calculations. The differences in the means of the experimental results were analyzed for their statistical significance with the one-way analysis of variance with an overall significance level of α = 0.05. The independent two-sample t test was also used.

RESULTS

Time-dependent Effects of AAT and AAT/LPS on TNFα and IL-10 Release—TNFα and IL-10 protein and mRNA were measured in monocytes 2, 6, and 18 h after exposure to LPS and AAT, either alone or in combination. At 2 h, both LPS and AAT induced TNFα protein, albeit at very modest levels, whereas AAT alone induced IL-10. Exposure of monocytes to LPS (10 ng/ml) and AAT (0.5 mg/ml) in combination, however, resulted in a more than additive increase in TNFα compared with either agent alone. No significant difference was found between effects of AAT alone and AAT and LPS in combination on IL-10 at 2 h (Fig. 1A). Thus, the effects of combinations of LPS and AAT at 2 h on TNFα protein appeared to be synergistic, whereas those on IL-10 reflected substantially an effect of AAT alone. At 6 h, combination of AAT with LPS resulted in significant inhibition of TNFα protein, whereas IL-10 levels appeared to be enhanced in an additive manner. At 18 h, inhibition of LPS-stimulated TNFα protein by AAT was further enhanced, whereas dramatic increase of IL-10 release was observed.

To explain further these complex changes in protein levels, we also performed mRNA analysis. At 2 h, we observed a remarkable increase in TNFα mRNA in response to combinations of AAT and LPS with moderate effects of both mediators alone. At 6 and 18 h, there was clear inhibition of LPS-stimulated TNFα RNA by AAT. For IL-10 we observed an increase in mRNA for combinations of AAT and LPS at 2 and 6 h. However, the changes in IL-10 mRNA did not mirror those in protein changes because significant IL-10 protein was observed at 2 h with AAT alone in the absence of detectable mRNA synthesis. The effects of AAT and LPS on IL-10 mRNA levels were transient with lower IL-10 mRNA levels in cells treated with LPS and AAT at 18 h than with LPS alone at this time point (Fig. 1B).

AAT Elevates Total Cellular cAMP Levels in LPS-treated Monocytes—Monocytes stimulated with LPS alone for up to 1 h showed no detectable rise in cAMP levels (2.6 ± 0.4 pmol/10⁷ cells in untreated monocytes (n = 10) and 2.45 ± 0.19 pmol/10⁷ in cells pretreated with LPS for 1 h (n = 8)). However, when monocytes were pretreated with LPS for 1 h followed by addition of AAT (0.1–4 mg/ml) for 2 min, cAMP levels increased significantly (82–190%, p < 0.001) compared with controls (Fig. 2A). Furthermore, when monocytes were pretreated with LPS (10 ng/ml, 1 h), the addition of constant concentration of AAT (0.5 mg/ml) at various time points yielded a rapid up-regulation of total cAMP that peaked after 2 min (Fig. 2B).
with control. Maximal cAMP responses to AAT occurred following incubation of cells with 1 mg/ml AAT for 2 min (2.6 ± 0.4 pmol/10^7 cells in untreated monocytes (n = 10 experiments) and 6.45 ± 1.2 pmol/10^7 in cells treated with AAT for 2 min (n = 8 experiments), p < 0.001). AAT was then rendered inactive as a proteinase (verified with neutrophil elastase inhibitor) by either oxidation with N-chlorosuccimide or heating to 60 °C for 10 h and tested for its ability to elevate cAMP. As shown in Fig. 3, both oxidized and polymerized forms of AAT triggered cAMP elevation in monocytes with a similar magni-

**FIGURE 1.** Release (A) and mRNA expression (B) of TNFα and IL-10 by human monocytes in response to exposure for 2, 6, and 18 h to LPS (10 ng/ml) in the presence or absence of AAT (0.5 mg/ml). Each bar represents a mean of four independent experiments ± S.E. Each curve is the mean value of two independent experiments.
tude of response. When human serum albumin was used as a negative control in this experimental model, no effects on cAMP levels were observed (data not shown). Moreover, when secretory leukocyte protease inhibitor (SLPI), another serine protease inhibitor with anti-inflammatory activities, was included for comparison, no effect on cAMP levels was observed (3.2 ± 0.54 pmol/10⁷ cells in untreated monocytes and 3.8 ± 0.6 pmol/10⁷ cells in cells treated with 1 mg/ml SLPI for 2 min (n = 3)). SLPI was further tested at a range of concentrations with cAMP measurements at various time points up to 30 min with no significant effect (data not shown). In addition, to examine whether the effects of AAT on cAMP were cell-specific, the influence of AAT on neutrophil cAMP levels was determined. Under the same experimental conditions, AAT had no effect on neutrophil cAMP levels (data not shown).

The Effects of AAT on Cellular cAMP Levels Are Mediated by Activation of Adenylate Cyclase—An important question regarding the AAT-stimulated rise in monocyte cAMP was whether it was because of effects on cAMP synthesis or catabolism. Forskolin and rolipram are substances that increase the concentrations of cellular cAMP by either direct activation of adenylate cyclase or by inhibition of PDE4, a major cAMP-catabolizing enzyme (33, 34). We therefore examined the effects of AAT on cAMP levels in combination with forskolin or rolipram by reasoning that a PDE4 inhibitor would enhance the cAMP response to an agent that induces its synthesis but not by an agent that prevents its catabolism, whereas a maximum response to forskolin might be enhanced by inhibition of cAMP catabolism but not by an inducer of adenylate cyclase activity.

Monocytes were treated with either 30 μM forskolin or 10 μM rolipram either alone or in combination with AAT before measuring intracellular cAMP levels. As illustrated in Fig. 4A, AAT (0.5 mg/ml) alone induced an ∼3-fold increase in total cAMP levels at 2 min, whereas pretreatment of monocytes with forskolin resulted in an ∼2-fold increase in total cAMP levels. In combination, the effects of forskolin and AAT on cAMP were not additive. This suggests that maximum activation of
adenylate cyclase had occurred with AAT. By contrast, pretreatment of the monocytes with rolipram alone resulted in an 87% \((p < 0.01)\) increase in cAMP level elevation and caused a significant augmentation in cAMP levels in response to AAT (52% increase, \(p < 0.01, n = 3\) experiments) compared with rolipram alone (Fig. 4B). Similarly, rolipram increased the forskolin effects on cAMP elevation (from 6.4 ± 0.8 pmol/10^7 cells in forskolin-treated monocytes to 9.45 ± 1.2 pmol/10^7 in cells treated with forskolin and rolipram in combination \((n = 3\) experiments), \(p < 0.01\)) (data not shown).

To further support the contention that the effects of AAT were due to activation of adenylate cyclase rather than inhibition of cAMP catabolism, we evaluated the effects of SQ22536, an inhibitor of adenylate cyclase, on AAT-stimulated cAMP. Preincubation of monocytes with 25 \(\mu\)M SQ22536 for 45 min almost totally inhibited the ability of AAT to induce a rise in monocyte cAMP (Fig. 5). This finding together with the data obtained with rolipram and the failure of AAT to directly inhibit purified PDE4 \textit{in vitro} (in contrast to 10 \(\mu\)M rolipram, which inhibited activity 86%, \(p < 0.001\) \((n = 3\) experiments)) strongly suggested that the effects of AAT on monocyte cAMP levels were primarily because of activation of adenylate cyclase.

\(\alpha_1\)-Antitrypsin Induces cAMP in Monocytes

\(\alpha_1\)-Antitrypsin (AAT) activates adenylate cyclase in monocytes, leading to an increase in cAMP levels. Pretreatment with AAT results in a significant elevation of cAMP compared to untreated cells. Rolipram, a cyclase activator, also increases cAMP levels, indicating that the effects of AAT on cAMP are due to activation of the cyclase rather than inhibition of cAMP catabolism.

![Figure 3: Comparisons of the effects of native (nAAT), temperature-inactivated (pAAT), and oxidized (oxAAT) (0.5 mg/ml) on cAMP rise at 2 min.

![Figure 4: AAT effects on induced cAMP rise in monocytes pretreated with forskolin (A) and rolipram (B).](image)

![Figure 5: Inhibitory effect of SQ22536 on the ability of AAT to raise cAMP.](image)

AAT Stimulates the Downstream Activation of cAMP-Dependent Protein Kinase A

AAT stimulates the downstream activation of cAMP-dependent protein kinase A (PKA), confirming the activation of adenylate cyclase. The increase in cAMP levels is consistent with the activation of PKA, which is involved in various cellular processes such as gene expression and cell growth.
(Fig. 6A) and protein levels (Fig. 6C). However, of note was that both AAT and forskolin alone induced a similar level of activation of PKA over 1 h (Fig. 5) despite very different temporal increases in cAMP.

The in Vitro Anti-inflammatory Activity of AAT on LPS-stimulated Monocytes Is Mediated by an Elevation in cAMP—To link the rise in cellular cAMP to the anti-inflammatory effects of AAT on TNFα and IL-10, we evaluated the effects of AAT, rolipram, and forskolin, either alone or in combination, on LPS-stimulated monocyte activation after 18 h. In accordance with the data obtained for cAMP, the inhibitory effects of AAT on LPS-induced TNFα release were significantly enhanced when added in combination with rolipram. Furthermore, rolipram also enhanced the release of LPS-induced IL-10 release by AAT (73% \( p < 0.001 \)) (Fig. 7, A and B). No significant changes in effects of AAT on LPS-induced TNFα release was observed in combination with forskolin (Fig. 8), and forskolin did not enhance the effects of AAT on LPS-stimulated IL-10 release (data not shown).

**DISCUSSION**

LPS (endotoxin) from Gram-negative bacteria induces monocyte/macrophage production of both pro-inflammatory cytokines, in particular TNFα, and anti-inflammatory cytokines, including IL-10. However, the overwhelming balance of LPS activity favors a pro-inflammatory response (35). The result in a clinical setting can be systemic inflammatory response often accompanied by severe tissue injury (36, 37). We and other investigators have reported that AAT, an endogenous inhibitor of serine proteases, may inhibit LPS-induced pro-inflammatory responses in vitro and in vivo by mechanisms that appear to be independent of inhibition of serine proteases (14, 38–40).

In this study we have explored the mechanism by which AAT modulates monocyte responses to LPS in vitro. We initially analyzed short term monocyte responses to LPS and AAT separately or in combination. As soon as 2 h after treatment, AAT

![FIGURE 6. AAT- and forskolin-mediated PKA activation in monocytes.](image)

**FIGURE 6.** AAT- and forskolin-mediated PKA activation in monocytes. A, monocytes were stimulated with AAT (0.5 mg/ml) and forskolin (50 μM) alone or in combination with and without adding H89 (20 μM) for 1 h. PKA activity was assayed as described under "Experimental Procedures." Bars represent mean ± S.E. from three separate experiments. ***, \( p < 0.001 \). B, Western blot shows representative experiment out of three performed. Blot with anti-actin antibodies was used as a protein loading control. C, Western blot shows representative experiment out of two performed. Blot with anti-actin antibodies was used as a protein loading control.

![FIGURE 7. Additive effects of AAT and rolipram on LPS-induced TNFα (A) and IL-10 release (B).](image)
induced IL-10 protein release. The discrepancy between mRNA and protein levels at this early time point appear to exclude effects on de novo protein synthesis and perhaps reflect release of pre-stored IL-10. However, at 2 and 6 h, a combination of AAT and LPS clearly increased IL-10 mRNA, and this may have been responsible for the large increase in IL-10 protein at 18 h if it were sustained. Paradoxically, perhaps at 18 h IL-10 mRNA levels returned to base line suggesting that a fairly dramatic fall in mRNA synthesis had occurred between 6 and 18 h. The mechanism behind this is unknown but may include autocrine modulation of IL-10 mRNA synthesis by IL-10 protein (41) and/or an increase in mRNA instability (42). At 2, 6, and 18 h there appeared to be a good correlation between TNFα mRNA and protein levels suggestive of effects on de novo TNFα synthesis. We are unable to explain the relevance of the short term increases in TNFα protein at 2 h by combinations of LPS and AAT, although they reflect a magnitude of response only 10% of the 18-h response to LPS alone. In the long term, the effects of AAT on the LPS-stimulated monocyte activation are predominantly anti-inflammatory with reduction of TNFα and enhancement of IL-10.

It has long been recognized that modulation of cellular cAMP can have a profound effect on leukocyte function (43). Cellular cAMP levels may be tightly regulated by synthesis through activation of adenylate cyclase or hydrolysis by cAMP phosphodiesterases (e.g. PDE4) (44).

Elevation of leukocyte cAMP is generally inhibitory in terms of pro-inflammatory cellular signaling. For example, exogenous cell-permeable cAMP analogues (e.g. dibutyryl cyclic AMP), inhibitors of PDE4 (e.g. rolipram), receptor agonists mediating the activation of adenylate cyclase (e.g. prostaglandin E2), and direct activator of adenylate cyclase (forskolin) are able to reduce the release of TNFα and enhance the release of the anti-inflammatory cytokine IL-10 in response to LPS (45–49).

Because we had observed similar functional activity by AAT on LPS-stimulated TNFα and IL-10 (14), we sought to determine whether AAT was operating by a similar mechanism, namely elevation of cellular cAMP.

Initial studies confirmed up-regulation of cAMP by AAT and to our surprise indicated that AAT was more efficacious than forskolin, a direct activator of adenylate cyclase. When we then showed that modulation of the cAMP response with rolipram and the adenylate cyclase inhibitor SQ23356 resulted in the expected enhancement and inhibition, respectively, of the cAMP response. In functional terms we demonstrated that the effects of AAT on cAMP- and LPS-stimulated TNFα and IL-10 release were enhanced by rolipram, also consistent with a mechanism involving the activation of adenylate cyclase by AAT rather than inhibition of PDE4.

Finally, we confirmed that the classical downstream signaling pathway for cAMP activation of PKA was responsible for mediating the effects of AAT. We found that despite very different time courses of cAMP accumulation, i.e. rapid for AAT but delayed for forskolin (50), the effects of both AAT and forskolin on PKA activation, the likely downstream effector of elevated cellular cAMP, were similar at 1 h. In this study we have not been able to identify the mechanism by which AAT actually activates adenylate cyclase. Although high affinity binding of AAT to the surface of cells has been reported (51), the receptor responsible remains elusive as do the immediate receptor proximal pathways. Interestingly, however, the ability of AAT to elevate cAMP was found to be cell-specific for monocytes, because AAT has no effect on neutrophil cAMP. A significant finding in this study was that AAT was able to elevate cAMP levels independently on its protease-inhibitory activity. Thus both oxidized and heat-inactivated forms of AAT, which lack inhibitory activity, caused elevation of cAMP levels similar to that of native AAT. We have shown previously that both forms are also able to modulate LPS-stimulated TNFα and IL-10 (14). This finding is exciting in that it confirms a mechanism by which physiologically modified forms of AAT, which are inactive as serine protease inhibitors and are presumed to have no anti-inflammatory activity, may still play an important and protective role at sites of inflammation. It is intriguing that a single endogenous protein can express complementary anti-inflammatory activity by two mechanisms, namely elevation of IL-10 and inhibition of TNFα. Although IL-10 has been reported to inhibit TNFα directly, previous data from this laboratory data suggest that autocrine inhibition of TNFα by IL-10 is not the mechanism responsible for the inhibitory effects of AAT (14).

Thus, although IL-10 antibody alone can enhance LPS-stimulated TNFα production by monocytes, suggestive of an autocrine modulation by IL-10, the inhibitory effects of AAT are similar whether IL-10 antibody is present or not (data not shown). Our findings are in accord with Seldon et al. (52) who showed that agents that elevate cAMP and inhibit TNFα in monocytes do so by a mechanism independent of induction of IL-10.

As a serine protease inhibitor AAT can block much of the destructive proteolytic activity from activated neutrophils, but as an elevator of cAMP, AAT may also block the accumulation and activation of monocytes by modulating the production of pro- and anti-inflammatory cytokines. It is worthwhile
noting that the pluripotential anti-inflammatory effects of AAT are not unique to this serine protease inhibitor. SLPI, for example, has also been reported to exert anti-inflammatory effects independent of inhibition of serine proteases (53), and antithrombin III has been shown to inhibit TNFα stimulation of E-selectin expression in endothelial cells. Like AAT, the effects of antithrombin III were because of elevation of cAMP (54). In this study we have shown that in contrast to AAT, SLPI increases neutrophil but not monocyte cAMP suggesting that the proximal signaling mechanisms for SLPI and AAT differ and thereby confer cellular specificity of anti-inflammatory activity.

In vivo, it is unclear which activity of AAT is most significant in suppressing inflammation. The protective role of AAT in smoke-induced emphysema is classically associated with a maintenance of a protease anti-protease balance (39, 55). However, AAT also reduces bacterial endotoxin and TNFα-induced lethality in vivo (11). In man, Prolastin® therapy has been shown to reduce LTB4 levels in patients with AAT-deficient emphysema (56, 57). Comparative studies in vivo looking at the efficacy of native and modified forms of AAT and low molecular weight serine protease inhibitors may help address the question regarding the dominant anti-inflammatory mechanism of AAT in vivo. An important question regarding our findings in isolated blood monocytes is whether they can be extended to effects on blood monocytes or resident and/or newly recruited tissue monocytes/macrophages, i.e. are the effects we see in vitro relevant to the in vivo situation or just an artifact of the way we have prepared the cells in vitro.

We have not performed such studies because of the notorious difficulty in working with a cell with the relevant phenotype. Therefore, studies on macrophages isolated from patients with inflammatory disease are necessary. However, an intriguing finding by Osawa et al. (58) that macrophages stimulated with LPS undergo a sensitization of their cAMP response to exogenous agonists suggests that macrophage-like cells may be potentially more sensitive to the inhibitory effects of AAT. In summary, our studies suggest a mechanism by which AAT may express anti-inflammatory activity in vitro, namely via elevation of cellular cAMP. These novel findings suggest that the effects of AAT in vivo are not simply related to modulation of serine protease activity but that more complex cAMP-regulated inflammatory mechanisms may be involved.

Acknowledgment—We thank Dr. Robert Virtala for helping with reverse transcription-PCR analysis.

REFERENCES

1. Turino, G. M., Seniormir Garg, B. D., Keller, S., Levi, M. M., and Mandl, I. (1969) Science 165, 709–711
2. Janciuksiene, S. (2001) Biochim. Biophys. Acta 1535, 221–235
3. Travis, J., Shieh, B. H., and Potempa, J. (1988) Tokai J. Exp. Clin. Med. 13, 313–320
4. Potempa, J., Korzus, E., and Travis, J. (1994) J. Biol. Chem. 269, 15957–15960
5. Gettins, P. G. (2002) Chem. Rev. 102, 4751–4804
6. Mahadeva, R., and Lomas, D. A. (1998) Thorax 53, 501–505
7. Lomas, D. A., and Carrell, R. W. (2002) Nat. Rev. Genet. 3, 759–768
8. Carrell, R. W., and Lomas, D. A. (2002) N. Engl. J. Med. 346, 45–53
9. Hutchison, D. C. (1988) Am. J. Med. 84, 3–12
10. Eriksson, S. (1999) J. Hepatol. 30, Suppl. 1, 34–39
11. Libert, C., Van Molle, W., Brouckaert, P., and Fiers, W. (1996) J. Immunol. 157, 5126–5129
12. Arora, P. K., Miller, H. C., and Aronson, L. D. (1978) Nature 274, 589–590
13. Knappstein, S., Ide, T., Schmidt, M. A., and Heusipp, G. (2004) Infect. Immun. 72, 4344–4350
14. Janciuksiene, S., Larsson, S., Larsson, P., Virtala, R., Jansson, L., and Stevens, T. (2004) Biochem. Biophys. Res. Commun. 321, 592–600
15. Churg, A., Wang, R. D., Tai, H., Wang, X., Xie, C., Dai, J., Shapiro, S. D., and Wright, J. L. (2003) Am. J. Respir. Crit. Care Med. 167, 1083–1089
16. Petracek, I., Fijalkowska, I., Zhen, L., Medler, T. R., Brown, E., Cruz, P., Choe, K. H., Taraseviciene-Stewart, L., Scerbavicius, R., Shapiro, L., Zhang, B., Song, S., Hicklin, D., Voelkel, N. F., Flotte, T., and Tuder, R. M. (2006) Am. J. Respir. Crit. Care Med. 173, 1222–1228
17. Martorana, P. A., Beume, R., Lucattelli, M., Wollin, L., and Lungarella, G. (2005) Am. J. Respir. Crit. Care Med. 172, 848–853
18. Nicosia, S., and Patrono, C. (1989) FASEB J. 3, 1941–1948
19. Chung, K. F. (2005) Sci. STKE 2005, PE47
20. Houslay, M. D. (1998) Semin. Cell Dev. Biol. 9, 161–167
21. Tasken, K., and Aandahl, E. M. (2004) Physiol. Rev. 84, 137–167
22. Mei, F. C., Qiao, J., Tsygankova, O. M., Meinikoth, J. L., Quilliam, L. A. and Cheng, X. (2002) J. Biol. Chem. 277, 11497–11504
23. Yoshimura, T., Kurita, C., Nagao, T., Usami, E., Nakao, T., Watanabe, S., Kobayashi, J., Yamazaki, F., Tanaka, H., and Nagai, H. (1997) Gen. Pharmacol. 29, 633–638
24. Seldon, P. M., Barnes, P. J., Meja, K., and Giembycz, M. A. (1995) Mol. Pharmacol. 48, 747–757
25. Guha, M., and Mackank, N. (2001) Cell. Signal. 13, 85–94
26. Bryce, P. J., Dascombe, M. J., and Hutchinson, I. V. (1999) Immunopharmacology 41, 139–146
27. Barnette, M. S., and Underwood, D. C. (2000) Curr. Opin. Pulm. Med. 6, 164–169
28. Gao, Y., and Raj, J. U. (2001) Eur. J. Pharmacol. 418, 111–116
29. Banner, K. H., and Trevethick, M. A. (2004) Trends Pharmacol. Sci. 25, 430–436
30. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
31. Sheng, T., Chu, S., Zhang, X., and Xie, J. (2006) J. Biol. Chem. 281, 9–12
32. Seamon, K. B., and Daly, I. W. (1981) J. Cyclic Nucleotide Res. 7, 201–224
33. Hatzelmann, A., and Schudt, C. (2001) J. Pharmacol. Exp. Ther. 297, 267–279
34. Morrison, D. C., and Ryan, J. L. (1987) Annu. Rev. Pharmacol. Toxicol. 27, 417–432
35. Chow, J. C., Young, D. W., Golenbock, D. T., Christ, W. J., and Gusovsky, F. (1999) J. Biol. Chem. 274, 10689–10692
36. Barton, G. M., and Medzhitov, R. (2003) Science 300, 1524–1525
37. Lewis, E. C., Shapiro, L., Bowers, O. J., and Dinarello, C. A. (2005) J. Exp. Med. 201, 1209–1220
38. Moore, K. W., O’Garra, A., de Waal Malefyt, R., Vieira, P., and Mosmann, T. R. (1993) Annu. Rev. Immunol. 11, 165–190
39. Bourne, H. R., Lichtenstein, L. M., Melmon, K. L., Henney, C. S., Weinstein, Y., and Shearer, G. M. (1974) Science 184, 19–28
40. Houslay, M. D., Schafer, P., and Zhang, K. Y. (2005) Drug Discov. Today 10, 1503–1519
41. Kunkel, S. L., Spengler, M., May, M. A., Spengler, R., Larrick, J., and Reimick, D. (1988) J. Biol. Chem. 263, 5380–5384
42. Ollivier, V., Parry, G. C., Cobb, R. R., de Prost, D., and Mackman, N. (1996) J. Biol. Chem. 271, 20828–20835
43. Ouagued, M., Martin-Chouly, C. A., Brinchault, G., Leportier-Comoy, C., Depince, A., Bertrand, C., Lagente, V., Belleguic, C., and Pruniaux, M. P. (2005) Palm. Pharmacol. Ther. 18, 49–54
**α₁-Antitrypsin Induces cAMP in Monocytes**

48. Kambayashi, T., Jacob, C. O., Zhou, D., Mazurek, N., Fong, M., and Straussmann, G. (1995) *J. Immunol.* **155**, 4909–4916

49. Shames, B. D., McIntyre, R. C., Bensard, D. D., Pulido, E. J., Selzman, C. H., Reznikov, L. L., Harken, A. H., and Meng, X. (2001) *J. Surg. Res.* **99**, 187–193

50. Sitaraman, S. V., Merlin, D., Wang, L., Wong, M., Andrew, T., Gewirtz, A. T., Si-Tahar, M., and Madara, J. L. (2001) *J. Clin. Investig.* **107**, 861–869

51. Joslin, G., Griffin, G. L., August, A. M., Adams, S., Fallon, R. J., Senior, R. M., and Perlmutter, D. H. (1992) *J. Clin. Investig.* **90**, 1150–1154

52. Seldon, P. M., Barnes, P. J., and Giembycz, M. A. (1998) *Cell Biochem. Biophys.* **29**, 179–201

53. Zhang, Y., DeWitt, D. L., McNeely, T. B., Wahl, S. M., and Wahl, L. M. (1997) *J. Clin. Investig.* **99**, 894–900

54. Uchiba, M., Okajiima, K., Kaun, C., Wojta, J., Bernd, R., and Binder, B. R. (2004) *Thromb. Haemostasis* **92**, 1420–1427

55. Dhami, R., Gilks, B., Xie, C., Zay, K., Wright, J. L., and Churg, A. (2000) *Am. J. Respir. Cell Mol. Biol.* **22**, 244–252

56. Stockley, R. A., Hill, A. T., Hill, S. L., and Campbell, E. J. (2000) *Chest* **117**, S291–S293

57. Stockley, R. A. (2000) *Thorax* **55**, 614–618

58. Osawa, Y., Lee, H. T., Hirshman, C. A., Xu, D., and Emala, C. W. (2006) *Am. J. Physiol.* **290**, C143–C151