α_1 -Antitrypsin Modulates Lung Endothelial Cell Inflammatory Responses to TNF- α

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α_1 -Antitrypsin (A1AT) is an acute-phase reactant, but also a major protective factor against the development of chronic obstructive pulmonary disease, a complex disease with sustained chronic inflammation. The lung-protective effects of A1AT have been attributed to the inhibition of proteases involved in lung matrix fragmentation, macrophage activation, and endothelial-cell apoptosis. More recently, A1AT has been shown to directly interact with or modulate the actions of cytokines such as TNF- α or IL-1 in inflammatory cells, but its effect on the lung endothelium, an active participant in the amplification and resolution of inflammation, has received little attention. An important role of A1AT in modulating lung endothelial inflammatory responses is expected, given the high concentrations of circulating A1AT during inflammation and its active uptake by endothelial cells. We investigated the role of A1AT in primary lung microvascular endothelial cell activation by relevant cytokines such as TNF- α or IL-1 β . Despite an initial marked augmentation of TNF- α self-induced transcription, A1AT inhibited TNF- α receptor 1 up-regulation and significantly reduced TNF- α secretion, effects that were associated with inhibition of TNF- α -converting enzyme activity. Furthermore, A1AT inhibited calpain activity, whose activation by TNF- α contributed to decreased intracellular A1AT concentrations. These data indicate that A1AT initially facilitates acute responses of the endothelium to TNF- α , followed by selective inhibition of TNF- α -induced self amplification, which may assist the vasculature in the resolution of chronic inflammation.

Keywords: proteinase inhibitor; serpin; vascular; inflammation; COPD

Persistent pulmonary inflammatory responses are involved in the pathogenesis of several lung diseases, including chronic obstructive pulmonary diseases (COPD) such as emphysema. Endothelial cells lining the lung vasculature constitute a key component of the inflammatory response. They are activated by proinflammatory cytokines to increase the expression of adhesion molecules necessary for the tethering and extravasation

of white blood cells into pulmonary tissue (1, 2), and to up-regulate cytokines such as TNF- α and their respective receptors such as TNF receptor 1 (TNF-RI) (3), leading to amplification of the inflammatory response. Endothelial cells are constantly exposed to circulating α_1 -antitrypsin (A1AT), which in acute inflammation is markedly increased as a main acute-phase reactant. However, the effect of A1AT on the function of the vascular endothelium during inflammation remains unknown. A1AT deficiency is a major risk factor for the development of emphysema, in large part because of unopposed protease activity targeting the lung matrix. In addition, A1AT inhibits apoptosis when taken up by the lung endothelium (4, 5), further protecting alveolar integrity. Although a more direct anti-inflammatory role of A1AT via the inhibition of the cytokine-induced activation of inflammatory cells (6–10) has recently been discovered, the effect of A1AT on the endothelial responses to inflammatory cytokines is not well characterized. Moreover, A1AT-deficient individuals run an increased risk of lung destruction with a peculiar panacinar and lower lobe involvement, and are also at increased risk of systemic vasculitis (11), suggesting a potential predominant vascular dysfunction in these patients. Therefore, we hypothesized that A1AT plays an active role in the modulation of the inflammatory responses of the lung endothelium to TNF- α , one of the principal cytokines implicated in chronic inflammatory conditions such as COPD.

A1AT is a serine protease inhibitor (serpin) acute-phase reactant mainly produced in the liver and secreted in the circulation, where it inhibits leukocyte proteases, attenuating tissue injury and inflammation (12). Individuals with A1AT deficiency demonstrate low circulating A1AT concentrations, attributable to mutations in the A1AT gene *SERPINA1*, and they develop lung disease earlier in life compared with individuals manifesting COPD with normal A1AT circulating concentrations (13, 14). However, cigarette smoking, the major etiological factor in the development of COPD, can decrease the amount of functional A1AT by post-translational modifications of the serpin, even in A1AT-sufficient patients (15–17). Oxidants and other noxious components of cigarette smoke can directly injure endothelial cells via apoptosis, or trigger an inflammatory response leading to increased circulating concentrations of proinflammatory cytokines such as TNF- α (18). These cytokines activate their receptors to stimulate nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activity via NF- κ B inhibitor, alpha ($I\kappa$ B α) degradation. The translocation of NF- κ B increases the transcription of inflammatory genes, including that of TNF- α , causing an increased secretion of TNF- α from endothelial cells via the proteolytic cleavage of membrane-bound pro-TNF- α by the TNF- α converting enzyme (TACE) (19). Evidence indicates that in addition to the inhibition of neutrophil serine proteases, A1AT exerts anti-inflammatory effects on neutrophils by directly binding and inhibiting TACE (20). The anti-inflammatory signaling of A1AT, resulting in reduced pulmonary TNF- α

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concentrations, has also been observed in airway macrophages (7) and other inflammatory cells (8) (21). However, the role of A1AT in endothelial cell responses to inflammation remains unclear. We and others have shown that A1AT performs an antiapoptotic function in lung endothelial cells by inhibiting executioner caspases 3, 6, and 7 (4, 22, 23). This activity requires an active uptake of A1AT by the lung endothelium, which normally does not produce A1AT (24). Although reports indicate inhibitory effects of A1AT on the TNF- α -induced transcriptome (25), the antiapoptotic effect of A1AT may actually be permissive to acute inflammatory signaling in endothelial cells (26).

Using primary lung microvascular cells, we demonstrate a complex effect of A1AT on the response of vascular endothelial cells to inflammation, which is cytokine-specific and which ultimately attenuates TNF- α self-amplification, in association with inhibition of TACE and calpain activities.

MATERIALS AND METHODS

Reagents

Reagents were acquired from Sigma (St. Louis, MO), unless otherwise specified. Purified human A1AT used in all experiments was obtained from Sigma, except that used in TACE activity assays (Baxter Healthcare, Deerfield, IL) and in calpain activity assays (Grifols, Barcelona, Spain). Human recombinant TNF- α was from R&D Systems (Minneapolis, MN), antibodies anti-I κ B α and anti-TNF-RI were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-A1AT was from Bethyl (Montgomery, TX), and anti-vinculin was purchased from Calbiochem (La Jolla, CA).

Cell Cultures

Primary rat lung microvascular endothelial cells (RLMVECs), a kind gift from Troy Stevens (University of South Alabama), were maintained in Dulbecco's Modified Eagle's Medium, 10% FBS, and 1% penicillin/streptomycin. Primary human pulmonary artery endothelial cells (Life Technologies, Grand Island, NY) were maintained in Medium 200/LSGS Supplement (Life Technologies). Primary human dermal microvascular endothelial cells, obtained from Mathias Clauss (Indiana University), were maintained in endothelial basal medium 2 (EBM2) and 5% FBS. Human lung microvascular endothelial cells (Lonza, Walkersville, MD) were cultured in Vasculife VEGF-Mv (Cell Systems, Troisdorf, Germany). Cells were maintained at 37°C in 5% CO₂. Before treatment, confluent monolayers were maintained in serum-free medium for 2 hours (24), and then pretreated with A1AT (100 μ g/ml) for the indicated times before exposure to cytokines. Treatment groups were harvested simultaneously, and were exposed to A1AT for similar durations, and for variable times to cytokines. Lysates were prepared in radio immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors.

Western Blotting

Proteins, quantified by bicinchoninic acid assay (BCA), were equally loaded onto 10% SDS-PAGE, followed by immunoblotting (4) and densitometric assessment with ImageJ (National Institutes of Health, Bethesda, MD).

Real-Time PCR

Total RNA was isolated with RNEasy Plus Kit (Qiagen, Valencia, CA) and reverse-transcribed into cDNA, using the High-Capacity Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA), with 50 ng of cDNA/reaction. DNA amplification was monitored via the incorporation of SYBR green. Our validated (27) rat primer sets included intercellular adhesion molecule-1 (ICAM-1; 5'-TTTCGATCTCCGAC TAGGG-3' and 5'-AGCTTCAGAGGCAGGAAACA-3'), TNF- α (5'-AAATGGGCTCCCTCTCATCAGTTC-3' and 5'-TCTGCTTG GTTGGTTTGCTACGAC-3'), and glyceraldehyde 3-phosphate dehydrogenase (5'-TGCCAAGTATGATGACATCAAGAAG-3' and 5'-AGCCAGGATGCCCTTTAGT-3').

Secreted Cytokines

Supernatants (1 ml) were concentrated via Nanosep column (Pall, Ann Arbor, MI). TNF- α , IL-1 β , and IL-6 were measured by ELISA (Thermo Scientific, Rockford, IL) and by a Quantibody Array performed by Raybiotech, Inc. (Norcross, GA).

TACE Activity

Plasma membranes were isolated using Membrane Protein Extraction Kit (Biovision, Mountain View, CA). TACE activity was determined with Sensolyte 520 TACE Activity Assay Kit (Anaspec, Fremont, CA) at 490 nm/520 nm.

Luciferase Assay

RLMVECs were transfected with Fugene HD/DNA (at a ratio of 3:2; Promega, Madison, WI), using pHTS-NF- κ B-luc (Biomx, San Diego, CA) and with renilla luciferase to normalize for transfection efficiency or with pHTS-MCS empty vector (Biomx) and renilla (28). Luciferase activity was measured with Dual-Luciferase Reporter Assay (Promega).

NF- κ B DNA binding was measured with Trans Am NF- κ B p65 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA).

A1AT Intracellular Uptake

A1AT was labeled using Alexa Fluor 488 Labeling Kit (Molecular Probes, Eugene, OR), and its concentration was determined with NanoDrop (Thermo Scientific). To quench noninternalized labeled A1AT, cells were washed, incubated with trypan blue (0.04%, for 1 min), rinsed, and paraformaldehyde (1%)–fixed. Internalized A1AT was measured by flow cytometry (Beckman Coulter, Fullerton, CA) (24).

The calpain activity assay was performed with a fluorometric kit (Abcam, Cambridge, United Kingdom), detecting cleavage of the calpain substrate, Ac-LLY-AFC (400/505 nm), with SpectraFluor Plus Plate Reader (Tecan, Crailsheim, Germany). Calpain activity was calculated according to the difference between calpain inhibitor-treated and untreated mixtures, to exclude the contributions of noncalpain enzymes to substrate cleavage.

Statistical Analysis

Statistical analysis was performed using SigmaStat (SigmaPlot, San Jose, CA), version 3.5. Differences between groups were compared using an unpaired Student *t* test or ANOVA with the Student-Newman-Keuls *post hoc* test. Data are expressed as means $\pm$ SEMs. Statistically significant differences were accepted at *P* < 0.05.

RESULTS

Effect of A1AT on Transcriptional Responses of Pulmonary Endothelial Cells to TNF- α

Endothelial cell responses to TNF- α are complex, and are both species-specific and context-specific, ranging from inflammatory activation to apoptosis (29, 30). Similar to human cells (31), primary rat lung microvascular endothelial cells treated with recombinant human TNF- α (10 ng/ml; up to 2 h) demonstrated prompt I κ B α degradation. The magnitude and kinetics of I κ B α degradation, an indication of NF- κ B activation, were similar regardless of whether cells were cultured in serum (10% FBS) or serum-free conditions (Figure E1 in the online supplement). This response, corroborated by lack of apoptosis and by barrier dysfunction (measured by lack of increase in caspase-3 activity and by a significant decrease of transendothelial electrical resistance, respectively; data not shown), suggested that TNF- α -treated rat lung microvascular endothelial cells undergo proinflammatory activation. Because FBS may contain variable amounts of endogenous (bovine) A1AT that may confound the results, in the remaining experiments, to study the specific effects of A1AT, we treated endothelial cells with recombinant human

TNF- α under serum-free conditions. Primary rat lung microvascular endothelial cells were pretreated with A1AT for 4 hours, to allow for the intracellular uptake of A1AT in the endothelium (24), followed by TNF- α treatment. A1AT treatment noticeably reduced the baseline concentrations of I κ B α protein in comparison with vehicle-treated cells (Figures 1A and 1B). Furthermore, A1AT treatment enhanced TNF- α -induced I κ B α degradation at 5 minutes (Figure 1C). Although on several Western blots (Figure 1A) A1AT appeared to enhance I κ B α recovery after TNF- α treatment, this effect was not statistically significant (Figure 1C). Consistent with these results, A1AT supplementation did not inhibit TNF- α -induced NF- κ B activation in lung endothelial cells, as detected by NF- κ B DNA binding or by luciferase-measured transactivation assay (Figure E2).

As previously reported (25), treatment with TNF- α significantly stimulated the transcription of TNF- α mRNA by lung endothelial cells (by 6- to 10-fold compared with control values) in a typical oscillatory manner (32, 33) (Figures 2A and 2B). Short-term treatment with A1AT markedly potentiated, by more than 6-fold, the TNF- α -induced up-regulation of TNF- α mRNA in both rat (Figure 2A) and human (Figure 2E) endothelial cells, but this effect decreased in potency with increased pre-exposure time to A1AT (> 48 hours; Figure 2B). We evaluated supernatants and cell lysates to ensure that A1AT protein did not undergo degradation in cell culture conditions over time (Figure E3). Unlike the significant acute effect of enhanced TNF- α transcription, A1AT did not significantly alter TNF- α -induced ICAM-1 gene transcription, a proinflammatory adhesion molecule and a marker of endothelial cell activation (Figure 2C). Furthermore, the marked impact of short-term A1AT treatment on TNF- α transcription was specific to TNF- α signaling, because under similar conditions, A1AT did not facilitate IL-1 β -induced TNF- α gene transcription (Figure 2D).

A1AT Inhibits TNF- α Secretion from Lung Endothelial Cells Treated with TNF- α

We next measured the effects of A1AT on the secretion of TNF- α protein from the endothelial cell monolayer into supernatants after TNF- α treatment. Despite a stimulatory effect on

TNF- α -induced self-gene expression, A1AT significantly inhibited TNF- α -induced TNF- α protein secretion by lung endothelial cells (Figure 3A). Interestingly, brief exposures of A1AT exerted variable effects on endothelial cells, occasionally enhancing the baseline TNF- α secretion, but this did not reach statistical significance, even after repeated independent experiments (Figure 3B). Nevertheless, extended exposure to A1AT significantly inhibited basal TNF- α secretion from endothelial cells (Figure 3B). The A1AT inhibitory effect on TNF- α -induced self-secretion was apparent throughout the kinetics of A1AT treatment (Figure 3B). Interestingly, unlike the case for inflammatory cells, TNF- α did not induce robust IL-1, IL-6, or even ICAM secretion in lung microvascular endothelial cells, because their concentrations were either undetectable or marginal when measured by ELISA or Quantibody arrays (data not shown).

A1AT Decreases TNF- α -Converting Enzyme Activity and TNF-RI Protein Abundance at Baseline and in Response to TNF- α

Because extracellular TNF- α secretion depends on the activation of TACE, which cleaves pro-TNF- α at the plasma membrane, we investigated the effects of A1AT on TACE activity in lung endothelial cells. After A1AT treatment, cells were harvested at the indicated times, and were fractionated to obtain plasma membranes. TACE activity was measured in endothelial plasma membranes, using a specific fluorescently tagged substrate. Treatment with A1AT significantly inhibited TACE activity in endothelial cells, both after short (1-h) and prolonged (24-h) exposures (Figure 4A).

The reduction of TNF- α secretion, associated with an inhibition of TACE, may serve to inhibit the augmentation of the inflammatory response by endothelial cells. However, in addition to cleaving pro-TNF- α , TACE also regulates the availability of TNF-RI on the cell surface by cleaving it and causing its membrane shedding, which serves to down-regulate inflammation (34–36). Therefore, the inhibition of TACE by A1AT may enhance TNF-RI expression and therefore continue to amplify inflammation. In contrast, using Western blotting, we observed that endothelial cells treated with A1AT exhibited a reduced

Figure 1. Temporally distinct effects of α_1 -antitrypsin (A1AT) on NF- κ B inhibitor, alpha (I κ B α) in untreated or TNF- α -challenged lung endothelial cells. (A) I κ B α protein expression in endothelial cells treated with A1AT or its vehicle (PBS), 4 hours before challenge with recombinant human (rh) TNF- α . (B) Expression levels of endothelial I κ B α quantified by densitometry at baseline and in response to A1AT treatment (100 μ g/ml, 4 h). (C) Expression levels of endothelial I κ B α in cells treated with TNF- α (10 ng/ml, 5–120 min), with or without pretreatment with A1AT (100 μ g/ml, 4 h). Open bars show the relative change of I κ B α expression in cells treated with TNF- α (and PBS as A1AT vehicle), compared with cells treated with vehicle alone (1% BSA in PBS as TNF- α vehicle), measured by densitometry. Solid bars show fold change in I κ B α expression in cells pretreated with A1AT and then TNF- α , compared with cells treated only with A1AT. Data represent means $\pm$ SEMs ($n = 2$ –3). Ctl, control.

Figure 2. A1AT enhances TNF- α -induced self-transcription. (A–C) TNF- α (A and B) and intercellular adhesion molecule-1 (ICAM-1 [C]) mRNA transcription was measured by real-time PCR in rat lung endothelial cells pretreated with A1AT (solid bars, 100 μ g/ml, 4–48 h) or with vehicle control (open bars) before TNF- α (10 ng/ml, 30–120 min [A] or 30 min to 48 h [B and C]) or (D) IL-1 β (10 ng/ml, 30–120 min) exposure. (E) TNF- α gene expression in human pulmonary artery endothelial cells pretreated with A1AT (solid bars, 100 μ g/ml, 4 h) or with vehicle control (open bars), followed by TNF- α (10 ng/ml, 5–120 min). Data represent means $\pm$ SEMs ($n = 2$ –3); RQ, relative quantification.

abundance of TNF-RI protein (Figure 4B). Furthermore, compared with endothelial cells treated with only TNF- α , which expectedly increased the abundance of TNF-RI, A1AT pretreatment both delayed the induction (from 5 to 120 min) and reduced the net amount of TNF-RI protein expression in response to the cytokine (Figure 4B). These results suggest that the effect of A1AT on TNF-RI may be the result of its inhibition of TNF- α self-amplification via the reduction of TACE-mediated soluble TNF- α secretion.

The inhibitory effect of A1AT on I κ B α , TACE activity, and TNF-RI is presumed to occur intracellularly, because A1AT was taken up by endothelial cells at the time points studied. Although the precise intracellular fate of A1AT in endothelial cells remains to be elucidated, the intracellular availability of the proteinase inhibitor, and therefore its activity, may be altered by cytokine treatment. We first determined that TNF- α , similar to IL-1 β , does not inhibit the rate of A1AT uptake by lung endothelial cells, as determined by flow cytometry (Figure 5A). However, TNF- α decreased intracellular A1AT concentrations in rat lung microvascular endothelial cells in the presence (Figure 5B) or absence (Figure E3B) of residual supplemented A1AT in the supernatants. This effect is not species-specific or organ-specific, because it was also observed in human lung microvascular endothelial cells and in human dermal microvascular endothelial cells (Figure E4). This effect was prevented by a potent calpain inhibitor, calpain inhibitor II (Figure 5C), but not by carbobenzoxy-Leu-Leu-leucinal (MG132) or chloroquine

(data not shown), suggesting that TNF- α promotes A1AT intracellular proteolysis via calpain-dependent degradation, but not via proteasome or lysosome pathways. Interestingly, in neutrophils and in cell-free systems, A1AT has been reported to inhibit calpain activity (37). We reproduced this effect in primary human lung endothelial cells, where A1AT treatment markedly (by $\sim$ 50%) inhibited endogenous calpain activity (Figure 5D). The opposing effects of TNF- α and A1AT on calpain activation and the negative feedback between calpain and A1AT suggest the existence of a regulatory loop whereby A1AT helps resolve inflammation by inhibiting TNF- α secretion. However, persistent proinflammatory stimulation may allow TNF- α to override the effects of A1AT on inflammation by increasing its proteolytic degradation, whereas supplementation with A1AT may interrupt this effect (a schematic is presented in Figure 6).

DISCUSSION

Our data indicate that A1AT modulates the inflammatory responses of lung endothelial cells to the proinflammatory cytokine TNF- α in a complex manner. As an acute-phase reactant, A1AT inhibited the expression of I κ B α , which may have accounted for a brief and modest secretion of TNF- α and markedly enhanced TNF- α transcription. With time, however, A1AT helped extinguish the inflammatory self-amplification of TNF- α by inhibiting its secretion and receptor expression. This effect on endothelial cells is somewhat similar to that on monocytes,

Figure 3. A1AT inhibits TNF- α -induced endothelial cell secretion of TNF- α . (A) TNF- α concentration in supernatants of endothelial cells treated with A1AT (10–500 μ g/mL, 4 h) only (open bars), or with TNF- α (10 ng/mL, 1 h, solid bars). (B) TNF- α concentration in supernatants of endothelial cells treated with A1AT (100 μ g/mL, 1–24 h, open bars), compared with cells pretreated with A1AT before TNF- α (10 ng/mL, 1 h, solid bars). Data represent means $\pm$ SEMs ($n \geq 3$).

where short-term A1AT treatment (2 h) briefly stimulated TNF- α gene expression and secretion, whereas prolonged A1AT exposure (18 h) inhibited TNF- α gene expression (8). Although we could not detect an inhibition of TNF- α transcription after long-term A1AT treatment, we did note less robust increases in TNF- α mRNA at later time points (52 h). Despite lack of degradation of A1AT for up to 24 hours, we cannot rule out reduced potency of A1AT attributable to post-translational modifications in the supernatant, such as oxidation. However, the A1AT-induced inhibition of TNF-induced self-secretion persisted even after prolonged incubation, suggesting the protein was still active. Because A1AT did not inhibit transcriptional responses induced by TNF- α , the anti-inflammatory effects of A1AT on TNF- α release and TNF receptor expression are likely post-translational. In particular, the A1AT-induced inhibition of TACE may explain the reduction of TNF- α secretion, whereas the A1AT-induced inhibition of calpain may enhance the availability of intracellular A1AT to mitigate inflammation. A similar mechanism of A1AT-mediated protection has been described in pancreatic β cells, where A1AT inhibits toxicity by preventing TNF- α release, likely through an inhibition of TACE activity, because a TACE inhibitor protected β cells to a degree similar to that of A1AT (38). The mechanism by which A1AT inhibits TNF-RI expression remains unclear, and may

Figure 4. A1AT inhibits TNF- α converting enzyme (TACE; ADAM-17) activity and TNF receptor 1 (TNF-RI) expression. (A) Baseline TACE activity in the plasma membrane of endothelial cells treated with vehicle control (PBS, 1–24 h, open bars) or after A1AT stimulation (100 μ g/mL, 1–24 h, solid bars). (B) TNF-RI protein expression in endothelial cells treated with TNF- α alone (10 ng/mL, 5–120 min) or with A1AT (100 μ g/mL, 4 h) before TNF- α treatment. Data represent means $\pm$ SEMs ($n = 2-3$).

involve a yet to be identified sheddase, or the inhibition could be explained by reduced TNF- α secretion and the extinguished transcriptional activation of TNF- α -dependent genes, including TNF-RI. The initial proinflammatory effects of A1AT may skew the response of cells to TNF toward a prosurvival, anti-apoptotic phenotype, which we and others previously attributed to A1AT treatment (4, 5, 39, 40). This link, and the role of calpain inhibition in the antiapoptotic effects of A1AT, should be explored in future studies.

Our results are in agreement with previous reports that endogenous A1AT decreases circulating concentrations of proinflammatory cytokines such as TNF- α , IL-1 β , IL-6, and IL-8 in whole blood (9), and that A1AT inhibits the secretion of TNF- α in cultured alveolar macrophages from mice exposed to cigarette smoke (7). Although we did not detect IL-6 and IL-1 β secretion, and IL-1 α mRNA was not affected by our treatment, our sample volume and timing of TNF- α treatment may not have allowed us to see changes in these cytokines. Interestingly, in other cell types such as human umbilical-vein endothelial cells, which underwent apoptosis in response to TNF- α , A1AT inhibited the release of full-length and calpain-processed IL-1 α in apoptotic bodies (41). These mechanisms for the attenuation of TNF- α amplification may account for the anti-inflammatory effects of A1AT administration, and also for a propensity toward chronic, unabated inflammatory responses under conditions of A1AT deficiency. Furthermore, given the reduced anti-inflammatory actions of both oxidized and polymerized A1AT on alveolar macrophages (21), such alterations in A1AT structure may impair the interactions of serpin with proteases such as TACE and calpain, and may also diminish A1AT anti-inflammatory activity in endothelial cells. Notably, various studies have identified anti-inflammatory A1AT properties that do not involve the inhibition of elastase or even other proteases, and these protective characteristics may extend the potential therapeutic benefits of A1AT beyond emphysema treatment (42). A1AT prevents neutrophil chemotaxis via mechanisms that are likely both antiprotease-mediated and nonprotease-mediated, because A1AT inhibits TACE in neutrophils and pancreatic β cells, and binds to IL-8 and to heat shock protein-70 to inhibit

Figure 5. Mutual A1AT–TNF- α effects involving calpain activation. (A) Flow cytometry analysis of Alexa Fluor-488 (AF488)-A1AT uptake in endothelial cells pretreated with vehicle control (veh, 1 h, gray bar), TNF- α (10 ng/ml, 1 h, solid bar), or IL-1 β (10 ng/ml, 1 h, solid bar) before treatment with A1AT (100 μ g/ml, 1 h). (B and C) TNF- α induces calpain-dependent degradation of A1AT in endothelial cells. A1AT intracellular concentrations in endothelial cells pretreated with A1AT (100 μ g/ml, 4 h), followed by TNF- α in the absence (B) or presence (C, 10 ng/ml, 1 h pretreatment) of a calpain inhibitor. Data represent means $\pm$ SEMs ($n = 3$). (D) A1AT inhibition of endothelial calpain activity. Calpain activity was measured by calpain activity assay (fluorescence units normalized by protein concentration) in lung endothelial cells treated with A1AT (2 mg/ml, 1 h, $n = 2$). A.U., arbitrary units; Cal II (calpain inhibitor II).

their proinflammatory actions (20, 38, 43). Moreover, A1AT deficiency promotes HIV infection in whole human blood, and A1AT inhibits HIV viral infection and replication, associated with suppression of NF- κ B activation (44–47).

The lack of an inhibitory effect of A1AT on NF- κ B DNA binding or transactivation noted in this report may be cell type-specific, because Churg and colleagues have shown that A1AT inhibits NF- κ B DNA binding in whole-lung homogenates (48), whereas Greene and colleagues demonstrated that A1AT activates NF- κ B in bronchial epithelial cells (39). However, our study does not rule out the possibility that other genes of the more than 400 that are regulated by NF- κ B may be inhibited by A1AT, or that A1AT may interfere with the binding of NF- κ B coactivators or repressors. Intriguingly, the permissive role of A1AT in inflammatory gene expression among rat lung endothelial cells is in concordance with previous findings that combined A1AT and LPS treatment synergistically and acutely increased TNF- α gene expression (49). One might suspect that LPS contaminated our A1AT preparation, given the magnitude of TNF- α mRNA stimulation and the decreased baseline I κ B α concentrations in response to A1AT treatment. However, such LPS contamination would have further increased TNF- α secretion, and would have caused increased NF- κ B activation and extended I κ B α degradation, effects not seen in our experiments. Furthermore, the prolonged exposure to A1AT diminished its stimulatory effect on TNF- α transcription, which would also argue against LPS contamination, and instead suggests agreement with a previous report showing that prolonged A1AT (18 h) exposure inhibits the transcriptional responses induced by TNF- α (25). Finally, pharmacological-grade purified A1AT, which should be LPS-free, exerted similar effects.

After uptake, A1AT underwent proteolytic degradation in part, which was enhanced by cytokine treatment. Given the dual

effect of A1AT inhibition on calpain activity and of calpain on A1AT degradation, a precise titration of intracellular A1AT degradation and uptake rates may guide the proinflammatory versus anti-inflammatory responses of endothelial cells. Although

Figure 6. Schematic of proposed effects of A1AT on TNF- α signaling in lung endothelial cells. Lung endothelial cells take up A1AT from the circulation, which in unchallenged cells (left) decreases the protein concentration of I κ B α , and may facilitate the early release of TNF- α , which is eventually restrained by the A1AT inhibition of TACE activity (blue shape). A1AT also decreases TNF-RI (grey shape) concentrations and calpain activity, likely preventing self-propagating autocrine signaling of the secreted TNF- α and preventing apoptosis. In cells stimulated by excess circulating TNF- α , such as occurs during inflammation (right), A1AT initially facilitates TNF- α -induced I κ B α degradation and self-transcriptional activation, but inhibits TNF- α secretion, likely via TACE inhibition. In addition, the inhibitory effect of A1AT on calpain activity may prevent its own degradation induced by TNF- α . These effects could explain both the acute-phase reaction effects of A1AT, as well as a potential critical role in the resolution of inflammation.

endothelial cells are continuously exposed to circulating concentrations of A1AT that increase during acute stress, other factors could influence net intracellular A1AT concentrations, such as cigarette smoking, which markedly reduces A1AT uptake (24) or, as described in this report, cytokines, which increase A1AT degradation.

Our study revealed a direct effect of A1AT on endothelial cell responses to inflammatory cytokines, potentially mitigating inflammation. This effect, added to its potent antiapoptotic function, emphasizes the role of A1AT as a vasculoprotective factor. Therefore, quantitative or qualitative A1AT deficiencies may instigate chronic inflammation and an increased apoptosis of microvascular endothelial cells, which, in turn, may contribute to the development of diseases such as emphysema and vasculitis, which are prevalent conditions of elusive pathogenesis in A1AT-deficient individuals.

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