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Cell Injury, Repair, Aging and Apoptosis

α-1 Antitrypsin Inhibits Caspase-3 Activity, Preventing Lung Endothelial Cell Apoptosis

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α-1 Antitrypsin (A1AT) is an abundant circulating serpin with a postulated function in the lung of potently inhibiting neutrophil-derived proteases. Emphysema attributable to A1AT deficiency led to the concept that a protease/anti-protease imbalance mediates cigarette smoke-induced emphysema. We hypothesized that A1AT has other pathobiological relevant functions in addition to elastase inhibition. We demonstrate a direct prosurvival effect of A1AT through inhibition of lung alveolar endothelial cell apoptosis. Primary pulmonary endothelial cells internalized human A1AT, which co-localized with and inhibited staurosporine-induced caspase-3 activation. In cell-free studies, native A1AT, but not conformers lacking an intact reactive center loop, inhibited the interaction of recombinant active caspase-3 with its specific substrate. Furthermore, overexpression of human A1AT via replication-deficient adeno-associated virus markedly attenuated alveolar wall destruction and oxidative stress caused by caspase-3 instillation in a mouse model of apoptosis-dependent emphysema. Our findings suggest that direct inhibition of active caspase-3 by A1AT may represent a novel anti-apoptotic mechanism relevant to disease processes characterized by excessive structural cell apoptosis, oxidative stress, and inflammation, such as pulmonary emphysema. *(Am J Pathol 2006, 169:1155–1166; DOI: 10.2353/ajpath.2006.060058)*

α-1 Antitrypsin (A1AT) is a prototypical serine protease inhibitor (serpin) with potent anti-neutrophil protease activities, such as against elastase and proteinase-3. The mechanism of protease inhibition by A1AT involves an exposed reactive loop, where the link of the P1-P1′ amino acids methionine and serine, respectively, is cleaved by the target protease, resulting in covalent binding between the serpin and its substrate, with irreversible trapping of the bound protease into the β-sheet A.[1] Significant decreases in serum levels of human A1AT (hA1AT) have been associated with the development of emphysema,[2] a chronic obstructive pulmonary disease characterized by permanent destruction of the small airways.[3] A1AT deficiency is the most frequent form of genetically determined emphysema, usually the result of ^{342}glutamic acid to lysine substitution (PiZ variant) or ^{264}glutamic acid to valine (PiS variant).[1] Approximately 60,000 Americans are predicated to be A1AT-deficient.[4] In patients with A1AT deficiency, cigarette smoking triggers the disease decades earlier than in chronic obstructive pulmonary disease patients with normal serum A1AT levels (PiM variant). Because A1AT is an effective elastase inhibitor, emphysema in A1AT deficiency is believed to occur as a result of increased, unopposed destruction of the lung matrix by the neutrophil serine proteases elastase and proteinase-3 engaged by cigarette smoking.[5] The decreased levels or activity of A1AT are attributed in part to excessive polymerization of the mutant protein[6] and to post-translational changes induced by oxidative stress.[7,8] Polymerized serpin may get entangled in hepatocytes as it is synthesized and posttranslationally modified by glycosylation, contributing to low levels of circulating A1AT, less than 0.3 mg/ml for the PiZ patients (normal, 1 to 2.48 mg/ml).[9]

The correlation between reduced serum levels of A1AT and pulmonary disease has been strengthened by the observations that mouse strain susceptibility to cigarette smoke-induced emphysema is inversely correlated with serum A1AT levels[10] and that A1AT supplementation had a beneficial effect in cigarette smoke-induced emphy-

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Table 1. Primary Antibodies and Their Sources

Primary antibody	Source	Dilution	Application	Figure
α-1 Antitrypsin goat polyclonal	Santa Cruz Biotechnology, Inc., Santa Cruz, CA	1:10,000	IB endothelial cells	2, 4
α-1 Antitrypsin sheep polyclonal	Enzyme Research Laboratories, South Bend, IN	1:10,000	IB endothelial cells	S2
Active caspase-3 rabbit polyclonal	Cell Signaling Technology, Beverly, MA	20 mg/ml	IP endothelial cells	2
Caspase-3 rabbit monoclonal	Cell Signaling Technology	1:2000 or 1:5000	IB endothelial cells	2, S2
		20 mg/ml	IP endothelial cells	3
		1:200	IF endothelial cells	3
Active caspase-3 rabbit polyclonal	Abcam, Cambridge, MA	1:200	IF endothelial cells	3
Caspase-3 rabbit polyclonal	Cell Signaling Technology	1:200	IF endothelial cells	3
Caspase-3 mouse monoclonal	Santa Cruz Biotechnology	1:1000	IB endothelial cells	2
Lamin rabbit polyclonal	Santa Cruz Biotechnology	1:5000	IB endothelial cells	Not shown
Actin mouse monoclonal	Santa Cruz Biotechnology	1:500	IB endothelial cells	2

sema in mice,[11] providing the rationale of A1AT augmentation in A1AT-deficient patients.[4]

As new functions of A1AT are uncovered, including a procellular survival effect in models of serum deprivation and ischemia-reperfusion injury,[12–14] and with the discovery of excessive alveolar cell (endothelial cell) apoptosis as an important mechanism of emphysema development,[15–20] we hypothesized that A1AT has a direct anti-apoptotic effect on lung. To address the mechanisms underlying our recently described protective effects of hA1AT against a model of apoptosis-dependent emphysema induced by blockade of vascular endothelial growth factor,[21] we investigated whether A1AT protects against apoptosis of structural parenchymal cells, in particular lung microvascular endothelial cells. We documented that A1AT and caspase-3 interact in cultured primary pulmonary endothelial cells and in cell-free systems and tested whether A1AT opposes the functional effects of capase-3 in the lung *in vivo*. Our results implicate an anti-apoptotic effect of A1AT in alveolar endothelial cells via direct caspase-3 inhibition.

Materials and Methods

Chemicals and Reagents

Primary antibodies are listed in Table 1. Recombinant active caspase-3, recombinant active caspase-8, and A1AT purified from human plasma were from Calbiochem (EMD Biosciences, San Diego, CA). His-tagged caspase-3 was from Chemicon, Temecula, CA. All other reagents were from Sigma-Aldrich, St. Louis, MO, unless otherwise specified.

Cell Culture Experiments

Primary mouse lung microvascular endothelial cells were kindly provided by Dr. Patty Lee (Yale University, New Haven, CT), human lung microvascular cells were from Clonetics, San Diego, CA, and rat lung microvascular cells were a kind gift from Dr. Troy Stevens (University of Alabama, Birmingham, AL). Experiments were performed up to passage 18. Cells were maintained in complete culture medium consisting of Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY), 20% fetal bovine serum, and penicillin/streptomycin (100 U/ml) at 37°C in 5% CO_2 and 95% air. Experiments were performed at 80 to 100% confluence. Two hours before the addition of A1AT, the medium was replaced with serum-free medium, in which the cells were maintained for the duration of the experiments. Cells were pretreated with A1AT for a time ranging from 2 to 16 hours before the addition of staurosporine and harvested 2 hours later. A1AT pretreatment duration was 2 or 16 hours before UV (6 mJ/cm^2) or tumor necrosis factor-α (20 ng/ml) exposure, respectively, and cells were harvested 16 hours later. The UV radiation was generated with an HL2000 Hybrylinker crosslinker with 8W short-wave UV (254 nm) tubes.

Cell-Free Experiments

Native or polymerized (by heat inactivation at 60°C for 2 hours) purified hA1AT was incubated with recombinant active caspase-3 (Calbiochem, La Jolla, CA) or recombinant caspase-8, and caspase activity was measured using a caspase-3 activity kit with either fluorescence (Promega, Madison, WI) or colorimetric readout (Calbiochem). We measured the interaction of A1AT with active caspase-3 in a functional assay of caspase-3 activity. We added active caspase-3 (4 U for most experiments; we also tested 5 U and 10 U) to wells containing buffer with increasing amounts of A1AT. We then added a fluorescently labeled tetrapeptide (Z-DEVD-R110, 25 μmol/L), which is a specific substrate for caspase-3. For these assays, human A1AT, salt-free lyophilized powder, was first resuspended in water and then incubated with recombinant caspase-3 in caspase-3 buffer (50 mmol/L HEPES, pH 7.4, 0.1% Chaps, 0.1 mmol/L ethylenediaminetetraacetic acid, and 1 mmol/L dithiothreitol) at room temperature for 15 minutes before the addition of the caspase-3 substrate. Fluorescence was measured at room temperature for 30 to 90 minutes. We calculated the slope of OD (fluorescence signal) versus time and the ratios of fluorescent signal in the presence and absence of A1AT in the reaction. As control, we used similar volumes of water or phosphate-buffered saline (PBS) as the reactions containing A1AT. The elastase inhibitory activity of

A1AT was assessed in parallel with that of caspase activity, using aliquots from the same batch of A1AT, to ensure that the dilutions of A1AT tested in the caspase-3 assays had preserved their serpin activity. The elastase activity assays were performed by A1AT incubation with purified active porcine pancreatic elastase (2 U) followed by the addition of fluorescently labeled substrate (DQ-elastin, 25 μg/ml) in a fluorescent elastase activity assay using the Enzchek elastase assay kit (Molecular Probes, Eugene, OR). The reaction was allowed to occur at room temperature, and measurements of fluorescence were performed every 5 to 10 minutes for up to 2 to 3 hours. Conformers of A1AT were generated following established methods[22] and tested for loss of activity against elastase before caspase-3 inhibitory assays. The conformers' concentrations are given as native A1AT that was input into their generation. The proteinases used to generate cleaved conformers[22] were not removed from the modified A1AT solution, but the mixture was allowed to incubate for >3 hours, which we predict might have led to the inactivation of any remaining active proteinase. The remaining proteinase did not have any significant influence on the results, because the negative control used identically treated mixtures (minus the A1AT) as part of the caspase-3 activity assays. Conformer activity was compared with these controls to exclude nonspecific effect of residual active proteinase. Dose-response inhibitory activities of A1AT against both caspase and elastase were checked in duplicate for a minimum of three independent experiments.

Apoptosis

Caspase-3 activity was measured in cell lysates or cell-free mixtures using a fluorescence-based assay (Promega) as reported.[18] Caspase-8 activity was measured with a specific rhodamine-labeled caspase-8 substrate (Z-IETD-R110; Molecular Probes). The amount of intracytoplasmic cytochrome *c* was measured with a cytochrome *c* ELISA kit from Calbiochem, following the manufacturer's protocol, using a plate reader (Molecular Dynamics, Sunnyvale, CA) set at 450 nm.

Western Blotting

Endothelial cell lysates were loaded in equal amounts (10 μg protein, unless otherwise noted) determined by Bradford assay (Pierce Biotechnology, Rockford, IL). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Novex, San Diego, CA), or native PAGE, followed by immunoblotting as previously described.[23] The chemiluminescent signals were quantified by densitometry (ImageQuant; Amersham) and normalized by actin. His-specific antibody was from Zymed (South San Francisco, CA).

Immunofluorescence and Co-Immunoprecipitation Assay

Mouse lung endothelial cells were exposed to serum-depleted medium, or serum-depleted medium with hA1AT (0.05 mg/ml) 2 to 24 hours before treatment with staurosporine (600 nmol/L, 2 hours). For immunofluorescence, cells were grown on coverslips, and hA1AT was added to live cells after being labeled with Dylight (procedure described below). Coverslips were fixed with 3% formaldehyde and visualized with the fluorescence or confocal microscope. Co-staining was performed in fixed cells that were then permeabilized with 0.25% Triton X and stained with antibodies specific for procaspase-3 or active caspase-3 (Cell Signaling, Beverly, MA), or isotype control antibody, as described.[24] For co-immunoprecipitation assays of caspase-3 and A1AT, mouse lung endothelial cells were cultivated in Dulbecco's modified Eagle's medium and then exposed to serum-depleted medium or serum-depleted medium supplemented with hA1AT (0.05 mg/ml) 24 hours before treatment with staurosporine (600 nmol/L, 2 hours). Cell lysates were collected in RIPA buffer, diluted in PBS, and precleared with protein A-Sepharose. In control experiments, mouse IgG was also used for preclearance. The supernatant was incubated with polyclonal rabbit anti-caspase-3 antibody at 4°C, 16 hours, followed by protein A-Sepharose (2 hours). Monoclonal rabbit anti-caspase-3 and mouse monoclonal anti-caspase-3 antibody were also tested (Table 1). After centrifugation pellets were suspended in sample buffer with reducing agent, boiled for SDS-PAGE, and Western-immunoblotted with caspase-3 and A1AT antibodies (Table 1). Rabbit serum and mouse IgG were used as controls for Western blotting and no cross-reactivity was observed. When detection of immunoprecipitated protein was performed with the same antibody source used as that used for immunoprecipitation (ie, rabbit monoclonal anti-caspase-3), IgG bands were observed at 45 kd as expected and clearly distinct from the expected molecular weight of caspase-3 or A1AT (data not shown). To test the specificity of the primary anti-A1AT antibody, the presence of A1AT in the precipitate was verified with goat antibody anti-A1AT (G-17) and with the same antibody blocked with 20-fold molar excess of G-17 peptide (Santa Cruz Biotechnology, Santa Cruz, CA).

Labeling Studies for hA1AT

hA1AT (5 mg/ml stock) was labeled with DyLight 547 NHS ester (Pierce) following the manufacturer's protocol and used at a final concentration of 0.05 mg/ml. The dye itself or dye excess eluted from Bio Spin column was used as controls.

Cell Fractionation

A nuclear/cytosol fractionation kit (BioVision) was used and the subcellular fractions were tested for the presence of hA1AT by Western blotting (Table 1).

Isothermal Titration Calorimetry

Isothermal calorimetry measured the heat released by interacting liquid solutions using VP-ITC Microcal (noise level, 1 ncal/second). Caspase-3 peptide (RGTELDCGIETD, corresponding to amino acids 164 to 175; lyophilized, resus-

pended in water) or purified elastase (lyophilized and then resuspended in water) was injected into a thermally controlled cell containing hA1AT (lyophilized and then resuspended in water). Injection amount and waiting time were adjusted according to the relaxation time of reactants and the response-function of the isothermal calorimetry. The free energy ΔG^0, enthalpy ΔH^0, and entropy ΔS^0 change on binding were related through the thermodynamic equation $\Delta G^0 = -RT \ln K = \Delta H^0 - T\Delta S^0$.

Label Transfer Array

The biotin label transfer assay was performed according to the manufacturer's (Pierce) instructions. A1AT was labeled with a tri-functional cross-linking reagent sufo-SBED that contains sulfonated *N*-hydroxysuccinimide active ester, photo-activable aryl azide, and biotin, used for identification. After separation of an unbound label on the column (Pierce), labeled hA1AT was incubated for 60 minutes with human recombinant caspase-3 at a 5:1 ratio, in the presence of 20% albumin. After UV exposure for 15 minutes to complete cross-linking, the mixture was then reduced with dithiothreitol, yielding biotinylated caspase-3. The transfer of biotin label from A1AT onto caspase-3 as a result of transient interaction was tested by Western immunoblotting using Neutravidin-horseradish peroxidase, rabbit monoclonal antibody against caspase-3, and sheep polyclonal antibody against A1AT.

Animal Studies

Animal studies were approved by the Animal Care and Use Committee and Animal Care of The Johns Hopkins University School of Medicine. Male C57Bl/6 mice (3 months old, 25 g) were from The Jackson Laboratory, Bar Harbor, ME. The experiment was performed in the same shipment lot, $n = 4$ to 6 mice/group.

AAV Constructs and Administration

All experiments were performed with a recombinant adeno-associated virus vector construct, rAAV-CB-AAT. The detailed structure of this construct has previously been reported.[25] In brief, it consists of adeno-associated virus (AAV) serotype 2 inverted terminal repeats flanking an expression cassette that drives hA1AT expression from a hybrid cytomegalovirus enhancer/β-actin promoter. This DNA cassette was pseudotyped into AAV serotype 5 capsids, using a published co-transfection method with purification on an iodixanol gradient.[26,27] All vector preparations were titrated using a DNA dot-blot hybridization method,[26] and doses were based on vector genome equivalents. Mice received 9.6×10^{10} particles of UF11 622 or 9.6×10^{10} particles of A1AT-AAV (pTR2-CB-AAT) intramuscularly, diluted in sterile 0.9% NaCl.

Active Caspase-3 Instillation

The intratracheal administration of active caspase-3 was performed in mice by injection of 50 μl of PBS solutions containing either active caspase-3 (0.8 μg; MBL) + Chariot (1 μl; Active Motif, Carlsbad, CA) or Chariot alone (1 μl).[28] The preparation of trachea, the intratracheal delivery, and lung processing were performed as previously described.[29]

Morphometric Analysis

The left lung was inflated and fixed, followed by paraffin-embedding, hematoxylin and eosin staining, and standardized morphometry, performed on coded slides as described.[29]

Oxidative Stress Measurements

The hA1AT protein was oxidized by incubation with either hydrogen peroxide or cigarette smoke extract, the latter obtained as previously described.[18,30] The catalase activity was measured in the lung tissue lysates with a catalase assay kit (Cayman Chemical, Ann Arbor, MI), following the manufacturer's instructions, using bovine liver catalase as a positive control.

Statistical Analysis

The statistical analysis was performed with SPSS for windows software package (SPSS Inc.). The differences between groups were compared using unpaired Student's *t*-test or one-way analysis of variance with Student-Newman-Keuls post hoc test. All of the data are expressed as mean ± SEM. Statistical difference was accepted at $P < 0.05$.

Results

Inhibition of Primary Lung Microvascular Endothelial Cell Apoptosis by hA1AT

hA1AT supplementation before the addition of staurosporine, a broad protein kinase inhibitor that induces endothelial cell apoptosis, significantly inhibited staurosporine-induced apoptosis of primary mouse and human (not shown) lung endothelial cells in culture, as measured by caspase-3 activity, cytochrome *c* release, and assessment of 4,6-diamidino-2-phenylindole (DAPI)-stained nuclei (Figure 1, a–c, respectively). Pretreatment with A1AT also inhibited UV (6 mJ/cm2) or tumor necrosis factor-α (20 ng/ml with cycloheximide 100 μg/ml)-induced apoptosis, as measured by cytochrome *c* release and capase-3 activity, respectively (Supplementary Figure 1 at *http://ajp.amjpathol.org*). To evaluate whether the prosurvival effect of A1AT, a 52-kd circulating protein, was exerted at the plasma membrane or intracellular membrane, endothelial cells were incubated with fluorescently labeled hA1AT at similar concentrations found to inhibit their apoptosis (50 μg/ml). Labeled hA1AT permeated primary lung endothelial cells, localizing intracellularly but not in the nucleus or on plasma membrane fractions, as demonstrated by cell fractionation (Figure 2a), fluorescence microscopy (Figure 2, b–d), and confocal mi-

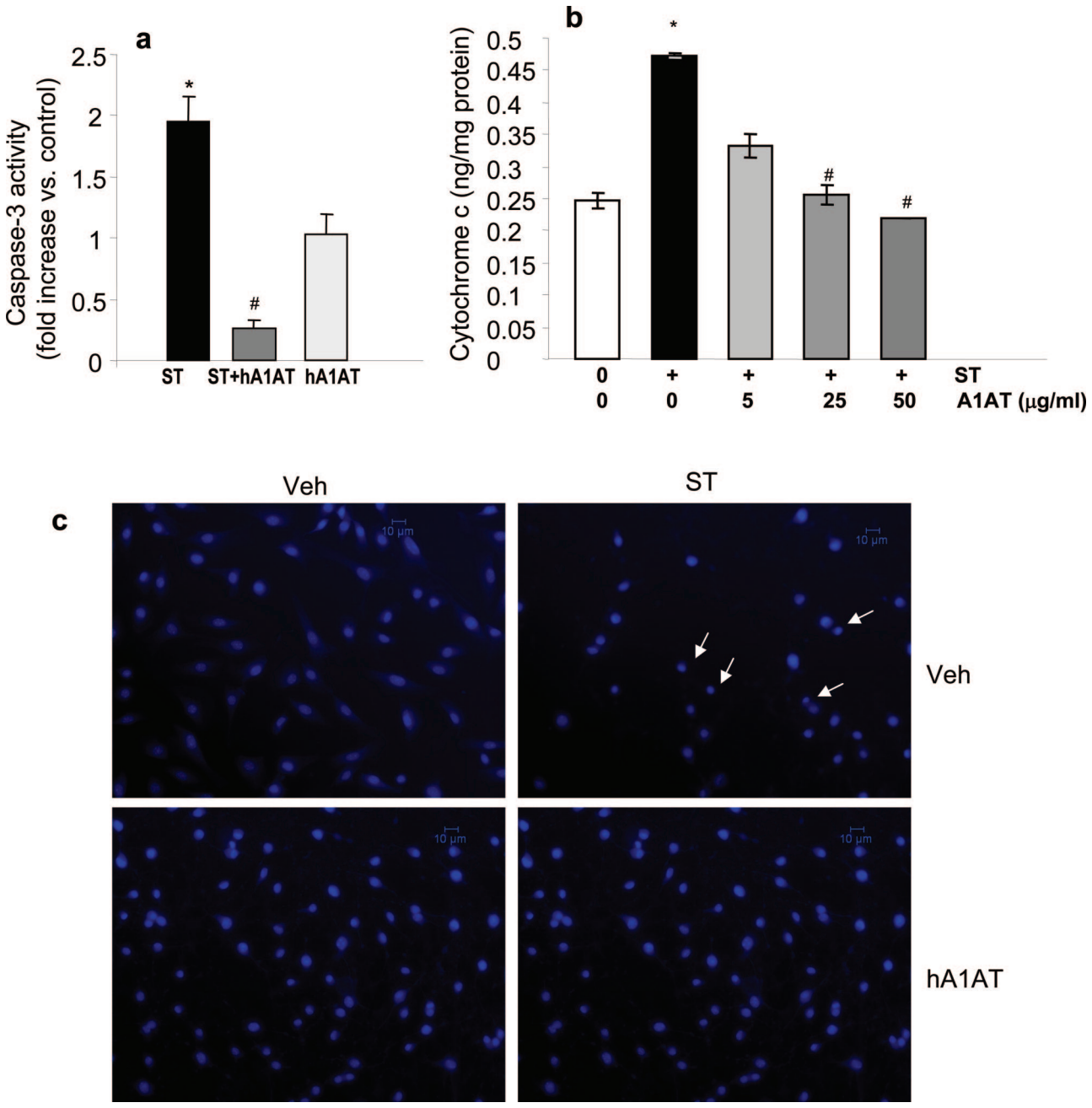

Figure 1. Antiapoptotic effects of hA1AT in lung microvascular endothelial cells. **a:** Caspase-3 activity (fold increase versus vehicle-treated control) in mouse lung endothelial cells exposed to staurosporine (ST, 600 nmol/L; 2 hours) in the presence of purified hA1AT (0.05 mg/ml, 16 hours; mean + SEM, $n = 3$, $P < 0.05$ versus ctl* or versus ST#). **b:** Cytochrome *c* release (normalized by protein concentration) induced by staurosporine in the presence of increasing concentrations of A1AT (pretreatment for 16 hours; mean + SEM, $n = 2$, $P < 0.05$ versus ctl* or ST#). **c:** DAPI-stained nuclei in blue in mouse lung endothelial cells exposed to staurosporine (ST, 600 nmol/L; 2 hours) in the presence of purified hA1AT [0.05 mg/ml, 16 hours, or vehicle (Veh)]. Note a reduction of total number of attached cells, which now show small nuclear size with pyknotic morphology in the ST-treated cells (**arrows**), and in contrast, a preserved number of nuclei of normal morphology in cells pretreated with hA1AT. Representative experiment, $n = 4$. Scale bar = 10 μm.

croscopy (Figure 2e). In contrast, polymeric hA1AT, obtained by heating the native hA1AT in PBS at 60°C for 2 hours and verified by gel electrophoresis, was not internalized by endothelial cells at 2 hours and 4 hours but rather remained attached to the plasma membrane (not shown). The finding of a granular and homogeneous pattern of labeled hA1AT expression suggested both a cytoplasmic and endosomal distribution of the protein, respectively.

Interaction of hA1AT with Caspase-3 in Lung Endothelial Cells

We next investigated whether hA1AT directly interacts with the apoptotic machinery of the lung endothelial cells, in particular with the executioner caspase-3. DyLight-labeled hA1AT did not co-localize with immunolabeled IgG or with procaspase-3 in nonstimulated cells. However, discrete ar-

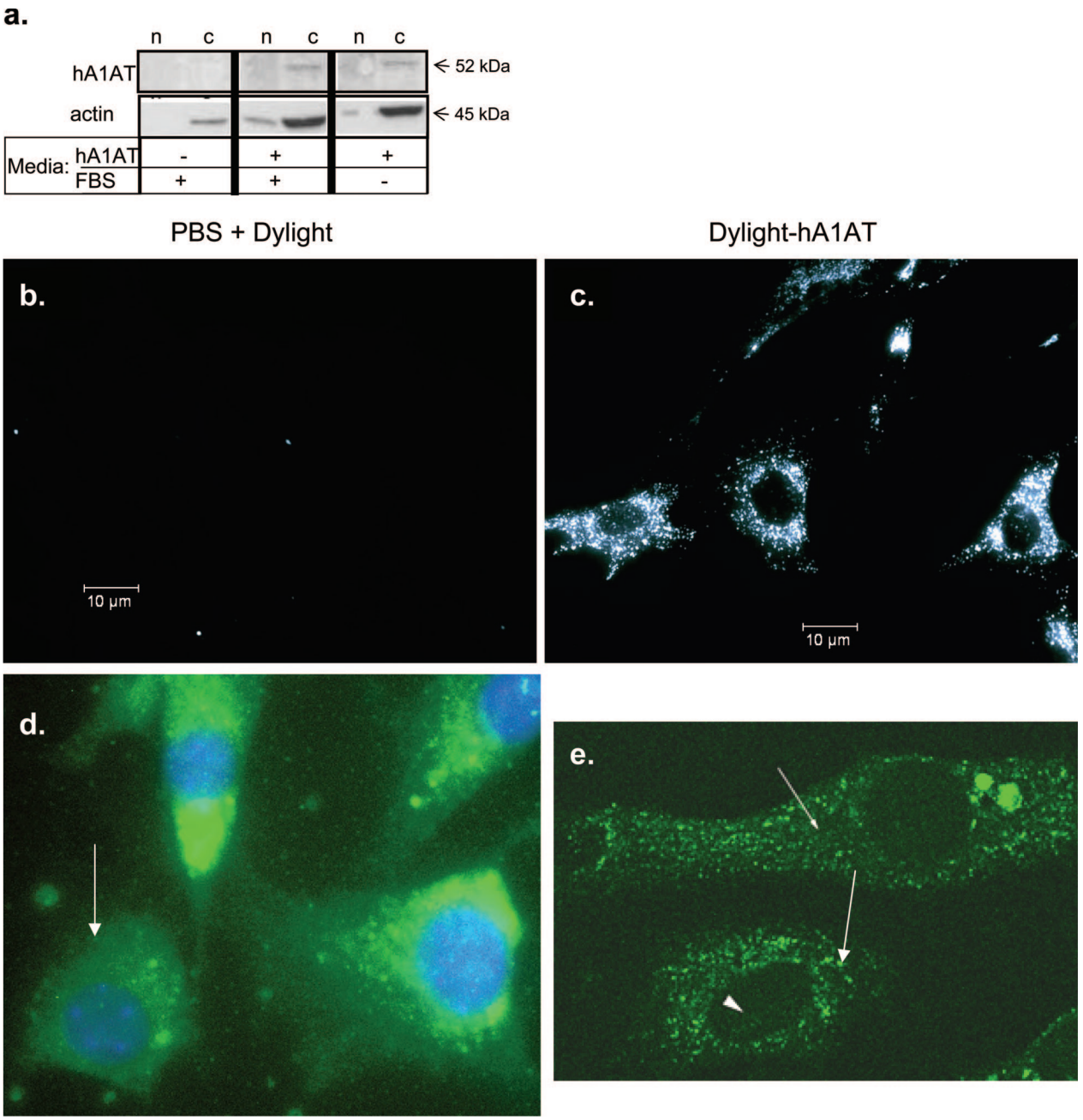

Figure 2. a: Immunoblots of nuclear (n) and cytoplasmic (c) fractions of mouse cells obtained after incubation with purified hA1AT (0.05 mg/ml, 24 hours) in the presence or absence of fetal bovine serum in the media, followed by washing and removal of the media. Note the hA1AT uptake in the cytoplasmic fraction, confirmed by the presence of actin immunostaining. Importantly, there was no cross-reactivity with the endogenous mouse or the serum bovine A1AT. $n = 1$ experiment. **b–e:** Fluorescence micrographs of mouse cells after incubation with Dylight alone (**b**) or with Dylight-labeled h1AT (0.05 mg/ml, 4 hours, **c**). In **d**, cells were counterstained with DAPI in blue to visualize nuclei 2 hours after the addition of hA1AT. Note hA1AT uptake in the cytoplasm (**arrow**) (in **b–e**, assays were performed in nonpermeabilized, live cells, representative of $n = 3$ experiments). **e:** Confocal microscopy of mouse endothelial cells treated with Dylight-hA1AT (0.05 mg/ml, 16 hours) confirming the presence of hA1AT intracellular (cytoplasm, **arrow**; nucleus, **arrowhead**; $n = 2$ experiments).

eas of co-localization of hA1AT and active caspase-3 by immunofluorescence microscopy (Figure 3a) suggested an interaction between the hA1AT with the proapoptotic cysteine protease caspase-3 in mouse and rat microvascular pulmonary endothelial cells treated with staurosporine. This interaction was further tested biochemically as mouse lung endothelial cells pretreated with hA1AT and exposed to staurosporine were evaluated by immunoprecipitation with active caspase-3 antibodies followed by immunoblotting with hA1AT-specific antibodies. This study confirmed an interaction between hA1AT and caspase-3 (Figure 3b) in lung primary endothelial cells.

Interaction of hA1AT with Caspase-3 in Cell-Free Systems

The presence and degree of protein-protein interaction between hA1AT and caspase-3 were further measured in

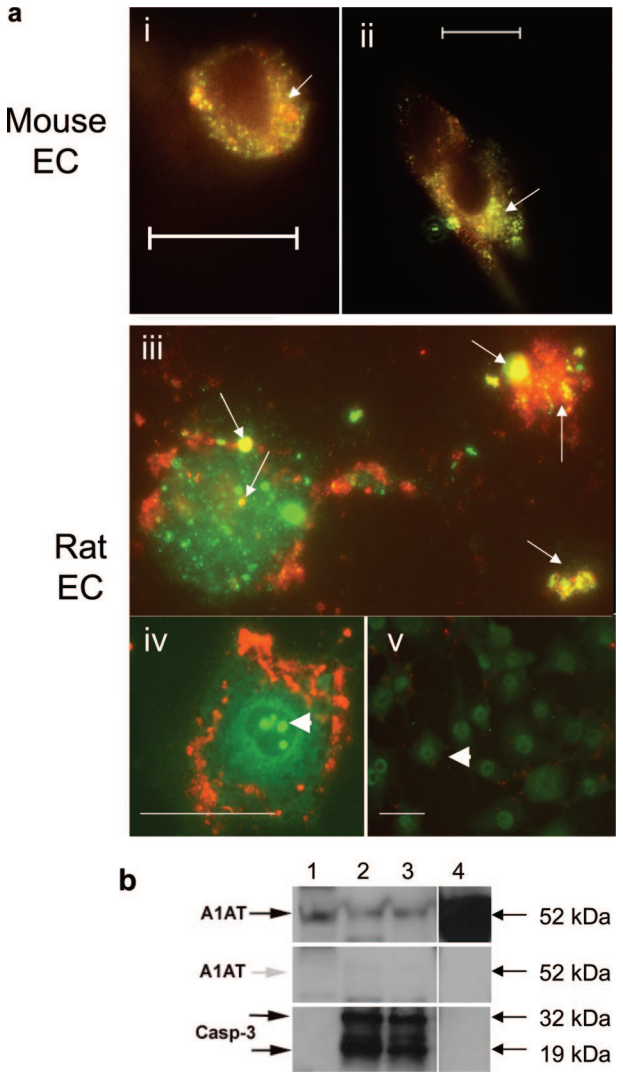

Figure 3. hA1AT interaction with active caspase-3 in cultured lung endothelial cells. **a:** Co-immunofluorescence of hA1AT and caspase-3 in mouse and rat microvascular lung endothelial cells. **i** and **ii:** hA1AT (green) co-localizing (yellow, **arrow**) with active caspase-3 (red) in mouse endothelial cells. **iii:** hA1AT (red) co-localizing (yellow, **arrow**) with active caspase-3 (green) in apoptotic cells (staurosporine-treated, 600 nmol/L, 2 hours; $n = 2$ experiments), but not with procaspase-3 (**iv**, **arrowhead**; untreated) or with rabbit IgG (**v**, **arrowhead**; untreated). **b:** Co-immunoprecipitation studies in mouse cells (treated with ST, 600 nmol/L, 2 hours; $n = 2$) with antibodies against caspase-3 for immunoprecipitation followed by immunoblotting for hA1AT before (top) and after (middle) incubation with A1AT blocking peptide. After stripping and reprobing, immunoprecipitated caspase-3 was confirmed by blotting for active caspase-3 (bottom). **Lane 1**: Starting material: diluted total cell lysate. **Lane 2**: Immunoprecipitate with active caspase-3-specific monoclonal antibody. **Lane 3**: Immunoprecipitate with a polyclonal antibody specific for active and procaspase-3. **Lane 4**: hA1AT protein control. Note that both caspase-3 antibodies precipitated hA1AT (**lanes 2** and **3**, top) and the lack of hA1AT detection in the presence of a specific hA1AT-blocking peptide.

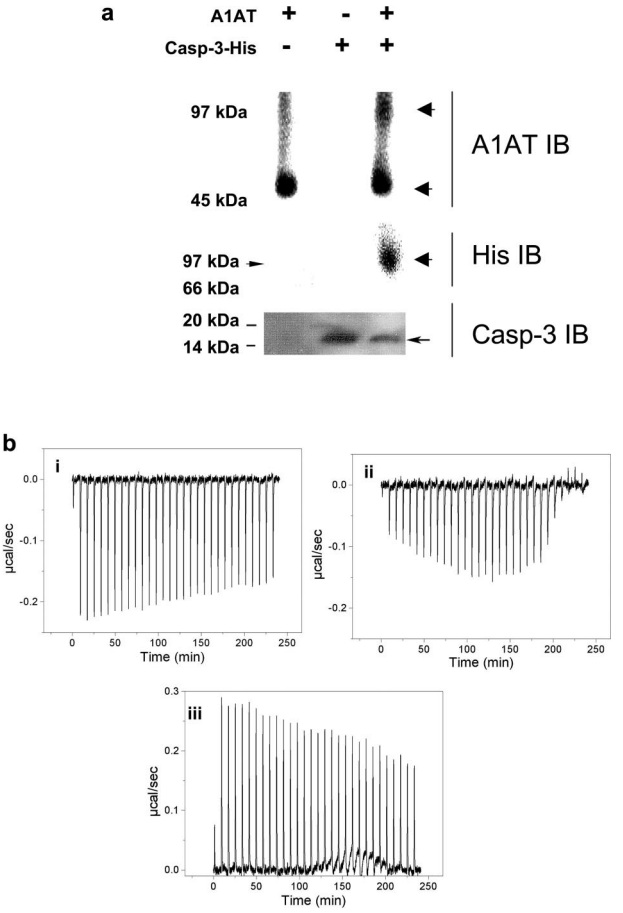

Figure 4. hA1AT interaction with active caspase-3 in cell-free systems. **a:** Native gel electrophoresis of the complex of hA1AT with histidine (His)-tagged active caspase-3. Top: immunoblot with hA1AT antibody highlighting the presence of an estimated 97-kd complex revealing the presence of His-caspase-3, after stripping and reprobing with His antibody (middle); (because of the nondenaturing conditions of migration, the molecular weight of the markers may not be accurate). The SDS-PAGE (bottom) reveals His-tagged active caspase-3 by Western blot, representative of two experiments). **b:** Isothermal calorimetry of the hA1AT-caspase-3 interaction. Exothermic enthalpies measured on hA1AT (0.098 mmol/L/injection) incubation with **i**) active caspase-3 peptide (0.0089 mmol/L, $n = 1$), **ii**) elastase (0.0097 mmol/L, $n = 3$), as positive control, or **iii**) PBS, as negative control ($n = 3$). Note the incubation with buffer generated an endothermic interaction.

cell-free systems using nondenaturing gel electrophoresis and titration calorimetry, a state-of-the-art technique for detection of intermolecular interactions.[31–33] A complex containing hA1AT and caspase-3 was detected by incubating hA1AT with His-tagged caspase-3 for 1 hour, followed by electrophoresis and immunoblotting sequentially for A1AT and then His-tag (Figure 4a). Calorimetric measurement of the interaction strength between A1AT and caspase-3 was compared with the enthalpy of the interaction of hA1AT with purified elastase. hA1AT showed similar binding interaction with the active caspase-3 peptide as with elastase. Exothermic reaction enthalpies per injection are shown in Figure 4b. The control measurement of hA1AT injection into PBS buffer alone showed that dilution of hA1AT was endothermic (Figure 3b, iii), excluding the possibility that measurements resulted from nonspecific interactions with the buffer.

The physical interaction between hA1AT and caspase-3 interaction was further assessed by label transfer,[34] in which a biotin-label was transferred from hA1AT (bait) to caspase-3 (prey) after UV-facilitated cross linking and reduction (schematic in Supplementary Figure 2a and Supplementary Figure 2b at *http://ajp.amjpathol.org*).

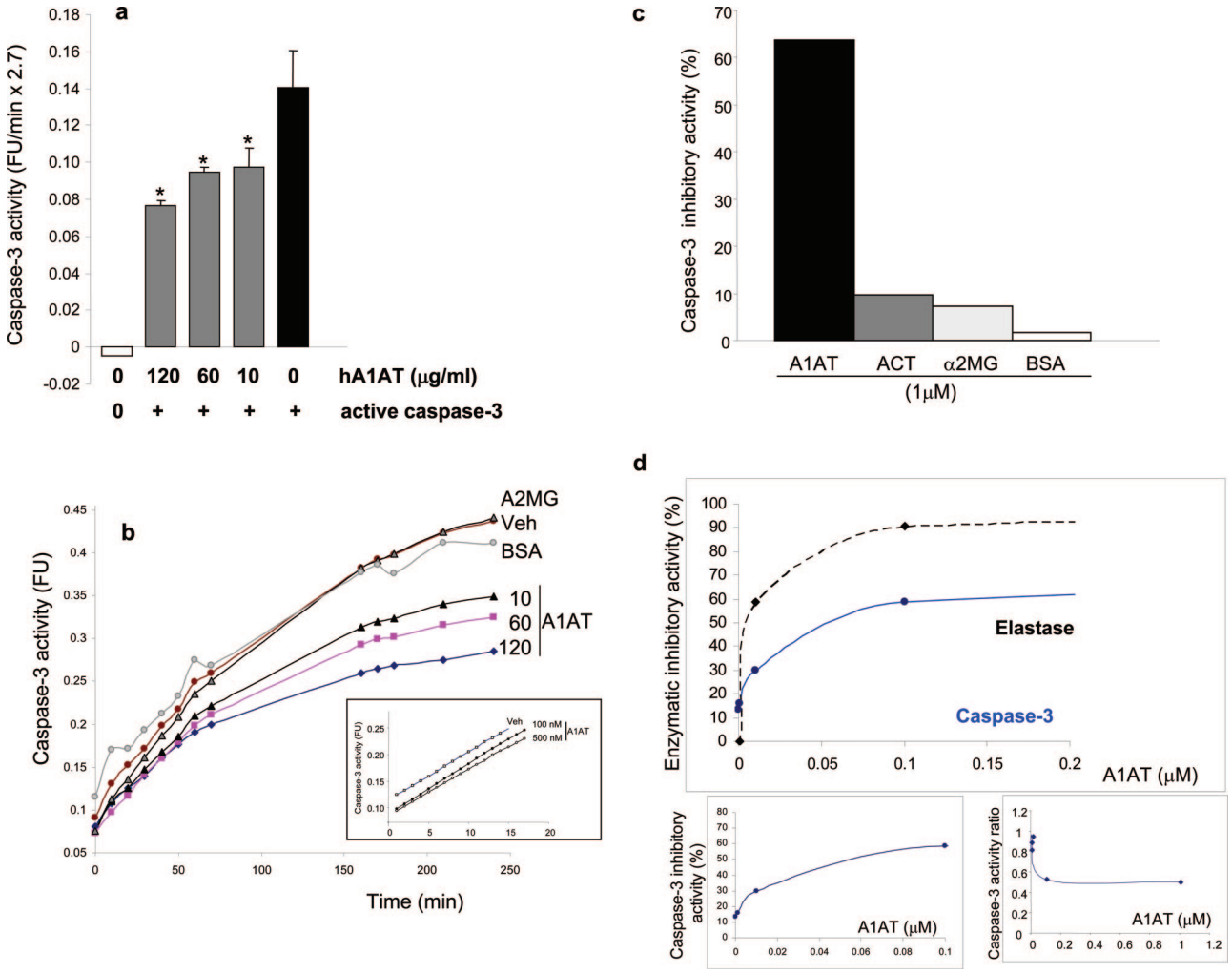

Figure 5. Functional effects of hA1AT on caspase-3 activity in cell-free systems. **a:** Dose-dependent inhibitory effect of hA1AT on the enzymatic activity (expressed in fluorescence signal per unit of time) of recombinant active caspase-3 on the specific substrate DEVD ($P < 0.05$). **b:** Kinetic curves of caspase-3 activity (cleavage of DEVD substrate) measured after incubating recombinant human active caspase-3 with vehicle (**black triangles**) or with bovine serum albumin (**gray circles**, 120 mg/ml, BSA), α-2 macroglobulin (**red squares**; 60 mg/ml, A2MG), or increasing concentrations of hA1AT. **Inset** showing the effects on caspase-3 activity for two lower concentrations of A1AT (100 and 500 nmol/L) compared with vehicle (Veh). **c:** Effect of hA1AT on caspase-3 activity (percent inhibition of maximum) compared with α1-antichymotrypsin (ACT), α2-macroglobulin (α2MG), or serum albumin (BSA) (mean, $n = 2$). **d:** Inhibitory effect of hA1AT on the enzymatic activity of recombinant active caspase-3 versus purified elastase (percent inhibition of maximum activity, representative of $n = 20$). In the **inset** we reported data as ratios of caspase-3 activity (fluorescence units) when incubated with A1AT versus the activity of uninhibited caspase-3, representing the reduction factors for A1AT.

We then investigated the functional effect of the interaction of hA1AT with caspase-3 in a cell-free *in vitro* system. Purified hA1AT, but not bovine serum albumin, α-anti-chemotrypsin, or human α2-macroglobulin (shown to inhibit apoptosis in serum-deprived cultured smooth muscle cells[14]), inhibited dose dependently caspase-3 activity (Figure 5, a–c). The enzymatic activity of recombinant human caspase-3 was measured in fluorometric assays using a specific peptide substrate, DEVD. Specificity of the anti-caspase-3 effect was further suggested by a lack of recombinant human caspase-8 inhibition by hA1AT in a similar assay (not shown). Parallel measurements of caspase-3 and elastase activities, calculated from the slopes of kinetic interactions of active caspase-3 or elastase enzyme with their respective substrate, demonstrated that the inhibitory effect of hA1AT on caspase-3 activity in cell-free conditions is less potent than on elastase activity (Figure 5d). The reduction factors for A1AT against caspase-3 were less than 1 (Figure 5d, inset), showing an inhibitory role of A1AT. The reduction factor is the ratio of caspase-3 activity when incubated with A1AT versus the activity of uninhibited caspase-3. For example, 0.2 μmol/L A1AT reduced caspase-3 activity by 50%, representing an inhibitory effect that is $\sim 10^2$ less than a specific caspase-3 inhibitor, Ac-DEVD-cho. These results altogether suggest an inhibitory, likely noncovalent, interaction between A1AT and the active caspase-3.

Effect of A1AT Conformers on the hA1AT Interaction with Caspase-3

Because posttranslational modifications have been shown to alter the interaction of A1AT with enzymatic substrates, we compared the A1AT conformers' effect on

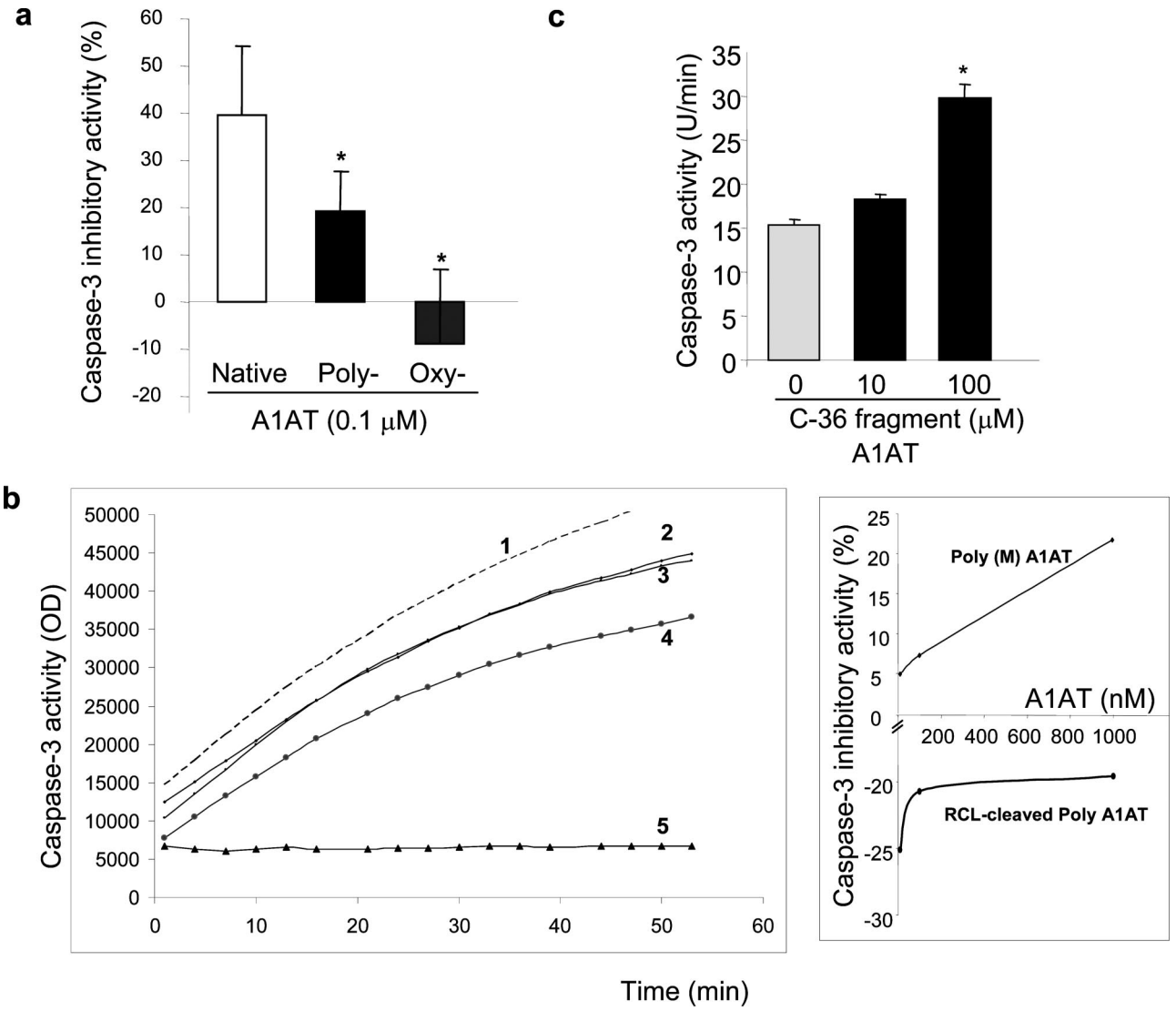

Figure 6. Effect of posttranslational modifications of A1AT on its anti-caspase-3 activity. **a:** Effect of polymerization (poly A1AT; 60°C for 2 hours) and oxidation, obtained by exposure to cigarette smoke extract (Oxy−), on the caspase-3 inhibitory effect of hA1AT (percent inhibition of maximum activity) compared with native hA1AT ($n = 5$, $^*P < 0.05$). **b:** Effects A1AT conformers on caspase-3 activity [activity of uninhibited caspase-3 in buffer is noted as vehicle (3) and is superimposed on the RCL-cleaved poly A1AT (2) curve]. The reactive center loop (RCL)-cleaved A1AT (via *Staphylococcus aureus* protease V8) and the polymerized RCL-cleaved A1AT (1) (via papain) ($n = 2$) were tested against controls that included the above proteases (5) and against polymerized noncleaved hA1AT, Poly(M) (4). The right **inset** depicts the concentration-dependence of the inhibitory activity against caspase-3 for the (M) polymers and for the RCL-cleaved polymers of A1AT. **c:** Effect of the cleaved hA1AT peptide, the C-36 fibrillary fragment, on caspase-3 activity (mean + SEM, $n = 3$; $^*P < 0.05$).

caspase-3 activity to that of the native serpin. Both heat-inactivation of native A1AT,[35] which typically promotes polymerization, and oxidative stress, via exposure to cigarette smoke extract or to hydrogen peroxide, significantly diminished the anti-caspase effect of hA1AT (Figure 6a and data not shown). Because both polymerization and oxidation alter the availability and binding of the reactive center loop of A1AT to elastase, we tested whether an intact reactive center loop is likewise essential for inhibiting caspase-3. A loss of anti-caspase 3 activity was noted with reactive center loop-cleaved A1AT conformers (Figure 6b) and with the A1AT cleavage product C-36, a peptide lacking the reactive center loop (Figure 6c).[36,37] In fact, higher concentrations of the C-36 peptide augmented caspase-3 activity *in vitro* (Figure 6c). These complementary studies documenting the impact of conformers of A1AT on caspase-3 activity argue for the specificity of this interaction and for a putative role for the reactive center loop in mediating the hA1AT inhibition of caspase-3.

Effect of the hA1AT on the Caspase-3-Triggered Lung Injury in Vivo

The anti-caspase-3 effect of A1AT was further tested *in vivo* in the mouse lung. hA1AT augmentation by intramuscular administration of hA1AT-AAV serotype 5, which leads to persistent hA1AT expression,[27] inhibited apoptotic airspace destruction and oxidative stress caused by subsequent (2 weeks after transduction) direct instillation of active caspase-3 into the

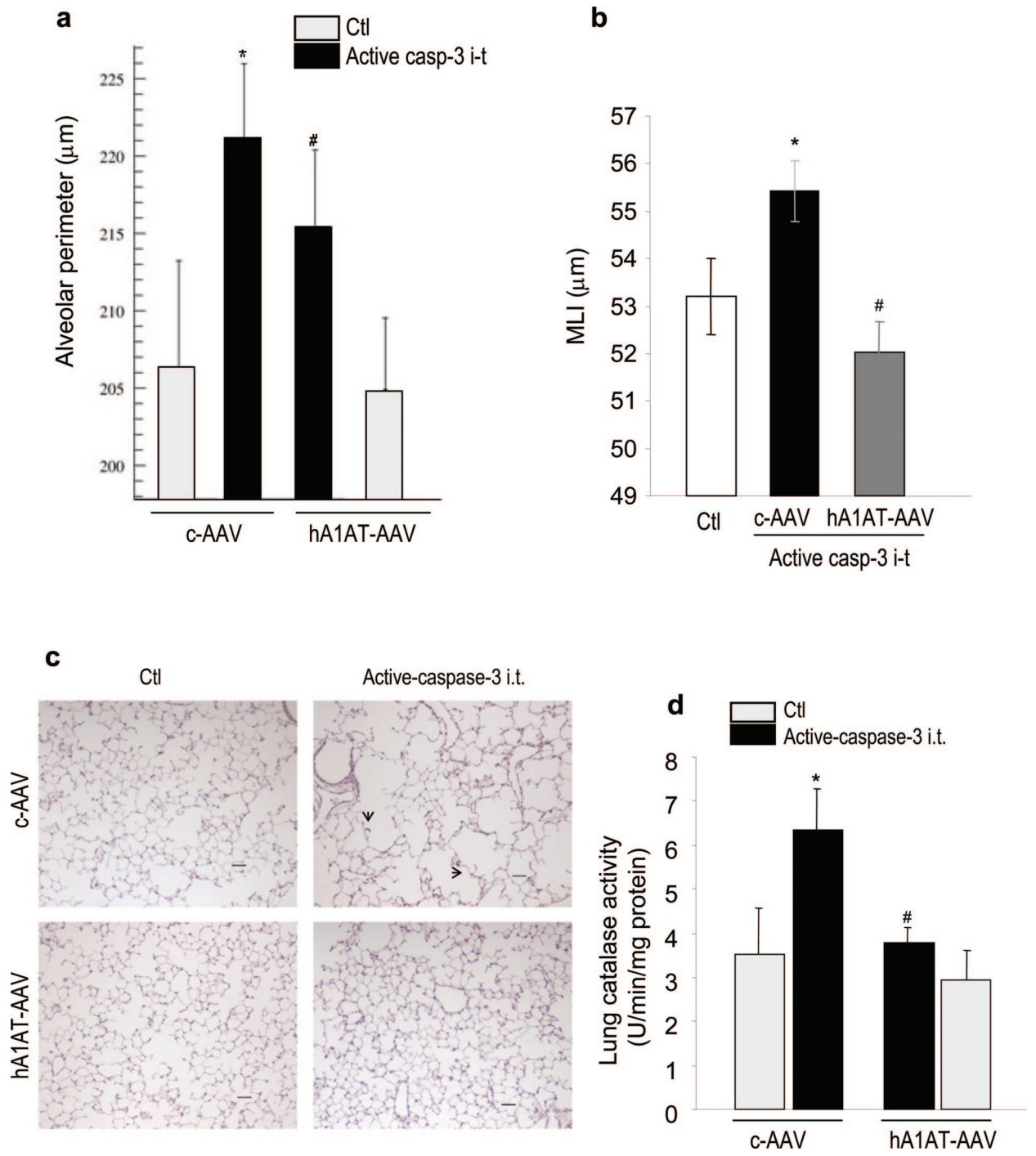

Figure 7. Protective effect of hA1AT on alveolar space destruction and oxidative stress triggered by lung instillation of active caspase-3 in mice. **a:** Morphometric measurements of alveolar perimeters, and **b:** mean linear intercept 48 hours after intratracheal caspase-3 or Chariot vehicle (Ctl) administration in the presence of hA1AT-expressing adeno-associated virus (hA1AT −AAV) or control virus (c-AAV) given by intramuscular injection (mean + SD, $P < 0.05$ versus c-AAV* and versus hA1AT-AAV^{+} caspase-3, analysis of variance, posthoc *t*-test, $n = 4$ to 5 mice per group). **c:** Histology of H&E-stained lung alveolar tissue in mice receiving intratracheal chariot vehicle (Ctl) or caspase-3 in the presence of control virus (c-AAV), or hA1AT-expressing adeno-associated virus (hA1AT −AAV). Note the airspace enlargement in the mice receiving active caspase-3 and empty vehicle (**arrows**), which is attenuated in the presence of overexpressed hA1AT. **d:** Lung catalase activity 2 days after intratracheal instillation of active caspase-3 or Ctl (mean + SEM, $^{*}P < 0.05$ versus ctl $^{\#}P < 0.05$ versus caspase-3). Scale bar = 50 μm.

mouse lung[28] (Figure 7, a–d). The corresponding decrease in mean linear intercept induced by A1AT overexpression was of ~4 μm, from 55.4 μm (±7.8) in caspase-3-instilled, empty virus-expressing lung to 51.3 μm (±6.5) in caspase-3-instilled, hA1AT-expressing lung. The protection against alveolar enlargement achieved by hA1AT overexpression, although incomplete (Figure 7, a and b), was remarkable, as active caspase-3 acts quickly and effectively as executioner of apoptosis and trigger of airspace enlargement.[28] These results suggest a potent anti-apoptotic effect of hA1AT in the lung.

Discussion

Our studies support a novel anti-apoptotic function of hA1AT in the lung, partly accounted for by the inhibition of active caspase-3. These findings therefore broaden the lung protective roles of A1AT beyond its effects on neutrophil-derived proteases. Our studies underscore the benefits of A1AT supplementation in the clinically relevant setting of emphysema and provide the framework for future studies addressing how A1AT is internalized by cells and mechanisms involved in its inhibition of intracellular enzymes.

A1AT is among several serum proteins that can rescue serum withdrawal-induced apoptosis.[14] These tonic pro-cell survival effects of A1AT occur by an unknown mechanism. Our results indicate that the anti-apoptotic effects of hA1AT may be direct, intracellular, and mediated by noncovalent interactions with caspase-3. hA1AT is an acute phase reactant, but its anti-caspase 3 activity was not shared by human α2-macroglobulin, another acute phase reactant shown to protect against *in vitro* and *in vivo* apoptosis. hA1AT inhibits serine proteases via suicidal binding at the reactive center loop,[38] and, likewise, an intact serpin reactive center loop was required for the A1AT-caspase-3 interaction. The functional differences of distinct hA1AT domains were underscored by experimental results with the proteolytic cleavage product of A1AT, the amyloidogenic C-36 fragment, which not only failed to inhibit caspases-3 activity in a cell-free system, but at higher concentration it appeared to enhance it. A similar behavior for this peptide was observed by others in monocytes, where C-36 induced cytochrome *c* and caspase-3 activation,[39] but the physiological significance of this finding in the lung is not yet clear.

Three other serpins, poxvirus CrmA,[40] the endogenous proteinase inhibitor-9,[41] and plasminogen activator inhibitor type-1[42] are endogenous caspase inhibitors, but the mechanism by which they inhibit caspase-3 is not yet fully elucidated. Similarly, the molecular mechanisms involved in the interaction between A1AT and active caspase-3 are not known, as, unlike the prototypical caspase-3 inhibitor XIAP, its structure does not contain a BIR2 domain (NCBI Blast). Furthermore, the A1AT molecule does not have a DXXD site, and we were unable to detect cleavage of A1AT after its interaction with caspase-3 *in vitro* (not shown). Although the data indicate specific inhibition of the active caspase-3 (a cysteine protease), we cannot exclude A1AT binding and inhibiting procaspase-3 or other intracellular serine proteases implicated in apoptosis.[43] Furthermore, we speculate that the lower affinity for caspase-3 (localized primarily intracellularly) compared with elastase (an extracellular enzyme) in the *in vitro* studies may reflect the requirement for different activities and effective concentrations of the A1AT at intracellular versus extracellular locations, respectively.

An important development from our studies will be the investigation of the mechanisms by which hA1AT (native or polymerized) elicits intracellular responses in cells other than hepatocytes (the major synthetic source of A1AT). The nonserpin actions of hA1AT may involve receptor-dependent and/or -independent effects. Although the lung itself is not a major source of A1AT synthesis, local production or accumulation of A1AT[44] may exert important roles. For example, enhanced alveolar cell apoptosis in A1AT deficiency might result from decreased cytoplasmic A1AT levels, and/or accumulation of polymerized or functionally impaired, oxidized A1AT. It remains to be elucidated which pool of A1AT, serum-derived or locally produced, regulates caspase-3 activation in alveolar cells in the lung and which alveolar cells—type II, endothelial, or myofibroblast—can internalize A1AT and thus be protected against apoptosis.

Our findings reveal that supplementation with hA1AT partially prevents apoptosis-dependent emphysema[28] and alveolar cell oxidative injury caused by direct intratracheal instillation of active caspase-3 in mice. These results confirm our observations that intramuscular or intratracheal hA1AT transduction via AAV vectors, while effectively increasing hA1AT protein expression in human alveolar cells, protected against alveolar cell apoptosis, lung oxidative stress, and alveolar wall destruction caused by vascular endothelial growth factor receptor blockade,[21] another murine model of apoptosis-dependent emphysema.[18] Because apoptosis is progressively recognized as a central mechanism of alveolar destruction and skeletal muscle cell loss, it will be important to elucidate how and whether posttranslational modifications of A1AT are involved in the pathogenesis of lung and systemic manifestations of chronic obstructive pulmonary disease.

In summary, our findings revealed that hA1AT may have a novel intracellular anti-proteolytic activity by binding and inactivating active caspase-3, protecting primary lung microvascular endothelial cell from apoptosis, thus broadening its impact in the development of lung disease.

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