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Alpha-1-antitrypsin inhibits nitric oxide production

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ABSTRACT

NO is an endogenously produced gas that regulates inflammation, vascular tone, neurotransmission, and immunity. NO production can be increased by exposing cells to several endogenous and exogenous proinflammatory mediators, including IFN-γ, TNF-α, IL-1β, and LPS. As AAT has been shown to inhibit cell activation and suppress cytokine production associated with proinflammatory stimulation, we examined AAT for NO-suppressive function. In RAW 264.7 murine macrophagic cells, physiological AAT concentrations significantly inhibited combined LPS- and IFN-γ-induced NO synthesis, and NO synthesis inhibition was associated with decreased expression of iNOS, suppressed NF-κB activation, and reduced translocation of extracellular AAT into the interior of RAW 264.7 cells. CE-2072, a synthetic inhibitor of serine proteases, also suppressed NO production, iNOS expression, and NF-κB activation. However, AAT did not alter activation of intracellular MAPKs. In subjects with genetic AAT deficiency, exhaled NO was increased significantly compared with exhaled NO in healthy controls. These in vitro and in vivo studies suggest that AAT is an endogenous inhibitor of NO production. Administering AAT or AAT-like molecules may have use as a treatment for diseases associated with excessive NO production.
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Introduction

Many illnesses are associated with increased NO synthesis, including septic shock, asthma, COPD, fibrosing lung diseases, type 1 diabetes mellitus, inflammatory arthritides, and myocardial reperfusion injury [1–3]. NO is generated predominantly by a family of intracellular NOS isoenzymes that produce NO through oxidation of the guanidino nitrogen of L-arginine. NOS isoenzymes include nNOS (or NOS1), iNOS (or NOS2), and eNOS (or NOS3). iNOS is thought to produce the increased NO observed during many human diseases [4, 5]. iNOS is synthesized primarily in human or murine macrophages and neutrophils, although endothelial cells may also produce iNOS [6–9]. Whereas iNOS protein levels are low or absent in unstimulated cells, iNOS expression increases rapidly in vitro or in vivo following exposure to proinflammatory stimuli that include LPS, TNF-α, IL-1β, IFN-α, IFN-β, IFN-γ, or hypoxia [10–12]. The increased iNOS expression is regulated at the level of transcription. Secondary pathways for NO generation have also been described, including the conversion of nitrate or NO_2^- to NO [13]. However, these alternative pathways are not known to generate the increased NO observed in human illness [1–3].

Endogenous inhibitors antagonize the activity of many proinflammatory molecules. For example, the IL-1R antagonist blocks IL-1 activity, and TNF soluble receptors inhibit TNF-α activity [14, 15]. These endogenous inhibitors are thought to limit the magnitude and spread of inflammation and to assist in terminating the inflammatory response when it is no longer needed. As NO possesses proinflammatory properties and is associated with disease pathogenesis, endogenous inhibitors of NO synthesis are likely available to contain NO activity. One candidate endogenous NO synthesis suppressor is ADMA, which is a naturally occurring amino acid derivative found in the circulation and a competitive inhibitor of NO synthases. Administration of exogenous ADMA is considered as a treatment for diseases associated with excessive NO production, such as septic shock and arthritis [16]. However, the NO-suppressive role of ADMA in vivo is uncertain, as ADMA inhibits NO production in vitro at concentrations approaching eight to 10 times larger than those normally found in the circulation in healthy persons [17].

Abbreviations: AAT=α-1-antitrypsin, ADMA=asymmetric dimethylarginine, AEBSF=4-(2-aminoethyl) benzene sulfonyl fluoride, COPD=chronic obstructive pulmonary disease, FE_{NO}=fractional concentration of NO in exhaled air, NO_2^-=nitrite, NP-40=Nonidet P-40, *p*=phosphorylated, p38=p38 (C-20) MAPK, ppb=parts/billion

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Serine protease activity appears to enhance NO production. For example, a synthetic serine protease inhibitor reduced NO production and iNOS expression in RAW 264.7 macrophagic cells [18]. In separate studies, synthesized chloromethylketone derivatives that irreversibly inactivate serine proteases reduced NO production in vitro [19, 20]. However, these synthetic compounds can also block activities of PKC, adenylate cyclase, cAMP-dependent protein kinase, and cGMP-dependent protein kinase [20]. Moreover, these synthetic molecules have no role as endogenous NO suppressors. These observations led us to consider the possibility that endogenous inhibitors of serine proteases may function as natural NO suppressors.

AAT is a 52-kDa serine protease inhibitor synthesized primarily in the liver and secreted into the circulation with a half-life of 4–5 days [21]. AAT is the most abundant circulating serine protease inhibitor with serum concentrations of 1.5–3.5 mg/mL using radial immunodiffusion assay (0.8–2.0 mg/mL by nephelometry) during health, with levels increasing up to fourfold during acute inflammation [22–25]. Several observations suggest an anti-inflammatory function for AAT. For example, AAT reduces LPS- or IFN-γ-induced TNF-α secretion in isolated human peripheral blood leukocytes [25], AAT suppresses LPS-induced TNF-α and IL-1β production in human monocytes [26–28], and AAT inhibits *Staphylococcus epidermidis*-stimulated cytokine production in human whole blood cultures [29]. As synthetic serine protease inhibitors suppress NO production, and AAT is an endogenous serine protease inhibitor with anti-inflammatory properties, we investigated the effect of AAT on NO production in vitro and in vivo.

MATERIALS AND METHODS

Reagents

AAT was purified from Cohn fraction IV-1 from the plasma of healthy volunteers [30]. Successive anion and cation exchange chromatography yielded functionally active AAT, as assessed by porcine elastase inhibition assay and array nephelometric antigen analysis. The AAT was >94% pure, as determined by SDS-PAGE analysis and HPLC. The AAT vehicle (diluent) consisted of 0.15 M NaCl and 0.02 M NaH_2PO_4, pH 7.05. CE-2072 is a 700-Da peptide-based, selective inhibitor of the endogenous serine proteases neutrophil elastase and proteinase-3 (provided by Cortech, Denver, CO, USA) [31]. The CE-2072 vehicle (diluent) is DMSO. HSA was obtained from Bayer (Elkhart, IN, USA). LPS purified from *Salmonella typhimurium* was obtained from Sigma (St. Louis, MO, USA). Murine IFN-γ was purchased from R&D Systems (Minneapolis, MN, USA). Culture medium consisted of RPMI-1640 medium (BioWhittaker, Walkersville, MD, USA), supplemented with 10% (v/v) heat-inactivated FBS (Atlanta Biologicals, Lawrenceville, GA, USA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Sigma). Rabbit anti-iNOS polyclonal antibody was purchased from Alexis Biochemicals (San Diego, CA, USA). A rabbit polyclonal antibody that detects ERK1/2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A rabbit polyclonal antibody directed against the p38 was also purchased from Santa Cruz Biotechnology. The ERK1/2 and p38 antibodies interact with total (phosphorylated and unphosphorylated) MAPK isoforms. A rabbit polyclonal antibody directed against *p*-ERK1/2 was obtained from Promega (Madison, WI, USA), and a rabbit polyclonal antibody directed against *p*-p38 was purchased from New England Biolabs (Beverly, MA, USA). A rabbit polyclonal antibody directed against β-actin was obtained from Cell Signaling Technology (Danvers, MA, USA). Recombinant c-$Jun_{1–79}$-GST was provided by Dr. Gary Johnson (University of Colorado Denver Anschutz Medical Campus, Denver, CO, USA), and GSH-sepharose beads were purchased from Pharmacia (Piscataway, NJ, USA). Goat anti-human FITC-conjugated AAT antibody was purchased from Bethyl Laboratories (Montgomery, TX, USA). Quantification of cell culture protein concentration was performed using a BCA-based protein assay kit (Pierce, Rockford, IL, USA).

RAW 264.7 cell cultures

RAW 264.7 murine macrophagic cells were provided by Dr. William Murphy (University of Kansas Medical Center, Kansas City, KS, USA) [32] and were used during Passages 25–30. The RAW 264.7 cells tested negative for *Mycoplasma* contamination (PlasmoTest, InvivoGen, San Diego, CA, USA). All RAW 264.7 cell cultures were incubated at 37°C in a 5% CO_2 atmosphere, and LPS or IFN-γ was added to cultures at final concentrations of 1 ng/mL and 10 U/mL, respectively.

NO production in RAW 264.7 cells

During log-phase growth, 2×10^5 RAW 264.7 cells and 1.0 mL culture medium were added to culture wells in 24-well polystyrene tissue-culture plates. Following 24 h of incubation, the RAW 264.7 cells adhered to the culture wells. The culture medium was then aspirated and replaced with fresh culture medium alone, with culture medium containing AAT or CE-2072 or with culture medium containing AAT diluent or CE-2072 diluent. AAT or CE-2072 diluents were added at final concentrations identical to those in the highest AAT (3 mg/mL) or CE-2072 (60 μM) concentrations used. In separate control experiments, 3 mg/mL HSA was added to cultures. After 1 h of incubation, LPS, IFN-γ, or combined LPS and IFN-γ were added to wells except for medium-alone (control) wells. The cultures were then incubated for 18 h. As the NO half-life extends only to several seconds [33], the stable NO metabolite NO_2^- was quantified in culture supernatants using the Greiss reagent as a measure of NO production [34]. Cytotoxicity was measured using a LDH release assay (CytoTox 96 nonradioactive cytotoxicity assay, Promega) and by trypan blue dye exclusion (Mediatech, Herndon, VA, USA).

iNOS Western blot

One million RAW 264.7 cells were added to separate wells in six-well polystyrene tissue-culture plates and incubated in culture medium alone, in culture medium with combined LPS and IFN-γ as stimulus, or in culture medium with combined stimulus in the presence of 3 mg/mL AAT, AAT diluent, or CE-2072 (3.75–60 μM). AAT, AAT diluent, or CE-2072 was added 1 h prior to combined stimulus. All final cell-culture volumes were 2.0 mL, and the cultures were incubated for 18 h after addition of stimulus. Culture supernatants were aspirated and the adherent cells lysed using 0.5 mL Western blot lysis buffer [50 mM Tris/HCl, pH 8.0, 137 mM NaCl, 10% (v/v) glycerol, 1% (v/v) NP-40, 1 mM NaF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 2 mM Na_3VO_4, and 1 mM PMSF]. The cell lysate solutions were transferred into 1.5 mL polypropylene tubes and the samples centrifuged at 20,000 *g* for 10 min. Cell lysate proteins were quantified, and 20 μg protein aliquots from each culture were electrophoresed into SDS-PAGE gels and the separated proteins transferred onto nitrocellulose membranes. The membranes were probed using a rabbit iNOS polyclonal antibody as described previously [35]. The blots were then stripped by immersion in 1% SDS/0.08% β-ME/0.05M Tris, pH 6.8, buffer for 1 h at 50°C. The membranes were then reprobed with a rabbit β-actin polyclonal antibody to visualize protein loading in each lane.

NF-κB EMSA

RAW 264.7 cells were added to separate wells in six-well polystyrene tissue-culture plates (1×10^6 cells in a final volume of 2.0 mL) and incubated in culture medium alone or in culture medium containing 3 mg/mL AAT or 60 μM CE-2072. Following 1 h of incubation, combined LPS and IFN-γ were added to each well, except for medium-alone wells. After 1 h of incubation, the supernatants were removed and the cells mechanically detached using a cell scraper and resuspended immediately in 1 mL ice-cold PBS.

The cell suspensions were transferred into 1.5 mL microfuge tubes and centrifuged at 500 *g* for 5 min at 4°C. After removing the supernatants, the cell pellets were washed with ice-cold PBS and resuspended in 0.5 mL ice-cold EMSA lysis buffer [10 mM HEPES, pH 7.8, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.12% NP-40 (v/v), 2 mM DTT, 1 mM PMSF, 0.5 mM AEBSF, and 10 μg/mL each of aprotinin, leupeptin, and pepstatin A]. Following 10 min incubation on ice, the samples were briefly agitated and centrifuged at 1500 *g* for 10 min at 4°C. The supernatants were discarded and the pellets (containing nuclear protein) washed twice in EMSA lysis buffer. The pellets were resuspended in an extraction buffer containing 0.4 M NaCl, 10 mM HEPES, pH 7.8, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT, 1 mM PMSF, 0.5 mM AEBSF, and 10 μg/mL each of aprotinin, leupeptin, and pepstatin A. The samples were incubated on ice with gentle mixing for 20 min, followed by centrifugation at 20,000 *g* for 10 min at 4°C. The nuclear protein-containing supernatants were removed, nuclear protein concentrations were measured using a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA), and the samples were frozen at −70°C until assayed.

A double-stranded oligonucleotide probe (Promega) containing a NF-κB-binding region was end-labeled using ^{32}P-γ-ATP. Nuclear protein (2 μg) from each culture was incubated at room temperature for 15 min with 10 fmol labeled probe in 20 μl binding reaction mixture consisting of 20 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1% NP-40, 5% glycerol, and 0.8 μg polydeoxyinosinic/deoxycytidylic acid. In parallel (cold probe) competition experiments, nuclear protein from cells stimulated with combined LPS and IFN-γ was incubated with a tenfold molar excess of unlabeled (cold) NF-κB probe. All samples were then separated in a 6% polyacrylamide gel and the gels dried onto Whatman paper and exposed to X-ray film overnight at −70°C.

RAW 264.7 cell experiments were repeated using culture conditions described above. These cultures were assessed for NF-κB activation using a quantitative ELISA-based assay as instructed by the manufacturer (TransAM NF-κB p65 activation assay, Active Motif, Carlsbad, CA, USA).

AAT internalization into RAW 264.7 cells

RAW 264.7 cells (2.5×10^5) were pipetted into each of four polystyrene tissue-culture chambers mounted on a single detachable glass microscope slide (Nunc, Naperville, IL, USA). One chamber of each culture slide contained medium alone, and three chambers contained culture medium with AAT at a final concentration of 3 mg/mL. After 1 h of incubation, combined LPS and IFN-γ stimulation was added to one AAT-containing chamber. The final volume in each chamber was 1.0 mL. Cultures were incubated for 18 h, supernatants were aspirated, the adherent RAW 264.7 cells were washed four times with PBS, and the cells were fixed by adding 0.5 mL 4% paraformaldehyde (Fisher, Fair Lawn, NJ, USA) for 10 min at room temperature. The paraformaldehyde was aspirated, and cells were washed four times with PBS. RAW 264.7 cells in two chambers were permeabilized (one chamber with AAT alone and one containing AAT with combined LPS and IFN-γ) by adding 0.5 mL 0.1% Triton X-100 (Fisher) in PBS at room temperature for 2.5 min. Cells in the chamber exposed to medium alone and one of the AAT-only culture served as nonpermeabilized controls and received 0.5 mL PBS in place of the Triton X-100. Supernatants were removed and all chambers washed four times with 0.5 mL PBS with 0.1% BSA. A 1.0-mL solution containing FITC-conjugated goat anti-human AAT antibody (diluted 1:200 in PBS/0.1% BSA) was added to all chambers except the chamber containing culture medium alone (this chamber received 1.0 mL PBS/0.1% BSA). Each chamber slide was incubated for 20 min at room temperature, the chamber contents aspirated and washed four times with PBS/0.1% BSA, and the glass slides with adherent cells detached from the overlying chambers. DAPI-mounting medium (100 μL; Vector Laboratories, Burlingame, CA, USA) was applied on each slide and spread using a cover slip to expose all adherent cells to DAPI. The cells were imaged using a Nikon E600 fluorescent microscope, photographed with a Diagnostic Instruments Spot RT Slider camera, and analyzed using Image-Pro version 4.5 software (Media Cybernetics, Bethesda, MD, USA).

MAPK Western blot

One million RAW 264.7 cells were pipetted into separate wells in six-well polystyrene tissue-culture plates with culture medium alone, 3 mg/mL AAT, or AAT diluent. After 1 h incubation, LPS, IFN-γ, or combined LPS and IFN-γ were added to the cultures except for medium-alone cultures. All final culture volumes were 2.0 mL. After 1 h of incubation, culture supernatants were aspirated and the adherent cells lysed using 0.5 mL Western blot lysis buffer (as described for iNOS Western blot). The cell lysate solutions were transferred into 1.5 mL polypropylene tubes and centrifuged at 20,000 *g* for 10 min. Protein aliquots (20 μg) of lysate solution from each culture were run into SDS-PAGE gels and the separated proteins transferred onto nitrocellulose membranes. Primary antibodies that specifically recognized *p*-ERK1/2 or *p*-p38 (activated) MAPK proteins were diluted in 1% BSA and incubated with membranes for 1 h. Bound primary antibodies on each membrane were detected using HRP-conjugated secondary anti-rabbit antibody and ECL. To assess loading of proteins, the primary MAPK antibodies were stripped from each membrane (as described for iNOS Western blot experiments), and each membrane was reprobed with rabbit polyclonal antibodies directed against total ERK1/2 or p38.

Assessment of JNK activity

RAW 264.7 cells were cultured under the same conditions as described above for MAPK Western blot studies. Cell lysates were obtained as described for MAPK Western blot methods, and JNK function was detected by measuring radioactive phosphate incorporation into a c-Jun_{1-79}-GST substrate as described previously [34].

Exhaled NO measurement

NO concentration in exhaled air from human subjects was measured using a rapid response chemiluminescent analyzer (Model 280, GE Instruments, Boulder, CO, USA) with a lower limit of detection of 1 ppb NO. The breathing circuit consisted of a mouthpiece connected to a Hans Rudolph valve in which inhaled ambient air was passed through a filter to remove exogenous NO. Daily calibration was performed with a zero gas devoid of NO and a standard 25-ppb NO gas (Scotts Specialty Gases, Plumsteadville, MA, USA). The NO sampling rate was 200 mL/min. After obtaining Institutional Review Board-approval and informed consent, nine healthy control subjects and five subjects with documented AAT deficiency were studied. The healthy control group comprised six (67%) males with a group mean age of 46.1 ± 3.3 years (mean±SEM). The AAT-deficient subjects were homozygous for the mutant Z-type AAT variant (PiZZ; determined using isoelectric focusing). Serum AAT concentration in each AAT-deficient subject was significantly below the normal assay range (data not shown). The AAT-deficient group comprised four (80%) males with a group mean ± SEM age of 50.4 ± 4.3 years. All AAT-deficient patients had COPD and were receiving i.v. AAT supplementation. These subjects were studied immediately prior to AAT infusion, when serum AAT concentrations were at the nadir. In a separate study using an identical experimental protocol, exhaled NO measurements were obtained from 15 subjects with COPD in the absence of AAT deficiency [36]. The COPD group comprised 10 (67%) males with a group mean ± SEM age of 59.3 ± 5.7 years.

Exhaled NO concentration was measured using a restricted breath technique according to established recommendations [37]. Subjects exhaled into a resistance while targeting a fixed mouth pressure displayed on a monitor, which closed the velum and excluded nasal NO from exhalations. The fixed mouth pressure and resistance created a constant exhalation airflow rate of 50 mL/s. The FE_{NO} (expressed as ppb) showed a washout phase followed by a steady NO plateau measurement. The NO plateau values were defined as the FE_{NO} in each measurement. Repeated exhalations in each subject were performed until three FE_{NO} values within 5% of each other were obtained. The mean of these three measurements was defined as the final FE_{NO} (exhaled NO) for each subject.

Statistical analysis

Replicate experiments were independent, and summary results are presented as the mean ± SEM or as the mean + SEM. In cell culture experiments shown in **Fig. 1**, differences in NO_2^- concentration were compared using the Freedman test with Dunn's Multiple Comparison assessment (Prism, GraphPad Software, La Jolla, CA, USA). Percent inhibitory effects of AAT, CE-2072, or respective diluents for data shown in Fig. 1 were derived by first calculating percent NO_2^- production in each sample using the following formula: (sample NO_2^-−medium-alone NO_2^-/combined stimulus-alone NO_2^-−medium-alone NO_2^-) × 100.

With the use of this calculation, percent NO_2^- production in the combined stimulus-alone samples (LPS and IFN-γ) was set to 100% in each experiment. The percent inhibition of NO_2^- for each sample was calculated by subtracting the percent NO_2^- production from 100%. The percent inhibition for each experimental condition represented in Fig. 1 was calculated as the mean of percent inhibitions in replicate experiments. For the statistical analysis of exhaled NO levels (see Fig. 6), the group differences were compared using Tukey's honestly significant difference test for multiple comparisons [38]. Differences between groups (see Figs. 1 and 6) were considered significant for $P < 0.05$.

RESULTS

Effect of AAT and CE-2072 on stimulated NO production in RAW 264.7 cells

The amount of NO was small or undetectable in cells cultured in medium alone or exposed to LPS or IFN-γ alone (Fig. 1A; three bars on the left). In contrast, a marked increase in NO production was observed in cells exposed to combined LPS and IFN-γ stimulation. Combined stimulation of cells in the presence of 0.1–3 mg/mL AAT resulted in a significant dose-dependent reduction in NO compared with combined stimulation alone. Compared with combined stimulation alone, an inhibitory effect of 81% was observed with 1 mg/mL AAT ($P<0.001$), and a maximum inhibitory effect of 93% was observed using 3 mg/mL AAT ($P<0.001$).

The AAT diluent was added to combined LPS- and IFN-γ-stimulated cultures at a concentration corresponding to diluent in 3 mg/mL AAT. As shown in Fig. 1A (far right bar), no significant inhibitory effect of the AAT diluent on stimulated NO production was detected. In separate experiments, we assessed the effect of a non-AAT human protein by adding 3 mg/mL HSA to RAW 264.7 cultures 1 h prior to combined LPS and IFN-γ stimulation. HSA did not affect stimulated NO production (three separate experiments; data not shown). We also investigated the AAT effect on cell viability by quantifying LDH release from RAW 264.7 cells and exclusion of trypan blue vital dye. With the use of both assays, 3 mg/mL AAT did not affect the viability of combined LPS and IFN-γ-stimulated cells (data not shown).

As AAT is the prototype endogenous serine protease inhibitor [21], we attempted to replicate the NO inhibitory effects of AAT shown in Fig. 1A using CE-2072, a synthetic inhibitor of serine proteases. As shown in Fig. 1B, CE-2072 inhibited combined LPS and IFN-γ-induced NO production dose-dependently. A significant 25% inhibition was observed at 3.75 μM CE-2072 ($P<0.001$), and a maximum 89% inhibition was obtained using 60 μM CE-2072 ($P<0.001$).

The CE-2072 diluent (DMSO) was added to combined LPS and IFN-γ-stimulated cultures at a concentration corresponding to DMSO in 60 μM CE-2072. As shown in Fig. 1B (far right bar), addition of the CE-2072 diluent to stimulated cultures resulted in a small but statistically significant reduction in NO compared with cultures with combined stimulus alone (13% inhibition; $P<0.001$).

Effect of AAT and CE-2072 on iNOS expression in RAW 264.7 cells

NO production in RAW 264.7 cells following stimulation with LPS and IFN-γ is associated with increased expression of intracellular iNOS [39, 40]. Therefore, we investigated the effect of AAT on iNOS protein expression. As shown in **Fig. 2A**, cultures stimulated with LPS and IFN-γ (lane 2) showed a marked increase in iNOS protein compared with cells cultured in medium alone (lane 1). The presence of AAT substantially inhibited stimulated iNOS protein production (lane 3). In contrast, the AAT diluent had no significant effect on stimulated iNOS expression (lane 4).

We also investigated the effect of CE-2072 on iNOS protein expression in RAW 264.7 cells. Compared with cultures stimulated with combined LPS and IFN-γ, iNOS was dose-dependently suppressed by increasing concentrations of CE-2072 (Fig.

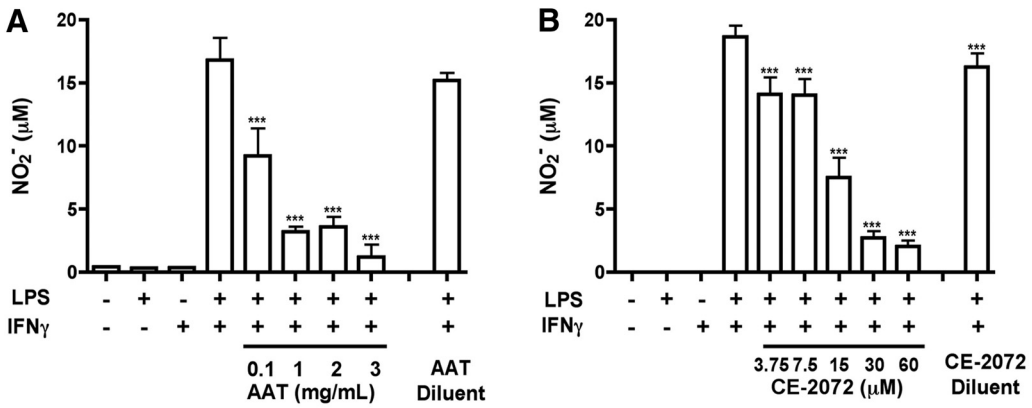

Figure 1. Effect of AAT or CE-2072 on stimulated NO production in RAW 264.7 cells. RAW 264.7 cells were incubated for 18 h with culture medium alone (far left bars); with LPS, IFN-γ, and combined LPS and IFN-γ; or with combined LPS and IFN-γ in the presence of AAT or CE-2072 (AAT and CE-2072 concentrations indicated below the horizontal axes). Stimulation with combined LPS and IFN-γ was also conducted in the presence of AAT or CE-2072 diluents (far right bars). Vertical axes show supernatant levels of the stable NO metabolite NO_2^-. Results are shown for experiments using AAT (A; three separate experiments) or CE-2072 (B; four separate experiments). Data are presented as means + SEM. ***$P < 0.001$ compared with cells with combined LPS and IFN-γ alone.

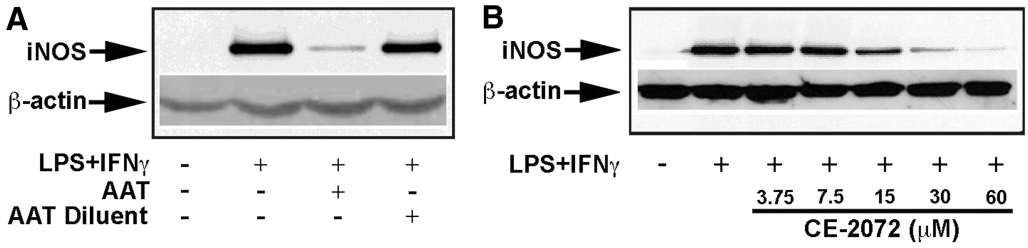

Figure 2. Effect of AAT or CE-2072 on stimulated iNOS expression in RAW 264.7 cells. RAW 264.7 cell Western blots were probed for iNOS protein after 18 h of incubation. (A) Cells were incubated with culture medium alone (lane 1), with combined LPS and IFN-γ as stimulus (lane 2), or with combined stimulus in the presence of 3 mg/mL AAT (lane 3) or AAT diluent (lane 4). (B) RAW 264.7 cells were incubated with culture medium alone (lane 1), with LPS and IFN-γ (lane 2), or with LPS and IFN-γ in the presence of CE-2072 at the concentrations indicated (lanes 3–7). β-Actin was probed to demonstrate equivalent loading of protein into each lane in A and B. Results shown are representative of three separate experiments.

2B; compare lanes 3–7 with lane 2). Inhibition was detectable at 15 μM CE-2072 (lane 5), and near-complete suppression was obtained using 60 μM CE-2072 (lane 7).

Effect of AAT and CE-2072 on NF-κB activation in RAW 264.7 cells

Two NF-κB-binding sites on the 5′-flanking region of the *iNOS* promoter are critical for initiating *iNOS* transcription following stimulation with combined LPS and IFN-γ [39, 40]. As AAT can inhibit NF-κB activation [30, 41, 42], we assessed the effect of AAT and CE-2072 on NF-κB activation in RAW 264.7 cells using the EMSA. As shown in **Fig. 3**, cells incubated in culture medium alone (lane 1) showed little nuclear NF-κB that bound labeled probe. In cells stimulated with combined LPS and IFN-γ, there was a substantial increase in NF-κB-containing complexes (lane 2). Stimulation in the presence of 3 mg/mL AAT (lane 3) or 60 μM CE-2072 (lane 4) resulted in significant reduction of NF-κB complexes compared with stimulus alone. The specificity of the NF-κB-probe interaction was confirmed by incubating nuclear extract from stimulated cells with labeled probe in the presence of a tenfold excess of unlabeled oligonucleotide probe (cold probe). As shown in lane 5, cold probe blocked the formation of complexes consisting of nuclear NF-κB and the ^{32}P-labeled oligonucleotide probe. In separate experiments, using an ELISA-based assay to quantify activated NF-κB, AAT significantly suppressed NF-κB activation in stimulated RAW 264.7 cells (data not presented).

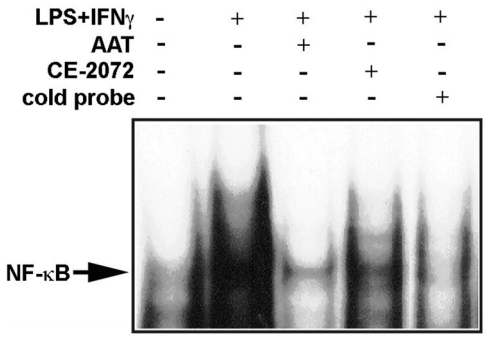

Figure 3. Effect of AAT or CE-2072 on NF-κ B activation in RAW 264.7 cells. With the use of an EMSA, cells were incubated with culture medium alone (lane 1), with combined LPS and IFN-γ as stimulus (lane 2), or with combined stimulus in the presence of 3 mg/mL AAT (lane 3) or 60 μM CE-2072 (lane 4). Nuclear proteins were isolated and incubated with a ^{32}P-labeled oligonucleotide probe containing a consensus sequence that binds nuclear NF-κB. The NF-κB-probe complexes were separated and subjected to autoradiography. In lane 5, nuclear protein obtained from combined LPS and IFN-γ-stimulated cells was exposed to labeled probe and an excess of nonradiolabeled NF-κB probe (cold probe). The arrow indicates activated NF-κB complexes. Results shown are representative of three separate experiments.

Internalization of AAT into RAW 264.7 cells

We considered the possibility that AAT accesses the interior of RAW 264.7 cells and interacts with intracellular molecules that result in NF-κB inhibition. Permeabilization of RAW 264.7 cells allowed detection of intracellular AAT using a FITC-labeled anti-AAT antibody, and confocal immunofluorescence microscopy was used to demonstrate AAT internalization into the cells. Cells incubated with culture medium alone showed no background immunofluorescence (**Fig. 4A**). In Fig. 4B, cells were incubated with AAT alone, and AAT was washed from the culture supernatants after 18 h. These nonpermeabilized cells exposed to FITC-conjugated anti-AAT antibody showed very little signal. As shown in Fig. 4C and D, permeabilized cells cultured in the absence (Fig. 4C) or presence (Fig. 4D) of combined LPS and IFN-γ stimulation demonstrated substantial intracellular localization of FITC-conjugated anti-AAT antibody bound to AAT. Stimulation with combined LPS and IFN-γ increased amounts of intracellular AAT compared with cells cultured with AAT alone, as confirmed using Image-Pro version 4.5 software (not presented).

Effect of AAT on MAPK phosphorylation and activation

As MAPKs transmit signals within cells through three principal MAPK family members comprised of ERK (ERK1 and ERK2), p38, and JNK, we considered the possibility that AAT inhibition of stimulated NO associated with modulation of MAPK activity in RAW 264.7 cells. A marker of MAPK activation is phosphorylation of MAPK proteins. As shown in **Fig. 5A** and **B**, little or no *p*-ERK1/2 or *p*-p38 was detectable in unstimulated cells (lane 1) or in cells exposed to IFN-γ alone (lane 3). However, in cells exposed to LPS alone (lane 2) or to combined LPS and IFN-γ (lane 4), there were significant increases in the amounts of *p*-ERK1/2 and *p*-p38. In cells stimulated with combined LPS and IFN-γ in the presence of 3 mg/mL

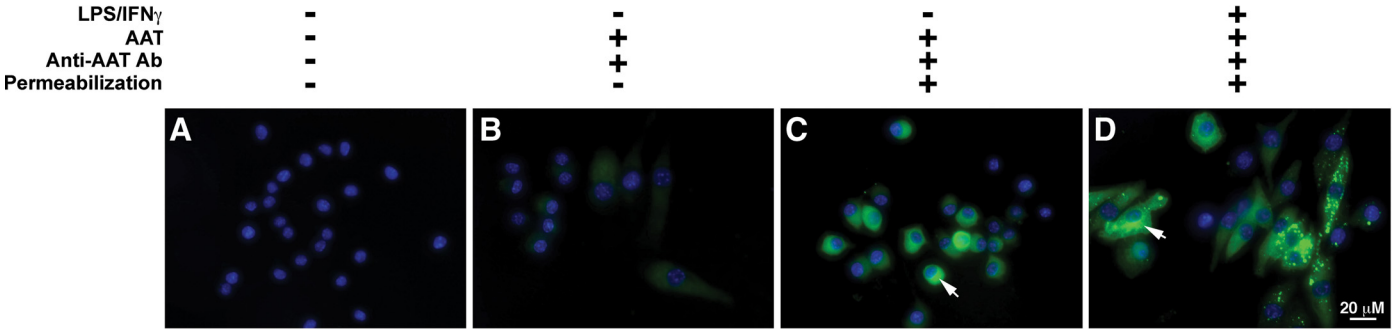

Figure 4. AAT internalization into RAW 264.7 cells. Nonpermeabilized RAW 264.7 cells were incubated for 18 h with culture medium alone (A) or with 3 mg/mL AAT and anti-AAT antibody (B). Permeabilized RAW 264.7 cells were incubated with AAT and anti-AAT antibody (C) or with AAT and anti-AAT antibody in the presence of combined LPS and IFN-γ (D). Results shown are representative of three separate experiments. Arrows indicate intracellular AAT (green), and the blue signal indicates nuclear (DAPI) staining. The bar at the bottom of D shows scale.

AAT, there was only a modest reduction in *p*-ERK1/2, and no substantial AAT effect was observed on *p*-p38 (compare lanes 6 and 4). The same membranes were also probed for total ERK1/2 and p38 proteins (lower immunoblots in Fig. 5A and B). As depicted, the amounts of ERK1/2 and p38 demonstrated approximately equal loading for each experimental condition.

The AAT effect on JNK phosphorylation in RAW 264.7 cells was assessed using an in vitro kinase activity assay. As shown in Fig. 5C, compared with cells incubated in culture medium alone (lane 1), c-Jun-GST phosphorylation was increased substantially following exposure of cells to LPS (lane 2) or to LPS and IFN-γ (lane 4). IFN-γ alone did not increase c-Jun-GST phosphorylation compared with medium alone (compare lanes 3 and 1). Comparing lanes 6 and 4 shows that AAT did not notably affect combined LPS and IFN-γ-induced *p*-JNK activation. The AAT diluent had no significant effect on activation of any of the MAPKs (compare lanes 5 and 4 in Fig. 5A–C).

We also tested CE-2072 for effect on MAPK activation in RAW 264.7 cells. Culture conditions and evaluation of ERK, p38, and JNK activation were performed using the same methods as described for Fig. 5A–C. CE-2072 did not affect activation of ERK1/2, p38, or JNK (data not shown).

Exhaled NO in healthy volunteers and in AAT-deficient subjects

As AAT inhibited NO production in vitro, we anticipated association between reduced endogenous AAT levels and increased NO production in humans in vivo. We measured exhaled NO concentrations in nine healthy volunteers (controls) and in five subjects with a specific genetic abnormality resulting in reduced circulating and tissue levels of AAT (AAT deficiency). As depicted in **Fig. 6**, the mean exhaled NO concentration in the nine controls was 17.1 ppb. In contrast, mean exhaled NO was 44.2 ppb in the AAT-deficient group, which is 2.6 × the mean level in controls ($P<0.05$). Exhaled NO was also measured in 15 stable patients with smoking-associated COPD who did not have AAT deficiency (COPD), and a mean exhaled NO concentration of 26.1 ppb was obtained. There was no significant difference between mean exhaled NO in the COPD group compared with mean exhaled NO in controls or in AAT-deficient subjects.

DISCUSSION

NO possesses antimicrobial and tumoricidal effects and participates in neurotransmission, bronchodilation, and vasodilation [1, 2, 43, 44]. However, excessive NO production can be detrimental, especially during inflammation or sepsis. Harmful NO effects may include hypotension, cardiac depression, or immunosuppression [4, 5, 45, 46]. We surmised that the presence of endogenous inhibitors of NO synthesis in blood may contain NO effects in tissues. As the endogenous serine protease inhibitor AAT has documented anti-inflammatory activities [25–29] and is present in the circulation at high concentrations during health, we considered the possibility that AAT functions as a circulating inhibitor of NO production.

In murine RAW 264.7 macrophagic cells stimulated with combined LPS and IFN-γ, physiological AAT concentrations significantly inhibited NO production. AAT biological effects are thought to result from inhibition of endogenous serine proteases such as elastase, proteinase-3, trypsin, chymotrypsin, plasmin, thrombin, plasminogen, kallikrein, and clotting factor Xa [21, 47]. However, AAT may possess biological effects separate from serine protease inhibition [27, 29]. Therefore, we used the synthetic serine protease inhibitor CE-2072 to show that serine protease inhibition may be a mechanism of AAT-induced NO suppression. CE-2072 also significantly reduced stimulated NO production in RAW 264.7 cells using molar concentrations similar to AAT molar concentrations. Therefore, the NO-suppressive effects of AAT and CE-2072 suggest that serine protease inhibition is a mechanism of AAT NO suppression. As the inhibitory activity of CE-2072 is restricted to the serine proteases elastase and proteinase-3 [31], elastase or proteinase-3 activation may induce NO production in RAW 264.7 cells.

AAT inhibitory effects on NO production in RAW 264.7 cells were not a result of cytotoxicity and were not attributable to the nonspecific presence of human protein. Although the AAT diluent did not affect stimulated NO production, the CE-

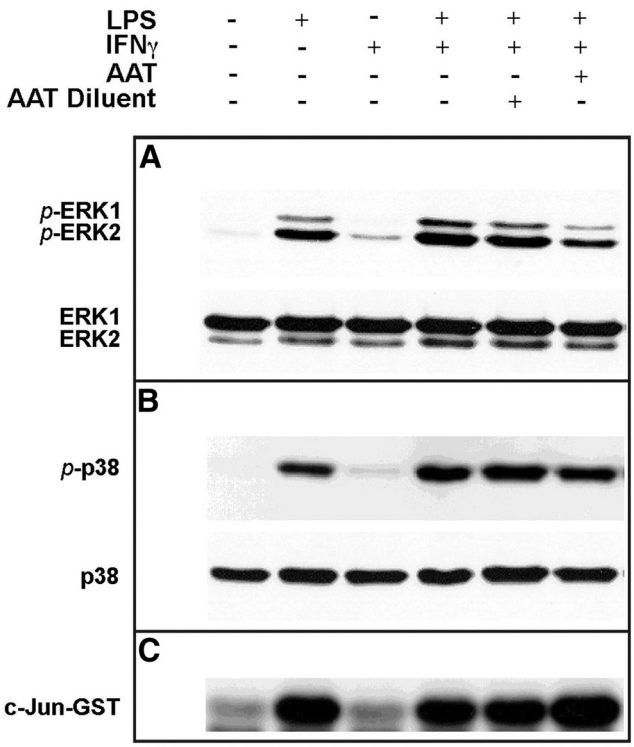

Figure 5. AAT effect on MAPK activation in RAW 264.7 cells. RAW 264.7 cells were incubated with culture medium alone (lane 1), with LPS (lane 2) or IFN-γ (lane 3), with combined LPS and IFN-γ stimulation (lane 4), or with combined stimulus in the presence of AAT diluent (lane 5) or 3 mg/mL AAT (lane 6). Nuclear-free cell lysate proteins were separated by electrophoresis, and Western blot membranes were probed with antibodies to phosphorylated and total MAPK proteins. Separate lysates were used for in vitro c-June kinase activity assay (see Materials and Methods). (A) Bands indicate *p*-ERK1/2 and total ERK1/2; (B) bands indicate *p*-p38 and total p38 protein; (C) bands show radiolabeled c-Jun-GST product that indicates JNK function in the cell lysates. Results shown in A–C are representative of three separate experiments.

2072 diluent (DMSO, added at the same concentration present in 60 μM CE-2072) showed a small but statistically significant 13% inhibitory effect on stimulated NO production (Fig. 1B). This degree of inhibition was small compared with the 89% inhibition observed using 60 μM CE-2072, which included the same DMSO concentration. A reanalysis of the data shown in Fig. 1B revealed that NO suppression in stimulated RAW 264.7 cells using each CE-2072 concentration tested was significantly larger than the inhibitory effect of the DMSO diluent alone ($P<0.01$ for each comparison).

The mechanism of AAT-induced NO suppression in RAW 264.7 macrophagic cells was examined. AAT and CE-2072 reduced combined LPS- and IFN-γ-stimulated iNOS expression. These results suggest that AAT-induced NO suppression involved blockade of iNOS production and that serine protease inhibition may account for this effect.

NF-κB activation can induce iNOS expression by binding to recognition sequences in the *iNOS* promoter [12, 32, 39, 40, 48]. As AAT has been shown to inhibit NF-κB activation [30, 41, 42], reduced NF-κB activity may explain AAT inhibition of iNOS synthesis and NO production. Our studies revealed that AAT substantially reduced NF-κB activation in stimulated RAW 264.7 cells, suggesting that NF-κB inhibition is a mechanism of AAT-induced NO suppression. As CE-2072 also inhibited NF-κB activation, inhibition of endogenous serine proteases may account for reduced NF-κB activation.

As activation of the MAPK family members ERK, p38, and JNK have been shown to induce *iNOS* transcription [34, 49, 50], we evaluated these MAPKs for roles in AAT-induced NO suppression in our studies. Activation of ERK1/2, p38, or JNK was not notably affected by AAT, suggesting that AAT suppression of NO does not involve altered MAPK activation. Further research is necessary to determine whether AAT affects other signaling pathways that mediate iNOS expression.

As AAT is not known to initiate signaling events from the exterior of cells, we considered the possibility that AAT inhibits NO production through intracellular activities. Although previous reports document internalization of exogenously added AAT into human $CD4^+$ T cells, porcine pulmonary artery endothelial cells, and mouse lung cells [42, 51, 52], no prior studies showed AAT internalization into macrophagic cells. We observed internalization of extracellular AAT into stimulated RAW 264.7 macrophagic cells, suggesting that suppression of stimulated NO production is a result of AAT events occurring within cells. Specifically, blockade of NF-κB activation following AAT internalization may explain the NO-suppressive effect of AAT. Our data are consistent with those in Zhou et al. [42], who showed that AAT can enter $CD4^+$ T cells and suppress NF-κB activation by inhibiting degradation of the cytoplasmic NF-κB inhibitor IκBα. Collectively, our experiments examining NO production in RAW 264.7 macrophagic cells suggest that extracellular AAT gains access to the intracellular compartment and suppresses NF-κB activation. This likely results in subsequent suppression of iNOS synthesis and decreased NO production.

We attempted to extend our in vitro studies to an in vivo investigation in humans. As lung epithelial cells possess iNOS

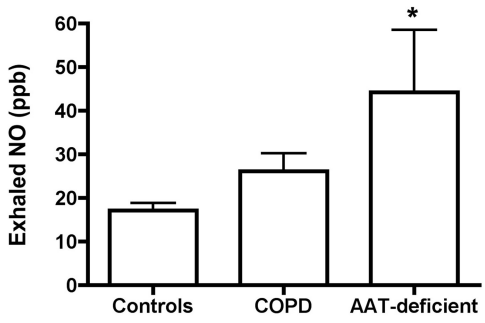

Figure 6. NO quantification in exhaled air. NO concentration was measured in exhaled air and expressed as fractional concentration in ppb. Exhaled NO is shown for healthy subjects (Controls; $n=9$), for patients with smoking-associated COPD (COPD; $n=15$), and for individuals with AAT deficiency (AAT-deficient; $n=5$). Means + SEM NO concentrations are shown on the vertical axis. $^*P < 0.05$ compared with controls.

[53], and lung tissues generate NO [54], NO can be quantified noninvasively in exhaled air [55]. It is of interest that lung epithelial iNOS appears to be the principal source of exhaled NO [53], as we demonstrated an AAT-suppressive effect on iNOS synthesis in RAW 264.7 cells. We measured exhaled NO in healthy volunteers and in patients with AAT deficiency. The AAT-deficient subjects were homozygous for the mutant Z-type AAT variant that contains a single-point mutation at aa 342 (Glu→ Lys). This mutation results in serum AAT concentrations <15% of normal, often leading to premature emphysema that is thought to be a result of unopposed protease activity in lung tissues [21, 56]. As our in vitro results showed that AAT can function as a NO inhibitor, we anticipated increased exhaled NO in persons with AAT deficiency. As predicted, AAT-deficient subjects had a significantly elevated mean exhaled NO level compared with healthy controls. Subjects with smoking-associated COPD who were not AAT-deficient had a mean exhaled NO concentration between those of the healthy control group and the AAT-deficient group.

Few previous studies investigated exhaled NO in AAT-deficient subjects. Two groups reported unexpected low levels of exhaled NO in subjects with AAT deficiency. Machado et al. [57] noted significantly reduced exhaled NO in 19 persons with AAT deficiency compared with 22 healthy control subjects. In the second report, Malerba et al. [58] showed lower exhaled NO levels in nine AAT-deficient persons compared with levels in 19 healthy controls, and exhaled NO was also lower in AAT-deficient persons compared with levels in nine matched persons with normal AAT phenotypes. Machado et al. [57] proposed two mechanisms to explain reduced exhaled NO in AAT-deficient subjects. The first mechanism proposes that NO produced in the lungs in AAT-deficient subjects is consumed by interaction with neutrophil-induced superoxide [57, 59]. The second mechanism proposes that neutrophil-derived enzymes metabolize NO_2^- [57, 60]. As NO^{2-} is a known NO precursor [13, 57] that may serve as a NO storage pool in the lung [57, 61], neutrophil-induced NO^{2-} depletion may reduce exhaled NO in AAT-deficient subjects. Both proposed mechanisms rely on increased activity of pulmonary neutrophils. It is therefore of note that increased neutrophils have been observed in lung tissues from AAT-deficient subjects [62–64]. Either mechanism may explain low exhaled NO, despite increased lung NO synthesis in AAT-deficient subjects.

A more recent publication from Malerba et al. [65] examined exhaled NO in 31 AAT heterozygous persons who harbored normal AAT protein and the AAT-deficiency protein. These AAT heterozygous subjects had significantly lower plasma AAT concentrations compared with plasma AAT in 30 healthy control subjects. In contrast to the two studies discussed above, exhaled NO was increased significantly in the AAT heterozygous group compared with healthy controls. Furthermore, there was a statistically significant association between reduced serum AAT levels and increased exhaled NO in the AAT heterozygous subjects. These results suggest that AAT functions as a dose-dependent suppressor of exhaled NO and supports our data showing increased exhaled NO in AAT-deficient persons.

Assessing our results in the context of prior studies of exhaled NO in AAT-deficient persons suggests two reasons for discrepancies. First, as discussed above, increased pulmonary neutrophils in AAT-deficient patients in the Machado et al. study [57] and the first Malerba et al. study [58] may have suppressed exhaled NO. Second, exogenous AAT given to AAT-deficient patients may have also suppressed exhaled NO in these studies. Clinical guidelines suggest that i.v. AAT replacement therapy be considered in AAT-deficient patients with obstructive lung disease [66]. Machado et al. [57] noted that some (but not all) of the 19 AAT-deficient patients who they studied were receiving AAT replacement therapy, and no mention is made regarding the timing of exhaled NO measurements relative to AAT infusion. In the first Malerba et al. study [58], it is not disclosed whether AAT-deficient persons received exogenous AAT replacement therapy. Therefore, exogenous AAT, given to AAT-deficient persons in these two studies, may have suppressed exhaled NO to levels below those of control subjects. If exogenous AAT administration were associated with a decrease in exhaled NO in these studies, the results may be consistent with a suppressive role for AAT in exhaled NO. In the more recent Malerba et al. study [65] of AAT heterozygous patients that showed association between reduced AAT and increased exhaled NO, no mention is made of AAT replacement therapy. However, clinical guidelines do not recommend AAT replacement treatment for AAT heterozygotes, and it is unlikely that these subjects received AAT infusions. In our study, we minimized the effect of AAT replacement therapy on exhaled NO in AAT-deficient subjects by measuring NO immediately before i.v. AAT administration. Therefore, AAT concentrations were at the nadir.

Several limitations apply to our studies. The applicability of our in vitro experiments in RAW 264.7 murine macrophagic cells to NO production in primary cells or to human cells is uncertain. Human monocytes and macrophages produce only low levels of NO in vitro [67], and the regulation of NO production in human monocytes is thought to be different from that in mouse monocytes [68]. Although AAT suppressed expression of iNOS in our studies, we did not examine the AAT effect on eNOS (NOS3) or nNOS (NOS1). Our experiments showing AAT-suppressive effects on iNOS expression and NF-κB activation in RAW 264.7 cells describe AAT effects at only one time-point, and different AAT effects may be obtained following AAT addition at additional time-points following stimulation. The minimal effects of AAT on MAPK activation were also obtained at one time-point. As an AAT-suppressive effect on MAPK activation at other time-points has not been determined, further investigation is warranted. Furthermore, the effect of AAT on other signaling pathways that may affect NO production, was not assessed.

In the exhaled NO studies, the study groups contained small numbers of subjects, and we could not match the clinical characteristics of the study groups. However, the three study groups were similar in sex distribution and age. Also, a complete understanding of the AAT effect on exhaled NO in humans requires more detailed descriptions of pulmonary neutrophil concentrations and exogenous AAT administration in AAT-deficient persons. Additional studies are needed to estab-

lish an inhibitory role for AAT in NO synthesis in vivo in humans.

AAT, the most abundant circulating serine protease inhibitor, may function as an endogenous suppressor of NO production. AAT, AAT derivatives, or synthetic molecules with AAT biological activity may have use in treating conditions where increased NO is thought to participate in pathogenesis. AAT has several properties that make it an attractive candidate for treating NO-related disease. AAT is currently available for clinical use, and AAT has been given as i.v. replacement therapy to treat AAT-deficient patients since 1988 with a very favorable safety record [22]. In a review of AAT infusion therapy, no deaths attributable to AAT were reported, and only two adverse events were recorded for each 5 years of AAT use [69].

AUTHORSHIP

All authors contributed to the design of the experiments, analysis of the data, and writing of the manuscript. E.D.C., G.B.P., P.E.S., and C.L.B. performed the in vitro experiments. P.E.S. performed the exhaled NO experiments.

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