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α1-Antitrypsin Combines with Plasma Fatty Acids and Induces Angiopoietin-like Protein 4 Expression

Eileen Frenzel,*† Sabine Wrenner,**† Britta Brügger,§ Sandeep Salipalli,*† Stephan Immenschuh,§ Nupur Aggarwal,*† Ralf Lichtinghagen,§ Ravi Mahadeva,‖ A. Mario Q. Marcondes,‡‡,* Charles A. Dinarello,††,†‡ Tobias Welte,*† and Sabina Janciauskiene*†

α1-Antitrypsin (A1AT) purified from human plasma upregulates expression and release of angiopoietin-like protein 4 (Angptl4) in adherent human blood monocytes and in human lung microvascular endothelial cells, providing a mechanism for the broad immune-regulatory properties of A1AT independent of its antiprotease activity. In this study, we demonstrate that A1AT (Proelastin), a potent inducer of Angptl4, contains significant quantities of the fatty acids (FA) linoleic acid (C18:2) and oleic acid (C18:1). However, only trace amounts of FAs were present in preparations that failed to increase Angptl4 expression, for example, A1AT (Zemaïra) or M-type A1AT purified by affinity chromatography. FA pull-down assays with Western blot analysis revealed a FA-binding ability of A1AT. In human blood-adherent monocytes, A1AT-FA conjugates upregulated expression of Angptl4 (54.9-fold, p < 0.001), FA-binding protein 4 (FABP4) (11.4-fold, p < 0.001), and, to a lesser degree, FA translocase (CD36) (3.1-fold, p < 0.001) relative to A1AT devoid of FA (A1AT-0). These latter effects of A1AT-FA were blocked by inhibitors of peroxisome proliferator-activated receptor (PPAR) β/δ (ST247) and PPARγ (GW9662). When compared with controls, cell pretreatment with ST247 diminished the effect of A1AT-LA on Angptl4 mRNA (11.6-versus 4.1-fold, p < 0.001) and FABP4 mRNA (5.4-versus 2.8-fold, p < 0.001). Similarly, preincubation of cells with GW9662 inhibited inducing effect of A1AT-LA on Angptl4 mRNA (by 2-fold, p < 0.001) and FABP4 mRNA (by 3-fold, p < 0.001). Thus, A1AT binds to FA, and it is this form of A1AT that induces Angptl4 and FABP4 expression via a PPAR-dependent pathway. These findings provide a mechanism for the unexplored area of A1AT biology independent of its antiprotease properties.

The online version of this article contains supplemental material.

α1-Antitrypsin (A1AT), angiopoietin-like protein 4 (Angptl4), peroxisome proliferator-activated receptor (PPAR), serum albumin; LA, linoleic acid; LDL, low-density lipoprotein; OA, oleic acid; PPAR, peroxisome proliferator-activated receptor.

Address correspondence and reprint requests to Prof. Sabina Janciauskiene, Department of Respiratory Medicine, Hannover Medical School, Member of the German Center for Lung Research, 30626 Hannover, Germany; 30625 Hannover, Germany. E-mail address: janciauskiene.sabina@mh-hannover.de

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Abbreviations used in this article: A1AT, α1-antitrypsin; Angptl4, angiopoietin-like protein 4; DMF, dimethylformamide; FA, fatty acid; FABP4, FA-binding protein 4; HDL, high-density lipoprotein; HIF-1α, hypoxia-inducible factor-1α; HSA, human serum albumin; LA, linoleic acid; LDL, low-density lipoprotein; OA, oleic acid; PPAR, peroxisome proliferator-activated receptor.

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Address correspondence and reprint requests to Prof. Sabina Janciauskiene, Department of Respiratory Medicine, Hannover Medical School, Member of the German Center for Lung Research, 30626 Hannover, Germany. E-mail address: janciauskiene.sabina@mh-hannover.de

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none are known to bind FAs in such large amounts as albumin (17). Therefore, a mechanism for increased expression of Angptl4 by proteins carrying FAs remains uncharacterized.

Extensive studies show that α1-antitrypsin (A1AT) purified from human plasma possesses anti-inflammatory and immuno-regulatory properties across a broad spectrum of animal models for systemic or local inflammation (20, 21). Although A1AT is commonly used to treat patients with inherited Z (Glu42Lys) deficiency, A1AT has been administered to nondiﬀicient patients with recent onset type 1 diabetes (22) and ST-elevated myocardial infarction (23). Those studies revealed a distinct anti-inflammatory profile of reduced IL-1β and C-reactive protein levels. In a small cohort of emphysema patients receiving therapy with A1AT (Prolastin), we recently reported that plasma Angptl4 levels correlate with recent onset type 1 diabetes (22) and ST-elevated myocardial infarction (23). Those studies revealed a distinct anti-inflammatory profile of reduced IL-1β and C-reactive protein levels.

A1AT strongly upregulates expression and release of Angptl4 in human blood-adherent monocyte and in primary human lung microvascular endothelial cells (24). However, it came to our attention that the magnitude by which preparations of A1AT stimulated expression of Angptl4 varied significantly. These inconsistencies between pharmaceutical preparations of seemingly the same protein suggested variability in their constituents. In this work, we provide unexpected evidence that A1AT binds FAs (LA and OA) and only FA-bound forms of A1AT induce Angptl4.

Materials and Methods

A1AT preparation

Clinical grade A1AT preparations, Zemaira (CSL Behring) and Prolastin (Grifols), were used in all experiments. Zemaira is also termed A1AT-0 to indicate that it is FA free.

Purification of A1AT from human serum

Human serum was collected from volunteers with PiMM and PiZZ genotype of A1AT. Serum M-A1AT and Z-A1AT were isolated by affinity chromatography using A1AT-specific α1-Antitrypsin Select matrix (GE Healthcare Life Sciences), according to the manufacturer’s recommendations. In brief, pooled serum (PiMM or PiZZ) was diluted 1:3 with 20 mM Tris/HC1 and 150 mM NaCl (pH 7.4) binding buffer and loaded onto α1-Antitrypsin Select columns. Serum A1AT binds to the ligand, and unbound impurities were washed away with binding buffer. M- and Z-A1AT were eluted with 2 M MgCl2 in 20 mM Tris/HC1 (pH 7.6). A1AT concentration in elution fractions was determined from OD280 measured on NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific). Pools of M- or Z-A1AT-containing fractions were concentrated, and buffer was changed to PBS using 10-kDa cutoff centrifics (Merck Millipore). M- and Z-A1AT purities were confirmed on Coomassie-stained gels.

Lipid analysis of A1AT preparations

In collaboration with the Institute of Clinical Chemistry of Hannover Medical School, commercial A1AT preparation (Zemaira) and affinity-isolated M- and Z-A1AT were probed for the content of cholesterol, triglycerides, low-density lipoprotein (LDL), and high-density lipoprotein (HDL). For analysis, the cobas 8000 modular analyzer series (Roche) was used.

Preparation of A1AT- and human serum albumin–FA complexes

A1AT-0 (Zemaira) and, in some experiments, FA-free human serum albumin (HSA; Sigma-Aldrich) were spiked with LA or OA (Sigma-Aldrich). For spiking, proteins were directly mixed with LA or OA (1:2.4 molar ratio) and incubated for 3 h at 37°C in a water bath. After incubation, unbound FAs were removed using 10-kDa cutoff membranes (Millipore). Potential protein–FA complexes were resolved in sterile PBS. FAs alone prepared under the same conditions were used as controls. For all experiments, preparations were directly used and kept no longer than 48 h at 4°C.

FA analysis

Lipid extraction was performed, as described previously (25). Briefly, 80 µg protein was subjected to an acidic Bligh and Dyer extraction in the presence of 200 pmol C17:0 FA. Evaporated lipid extracts were resuspended in methanol. Samples subjected to mass spectrometry analysis in negative ion mode were diluted 1:2 in 0.05% triethylamine in methanol. Mass spectrometric analysis of lipids was performed on a Q Exactive from Thermo Scientific. Samples were automatically injected via a TriVersa NanoMate device (Advion). Spray voltage was set to 1500 V with a capillary temperature of 200°C. Full mass spectrometry scans (m/z 200–1000 Da) were obtained with automatic gain control target of 1 × 106 ions and maximal injection time of 200 ms.

Pull-down assay with FA beads

Coupling of FA to agarose beads was performed according to Beck-García et al. (26). Shortly, in a first step, the carbonyl group of the FA was activated by incubation of 46 µmol FA with 92 µmol N,N-diisopropyl-ethylamine and 35 µmol O-(benzotriazol-1-yl)-tetramethyluronium tetra-fluoroborate in 1 ml dimethylformamide (DMF) for 6 h at room temperature on a rotating mixer. Then 500 µl (bed volume) α-aminoehexyl-agarose beads in DMF were added and incubated overnight at room temperature on a rotating mixer. Coupled beads were washed twice with DMF and three times with PBS and were diluted 1:10 with unbound α-aminoehexyl-agarose beads. For the pull-down assay, 400 µl protein solution in PBS was incubated with 15 µl diluted beads (bed volume) for 2 h at 4°C on a rotating mixer. After washing beads five times with ice-cold PBS, bound protein was eluted from the beads by addition of 2-fold SDS loading buffer and heating at 95°C for 5 min. Samples were separated on 7.5% polyacrylamide SDS gels, followed by Western blot analysis.

Electrophoresis (SDS-PAGE) and Western blot analysis

For protein analysis, samples were run on 7.5 or 10% native or NaDodSO4–polyacrylamide gels (SDS-PAGE). For visualization of separated proteins, gel was stained with 0.1% Coomassie blue R250 in 10% acetic acid, 50% methanol, and 40% H2O and destained with 10% acetic acid, 50% methanol, and 40% H2O. From other gels, proteins were transferred onto polyvinylidene fluoride membrane by semidyling Western blotting. For specific detection, following the primary Abs were used: rabbit polyclonal anti-A1AT (DAKO), mouse monoclonal anti-A1AT (clone B9; Santa Cruz Biotechnology), mouse monoclonal antipolymeric A1AT (clone 2C1; Hycult Biotech), mouse monoclonal anti–FA-binding protein 4 (FABP4; clone 1105CT1-1; Antibodies Online), rabbit polyclonal anti-ERK1/2 and mouse monoclonal anti–

Isolation of PBMCs

Human PBMCs were isolated from peripheral blood of healthy volunteers using Lymphosep discontinuous gradient centrifugation, according to the manufacturer’s instructions. PBMCs were resuspended in RPMI 1640 with 2 mM N-acetyl-l-alanyl-l-glutamine (Life Technologies, Life Technologies) supplemented with 1% nonessential amino acids, 2% sodium pyruvate, and 20 mM HEPES and plated at a density of 4–10 × 106 cells/ml. Cells were incubated for 75 min at 37°C and 5% CO2 to allow monocytes to adhere to the cell culture plates. Afterward, nonadherent cells were removed by washing with PBS containing Mg2+ and Ca2+ (Life Technologies, Life Technologies, and fresh medium without FCS was added. After 24 h, adherent monocytes were used for experiments.

Isolation of human peripheral blood monocytes

Primary monocytes were isolated by negative isolation from freshly prepared PBMCs using MACS magnetic separation system with Human Monocyte Isolation Kit II (Miltenyi Biotech), as described previously (27). Cells were >98% positive for CD14 as determined by flow cytometry.

Experimental conditions

Human blood-adherent monocytes or purified monocytes were incubated directly or pretreated for 30 min with specific MAPK/ERK kinase inhibitor U0126 (10 µM; Sigma-Aldrich); with GW9662, a selective, irreversible PPARγ antagonist; or with ST247 (1 µM; Sigma-Aldrich), a selective inverse agonist of PPARβ/δ. Afterward, cells were cultured alone or together with the aforementioned A1AT preparations (0.5 or 1 mg/ml) or HSA (1 mg/ml) for various time points up to 24 h. At the end of incubation time, cell supernatants and lysates were collected for further analysis. In some experiments cells, were subjected to fractionation using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific).
Detection of cytotoxicity (LDH assay)

Treatment-associated cytotoxicity was determined using the Cytotoxicity Detection Kit (LDH) from Roche, according to the manufacturer’s protocol. In brief, the assay quantified LDH released from ruptured or dead cells into the culture supernatant by a colorimetric reaction. Cells were treated according to the experimental setting, and cell supernatants were collected at the end of incubation time. Total cell lysate was used as high control. For low control and background control, supernatant from untreated cells and assay medium alone were used, respectively. Absorbance of colorimetric product of LDH reaction was measured at 490 nm using Infinite M200 microplate reader (Tecan). Measurements were carried out in triplicates.

Specific gene expression analysis by RT-PCR

Gene expression analysis was assessed, as described earlier (24). Briefly, total RNA was isolated using the RNeasy Mini Kit (Qiagen), according to the manufacturer’s instructions. cDNA was synthesized by reverse transcription using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Life Technologies). Expression levels of Angpt4, FABP4, and FA translocase (CD36) were analyzed by RT-PCR using the TaqMan Gene Expression Assay (Applied Biosystems, Life Technologies). The expression of the housekeeping gene, GAPDH, was used for normalization. All primers were purchased from Applied Biosystems. Relative gene expression was calculated according to the ΔΔ cycle threshold method.

Analysis of CD36 surface expression by flow cytometry

In all flow cytometry experiments, 2 × 10^5 cells per condition were used. Surface expression of CD14, CD16, and CD36 was assessed in cells kept as control or preincubated with appropriate substance for 6 h. Cells were then labeled with FITC-conjugated anti-human CD14 mAb, PE-conjugated anti-human CD16 mAb, and allophycocyanin-conjugated anti-human CD161 mAb, or the corresponding isotype controls (all mouse IgG1 mAb) (Immunotools, Friesoythe, Germany) for 35 min at 4˚C. One set of cells was kept unstained for gating purpose. After labeling, cells were washed with FACS buffer (PBS, 1% BSA [pH 7.4]), resuspended in PBS with 0.5% BSA, and examined using FACS Calibur (BD Biosciences). Data were analyzed using FACSdiva software (BD Biosciences).

In vitro measurement of elastase activity

The A1AT preparations were tested for their inhibitory activity toward pancreatic elastase (Sigma-Aldrich). For quantification of elastase activity, degradation of elastase substrate N-succinyl-Ala-Ala-Ala-p-nitroanilide (Sigma-Aldrich) was followed spectrophotometrically, as described previously (28). In brief, A1AT preparations were preincubated with elastase at a molar ratio of 1:2.6 at 37˚C in 0.1 M Tris buffer (pH 8). After 5 min, elastase substrate to a final concentration of 83 μM was added, and absorbance was followed at 405 nm for 3 min on Infinite M200 microplate reader (Tecan). Sample containing substrate and buffer alone was used for blank reduction. Elastase inhibition was calculated relative to samples containing only elastase and substrate.

Quantitative analysis of Angpt4

Cell culture supernatants collected from monocyes treated with different A1AT preparations for 24 h were analyzed for the concentration of Angpt4 protein using Duoset ELISA kit (R&D Systems) or angiopoietin-like 4 (ARP4) human ELISA kit (Abcam). Detection limit for Duoset ELISA was 1.25 ng/ml, and for ARP4 ELISA <20 pg/ml.

Statistical analysis

The differences in the means of experimental results were analyzed for their statistical significance using one-way ANOVA combined with a multiple-comparison procedure (Scheffe multiple range test), with an overall significance level of p = 0.05. An independent two-sample t test was also used. Statistical Package (SPSS for Windows, release 21.0) was used for the statistical calculations.

Results

Analysis of FA content in different A1AT preparations

FA spectra in the A1AT solutions were determined by nanoelectrospray ionization tandem mass spectrometry, as previously described (25). The FA anion fragments found at m/z 279 and 281 represent the anions of LA and OA, respectively. For quantitative determination of LA and OA, an internal standard was added prior to lipid extraction. As FA standard, FA 17:0 was used that does not occur in a significant amount in the analyzed samples. As shown in Table I, A1AT (Prolastin) contained significant amounts of FAs C18:2 (LA) and C18:1 (OA), whereas A1AT (Zemaira) contained only trace amounts of LA and OA (Table I). Other studies (see Materials and Methods) confirmed that A1AT (Zemaira) contains no detectable amounts of other lipids, such as HDL, LDL, cholesterol, or triglycerides. In light of the findings presented above, we prepared M- and Z-A1AT from pooled PiMM and PiZZ plasma by using one-step α-1 Antitrypsin Select affinity chromatography without employing organic solvents, detergents, or heat inactivation steps (Supplemental Fig. 1A). Affinity-purified M-A1AT contained only trace amounts of LA and OA, whereas Z-A1AT contained 10 times or more LA and OA than M-A1AT (Table I). Furthermore, FA-free Zemaira (A1AT-0) was preincubated with LA (at 1:2.4 molar ratio A1AT:LA) for 3 h at 37˚C. Unbound LA was removed by repeated filtration using Centricon centrifugal filter with molecular mass cutoff of 10 kDa. Analysis of FA content revealed that A1AT-LA (Zemaira preincubated with LA) contained more LA than A1AT (Prolastin) (Table I).

Analysis of interactions between A1AT-0 and FA using a FA pull-down assay

Based on the above findings, we prepared LA- and OA-coupled agarose beads, and, using pull-down assays, investigated a putative interaction between A1AT and FA. HSA, a known FA-binding protein (19), was chosen to validate the FA pull-down assay. FA bead-bound proteins were separated by SDS-PAGE and visualized on Western blots by using specific mAbs against human A1AT or HSA. As shown in Fig. 1A, both LA- and OA-coupled beads enriched HSA-0 from 1 mg/ml solution with a similar efficiency. Control beads produced in a coupling reaction without addition of FA were used to evaluate nonspecific binding to ω-aminohexyl-agarose. Nonspecific binding of HSA to control beads was negligibly low (Fig. 1A). Similar to HSA-0, A1AT-0 bound to LA- and OA-coupled beads from 1 mg/ml A1AT solution (Fig. 1A). Nonspecific binding of A1AT to control beads was low.

Relative affinities of HSA and A1AT for LA binding

We next performed competition experiments of A1AT binding to LA-coupled beads in the presence of increasing concentrations of HSA and vice versa. Fig. 1B shows that HSA-0 dose dependently competes with A1AT-0 for binding to FA. Similarly, A1AT also competed for HSA binding to LA beads. When albumin was added into the mixture in a molar ratio of 1:0.8 (A1AT to HSA),

<table>
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<th>Sample</th>
<th>LA pmol/μg Protein</th>
<th>Mean</th>
<th>SD</th>
<th>n</th>
<th>OA pmol/μg Protein</th>
<th>Mean</th>
<th>SD</th>
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<tr>
<td>A1AT (Zemaira)</td>
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<td>nd</td>
<td></td>
<td>6</td>
<td>6</td>
<td>nd</td>
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<tr>
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<td>0.88</td>
<td>6</td>
<td>1.10</td>
<td>0.55</td>
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<tr>
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<td>0.55</td>
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<td>2.65</td>
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<tr>
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<td>0.02</td>
<td>6</td>
<td>0.02</td>
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<td>Z-A1AT affinity</td>
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* nd, Not detected.
A rotating mixer with $\omega$ the amount of bead-bound A1AT was reduced to 66% agarose beads. (A) FIGURE 1. Pull down of FA-free HSA-0 and A1AT-0 by FA-coupled beads. (B) For competition experiments, target protein was mixed with competing protein in the concentrations, as indicated in the figure before addition of beads. One representative blot of three independent experiments is shown. Densities were evaluated using ImageJ. Results are given in mean ± SD.

the amount of bead-bound A1AT was reduced to 66% compared with 100% when A1AT-0 was added alone (Fig. 1B). Under similar experimental conditions, the addition of A1AT-0 in a molar ratio of 1:1.2 (HSA to A1AT) resulted in the loss of 41% bead-bound albumin. HSA-0 and A1AT-0 prevented their respective binding to LA beads by ~73% when added in a molar excess of 8-fold for HSA-0 and 12-fold for A1AT-0. Noticeably, with 33-fold molar excess of A1AT and 36-fold molar excess of HSA, both A1AT and HSA were capable of almost completely hindering binding of the other protein to LA beads (Fig. 1B).

Despite the high molar excess of HSA versus A1AT (37.9 mg/ml albumin versus 1.44 mg/ml A1AT, in PiMM serum), LA- and OA-bound beads captured and pulled down not only albumin, but also M-A1AT. Likewise, FA beads were capturing lower but clearly detectable amounts of Z-A1AT from PiZZ serum (Fig. 2).

Qualitative analysis of A1AT preparations

It remained unclear whether the A1AT complex formation with FA alters the molecular form of A1AT protein. As illustrated in Fig. 3A, a polyclonal anti-A1AT Ab showed a similar pattern for A1AT-0 and A1AT-FA preparations, that is, monomer and distinct higher molecular mass forms of A1AT. However, when the specific anti-A1AT (2C1) polymer Ab was used, 110-kDa and larger polymers were detected in A1AT-LA and A1AT-OA preparations (Fig. 3A). Qualitative characterization of M- and Z-type A1AT isolated from human serum by affinity chromatography revealed that the M-A1AT protein mainly appears as a monomer, whereas Z-A1AT, which contains FAs, comprises a mixture of a monomeric and several polymers of different sizes (Fig. 3B). Importantly, mixing A1AT with LA did not change the property of A1AT to inhibit elastase activity (Supplemental Fig. 1B). In line with previously published data (29), the antielastase activity of affinity-purified M-A1AT was similar to that of commercial serum-derived A1AT-0 (Zemaira), whereas affinity-purified Z-A1AT showed lower antielastase activity (Supplemental Fig. 1B).

Time-dependent effects of A1AT-0 and A1AT-FA on Angptl4 and FABP4 expression

In the current study, adherent blood monocytes were used to compare the effect of A1AT-0 (Zemaira) with A1AT-LA (Zemaira LA complex) on Angptl4 expression. As demonstrated in Fig. 4A, exposure of cells to A1AT-LA for 2 h resulted in a 10-fold induction of Angptl4 expression, whereas, at 6 h, Angptl4 expression was maximal relative to untreated or A1AT-0–treated cells. After 24 h, Angptl4 expression in A1AT-LA–treated cells still remained higher (4-fold; NS) than in untreated controls or A1AT-0–treated cells. As demonstrated in Fig. 4B, adherent blood monocytes incubated with A1AT-LA increased FABP4 expression in a time-dependent manner. After only 4 h, there was a 3-fold induction of FABP4 expression ($p = 0.02$) relative to untreated controls (Fig. 4B). However, unlike Angptl4, the maximal increase of FABP4 (9.7-fold, $p < 0.001$) occurred after 24 h. A1AT-OA significantly induced transient expression of Angptl4 and FABP4 genes albeit with lower magnitude than A1AT-LA (Supplemental Fig. 3). Both Angptl4 and FABP4 mRNA levels did not change in cells treated with A1AT-0 (Fig. 4B, Supplemental Fig. 2). It is noteworthy that exposure to A1AT-FA did not affect cell viability as measured by lack of release of LDH (data not shown). Because A1AT-LA maximally induces Angptl4 expression and significantly upregulated FABP4 at 6 h, further experiments used this time point.

Effects of A1AT-0 and A1AT-FA on Angptl4, FABP4, CD36, and hypoxia-inducible factor–1α expression

At mRNA level, FABP4 was shown to correlate with CD36 in cells of monocyte/macrophage lineage (30). CD36 is a key protein involved in regulating the uptake of FA (31). In the following set of experiments, we compared effects of A1AT-LA and A1AT-OA on

FIGURE 1. Pull down of FA-free HSA-0 and A1AT-0 by FA-coupled agarose beads. (A) FA-free HSA-0 or A1AT-0 (Zemaira) was mixed on a rotating mixer with $\omega$-aminohexyl-agarose beads coupled to LA, OA, or control (co.) beads exposed to coupling reaction in absence of FA. After incubation for 2 h, the beads were washed thoroughly with PBS to remove unbound proteins, SDS sample buffer was added, and bead-bound proteins were released by heating at 95°C for 5 min. For competition experiments, target protein was mixed with competing protein in the concentrations, as indicated in the figure before addition of beads. One representative blot of three independent experiments is shown. Densities were evaluated using ImageJ. Results are given in mean ± SD.

FIGURE 2. FA pull down of A1AT and HSA from PiMM and PiZZ serum. Pooled serum samples were prediluted with 3 vol PBS and incubated with LA, OA, or control (co.) beads for 2 h on a rotating mixer. Beads were washed thoroughly with PBS to remove unbound proteins, SDS sample buffer was added, and bead-bound proteins were released by heating at 95°C for 5 min. Sample load was 15 μl for A1AT blot and 5 μl for HSA blot. To facilitate comparability of the blots, 200 ng target proteins were applied. Abs do not show any cross-reactivity with competing proteins.

FIGURE 3. A1AT LA and OA preparations were detected in A1AT-LA and A1AT-OA preparations (Fig. 3A). Qualitative characterization of M- and Z-type A1AT isolated from human serum by affinity chromatography revealed that the M-A1AT protein mainly appears as a monomer, whereas Z-A1AT, which contains FAs, comprises a mixture of a monomeric and several polymers of different sizes (Fig. 3B). Importantly, mixing A1AT with LA did not change the property of A1AT to inhibit elastase activity (Supplemental Fig. 1B). In line with previously published data (29), the antielastase activity of affinity-purified M-A1AT was similar to that of commercial serum-derived A1AT-0 (Zemaira), whereas affinity-purified Z-A1AT showed lower antielastase activity (Supplemental Fig. 1B).

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Effects of A1AT-0 and A1AT-FA on Angptl4, FABP4, CD36, and hypoxia-inducible factor–1α expression

At mRNA level, FABP4 was shown to correlate with CD36 in cells of monocyte/macrophage lineage (30). CD36 is a key protein involved in regulating the uptake of FA (31). In the following set of experiments, we compared effects of A1AT-LA and A1AT-OA on

FIGURE 1. Pull down of FA-free HSA-0 and A1AT-0 by FA-coupled agarose beads. (A) FA-free HSA-0 or A1AT-0 (Zemaira) was mixed on a rotating mixer with $\omega$-aminohexyl-agarose beads coupled to LA, OA, or control (co.) beads exposed to coupling reaction in absence of FA. After incubation for 2 h, the beads were washed thoroughly with PBS to remove unbound proteins, SDS sample buffer was added, and bead-bound proteins were released by heating at 95°C for 5 min. For competition experiments, target protein was mixed with competing protein in the concentrations, as indicated in the figure before addition of beads. One representative blot of three independent experiments is shown. Densities were evaluated using ImageJ. Results are given in mean ± SD.
Angptl4, FABP4, and CD36 expression at 6 h. To exclude the putative interference of free FA, adherent peripheral monocytes were treated with LA or OA solutions produced under the same conditions as A1AT-FA complexes (see Materials and Methods). As illustrated in Fig. 5, A1AT-LA preparation markedly increased mRNA levels of Angptl4 (54.9-fold, \( p < 0.001 \)), FABP4 (11.4-fold, \( p < 0.001 \)), and CD36 (3.1-fold, \( p < 0.001 \)) relative to cells treated with A1AT-0 or FA alone. Similarly, when compared with A1AT-0–treated cells, A1AT-OA induced expression of Angptl4 by 10.7-fold (\( p < 0.001 \)), FABP4 by 2.9-fold (\( p < 0.001 \)), and CD36 by 2-fold (\( p < 0.001 \)).

We previously found that A1AT (Prolastin) induces hypoxia-inducible factor–1α (HIF-1α) expression in a time-dependent manner; however, blocking HIF-1α expression with CAY10585, a small molecule inhibitor of HIF-1α accumulation and gene transcriptional activity, had no effect on A1AT (Prolastin)-induced Angptl4 expression (24). We then asked whether FA-bound A1AT has any effect on HIF-1α expression. When compared with controls, treatment of adherent peripheral monocytes with A1AT-LA resulted in an upregulation of HIF-1α mRNA by 1.6 (0.34)-fold, \( p < 0.01 \), \( n = 3 \) independent experiments with 15 repeats. However, milder induction in HIF-1α expression was found in A1AT-OA–treated cells (1.2 [0.38]-fold, NS relative to controls, \( n = 3 \) independent experiments, 21 repeats).

Effects of A1AT-0 and A1AT-FA on Angptl4 and related gene expression in CD14-positive peripheral blood monocytes

In the next set of experiments, the effect of A1AT-0 and A1AT-FA complexes was studied in human peripheral blood monocytes isolated by negative selection (purity >98% CD14-positive cells). At 6 h, A1AT-LA and A1AT-OA both increased Angptl4 gene expression by 28-fold and 23-fold (\( p < 0.001 \)) relative to controls. Concomitantly, when compared with controls, cell treatment with A1AT-LA and A1AT-OA resulted in an increased FABP4 expression by 8.9- and 6.4-fold, respectively, \( p < 0.001 \) (Supplemental Fig. 3B). As demonstrated in Supplemental Fig. 3C, both A1AT-FA preparations also increased CD36 mRNA (A1AT-LA by 3-fold and A1AT-OA by 1.7-fold, \( p < 0.001 \), relative to controls). A1AT-0 did not show any effects on Angptl4, FABP4, or CD36 mRNA levels (Supplemental Fig. 3). These results clearly indicate the contribution of CD14-positive monocytes to A1AT-FA–induced Angptl4, FABP4, and CD36 gene expression.

Effect of A1AT-FAs on Angptl4, FABP4, and CD36 protein levels

In accordance with our gene expression, we observed increased protein levels of Angptl4, FABP4, and CD36 in A1AT-FA–treated

FIGURE 3. Qualitative analysis of molecular forms of A1AT found in A1AT preparations by nondenaturing SDS-PAGE following Western blot analysis using rabbit polyclonal anti-A1AT and mouse anti-A1AT polymer (2C1) mAbs. (A) A1AT-LA and -OA complexes were prepared by 3-h incubation at 37˚C following separation from free FA with centricon-10 (MWCO 10 kDa). Each blot is representative of \( n = 3 \) independent experiments. (B) M- and Z-A1AT pools from α-Antitrypsin Select affinity purification were analyzed by Western blot. Each blot is representative of \( n = 3 \) independent experiments. The black lines indicate where parts of the image were joined.

FIGURE 4. Time-dependent effect of A1AT-0 and A1AT-LA on Angptl4 and FABP4 expression of human adherent blood monocytes. Cells were treated with A1AT-0 or A1AT-LA (1 mg/ml) for different periods of time. Gene expression levels of Angptl4 (A) and FABP4 (B) were analyzed by RT-PCR and normalized to GAPDH. Each point represents mean ± SD of \( n = 3 \) independent experiments, each with three repeats.
adherent peripheral monocytes for 6 h as compared with untreated control cells and cells treated with A1AT-0 (Fig. 6). When compared with Angptl4 levels in supernatant of control cells (13.9 ± 11.6 pg/ml), treatment with A1AT-FAs remarkably increased the release of Angptl4 (A1AT-LA, 59.3 ± 12.4 pg/ml, p = 0.013; A1AT-OA, 66.1 ± 19.1 pg/ml, p = 0.005) (Fig. 6A). Analysis of nuclear and cytoplasmic fractions of adherent peripheral monocytes revealed increased cytoplasmic FABP4 protein concentration in cells treated with A1AT-LA or A1AT-OA, but not in control cells or those treated with A1AT-0 (Fig. 6B). Notably, nuclear translocation of FABP4 was not found (Fig. 6B). Flow cytometry analysis showed increased CD36 surface levels on A1AT-LA (162 ± 23 mean fluorescence intensity in percentage of control, p < 0.001) and A1AT-OA (153 ± 12 mean fluorescence intensity in percentage of control, p = 0.012)–treated cells compared with control cells. According to our results, no significant shift in monocyte populations occurred when cells were treated with A1AT-0 or A1AT-FA (Fig. 6C).

Effects of affinity-purified M-A1AT and Z-A1AT on Angptl4 and related gene expression

To demonstrate physiological relevance of A1AT-FA complexes and to confirm whether endogenous A1AT carrying different concentrations of LA and OA has diverse effects on expression of genes related with lipid homeostasis, we treated adherent blood monocytes with different naturally occurring A1ATs, namely affinity-purified M-A1AT binding only small amounts of FAs and Z-A1AT carrying considerable amounts of both LA and OA (Table I). As demonstrated in Fig. 7A, 7C, and 7D, M-A1AT did not affect Angptl4, FABP4, and CD36 gene expression, whereas Z-A1AT significantly induced expression of Angptl4, FABP4, and CD36 by 4-, 3.7-, and 2-fold, respectively, as compared with controls. In support of these latter, Z-A1AT also markedly induced the release of Angptl4 protein, whereas M-A1AT had only a negligible effect (Fig. 7B).

Effects of GW9662 and ST247 on A1AT-LA–induced Angptl4 and FABP4 expression

We previously found that preincubation of adherent blood monocytes for 30 min with GW9662, a selective and irreversible PPARγ antagonist, dramatically lowers the ability of A1AT (Prolastin) to induce Angptl4 expression (24). As shown in Fig. 8A, cells pretreated with GW9662 (10 μM) significantly diminished the effect of A1AT-LA on Angptl4 mRNA compared with A1AT-LA effect without the inhibitor. Similarly, preincubation of cells with GW9662 also significantly diminished inducing effect of A1AT-LA on FABP4 mRNA (6.2- versus 2.5-fold, p < 0.001) (Fig. 8B). Angptl4 expression is not exclusively regulated by PPARγ. Others report that Angptl4 is also regulated by the subtype PPARβ/δ (32), and that FAs induce Angptl4 expression via PPARβ/δ (33). Therefore, the effect of ST247, a selective and inverse agonist of PPARβ/δ, was studied. When compared with nonpretreated cells, cell pretreatment with ST247 (1 μM) clearly diminished the effect of A1AT-LA on Angptl4 mRNA (11.6- versus 4.1-fold, p < 0.001) and FABP4 mRNA (5.4- versus 2.8-fold, p < 0.001), respectively (Fig. 7C, 7D). Cell pretreatment with ST247 also significantly lowered A1AT (Prolastin)-induced Angptl4 and FABP4 expression relative to nonpretreated cells (Angptl4, 9.7- versus 2.6-fold, p = 0.003; FABP4, 4.6- versus 2.4-fold, p < 0.001) (Fig. 7C, 7D). Hence, A1AT-FA induces Angptl4 through the PPAR pathway.

Effects of A1AT-0 and A1AT-LA on transient ERK1/2 phosphorylation

PPAR activity is regulated by ERK, MAPK, and PPAR ligands showing varying effects on the activity of ERK (34). Our previous studies revealed that A1AT (Prolastin) induces a rapid and transient activation of the MEK-ERK1/2 pathway (24). Elevated phosphorylation of ERK1/2 was noted as early as 15–30 min following treatment with A1AT; however, this increase was not detected after 1 h. To address whether transient MEK-ERK1/2 activation by A1AT (Prolastin) is due to complexes of A1AT with FAs (Table I), adherent blood monocytes were treated for 30 min with A1AT-0 or A1AT-LA. As illustrated in Fig. 9A, both A1AT-0 and A1AT-LA induced ERK1/2 phosphorylation, suggesting that this effect of A1AT is independent of FA content. To observe an influence of ERK1/2 phosphorylation on A1AT-LA–induced Angptl4 expression, we preincubated cells with MEK/ERK1/2 inhibitor, U0126. Consistent with our previous data, the A1AT-induced increase in ERK phosphorylation was absent by a 30-min preincubation with U0126. Concomitantly, we observed a significant reduction of the stimulating effect of A1AT-LA on Angptl4 mRNA (Fig. 9B).

Effects of GW9662, ST247, and U0126 on A1AT-OA–induced Angptl4 and FABP4 expression

Similarly to the A1AT-LA, targeting of PPARs with GW9662 and ST247 as well as blocking ERK phosphorylation using U0126, strongly decreased A1AT-OA–induced Angptl4 and FABP4 mRNA levels (Supplemental Fig. 4), suggesting that A1AT-FA–induced Angptl4 expression is dependent on ERK-regulated PPAR activation.
Discussion

The clinical importance of A1AT is highlighted in individuals with inherited PiZZ (Glu342Lys) A1AT deficiency (plasma levels < 0.7 g/l, whereas normal values range between 1 and 2 g/l) due to the polymerization and defective secretion of Z-A1AT protein. These individuals have an increased risk of developing early-onset emphysema and liver and pancreatic diseases at any age and, in rare cases, panniculitis and vasculitis (35). Therapy with A1AT isolated from pooled human plasma is used to treat patients with inherited A1AT deficiency–related emphysema, and several preparations of A1AT are available. These A1AT preparations are administered i.v. every week, typically at a dose of 60 mg/kg body weight. They are well tolerated with no evidence of virus transmission and show very similar effects in maintaining serum levels of A1AT. Differences in manufacturing process are known to result in analytical differences in the A1AT preparations. For example, Cowden et al. (36) demonstrated that A1AT (Zemaira) is 99% pure, contains the least contaminating proteins, and has high activity as an elastase inhibitor. By contrast, A1AT (Prolastin) has only 60% purity and contains significant amounts of inhibitory-inactive forms of A1AT. The significance to the patient, if any, of these differences in A1AT preparations is not known.

We previously reported that clinical-grade preparations of A1AT, such as Prolastin (Grifols) and Aralast (Baxter), as well as A1AT purchased from Sigma-Aldrich, induce Angptl4 expression in human adherent monocytes and primary lung endothelial cells (24). However, under the same experimental conditions, A1AT (Zemaira) did not affect Angptl4 mRNA and protein release. This striking discrepancy between the plasma-purified preparations of A1AT prompted us to investigate this further.

One of the biological functions of Angptl4 is the regulation of lipid metabolism (37–39). The expression of Angptl4 is also regulated through a synergistic induction of the lipid-sensing PPARs α, β, and γ (40). Among the lipids, FA are the best-recognized inducers of Angptl4 expression (16, 41–43). Although FA are an energy source, they are also recognized as regulators of inflammation, and it has been suggested that PPARs play an important role in FA-dependent gene regulation (44).

Depending on purification methods, therapeutic plasma protein products can contain variable amounts of FAs and lipids (45). Thus, we suspected that FA content might explain A1AT-induced Angptl4 expression. Indeed, lipodomic analysis revealed that A1AT (Prolastin) contains significant amounts of FA, specifically LA (C18:2) and OA (C18:1), two of the most abundant free FA in human plasma (46). In contrast, A1AT (Zemaira) was FA free—only trace amounts of LA and OA were detected. The presence of FA in A1AT (Prolastin) preparations pointed to a putative property of A1AT to bind FA.

The solubility of FA in aqueous solutions, such as blood plasma, is far below 0.3 mM (47); therefore, FA-binding and transporting proteins guarantee a sufficient transport of FA to the FA-consuming organs. The best-characterized candidate for transport is serum albumin. Due to the high FA-binding capacity of albumin, it is assumed that even substantially reduced albumin levels during acute-phase reaction can sufficiently perform this function. In contrast, it is likely that a low-albumin FA-binding capacity is compensated by other acute-phase plasma proteins with FA-
binding properties. To test whether A1AT, a positive acute-phase protein, is a FA-binding protein, we employed a FA bead-based pull-down assay (26). When LA- or OA-coupled beads were preincubated with A1AT-0 or HSA-0 (as a positive control), FA-coupled beads pulled down A1AT and HSA with similar efficiency. Neither A1AT nor HSA demonstrated nonspecific binding to uncoupled agarose beads. Both proteins in a concentration-dependent manner competed for the binding to the FA-coupled beads. We next took an unbiased approach by employing the LA bead pull-down assay for human plasma. Incubation of LA-coupled agarose beads with pooled plasma from MM (normal)- or ZZ (deficient)-A1AT subjects resulted in a specific binding of M- and Z-A1AT protein with LA. Plasma A1AT bound to LA despite the fact that normal serum contains ∼26 times more albumin than A1AT and that albumin-binding affinity to LA is high. Moreover, when we employed plasma from ZZ-A1AT individuals, containing only 10% of A1AT relative to normal MM plasma, binding of A1AT to FA was still measurable. In fact, lipidomics analysis revealed that affinity-purified plasma Z-, but not M-A1AT, contains significant amount of FAs, LA and OA. The Z-A1AT protein is characterized by an increased exposure of hydrophobic regions to the solvent and therefore has enhanced tendency to hydrophobic interactions and oligomeric assemblies (48). As a consequence, Z-A1AT may spontaneously interact with free FAs and form protein–FA polymers. In contrast, the amino acids involved in the polymerization are completely buried in native, M-type A1AT (49). Likewise, M-A1AT probably binds FA only under interaction-favoring conditions, such as during increased free FA concentrations and/or decrease in albumin concentration during acute-phase reaction. These data constitute good evidence that A1AT exists in FA-free and FA-bound forms and therefore might express different biological activities.

Angptl4 is a potent anti-angiogenic and anti-inflammatory factor, which is under the transcriptional control of PPARγ (50). FABP4 and CD36 are also PPARγ target genes involved in lipid metabolism, and PPARγ activation was found to induce FABP4 mRNA in human monocytes (51). As expected, GW9662, a selective and irreversible PPARγ antagonist (52), significantly inhibited A1AT-FA–induced Angptl4, FABP4, and CD36 expression, confirming involvement of PPARγ pathway. However, Angptl4 expression can also be regulated by other PPAR isotypes. For example, Koliwad et al. (53) reported that in rat hepatoma cells FA induce Angptl4 mRNA via PPARα, in mouse intestinal cells via PPARβ/δ, whereas in human myocytes both PPARα and PPARβ/δ most likely play a role. Krey et al. (54) showed that the affinity of PPARδ for LA is higher than for OA, and that LAs are the most potent natural ligands for PPARδ. These findings imply that Angptl4 is one of the PPARβ/δ target genes. ST247 is an inverse agonist of PPARβ/δ, which has the ability to enter cells and to inhibit the transcriptional activity of PPARβ/δ, but it does not affect activated PPARγ. Likewise, preincubation of adherent blood monocytes with ST247 resulted in a significant inhibition of A1AT-LA–induced Angptl4 and FABP4 expression. Hence, both GW9662 and ST247 inhibit effect of A1AT-FA on Angptl4 expression, supporting a notion that Angptl4 transcription is regulated by several members of the PPAR family.
Angptl4 transcription, at least partially, can be regulated by ERK-mediated PPAR activation (56). Knowing that A1AT can be in FA-free or FA-bound forms, we wanted to reinvestigate effects of A1AT on ERK1/2 activation. We previously published that A1AT (Prolastin) induces transient ERK1/2 phosphorylation and that blocking the ERK1/2 pathway with MEK inhibitor (UO126) markedly diminishes effect of A1AT on Angptl4 expression (24). In this study, in adherent blood monocyte cultures we show that both A1AT-0 and A1AT-FA induce a rapid and transient ERK1/2 activation. Thus, the FA-bound form of A1AT does not seem to be

**FIGURE 8.** A1AT-LA–induced expression of Angptl4 and FABP4 is related to PPARγ and PPARβ/δ activity. Cells were pretreated for 30 min with 10 μM GW9662, an irreversible PPARγ antagonist (A and B), or with 1 μM ST247, a selective and inverse agonist of PPARβ/δ (C and D), prior to addition of 1 mg/ml A1AT-0, A1AT-LA, LA preparation, or Prolastin for another 6 h. Expression levels of Angptl4 (A and C) and FABP4 (B and D) were determined by RT-PCR and normalized to GAPDH. In (A) and (B), each point represents mean ± SD of n = 3 independent experiments, each with three repeats. In (C) and (D), each point represents mean ± SD of n = 2 independent experiments, each with three repeats.

**FIGURE 9.** (A) Transient activation of MEK-ERK1/2 pathway is independent on A1AT complexation with LA. Western blot of cell lysates prepared from adherent blood monocytes illustrates phosphorylation of ERK1/2 at 30 min in response to treatment with 1 mg/ml A1AT-0 or A1AT-LA. Blots were stained for phosphorylated and total ERK1/2. β-Actin was used as a loading control. Preincubation of cells with MEK/ERK1/2 inhibitor UO126 (10 μM) completely abolished ERK1/2 activation in all samples. Presented blot is representative of n = 4 repeated experiments. (B) Effect of ERK1/2 phosphorylation on A1AT-LA–induced Angptl4 expression. Adherent PBMCs were preincubated for 30 min with UO126 (10 μM) prior to addition of 1 mg/ml A1AT-0 or A1AT-LA for another 6 h. Angptl4 mRNA levels were assessed by RT-PCR and normalized to GAPDH. Each point represents mean ± SD of n = 3 independent experiments, each with three repeats.
or no clinical symptoms of any disease. This shows that clinical cirrhosis, lung and liver diseases simultaneously during adulthood, emphysema (67). However, clinical phenotypes in Z-A1AT defi-
whereas the lack of active protein leaves lung parenchyma endoplasmic reticulum of hepatocytes leading to liver disease, A1AT-FA complexes as exogenous PPAR ligands.

FA levels and/or FA–protein complexes and warrants further findings in vivo (24). This latter might be mediated by plasma-free expression (64). However, there is a paucity of data on FA-binding proteins/translocases in FA uptake, intracellular trafficking, and signaling. Relevant to the putative efficacy of A1AT replacement therapy to reduce lung inflammation via inhibition of neutrophil elastase, the interaction between FA and A1AT has no effect on the antielastase activity of A1AT protein. This suggests that FA binding does not interfere with inhibitory conformation of the reactive loop of A1AT. However, aside from its elastase-inhibitory function, A1AT expresses other anti-inflammatory activities (65).

The interest of health care providers in A1AT preparations re-
cently has increased because of the beneficial effects of A1AT therapy in single cases and in small cohorts with clinical conditions other than lung emphysema (66). Novel data provide evidence that therapy with A1AT modulates or prevents tissue injury in exper-
imental animal models of human diseases, including graft-versus-
host disease, rheumatoid arthritis, autoimmune diabetes, and renal ischemia–reperfusion injury, among others (20). Nevertheless, despite these many effects ascribed to the A1AT protein, the is ischemia–reperfusion injury, among others (20). Nevertheless, the mechanisms of its effects remain incompletely understood. Our data illustrate that A1AT might occur in FA-free and FA-bound forms, which express diverse effects on the regulation of Angpt4 expression, an inhibitor of plasma triglyceride clearance and anti-
flammatory protein. Notably, we observed high donor-dependent variability in Angpt4 mRNA levels, which is in accordance with findings in vivo (24). This latter might be mediated by plasma-free FA levels and/or FA-protein complexes and warrants further investigations. In addition, it is of interest to consider the role of A1AT-FAC complexes as exogenous PPAR ligands.

According to the current theory, Z-A1AT forms polymers in the endoplasmic reticulum of hepatocytes leading to liver disease, whereas the lack of active protein leaves lung parenchyma unprotected against neutrophil elastase, leading to early-onset emphysema (67). However, clinical phenotypes in Z-A1AT defi-
ciency can be expressed in different ways, including early-onset (at age of 30 y) pulmonary emphysema, childhood or adult liver cirrhosis, lung and liver diseases simultaneously during adulthood, or no clinical symptoms of any disease. This shows that clinical phenotypes are driven by additional factor(s). Our finding that Z-A1AT contains FAs and upregulates expression of Angpt4 and FABP4 suggests a novel role for Z-A1AT-FA polymers in lipid metabolism and inflammation. For example, FABP4 is expressed in adipocytes, monocytes/macrophages, and human bronchial epithelial cells (68, 69) and plays a role in cholesterol ester accu-
mulation, uptake of FAs, and cholesterol. Several studies have shown that abolishing expression of FABP4 protects against atherosclerosis (70) and macrophage activation (71). In contrast, the induction of FABP4 in human bronchial epithelial cells was re-
lated to inflammation and the development of asthma (72). Based on these examples above, it is possible to hypothesize that, in particular circumstances, Z-A1AT-FA polymers may constantly activate Angpt4 and FABP4, and thus express proinflammatory effects. Taken together, our data provide new insights about Z- and M-A1AT properties, which might add to the knowledge about the biological mechanisms behind Z-A1AT–related pathologies.

Our finding that A1AT binds FA and regulates Angpt4 expression opens a new field for investigations of A1AT role in health and disease and provides a new opportunity for evaluating effects of A1AT therapy.

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References


