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Commercial α1-antitrypsin preparations markedly differ in their potential to inhibit the ATP-induced release of monocytic interleukin-1β

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ABSTRACT

The acute phase protein α1-antitrypsin (AAT) inhibits numerous proteases, specifically neutrophil elastase. Patients with an AAT deficiency due to mutations frequently develop early onset emphysema. The commercial preparations of human plasma AAT are clinically used as biopharmaceuticals to protect the lung tissue of AAT-deficient patients from damage caused by neutrophil elastase. Accordingly, preparations of AAT are validated for their anti-elastase activity. However, several anti-inflammatory effects of AAT were described, some of them being independent from its anti-protease function. We recently demonstrated that AAT isolated from the blood of healthy persons efficiently inhibits the ATP-induced release of interleukin-1β by human monocytes. This finding is of therapeutic relevance, because IL-1β plays an important role in numerous debilitating and life-threatening inflammatory diseases. As anti-inflammatory functions of AAT are of increasing clinical interest, we compared the potential of two widely used AAT preparations, Prolastin® and Respreeza®, to inhibit the ATP-induced release of IL-1β using human monocytic U937 cells. We detected marked functional differences between both medicaments. The AAT preparation Respreeza® is less active compared to Prolastin® regarding the inhibition of the ATP-induced release of monocytic IL-1β. Chemical oxidation of Respreeza® restored this anti-inflammatory activity, while destroying its anti-protease function. Our data suggest that the anti-inflammatory potential and the anti-protease function of AAT can be fully uncoupled. In the light of the increasing clinical interest in anti-inflammatory functions of AAT, commercial AAT preparations should be carefully reinvestigated and optimized to preserve the dual anti-protease and anti-inflammatory activity of native AAT.

1. Introduction

In healthy persons the serine protease inhibitor α1-antitrypsin (AAT, SERPINA1) is constitutively produced by hepatocytes and is present in human blood plasma at concentrations ranging from 0.9 to 1.75 mg/ml [1]. AAT inhibits the function of numerous proteases including neutrophil elastase (NE), proteinase-3, trypsin, kallikrein-7, kallikrein-14, and matriptases [2]. Circulating pro-inflammatory cytokines, namely interleukin (IL)-1 and IL-6, can further enhance the hepatic expression of AAT and increase plasma levels up to 4 times [1]. Therefore, AAT is classified as an acute phase protein.

AAT deficiency is a common genetic disease that mainly causes early onset lung emphysema and, depending on the underlying mutation, liver cirrhosis due to accumulation of misfolded protein in the endoplasmic reticulum of hepatocytes [3]. Traditionally, the pulmonary manifestation of AAT deficiency is explained by a reduced anti-protease activity that enables degradation of elastic fibers by elastases released during neutrophil degranulation resulting in structural damage of the lung. Additionally, AAT-deficient patients are prone to develop inflammatory diseases such as panniculitis, anti-neutrophil cytoplasmic antibody-associated vasculitis, autoimmune diabetes, or metabolic disorders [4,5]. Increased pro-inflammatory cytokine levels and neutrophil counts were found in the lungs of AAT-deficient patients suffering from emphysema and/or airway obstruction, when compared to patients suffering from chronic obstructive pulmonary disease in genetically healthy persons [6,7]. These data suggest that the pathological manifestations of AAT-deficiency are not exclusively caused by deficiencies in anti-protease functions but may also contain an important inflammatory

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component.

Potent anti-inflammatory effects of AAT were described both *in vitro* and *in vivo*. Pro-inflammatory effects of the pyrogen lipopolysaccharide (LPS) are blunted by AAT, chemotaxis and leukocyte adhesion are reduced and even bactericidal functions of AAT were reported [8–13]. Gene expression of Toll-like receptor 4, pro-inflammatory cytokines IL-1β and tumor necrosis factor (TNF)-α are down-regulated, and the expression of anti-inflammatory factors such as IL-10 and IL-1 receptor antagonist are up-regulated [14–16]. AAT protects from inflammation in diverse experimental settings such as ischemia/reperfusion injury, rheumatoid arthritis, rejection of allogeneic islets, or graft-versus-host-disease [17–20].

Our laboratory recently described a novel anti-inflammatory function of AAT [21]. AAT potently reduces the ATP-induced secretion of IL-1β by monocytes and lung tissue slices in the presence of physiological AAT concentrations [21]. AAT signals via CD36, activates the calcium-independent phospholipase A2β and induces the secretion of a yet unknown nicotinic agonist. Stimulation of nicotinic acetylcholine receptors leads to an inhibition of the ionotropic functions of the ATP-sensitive P2X7 receptor. Accordingly, the assembly of the NLRP3 inflammasome is strongly inhibited, and the release of IL-1β is potently reduced [21]. Interestingly, this and other anti-inflammatory functions of AAT are independent of its anti-protease activity [22,23].

AAT preparations were developed to protect the lung tissue of AAT-deficient patients from further damage caused by proteases. The deceleration of the pulmonary disease process by AAT augmentation therapy, however, turned out to be modest [24]. In contrast, AAT substitution for the treatment of extrapulmonary inflammatory manifestations of AAT deficiency seems to be more promising [25]. In addition, AAT supplementation is a promising emerging treatment option for severe inflammatory conditions, such as graft versus host disease, in genetically AAT-sufficient patients [25–27]. As a matter of course, process technologies to produce commercial AAT preparations, are optimized to preserve the anti-protease activity of AAT and do not consider its anti-inflammatory potential.

As the anti-inflammatory functions of AAT preparations may turn out to be center stage, we compared the potential of two widely used AAT preparations, Prolastin® and Respreeza®, to inhibit the ATP-induced release of monocytic IL-1β *in vitro*. We detected marked functional differences between both medicaments and provide evidence that the oxidation state of AAT plays an essential role.

2. Materials and methods

2.1. Reagents

LPS (*E. coli*, L2654) and NE were obtained from Sigma-Aldrich (Merck, Taufkirchen, Germany), 2′(3′)-O-(4-benzoylbenzoyl)adenosine 5′-triphosphate triethylammonium salt (BzATP) from Jena Bioscience (Jena, Germany), Prolastin® from Grifols (Frankfurt, Germany), and Respreeza® from CSL Behring (Marburg, Germany).

2.2. Oxidation of AAT (oxAAT)

AAT was oxidized as previously described [8]. In sum, *N*-chlorosuccinimide (Sigma-Aldrich) was incubated with AAT for 30 min at ambient temperature at a molar ratio of 25:1. Thereafter, the mixture was ultrafiltrated to remove the excess of *N*-chlorosuccinimide (NCS). The efficiency of oxidation was controlled by testing anti-elastase activity. For this purpose, human NE was dissolved in 50 mM sodium acetate buffer, pH 5.5, containing 200 mM NaCl and stored at −20 °C. AAT and NE were diluted at a molar ratio of 1:2 in 100 mM Tris/HCl buffer, pH 7.0, containing 500 mM NaCl and incubated for 30 min at 37 °C. The reaction was stopped by adding Laemmli's sample buffer and heating at 90 °C for 5 minutes [28]. Thereafter, samples were separated by SDS-PAGE in 10% polyacrylamide gels [28] followed by staining with Comassie Brilliant Blue R-250 (Sigma-Aldrich).

2.3. U937 cells

Human monocytic U937 cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Rockford, IL, USA) supplemented with 10% fetal bovine serum (FBS Superior, Biochrom, Berlin, Germany) and 2 mM Glutamax® (Gibco) at 37 °C, 5% CO_2. Cells were primed with LPS (1 μg/ml) for 5 h and stimulated with BzATP (100 μM) for 30 min. Where applicable, AAT preparations were applied immediately before adding BzATP. Thereafter, cell-free cell culture supernatants were kept at −20 °C until measurement of IL-1β and lactate dehydrogenase activity (LDH).

2.4. Enzyme-linked immuno sorbent assay (ELISA)

IL-1β concentrations were determined by the Human IL-1beta DuoSet (R&D systems, Minneapolis, MN, USA) in combination with the DuoSet ancillary kit (R&D systems) according to manufacturer's instructions. The limits of detection ranged from 1.95 to 125 pg/ml IL-1β.

2.5. Cell viability

Cell viability was estimated by measurement of LDH concentrations using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA). The maximal LDH activity in the cells under investigation was determined by freezing and thawing of the same number of untreated cells.

2.6. SDS-PAGE

Proteins were denatured and separated by SDS-PAGE on 10% polyacrylamide gels along with dual color precision plus protein standards (Bio-Rad, Hercules, CA, USA). Gels were stained with Comassie Brilliant Blue R-250.

2.7. Statistics

Statistical analyses were performed using SPPS® (Version 23, IBM®, Armonk, NY, USA). Multiple data groups were first analyzed using Kruskal-Wallis test followed by two-tailed Mann-Whitney rank sum test. Determination of IC_{50} values and analysis of the dose–response relationship has been performed with R (Version 3.5.2.) by applying the function drm and the Weibull 2 model.

3. Results

3.1. Inhibition of the BzATP-induced release of IL-1β with AAT preparations

Untreated U937 cells and those treated with LPS alone released very low amounts of IL-1β into the supernatant (Fig. 1A). Application of BzATP to LPS-primed cells resulted in increased IL-1β concentrations ranging from 31.02 to 86.45 pg/ml that were set to 100% ($n = 8$, Fig. 1A). As expected [21], application of the physiological concentration of 1 mg/ml Prolastin® inhibited the BzATP-induced release of IL-1β by 95% (AAT-P, $n = 4$, $p = 0.004$, Fig. 1A). Application of the same dose of Respreeza®, however, led to a reduction of the BzATP-induced IL-1β release only by 19% (AAT-R, $n = 4$, Fig. 1A). Compared to Prolastin®, the efficacy of Respreeza® to inhibit BzATP-induced IL-β release is significantly lower ($p = 0.029$, $n = 4$, Fig. 1A). Neither LPS, BzATP nor both AAT preparations evoked changes in LDH activity in cell culture supernatants (Fig. 1B).

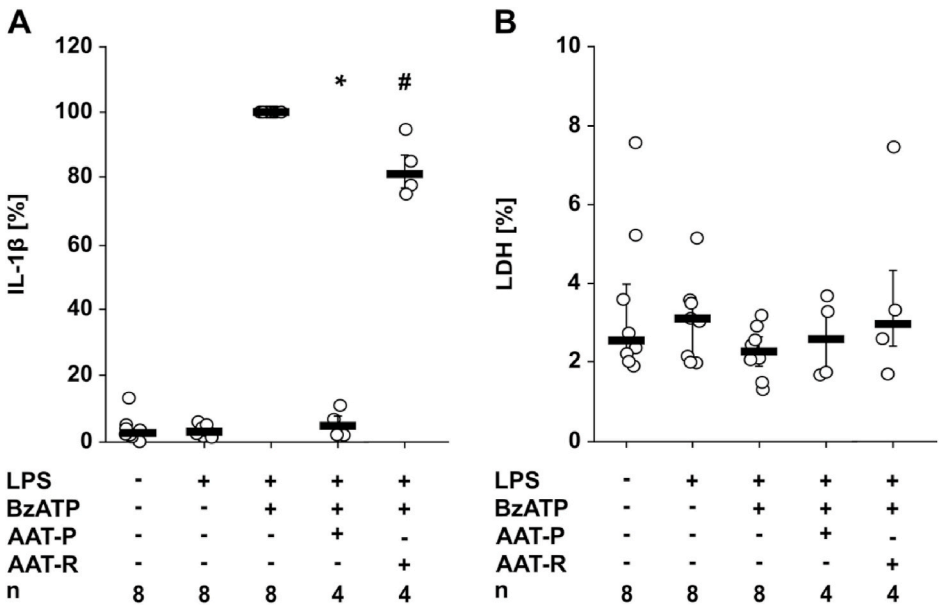

Fig. 1. Prolastin®, but not Respreeza®, strongly inhibits the BzATP-dependent release of interleukin (IL)-1β by human monocytic U937 cells. **(A, B)** Lipopolysaccharide (LPS)-primed (1 μg/ml, 5 h) U937 cells were stimulated with 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate (BzATP, 100 μM) in the presence of 1 mg/ml Prolastin® (AAT-P) or 1 mg/ml Respreeza® (AAT-R). IL-1β **(A)** and lactate dehydrogenase (LDH) **(B)** released into the cell culture supernatant were measured after 30 min. IL-1β concentrations obtained with BzATP treatment alone were set to 100% and all other values were calculated accordingly **(A)**. LDH values were normalized to values obtained from lysed cells **(B)**. Data are presented as individual data points, bars represent median and whiskers encompass the 25th to 75th percentile. N-numbers of independent experiments are indicated in the graphs. Experimental groups were compared by Kruskal-Wallis test followed by Mann-Whitney rank sum test (*$p \leq 0.05$ compared to supernatants from cells treated with LPS and BzATP; #$p \leq 0.05$ compared to supernatants from cells treated with LPS, BzATP and Prolastin®).

3.2. Oxidation of AAT preparations

Oxidation of AAT preparations was performed with NCS. As oxidation destroys the anti-protease activity of AAT [29], complex formation with NE followed by denaturing SDS-PAGE was investigated as a technical control. A distinct protein band with an estimated molecular mass of 52 kDa was visible in samples without NE (Fig. 2A and B). AAT/NE complexes were detected as an additional band with an estimated molecular mass of 77 kDa (Fig. 2A and B). This band was absent from oxidized AAT incubated with NE and only diverse weak bands of degradation products were detected (Fig. 2A and B).

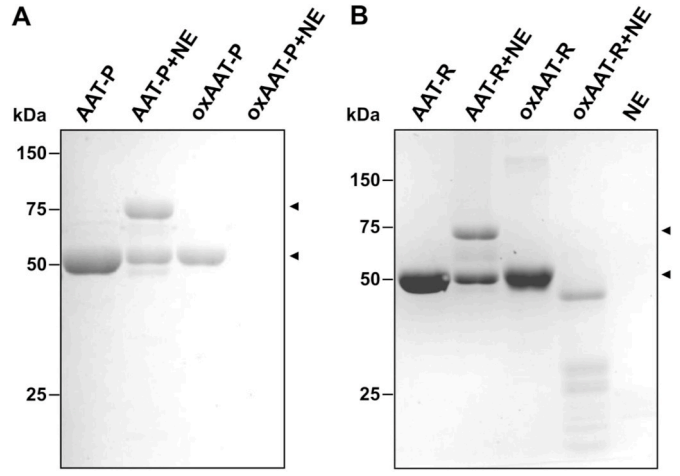

Fig. 2. Oxidation of AAT preparations prevents complex formation with neutrophil elastase (NE). Samples were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide) and stained with Brilliant Blue **(A, B)**. Native (AAT-P) and oxidized Prolastin® (oxAAT-P) **(A)** as well as native (AAT-R) and oxidized Respeeza® (oxAAT-R) **(B)** were incubated with NE before electrophoresis. In the last lane, NE was applied alone. Arrow heads are pointing to AAT (52 kDa), oxAAT (52 kDa) and to a covalent complex of AAT and NE (77 kDa).

3.3. Inhibition of the BzATP-induced IL-1β release with oxidized AAT preparations

Next, the effect of oxidized AAT preparations on the BzATP-induced IL-1β release from U937 cells was investigated and dose-response relationships were determined. The dose-response relationship of native Prolastin® has already been investigated and revealed an IC_{50} of 0.21 mg/ml [21]. Here, oxidized Prolastin® dose-dependently inhibited the BzATP-induced IL-1β release with an IC_{50} of 0.09 mg/ml (n = 4, Fig. 3A). Native Prolastin® at a concentration of 1 mg/ml, that was included as a positive control, reduced the BzATP-induced IL-1β release to 6% and thereby did not differ significantly from cells treated with the same dose of oxidized Prolastin® (n = 6, p = 0.762, Fig. 3A). Neither native Prolastin® nor oxidized Prolastin® increased LDH levels in supernatants of BzATP-stimulated U937 cells (≤10% LDH activity, Fig. 3B).

Finally, the inhibition of the BzATP-induced IL-1β release by Respreeza® (AAT-R) and oxidized Respreeza® (oxAAT-R) was investigated. Since Respreeza® showed only a low activity in the native state (Fig. 1A), the dose-response relationship of native Respreeza® was not investigated. Interestingly, oxidized Respreeza® inhibited the BzATP-mediated IL-1β release in a dose-dependent manner with an IC_{50} of 0.04 mg/ml (0.01, 0.1, 0.5, 1 and 2 mg/ml oxidized Respreeza®, n = 4, Fig. 4A). Native Respreeza® at a concentration of 1 mg/ml reduced the BzATP-induced IL-1β release by 20% and differed significantly from the same dose of oxidized Respreeza® that exerted a strong inhibitory effect (n = 6, p = 0.01, Fig. 4A). Neither native Respreeza® nor oxidized Respreeza® increased the LDH activity in supernatants of BzATP-stimulated U937 cells (≤10% LDH activity, Fig. 4B).

4. Discussion

We have demonstrated before, that the commercial AAT preparation Prolastin® and AAT isolated from the blood of healthy donors or patients, who underwent cardiopulmonary bypass combined with open cardiac surgery, efficiently inhibit the BzATP-induced release of IL-1β by human monocytic U937 cells [21,23]. In addition, we showed, that chemical oxidation of AAT from healthy donors impairs its anti-protease function but does not change its ability to inhibit IL-1β release [21]. We demonstrate here that oxidized Prolastin® functions as an efficient inhibitor of BzATP-induced release of monocytic IL-1β. This indicates that AAT lacking anti-protease activity can efficiently exert anti-inflammatory functions. Similar results regarding other

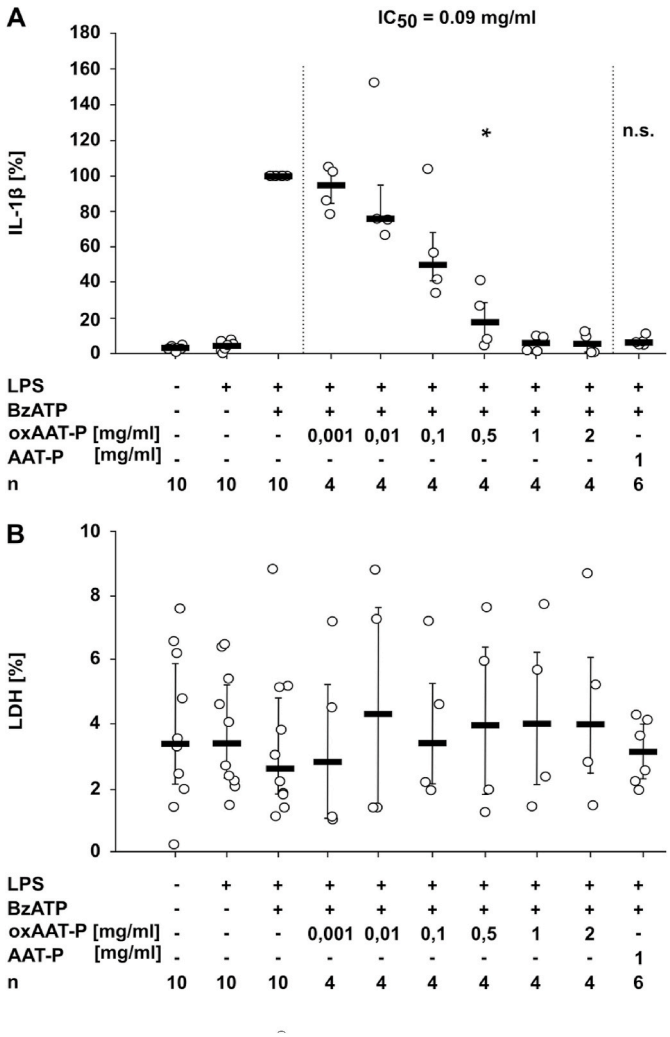

Fig. 3. Native and oxidized Prolastin® inhibits the ATP-dependent release of interleukin (IL)-1β from U937 cells. **(A, B)** Human lipopolysaccharide (LPS)-primed monocytic U937 cells were stimulated with 2′(3′)-O-(4-benzoylbenzoyl) adenosine-5′-triphosphate (BzATP, 100 μM) in the presence 1 mg/ml of native (AAT-P) or different concentrations of oxidized Prolastin® (oxAAT-P). IL-1β **(A)** and lactate dehydrogenase (LDH) **(B)** released to the supernatant were measured after 30 min. IL-1β concentrations obtained with BzATP treatment alone were set to 100% and all other values were calculated accordingly **(A)**. LDH values were normalized to values obtained from lysed cells **(B)**. Data are presented as individual data points, bars represent median and whiskers encompass the 25th to 75th percentile. N-numbers of independent experiments are indicated in the graphs. Experimental groups were compared by Kruskal-Wallis test followed by Mann-Whitney rank sum test (*$p \leq 0.05$ compared to supernatants from cells treated with LPS and BzATP alone; n.s., not significant, $p \geq 0.05$ compared to supernatants from cells treated with LPS, BzATP and 1 mg/ml oxidized Prolastin®).

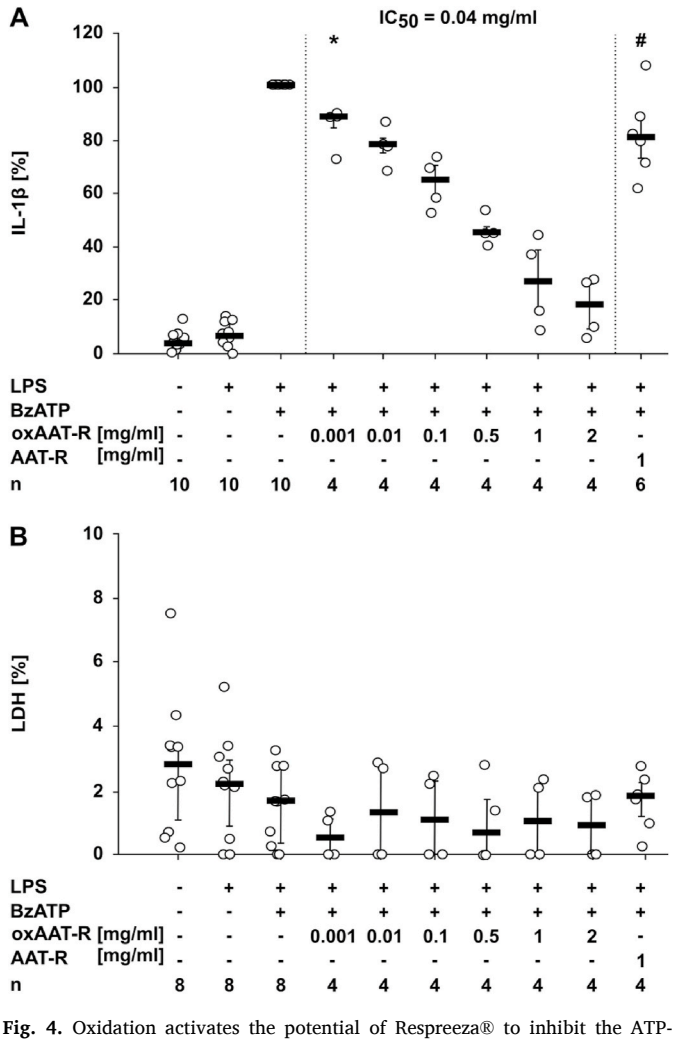

Fig. 4. Oxidation activates the potential of Respreeza® to inhibit the ATP-induced release of interleukin (IL)-1β from human monocytic U937 cells. **(A, B)** Lipopolysaccharide (LPS)-primed (1 μg/ml, 5 h) U937 cells were stimulated with 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate (BzATP, 100 μM) in the presence of 1 mg/ml native Respreeza (AAT-R) or different concentrations of oxidized Respreeza® (oxAAT-R). IL-1β **(A)** and lactate dehydrogenase (LDH) **(B)** released to the supernatant were measured after 30 min. IL-1β concentrations obtained with BzATP treatment alone were set to 100% and all other values were calculated accordingly **(A)**. LDH values were normalized to values obtained from lysed cells **(B)**. Data are presented as individual data points, bars represent median and whiskers encompass the 25th to 75th percentile. N-numbers of independent experiments are indicated in the graphs. Experimental groups were compared by Kruskal-Wallis test followed by Mann-Whitney rank sum test (*$p \leq 0.05$ compared to supernatants from cells treated with LPS and BzATP; # $p \leq 0.05$ compared to supernatants from cells treated with LPS, BzATP and 1 mg/ml oxidized Respreeza®).

anti-inflammatory properties of AAT have been shown by other laboratories [8,30]. The important new finding of our study is that anti-inflammatory and anti-protease functions of AAT can be fully uncoupled. The AAT preparation Respreeza® is less active compared to Prolastin® regarding the inhibition of the BzATP-induced release of monocytic IL-1β but is characterized by an efficient anti-protease activity.

We suggest the existence of at least three different functional states of AAT: (1) AAT with anti-protease activity alone (Respreeza®), (2) AAT with anti-protease activity and the potential of inhibiting monocytic IL-1β release (Prolastin® and AAT purified from human plasma) and (3) AAT with IL-1β release inhibiting activity in the absence of anti-protease activity (all oxidized AAT preparations investigated). State 1 is presumably an artificial state of AAT that seems to form during the purification of Respreeza®. It remains to be investigated if Respreeza® is activated *in vivo* and transformed to state 2 after infusion into the circulation. State 2 most probably corresponds to naturally occurring AAT circulating in healthy persons. We can, however not exclude, that AAT is modified during the production of Prolastin® and/or during its purification in our laboratory [21]. AAT in state 3, as it was investigated in this study, is also an artificial form of AAT because of its oxidation with N-chlorosuccinimide. Functionally it may correspond to naturally occurring oxidized AAT that forms due to oxidative stress [31]. Endogenous AATs from ZZ, SZ or MZ-AAT-deficient patients are other variants, which need to be tested regarding their capacity to control the

BzATP-induced release of monocytic IL-1β.

Respreeza® and Prolastin® were compared in two studies regarding their anti-protease function, purity, structure, and chemical modifications [32,33]. It should be mentioned that these analyses were performed in the laboratories of the company producing Respreeza®. Among all AAT preparations tested, Respreeza® and Zemaira® (the US brand name of the same product) had the highest purity and specific anti-protease activity [10]. Prolastin® contained detectable levels of numerous other proteins that were absent from Respreeza® [10]. These differences, however, do not provide a plausible explanation of our findings.

In contrast to Prolastin®, the purification process of Respreeza® includes chemical reduction by dithiothreitol and separation by hydrophobic interaction chromatography [33]. Both steps might be responsible for a reduction of the anti-inflammatory properties of Respreeza®. Hydrophobic interaction chromatography might detach of non-covalently interacting fatty acids from AAT. These fatty acids in association with AAT were shown to be essential for the induction of the pleiotropic angiopoietin-like protein 4 by human peripheral blood mononuclear leukocytes [34]. As we demonstrated before, that the AAT-mediated control of monocytic IL-1β release involves the fatty acid receptor CD36 [21], we first tested in a small number of pilot experiments if a reconstitution of Respreeza® with fatty acids linoleic acid or oleic acid [34] reactivates this inhibitory activity. However, we found that complexes of Respreeza® and linoleic acid or oleic acid were inactive (data not shown).

To investigate if reduction by dithiothreitol is responsible for the inhibitory function on IL-1β release, we chemically oxidized Respreeza® and obtained a preparation that efficiently inhibited the BzATP-induced release of IL-1β by LPS-primed monocytes but was devoid of anti-protease function. Treatment with dithiothreitol removes the cysteine group from C232, a modification that was shown to be irrelevant for the anti-protease function of AAT [33,35]. Incubation with the oxidant *N*-chlorosuccinimide will, however, not cysteinylate a reduced C232 position, suggesting that this cyteinylgroup is not essential for the AAT function under investigation. Nine methionines are present in the amino acid sequence of AAT that are potentially reduced by dithiothreitol and oxidized by *N*-chlorosuccinimide. Two of these methionine groups (Met358 and Met352) are located in the reactive center that is essential for the anti-protease function of AAT [29,36].

Because Prolastin® and AAT isolated from healthy persons exhibit anti-protease function, we assume that the oxidation of these methionines is not essential for the capacity to inhibit the BzATP-induced release of IL-1β. We suggest that at least a partial oxidation of the remaining seven methionines is essential for an efficient inhibition of the BzATP-induced IL-1β release. The capacity of oxidized AAT to inhibit NE was included as a technical control of AAT oxidation. We explicitly do not suggest the use of chemically oxidized AAT as a medicament. Therefore, the properties of oxidized AAT including inhibition of other proteases have not been further investigated.

This study has numerous limitations and only gives a first glimpse on potential pitfalls associated with the use of AAT preparations in basic research and their clinical application. We use human monocytic U937 cells, which have lost numerous traits of their natural counterparts and entail all the inherent drawbacks typical for cell lines. No tests in primary cells and no testing in living organisms were included. However, we demonstrated before, that primary human and murine monocytes closely resemble U937 cells regarding the AAT-mediated control of ATP-induced IL-1β release [21], but admittedly we fully ignore all kinds of complex interactions occurring *in vivo*. Further, we investigate only one of several known anti-inflammatory functions of AAT. Many more studies are needed to compare AAT preparations in different experimental settings. Finally, there are more AAT preparations on the market that also should be tested in our experimental setting.

Despite the limitations of this study, the relevance of our finding is high, especially in the light of the increasing awareness of the clinical importance of anti-inflammatory functions of AAT. When aiming at a supplementation of AAT-deficient patients with AAT that exerts all the pleiotropic functions discovered so far, the process technologies as well as the functional tests of all AAT-based medicaments need to be scrutinized and, where necessary, improved. The same holds true for the off-target use of AAT preparations for the prevention or treatment of inflammatory diseases in AAT-sufficient patients.

Author statement

Alisa Agné: investigation, formal analysis, visualization; writing review & editing; **Katrin Richter:** writing review & editing, formal analysis, visualization; **Winfried Padberg:** conceptualization, writing review & editing, supervision; **Sabina Janciauskiene:** conceptualization, writing review & editing, methodology, resources; **Veronika Grau:** conceptualization, project administration, writing original draft, writing review & editing, validation, funding acquisition.

Declaration of competing interest

The authors declare that they have no competing interests.

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