2-O, 3-O Desulfated Heparin Blocks High Mobility Group Box 1 Release by Inhibition of p300 Acetyltransferase Activity

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Abstract

High mobility group box 1 (HMGB1) is an alarmin released from macrophages after infection or inflammation and is a biomarker of lung disease progression in patients with cystic fibrosis. We reported that 2-O, 3-O desulfated heparin (ODSH) inhibits the release of HMGB1 from murine macrophages triggered by neutrophil elastase both *in vivo* and *in vitro*. HMGB1 shuttles between the nucleus and the cytoplasm. When acetylated at lysine residues in the nuclear localization signal domains, HMGB1 is sequestered in the cytoplasm and is fated for secretion. In this study, we investigated the mechanism by which ODSH blocks HMGB1 secretion. We tested whether ODSH inhibits the activity of p300, a histone acetyltransferase that has been linked to HMGB1 acetylation and release. ODSH inhibited both neutrophil elastase and LPS-triggered HMGB1 release from the murine macrophage cell line RAW264.7 in a concentration-dependent manner. Fluorescein-labeled ODSH was taken up by RAW264.7 cells into the cytoplasm as well as the nucleus, suggesting an intracellular site of action of ODSH for blocking HMGB1 release. ODSH inhibited RAW264.7 cell nuclear extract, human macrophage nuclear extract, and recombinant p300 HAT activity *in vitro*, resulting in the failure to

acetylate HMGB1. *In silico* molecular modeling predicted that of the numerous possible ODSH sequences, a small number preferentially recognizes a specific binding site on p300. Fluorescence binding studies showed that ODSH bound p300 tightly (dissociation constant ~ 1 nM) in a highly cooperative manner. These results suggest that ODSH inhibited HMGB1 release, at least in part, by direct molecular inhibition of p300 HAT activity.

Keywords: p300; heparin; high mobility group box 1

Clinical Relevance

We report that a modified heparin, 2-O, 3-O desulfated heparin (ODSH), inhibits high mobility group box 1 release from macrophages via inhibition of a histone acetyltransferase, p300, required for acetylation. This is a novel mechanism by which ODSH blocks inflammation, and this finding supports the development of ODSH as an antiinflammatory therapy for cystic fibrosis.

High mobility group box 1 (HMGB1) is found at high concentrations in the airways of patients with chronic inflammatory lung diseases. Increased airway HMGB1 is associated with bronchopulmonary

dysplasia in preterm infants (1), severe asthma in children (2), and chronic obstructive pulmonary disease in adults (3, 4). HMGB1 is a biomarker for lung disease progression in patients with cystic

fibrosis (CF) (4, 5). Furthermore, a polymorphism in the gene *Ager*, which encodes the HMGB1 receptor, receptor for advanced glycation end products (RAGE), results in increased RAGE expression

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and is associated with more severe lung disease in CF (6).

HMGB1 is a multifunctional protein that shuttles between the nucleus and the cytoplasm (7). In the nucleus, HMGB1 functions as a nonhistone chromatin binding protein that regulates gene expression. However, in the cytoplasm, if HMGB1 is modified by lysine acetylation at the nuclear localization domains, it is blocked from reentry into the nucleus and is fated for secretion. Histone acetyltransferases (HAT), including p300, catalyze the lysine acetylation required for HMGB1 secretion (8). Once secreted, HMGB1 acts as an alarmin and activates RAGE or facilitates ligand activation of Toll-like receptor (TLR)-2 and TLR-4 (9). HMGB1 ligation of RAGE, TLR-2, or TLR-4 results in the activation of nuclear factor- κ B and neutrophilic inflammation (10). HMGB1 also contributes to chronic neutrophilic inflammatory airway disease

by blunting macrophage phagocytosis and clearance of bacteria (11), inhibiting phagocytosis of apoptotic neutrophils (12), promoting neutrophil extracellular trap formation (13), and inducing dendritic cell-mediated Th17 polarization (14). Although HMGB1 is a potential therapeutic target for reducing excessive inflammation in chronic airway diseases, there are currently no therapies that target HMGB1 inhibition.

We and others have reported that a partially modified heparin, 2-O, 3-O desulfated heparin (ODSH), is a potent antiprotease and antiinflammatory agent with anti-HMGB1 properties (15, 16). Loss of the 2-O and 3-O sulfate residues markedly reduces the anticoagulant activity of ODSH, which endows ODSH with a better safety profile as a therapeutic agent than its parent compound heparin for patients with CF. ODSH blocks HMGB1 *in vivo* in both a neutrophil elastase

(NE)-induced intratracheal inflammatory model (17) and a *Pseudomonas aeruginosa* pneumonia model (18). However, the mechanism of how ODSH blocks HMGB1 secretion is unknown. Therefore, we tested whether ODSH inhibits p300 HAT activity and thus prevents HMGB1 secretion from macrophages.

Materials and Methods

See online supplement for details.

ODSH and NE or LPS Treatment of Raw264.7 Cells

RAW264.7 cells (TIB-71; ATCC, Rockville, MD) were preincubated in serum-free media with ODSH (0–7.5 μ M, 30 min, gift from Dr. Stephen Marcus, Cantex Pharmaceuticals, Weston, FL) and then incubated with NE (0.5 μ M, 4 h) (SE563; Elastin Products, Owensville, MO) (17). For LPS treatments, RAW264.7 was preincubated

Figure 1. 2-O, 3-O desulfated heparin (ODSH) inhibited neutrophil elastase (NE)- and LPS-induced high mobility group box 1 (HMGB1) release. RAW264.7 cells in serum-free media were pretreated with ODSH (0.5, 2, or 7.5 μ M) for 30 minutes, and then treated with NE (0.5 μ M) for 4 hours. (A) Conditioned media were collected and tested for HMGB1 expression using Western analysis. Densitometric analysis of autoradiographs from three experiments (mean $\pm$ SEM; $n = 3$) was summarized graphically and is expressed as relative values compared with control treatment (Ctrl). (B) $^{+}$ HMGB1 levels after NE treatment were significantly greater than Ctrl or HMGB1 levels after 7.5 μ M ODSH treatment alone ($P < 0.05$); *HMGB1 levels after NE + 0.5 or NE + 2 μ M ODSH were significantly lower than HMGB1 levels after NE treatment alone ($P < 0.05$); **HMGB1 levels after NE + 7.5 μ M ODSH were significantly lower than HMGB1 levels after NE treatment alone ($P < 0.01$). (C) RAW264.7 cells were treated with LPS $\pm$ ODSH (0.5, 2, or 5 μ M) in OPTI-MEM media for 24 hours. Conditioned media were collected, and Western analysis for HMGB1 was performed as described in MATERIALS AND METHODS. Molecular weight markers are shown (KD). (D) Densitometric analysis of autoradiographs from three experiments (mean $\pm$ SEM; $n = 3$) was summarized graphically and is expressed as relative values compared with Ctrl. $^{+}$ HMGB1 levels after LPS treatment were significantly greater than Ctrl or HMGB1 levels after 5 μ M ODSH treatment alone ($P < 0.01$); *HMGB1 levels after LPS + 2 μ M ODSH treatment were significantly lower than HMGB1 levels after LPS treatment alone ($P < 0.05$); **HMGB1 levels after LPS + 5 μ M ODSH were significantly lower than HMGB1 levels after LPS treatment alone ($P < 0.01$).

in serum-free OPTI-MEM I with ODSH (0–5.0 μ M, 1 h) and then incubated with LPS (100 ng/ml, 24 h) (L9764; Sigma Aldrich, St. Louis, MO). Conditioned media (38 μ l) from NE and LPS experiments were separated on a 4–20% polyacrylamide gel electrophoresis for HMGB1 Western analyses.

Fluorescein Labeling of ODSH

Fluorescein conjugation of ODSH was performed using fluoresceinamine (FA) and acid-amine coupling technology, as described previously. The fluorescein-labeled ODSH (FA-ODSH) was characterized using size exclusion chromatography, as described previously (19).

Cell Uptake and Localization of FA-ODSH by Confocal Microscopy Imaging

RAW264.7 cells were incubated with FA-ODSH (100 μ g) for 2 or 24 hours, fixed, and counterstained with 4',6-diamidino-2-phenylindole for detection of nuclei. Slides were examined using confocal laser scanning microscopy (LSM 700; Zeiss, Oberkochen, Germany), and fluorescent images were analyzed with Zen 2012 software.

Human Blood Monocyte-Derived Macrophages

Heparinized blood (20 ml) from healthy volunteers was obtained after institutional review board-approved informed consent. Monocytes were isolated and cultured in granulocyte-macrophage colony-stimulating factor to differentiate to macrophages, as reported previously (20).

Macrophage Nuclear Extract- or p300-Induced HMGB1 Acetylation *In Vitro*

The effect of preincubation with ODSH (8.5–850 μ M) on recombinant HMGB1 (2 μ g, H4652; Sigma) acetylation after incubation with RAW264.7 (45 μ g), human blood monocyte-derived macrophages (hBMDM) (25 μ g) cell nuclear extract, or recombinant p300 (50 ng) and acetyl CoA (Active Motif, Carlsbad, CA) was determined by Western analysis for acetylysine.

Western Analyses for HMGB1 and Acetylysine

HMGB1 Western analysis was performed as reported previously (17). For acetylysine detection, recombinant HMGB1 was collected by Dynabeads (10103; Thermo Fisher, Waltham, MA), separated by 4–20%

polyacrylamide gel electrophoresis, and transferred to nitrocellulose. Membranes were incubated with antiacetylysine antibody (1:1000, rabbit mAb, 9814; Cell Signaling, Danvers, MA) overnight at 4°C, followed by goat antirabbit IgG-horse radish peroxidase (1:5,000, 7074; Cell Signaling), and detection was performed using Western Lightning Plus ECL (NEL105001EA; Perkin Elmer, Waltham, MA). Densitometric analysis was performed with Image Quant TL software (GE Healthcare, Pittsburgh, PA).

In Silico Molecular Modeling of ODSH and p300 Interaction

Molecular modeling of the ODSH-p300 complex was performed using GOLD v5.2 software with a 3BIY crystal structure of p300 (21) and a library of 1,728 ODSH sequences, following the strategy described earlier for glycosaminoglycans (GAGs) (22, 23) (*see online supplement*).

Affinity of ODSH for p300

The affinity of ODSH for p300 was measured in a spectrofluorimetric assay by titrating ODSH as a function

of p300 concentration in 20 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid, and 0.1% PEG8000 at 25°C, following the strategy described earlier for GAGs (24–26) (*see online supplement*).

Statistical Analysis

Western and HAT activity data were compared by one-way nonparametric analysis of variance with *post hoc* comparisons by Tukey test (Graph Pad Prism 5; GraphPad Software Inc., La Jolla CA).

Results

NE And LPS Activated HMGB1 Release and ODSH Blocked HMGB1 Release from Raw264.7 Cells

Previously, we reported that intratracheal administration of NE to BALB/c mice increased HMGB1 release into the bronchoalveolar fluid and that NE treatment of RAW264.7, a mouse macrophage cell line, activated HMGB1

Figure 2. Fluoresceinamine-labeled ODSH (FA-ODSH) was taken up by RAW264.7 cells into the cytoplasm and nucleus. RAW264.7 cells cultured on glass coverslips (5×10^5 cells) were exposed to FA-ODSH (100 μ g/ml) for 2 or 24 hours, washed with phosphate-buffered saline twice, and fixed with 500 μ l paraformaldehyde; cells were stained with 4',6-diamidino-2-phenylindole and fixed with mounting media. Confocal microscopy images were obtained with Zeiss LSM 700. (A) The presence of FA-ODSH in the cytoplasm was seen at 2 hours of incubation (green with white arrows) and (B) morphology of corresponding image is shown by merging the green fluorescence (FA-ODSH) + 4',6-diamidino-2-phenylindole (blue nuclear stain) + differential interference contrast. (C) At 24 hours, FA-ODSH was present in the nucleus (green with white arrowheads). (D) Corresponding merged image. White arrows indicate FA-ODSH in cytoplasm but not the nuclei; white arrowheads indicate FA-ODSH in the nuclei. Images are representative of $n = 3$ experiments.

secretion into cell culture media. Importantly, we demonstrated that ODSH blocks NE release of HMGB1, at least in part, by inhibiting NE activity (17). In this report, we tested whether ODSH inhibited HMGB1 release from RAW264.7 cells when triggered by NE or by LPS, an inflammatory mediator. We show that ODSH inhibited both NE- and LPS-activated HMGB1 release in a concentration-dependent manner (Figure 1). Importantly, HMGB1 release in these experiments was not caused by cell necrosis (as determined by LDH release assay, data not shown). Therefore, these data support the concept that ODSH blocked HMGB1 secretion by mechanisms other than its antiprotease activity. Specifically, we propose that HMGB1 release was caused by acetyllysine modification catalyzed by HAT,

specifically by p300/creb binding protein-associated factor, creb binding protein, or p300 HAT (8) and that ODSH blocked this modification by inhibiting HAT activity.

ODSH Was Taken Up by the Raw264.7 Murine Macrophage Cell Line

To determine whether ODSH can directly inhibit HAT, we tested whether ODSH is taken up by macrophages, using ODSH covalently linked to fluorescein molecules (i.e., FA-ODSH). RAW264.7 cells were exposed to FA-ODSH over time, and the cells were analyzed by confocal microscopy (Figure 2). By 2 hours, FA-ODSH was taken up into the cytoplasm, and by 24 hours, FA-ODSH was visualized in the nucleus. These data demonstrate that ODSH gained access to macrophage intracellular compartments, including the

nucleus; thus, ODSH may directly interact with and inhibit HAT.

ODSH Inhibited Nuclear Extract HAT Activity and Specifically Inhibited p300 Acetylation of HMGB1 Lysine Residues

Because HAT catalyze the acetylation of lysine residues required for HMGB1 release, these enzymes are a logical target for inhibiting HMGB1 acetylation and cellular release. Heparin is an inhibitor of HAT activity (27). Therefore, we hypothesized that ODSH would also inhibit HAT, blocking HMGB1 acetylation and release. RAW264.7 cell nuclei were isolated, and lysate protein was prepared. First, using a spectrophotometric assay of histone H3 acetylation, we tested whether ODSH blocked RAW264.7 nuclear HAT activity. We determined that ODSH inhibited HAT

Figure 3. ODSH inhibited HMGB1 acetylation induced by RAW264.7 nuclear lysates and by human blood monocyte-derived macrophage (hBMDM) nuclear lysates in a concentration-dependent manner. (A) RAW264.7 cell or (B) hBMDM nuclear extract was incubated with recombinant HMGB1-his-tag, acetyl-CoA (Active Motif, Carlsbad, CA), and ODSH (8.5–850 μM), at room temperature for 2 hours. The reaction mixture was collected using His-tagged Dynabeads and was resuspended in loading buffer. An aliquot (20 μl) was separated by 4–20% polyacrylamide gel electrophoresis for Western analyses for acetyllysine and recombinant HMGB1. Western blots are representative of three separate experiments for RAW264.7 cells and hBMDM. Molecular weight markers are shown. (C and D) Graphic summaries of densitometric analyses of autoradiographs were expressed as the ratio of acetyllysine to HMGB1 normalized to the no-ODSH control treatment condition (mean ± SEM; $n = 3$). ***ODSH significantly inhibited RAW264.7 nuclear extract-induced HMGB1 acetylation ($P < 0.001$); *ODSH significantly inhibited hBMDM nuclear extract-induced HMGB1 acetylation ($P < 0.05$). CoA, coenzyme A.

activity in a concentration-dependent manner (see Figure E1 in the online supplement). Next, we developed an assay of HMGB1 lysine acetylation to test whether ODSH specifically inhibited HAT-catalyzed lysine acetylation of HMGB1. Using recombinant HMGB1 incubated with an acetyl donor, acetyl-CoA, and a source of HAT activity, RAW264.7 (Figures 3A and 3C) or hBMDM nuclear lysate (Figures 3B and 3D), we used Western analysis to test whether ODSH blocked lysine acetylation of HMGB1. We demonstrated that RAW264.7 cell or hBMDM nuclear extract HAT activity was sufficient to catalyze HMGB1 lysine acetylation, and that ODSH inhibited the acetyllysine modification in a concentration-dependent manner. We then investigated whether p300 was a specific HAT target for ODSH. Several potential HAT are expressed in macrophages; we focused on p300 because this enzyme catalyzes HMGB1 lysine acetylation (8) and has been reported to interact with HMGB1 by atomic force spectroscopy (28). We discovered that ODSH inhibited recombinant p300 HAT activity (Figure 4A) and that p300-catalyzed HMGB1 lysine acetylation was inhibited by ODSH in a concentration-dependent manner (Figures 4B and 4C). Importantly, ODSH did not deacetylate HMGB1 by increasing HDAC activity (Figure E2). Thus, our results support a novel mechanism of ODSH that blocks HMGB1 release by inhibition of p300 HAT activity.

ODSH Interaction with P300 Using *In Silico* Molecular Modeling

To gain some insight into whether ODSH could interact directly with p300, we used an *in silico* combinatorial virtual library screening (CVLS) strategy. This *in silico* molecular modeling approach has been used extensively in the literature for studying interactions of GAGs with proteins of diverse types (22, 23, 29, 30). The structure of p300 shows several regions that are rich in basic amino acids. Typically, sulfated GAGs, such as ODSH, recognize and bind to Lys/Arg-rich domains on proteins (31), and the presence of multiple such regions makes their identification challenging. We implemented a simple method of identifying potential sites of binding by studying the recognition of a small group of ODSH hexasaccharide

Figure 4. ODSH inhibited p300 histone acetyltransferase (HAT) activity and p300-induced HMGB1 acetylation *in vitro* in a concentration-dependent manner. (A) To test whether ODSH inhibited p300 HAT activity, recombinant p300 HAT activity (50 ng/50 μ l total reaction volume; Active Motif) was assayed in the presence or absence of ODSH (0–850 μ M) using histone H3 as a substrate. Relative HAT activity was determined using a HAT activity assay kit (Active Motif) and was expressed as relative fluorescent units and normalized to the incubation control with no ODSH (0). Data summarize three experiments (mean $\pm$ SEM; $n = 3$). ***ODSH 85 and 850 μ M significantly inhibited p300 HAT activity ($P < 0.001$). (B) To evaluate whether ODSH inhibited p300-catalyzed lysine acetylation of HMGB1, recombinant p300 (50 ng/50 μ l total reaction volume; Active Motif) was incubated with recombinant HMGB1 (2 μ g), acetyl-CoA (Active Motif), and a concentration range of ODSH (8.5–850 μ M). An aliquot of reaction mixture (5 μ l) was separated by 4–20% polyacrylamide gel electrophoresis, and Western analyses were performed for acetyllysine and HMGB1. Graphic summary of densitometric analyses of Western autoradiographs are expressed as the ratio of acetyllysine/HMGB1 normalized to a no-ODSH control treatment condition (mean $\pm$ SEM; $n = 3$ experiments). (C) ***All ODSH treatments significantly decreased acetyllysine/HMGB1 levels compared with no ODSH treatment ($P < 0.001$).

sequences at domains containing Lys1291, Arg1312, Arg1342, Arg1391, Lys1484, and Arg1627, which were labeled as binding sites (BS) 1 through 6 (BS1–BS6) (Figure 5A), and which ensured coverage of the entire p300 HAT domain protein surface. Using our CVLS strategy, these sequences were docked in triplicate onto all six sites (BS1–BS6). These initial studies showed that ODSH sequences interacted well in BS1 and BS5, as evidenced by GOLDScores >70 (not shown), while also displaying a low root mean square deviation of $<2.5\text{\AA}$ (not shown) between the best docked solutions. This eliminated other sites from consideration. To further parse the most favored site of binding for ODSH, we applied our CVLS strategy to a

library of 1,728 hexasaccharide sequences to sites BS1 and BS5 of p300 (see online supplement). This resulted in 13 and 7 ODSH hexasaccharide sequences recognizing BS1 and BS5, respectively. Of these, ODSH sequences binding in BS1 displayed a much higher GOLDScore (≥ 100), which implied that the preferred site of ODSH interaction onto p300 is likely BS1 (Figure 5B). Analysis of these sequences using UCSF Chimera (32) indicated the presence of multiple hydrogen bonds (e.g., with Lys1287, Lys1291, Arg1292, Ser1295, Thr1300, Asn1304, Asn1307, Arg1311, Arg1324, and His1659) of BS1 (Figure 5B). Thus, the *in silico* CVLS strategy predicted strong interaction between p300 HAT and ODSH.

ODSH Interaction with p300 Using Spectrofluorometry

To investigate the interaction of p300 with ODSH in solution, we used the change in the intrinsic fluorescence of p300 on titration with ODSH at pH 7.4 and 25°C. This technique has been used extensively in the literature for studying direct protein–ligand interactions, especially for GAGs (24, 25). For a typical GAG-protein system, a saturable, hyperbolic change in fluorescence emission suggests physical interaction between the two binding partners. For the ODSH-p300 system, a sigmoidal change in fluorescence was observed (Figure 6), which is characteristic of a cooperative phenomenon in which the initial binding of ODSH to p300 is followed by a conformational change in the protein, resulting in tight binding. This profile was well fitted using the standard, three-parameter Hill equation I (see online supplement), which gave a maximal fluorescence change of $129 \pm 6\%$, a Hill coefficient of 3.1 ± 0.5 , and a dissociation constant of 1.4 ± 0.1 nM. This indicates extremely tight binding of ODSH to p300, which was also predicted on the basis of the molecular modeling results, as described above. The measured Hill coefficient of 3 suggests a strongly cooperative interaction, which alludes to the possibility of highly specific recognition of ODSH by p300.

Discussion

HMGB1 is a major inflammatory mediator in the airways of patients with chronic inflammatory airway diseases including bronchopulmonary dysplasia (1), asthma (2), chronic obstructive pulmonary disease (3), and CF (4, 5). Classically, HMGB1 is either secreted by macrophages and other airway cells after infection (11), inflammation (17), and oxidative stress (33, 34), or released from necrotic cells, attracting neutrophils to necrotic tissues (35). Neither of the inflammatory stimuli used to activate HMGB1 release from RAW264.7 cells caused macrophage cell death, so HMGB1 was released by signaling mechanisms, resulting in secretion. LPS induces HMGB1 translocation from the nucleus to cytoplasmic endolysosomes, where phosphatidylcholine induces secretion (36). The mechanism of NE-induced HMGB1 release is not yet known but may be triggered by NE-induced

Figure 5. *In silico* molecular modeling of ODSH and p300 domains of interaction using the combinatorial virtual library screening (CVLS) strategy. (A) Initial screening was performed with a small group of ODSH sequences to detect binding to p300 HAT domains containing Lys1291, Arg1312, Arg1342, Arg1391, Lys1484, and Arg1627. Six potential ODSH binding sites (BS) were identified, BS 1–6. (B) Results from CVLS screening of 1,728 ODSH sequences interacting with BS1 of p300. Shown are CVLS technology-predicted interactions of 13 common ODSH sequences (green-colored sticks) interacting with p300 BS1. The interacting residues are H1659, K1287, R1292, K1291, N1307, R1311, N1304, T1309, and S1295. Elements are denoted as follows: red, oxygen; dark blue, nitrogen; and light blue, carbon. NRE, nonreducing end of ODSH sequences; RE, reducing end of ODSH sequences.

oxidative stress (37) and is currently under investigation.

HMGB1 is recognized as a biomarker of lung disease progression in CF (4, 5). However, there are currently no approved therapies to block HMGB1-induced inflammation. Importantly, inhibition of

HMGB1 in mouse models ameliorates lung disease. Inhibition of HMGB1 with neutralizing anti-HMGB1 antibodies in a mouse model of *P. aeruginosa* pneumonia rescues macrophage phagocytic function and increases mouse survival (11). Anti-HMGB1 also blocks allergen-induced

Figure 6. Representative fluorescence titration for the measurement of affinity of ODSH for p300. The interaction of ODSH with p300 was studied by monitoring the change in fluorescence ($\Delta F/F_0$) as a function of ODSH concentration in 20 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl at 25°C. The increase in tryptophan fluorescence ($\lambda_{EX} = 295$ nm, $\lambda_{EM} = 340$ nm) was fitted using Equation 1 (solid line) (see online supplement) to derive the equilibrium dissociation constant (K_D) of interaction.

Figure 7. Hypothetical schema of ODSH mechanisms to inhibit release of HMGB1. HMGB1 acetylation and cellular release require p300 catalytic activity. ODSH inhibits p300 activity by binding to the catalytic domain via an allosteric mechanism. ODSH also inhibits NE protease activity, which is required for NE-induced HMGB1 release. Ac-HMGB1, Acetyl-HMGB1.

airway inflammation and remodeling in an ovalbumin-sensitized model of mouse asthma (38). These studies support the concept that HMGB1 is increased in inflammatory lung diseases and that inhibition of HMGB1 may prevent lung inflammation and injury.

One class of molecules that may be effective in inhibiting HMGB1 release and ligation of RAGE are GAGs, which include heparin and heparan sulfate. Heparin and heparan sulfate have sulfate residues that bind to positively charged domains of many proteins and are critical in mediating their antiinflammatory activity. Importantly, the length of the GAGs, the sites of sulfation, and the length of target proteins markedly affect the kinetics and thermodynamics of their interactions (39, 40). Heparin has been reported to have antiprotease, antiinflammatory (41–43), and anti-HMGB1 (16) properties. Heparin directly blocks HMGB1 and LPS complexes from activating receptors at the macrophage plasma membrane (44). Furthermore, heparin binds to HMGB1, changing its conformation to decrease its interaction with RAGE (45). Heparin treatment increases the survival of mice with LPS-HMGB1-induced shock (44), and improves the survival of rats with endotoxin-induced acute lung injury (46). Finally, heparin is known to inhibit HAT activity, which would block HMGB1 lysine acetylation and

thus HMGB1 secretion (27). This action of heparin may also diminish proinflammatory cytokine expression by epigenetic regulation. However, for patients with CF, heparin is not an acceptable airway therapy because of the risk of pulmonary hemorrhage in patients with bronchiectasis. Therefore, we investigated the effect of a modified heparin, ODSH, which has little residual anticoagulant activity and does not cause heparin-induced thrombocytopenia (15, 16).

In this report, we demonstrated that ODSH inhibited total nuclear HAT activity, and, specifically, p300 HAT activity. We also showed that ODSH inhibition of p300 HAT activity blocked HMGB1 acetylation and secretion in a concentration-dependent manner (Figure 7). ODSH did not increase RAW264.7 cell histone deacetylase activity (Figure E2), so the effect on HMGB1 lysine acetylation was not caused by altered deacetylation. Total HAT activity was inhibited only partially, but HMGB1 acetylation was inhibited completely by ODSH. RAW264.7 cell nuclei likely contain multiple HAT species, potentially 13 enzymes in addition to p300. Although all HAT enzymes catalyze lysine acetylation of histone H3 and share a common catalytic core, they also have unique amino and carboxy termini that influence substrate specificity and probably also affect ODSH binding (47). Therefore, we speculate that p300 is required for HMGB1 lysine acetylation and that its high avidity for ODSH binding leads to complete inhibition of HMGB1 acetylation. In contrast, other HAT enzymes are sufficient for histone H3 lysine acetylation, but may have low affinity for ODSH, which would explain why HAT activity for H3 acetylation is maintained even in the presence of ODSH.

Importantly, we demonstrated by confocal microscopy that ODSH was taken up by RAW264.7 cells, first to the cytoplasm and then to the nucleus. This colocalizes ODSH to the site of p300 activity. Consistent with our findings, heparan sulfate proteoglycans have been localized to the nucleus of fibroblasts (48) and other cell types (49). Another GAG, syndecan-1, requires heparan sulfate chains to translocate to the nucleus of bone marrow stromal tumor cells, where it inhibits p300 HAT activity, confirming the model of nuclear translocation of GAGs to where they inhibit nuclear p300 HAT activity

(50). Cellular uptake and intracellular localization of heparin-like polysaccharides are determined by sulfation patterns (19); the loss of the 2-O and 3-O sulfate residues does not prevent ODSH translocation to the nucleus.

To investigate the possibility of direct interaction of ODSH with p300, we used two complementary approaches: an *in silico* CVLS strategy and a fluorescence-based biophysical approach. Using a library of a large number of ODSH sequences and the crystal structure of the p300 catalytically active domain, we predicted that ODSH would preferentially recognize a specific region on p300. These predictions were confirmed through biophysical experiments with FA-ODSH. In fact, the affinity of ODSH was measured to be ~ 1 nM, which is perhaps the highest of affinities recorded in the literature for a GAG-protein system. However, the inhibition potency of ODSH appears to be in the micromolar range. One possibility for this apparent discrepancy is that inhibition may require ODSH engaging two sites on p300. In fact, the *in vitro* fluorescence experiments show a classic sigmoidal fluorescence change, which is indicative of a two-step induced-fit conformational change phenomenon (48). It is likely that this induced-fit phenomenon involves a full-length ODSH chain binding to BS1 first (high affinity), followed by an interaction with BS5. Advanced experiments, such as site-directed mutagenesis or p300-ODSH cocrystallography, would be needed to confirm this expectation. However, these results imply that p300 probably interacts with ODSH through a specific cluster of residues, especially lysine, in the nuclear activation loop (51).

The impact of ODSH inhibition of p300 activity is magnified by the interaction of ODSH with other proteins relevant to the HMGB1/RAGE axis. ODSH may interact directly with HMGB1 at the positively charged residues in the nuclear localization domain (16). Indeed, heparin changes the conformation of HMGB1 and reduces its affinity for RAGE, resulting in decreased tumor necrosis factor α and decreased interleukin 6 secretion (45). ODSH also binds to RAGE (16), blocking HMGB1 ligation at this receptor. Importantly, ODSH has other antiinflammatory activities (16), including inhibition of NE and cathepsin G, inhibition of nuclear factor- κ B activation, and inhibition of

cellular binding to L-selectin and P-selectin (16). This report adds a novel antiinflammatory property to the ODSH repertoire: inhibition of p300 HAT activity. We believe that ODSH could be a promising multifunctional,

antiinflammatory agent that inhibits NE protease activity, p300 activity, and HMGB1-RAGE ligation. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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