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Hyaluronan and halogen-induced airway hyperresponsiveness and lung injury

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Abstract

Chlorine (Cl₂) and bromine (Br₂) are produced in large quantities throughout the world and used in the industry and the sanitation of water. They pose a significant threat to public health when released into the atmosphere during transportation and industrial accidents as well as acts of terrorism. In this review, we discuss evidence showing that Cl₂, Br₂, and products formed by their interaction with biomolecules fragment high-molecular-weight hyaluronan (HMW-HA), a key component of the interstitial space and also present in epithelial cells, to form proinflammatory, low-molecular-weight hyaluronan (LMW-HA) fragments, increasing intracellular calcium (Ca²⁺) and activating Ras homolog family member A (RhoA) in airway smooth muscle, epithelial, and microvascular cells. These changes result in airway hyperresponsiveness (AHR) to methacholine and increased epithelial and microvascular permeability. The increase of intracellular Ca²⁺ is the result of the activation of the calcium-sensing receptor (CaSR) by Cl₂, Br₂, and their byproducts. Post-halogen administration of a commercially available form of HMW-HA to mice and airway cells reverses the increase of Ca²⁺ and the activation of RhoA and restores AHR to near-normal levels of airway function. These data establish the potential of HMW-HA as a countermeasure against Cl₂ and Br₂ toxicity.

Graphical abstract:

In this review, we discuss evidence showing that Cl₂, Br₂, and products formed by their interaction with biomolecules fragment high-molecular-weight hyaluronan, a key component of the interstitial space and also present in epithelial cells, to form proinflammatory, low-molecular-weight

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hyaluronan fragments, increasing intracellular calcium and activating Ras homolog family member A in airway smooth muscle, epithelial, and microvascular cells

Keywords

chlorine; bromine; chlorinated lipids; airway reactivity; membrane potential

Introduction and purpose

The purpose of our review is to discuss the mechanisms by which exposure of animals and humans to chlorine (Cl_2) and bromine (Br_2) results in acute airway injury, manifesting in airway hyperresponsiveness (AHR) to methacholine. This is a test used clinically to diagnose whether an individual has or is likely to develop asthma (1). These halogens, because of their high reactivity with biomolecules, interact with components of the airway lining fluid and apical membranes of the airway and alveolar epithelial cells (ECs). The mechanisms by which these halogens damage airway smooth muscle cells (ASMCs) and result in airway vasoconstriction before or after a subsequent challenge with methacholine or allergens have not been elucidated. Herein, we review our published work, as well as the work of others, showing that the halogens Cl_2 and Br_2 , and their hydration products, hypochlorous (HClO) and hypobromous (HBrO) acids, react with plasmalogens (a class of membrane glycerophospholipids containing alcohol with vinyl-ether bond at the sn-1 position, and enriched in polyunsaturated fatty acids at the sn-2 position of the glycerol backbone), which are key components of pulmonary surfactant and membrane lipids. These reactions form reactive intermediates, halogenated lipids, with a much longer half-life, detected in alveolar spaces, lung tissues, and plasma. These intermediates act as biomarkers and mediators of halogen injury. Halogens and halogenated lipids fragment hyaluronan or hyaluronic acid (HA), a key component of the interstitial space, and also present in ECs, to form the proinflammatory, low-molecular-weight (LMW)-HA fragments, capable of increasing intracellular calcium (Ca^{2+}) and activating Ras homolog family member A (RhoA), (2) increasing AHR, and alveolar permeability to proteins (2;3;4). The increase in Ca^{2+} is brought about by the activation of the calcium-sensing receptor (CaSR) of ASMCs by Cl_2 , Br_2 , and halogenated lipids. Finally, we will present evidence showing that post halogen administration of a commercially available form of high-molecular-weight (HMW)-HA to mice, reverses the toxic effects of halogens and restores AHR and alveolar permeability to normal levels. We will concentrate mainly on the pathophysiology of Cl_2 -induced lung injury and provide some data showing that similar results were obtained following exposure to Br_2 .

Exposure to toxic gases presents a significant threat to public health

The halogens Cl_2 and Br_2 are produced in large quantities throughout the world and used extensively in various manufacturing processes and the sanitation of water. They are very reactive gases and pose a significant threat to public health when released into the atmosphere in large quantities during transportation and industrial accidents, as well as acts of terrorism (5-7).

Worldwide production of Cl_2 and Br_2 exceeds millions of tons per year; the list of the producing countries is maintained by the World Chlorine Counsel and sustainable development (www.worldchlorine.com). Current industrial uses for Cl_2 and Br_2 include pulp bleaching, waste sanitation, organic compound and pharmaceutical manufacturing, drinking water treatment, and maintenance of pathogen-free swimming pools. Exposure to Cl_2 or Br_2 may lead to skin disorders (8), bronchospasm, acute lung injury (ALI), peribronchiolar abscess, delay in healing (9), and death from cardiorespiratory failure (10-14).

Cl_2 and Br_2 are produced in central facilities and transported to manufacturing plants by rail or trucks, raising the possibility that major accidents may occur. Indeed, industrial and transportation accidents have resulted in the release of these toxic gases into the atmosphere with disastrous consequences. For example, a Norfolk Southern train was diverted into a side rail by accident where it crashed into a parked tanker car filled with Cl_2 . The tanker ruptured spilling 11,500 gallons of Cl_2 , which engulfed the city of Graniteville, South Carolina. Eight persons died before reaching medical care. Of the 71 persons hospitalized for acute health effects because of Cl_2 exposure, one died in the hospital and 25 (35%) were admitted to intensive care with a median length of stay of 3 days (15). The number of hospital admissions for pulmonary and nonpulmonary causes increased significantly, following the release of Cl_2 in South Carolina (16). Eight to ten months after the Graniteville incident, cough and shortness of breath were still persistent in those victims (17). Preliminary evidence demonstrates that mill workers residing in Graniteville at the time of the chlorine spill experienced accelerated FEV_1 annual decline in 18 months after the chlorine incident (5). A long-term follow-up of the known victims is ongoing. Patients who survive ALI from any source often develop chronic lung disease with airflow obstruction, fibrosis (18), AHR, and impaired gas exchange (14;19-21). These patients often require hospitalization (6;16) and are predisposed to bacterial infections(22). AHR, also termed reactive airways dysfunction syndrome (RADS), has also been reported in individuals exposed to Cl_2 , and asthma-like symptoms can persist for long periods (15;23).

Other notable Cl_2 exposure incidents resulting in serious casualties include an industrial accident at a hazardous waste facility in Apex, NC, on October 5, 2006 and the malfunction of a Cl_2 delivery system in a Sacramento, CA waterpark on August 15, 2011, both of which led to numerous hospitalizations for severe respiratory complaints. On May 25, 2019, the release of Cl_2 at Birmingham, AL, water treatment plant resulted in 55 workers being transported to local emergency rooms. Exposures could be also encountered in swimming pools and through the mixing of domestic cleaning products (24). An estimated 9,000 calls occur annually to U.S. poison control centers for Cl_2 related exposures. (25).

Chlorine was used as a chemical warfare agent during the First World War when German forces waited for favorable meteorological conditions before opening cylinders of Cl_2 to surprise unsuspecting forces caught downwind of the suffocating greenish-yellow cloud. More recently, during the Iraqi conflict, Cl_2 cylinders have been bundled with traditional explosives, raising significant concerns regarding the possible reemergence of this agent as a chemical weapon against both combatants and civilians (26). Indeed, barrel bombs filled with Cl_2 gas were used recently in Syria against civilians with disastrous consequences (<https://www.bbc.com/news/world-middle-east-42944033/>). Cl_2 facilities are also implicated

as a potential terrorist target. The U.S. Department of Homeland Security considers Cl₂ production and storage facilities as potential high-risk targets that, according to the projections, could result in thousands of fatalities and up to 100,000 hospitalizations (the Homeland Security Council. Planning Scenarios: Executive Summaries. 004; 8–1).

Treatments following halogen gas exposure are mainly supportive, focusing on alleviating developed symptoms, such as bronchospasm, pulmonary edema, and upper and lower airway obstruction, including humidified oxygen administration, β_2 -agonists for bronchospasms and antibiotics for potential infections (27). In severe cases of acute respiratory distress syndrome (ARDS), supported ventilation with or without intubation and positive pressure ventilation may be necessary. Corticosteroids and sodium bicarbonate have been shown to alleviate ARDS following Cl₂ exposure in animal studies, but their efficacy in improving respiratory function following human exposures has not been established (3;28). Therefore, animal models of halogen gas inhalation induced acute lung injury and ARDS provide us a unique opportunity to elucidate its basic mechanisms and to test novel therapeutic agents to reduce morbidity and mortality.

Animal models of Cl₂-induced lung injury

Animal models in multiple species, including mice, rats, rabbits, pigs, sheep, and dogs (12,13;16;23;29-35), have been developed to study mechanisms of Cl₂ toxicity and test the efficacy of a variety of therapeutic agents, which when administered postexposure either resolve or mitigate the injury. Rodents, rabbits, and other small animals are exposed to Cl₂ in either environmental chambers or in the nose-only fashion. The advantages and disadvantages of each method have been described (36). Large animals, such as sheep, pigs, and dogs, are anesthetized and intubated at least during the exposure. Studies on large animals complement rodent studies and provide valuable information. However, the effects of anesthetics complicate the interpretation of the findings. Levels and duration of exposures can be based on studies (37) modeling the 2005 Graniteville Cl₂ accident using the hazard prediction and assessment capability (HPAC) plume modeling system. Persons within 0.5 km from the epicenter were exposed to 1000 ppm Cl₂ for 30 min and developed lethal cardiopulmonary injuries. Those within 2 km were exposed to 400–600 ppm for 30 min and developed severe but recoverable injuries. In agreement with these data, our data also show that exposure of adult male and female mice to 600 ppm for 45 min results in 90% mortality within 48 h (38;39) while mice exposed to 400–600 ppm for 30 min developed significant but recoverable injuries (19;20;29;40-43)

Mechanisms of Cl₂-induced toxicity

When inhaled, Cl₂ and Br₂ and their hydration products, HOCl and HOBr, react with lung plasmalogens to form halogenated aldehydes (44;45). Major molecular species of the reactions of Cl₂ with plasmalogens include 2-chloropalmitaldehyde (2-CIPALD) and 2-chlorostearaldehyde (2-CISALD), which are either reduced to alcohol or oxidized to 2-chloropalmitic acid (2-CIPA) and 2-chlorostearic acid (2-CISA), respectively. The fatty acids exist either in the esterified or free forms and may exert significant injury (45). We have shown the presence of significant levels of 2-CIPA and 2-CISA in the lungs and plasma of

mice even at 72 h post Cl₂ exposure (45). These species were also detected in the plasma of persons exposed to Cl₂ during the recent release of Cl₂ in Birmingham, AL (46), in concentrations at least 100-fold higher than what has been reported in patients with sepsis (due to the endogenous production of HOCl by activated neutrophils) (47). The corresponding brominated species were also identified in the lungs and plasma of mice post Br₂ exposure (44;48). Thus, these species along with additional ones formed by the reactions of HOCl with protein side chains, DNA, and lipids of the cells that line the airway epithelium; for instance, chloramines, byproducts of Cl₂(49), could activate inflammatory cascades through stimulation of mitogen-activated protein kinase (MAPK) (40) and activation of nuclear factor- κ B (NF- κ B) via reversible I κ B α (inhibitor of NF- κ B) oxidation. The halogen injury will be amplified by the inflammatory response occurring as early as 12–24 h postexposure. Chlorinated and brominated species also increase the fragility of red blood cells resulting in the release of hemoglobin, which is oxidized to heme (46). Free heme stimulates the production of reactive species in the cytoplasm and mitochondria and causes extensive injury to all components of the blood–gas barrier (50).

Mechanisms of Cl₂-induced AHR

Increased airway resistance and AHR are important pathological events of oxidative lung injury in general and Cl₂ toxicity in particular, and result in persistent asthma-like symptoms, exacerbation of allergic airway inflammation, (19;20;23) which may progress to lung fibrosis (18;31). The transient receptor potential A1 (TRPA1) family of channels, present in sensory airway neurons, play an important role in the development of AHR in response to low (<50 ppm) concentrations of Cl₂ (51). However, the mechanisms responsible for Cl₂-induced AHR (when inhaled at concentrations, likely to be encountered near industrial accidents) have not been investigated adequately. For a thorough discussion of the various therapeutic agents that show promise in reversing chlorine-induced lung injury when administered postexposure, please see reviews in Refs. 3,16, and 52.

Below we discuss our studies showing low and HMW-HA play an important role in the development (low-molecular-weight) and treatment (high-molecular-weight) of chlorine-induced injury to airways. We start with a general discussion of the biochemistry and metabolism of HA, the evidence that it plays a role in the development and progression of acute and chronic lung injury, and then we discuss the role of these two forms of HA in the development and treatment of chlorine lung injury.

Basic biochemistry and metabolism of HA

HA is a linear polymer formed by a repeating disaccharide structure of glucuronic acid and *N*-acetyl glucosamine consisting of up to 25,000 disaccharide units (MW = 1–10 million Daltons). Mammalian HA is synthesized at the plasma membrane (53) by a family of membrane-bound glycosyltransferases, HA synthases (HAS)-1, -2, and -3. HAS enzymes are evolutionarily conserved, highly homologous (55–70% protein identity)(54), and catalyze the addition of UDP-D-glucuronic acid and UDP-*N*-acetyl-D-glucosamine monomers in an alternating assembly to form HA polymers (55). The HAS isoforms differ in half-life, stability, rate of HA synthesis, and affinity for HA substrates, which impacts

their biological function (56) and, in particular, the molecular masses of their products. HAS1 and HAS2 synthesize larger polymers (2×10^5 to 2×10^6 Da), while HAS3 synthesizes shorter HA polymers (1×10^5 to 1×10^6 Da) (56). Because HA of different lengths also have different biological effects, differential expression of HAS enzymes may affect organ development, injury, and disease. *HAS* expression is regulated by a wide range of cytokines and growth factors (57). Dysregulation of *HAS* expression is relevant in disease and injury, consistent with the biological roles of HA in disease progression, wound healing, and tissue regeneration (57).

HA turnover is partly accomplished by hyaluronidases (HYALs). The HYAL family in mammals consists of hyaluronidases 1–4 (HYAL1–4), PH20, HYALP1, and possibly TMEM2 (58). HYALs are highly homologous endoglycosidases, which hydrolyze the β -1, for linkage of the HA molecule. HYALs functional range, however, is not identical; for example, HYAL1 to HYAL3 are primarily active at an acidic pH, while hyaluronidase PH20 has optimum activity at a neutral pH (58). HYAL1 and HYAL2 are the predominant isoforms functioning to catabolize HA in somatic tissues. HYAL1 and HYAL2 work together to catabolize HA into tetrasaccharides. HA is anchored to the cell surface through CD44 and HYAL2 and localized to lipid rafts in the cell membrane, where HA is first broken down to polymers of approximately 20 kDa (~50 disaccharide units). These fragments are internalized into lysosomes, where lysosomal HYAL1 further degrades the HA into tetrasaccharides (59).

Besides specific enzymatic degradation pathways, HA can also be fragmented by nonspecific pathways. This will be particularly relevant in the context of halogen-induced lung injury described below. Reactive oxygen species (ROS), including superoxide, hydrogen peroxide, nitric oxide, and peroxynitrite, and HClO and HBrO, degrade HA (2;4;60-62). Nonenzymatically produced, ambient ROS may be most relevant in environmental lung injury as described below; for example, inhaled ozone and Cl₂ can fragment HMW-HA into LMW-HA *in vitro*, while neutralization of ROS through superoxide dismutase or its mimetics decreases HA degradation and resulting inflammation (63;64).

HMW-HA is a major structural component of the extracellular matrix; it promotes cell survival, has antiangiogenic properties and anti-inflammatory effects on immune cells, some of these mediated via binding to its receptors, CD44 Toll-like receptor (TLR)-2 and TLR4 (65-68). On the contrary, LMW-HA (L-HA ~300 kDa) fragments, act as endogenous innate immune ligands, and promote inflammatory responses, angiogenesis, and epithelial-to-mesenchymal transition (57;65;66;69). LMW-HA fragments stimulate cytokine production and activate the innate immune response via binding to CD44 and TLR signaling in a myeloid differentiation primary response 88 (MyD88)– and NF- κ B–dependent fashion, whereas HMW-HA inhibits TLR2 signaling *in vitro* and *in vivo* (57). Binding of LMW-HA to CD44 is enhanced by inter- α -trypsin inhibitor (I α I), a serum protease inhibitor consisting of three polypeptides (70;71); the concentration of I α I in the bronchoalveolar lavage (BAL) increases considerably when blood gas permeability to plasma proteins increases (65;72). Increased concentrations of LMW-HA fragments have been found in a number of lung diseases, such as asthma, COPD, and pulmonary fibrosis. LMW-HA is both necessary and

sufficient for the development of AHR after exposure to ozone (65;73), ischemia-reperfusion injury,(74) and various other forms of injury. LMW-HA increases vascular permeability by activating RhoA and ROCK (its downstream kinase), inducing cytoskeletal reorganization, and inhibiting cell – cell contacts (75). On the contrary, the administration of HMW-HA protects from the development of lung injury in ozone exposure (73), bleomycin administration (76), smoke inhalation, and sepsis (77;78). Others have also shown that HMW-HA inhibits the development of AHR in various models of lung injury (73;79-81) and the development of pulmonary fibrosis (82). A type of HMW-HA (Yabro[®]; ~1,000 kDa) has been shown to prevent exercise-induced bronchoconstriction in patients with asthma (83). No protective effects were seen following the administration of LMW-HA (84). Several studies indicate that the relevant factor for HMW-HA and Yabro protection is their size and not the source from which HMW-HA is derived (66;77;78).

Role of HA in halogen toxicity

Increased airway resistance and AHR are important pathological events following exposure to Cl₂ and Br₂, and results in persistent asthma-like symptoms and exacerbation of allergic airway inflammation (19;20;23), which may progress to lung fibrosis (14;18;31). The TRPA1 channels, present in sensory airway neurons, play an important role in the development of AHR in response to low (<50 ppm) concentrations of Cl₂ (51). However, the mechanisms responsible for Cl₂-induced AHR (when inhaled at concentrations, likely to be encountered near industrial accidents) have not been investigated adequately. We performed a number of studies to test the hypothesis that LMW-HA, generated by the action of Cl₂, HOCl, and other secondary reactive intermediates on HMW-HA, was necessary and sufficient for the development of AHR in mice exposed to sublethal concentrations of Cl₂. In a series of experiments, Lazrak *et al.* showed the presence of LMW-HA in the BAL (Fig. 1) and peribronchial spaces of C57BL/6 mice were exposed to Cl₂ (400 ppm for 30 min) and returned to room air for up to 24 h (2;14). The increase in LMW-HA levels coincided with a significant increase in the expression of HA synthases, as well as hyaluronidases. There was also an increase in HA staining in the peribronchial space (Fig. 1). Concentrations of LMW-HA were also increased in the BAL fluid (BALF) of mice exposed to Br₂ (600 ppm for 30 min) and returned to air (50). Instillation of HMW-HA in the external nares post Cl₂ or Br₂ exposure decreased the extent of AHR significantly (Fig. 2).(2;50) The Cl₂-induced AHR was mitigated by instillation of an antibody against the IaI, which inhibits HA signaling (85). An additional, interesting finding was that mice infected with respiratory syncytial virus (RSV), the most common cause of lower respiratory tract infection in children (86), developed increased sensitivity to Cl₂ gas, which was attributed to the presence of LMW-HA, a proinflammatory agent.

A major byproduct of the hydrolysis of Cl₂, in addition to HOCl, is hydrochloric acid (HCl). HCl formed during Cl₂ inhalation does not have a major contribution to injury caused by Cl₂ and HOCl (87). At small concentrations, HCl is buffered by bicarbonate and unlike HOCl does not cause extensive lung injury. However, pulmonary aspiration of acid and/or gastric contents is considered to be a direct cause of ARDS (3),(88). There was a significant increase in LMW-HA mice IaI, as early as 1 h after HCl instillation, which persisted at least 24 h post HCl instillation (4). Intranasal instillation of HMW-HA post-HCl alleviated distal

lung epithelial injury at 24 h postexposure, as shown by considerably lower levels of plasma protein concentration and a decreased number of inflammatory cells in their BAL, as well as near-normal lung histology (Fig. 3) and AHR. In addition, HMW-HA reversed HCl-induced lung injury to the pulmonary microvasculature, as shown by measurements of the filtration coefficients (K_f), a product of endothelial permeability and surface area, and of pulmonary edema, as shown by the reduction in the lung wet/dry weights (Fig. 4), (4) as well as the increase in transendothelial resistance across human lung bronchial ECs. Instillation of HCl in $CD44^{-/-}$ mice resulted in significantly less injury, strongly suggesting that LMW-HA was contributing, at least to a large extent, to this injury (Fig. 4). In subsequent *in vitro* studies, it was shown that the exposure of mouse or human lung airway cells to Cl_2 or LMW-HA increased intracellular Ca^{2+} levels and RhoA activity in human airway smooth muscle (HASM) cells after exposure to Cl_2 . This resulted in activation of the RhoA downstream kinase ROCK2 and TMEM16, a Ca^{2+} -activated Cl^- channel present, resulting in membrane depolarization (50). Postexposure addition of HMW-HA reversed these effects (Fig. 5). Activation of RhoA contributes to the development of AHR, airway smooth muscle contractility, and increases microvascular permeability (89-91). Instillation of HCl in mice also activated lung RhoA and ROCK2, and postexposure instillation of HMW-HA returned these values to their control levels (4). Br_2 and Cl_2 , as well as LMW-HA, increased the expression of the CaSR 24 h later (Fig. 6) (50). The effect of LMW-HA on the CaSR was reversed when cells were treated with HMW-HA, added after the incubation with LMW-HA. The importance of the CaSR in promoting AHR in mice is demonstrated by the fact that instillation of its inhibitor (calcilytic) NPS2143 at 1 and 23 h post Br_2 exposure in the external nares resulted in normal AHR to methacholine (50).

HMW-HA reverses LMW-HA-induced CaSR expression by either inhibiting downstream signaling cascades or by displacing LMW-HA from its receptors. It is also possible that activation of the CaSR may activate TPRC or other Ca^{2+} channels on the surface of ASMCs and HMW-HA prevents this from happening.

It is interesting to note that the exposure of C57BL/6 mice to Br_2 (600 ppm for 30 min) also increases the concentration of LMW-HA in the BALF and AHR with similar mechanisms as for Cl_2 (50). However, whereas the AHR in Cl_2 -exposed mice is caused by an increase in the Newtonian resistance (i.e., the resistance of the large airways), Br_2 -induced AHR is due to the tissue resistance (parameter G of the constant phase equation) that reflects tissue viscoelasticity and possibly the resistance of the small airways, which normally account for less than 10% of the total airway resistance.

Conclusions

Developing countermeasures, which when administered post Cl_2 exposure, decrease acute and long-term injury, and mortality is a high priority of the CounterACT network. Yabro shows considerable promise in reversing acute lung injury to airways and distal lung regions when administered post Cl_2 exposure by counteracting the deleterious effects of LMW-HA (Fig. 7). LMW-HA activates innate and adaptive immunity and increases permeability and airway resistance by activating RhoA and ROCK2, while HMW-HA has strong anti-inflammatory and prohomeostasis functions. The reason for this difference may be because

of differences in receptor engagement, or cell uptake depending on size, but ultimately remains elusive. Recent work suggested that HMW-HA may create a transmembrane “picket fence” barrier, tethered on CD44 and the cellular cytoskeleton, which prevents ligands from reaching and activating their respective receptors, an effect that was abolished after HA degradation (92). HMW-HA also binds several extracellular proteins with strong anti-inflammatory potential, such as $\text{I}\alpha\text{I}$, which is associated with decreased endothelial injury and organ dysfunction in sepsis (93). Furthermore, HMW-HA is a recently recognized crucial constituent of the endothelial glycocalyx, and HA homeostasis is central to the maintenance of a healthy endothelial barrier and the avoidance of tissue injury (94). Finally, HA has well-described antimicrobial properties, inhibiting bacterial adhesion and promoting phagocytosis (95). Thus, HMW-HA acts along with its binding partners to reduce inflammation and promote the antibacterial properties of the host. A major theoretical concern, whenever anti-inflammatory applications of HMW-HA are being discussed, is its potential degradation into smaller, proinflammatory fragments. It is interesting to note, however, that in our study, HMW-HA retained its large MW despite being in a presumably prodegradation environment for several hours. This agrees with the existing literature on the use of HMW-HA in lung inflammation and suggests that pharmacologically-dosed HMW-HA either overwhelms or somehow escapes the degrading activity of the inflammatory milieu and, therefore, is safe to use in this setting.

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Figure 1.

Detection of HA and IaI in the BALF of Cl₂-exposed mice. (A) Mice were exposed to Cl₂ (400 ppm for 30 min) and returned to air. Values are means $\pm$ 1 standard error of the mean (SEM); a number of mice (*n*) in each group = 5; **P* < 0.01 compared with air and the value to its left at the same time point, respectively. (B) Agar gel electrophoresis of Yabro. Lanes: 1, HA Mega-HA™ Ladder (Hyalose, L.L.C.); 2, Select-HA™ HiLadder; 3, Yabro; 4, Yabro exposed to Cl₂ (400 ppm for 30 min) and stored at -4 °C for 24 h; 5, sonicated Yabro; and 6, Select-HA LoLadder. (C) Agar gel electrophoresis of concentrated BALF from air and Cl₂ exposed mice. Lanes: 1, Select-HA HiLadder; 2, Select-HA LoLadder; 3, 95% air and 5% CO₂ (air); 4 and 5, immediately post-Cl₂; 6, 6 h post-Cl₂; 7, 24 h post-Cl₂; and 8, as in 7, but the BALF was treated with hyaluronidase, which degrades HA. In all cases, proteins were visualized with Stains-All (Sigma, St. Louis, MO). (D–F.) Representative image of mouse airways in naïve state (D) or 6 h (E) and 24 h (F) after Cl₂ exposure. Increased HA staining (green, arrows) at 24 h in the peribronchial area surrounding ASMCs (red). Magnification: 200 $\times$ (reprinted from Ref. 2 with permission). Abbreviations: HA, total hyaluronan; IaI, inter- α -trypsin inhibitor.

Figure 2.

AHR in Cl₂-exposed mice was reversed by instillation of Yabro or an antibody against IaI. (A-D.) Mice were exposed to Cl₂ (400 ppm for 30 min) and returned to room air for 24 hours. Yabro (HMW-HA; 3 mg/mL; panels A and B) or a blocking antibody against IaI; panels C–D) were administered in the external at 0.5 and 23 h postexposure. Airway resistance (R; panels A and C) and Newtonian resistance (R_N; panels B and D) measured by flexiVent. Values are means $\pm$ 1 SEM; *N* = 6 (mice); * *P* < 0.05 compared with the corresponding air and Cl₂ values in the same group. (E) R, measured 1 h post-intranasal instillation of either Yabro or Cl₂-exposed H-HA. Values are means $\pm$ 1 SEM; *n* = 4 (mice); * *P* < 0.05 compared with the corresponding air or H-HA value in the same group (reprinted from Ref. 2 with permission).

Figure 3.

H-HA treatment or the lack of CD44 protein attenuates persistent lung injury and inflammation after HCl instillation. BALF protein (A), neutrophils (B), macrophages (C) and lung injury score (D) in WT or *CD44*^{-/-} mice at 24 h post-HCl or NS; WT mice were also treated with H-HA (Yabro). (E) H&E staining of lung tissue at 24 h after HCl instillation shows the presence of hyaline membranes and protein exudates in alveolar spaces, as well as MPO staining (third row: 10x; and fourth row:40x) in WT mice. Reprinted from Ref. 4 with permission.

Figure 4.

Beneficial effects of H-HA following HCL injury *in vivo*. (A and B) Airway resistance prior by flexiVent® at 24 h in after NS, HCl instillation or H-HA treatment post HCl exposure. (means $\pm$ 1 SEM; ** $P < 0.01$ or less when comparing the WT + HCl group with the corresponding WT + NS control at the same dose of methacholine. (C and D) Pulmonary filtration coefficients (K_f) and wet-to-dry lung weights 24 h after HCl instillation in WT mice; (E) Electrical resistance values (R_T) of confluent primary human bronchial epithelial cell monolayers seeded on ECSIS arrays before and L-HA or PBS followed H-HA or PBS. Reprinted from Ref. 4 with permission.

Figure 5.

Exposure of HASM cells to Cl₂ or LMW-HA activates Rho and causes depolarization. (A) HASM cells were exposed to Cl₂ and returned to 95% air/5% CO₂. RhoA activity and protein, levels were measured by GLISA/ELISA. Values are means ± SEM; * *P* < 0.01; (B and C) HASM cells were exposed to different concentrations of LMW-HA with either HMW-HA (Yabro), IgG, or anti-IαI antibody; RhoA activation was measured as above. * *P* < 0.01. (D and E) Measurements of the membrane potential of HASM cells 1 h postexposure to Cl₂ incubated with the reagents shown. **P* < 0.05 compared with air (E). Reprinted from Ref. 2 with permission.

Figure 6.

(A) hpASMC (ASMCs harvested from normal human lungs, cultured for 2–4 passages), were exposed to air (lanes 1–3) or Br_2 (100 ppm for 10 min; lanes 4–9). At 1 h post Br_2 exposure, saline (lanes 4–6) or Yabro (150 $\mu\text{g}/\text{mL}$; lanes 7–9). Western blots showing immunostaining with an antibody against the CaSR 24 h later. (B) Quantification of the 250-kDa band for the conditions shown. Individual values for each lane and means $\pm$ 1 SEM; $n = 5$ for each condition. (C) Saline (lanes 1–3) or LMW-HA (150 $\mu\text{g}/\text{mL}$; lanes 4–6) were added into the medium of phASMC followed 1 h later by saline (lanes 4–6) or Yabro (150 $\mu\text{g}/\text{mL}$; lanes 7–9). They were immunostained with an antibody against the CaSR at 24 h postexposure. (D) Quantification of the 250-kDa band (the active form of the CaSR) for the conditions shown. (E) pmASMCs (mouse ASMCs in primary culture) treated with saline (lanes 1 and 2) or 150 $\mu\text{g}/\text{mL}$ LMW-HA (lanes 3–6) for 24 h as described above. Western blots showing immunostaining with an antibody against the CaSR. (F and G) Quantitation of the 120- and 250-kDa bands of the CaSR. Data are individual values and means $\pm$ SE, $n = 5$ (reprinted from Ref. 50 with permission).

Figure 7.

The summary diagram depicting the damaging effects of LMW-HA and the protective effects of HMW-HA (Yabro) on the airway function.