Absence of the inflammasome adaptor ASC reduces hypoxia-induced pulmonary hypertension in mice

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Submitted 12 November 2014; accepted in final form 8 June 2015

Cero FT, Hillestad V, Sjaastad I, Yndestad A, Aukrust P, Ranheim T, Lunde IG, Olsen MB, Lien E, Zhang L, Haugstad SB, Løberg EM, Christensen G, Larsen K-O, Skjønsberg OH. Absence of the inflammasome adaptor ASC reduces hypoxia-induced pulmonary hypertension in mice. Am J Physiol Lung Cell Mol Physiol 309: L378–L387, 2015. First published June 12, 2015; doi:10.1152/ajplung.00342.2014.—Pulmonary hypertension is a serious condition that can lead to premature death. The mechanisms involved are incompletely understood although a role for the immune system has been suggested. Inflammasomes are part of the innate immune system and consist of the effector caspase-1 and a receptor, where nucleotide-binding oligomerization domain-like receptor pyrin domain-containing 3 (NLRP3) is the best characterized and interacts with the adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC). To investigate whether ASC and NLRP3 inflammasome components are involved in hypoxia-induced pulmonary hypertension, we utilized mice deficient in ASC and NLRP3. Active caspase-1, IL-1β, and IL-1β, which are regulated by inflammasomes, were measured in lung homogenates in wild-type (WT), ASC−/−, and NLRP3−/− mice, and phenotypical changes related to pulmonary hypertension and right ventricular remodeling were characterized after hypoxic exposure. Right ventricular systolic pressure (RVSP) of ASC−/− mice was significantly lower than in WT exposed to hypoxia (40.8 ± 1.5 mmHg vs. 55.8 ± 2.4 mmHg, P < 0.001), indicating a substantially reduced pulmonary hypertension in mice lacking ASC. Magnetic resonance imaging further supported these findings by demonstrating reduced right ventricular remodeling. RVSP of NLRP3−/− mice exposed to hypoxia was not significantly altered compared with WT hypoxia. Whereas hypoxia increased protein levels of caspase-1, IL-1β, and IL-1β in WT and NLRP3−/− mice, this response was absent in ASC−/− mice. Moreover, ASC−/− mice displayed reduced muscularization and collagen deposition around arteries. In conclusion, hypoxia-induced elevated right ventricular pressure and remodeling were attenuated in mice lacking the inflammasome adaptor protein ASC, suggesting that inflammasomes play an important role in the pathogenesis of pulmonary hypertension.

PULMONARY HYPERTENSION can be a life-threatening condition leading to right-sided heart failure and premature death (5, 40). The pathogenesis of the various forms of pulmonary hypertension is not fully known, and insight into disease mechanisms is important for the development of improved treatment options for this severe condition. The role and importance of inflammation and immune activation in the development of pulmonary hypertension are not fully understood; however, inflammation has emerged as an important player. Clinically, increased circulating levels of IL-1β and IL-18 have been observed in patients with pulmonary arterial hypertension, indicating that inflammasomes can be activated in this condition (18, 41). It is well known that innate immunity is activated in response to infection. However, in the last decade, new knowledge has emerged demonstrating that inflammatory pathways can also be activated as a result of cellular stress during sterile inflammation and that these inflammatory responses involve activation of inflammasomes (27). Inflammasomes are large, cytosolic multiprotein complexes consisting of an inflammasome sensor molecule [such as the nucleotide-binding oligomerization domain like receptor (NLR)], caspase-1, and often an adaptor protein called apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) (45, 49). Upon activation, inflammasome assembly triggers the activation of caspase-1, which in turn cleaves pro-IL-18 and pro-IL-1β, leading to the release of activated IL-1β and IL-18, which are important mediators of innate immune responses.

We have previously shown that mice exposed to hypoxia develop pulmonary hypertension concomitant with markedly increased levels of circulating IL-18, indicating hypoxia-induced activation of inflammasomes (24). This activation in turn might contribute to the development of pulmonary hypertension. To study a possible role for inflammasomes in pulmonary hypertension, we exposed mice deficient in ASC, a component in several inflammasomes, and mice deficient in...
NLR pyrin domain-containing 3 (NLRP3), which is involved in the NLRP3 inflammasome only, to hypoxia. We show here for the first time that ASC-deficient mice, but not NLRP3-deficient mice, develop less increase in right ventricle (RV) systolic pressure (RVSP) and reduced RV remodeling compared with wild-type (WT) controls, indicating that ASC is important for development of pulmonary hypertension.

MATERIALS AND METHODS

Mice. All investigations conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication, 8th Edition, 2011) and were approved by the Norwegian Animal Research Authority (ID 3991). WT C57BL/6 mice were obtained from Scanbred (Uppsala, Sweden). NLRP3−/− and ASC−/− mice (21, 56) were provided by K. Fitzgerald (UMass Medical School) and were backcrossed 10 generations onto the C57BL/6 background. All experiments were performed in a blinded manner.

Experimental protocol. Seven-week-old male WT, NLRP3−/−, and ASC−/− mice were placed in a tightly sealed chamber containing 10% oxygen under normobaric conditions (hypoxia group) or under normoxic conditions (control group) for 3 days, 1 mo, or 3 mo (39, 50). Anesthesia by inhalation of isoflurane was used during all invasive procedures. Blood was drawn from inferior vena cava, and the heart and lungs were rapidly excised. The atria were removed, and the RV was separated, weighed, and immediately snap frozen in liquid nitrogen and stored at −70°C. The blood was centrifuged at 2,000 g at 21°C for 20 min after 1.5 h. Serum and organs were collected after 3 days and 1 mo of exposure for ELISA, Western blot, and PCR analysis (n = 6). In animals exposed to 3 mo of hypoxia and control animals, MRI (n = 8) and cardiac catheterization were performed (n = 9–14). In addition, six animals from each group were collected from the early time point of 3 days and the late time point of 3 mo; the lungs were used for immunohistochemical analysis and were fixed and inflated by intratracheal instillation of 4% paraformaldehyde with a constant pressure of 25 cmH2O (13).

Assessment of hemodynamics and RV hypertrophy. Phenotypical characterization was performed after 3 mo of hypoxic exposure to evaluate the effect of NLRP3 and ASC knockout on the degree of pulmonary hypertension and RV hypertrophy. RVSP was measured as primary readout (n = 9–14). Under general anesthesia with inhalation of isoflurane, the RVSP was measured with a micropressure transducer (Samba Preclin 420 LP transducer; Samba Sensors, Västra Frölunda, Sweden) inserted into the RV via the right internal jugular vein (55). Data from 10 consecutive beats were recorded and analyzed using Diadem software (National Instruments, Austin, TX). MRI was used to measure RV wall thickness and RV volume. MRI experiments were performed on all 3-mo groups (n = 8) using a 9.4T preclinical MR system (Agilent Technologies, Santa Clara, CA) with high-performance gradient and RF coils dedicated to mouse imaging. Anesthesia was induced in an anesthesia chamber with a mixture of O2 and 4% isoflurane and further maintained by administration of a mixture of O2 and 1.5–2.0% isoflurane. Body temperature and heart and respiration rates were constantly monitored during experiments. Heated air was used to maintain animal body temperature at 37°C. MRI cine data sets were acquired using a motion-compensated gradient echo sequence. Seven to ten ventricular short-axis slices were acquired, covering the heart from base to apex. Central imaging parameters were as follows: echo/repetition time = 2.10/4.70 ms; field of view = 30 × 30 mm; acquisition matrix = 128 × 128 mm; slice thickness = 1.00 mm; flip angle = 15°; averages = 2. Acquisition was both ECG and respiration gated. MRI data were analyzed using Fiji (General Public License) and MatLab (The MathWorks, Natick, MA) with the operator blinded to animal groups (11). RV hypertrophy was also assessed by measuring RV weight normalized to tibia length. RVSP and RV weight were measured in mice exposed to 3 and 8 wk of hypoxia to examine the hemodynamic effects of NLRP3 and ASC depletion at earlier time points. For all invasive procedures, general anesthesia was induced and maintained by inhalation of isoflurane.

Western blotting. To investigate whether the inflammasome was differentially activated in WT, NLRP3−/−, and ASC−/− mice (n = 6), Western blot analysis was performed on total lung protein lysates using primary antibodies recognizing specifically the active forms of caspase-1 (20 kDa), IL-18 (18 kDa), and IL-1β (17 kDa). Frozen lung tissue from WT, NLRP3−/−, and ASC−/− mice in hypoxia groups from 3 days and 1 mo and their respective controls was homogenized using a Polytron 1200 homogenizer in T-PER tissue protein reagent buffer (Thermo Scientific, Rockford, IL) with protease and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany). Total lung homogenates were used because inflammasome components were expressed in infiltrating cells and airway epithelium. The supernatant was collected, and protein concentrations were quantified using micro-BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Sample buffer containing 4× SDS (50.0% sucrose, 7.5% SDS, 62.5 mM Tris-HCL, pH 6.8, 3.1% DTT, and 0.1% bromophenol blue) was added to protein extracts, and these were heated for 5 min at 4°C. Proteins were size fractionated on 8–16% Criterion TGX gels (Bio-Rad, Hercules, CA) and blotted on to PVDF membranes (GE Healthcare, Little Chalfont, United Kingdom). Blots were blocked in 8% nonfat dry milk or 3% BSA and incubated with primary and secondary antibodies in 2% milk or 3% BSA. The primary antibodies used were anti-caspase-1 (sc-1218) and anti-IL-18 (sc-7954) (both from Santa Cruz Biotechnology, Santa Cruz, CA) and anti-IL-1β (8698S; Cell Signaling Technology, Danvers, MA). Anti-vinculin (V9131; Sigma-Aldrich, St. Louis, MO) was used as a loading control. Horseradish peroxidase-conjugated secondary antibodies against mouse and rabbit were used (GE Healthcare), as well as anti-goat secondary antibody (R&D Systems, Minneapolis, MN). The membranes were developed using the ECL Plus Western blotting detection system (GE Healthcare) and visualized in the LAS-4000 mini from Fujifilm. Signals were quantified with ImageQuant software (GE Healthcare).

ELISA. Serum level of IL-1β was measured in all animal groups (n = 6) after 3 days of hypoxia using a commercially available ELISA kit (R&D Systems). All samples were analyzed in duplicate, and the ELISA plates were calculated. ELISA was also performed to measure the circulating content of IL-1β; however, these were below the detection limit (assay range 12.5–800.0 pg/ml, R&D Systems; data not shown).

Histology. For histological examinations, lungs from six animals in each group were fixed in 4% paraformaldehyde and embedded in paraffin. Embedded tissue was sectioned transversely (5 μm), and three slides from each animal were stained with hematoxylin and eosin, acid fuchsin orange G stain (AFOG), and Sirius red to assess collagen content around blood vessels. Image J software (NIH, Bethesda, MD) was used to measure the luminal area of small arteries stained with AFOG by subtracting the area of the lesser curvature from the greater curvature and dividing by the lesser curvature × 100 (29).

Immunohistochemistry. To study the presence of leukocytes, formalin-fixed paraffin-embedded serial sections of lungs from WT mice subjected to 3 days of hypoxia (n = 6) and their control group (n = 6) were incubated with primary antibodies against myeloperoxidase (MPO) (1:25) and CD3 (1:50; both from Abcam, Cambridge, MA). To evaluate the expression of NLRP3 and ASC protein in the lungs, sections were incubated with primary antibodies against NLRP3 and ASC (1:400; both from Enzo Life Sciences, Farmingdale, NY) for 1 h followed by the secondary peroxidase-conjugated antibody (Impress-Vector; Vector Laboratories, Burlingame, CA). Omission of the primary antibody served as a negative control. To measure the number of alveolar macrophages and their functional status, sections from WT, NLRP3−/−, and ASC−/− mice subjected to 3 days of hypoxia and their respective control groups (n = 6) were incubated with...
primary antibodies against F4/80 (1:100; eBioscience, San Diego, CA), inducible nitric oxide synthase (iNOS; 1:500) and CD206 (1:1,000) (both from Abcam). The number of macrophages positive for F4/80, iNOS, and CD206 were counted per 100 alveoli. Ten fields were assessed for each animal (magnification, ×20). To quantify muscularization of arteries, lung sections from WT, NLRP3−/−, and ASC−/− mice exposed to 3 mo of hypoxia and their controls (n = 6) were stained with the endothelial cell marker von Willebrand factor and smooth muscle α-actin as previously described (25). The total number of peripheral arteries at alveolar duct and wall level was counted as the number of arteries positive for von Willebrand factor per 100 alveoli. Five fields were assessed for each animal (magnification, ×200). Immunostaining with smooth muscle α-actin was used to quantify muscularization of arteries, which were categorized as fully (75–100% of medial layer covered by anti-smooth muscle α-actin staining) and partially (1–74% of medial layer covered by anti-smooth muscle α-actin staining; magnification, ×200) muscularized. Muscularization was measured as the percentage of fully and partially muscularized arteries to the total number of peripheral arteries (30). All microscopic assessments were performed in a blinded manner.

Analysis of gene expression. An RT-PCR system (ABI 7900HT Fast Real-Time PCR System; PE Biosystems, Foster City, CA) was used to measure mRNAs. The specific mRNA transcripts were quantified by 11 TaqMan Gene Expression assays (Applied Biosystems, Foster City, CA). Levels of hypoxia-inducible factor (HIF)-1α (Mm00468869_m1), IL-6 (Mm00446190_m1), matrix metalloproteinase (MMP)-9 (Mm00442991_m1), tumor necrosis factor-α (Mm00443258_m1), nitric oxide synthase (NOS) 2 (Mm00440502_m1), NOS3 (Mm00435217_m1), prostacyclin synthase (Mm00447271_m1), vaso-intestinal polypeptide (Mm00660234_m1), endothelin-1 (Mm00438656_m1), angiotensin-converting enzyme (Mm00802048_m1), thromboxane synthase (Mm00495553_m1), vascular endothelial growth factor (VEGF)-A (Mm01284499_m1), VEGF receptor (VEGFR)-2 (Mm01222421_m1), fibroblast growth factor-2 (Mm00433281_m1), platelet-derived growth factor (PDGF)-A (Mm01205760_m1), and PDGF-B (Mm00440677_m1) mRNA in the lungs of WT, NLRP3−/−, and ASC−/− mice exposed to 3 days of hypoxia and their respective control groups (n = 6) were determined. All samples were tested in duplicate, and average values were used for quantification. The average values were normalized to RPL32 mRNA (Mm02528467_g1) (Applied Biosystems).

Statistical analysis. All data are presented as means ± SE. Student’s t-test or Mann-Whitney rank-sum test was used when comparing two groups. To evaluate the differences between WT mice and NLRP3−/− mice, and between WT mice and ASC−/− mice exposed to hypoxia, two-way ANOVA was performed, correcting for multiple comparisons using Student-Newman-Keuls pairwise procedures. A P value <0.05 was considered statistically significant.

RESULTS

Early and sustained inflammasome activation was observed in the lung in response to hypoxia. To investigate the time course of hypoxia-induced inflammasome activation in the lung, WT mice were exposed to 3 days and 1 mo of hypoxia. Western blot analysis revealed that hypoxia rapidly induced inflammasome activation, as shown by increased levels of active caspase-1, mature IL-18, and mature IL-1β after 3 days of hypoxic exposure (all P < 0.05, Fig. 1, A and C). After 1 mo of hypoxia, levels of active caspase-1 (P < 0.01) and of mature IL-18 and mature IL-1β (both P < 0.05, Fig. 1, B and C) were still increased, indicating sustained inflammasome activation in the hypoxic lung. To investigate in which cell type the inflammasome activation occurred, immunohistological examinations were performed on lung tissue sections from mice exposed to 3 days of hypoxia and their normoxic controls. In lungs from mice breathing normal air, staining of NLRP3 and ASC was found mainly in the airway epithelium, in accordance with previous studies (48). Three days of hypoxic exposure, however, revealed infiltration of leukocytes, predominantly neutrophils shown by positive MPO staining, and also T cells shown by positive CD3 staining aggregating around pulmonary blood vessels (Fig. 2). The positive staining of both NLRP3 and ASC in these cells (Fig. 3) indicates that infiltrating inflammatory cells together with airway epithelium might be the cellular sources of the increased inflammasome activity in the hypoxic lung. Hypoxia did not induce any significant changes in the number of F4/80+ macrophages in WT (13.5 ± 0.5 in hypoxia vs. 14.6 ± 0.6 in normoxia group, P = 0.14), NLRP3−/− (14.4 ± 0.7 in hypoxia vs. 13.4 ± 0.5 in normoxia, P = 0.27), or ASC−/− mice (13.2 ± 0.5 in hypoxia vs. 13.4 ± 0.6 in normoxia, P = 0.79). No significant differences were found between the ASC−/− and WT hypoxia groups (P = 0.41). There were no changes in the number of iNOS+ macrophages, representing the classically activated (M1) macrophage, in WT (5.4 ± 0.4 in hypoxia vs. 5.9 ± 0.4 in normoxia, P = 0.38), NLRP3−/− (5.8 ± 0.4 in hypoxia vs. 5.7 ± 0.3 in normoxia, P = 0.75), or ASC−/− mice (5.4 ± 0.4 in hypoxia vs. 5.8 ± 0.4 in normoxia, P = 0.53). The number of alternatively activated (M2) macrophages, staining positive for CD206, were not significantly altered in WT (6.1 ± 0.5 in hypoxia vs. 5.9 ± 0.5 in normoxia, P = 0.82), NLRP3−/− (6.5 ± 0.5 in hypoxia vs. 5.9 ± 0.4 in normoxia, P = 0.39), or ASC−/− mice (6.4 ± 0.5 in hypoxia vs. 6.2 ± 0.4 in normoxia,
No significant changes were found in the number of iNOS- or CD206-positive macrophages between the ASC and WT hypoxia groups ($P = 0.95$ and $0.89$, respectively). Macrophages are regarded as the prototypical NLRP3 inflammasome cells, and it is not inconceivable that the function of pulmonary macrophages will differ between the different genotypes although we could not reveal such differences.

Chronic hypoxia-induced pulmonary hypertension with RV remodeling in WT mice. Pilot studies in hypoxic WT mice ($n = 7$) showed that the degree of pulmonary hypertension was more pronounced after 3 mo than after 1 mo ($57.9 \pm 2.3$ mmHg vs. $40 \pm 1.8$ mmHg, $P < 0.05$); hence the 3-mo time point was chosen for more extensive characterization of the phenotype. In WT mice, chronic hypoxic exposure induced pulmonary hypertension compared with the control group, as shown by increased RVSP ($55.8 \pm 2.4$ mmHg vs. $30.5 \pm 0.8$ mmHg, $P < 0.001$, Fig. 4A). Increased RV thickness and increased RV weight relative to tibia length (both $P < 0.001$, Fig. 4, B and C, respectively) were also found, indicating RV hypertrophy. In addition, MRI demonstrated that chronic hypoxia induced
enlarged RV volume (53.9 ± 3.0 mm³ in WT hypoxia vs. 41.6 ± 4.6 mm³ in WT normoxia, P < 0.05, Fig. 4, D and E).

**Pulmonary hypertension and RV remodeling were attenuated in ASC−/− mice.** RVSP of ASC−/− mice exposed to 3 mo of hypoxia was lower than in the WT hypoxia group (40.8 ± 1.5 mmHg vs. 55.8 ± 2.4 mmHg, P < 0.01, Fig. 4A), demonstrating attenuated pulmonary hypertension in mice lacking the inflammasome component ASC. Consistent with reduced pulmonary hypertension, ASC−/− mice exposed to hypoxia developed less RV hypertrophy, as shown by decreased RV wall thickness and decreased RV weight compared with the WT hypoxia group (0.64 ± 0.01 mm vs. 0.72 ± 0.01 mm, P < 0.05 and 1.42 ± 0.07 mg/mm vs. 1.74 ± 0.09 mg/mm, P < 0.05, Fig. 4, B and C, respectively). Furthermore, hypoxia did not induce RV volume dilatation in these mice compared with WT mice (Fig. 4, D and E). Reduced pulmonary hypertension in ASC−/− mice was demonstrated also after 3 and 8 wk of hypoxia, shown by reduced RVSP and RV weight compared with WT mice (Table 1). In contrast to ASC−/− mice, RVSP of NLRP3−/− exposed to hypoxia was not altered compared with the WT hypoxia group (52.4 ± 2.3 mmHg vs. 55.8 ± 2.4 mmHg, P = 0.3, Fig. 4A). RV wall thickness was, however, decreased compared with the WT hypoxia group (0.62 ± 0.02 mm vs. 0.72 ± 0.02 mm, P < 0.05, Fig. 4B) although RV volumes and RV weight were not different (60.9 ± 2.8 mm³ vs. 53.9 ± 3.0 mm³ and 1.68 ± 0.05 mg/mm vs. 1.74 ± 0.10 mg/mm, respectively, Fig. 4, C and D). Altogether, our results show that the ASC−/− mice developed a lesser degree of pulmonary hypertension than both WT and NLRP3−/− mice and reduced RV hypertrophy induced by hypoxic exposure. Although there was some improvement in RV thickness also in the NLRP3−/−, the changes were clearly more moderate than in the ASC-deficient mice.

**ASC−/− mice displayed blunted pulmonary activation of the inflammasome in response to hypoxia.** Lung tissue from NLRP3−/− and ASC−/− mice exposed to hypoxia was examined to investigate the degree of inflammasome activation. ASC−/− mice, in contrast to WT mice, showed no significant changes in levels of caspase-1, IL-18, or IL-1β in the hypoxia group compared with the normoxia group, neither after 3 days nor after 1 mo of hypoxic exposure (Fig. 5, A–C), showing absent inflammasome activation in ASC−/− mice. In NLRP3−/− mice, the protein levels of active caspase-1 at both 3 days and 1 mo of hypoxia were increased (both P < 0.05, Fig. 5, D–F), whereas the hypoxia-induced increase in IL-18 was not significant and the increase in IL-1β was only significant after 1 mo, illustrating some inhibitory effects compared with WT mice (Fig. 5).

**Lack of circulating IL-18 in ASC−/− mice.** We have in a previous study shown increased levels of circulating IL-18 in mice exposed to hypoxia (24). Thus we investigated the effects of lack of NLRP3 and ASC on serum levels of IL-18. Consistent with no inflammasome activation in lungs of hypoxic ASC−/− mice (Fig. 5, D–F), we found no increase in circulating IL-18 in these mice after 3 days of hypoxia (Fig. 6). In contrast, both WT and NLRP3−/− mice had increased circulating IL-18 levels compared with their normoxic control groups (both ~2-fold, P < 0.01), suggestive of hypoxia-induced inflammasome activation in these mouse lines. The production sites for IL-18 in hypoxia remain to be investigated.

**Reduced muscularization of arteries and collagen deposition around arteries in ASC−/− mice after hypoxia exposure.** Several studies have shown increased muscularization of pulmonary arterial walls and collagen accumulation within pulmonary arteries in pulmonary hypertension (36, 44). A role for inflammatory cytokines in this process has been suggested (28, 35). To investigate possible mechanisms involved in the reduced pulmonary hypertension in ASC−/− mice, muscularization of peripheral pulmonary arteries was assessed in WT, ASC−/−, and NLRP3−/− mice exposed to chronic hypoxia. Consistent with elevated RVSP in hypoxic WT mice, a higher number of muscularized distal arteries at the alveolar duct and the alveolar wall level were found compared with normoxic WT group (72.8 ± 2.7% vs. 42.7 ± 2.7%, P < 0.01, Fig. 7). Interestingly, ASC−/− mice showed less increase in the number of muscularized distal arteries compared with WT mice exposed to hypoxia (48.7 ± 2.4% vs. 72.8 ± 2.7%, P < 0.05, Fig. 7), consistent with our findings of reduced RVSP in mice lacking ASC. There were no changes in density of peripheral arteries in hypoxic WT, NLRP3−/−, or ASC−/− mice compared with their control groups (7.0 ± 0.3 vs. 6.7 ± 0.2 arteries/100 alveoli, 7.0 ± 0.3 vs. 6.6 ± 0.2 arteries/100 alveoli, and 6.8 ± 0.1 vs. 6.6 ± 0.2 arteries/100 alveoli). AFOG staining revealed collagen accumulation in the adventitial layer of medium-sized and small pulmonary arteries in both WT and NLRP3−/− mice after 3 mo of hypoxic exposure, whereas ASC−/− mice had visually less accumulation of collagen (Fig. 8A). Significantly reduced adventitial collagen deposition was measured in small pulmonary arteries by luminosity in ASC−/− exposed to hypoxia compared with WT mice (Fig. 8B). Sirius red showed mainly green-yellow staining around blood vessels in WT controls, indicating assembly of

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**Table 1. RV weights and RVSP after 3 and 8 wk of hypoxia**

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<tr>
<th></th>
<th>WT Control</th>
<th>WT Hypoxia</th>
<th>NLRP3−/− Control</th>
<th>NLRP3−/− Hypoxia</th>
<th>ASC−/− Control</th>
<th>ASC−/− Hypoxia</th>
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<td>N</td>
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<tr>
<td>RVW/TL, mg/mm</td>
<td>0.95 ± 0.1</td>
<td>1.5 ± 0.2†</td>
<td>0.8 ± 0.1</td>
<td>1.4 ± 0.2†</td>
<td>1.0 ± 0.06</td>
<td>1.2 ± 0.05‡</td>
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<tr>
<td>RVSP, mmHg</td>
<td>28.2 ± 1.4</td>
<td>41.3 ± 2.3†</td>
<td>28.0 ± 1.7</td>
<td>38.5 ± 2†</td>
<td>28.8 ± 1</td>
<td>36 ± 1.8‡</td>
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<td>Weeks</td>
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<tr>
<td>RVW/TL, mg/mm</td>
<td>1.2 ± 0.3</td>
<td>2.13 ± 0.3†</td>
<td>1.1 ± 0.1</td>
<td>1.9 ± 0.2†</td>
<td>1.0 ± 0.05</td>
<td>1.5 ± 0.2‡</td>
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<tr>
<td>RVSP, mmHg</td>
<td>29.6 ± 0.4</td>
<td>45.3 ± 2.3†</td>
<td>30.2 ± 0.4</td>
<td>44.8 ± 1.7†</td>
<td>30 ± 0.4</td>
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Values are presented as means ± SE. WT, wild-type; NLRP3, nucleotide-binding oligomerization domain-like receptor pyrin domain-containing 3; ASC, adaptor protein apoptosis-associated speck-like protein containing a caspase-recruitment domain; RVW, right ventricular weight; TL, tidal length; RVSP, right ventricular systolic pressure. *P < 0.05, †P < 0.01 hypoxia vs. control group. ‡P < 0.05 2-way ANOVA comparing WT vs. NLRP3−/− mice and WT vs. ASC−/− mice.
thin collagen fibers (47), whereas hypoxic animals had predominantly orange-red staining, showing accumulation of thicker collagen fibers in all groups. Again, similar to AFOG staining, Sirius red staining showed that ASC-deficient mice had less collagen deposition than WT and NLRP3-deficient mice exposed to hypoxia (Fig. 8).

**Pulmonary gene expression.** To investigate mechanisms for the reduction in pulmonary hypertension found in mice depleted of ASC, mediators known to be involved in the development of pulmonary hypertension were examined. The pulmonary gene expressions are presented in Table 2, and three mediators of known importance that could contribute to lower pulmonary arterial pressure in ASC mice are presented in Fig. 9 (3, 14, 15, 32, 42, 46).

**DISCUSSION**

In the current study, we have investigated the role of the inflammasome in the development of pulmonary hypertension induced by chronic hypoxic exposure. Mice lacking ASC, a shared component among several inflammasomes, developed less pulmonary hypertension and displayed less remodeling of the RV than WT mice when exposed to chronic hypoxia. In contrast, mice lacking NLRP3, a component specific to the NLRP3 inflammasome, showed modest or no improvement in central parameters of hypoxia-induced pulmonary hypertension. Protein analysis in lungs and serum revealed blunted inflammasome activation in ASC/−/− mice with only modest or no reduction in NLRP3/−/− mice, shown by active caspase-1, IL-18, or IL-1β levels. Finally, ASC/−/− mice displayed less remodeling of the pulmonary vasculature, i.e., reduced muscularization and less fibrosis in the adventitial layer of pulmonary arteries. Our findings suggest a role for ASC-dependent inflammasomes in the development of hypoxia-induced pulmonary hypertension.

In the present study, we show that ASC-deficient mice had reduced RVSP compared with WT mice when exposed to chronic hypoxia, demonstrating reduced pulmonary hypertension. To our knowledge, no previous studies have shown a link between ASC and development of pulmonary hypertension, making this a novel finding. Interestingly, we also observed a clear effect on RV remodeling in ASC/−/− mice. RV weight was significantly lower than in WT, indicating less cardiac hypertrophy. In fact, using MRI, we found a distinct phenotype of the RV in WT mice exposed to chronic hypoxia, consisting
of an enlarged ventricle with increased thickness of the RV free wall and increased RV volume. RV dilatation was completely absent in the ASC−/− hypoxia group. Thus, in the presence of ASC deficiency, development of pulmonary hypertension was diminished and RV remodeling and dilatation were improved.

The main known role of the inflammasomes is to produce active caspase-1, which in turn converts pro-IL-1β into their mature forms. In the present study, we show that hypoxia-induced pulmonary hypertension in WT mice is characterized by increased levels of active caspase-1, mature IL-18, and mature IL-1β in the lung, suggesting that inflammasome activation is a feature of the pathology. Consistent with ASC being a central protein, the reduction in several markers of pulmonary hypertension that was seen in ASC-deficient mice was accompanied by blunted inflammasome activation, i.e., protein levels of caspase-1, IL-18, and IL-1β in pulmonary tissue and IL-18 levels in serum. IL-18 and IL-1β have previously been associated with pulmonary hypertension by us (24) and others (17, 18, 53). Our findings in the present study further support a role for these two cytokines in development of pulmonary hypertension and are consistent with a beneficial effect of inhibiting IL-1β in monocrotaline-induced pulmonary hypertension (53).

Muscularization of the small pulmonary arteries and collagen deposition as part of small pulmonary artery remodeling are important contributors to increased vascular resistance in pulmonary hypertension (20). Herein, we found that both the degree of muscularization and the accumulation of collagen around pulmonary arteries were diminished in ASC-deficient mice compared with WT mice, suggesting an involvement of the inflammasome in these pathological processes in hypoxic pulmonary hypertension. Hypoxia can induce synthesis of IL-6 (8, 42), an important cytokine in perivascular inflammation and vascular remodeling. IL-6 is capable of switching the process...
of acute inflammation to a chronic profibrotic state, thereby facilitating pulmonary vascular remodeling (34). Mice deficient in ASC had reduced expression of IL-6 induced by hypoxia, which could be a mediator of the diminished vascular remodeling observed in our study. MMPs also play a role in vascular remodeling (37), and a positive correlation has been shown between MMP-9 and the severity of experimental pulmonary hypertension (26, 43). In this study, a lower expression of MMP-9 might contribute to the reduced vascular remodeling found in mice depleted of ASC. The master transcriptional regulator HIF-1α is involved in hypoxia-induced pulmonary vascular remodeling (3), and herein we found lower expression of HIF-1α in ASC deficiency, indicating that HIF-1α might play a role in the reduced pulmonary hypertension observed in mice lacking ASC. In addition, both IL-1β and IL-18 have been shown to induce increased collagen production and proliferation of fibroblasts from various origins (12, 16, 33). Furthermore, studies have demonstrated that both cytokines can induce vascular smooth muscle proliferation and migration (6, 23, 34). Thus it is not unlikely that the lack of muscularization and fibrosis observed in ASC−/− mice is related to impaired IL-18 and IL-1β production.

In contrast to ASC-deficient mice, NLRP3−/− mice did not display a clear reduction in pulmonary hypertension. Although we found a significant reduction in RV thickness, a significant reduction in RVSP could not be demonstrated. In addition, the changes in RV weight and volume were similar to the WT hypoxia group. Furthermore, in the lungs from NLRP3−/− mice, we found increased levels of active caspase-1, whereas the intrapulmonary increase in IL-18 was not significant and the increase in IL-1β was significant only after 1 mo, suggesting that disruption of the NLRP3 gene was not sufficient to prevent hypoxia-induced caspase-1 activity. Histological examinations demonstrated the same degree of muscularization and accumulation of collagen in pulmonary arteries compared with WT mice. Hence, although Villegas et al. (51) have previously inhibited reactive oxygen species production in alveolar hypoxia and found an association between hypoxia-induced pulmonary hypertension and activation of the NLRP3 inflammasome, NLRP3 seemed to be less important for the development of hypoxia-induced pulmonary hypertension than ASC. A possible explanation for this finding is that ASC is involved in several inflammasomes, such as the Aim2, NLRP1, NLRC4, NLRP6, NLRP7, and NLRP12 inflammasomes (22, 38, 45), and future studies will have to determine whether these

| Table 2. Pulmonary gene expression after 3 days of hypoxia |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | WT Control     | WT Hypoxia     | NLRP3−/− Control | NLRP3−/− Hypoxia | ASC−/− Control | ASC−/− Hypoxia |
| n               | 6              | 6              | 6               | 6               | 6              | 6               |
| TNF-α           | 100 ± 6.8      | 129 ± 18.2     | 112 ± 11.6      | 127 ± 10.9      | 87 ± 8.7       | 96 ± 11.4       |
| NOS2            | 100 ± 8.3      | 180 ± 21.0†‡   | 83 ± 3.2        | 169 ± 16.0††    | 79 ± 4.7       | 194 ± 33.0*     |
| NOS3            | 100 ± 9.2      | 126 ± 9.0      | 81 ± 2.8        | 124 ± 6.2††     | 105 ± 6.2      | 121 ± 11.0      |
| PTGIS           | 100 ± 6.3      | 107 ± 6.8      | 98 ± 6.8        | 155 ± 9.3†‡     | 100 ± 4.9      | 109 ± 11.0      |
| VIP             | 100 ± 12.3     | 88 ± 7.0       | 91 ± 5.9        | 113 ± 15.0      | 85 ± 5.1       | 82 ± 8.5        |
| Endothelin-1    | 100 ± 5.6      | 133 ± 15.0     | 71 ± 2.1        | 256 ± 41.7†‡    | 112 ± 17.0     | 133 ± 18.6      |
| ACE             | 100 ± 4.7      | 106 ± 7.0      | 107 ± 5.1       | 97 ± 5.0        | 108 ± 7.3      | 112 ± 5.2       |
| TxA synthase    | 100 ± 4.9      | 91 ± 6.3       | 102 ± 5.6       | 104 ± 4.0       | 97 ± 4.4       | 90 ± 5.3        |
| VEGF-A          | 100 ± 10.7     | 80 ± 9.0       | 86 ± 10.7       | 88 ± 5.7        | 91 ± 5.5       | 94 ± 6.1        |
| VEGFR-2         | 100 ± 7.6      | 76 ± 2.9†      | 101 ± 5.2       | 74 ± 1.6†       | 100 ± 2.2      | 76 ± 4.3†       |
| FGF-2           | 100 ± 6.8      | 121 ± 8.0      | 79 ± 2.6        | 139 ± 4.9†‡     | 95 ± 4.8       | 120 ± 12.3      |
| PDGF-A          | 100 ± 6.8      | 129 ± 8.5†     | 92 ± 7.0        | 101 ± 5.4       | 82 ± 2.9       | 118 ± 8.7*‡     |
| PDGF-B          | 100 ± 5.1      | 126 ± 6.4†‡    | 82 ± 8.4        | 114 ± 4.9†‡     | 88 ± 3.3       | 108 ± 8.1       |

Values are presented as means ± SE. TNF-α, tumor necrosis factor-α; NOS2, nitric oxide synthase 2; PTGIS, prostacyclin synthase; VEGF-A, vascular endothelial growth factor; VEGF receptor-2; FGF-2, fibroblast growth factor-2; PDGF-A, platelet-derived growth factor-A. *P < 0.05, †P < 0.01 hypoxia vs. control group. ‡P < 0.05 2-way ANOVA comparing WT vs. NLRP3−/− mice and WT vs. ASC−/− mice.

Fig. 9. mRNA levels of hypoxia-inducible factor (HIF)-1α (A), IL-6 (B), and matrix metalloproteinase (MMP)-9 (C) mRNA levels in WT, NLRP3−/−, and ASC−/− mice (n = 6) exposed to 3 days of hypoxia, breathing normal air, or normoxia. Results were obtained by real-time RT-PCR and are normalized to mRNA levels of RPL32. Data are shown as means ± SE, *P < 0.05, **P < 0.01.
inflammasomes play a role in the development of pulmonary hypertension. Such studies can also provide information regarding putative activators of the inflammasomes, such as reactive oxygen species and endogenous molecules like high-mobility group box 1 that act via Toll-like receptor 4 in hypoxia-induced pulmonary hypertension (4, 51). Finally, it cannot be excluded that inflammasome-independent effects of ASC may be involved. Altogether, the present work suggests that the activation of inflammasomes in the lungs is of importance for development and progression of pulmonary hypertension and that ASC is necessary for pulmonary IL-18 and IL-1β activation in response to hypoxic exposure.

Although we show a crucial role for ASC in the development of pulmonary hypertension in response to hypoxia, different disease mechanisms might be of importance in pulmonary hypertension of other etiologies. However, hypoxia is a complication of pulmonary diseases, affecting large patient groups, such as those with chronic obstructive pulmonary disease (COPD), for which our findings are highly relevant. IL-1β and IL-18 have been shown to be involved in the development of COPD (7, 19), and these patients have increased levels of caspase-1 in their lung tissue (10), indicating a role for the inflammasomes in the pathogenesis of COPD (9). Patients suffering from pulmonary hypertension have increased resistance in the pulmonary vascular bed, which imposes increased work load on the RV, that ultimately might result in RV dilatation (2, 31, 52), a process relevant also to hypoxia-induced pulmonary hypertension.

In summary, the present study shows for the first time that mice lacking ASC, a shared component of various inflammasomes, display attenuated pulmonary hypertension and RV remodeling in response to hypoxia, accompanied by blunted inflammasome activation, i.e., decreased caspase-1, IL-18, and IL-1β levels. Medical treatments that exist for pulmonary hypertension are palliative rather than curative (2), demonstrating a need for discovering new treatment modalities (1). The inflammasome may constitute a novel target for future treatment of pulmonary hypertension.

ACKNOWLEDGMENTS

We are grateful to Solveig Sirnes, Almira Basic, and Ingeborg Løstegaard Goverud for skilful laboratory work and to Vidar Magne Skulberg for expert technical support.

GRANTS

This work was supported by Trelasthandler A. Delphin og Hustrus legacy, Research Council of Norway, Stiftelsen Kristian Gerhard Jebsen, Anders Jahre’s Fund for the Promotion of Science, the South-Eastern Regional Health Authority, Norway, and the Simon Fougnier Hartmanns Family Fund, Denmark.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

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