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Lung volume quantified by MRI reflects extracellular-matrix deposition and altered pulmonary function in bleomycin models of fibrosis: effects of SOM230

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Egger C, Gérard C, Vidotto N, Accart N, Cannelt C, Dunbar A, Tiganzi B, Piaia A, Jarai G, Jarman E, Schmid HA, Beckmann N. Lung volume quantified by MRI reflects extracellular-matrix deposition and altered pulmonary function in bleomycin models of fibrosis: effects of SOM230. Am J Physiol Lung Cell Mol Physiol 306: L1064–L1077, 2014. First published April 11, 2014; doi:10.1152/ajplung.00027.2014.—Idiopathic pulmonary fibrosis is a progressive and lethal disease, characterized by loss of lung elasticity and alveolar surface area, secondary to alveolar epithelial cell injury, reactive inflammation, proliferation of fibroblasts, and deposition of extracellular matrix. The effects of oropharyngeal aspiration of bleomycin in Sprague-Dawley rats and C57BL/6 mice, as well as of intratracheal administration of ovalbumin to actively sensitized Brown Norway rats on total lung volume as assessed noninvasively by magnetic resonance imaging (MRI) were investigated here. Lung injury and volume were quantified by using nongated or respiratory-gated MRI acquisitions (ultrashort echo time (UTE)) or gradient-echo techniques. Lung function of bleomycin-challenged rats was examined additionally using a flexiVent system. Postmortem analyses included histology of collagen and hydroxyproline assays. Bleomycin induced an increase of MRI-quantified total lung volume, lung dry and wet weights, and hydroxyproline content as well as collagen amount. In bleomycin-treated rats, gated MRI showed an increased volume of the lung in the inspiratory and expiratory phases of the respiratory cycle and a temporary decrease of tidal volume. Decreased dynamic lung compliance was found in bleomycin-challenged rats. Bleomycin-induced increase of MRI-detected lung volume was consistent with tissue deposition during fibrotic processes resulting in decreased lung elasticity, whereas influences by edema or emphysema could be excluded. In ovalbumin-challenged rats, total lung volume quantified by MRI remained unchanged. The somatostatin analog, SOM230, was shown to have therapeutic effects on established bleomycin-induced fibrosis in rats. This work suggests MRI-detected total lung volume as readout for tissue-deposition in small rodent bleomycin models of pulmonary fibrosis.

bleomycin; compliance; elastance; fibrosis; imaging; magnetic resonance imaging (MRI); SOM230; ultrashort echo time (UTE)

IDIOPATHIC PULMONARY FIBROSIS (IPF) and its underlying histology, defined as usual interstitial pneumonia (UIP), is considered the prototype of lung diseases dominated by fibrosis, which can be induced by a large number of systemic diseases, genetic conditions, and inhalation/exposure to variable materials, including drugs, resulting in scarring and permanent lung remodeling (20, 22, 29). IPF remains the most common disease, with an unknown cause, occurring slightly more commonly in older men than women and with a progressive nature associated with a poor prognosis, despite any treatment. Histopathologically it is characterized by alternating zones of interstitial fibrosis, inflammation, honeycomb changes, and normal lungs (all aspects of UIP) (26, 43).

Although the pathway of remodeling during fibrotic processes is still not completely understood, it is so far known that the lung structure is altered by loss of alveolar surface area, secondary to alveolar epithelial cell injury, and loss of lung elasticity, secondary to interstitial inflammation, proliferation/migration of fibroblasts, and deposition of extracellular matrix, primary collagen, and proteoglycans (14, 15, 23, 30, 39, 57, 59). These changes in lung structure lead to a severe impairment of lung function related to a decreased elasticity. Indeed, increased lung elastance as well as decreased compliance (the reciprocal of elastance) have been shown in humans (19, 26, 38) and in small rodent pulmonary fibrosis models (1, 7, 12, 15, 30, 53, 57, 58). Moreover, changes in tidal volume and breathing cycle times have been shown in a mouse fibrosis model (33).

Treatment with bleomycin, an antibiotic used against gram-negative bacteria (55) and with chemotherapeutic properties (5, 27), leads to pulmonary fibrosis in ~18% of patients (40, 47). Therefore, bleomycin has become the primary substance used to model pulmonary fibrosis in small rodents (36, 37, 46). Several studies have shown that bleomycin instilled directly into the lung of small rodents elicits an early inflammatory and a late fibrotic response (13, 21, 48, 52). Application of bleomycin causes an acute inflammatory reaction and fibrotic changes that mimic human fibrotic lung disease both histologically and physiologically (23, 36).

The availability of the bleomycin model of pulmonary fibrosis provides the opportunity to investigate in vivo novel pharmacological approaches aiming to treat this crippling disease (37). Noninvasive readouts are of importance in the frame of pharmaceutical research, since the ongoing mechanisms during the development of pulmonary fibrosis are still not
completely known. Moreover, target identification and development of therapeutics against pulmonary fibrosis are challenging. The use of magnetic resonance imaging (MRI) to noninvasively follow the course of lung injury induced by bleomycin administration to mice (3, 16) and rats (2, 16, 24, 25) has been reported earlier. The ability of MRI to noninvasively quantify lung injury in bleomycin-treated animals facilitates in vivo pharmacological studies in the model. Repetitive measurements open new avenues in testing compounds as the responses at several time points during the course of treatment can be easily compared. Specifically, studies at the chronic phase, when fibrosis is already established, become amenable.

The present work shows that remodeling of lung tissue induced by oropharyngeal aspiration of bleomycin in rats and mice leads to an increase of lung tissue weight and total lung volume, the latter quantified noninvasively by MRI. The characterization of lung volume changes in the bleomycin model was motivated by the fact that they may impact lung function. For comparison, lung volumes were also determined by MRI in actively sensitized, ovalbumin-challenged Brown Norway rats, representing a model of acute pulmonary inflammation.

As proof-of-concept, the methodology has been applied to study the effects of the somatostatin analog pasireotide (SOM230) on established lung injury elicited by bleomycin in rats. SOM230 is a multireceptor-targeted somatostatin analog with high binding affinity for the somatostatin receptors 1, 2, 3, and 5 (sstr1, sstr2, sstr3, and sstr5), including a 39- and 30-fold higher binding affinity for sstr5 and sstr1, respectively, than the somatostatin analog octreotide, which binds primarily to sstr2 (45). It is approved for the treatment of Cushing’s disease (18, 41). Motivations to test SOM230 in the rat bleomycin model were 1) to assess if SOM230 reduces lung volume in bleomycin-treated rats, 2) to assess if sst2 in IPF patients was determined by 111In-octreotide scintigraphy, the lung uptake of the radioactive probe correlating with the alteration of lung function and with the intensity of alveolitis (28); 3) a nonrandomized, noncontrolled study on 25 IPF patients treated over 48 wk indicated that long-acting octreotide is well tolerated and provided a proof-of-concept that a somatostatin analog can slow down the progression of lung fibrosis (11); and 4) when administered preventively, SOM230 attenuated bleomycin-induced fibrosis in mice (6).

MATERIALS AND METHODS

Studies were performed in conformity with the Swiss Animal Welfare Law and specifically under animal license by the Cantonal Veterinary Office in Basel. The flexiVent analyses were conducted in accordance with the guidelines of the United Kingdom Home Office on the operation of animals (Scientific Procedures) Act 1986 and were approved by the local ethical review process.

Animals and study protocols. Seven- or 8-wk-old (age at the beginning of the study) male Sprague-Dawley rats (n = 105, Elevage Janvier, Saint Berthevin, France), male Brown Norway rats (n = 12, Iffa-Credo, Saint-Germain-sur-L’Arbresle, France), or male C57BL/6 mice (n = 21, Elevage Janvier) were used throughout the study. Animals were kept at an ambient temperature of 22 ± 2°C under a 12-h normal-phase light-dark cycle and fed NAFAG pellets (Nähr- und Futtermittel, Gossau, Switzerland). Drinking water and food were freely available.

Summaries of the studies performed and how many animals participated in each of them are presented in Table 1.

Oropharyngeal aspiration of saline or bleomycin in rats or mice. Sprague-Dawley rats were lightly anesthetized with 2.5% isoflurane (Abbott, Cham, Switzerland) delivered in a plastic box, and bleomycin hydrochloride (2 or 3 mg/kg; Teva, Basel, Switzerland) in 100 μl of saline (0.9%) or vehicle [100 μl of saline (0.9%)] was administered preventively, SOM230 attenuated bleomycin-induced fibrosis in mice (6).

Table 1. Summary of different studies performed in this work

<table>
<thead>
<tr>
<th>Study No.</th>
<th>Objectives</th>
<th>Field Strength</th>
<th>Species and Strain</th>
<th>No. of Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MRI assessment of total lung volume in naive rats from 7 to 11 mo of age under influence of anesthesia</td>
<td>4.7 T</td>
<td>Male Sprague-Dawley rats</td>
<td>6 naive rats</td>
</tr>
<tr>
<td>2</td>
<td>MRI assessment of pulmonary injury and total lung volume in bleomycin-challenged rats</td>
<td>7.0 T</td>
<td>Male Sprague-Dawley rats</td>
<td>18 saline-challenged rats, 27 bleomycin-challenged rats (18 for 2 mg/kg, 9 for 3 mg/kg)</td>
</tr>
<tr>
<td>3</td>
<td>MRI assessment of total lung volume in bleomycin-challenged rats: nongated and gated acquisitions</td>
<td>7.0 T</td>
<td>Male Sprague-Dawley rats</td>
<td>9 saline-challenged rats, 9 bleomycin (3 mg/kg)-challenged rats</td>
</tr>
<tr>
<td>4</td>
<td>MRI assessment of total lung volume in naive mice/effects of anesthesia</td>
<td>4.7 T</td>
<td>Male C57BL/6 mice</td>
<td>5 naive mice</td>
</tr>
<tr>
<td>5</td>
<td>MRI assessment of total lung volume in ovalbumin-challenged rats</td>
<td>4.7 T</td>
<td>Male Brown Norway actively sensitized rats</td>
<td>6 saline-challenged rats, 6 ovalbumin (0.3 mg/kg)-challenged rats</td>
</tr>
<tr>
<td>6</td>
<td>Assessment of SOM230 effects in bleomycin-challenged rats</td>
<td>4.7 T</td>
<td>Male Sprague-Dawley rats</td>
<td>6 saline-challenged rats, 9 bleomycin (3 mg/kg)-challenged rats, 9 bleomycin (3 mg/kg)-challenged rats treated with SOM230 (80 mg/kg)</td>
</tr>
</tbody>
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anesthetic chamber. Ovalbumin (0.3 mg/kg dissolved in saline, 0.2 ml per animal) or vehicle (saline, 0.2 ml per animal) was administered intratracheally and the animals were allowed to recover.

**SOM230 treatment.** Sprague-Dawley rats (n = 24) were challenged with either saline (n = 6) or bleomycin (3 mg/kg, n = 18) delivered via oropharyngeal aspiration. For the long-term application of SOM230 the long-acting release (LAR) formulation of the compound was used (pasireotide LAR, Novartis Pharma, Basel, Switzerland). SOM230-LAR suspensions were carefully prepared according to the manufacturer’s details. In brief, the microparticles consisted of poly(D,L-lactide-co-glycolide) PLGA polymer and were loaded with 25% SOM230. The LAR formulation was reconstituted with the appropriate vehicle and rapidly administered once according to the manufacturer’s description. At day 9 after bleomycin, nine rats received a single subcutaneous administration of SOM230-LAR (80 mg/kg active compound). The dose of SOM230-LAR was chosen based on earlier experience in other models (44). MRI acquisitions were performed at baseline and at days 8, 14, and 21 after saline/bleomycin challenge.

**MRI.** During MRI signal acquisitions, animals were anesthetized and placed in prone position in a Plexiglas cradle. Body temperature was kept at 37 ± 1°C with a heating pad. Anesthesia was maintained with isoflurane (2% for rats, 1.5% for mice) in a mixture of O2:N2O (1:2), administered via a nose cone. Measurements were performed on spontaneously breathing animals. Measurements were carried out with either a Biospec 47/40 or a 70/30 spectrometer (Bruker Medical Systems, Ettlingen, Germany) operating at 4.7 or 7.0 T, respectively. The operational software of the scanners was Paravision (Version 5.1, Bruker).

A three-dimensional (3D) ultrashort echo time (UTE) sequence (49, 50, 56, 60) with the following parameters was used for acquisitions on rats and mice (whenever different, parameters for mice are provided in parentheses): repetition time (TR) 2.7 ms, echo time 0.2 ms, flip angle of the excitation pulse 2° (3°), 51360 (77086) projections, field-of-view (FOV) 50 × 50 × 70 (30 × 30 × 50) mm², matrix 128 × 128 × 128 (192 × 192 × 192), 5 (2) averages. The acquisition times for the 3D acquisitions were of 11.6 and 6.9 min for rats and mice, respectively. A gradient-echo sequence with the following parameters was used for respiration-gated acquisitions to assess lung volume at the inspiratory and expiratory phases: TR 15.0 ms, TE 2.5 ms, flip angle of the excitation pulse 10°, FOV 50 × 50 × 50 mm², matrix size 256 × 128, slice thickness 2.0 mm, and 4 averages. Eighteen to 24 consecutive transverse slices covered the entire lung. Gradient-echo images (TR 5.6 ms, TE 2.7 ms, flip angle of the excitation pulse 15°, FOV 60 × 60 × 60 mm³, matrix size 256 × 128, slice thickness 1.5 mm, 18 slices, 60 averages) were acquired from ovalbumin-challenged Brown Norway rats. A birdcage resonator of 72-mm diameter for rats, 32-mm diameter for mice (Bruker) was used for excitation and detection.

**Respiratory-gated acquisitions.** Most acquisitions were performed in spontaneously breathing animals, without gating. In selected experiments, however, images from rats were acquired at the inspiratory and expiratory phases of the respiratory cycle. In this case, acquisition was triggered with a small animal monitoring and gating system (model 103-IBP-50, SA Instruments, Stony Brook, NY). The breathing rate was monitored and kept between 50 and 60 strokes/min (regulation via anesthesia level). For obtaining images of the lung at the inflated state, data acquisition was performed for a duration of ~200 ms with respect to the inspiration peak of the respiratory cycle. The inverted trigger signal of the breathing cycle was used to acquire data to reconstruct images reflecting the lung in the deflated state.

**MR image evaluation.** The volume of fluid signals was quantified using a semiautomated segmentation procedure (ImgTool) implemented in the IDL (Interactive Data Language Research Systems, Boulder, CO) environment on a Linux system. This procedure has been extensively described earlier (4, 16). Segmentation parameters were the same for all analyzed images, choosing to segment regions corresponding to high-intensity signals. For a given time point, the total volume of high-intensity signals was calculated by adding the areas obtained for each of the images covering the whole lung and multiplying the sum by the slice thickness. High-intensity signals present in baseline images were mainly due to vessels.

ImgTool was also used to evaluate lung volume (defined in this work as total volume of the lung, including tissue, air, and airways). For this purpose, the segmentation parameters were changed to select the whole lung in each slice. Lung volume was obtained by adding the segmented lung areas in the individual slices and multiplying the sum by the slice thickness.

**Assessment of lung function in Sprague-Dawley rats: flexiVent.** Measurements of lung function, including total lung capacity (TLC) and dynamic compliance as a readout of tissue elasticity, were obtained using the flexiVent (Scireq, Montreal, Canada) system. Animals were anesthetized with medametidine (1 mg/ml) and ketamine (100 mg/ml), tracheotomized, and mechanically ventilated. The computer-controlled small animal ventilator ventilated the rats quasi-sinusoidally with a tidal volume of 10 ml/kg at a frequency of 85 breaths/min and a positive end-expiratory pressure of 2 cmH2O to achieve a mean lung volume close to spontaneous breathing.

To measure respiratory mechanics, mechanical ventilation was briefly paused and a predefined pressure/volume perturbation applied to the airways. Readouts were obtained in response to a sinusoidal (single) forced oscillation waveform. TLC perturbation, which reflects total lung volume or the extent to which airways expand when a pressure of 25 cmH2O is applied, was performed prior to snapshot perturbation as a measure of dynamic compliance, or the degree of elasticity of the respiratory system (including central and peripheral airways). The TLC perturbation normalized the lungs prior to the snapshot perturbation. All measurements were carried out until three acceptable measurements (coefficient of determination > 0.95) were recorded for each animal, of which an average was calculated.

**Postmortem analyses.** Animals were euthanized with an overdose of thiopental (Pentothal, Abbott; 250 mg/kg ip, 0.2 ml) immediately after the last MRI acquisition. For histological analysis, the trachea was ligated to avoid total collapse of the lung. For determination of wet weight, dry weight, and hydroxyproline content, lung lobes were removed without trachea and bronchi.

**Histology.** Histological analysis was performed as described earlier (2, 16). Left lung lobes were immersed in 10% neutral buffered formalin for 24 h. Following fixation, lungs were trimmed, and four transverse sections were cut through the left lung (cromial, two median, and caudal levels) to include the main bronchi as well as the pulmonary alveoli. Slices were then dehydrated through increasing graded series of ethyl alcohol and embedded in one block of paraffin wax. Serial histological sections (5 μm) were obtained from each block and put on glass slides. Sections were stained with picrosirius red for the identification of collagen fibers and counterstained with Weigert’s hematoxylin for nuclei detection.

Picrosirius-stained slides from rat lungs were scanned at a magnification of ×400 (resolution of 0.25 μm/pixel) by using the Aperio ScanScope XT systems (Aperio Technologies, Vista, CA). External limits of sections were manually delimited. Areas around main bronchia and arteries (diameter ≥300 μm) were excluded from the analyses. The Spectrum Analysis algorithm package and ImageScope analysis software (version 9; Aperio Technologies) were applied for picrosirius red staining quantification by a color deconvolution method. Results were expressed as the percentage of picrosirius-positive staining on the total stained tissue.

Collagen was quantified on histological slices from mouse lungs by use of the program “Histolab” (Microvision Instruments, Evry, France). Picrosirius-stained slides were examined with a light microscope (Eclipse E600, Nikon, Egg, Switzerland) connected to a CCD progressive scan video color camera (XCD-U100 CR, Sony, Tokyo, Japan). The whole surface of three slices of the left lung was captured at ×10 magnification. The color corresponding to picrosirius was
Determination of hydroxyproline in lung tissue samples. Lung tissue samples were weighed and dried in an oven at 90°C overnight, and the weight was recorded again. Dry tissues were then boiled in 0.5 ml of 6 M HCl at 120°C overnight (8–16 h) in Pyrex tubes with heat-resistant screw-on caps (article TES-830-70G; Fisher Scientific, Wohlen, Switzerland). After cooling down and adding 5 µl of phenolphthalein (1%), the samples were neutralized with NaOH 10 M (article S-5881; Sigma-Aldrich, Buchs, Switzerland) and 6 M HCl. Black precipitate and brown color was removed by adding 100 µl of carbon suspension [10 mg/ml activated charcoal (article C4386; Sigma-Aldrich) in water], centrifugation, and filtration. Five microliters of standard or hydrolyzed samples were pipetted in triplicate onto a 96-well plate. Five-microliter citrate acetate buffer (5% citric acid, 7.2% sodium acetate, 3.4% sodium hydroxide, 1.2% glacial acetic acid, distilled water, 0.8 ml citrate acetate buffer) were added to each well. The samples were then incubated at room temperature for 20 min. After adding 100 µl of Ehrlich’s reagent [2.5 g of 4-(dimethylamino)benzaldehyde, 9.3 ml of n-propanol, and 3.9 ml of 70% perchloric acid], the wells were incubated for 20 min at 65°C. After cooling down, the samples were measured at 550 nm on a spectrophotometer (Spectra-Max 340PC, Molecular Devices, Sunnyvale, CA), a standard curve from 5 to 100 µg hydroxyproline in water was created. Hydroxyproline data were expressed as micrograms per right lung lobe.

Quantitative real-time polymerase chain reaction. Removed lung tissue was stabilized in liquid nitrogen and stored at −80°C until use. Details concerning RNA purification, total RNA quantification, and determination of the expression of genes interest were provided in Ref. 16. Expression for each sample was normalized to HPRT (probe Mm03024075 m1) and compared with the vehicle-treated group, by calculating the weight of rat organs suggests that the rat lung can grow until the age of ~14 wk (Fig. 1B). For predicting the physiological increase in lung volume, an average lung density of 0.21 ± 0.019 mg/ml (means ± SD) was determined from the ratios between the lung wet weights and the corresponding MRI-detected lung volumes from an earlier work (16) and averaged threshold cycle value of a sample, normalized to HPRT and ΔCT2 is the average of ΔCT in the control group (vehicle group), also normalized to the housekeeping gene, resulting in the relative fold induction. The expression of HPRT was the same in all analyzed groups.

Statistics. For statistical analysis the software SigmaPlot (Systat Software, San Jose, CA) has been used. One-way ANOVA and t-tests were performed for endpoint readouts and two-way repeated-measures ANOVA with Tukey tests were used for readouts with multiple measurements. The following abbreviations were used for the indication of significance: */#0.01 < P < 0.05; **/###0.001 < P < 0.01; ***/####P < 0.001. Significance was assumed for P < 0.05.

RESULTS

Study 1: influence of breathing rate and physiological growth on lung volume detected by nontriggered UTE-MRI. Nontriggered MRI acquisitions of the lung result in an averaged image over all respiratory phases (4). Factors such as, for instance, age and therefore growth of the lung or breathing rate of the animal might influence the evaluated lung volume. In a first step we investigated the possible influence of breathing rate, length of anesthesia, and rat age on the detected lung volume. Six healthy Sprague-Dawley rats were imaged by UTE-MRI once a week during 4 wk. They were 8 wk old at the first measurement. During all imaging sessions the breathing rate was monitored. No correlation was found between the lung volume assessed by MRI and the breathing rate (Fig. 1A).

A formula published by Mirfazaelian and Fisher (34) for calculating the weight of rat organs suggests that the rat lung can grow until the age of ~14 wk (Fig. 1B). For predicting the physiological increase in lung volume, an average lung density of 0.21 ± 0.019 mg/ml (means ± SD) was determined from the ratios between the lung wet weights and the corresponding MRI-detected lung volumes from an earlier work (16) and...
from other, unpublished studies. By introducing the average lung density into the formula of Mirfazaelian and Fisher, we predicted the increase of lung volume due to growth (Fig. 1C). The mean lung volumes of the six naive Sprague-Dawley rats assessed by UTE-MRI at different time points agreed well with the predicted lung volumes (Fig. 1C).

To investigate the possible influence of time in anesthesia on the assessed lung volume, a second image was acquired ~15 min after the first acquisition (resulting in a total anesthesia time of ~35 min, including induction time). When the animals were kept for a longer time in anesthesia, the detected lung volume decreased (Fig. 1D). Therefore, in the applications outlined below for nongated acquisitions, care was taken to maintain the total anesthesia time approximately the same or less than 15 min per session.

**Study 2: lung volume increase induced by bleomycin as assessed in rats from nongated MRI acquisitions.** Treatment of naive Sprague-Dawley rats with a single dose of bleomycin (3 mg/kg) led to a distinct lung injury as shown and quantified by MRI (Fig. 2). Whereas at baseline signals corresponded to vessels, additional signals were detected around main airways following bleomycin administration. In addition to injury, these animals showed enlarged lung volumes on nongated UTE-MRI images (Fig. 3). This can be noticed qualitatively by verifying in the 3D data sets that, following bleomycin administration, the lung borders were outside the areas of rectangles chosen to delimit the lung on baseline images.

Quantitative assessments of lung volumes in Sprague-Dawley rats (Fig. 4A) treated with either 2 or 3 mg/kg bleomycin showed within 7 days a significant increase in MRI-detected lung volume (Fig. 4B) that persisted until the end of the experiment (21 days after challenge). The determined lung volume of bleomycin-treated rats was significantly higher compared with the vehicle group, even though the lung volume of saline-treated healthy animals increased slightly with age, following the prediction of Mirfazaelian and Fisher (34) (Fig. 4C). Furthermore, bleomycin (3 mg/kg)-challenged rats developed a significant \( P < 0.001 \) decline in lung function, which was associated with a decrease in both total lung capacity and dynamic compliance, compared with saline-challenged control animals, at day 21 (Fig. 4D).

**Study 3: characterization of lung volume detected by MRI using nongated or gated acquisitions.** Twelve male Sprague-Dawley rats were examined repeatedly by MRI. Immediately after baseline measurements animals received either 100 \( \mu \)L of saline (0.9%) or bleomycin (2 mg/kg) via oropharyngeal aspiration. At each time point a nontriggered acquisition was
followed by two gated acquisitions to reflect inflated and deflated state, as described in MATERIALS AND METHODS.

Lung volumes assessed by MRI were significantly increased in bleomycin- compared with saline-challenged rats for non-triggered as well as respiration-triggered acquisitions (Fig. 5). In gated acquisitions, increased volumes were observed for both inflated and deflated lungs. Moreover, animals of the control group showed a slight increase in lung volumes that was comparable to the increase in lung volume of naive animals (Fig. 5, see also Figs. 1C and 4C). Seven days after challenge, the tidal volume (difference of lung volumes at inspiration and expiration) of bleomycin-challenged rats was decreased, and it finally recovered within another 7 days (Fig. 5).

At day 14 after saline or bleomycin administration, the 12 Sprague-Dawley rats were euthanized following the last MRI acquisitions and the lungs were harvested. The wet and dry weights of all five lung lobes were significantly higher in the bleomycin group (Fig. 6A). Similarly, the hydroxyproline content determined in all five lung lobes was significantly increased after bleomycin administration (Fig. 6B). The relative water content was the same in both groups (79.3% for saline, 79.2% for bleomycin group). The lung volume at expiration determined noninvasively by MRI was significantly correlated to the wet and dry lung weights determined postmortem, as well as to hydroxyproline content (Fig. 6C). Also, the lung volumes at expiration and those determined from nongated MRI acquisitions were highly significantly correlated (Fig. 6C).

Postmortem analyses of bleomycin-induced lung injury in Sprague-Dawley rats. Figure 7, A and B, summarizes histological evidence of collagen deposition in the lungs of rats at day 21 after oropharyngeal aspiration of bleomycin (3 mg/kg). Histology also revealed perivascula, peribronchial and parenchymal infiltration of inflammatory cells (data not shown). Lung alveolar obliteration corresponding to fibrosis areas induced by bleomycin ranged between -10 and 30% of the total lung sections. Minimal to moderate interstitial fibrosis, generally multifocal and centered around major bronchial branches and/or perivascularly (red arrows), bronchiectasis, and bronchial/bronchiolar epithelium hypertrophy/hyperplasia was observed in the animals that received the antibiotic. However, neither edema nor enhanced emphysema was present in the lung sections of bleomycin- compared with saline-treated animals. Similar histological observations were made earlier for a dose of 2 mg/kg of bleomycin, delivered via oropharyngeal aspiration to rats (16). In agreement with the histological evidence of collagen increase, lungs of rats treated with bleomycin (3 mg/kg) also showed an increased hydroxyproline content (Fig. 7C).

Study 4: bleomycin-induced lung injury and volume increase detected by MRI in C57BL/6 mice. Before interrogating possible effects of bleomycin on MRI-determined lung volumes, in analogy to the rat experiments, we verified in naive mice whether respiratory rate or anesthesia could affect the assessments. For this purpose, the animals were measured on 3 consecutive days. During each session, three acquisitions were performed, each at a different level of anesthesia, to achieve distinct breathing rates. An interval of 10 min was left between the acquisitions. No correlation was found between the assessed volumes from nongated acquisitions and the respiratory rates, which ranged from 25 to 100 bpm (Fig. 8A). Also, the repeated acquisitions were highly reproducible [coefficient of variation for five mice and nine individual measurements per animal: 0.06 ± 0.01 (means ± SD)], despite the extended anesthesia time of each imaging session (Fig. 8B).

Repeate oropharyngeal aspiration of bleomycin (6 × 0.1 mg/kg) resulted in lung injury characterized by signals detected primarily around main airways (Fig. 9A, arrows). The response detected by MRI in the mouse lung was similar in appearance to that observed in rats following administration of the antibiotic (Fig. 2A). Quantification of the MRI signals was summarized in Fig. 9B.
An increased lung volume was observed in repeatedly bleomycin-challenged mice, at day 14 after last dosing (Fig. 10A). Histology revealed increased picrosirius content in the left lung of these animals. Moreover, the wet and dry weight as well as the hydroxyproline content was increased in the right lungs of these mice, compared with saline-treated ones (Fig. 10, B–D).

Overall, these postmortem analyses were consistent with the development of fibrosis in the model, as examined extensively earlier (3, 16).

Study 5: ovalbumin-induced lung injury and volume assessment by MRI in actively sensitized Brown Norway rats. As a model of acute lung inflammation, actively sensitized Brown Norway rats were challenged intratracheally with ovalbumin (1 mg/kg). The allergen induced massive signals with a volume on the order of 1,500 µl (Fig. 11A), which had been characterized extensively earlier as due to perivascular/peribronchial edema related to inflammation in the model (4, 54). In contrast, despite the large volume of signals detected by MRI, the lung volume of these animals did not increase 24 and 48 h after challenge (Fig. 11B).

Study 6: effects of SOM230 on established bleomycin-induced lung injury and volume increase detected by MRI in Sprague-Dawley rats. Increased MRI signals and lung volume were detected at day 8 after bleomycin (Fig. 12A). Both the volumes of MRI signals and of the lungs were used to randomize the distribution of bleomycin-challenged rats, to have comparable mean values of these parameters in the different groups of animals at the beginning of SOM230 treatment (day 9 following bleomycin administration). Subcutaneous administration of SOM230-LAR at day 9 led to a significant reduc-
tion of the MRI signals quantified at days 14 and 21. Moreover, the lung volume of bleomycin-challenged, SOM230-LAR-treated animals reduced significantly, attaining values that were comparable to the volume of saline-challenged rats. Relative wet and dry lung weights were comparable at day 21 postbleomycin (Fig. 12B), indicating that edema was not prominent at this time point and corroborating earlier results (2, 16). Weights of lungs from bleomycin-challenged, SOM230-LAR-

Fig. 5. Lung volumes assessed in Sprague-Dawley rats from nontriggered acquisitions as well as from gated acquisitions at the inspiratory and expiratory phases. Tidal volumes were derived by subtracting volumes at inspiration and expiration. Values are expressed as means ± SD (n = 6 rats per group). The levels of significance *0.01 < P < 0.05, **0.001 < P < 0.01, b and ***P < 0.001 refer to ANOVA comparisons to baseline values in the same group. #0.01 < P < 0.05 and ##0.001 < P < 0.01 correspond to ANOVA comparisons as indicated.

Fig. 6. Postmortem analyses of rats that had been examined by MRI (data summarized in Fig. 5). A: wet and dry weights of the whole lungs (means ± SD, n = 6 per group) after autopsy. B: hydroxyproline content (means ± SD, n = 6 per group) in the corresponding lungs. The levels of significance P < 0.001 indicated in the graphs correspond to t-test comparisons. C: comparison between lung volumes assessed in vivo by MRI and the postmortem parameters. Coefficients of correlation are expressed by R.
treated rats were significantly lower (−16%) than those from bleomycin-challenged animals (Fig. 12B). Also picrosirius staining levels at day 21 after bleomycin were significantly lower (−26%) for SOM230-LAR-treated rats (Fig. 12C). Despite not being significant, at this time point there was a clear trend toward reduced hydroxyproline content (−13%) in the lungs of animals receiving SOM230-LAR compared with controls (Fig. 12C). Gene expressions for collagen (col1a1 and col3a1) at day 21 after bleomycin were also significantly reduced in SOM230-LAR-treated compared with untreated rats (Fig. 12D).

DISCUSSION

Despite substantial research during the last years, currently only a few noninvasive in vivo diagnostic tools for lung fibrosis exist. The availability of such methods and representative animal models are of great importance for the development of therapeutics against lung fibrosis. It has been shown in former studies that fibrosis can be modeled by the instillation of bleomycin into the lung (9, 36). This model has often been used to investigate the effect of potential therapeutics or the influence of genes on the development of lung fibrosis for target finding (10, 21, 51).

It has been shown earlier that proton MRI techniques are able to noninvasively follow the progression of bleomycin-induced lung injury in rats (2, 16, 24, 25) and mice (3, 16). The signals quantified by MRI in the lung showed high correlation to histological analysis of collagen and to hydroxyproline content. In the present work we aimed at validating lung volume as a readout that might reflect changes in lung function, since the improvement of lung function is a main aim for therapeutics against several lung diseases.

The lung volume detected by nontriggered MRI increased after instillation of bleomycin (Figs. 4, 5, and 10). An increase in lung volume has also been reported earlier for nontriggered MRI acquisitions of spontaneously respiring rats following an intratracheal administration of elastase (42), due to emphysema induced by this enzyme. No enhanced emphysema could be observed in histological sections of rat lungs after instillation of bleomycin compared with saline treatment. Also, the presence of edema was excluded by histology and by the fact that the relative water content in the lungs did not change upon...
instillation of bleomycin. Of importance, induction of pulmonary inflammation by instillation of ovalbumin (1 mg/kg) did not result in an increase of MRI-detected lung volume within 48 h despite massive presence of edema (Fig. 11). Breathing rate or anesthesia showed no or only slight influence on MRI-detected lung volume (Figs. 1 and 8) and could therefore not explain the present observations. Finally, the increase in lung volume after bleomycin administration was clearly more pronounced than that expected from the physiological growth curve (Fig. 4C). Therefore, the increase in lung volumes following bleomycin detected with nongated MRI acquisitions had to be related to either anatomical or lung function changes, or a combination of both.

Analogously to nongated images, respiratory-triggered acquisitions of bleomycin-challenged rats showed a significant increase in lung volume in both the inflated and deflated states (Fig. 5). Therefore, the increase in lung volume on nongated images of bleomycin-treated animals evolved from a significant increase of volume in both inflated and deflated states of the lung. Since neither enhanced emphysema nor edema was observed histologically in this model, the increase in lung volume after expiration might only be explained either by air that remained in the lung as a consequence of a loss in lung function or by tissue remodeling due to the deposition of collagen, elastin, and proteoglycans (14, 15, 23, 30, 57). The postmortem-assessed lung wet and dry weight (Figs. 6 and 10), as well as hydroxyproline content and picrosirius staining on histological samples (Figs. 7 and 10) as markers for collagen deposition, were all increased in bleomycin-treated animals. These observations indicate that the increase in MRI-assessed lung volumes in the bleomycin group (Figs. 4, 5, and 10), particularly at expiration (Fig. 5), were correlated to tissue remodeling induced by the antibiotic. This suggestion is reinforced by the correlation of lung volume at expiration to lung wet and dry weights as well as to hydroxyproline (Fig. 6). An examination of the linear fits reveals that an increase of the lung volume at expiration from 8 to 10 ml corresponded to a relative increase of the other readouts by the same amount (~25%). Moreover, the strong correlation of MRI-derived lung volumes at expiration and from nongated acquisitions (Fig. 6D) demonstrated that not only volume assessments at the expiratory phase but also the less time-consuming nongated assessments provide a readout for bleomycin-induced tissue deposition in rats. It is remarkable that the tidal volume, which was decreased on day 7 after bleomycin challenge, recovered within another 7 days (Fig. 5). Similar observations were made in bleomycin-challenged mice by use of barometric plethysmography (33). A tentative explanation of these results might

Fig. 9. UTE-MRI acquisitions from C57BL/6 mice: bleomycin-elicited responses. A: 2 representative slices extracted from 3D data sets acquired at baseline and after repeated bleomycin administration (6 × 0.1 mg/kg by oro-pharyngeal aspiration). The position of each slice was chosen at approximately the same anatomical position at the different time points. Lung injury primarily around main airways is indicated by arrows. B: quantification of MRI signals (means ± SD, n = 8 mice per group) related to vessels and to injury elicited by bleomycin in the lungs. The levels of significance ***P < 0.001 refer to ANOVA comparisons to baseline values in the same group. ##P < 0.001 and ###P < 0.001 correspond to ANOVA comparisons as indicated.
be that the increase in lung volume at expiration, leading initially to a loss of tidal volume, could be compensated over time by an increase of lung volume at inspiration.

Extracellular matrix deposition in the lung, as for instance due to collagen and proteoglycan accumulation, induced by bleomycin correlates to an increase in lung elastance (14, 15, 30, 39, 59) and therewith to a decrease in lung compliance (1, 31). Therefore, MRI-detected alterations in lung volume, which in this work were shown to be related to increases of lung wet and dry weight as well as of collagen content, may also be considered to reflect changes of lung function in the model. A decreased compliance was indeed measured in bleomycin-challenged rats (Fig. 4D). Since measurements are performed in spontaneously breathing animals, it is recommended to keep the animals anesthetized for less than 20 min in each imaging session, as prolonged anesthesia may impact the...
assessment of the total lung volume, at least in rats (Fig. 1D).
Isoflurane anesthesia leads to respiratory depression in small rodents (8). Increasing concentrations produce a progressive decrease in tidal volume and response to increases in arterial carbon dioxide concentration (32). Also, isoflurane has an effect on upper airway dilator muscle activity (17). It is conceivable that decreases in tidal volume might explain the reduction in total lung volume in rats with time after anesthesia onset. Differences in reduction in tidal volume between isoflurane-anesthetized rats and mice might explain the differences in lung volumes observed in rats as opposed to mice, with the duration of anesthesia. The fact that rats possess a larger fat mass compared with mice may be at the basis of this effect, because initial isoflurane deposition in fat and thus also in muscle would be higher in rats. Moreover, the physiological growth of the lung needs to be taken into account by the incorporation of appropriate control groups or by correcting the assessed lung volumes by using the formula of Mirfazaelian and Fisher (34).

In a proof-of-concept study, we have shown that SOM230-LAR administered therapeutically could reduce bleomycin-induced lung injury. As shown earlier, bleomycin at a dose of
3 mg/kg was able to induce multifocal early fibrosis already at day 7 (2). Therefore, fibrosis was already present at the beginning of the SOM230-LAR treatment, namely at day 9 postbleomycin. The increased MRI signal and lung volumes detected at this time point would be in agreement with fibrogenic changes resulting in deposition of matrix and distortion of lung structure starting at day 7 after bleomycin (35). The present results suggest therefore that SOM230 had a therapeutic effect on established lung fibrosis. This is of significance, because most compounds have been tested preventatively up to now, i.e., when administered before and/or during the early, inflammatory phase after bleomycin administration (35). Testing substances in the therapeutic phase as conducted in the present work is of more clinical relevance. The reduction in lung volume following SOM230-LAR treatment found here would be consistent with reduced extracellular matrix deposition as suggested by a reduced collagen content in the lungs of SOM230-LAR-treated rats. Despite the encouraging results obtained in this proof-of-concept study, more investigations are necessary to better understand the mechanism of action of SOM230 in the bleomycin model.

Because therapies aim primarily at improving lung function, incorporation of lung volume assessment by MRI into pharmacological testing in the bleomycin model could be of interest especially because of its noninvasive character, which allows repetitive assessments in the same animal thus contributing to the refinement of the experiments. Further pharmacological validation and comparison to functional tests is warranted to qualify this anatomical readout as noninvasive marker of changes in lung function in spontaneously breathing, bleomycin-challenged small rodents.

DISCLOSURES

All authors have been employed by Novartis Pharma AG during the execution of the work. C. Gérard, N. Vidotto, N. Accart, A. Dunbar, B. Tigan, A. Paia, G. Jarai, H. Schmid, and N. Beckmann are still employed by Novartis Pharma AG.

AUTHOR CONTRIBUTIONS


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