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The inhaled Rho kinase inhibitor Y-27632 protects against allergen-induced acute bronchoconstriction, airway hyperresponsiveness, and inflammation

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Schaafsma D, Bos IS, Zuidhof AB, Zaagsma J, Meurs H. The inhaled Rho kinase inhibitor Y-27632 protects against allergen-induced acute bronchoconstriction, airway hyperresponsiveness, and inflammation. *Am J Physiol Lung Cell Mol Physiol* 295: L214–L219, 2008. First published May 16, 2008; doi:10.1152/ajplung.00498.2007.—Recently, we have shown that allergen-induced airway hyperresponsiveness (AHR) after the early (EAR) and late (LAR) asthmatic reaction in guinea pigs could be reversed acutely by inhalation of the Rho kinase inhibitor Y-27632. The present study addresses the effects of pretreatment with inhaled Y-27632 on the severity of the allergen-induced EAR and LAR, the development of AHR after these reactions, and airway inflammation. Using permanently instrumented and unrestrained ovalbumin (OA)-sensitized guinea pigs, single OA challenge-induced EAR and LAR, expressed as area under the lung function (pleural pressure, P_{pl}) time-response curve, were measured, and histamine PC_{100} (provocation concentration causing a 100% increase of P_{pl}) values were assessed 24 h before, and at 6 and 24 h after, the OA challenge (after the EAR and LAR, respectively). Thirty minutes before and 8 h after OA challenge, saline or Y-27632 (5 mM) was nebulized. After the last PC_{100} value, bronchoalveolar lavage (BAL) was performed, and the inflammatory cell profile was determined. It was demonstrated that inhalation of Y-27632 before allergen challenge markedly reduced the immediate allergen-induced peak rise in P_{pl}, without significantly reducing the overall EAR and LAR. Also, pretreatment with Y-27632 considerably protected against the development of AHR after the EAR and fully prevented AHR after the LAR. These effects could not be explained by a direct effect of Y-27632 on the histamine responsiveness, because of the short duration of the acute bronchoprotection of Y-27632 (<90 min). In addition, Y-27632 reduced the number of total inflammatory cells, eosinophils, macrophages, and neutrophils recovered from the BAL. Altogether, inhaled Y-27632 protects against acute allergen-induced bronchoconstriction, development of AHR after the EAR and LAR, and airway inflammation in an established guinea pig model of allergic asthma.

airway inflammation; allergic asthma; guinea pig; Rho kinase

ALLERGIC ASTHMA IS AN INFLAMMATORY airways disease characterized by allergen-induced early (EAR) and late (LAR) bronchial obstructive reactions, associated with infiltration and activation of inflammatory cells in the airways and the development of airway hyperresponsiveness (AHR) to a variety of stimuli, including contractile agonists such as histamine (6, 12, 16).

Agonist-induced smooth muscle contraction is regulated to an important extent by phosphorylation of the 20-kDa myosin light chain (MLC_{20}) (28). MLC_{20} phosphorylation is initiated by an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and subsequent formation of Ca^{2+} calmodulin, resulting in activation of myosin light chain kinase (MLCK). The extent of MLC_{20} phosphorylation is determined by the balance between MLCK and myosin light chain phosphatase (MLCP) activities (42). It has been established that contractile stimuli may not exert their effects exclusively by increasing $[Ca^{2+}]_i$, but also by increasing the sensitivity of the contractile apparatus to Ca^{2+}. The Rho/Rho kinase pathway has emerged to be a key regulator of this Ca^{2+} sensitization (13, 15, 42). Activated Rho kinase inactivates the myosin binding subunit of MLCP by phosphorylation, thereby interfering with the equilibrium of MLCK and MLCP activities. This leads to an augmentation of MLC_{20} phosphorylation and hence an elevation of contraction at an established $[Ca^{2+}]_i$ (13, 48).

Only recently, Rho kinase has come forward to be a potential target in airways diseases, including allergic asthma (15). It has been demonstrated that Rho/Rho kinase-mediated Ca^{2+} sensitization is enhanced in acetylcholine-induced contraction of bronchial smooth muscle obtained from repeatedly allergen-challenged rats (8). Moreover, we have previously demonstrated that active allergic sensitization, without subsequent allergen exposure, was sufficient to increase the role of Rho kinase in guinea pig tracheal smooth muscle contraction ex vivo and airway responsiveness in vivo (37). In accordance, passive sensitization-induced nonspecific ASM hyperresponsiveness and specific allergen responsiveness in these preparations were found to be dependent on Rho kinase as well (39). The enhanced contribution of Rho kinase to airway responsiveness could involve increased expression of RhoA, as protein levels of this upstream activator of Rho kinase have been reported to be elevated both after allergic sensitization in guinea pigs (37) and after repeated allergen challenge in rats (8) and mice (9). Clearly, although Rho kinase is involved to some extent in the regulation of airway smooth muscle tone under control conditions (36, 38), an increased contribution of Rho kinase to airway responsiveness is evident under pathophysiological conditions. Further supporting such a pathophysiological (primed) role, we have very recently demonstrated that in addition to a bronchoprotective effect under basal conditions, inhalation of the Rho kinase inhibitor Y-27632 acutely reverses allergen challenge-induced AHR after the early (EAR) and late (LAR) asthmatic reaction in permanently instrumented, unanesthetized, and unrestrained guinea pigs (35). Moreover, it was found that an increased Rho kinase activity is involved in the development of the allergen-induced AHR, as indicated by an enhanced effectiveness of Y-27632 to acutely reverse the increased airway responsiveness to histamine and $PGF_{2\alpha}$, both after the EAR and the LAR (35).

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Thus far, investigations have mainly been focused on the acute effects of Rho kinase inhibitors on airway (hyper)responsiveness. In contrast, the present study focuses on the putative prophylactic effects of inhaled Y-27632 against the allergen-induced EAR and LAR, the development of AHR after these reactions, and airway inflammation, using a well-established guinea pig model of allergic asthma (25, 45).

MATERIALS AND METHODS

Animals. Outbred specified pathogen-free male Dunkin Hartley guinea pigs (Harlan, Heathfield, United Kingdom), weighing 600–700 g, were used in this study. The animals were actively IgE sensitized to ovalbumin (OA) as described previously (25, 45). In short, 0.5 ml of an allergen solution containing 100 μg/ml OA and 100 mg/ml $Al(OH)_3$ in saline was injected intraperitoneally, while another 0.5 ml was divided over seven intracutaneous injection sites in the proximity of lymph nodes in the paws, lumbar regions, and the neck. The animals were operated on 2 wk after sensitization and used experimentally in wk 4–8 after sensitization. The animals were group housed in individual cages in climate-controlled animal quarters and given water and food ad libitum, while a 12-h on/12-h off light cycle was maintained. All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation.

Measurement of airway function. Airway function was assessed in conscious, permanently instrumented, unrestrained guinea pigs, by online measurement of pleural pressure (P_{pl}) as described previously (25, 32). In short, a small saline-filled balloon catheter was surgically implanted inside the thoracic cavity. The free end of the catheter was driven subcutaneously to the neck of the animal, where it was exposed and attached permanently. Via an external saline-filled cannula, the pleural balloon catheter was connected to a pressure transducer (Ohmeda DTX; SpectraMed, Bilthoven, The Netherlands), a signal conditioner, and an online computer system, enabling continuous measurement of P_{pl} changes (in cmH_2O). We have previously demonstrated that changes in P_{pl} are linearly correlated with changes in airway resistance and hence can be used as a sensitive index for bronchoconstriction (32).

Provocation procedures. Provocations with OA and histamine (Sigma Chemical, St. Louis, MO) as well as administration of (+)-(R)-trans-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclohexane carboxamide (Y-27632; Tocris Cookson, Bristol, United Kingdom) were performed by inhalation of aerosolized solutions. Aerosols were produced by a DeVilbiss nebulizer (type 646; DeVilbiss, Somerset, PA) driven by an airflow of 8 l/min and resulting in an output of 0.33 ml/min. Provocations were carried out in a perspex cage (internal volume of 9 l) in which the guinea pigs could move freely (25, 32). Before the start of the experiment, the animals were habituated to the experimental conditions on three sequential days at least 1 wk after surgery, when preoperative weight had been restored. On the first day, the animals were placed in the provocation cage unconnected to the pressure transducer. After an adaptation period of at least 30 min, three consecutive provocations with saline were performed, each exposure lasting 3 min and separated by a 7-min interval. The next day, this procedure was repeated with the animals connected to the measurement system. Finally, a histamine challenge, as described below, for habituation was performed on the third day.

On the experimental days, following the habituation procedure, histamine and OA provocations were performed. All provocations were preceded by an adaptation period of at least 30 min, followed by two consecutive control provocations with saline. Baseline P_{pl} was calculated by averaging the P_{pl} of the last 20 min of the adaptation period.

To assess the airway reactivity to histamine, provocations were performed with an initial 25 μg/ml histamine solution in saline, followed by increasing dosage steps of 25 μg/ml. Histamine provocations lasted 3 min, separated by 7-min intervals. Animals were challenged until P_{pl} was increased by more than 100% above baseline for at least 3 consecutive minutes. P_{pl} returned to baseline value within 15 min after the last provocation. The provocation concentration causing a 100% increase of P_{pl} (PC_{100} value) was derived by linear interpolation of the concentration-P_{pl} curve and was used as a measure for airway reactivity toward the agonist. OA provocations were performed by inhalation of increasing aerosol concentrations of 0.5 and 1.0 mg/ml OA in saline for 3 min, separated by 7-min intervals. In the saline-treated control animals, allergen inhalations were discontinued when an increase in P_{pl} of more than 100% was observed, and the nebulized dose of OA was determined. Identical doses of OA were used in weight- and age-matched animals treated with Y-27632, as indicated below. Using these conditions, no antihistamines were necessary to prevent anaphylactic shock after allergen provocation.

Provocation protocol. Histamine PC_{100} values were assessed 24 h before OA challenge and at 6 and 24 h after the OA challenge, i.e., after the EAR and LAR asthmatic reaction, respectively (25, 33). At these latter time points, airway reactivity to histamine was assessed by starting with low concentrations of the agonist (6.25 μg/ml). Thirty minutes before and 8 h after OA challenge, saline or Y-27632 (5 mM) was nebulized during 5 min. Previous experiments with Y-27632 in this model have indicated that the Rho kinase inhibitor is highly effective at 30 min after inhalation (35, 37).

For the quantitative assessment of the EAR (between 0 and 5 h after allergen provocation) and LAR (between 8 and 23 h after allergen provocations), airway function was continuously measured during the whole procedure. Between the measurements of histamine PC_{100} values at 6 and 24 h, the animals were placed in their home cage (0.16 m^2) in which water and food were freely accessible and where they could move around freely. During this transfer, the animals remained connected to the measurement system.

In a separate set of experiments, the duration of the acute bronchoprotective effect of inhaled Y-27632 was examined. At 15 min after the assessment of basal histamine PC_{100}, Y-27632 was aerosolized (5 mM, nebulizer concentration) for 5 min. Subsequently, histamine PC_{100} values were reassessed at 0.3, 1.5, 3, 6, and 24 h after Y-27632 inhalation.

Bronchoalveolar lavage. One hour after assessment of the last PC_{100} value, animals were anaesthetized with 20 mg/ml Brietal-sodium, 35 mg/kg ketamine hydrochloride, and 6 mg/kg Rompun intraperitoneally, which ensured a fast, deep anesthesia. Using a tracheal cannula, the lungs were lavaged gently using 5 ml of sterile saline at 37°C, followed by three subsequent aliquots of 8 ml of saline. The recovered samples were placed on ice and centrifuged at 200 *g* for 10 min at 4°C. The combined pellets were resuspended to a final volume of 1.0 ml in PBS, and total cell numbers were counted using a coulter counter (Beckman). For cytological examination, cytospin preparations were stained with May-Grünwald and Giemsa stain (Sigma Chemical). A cell differentiation was performed by counting at least 400 cells in duplicate.

Data analysis. The magnitude of the EAR after allergen provocation was expressed as the initial maximal increase in P_{pl}, which is mainly attributed to the release of mast cell-derived histamine (34, 46), as well as the area under the P_{pl} time-response curve (AUC) between 0 and 5 h after provocation, calculated by trapezoid integration over discrete (5-min) time periods. The magnitude of the LAR was expressed as the AUC between 8 and 23 h after provocation. All data represent means ± SE from *n* separate experiments. Statistical significance of differences was evaluated using an unpaired two-tailed Student's *t*-test, a repeated measures one-way ANOVA followed by a Holm-Sidak posttest, or a one-way ANOVA on ranks followed by a Dunn's posttest as appropriate. Significance was accepted when $P < 0.05$.

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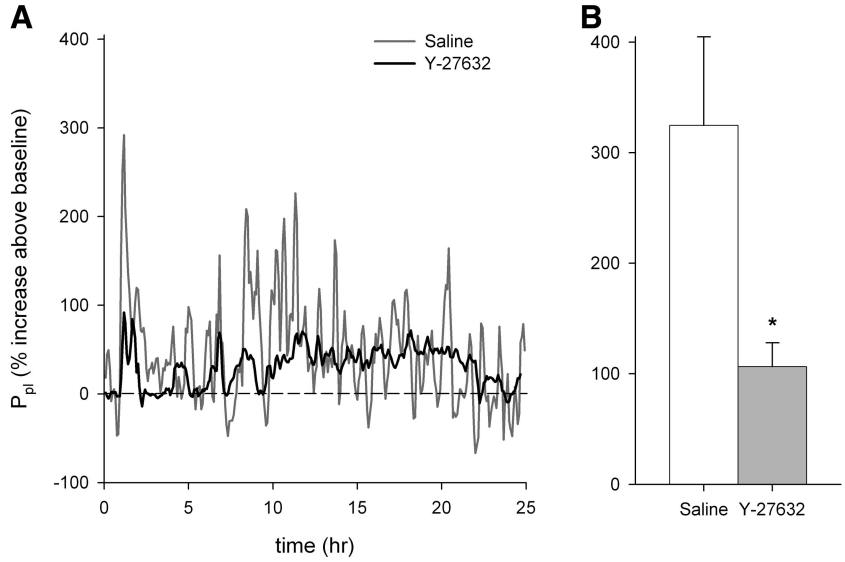

Fig. 1. *A*: representative online recordings of pleural pressure (P_{pl}) in sensitized, conscious, and unrestrained guinea pigs after allergen challenge at *time 0* h, the animals being treated with inhaled saline (gray line) or Y-27632 (5 mM nebulizer concentration; black line) at 30 min before and 8 h after the challenge. *B*: effects of pretreatment with inhaled saline or Y-27632 on the initial peak rise in response to allergen challenge. Results are presented as percentage increase in P_{pl} above baseline. Data represent means ± SE of 4–5 animals. $*P < 0.05$ compared with saline-treated animals.

RESULTS

In Fig. 1*A*, examples of online P_{pl} recordings of OA-challenged guinea pigs pretreated with either saline or Y-27632 are shown. Overall analysis of these recordings revealed that Y-27632-pretreated animals displayed a marked reduction of the initial peak response in P_{pl} (Fig. 1*B*), which is mainly attributed to the release of histamine in the airways. However, the AUC of the EAR was not significantly affected (2,562 ± 435% × 5 min in saline-treated animals and 2,579 ± 811% × 5 min in Y-27632-treated animals). Also, no effects of Y-27632 inhalation on AUC of the LAR were observed (9,663 ± 1,261% × 5 min and 10,224 ± 2,411% × 5 min, respectively).

As shown in Fig. 2, OA challenge induced a significant AHR in response to histamine after both the EAR and the LAR in the saline-treated control animals, as indicated by the reduced PC_{100} values after these reactions. Compared with the saline-treated animals, pretreatment with inhaled Y-27632 resulted in a marked reduction of the AHR after the EAR, as indicated by a significantly smaller decrease of the absolute PC_{100} value (Fig. 2*A*) and a significantly reduced ratio of PC_{100} pre-/postchallenge (Fig. 2*B*). Notably, in the Y-27632-pretreated animals, the AHR after the LAR was fully normalized to prechallenge conditions.

In separate experiments, it was established that the acute bronchoprotective effect of the Y-27632 inhalation on histamine-induced airways obstruction is abolished within 90 min (Fig. 3), indicating that the effects of Y-27632 inhalation on the AHR after the EAR (6 h after challenge) and LAR (24 h after challenge) cannot be accounted for by an acute bronchoprotection. Since inhibition of airway inflammation could be involved, BAL was performed 1 h after assessment of the last PC_{100} value to determine infiltration of inflammatory cells in the airways. In the saline-treated controls, OA challenge induced significant increases in total inflammatory cell number, eosinophils, macrophages, and neutrophils, which was significantly inhibited by pretreatment with inhaled Y-27632 (Fig. 4).

DISCUSSION

In the present study, we have demonstrated that inhalation of the Rho kinase inhibitor Y-27632 at 30 min before and 8 h after allergen challenge effectively prevents the development of AHR to histamine both after the EAR (measured at 6 h after

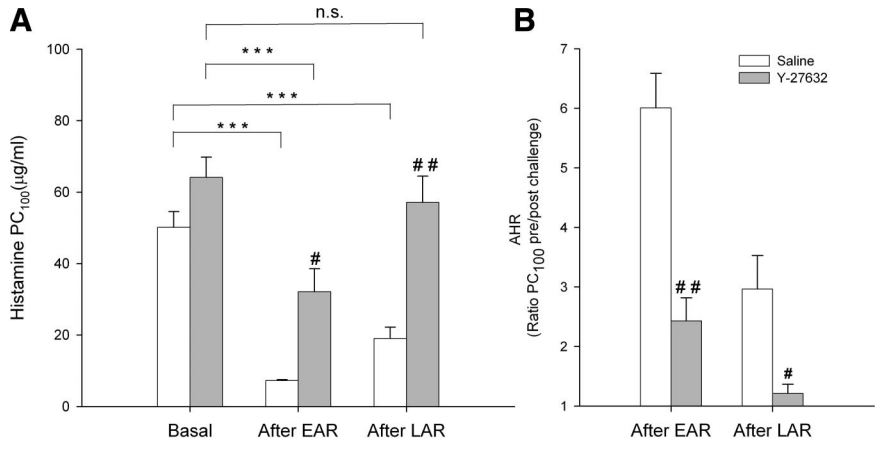

Fig. 2. Pretreatment with inhaled Y-27632 significantly prevents the development of allergen-induced airway hyperresponsiveness (AHR) after the early (EAR) and late (LAR) asthmatic reaction. *A*: effects of saline (white bars) and Y-27632 (5 mM nebulizer concentration; gray bars) inhalations, performed 30 min before and 8 h after allergen challenge on histamine PC_{100} values after the EAR (measured at 6 h after allergen challenge) and LAR (24 h after the challenge). Basal represents the PC_{100} values as assessed before saline or Y-27632 inhalation. *B*: the degree of AHR after the EAR and the LAR in saline (white bars)- and Y-27632 (gray bars)-treated animals, expressed as the ratio PC_{100} pre-/postchallenge. A ratio of 1 signifies normoresponsiveness. Data represent means ± SE of 6–7 animals. $***P < 0.001$ compared with basal. $\#P < 0.05$, $\#\#P < 0.01$ compared with saline.

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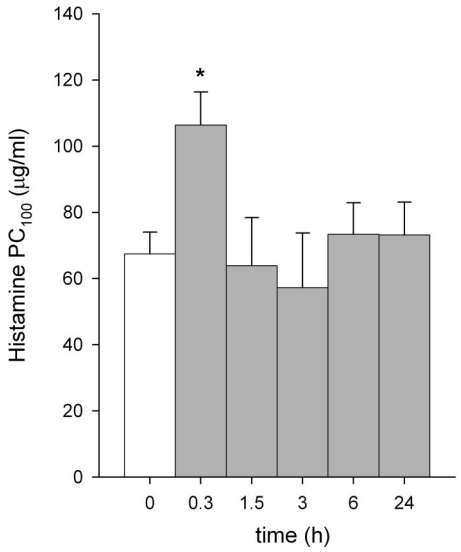

Fig. 3. Time course of the acute bronchoprotective effects of inhaled Y-27632 on histamine-induced bronchoconstriction. The white bar represents basal airway responsiveness to histamine, whereas the gray bars represent responsiveness to histamine as determined 0.3, 1.5, 3, 6, and 24 h after Y-27632 inhalation. Data represent means ± SE of 3–4 animals. *$P < 0.05$ compared with time point 0 (i.e., basal airway responsiveness before Y-27632 inhalation).

allergen challenge) and the LAR (measured at 24 h after allergen challenge). Since it was established that the bronchoprotective effect of the inhaled dose of Y-27632 against histamine disappears within 90 min, the observed effects cannot be explained by a direct inhibitory effect of the Rho kinase inhibitor on the histamine-induced airway constriction after the EAR and LAR. Rather, the observed prophylactic effects of Y-27632 inhalation might be explained by an inhibition of infiltration of inflammatory cells into the airways, as the numbers of total inflammatory cells, eosinophils, neutrophils, and macrophages were reduced in Y-27632-pretreated animals. What clearly distinguishes this study from previous work is the demonstration of the prophylactic effects of the Rho kinase inhibitor on the genesis of AHR after the EAR and the LAR, which is associated with decreased numbers of inflammatory cells recovered from the BAL.

Previous studies have indicated a possible relationship between infiltration and activation of particularly eosinophils and AHR. Thus, in BAL fluid obtained from asthmatic patients, the number of eosinophils is increased and is inversely correlated with the histamine PC_{20} in these patients (47). In addition, in biopsy studies, a positive correlation was found between (activated) $EG2^+$ eosinophils and AHR (7). Also, in mild asthmatics, a significant relationship between the amount of eosinophil-derived major basic protein and AHR to histamine was found (47). Accordingly, in our guinea pig model, a significant correlation was found between eosinophil peroxidase in BAL fluid and AHR at 6 h after allergen challenge (31). Although the role of the eosinophil in allergic asthma has been challenged, there is compelling evidence for the involvement of eosinophils in various processes that may induce AHR (22). As a source of basic granule proteins, growth factors, lipid mediators, proinflammatory cytokines, and chemokines, eosinophils have, for instance, been associated with the induction of epithelial damage, M_2 autoreceptor dysfunction on cholinergic nerves, mucus hypersecretion, deficiency of cNOS-derived NO, and airway remodeling (10, 14, 21, 22, 24, 26).

In agreement with our observations, recent studies using mouse models of allergic asthma have indicated an important role for Rho kinase signaling in pulmonary eosinophilia (19, 43). These findings could possibly be accounted for by a reduction in chemokine (eotaxin) and/or cytokine (IL-5, IL-13) production by Rho kinase inhibition (43), although the source of these inflammatory mediators and the precise mechanisms underlying the suppression by Rho kinase inhibition remain to be determined. The effects of Y-27632 on eosinophilia are consistent with in vitro findings demonstrating a crucial role for RhoA/Rho kinase signaling in the chemotaxis of eosinophils in response to the chemokine eotaxin (1) and in the migration of eosinophils through endothelial barriers (3).

In addition to its effects on airway eosinophilia, our current data indicate that Y-27632 inhalation reduces the number of infiltrated macrophages and neutrophils as well. Currently, the role of neutrophils and macrophages in the development of AHR is incompletely understood. However, neutrophils have been implicated in the development of allergen challenge-induced acute AHR (41). In addition, in vitro experiments have shown that supernatants from activated neutrophils induce hyperresponsiveness of human bronchial smooth muscle preparations in response to electrical field stimulation, with the site of action presumably being prejunctional on the parasympathetic nerve (4). In a murine model of allergic asthma, it was demonstrated that accumulation of neutrophils and activated

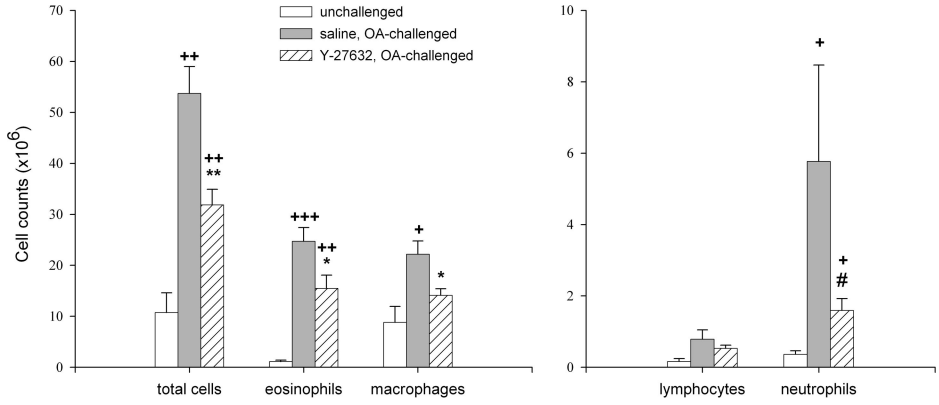

Fig. 4. The effects of saline (gray bars) and Y-27632 (hatched bars) inhalations performed 30 min before and 8 h after allergen challenge on the inflammatory cell profile in the bronchoalveolar lavage obtained at 25 h after the allergen challenge. Data represent means ± SE of 4–7 animals. *$P < 0.05$, **$P < 0.01$, #$P = 0.07$ compared with saline-treated, ovalbumin (OA)-challenged; +$P < 0.05$, ++$P < 0.01$, +++$P < 0.001$ compared with unchallenged.

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macrophages in the airway wall at least in part was related to AHR (2).

As for eosinophils, reduced infiltration of neutrophils and macrophages could be explained by the involvement of RhoA and Rho kinase on the migration of these cells (5, 20, 23, 30, 40). Some of these effects may be linked to effects on endothelial barrier integrity. Thus, actomyosin-mediated endothelial cell contraction is a key process in inflammatory cell-induced reduction in endothelial barrier function, which is required for tissue infiltration by inflammatory cells. Reduction of myosin phosphorylation in HUVEC by Rho kinase or MLCK inhibition preserves endothelial barrier integrity and prevents inflammatory cell transit (30). Although studied in vitro, these effects could be important in vivo as well, as it was demonstrated that in the lungs of a mouse model of acute lung injury induced by endotoxin, Y-27632 attenuated both edema and migration of neutrophils, likely by modulating endothelial cell contraction induced by inflammatory mediators, rather than through an effect on neutrophil sequestration (44). Although the infiltration of inflammatory cells into the airways appears to be Rho/Rho kinase dependent, the specific link to this pathway is complex and requires further study.

In the present study, we also demonstrated that inhalation of Y-27632 significantly reduces the acute allergen-induced bronchoconstriction as indicated by the considerable attenuation of the initial peak rise in P_{pl}, which is believed to be mediated by histamine (11, 27, 46). On the one hand, this might be explained by the bronchoprotective effect of Y-27632 inhalation on histamine responsiveness, as has also been observed previously (35). An additional mechanism by which Rho kinase inhibition could reduce this peak rise and perhaps even modulate airway inflammation and subsequent development of AHR might be through attenuating the release of mast cell-derived histamine. In vitro, using rat peritoneal mast cells, a role for Rho kinase in regulating histamine release has been reported (17). Several lines of evidence indicate that histamine may be involved in the development of allergen-induced airway inflammation and AHR. Thus, it has been described that histamine is capable of inducing the expression of adhesion molecules on endothelial cells (29) and potently increases vascular permeability. Accordingly, previous work from our laboratory showed that inhalation of the histamine H_1-receptor antagonist mepyramine strongly reduced the allergen-induced AHR, both after the EAR and the LAR, and airway inflammation in the same guinea pig model as used in the present study (34).

The inhibition of the initial allergen-induced constriction by Y-27632 was not associated with an inhibition of the magnitude of the EAR when expressed as the AUC. This could be explained by the relatively short duration of the effect of the Rho kinase inhibitor and the small contribution of the P_{pl} peak to the entire AUC. Nevertheless, this effect may be of importance as it represents the largest decrease in lung function after the allergen challenge. Similarly, a short duration of action could explain why a significant effect of Y-27632 on the AUC of the LAR was absent. Remarkably, however, the infiltration of inflammatory cells into the airways was markedly inhibited, which may suggest a dissociation between airway inflammation and the LAR. On the other hand, the effect of Y-27632 on allergen-induced AHR appears to be larger in magnitude than that on inflammatory cell infiltration. In this regard, it is interesting to note that using the same animal model, a similar tendency, particularly for eosinophils, was observed after administration of the antihistamine mepyramine (33), supporting our hypothesis that inhibition of histamine release could be involved in Y-27632-induced attenuation of allergen-induced AHR. The exact relationship between AHR and infiltrated cells in the airways is presently unknown. However, such a relationship will also depend on the activation state of the infiltrated cells, particularly eosinophils (30, 46), which may similarly be influenced by Y-27632 (18) and thus offers an explanation for the apparent dissociation.

Collectively, our previous (35) and current findings indicate a pivotal role for Rho kinase in regulating the development of the AHR after the EAR and LAR in response to allergen challenge. Thus, in addition to the acute reversal of AHR after the EAR (at 6 h) and the LAR (at 24 h after allergen challenge) by inhalation of Y-27632 at 5.5 and 23.5 h, respectively (35), the present study demonstrates that treatment with the inhaled Rho kinase inhibitor before allergen challenge protects against the development of AHR after these reactions. This effect is associated with attenuation of the acute allergen-induced bronchoconstriction and airway inflammation. Since the AHR after the EAR and LAR is inhibited presumably without a direct bronchoprotective effect, the anti-inflammatory effect of Y-27632 could be importantly involved. Further studies are indicated to unravel this relationship. Altogether, these findings support a pharmacotherapeutical interest for Rho kinase inhibitors in the treatment of allergic asthma.

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GRANTS

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