Biochemical and Biophysical Research Communications 386 (2009) 124–129

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

LPS-stimulated MUC5AC production involves Rac1-dependent MMP-9 secretion and activation in NCI-H292 cells

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ARTICLE INFO

Article history:
Received 24 May 2009
Available online 6 June 2009

Keywords:
NCI-H292 cells
Lipopolysaccharides (LPS)
Rac1
Reactive oxygen species (ROS)
Matrix metalloproteinase-9 (MMP-9)
MUC5AC, mucin 5AC
Mucin
Chronic obstructive pulmonary disease (COPD)

ABSTRACT

Chronic obstructive pulmonary disease (COPD) is an inflammatory process characterized by airway mucus hypersecretion. Previous studies have reported that lipopolysaccharides (LPS) stimulate mucin 5AC (MUC5AC) production via epidermal growth factor receptor (EGFR) in human airway cells. Moreover, this production was shown to depend on the expression and activity of matrix metalloproteinase 9 (MMP-9), which is increased in COPD patients' serum. In the present study we investigated the signaling pathway mediating LPS-stimulated secretion and activation of MMP-9, and the regulatory effects of this pathway on the production of MUC5AC in the human airway cells NCI-H292. Using specific inhibitors, we found that LPS-stimulated cells secreted and activated MMP-9 via EGFR. Our results also indicate that signaling events downstream of EGFR involved PI3K-dependent activation of Rac1, which mediated the NADPH-generated reactive oxygen species responsible for MMP-9 secretion and activation. Finally, we observed that EGFR/PI3K/Rac1/NADPH/ROS/MMP-9 regulate MUC5AC production in LPS-challenged NCI-H292 cells.

Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality [1]. Airflow obstruction, chronic bronchitis, and emphysema are the main features of COPD [2]. This chronic inflammatory process is characterized by pathologic abnormalities in the submucosal glands and surface epithelium, which lead to airway mucus hypersecretion [2]. Mucus is a viscoelastic gel that lines the respiratory tract epithelium and protects against infectious and environmental agents [3]. Mucus composition consists of water, salts, lipids, and several proteins, including mucin glycoproteins [3]. The highly inducible mucin 5AC (MUC5AC) constitutes the most abundant mucin in the airway secretions of humans [3,4].

Previous studies have shown that lipopolysaccharides (LPS), a major outer surface membrane component of Gram-negative bacteria such as *Pseudomonas aeruginosa*, stimulate MUC5AC production via epidermal growth factor receptor (EGFR) in human airway cells [4–7]. LPS-stimulated MUC5AC production was also shown to be dependent on the expression and activity of matrix metalloproteinase 9 (MMP-9), also known as gelatinase B [6,7]. Notoriously, MMP-9 has been found increased not only in sputum, but also in serum of COPD patients [8,9]. Furthermore, higher MMP-9 serum concentration is connected with higher airway obstruction and disease progression [9].

In the present study we investigated the signaling pathway mediating LPS-stimulated secretion and activation of MMP-9, and the regulatory effects of this pathway on the production of MUC5AC in the human airway cells NCI-H292.

Materials and methods

Antibodies and reagents. Mouse monoclonal anti-MUC5AC was from Sigma Chemical Co. (St. Louis, MO). Roswell Park Memorial Institute medium (RPMI 1640) and fetal calf serum were from GIBCO/Invitrogen (Carisbad, CA). CM-H_2DCFDA was from Molecular Probes (Burlington, Ontario, Canada). NSC23766 was from Tocris (Bristol, UK). Lipopolysaccharides (LPS) from *P. aeruginosa* and specific inhibitors of EGFR (AG1478), PI3K (LY294002), NADPH (DPI), ROS (NAC), and MMP (GM6001) were from Sigma Chemical Co.

Cell culture and treatments. NCI-H292 mucoepidermoid carcinoma cells from human lung (ATCC, Manassas, VA) were maintained in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml), and incubated

Abbreviations: AG, AG1478; DPI, diphenyleneiodonium; EGFR, epidermal growth factor receptor; GM, GM6001; LPS, lipopolysaccharides; LY, LY294002; MMP, matrix metalloproteinase; MUC5AC, mucin 5AC; NAC, *N*-acetylcysteine; NADPH, nicotinamide adenine dinucleotide phosphate; NSC, NSC23766; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; Veh, vehicle.

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doi:10.1016/j.bbrc.2009.05.136

at 37 °C in a 5% CO_2 humidified atmosphere. For experiments, NCI-H292 cells were seeded at the density of 5×10^5 cells per well in six-well plates. For LPS treatment, cells were incubated in serum-free medium (RPMI 1640) for 24 h. After that, cells were treated for the indicated time periods with 20 μg/ml LPS in phosphate-buffered saline (vehicle) and untreated cells served as control. To determine the effect of specific inhibitors, they were added to the cells 1 h before LPS, and they were maintained in the media throughout the experiment. The inhibitors and concentrations used in these studies were: 10 μM AG1478 (AG); 20 μM GM6001 (GM); 50 μM NSC23766 (NSC); 50 μM LY294002 (LY); 15 μM DPI; and 30 mM NAC.

MMP-9 activity assay. For measurement of active and total (pro and active) MMP-9 in the supernatants of NCI-H292 cells, the high-sensitivity MMP-9 Activity Biotrak Assay System kit (Amersham Biosciences, Piscataway, NJ) was used according to the manufacturer's instructions. This activity assay is based on an antibody-capture technique that sequesters the metalloproteinase. Bound endogenously active metalloproteinase activates a detection enzyme, which in turn activates a detectable chromogenic substrate. Total metalloproteinase activity (endogenously active plus the activatable pro-enzyme) is measured by the addition of α-aminophenylmercuric acetate, which artificially activates the inactive form of the metalloproteinase. Total-MMP-9 levels were expressed as a percentage of total-MMP-9 levels in LPS-stimulated cells, which were considered as 100%. Active-MMP-9 levels were expressed as a percentage of active-MMP-9 levels in LPS-stimulated cells, which were considered as 100%.

Rac1 activity assay. For measurement of Rac1 activation in cell lysates from NCI-H292 cells, equal amounts of protein per sample (determined by use of a Protein Assay Kit from Bio-Rad Laboratories, Hercules, CA) were analyzed using the specific Rac1 G-LISA™ Activation Assay kit (Cytoskeleton, Denver, CO) according to the manufacturer's instructions. This luminescence G-LISA™ is an ELISA based assay that allows measuring the GTP-bound (active) form of small G-proteins. Active Rac1 levels were expressed as fold increase over the vehicle-control.

ROS production. Free radical production was determined by incubating NCI-H292 cells in the presence of 10 μM CM-H_2DCFDA. Fluorescence was measured in a stirred cuvette at 37 °C in a Hitachi F-2000 (Hitachi Ltd., Tokyo, Japan) spectrofluorometer with excitation at 488 nm and emission at 530 nm. ROS production levels were expressed as fold increase over the vehicle-control.

MUC5AC ELISA. Levels of MUC5AC protein have been shown to increase after 6 h of LPS stimulation [7]. Here, we measured MUC5AC in cell culture supernatants and in cell lysates following 6 h of LPS challenge, by ELISA as previously described [10]. The total production of MUC5AC protein was the sum of MUC5AC protein in cell culture supernatant and in cell lysate. The amount of MUC5AC protein in each sample was normalized to total protein and expressed as fold increase over the vehicle-control.

Statistical analysis. Data correspond to at least three independent experiments, each of which was done in triplicate. Results are presented as mean ± standard error (SE). The data for each condition were subject to analysis of variance (ANOVA) followed by Dunnet post hoc test when comparing three or more conditions, or evaluated using Student's *t*-test when comparing only two conditions. Significant differences were considered with values of $p < 0.05$.

Results

EGFR is required for optimal LPS-stimulated MMP-9 secretion and activation

LPS-induced MMP-9 activity in NCI-H292 cells was previously demonstrated using gelatin zymography technique [7]. Here, we used a high-sensitivity assay to determine the effects of LPS stimulation on the secretion and activation of MMP-9 in these cells. MMP-9 is secreted by cells as a pro-enzyme (pro-MMP-9) and then activated in the pericellular space (active-MMP-9) to execute its proteolytic activity [11]. LPS-stimulated NCI-H292 cells exhibited a time-dependent increase in total (pro plus active) MMP-9 levels (Veh: 15.1 ± 1.2%, 2 h LPS: 31.9 ± 2.8%, 6 h LPS: 54.5 ± 4.7%, and 12 h LPS: 100.0 ± 7.8%; Fig. 1A). While, active MMP-9 levels were non-detectable in unstimulated cells, NCI-H292 cells stimulated with LPS also showed time-dependent increasing levels of active MMP-9 (28.4 ± 2.4%, 51.1 ± 4.5%, and 100.0 ± 7.8% for 2, 6, and 12 h, respectively; Fig. 1B).

EGFR was shown to regulate the transcript and protein levels of MMP-9 in NCI-H292 cells stimulated with LPS [7]. Here, we evaluated the influence of EGFR on the secretion and activation of MMP-9 in these cells stimulated with LPS. NCI-H292 cells pretreated with AG1478 (AG), an EGRF inhibitor, and stimulated with LPS for 6 h presented a 54.3% and 67.4% reduction in both secretion and activation of MMP-9 (AG/LPS: 45.7 ± 3.6% vs Veh/LPS: 100.0 ± 6.9% for total-MMP-9, and AG/LPS: 32.6 ± 2.7% vs Veh/LPS 100.0 ± 6.8% for active-MMP-9; Fig. 1C and D), indicating that EGFR was required for an optimal secretion and activation of MMP-9. As expected, GM6001 (GM), a MMP inhibitor, reduced the activation but not the secretion of MMP-9 in LPS-stimulated cells (14.2 ± 1.3% and 114.7 ± 5.2% for active and total-MMP-9, Fig. 1C and D).

LPS induces Rac1 activation through EGFR

Acting as molecular switchers cycling between a GDP-bound inactive state and a GTP-bound active state, members of the Rho family of small GTPases play an essential role in a variety of cellular events [12]. Rac1, a member of this family, was shown to become active in different cell types challenged with LPS [13–15]. We therefore examined activation of Rac1 in NCI-H292 cells. Stimulation of these cells with LPS induced up to 3.8 times increase in Rac1 activity (fold increases of 2.4 ± 0.4, 2.9 ± 0.4, 3.8 ± 0.4, and 3.5 ± 0.4 at 5, 10, 15, and 30 min, respectively; Fig. 2A).

We next evaluated the influence of EGFR on LPS-mediated activation of Rac1 in NCI-H292 cells. Cells pretreated with AG and stimulated with LPS for 15 min exhibited a 58.3% reduction in Rac1 activity (fold increase of AG/LPS: 1.5 ± 0.3 vs Veh/LPS: 3.6 ± 0.4; Fig. 2B), indicating that EGFR is necessary for LPS to activate Rac1. As control, NSC23766 (NSC) reduced Rac1 activity in both LPS-stimulated and unstimulated cells (fold increase of NSC/Veh: 0.6 ± 0.2 vs NSC/LPS: 1.1 ± 0.3; Fig. 2B).

PI3K mediates LPS-induced Rac1 activation

Phosphatidylinositol 3-kinase (PI3K) controls a wide variety of intracellular signaling pathways, including Rac1 activation by LPS [14,15]. To evaluate the role of PI3K in LPS-stimulated Rac1 activity in NCI-H292 cells, we used LY294002 (LY), a specific PI3K inhibitor. Cells pretreated with LY and stimulated with LPS for 15 min exhibited a 54.1% reduction in Rac1 activity (fold increase of LY/LPS: 1.75 ± 0.3 vs Veh/LPS: 3.7 ± 0.4; Fig. 2C), indicating that PI3K mediates LPS-induced Rac1 activation.

LPS-stimulated MMP-9 secretion and activation is mediated by Rac1

In addition to regulate Rac1 activity, PI3K was shown to participate in the transcription, protein expression and secretion of MMP-9 in several cell types [16–18]. Thus, we analyzed the effect of PI3K inhibition on MMP-9 secretion and activation

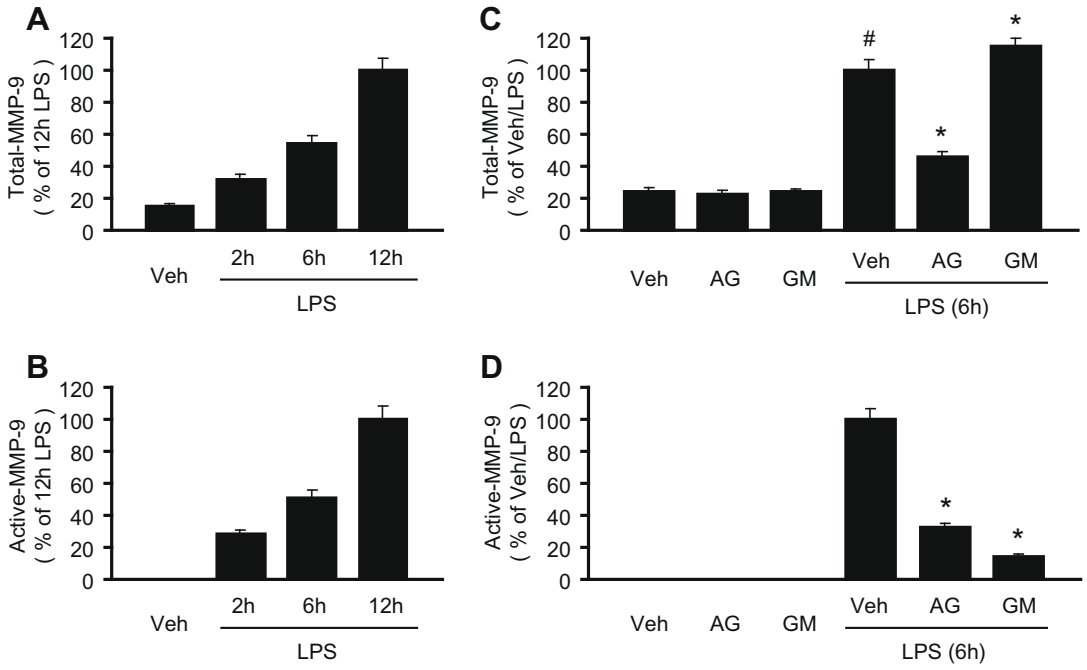

Fig. 1. EGFR is required for optimal LPS-stimulated MMP-9 secretion and activation. (A,B) NCI-H292 cells were stimulated with LPS for 2, 6, and 12 h. Total (A) and active (B) MMP-9 levels were measured in cell supernatants. (C,D) Specific inhibitor AG1478 (AG) was used to evaluate EGFR influence on total (C) and active (D) levels of MMP-9 in cell supernatants of NCI-H292 cells stimulated with LPS for 6 h. This stimulation period time was selected based on previous available data on MUC5AC production [7]. MMP specific inhibitor GM6001 (GM) was used as a control of MMP-9 activity. $^{*}p < 0.05$ compared to LPS-stimulated cells in the absence of inhibitors, $^{\#}p < 0.05$ compared to unstimulated vehicle-control cells.

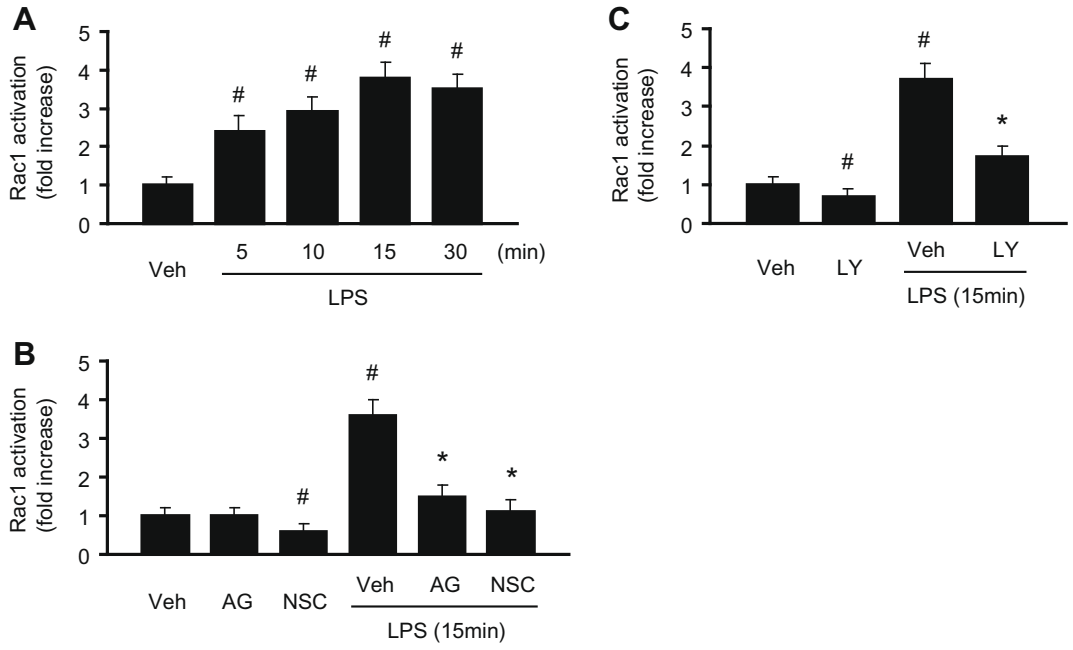

Fig. 2. LPS induces PI3K-mediated Rac1 activation through EGFR. (A) NCI-H292 cells were stimulated with LPS for 5, 10, 15, and 30 min. Rac1 activation levels were assessed in cell lysates. (B,C) NCI-H292 cells were stimulated with LPS for 15 min. Specific inhibitors AG1478 (AG) and LY294002 (LY) were used to evaluate EGFR (B) and PI3K (C) requirement for Rac1 activation. Specific inhibitor NSC was used as a control of Rac1 activity. Rac1 activation levels were assessed in cell lysates. $^{*}p < 0.05$ compared to LPS-stimulated cells in the absence of inhibitors, $^{\#}p < 0.05$ compared to unstimulated vehicle-control cells.

in NCI-H292 cells. Cells pretreated with LY and stimulated with LPS for 6 h presented a 53.8% and 68.3% reduction in both secretion and activation of MMP-9 (LY/LPS: 46.2 ± 2.9% vs Veh/LPS: 100.0 ± 6.6% for total-MMP-9, and LY/LPS: 31.7 ± 2.8% vs Veh/LPS: 100.0 ± 5.7% for active-MMP-9; Fig. 3A and B).

Subsequently, we proceeded to evaluate the involvement of Rac1 in these processes in NCI-H292 cells. Cells pretreated with NSC and stimulated with LPS for 6 h presented a 64.3% and 78.1% reduction in both secretion and activation of MMP-9 (35.7 ± 2.5% and 21.9 ± 2.0% for total and active MMP-9, Fig. 3A and B), indicating that Rac1 mediates the LPS effects on MMP-9.

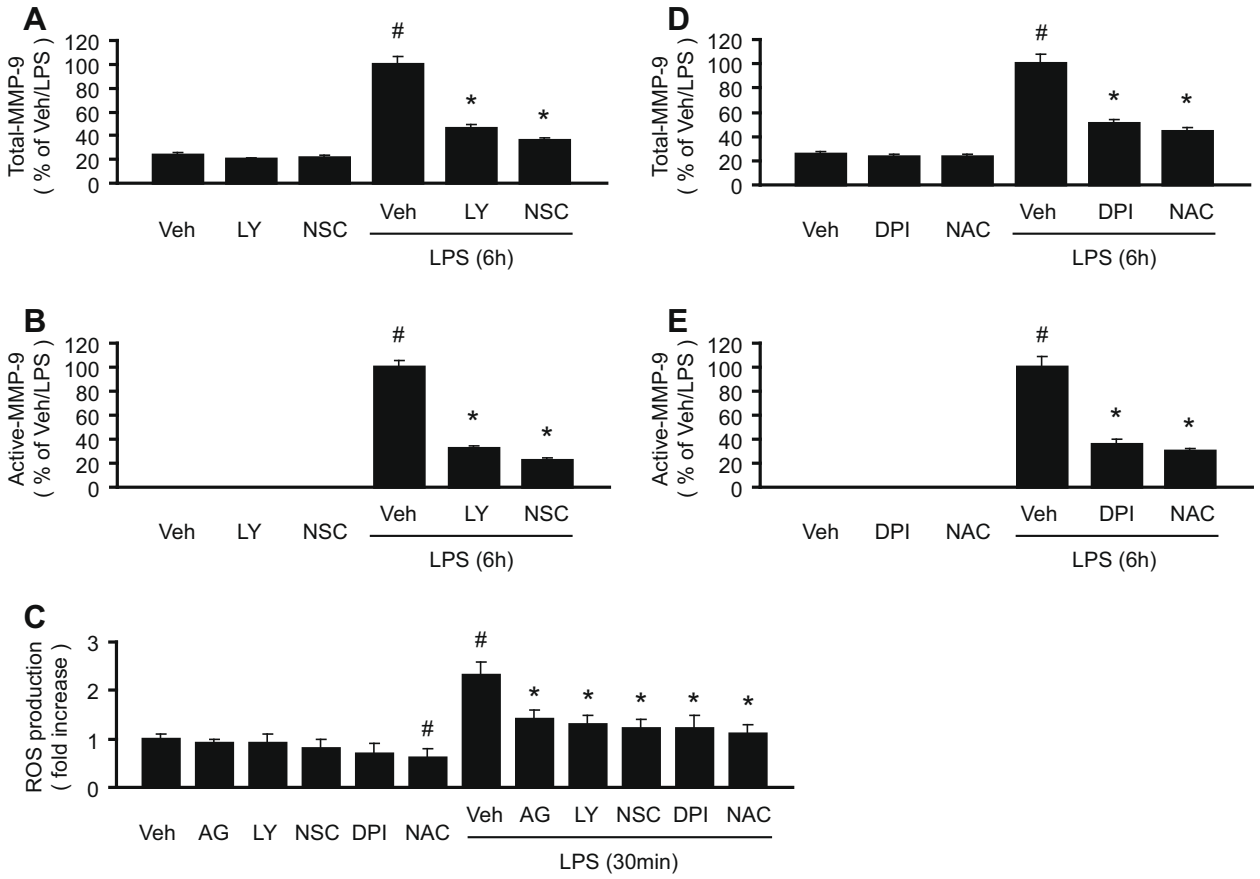

Fig. 3. Rac1 mediates LPS-stimulated MMP-9 secretion and activation through generation of ROS. (A,B) NCI-H292 cells were stimulated with LPS for 6 h. Total (A) and active (B) MMP-9 levels were measured in cell supernatants. Specific inhibitors NSC23766 (NSC) and (LY294002) LY were used to evaluate Rac1 and PI3K influence on total (A) and active (B) levels of MMP-9. (C) Reactive oxygen species (ROS) production in NCI-H292 cells. Specific inhibitors AG, LY, NSC, DPI, and NAC were used to evaluate EGFR, PI3K, Rac1, and NADPH influence on ROS generation. Specific inhibitor NAC was used as a control of ROS formation. (D,E) NCI-H292 cells were stimulated with LPS for 6 h. Total (D) and active (E) MMP-9 levels were measured in cell supernatants. Specific inhibitors DPI and NAC were used to evaluate NADPH and ROS influence on total (D) and active (E) levels of MMP-9. $^{*}p < 0.05$ compared to LPS-stimulated cells in the absence of inhibitors, $^{\#}p < 0.05$ compared to unstimulated vehicle-control cells.

NADPH-generated ROS act downstream of Rac1 to promote MMP-9 secretion and activation

Rac GTPase is the upstream signal protein for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent reactive oxygen species (ROS) generation, which has been shown to induce expression and secretion of MMP-9 in other cell types [19–21]. In Fig. 3C, we examined ROS production in NCI-H292 cells and determined the participation of upstream signal proteins using specific inhibitors. Basal ROS levels for unstimulated vehicle-control cells were substantially increased by stimulation with LPS for 30 min (1.0 ± 0.1 and 2.3 ± 0.3 fold increase, respectively). Pretreatment of these cells with either AG, or LY, or NSC, or diphenyleneiodonium (DPI), a NADPH oxidase inhibitor, all of them reduced LPS-stimulated ROS production in a 39.1%, 43.5%, 47.8%, and 47.8%, respectively (fold increases of 1.4 ± 0.2, 1.3 ± 0.2, 1.2 ± 0.2, and 1.2 ± 0.3). As control, we used the ROS inhibitor *N*-acetylcysteine (NAC), which diminished ROS levels in both unstimulated and LPS-stimulated cells (0.6 ± 0.2 and 1.1 ± 0.2 fold increase, respectively).

We then assessed the effects of NADPH and ROS inhibition on MMP-9 secretion and activation. Pretreatment of LPS-stimulated cells with either DPI or NAC produced a reduction of 49.6% and 56.3% in total-MMP-9, and 64.2% and 70.3% in active-MMP-9, respectively (DPI/LPS: 50.4 ± 3.6% and NAC/LPS: 43.7 ± 3.9% vs Veh/LPS: 100.0 ± 7.9% for total-MMP-9, and DPI/LPS: 35.8 ± 3.7% and NAC/LPS: 29.7 ± 2.8% vs Veh/LPS: 100.0 ± 8.7% for active-MMP-9; Fig. 3D and E), indicating that NADPH-generated ROS acted downstream of Rac1 promoting secretion and activation of MMP-9.

LPS-stimulated MUC5AC production is regulated by Rac1/ROS-induced MMP-9 activity

Lastly, we examined the requirement of the molecules we found involved in MMP-9 secretion and activation for LPS-stimulated production of MUC5AC in NCI-H292 cells. In Fig. 4, we show that stimulation of these cells with LPS for 6 h exhibited a 3.8 ± 0.5 fold increase over the vehicle-control (1.0 ± 0.1). Pretreatment of cells with either AG, or LY, or NSC, or DPI, or NAC, reduced LPS-stimulated MUC5AC production in a 57.9%, 52.6%, 65.6%, 57.9%, and 50.0%, respectively (fold increases of 1.6 ± 0.3, 1.8 ± 0.4, 1.3 ± 0.3, 1.6 ± 0.3, and 1.9 ± 0.4). As expected, GM also decreased the MUC5AC levels of LPS-stimulated cells (1.8 ± 0.3 fold increase). These results support the requirement of Rac1/ROS-induced MMP-9 activity for LPS-stimulated MUC5AC production.

Discussion

In the present study, we show that LPS stimulation of human airway cells NCI-H292 induced secretion and activation of MMP-9 via EGFR. Our results also indicate that signaling events downstream of EGFR involve PI3K-dependent activation of Rac1.

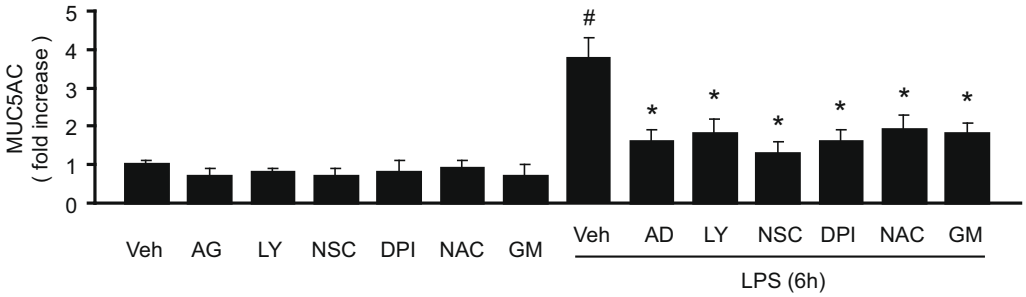

Fig. 4. LPS-stimulated MUC5AC production is regulated by Rac1/ROS-induced MMP-9 activity. NCI-H292 cells were stimulated with LPS for 6 h. MUC5AC levels were measured in cell supernatants and in cell lysates. The influence of specific inhibitors AG1478 (AG), LY294002 (LY), NSC23766 (NSC), DPI, and NAC on the total production of MUC5AC was assessed. $^{*}p < 0.05$ compared to LPS-stimulated cells in the absence of inhibitors, $^{\#}p < 0.05$ compared to unstimulated vehicle-control cells.

Through NADPH oxidase, activated Rac1 induces formation of ROS, which mediates secretion and activation of MMP-9. Once active, MMP-9 promotes production of MUC5AC in LPS-stimulated cells. Additionally, all the molecules involved in this pathway (EGFR, PI3K, Rac1, NADPH, ROS, and MMP-9) are required for MUC5AC production in NCI-H292 cells challenged with LPS.

Although MMP-9 was previously found to be required for LPS-stimulated production of MUC5AC in human airway cells [6,7], the underlying mechanism driving MMP-9 activity in these cells was not fully elucidated. Stimulation of NCI-H292 cells with LPS lead to a notorious increase in both total and active MMP-9, being this increase dependent of EGFR (Fig. 1). Rac1 is one of the signal cascades activated by EGFR in various cell types [22–24]. Moreover, PI3K was shown to mediate the signal between EGFR and Rac1 in these cell types [22–24]. Using specific inhibitors, our experiments revealed not only that Rac1 becomes active following LPS stimulation of NCI-H292 cells, but also that PI3K was the signaling molecule downstream of EGFR regulating Rac1 activity (Fig. 2).

ROS species are physiological compounds in cells, but also are risk factors for diverse respiratory diseases, such as COPD [25]. LPS, a highly potent proinflammatory substance, was shown to stimulate ROS generation in several cell types [26]. It is well established that Rac is the upstream signaling protein for NADPH oxidase-mediated ROS production [19]. In agreement, we found NADPH oxidase downstream of Rac1 to be mediating LPS-stimulated formation of ROS in NCI-H292 cells (Fig. 3). Furthermore, Rac1 and ROS showed to be responsible for MMP-9 secretion and activation (Fig. 3).

Consistently with previous studies implicating MMP-9 as well as ROS in MUC5AC production by human airway cells [6,7,27], inhibition of the molecules we found to be involved in the signaling pathway mediating LPS-stimulated MMP-9 secretion and activation, diminished the production of this mucin (Fig. 4).

In summary, our findings support the requirement of MMP-9 secretion and activation to increase LPS-mediated MUC5AC production. The critical role that we found for Rac1 in MMP-9 secretion and activation makes Rac1 attractive as a potential pharmacological target for COPD therapy. However, this tempting idea has to be carefully explored. Rac1 is also involved in other processes such as phagocytosis by professional phagocytes, which is required for an adequate bacterial clearance [28,29]. Thus, targeting of Rac1 in infection-related COPD will require developing a cell-specific delivery system, as it has been created for other cell types [30].

Acknowledgments

This work was supported by research grants given to Dr. Cosen-Binker by GlaxoSmithKline S.A. and KB Certification International.

To the memory of Mario Binker.

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