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# Omega-3 PUFA docosahexaenoic acid decreases LPS-stimulated MUC5AC production by altering EGFR-related signaling in NCI-H292 cells

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is an inflammatory process characterized by airway mucus hypersecretion. Lipopolysaccharides (LPS) are known to stimulate the production of mucin 5AC (MUC5AC) via epidermal growth factor receptor (EGFR) in human airway cells. Noteworthy, we have previously demonstrated that EGFR/Rac1/reactive oxygen species (ROS)/matrix metalloproteinase 9 (MMP-9) is a key signaling cascade regulating MUC5AC production in airway cells challenged with LPS.

Various reports have shown an inverse association between the intake of polyunsaturated fatty acids (PUFA) of the n-3 (omega-3) family or fish consumption and COPD. In the present study, we investigated the influence of docosahexaenoic acid (DHA), one of the most important omega-3 PUFA contained in fish oil, on the production of MUC5AC in LPS-challenged human airway cells NCI−-H292. Our results indicate that DHA is capable of counteracting MUC5AC overproduction in LPS-stimulated cells by abrogating both EGFR phosphorylation and its downstream signaling pathway. This signaling pathway not only includes Rac1, ROS and MMP-9, but also NF-κB, since we have found that ROS require NF-κB activity to induce MMP-9 secretion and activation.



## 1. Introduction

Chronic obstructive pulmonary disease (COPD), a major cause of morbidity and mortality, is characterized by airflow obstruction, chronic bronchitis, and emphysema [1,2]. This chronic inflammatory process exhibits pathologic abnormalities in the submucosal glands and surface epithelium, leading to airway mucus hypersecretion [2]. Mucus, a protective viscoelastic gel, consists of water, salts, lipids and several proteins, including mucin glycoproteins [3]. Amongst these, the highly inducible mucin 5AC (MUC5AC) constitutes the most abundant mucin in the airway secretions of humans [3,4].

Various reports have shown an inverse association between the intake of polyunsaturated fatty acids (PUFA) of the n-3 (omega-3) family or fish consumption and COPD among cigarette smokers [5,6]. Furthermore, studies involving current or former smokers found that the prevalence odds of COPD were inversely related to the content of docosahexaenoic acid (DHA, C22:6) in the phospholipid and cholesterol ester fractions of the plasma [7].

DHA, one of the most important omega-3 PUFA contained in fish oil, exhibits anti-inflammatory properties, including attenuation of the neutrophils' and monocytes' chemotactic responsiveness, and decrease in the secretion of leukotriene $B_4$, free radicals, interleukine-1 (IL-1), IL-6, IL-8, tumor necrosis factor alpha, platelet-activating factor and platelet-derived growth factors [8–10]. Although these effects are attributed to its ability to compete with arachidonic acid in the production of eicosanoids, thereby decreasing the production of pro-inflammatory cytokines and reducing immune cell functions [11], omega-3 PUFA may have other mechanisms to exert its beneficial influence on chronic airway inflammatory diseases. For instance, omega-3 PUFA significantly inhibits lipopolysaccharides (LPS)-stimulated nuclear

*Abbreviations:* COPD, chronic obstructive pulmonary disease; DHA, docosahexaenoic acid; EGFR, epidermal growth factor receptor; LPS, lipopolysaccharides; MMP, matrix metalloproteinase; MUC5AC, mucin 5AC; PUFA, polyunsaturated fatty acids; ROS, reactive oxygen species.

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factor-kappa beta (NF-κB) translocation as well as the expression and activity of matrix metalloproteinase 2 (MMP-2) and MMP-9 in peripheral blood mononuclear cells [10].

LPS are not only the major outer surface membrane molecules present in almost all Gram-negative bacteria, but also are bioactive components found in both the tobacco portion and filter tips of cigarettes as well as in both mainstream and sidestream smoke [12]. Previous studies have shown that LPS induces MUC5AC production via EGFR in airway cells [13–16]. Remarkably, we have demonstrated that this production involves Rac1-dependent secretion and activation of MMP-9 [16]. In the present study, we investigated the influence of DHA on the production of MUC5AC in human airway cells NCI-H292 challenged with LPS.

## 2. Materials and methods

### 2.1. Antibodies and reagents

Mouse anti-MUC5AC, anti-$Na^+/K^+$ ATPase and anti-α-tubulin antibodies, lipopolysaccharides (LPS) from *Pseudomonas aeruginosa*, docosahexaenoic acid (DHA), bovine serum albumin (BSA) and specific inhibitors for EGFR (AG1478), reactive oxygen species-ROS (*N*-Acetyl-L-cysteine, NAC), NF-κB (pyrridoline dithiocarbamate, PDTC) and MMP (GM6001) were from Sigma Chemical Co (St. Louis, MO). Rabbit anti-EGFR and anti-phospho-EGFR (Tyr845), and mouse anti-phospho-tyrosine antibodies were from Cell Signaling (Danvers, MA). Roswell Park Memorial Institute medium (RPMI 1640) and fetal calf serum were from GIBCO/Invitrogen (Carisbad, CA). CM-$H_2$DCFDA was from Molecular Probes (Burlington, Ontario, Canada). Specific inhibitor for Rac1 (NSC23766) was from Tocris (Bristol, UK).

### 2.2. 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 at 37 °C in a 5% $CO_2$ humidified atmosphere. For experiments, NCI-H292 cells were seeded at the density of $5 \times 10^5$ cells per well in six-well plates and were untreated (control) or treated with 50 μM DHA complexed with BSA (ratio 4:1) for 48 h. For the final 24 h of this treatment and throughout all the experiments, cells were incubated in serum free media with the same concentration of DHA. For treatments, cells were stimulated for the indicated time periods with 20 μg/ml LPS in phosphate-buffered saline-PBS (vehicle) and untreated cells served as control.

### 2.3. Cell lysate preparation

NCI-H292 cells were subjected to the indicated treatments, which were terminated by adding excess volume of ice-cold media. Cells were then washed twice with PBS. Subsequently, 200 μl reporter lysis buffer (Promega, Madison, WI), 4 μl protease inhibitor cocktail and 2 μl halt phosphatase inhibitor cocktail were added and the cells were pelleted by centrifugation (300 *g*, 4 °C). The protein concentration of the supernatant was measured with the Micro BCA Protein Assay Kit (Thermo Scientific, Waltham, MA).

### 2.4. Immunoblotting

Protein content of all samples was determined with the Micro BCA Protein Assay Kit. Samples of cell lysates supernatants were dissolved in Laemmli buffer and boiled for 5 min. Equal amounts of protein from cell lysates supernatants were loaded, separated by 8% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Waltham, MA). Blots were blocked for 1 h in Tris-buffered saline/0.1% Tween-20 containing 5% BSA and then incubated overnight with the corresponding primary antibody (1:1000 dilution) in Tris-buffered saline/0.1% Tween-20 containing 0.5% BSA at 4 °C. After three washes with Tris-buffered saline/0.1% Tween-20, the respective antigen–antibody complexes were identified by appropriate peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Amersham, Arlington Heights, IL). Quantification was performed by densitometry using Scion Image software (National Institutes of Health).

### 2.5. Immunoprecipitation

Lysates containing equal amounts of protein were clarified by centrifuging at 12,000 *g* for 10 min. Supernatants were precleared for 40 min using Protein G-Sepharose beads (Pharmacia Biotech, Buckinghamshire, UK) and then incubated with the corresponding first antibody for 1 h. Immune complexes were captured using Protein G-Sepharose beads, which were washed four times with lysis buffer containing 1 mM $Na_3VO_4$. Protein content of all samples was determined by Micro BCA Protein Assay Kit, and equal amounts of these samples were dissolved in Laemmli buffer, boiled for 5 min, separated on SDS-PAGE, and transferred to nitrocellulose membranes. Separated proteins and the respective antigen–antibody complexes were identified by appropriate peroxidase-conjugated secondary antibodies and enhanced chemiluminescence. Quantification was performed by densitometry using Scion Image software.

### 2.6. Rac1 activity

Active Rac1 was determined by a pull-down assay as previously described [17]. Briefly, serum-starved NCI-H292 cells were or were not stimulated with LPS and then collected in 800 μl of ice-cold lysis-buffer. Lysates were centrifuged to remove cellular debris. From each supernatant, 10 μl were removed to measure protein content using Protein Assay Kit (BioRad, Hercules, CA), 20 μl were removed to determine total Rac-1 in total lysate, and the rest of the volume was used for the pull-down assay. Lysates containing equal amount of proteins were then mixed with 15 μg of GST-PAK-PBD-beads. Samples for total Rac1 in total lysate and the pelleted beads were diluted in Laemmli sample buffer and boiled. The proteins were separated using SDS-PAGE (12% gel). After transfer to nitrocellulose membranes (BioRad), blots were blocked with bovine serum albumin, followed by incubation with Rac1 antibody. Binding of the antibody was visualized using peroxidase-coupled anti-mouse antibody, and enhanced chemiluminescence method (Amersham, Arlington Heights, IL). Equal loading was verified by reprobing the membranes corresponding to total lysate with anti-tubulin antibody (not shown). Quantification was performed by densitometry using Scion Image software (National Institutes of Health).

### 2.7. ROS production

Free radical production was determined by incubating NCI-H292 cells in the presence of 10 μM CM-$H_2$DCFDA. 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.

### 2.8. NF-κB activity assay

For determination of the p65 and p50 subunits of NF-κB in nuclear extracts from NCI-H292 cells, the NF-κB (p65) and NF-κB

(p50) Transcription Factor Assay Kits (Cayman Chemical Company; Ann Arbor, MI) were used according to the manufacturer's instructions.

### 2.9. MMP-9 activity

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 proenzyme) is measured by the addition of α-aminophenylmercuric acetate, which artificially activates the inactive form of the metalloproteinase.

### 2.10. MUC5AC production

MUC5AC was measured in cell culture supernatants and in cell lysates following LPS challenge, by ELISA as previously described [18]. The amount of MUC5AC protein in each sample was normalized to total protein. The total production of MUC5AC protein was the sum of MUC5AC protein in cell culture supernatant and in cell lysate.

### 2.11. 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 $p < 0.05$.

## 3. Results

### 3.1. DHA decreases LPS-stimulated EGFR transactivation

Tyrosine phosphorylation of EGFR by *P. aeruginosa* culture supernatants and LPS was previously reported [13,15]. Here, to evaluate the effects of DHA on EGFR phosphorylation in NCI-H292 cells stimulated with LPS, lysates were probed for EGFR phosphorylated on Tyr845, one of the sites of EGFR phosphorylation that is involved in activation of downstream signaling. DHA treatment resulted in 2.4 fold decrease in EGFR phosphorylation compared to LPS-stimulated cells in the absence of DHA (Fig. 1A and B).

EGFR was also immunoprecipitated from cell lysates followed by immunoblotting for total phosphorylated tyrosine residues. Consistent with the initial results, DHA decreased total EGFR tyrosine phosphorylation by 3 fold (Fig. 1C and D).

### 3.2. LPS-induced Rac1 activation and downstream ROS production via EGFR are abrogated by DHA

EGFR phosphorylation is characteristically associated with activation of downstream signaling, such as Rac1/ROS in cells stimulated with LPS [16]. Therefore, we first examined the influence of DHA on Rac1 activity in NCI-H292 cells stimulated with LPS. As it can be observed in Fig. 2A and B, DHA treatment resulted in 5.5 fold decrease in Rac1 activation compared to LPS-stimulated cells in the absence of DHA.

We next assessed the production of ROS. Treatment of LPS-challenged NCI-H292 cells with DHA decreased ROS production by 1.8 fold compared to LPS-challenged cells in the absence of DHA (Fig. 2C).

### 3.3. DHA diminishes LPS-induced NF-κB activation

The transcription factor nuclear factor-kappa beta (NF-κB) is one of the main signal-transduction molecules activated in response to oxidative stress [19]. Thus, we investigated the activation of NF-κB in NCI-H292 cells stimulated with LPS, as well as the influence of DHA treatment. Results presented in Fig. 3A and B revealed that both basal NF-κB (p65) and NF-κB (p50) activities in control cells were increased by stimulation with LPS (2.4 ± 0.4 and 2.0 ± 0.3 fold increase, respectively). As expected, LPS-stimulated NCI-H292 cells treated with the ROS inhibitor NAC exhibited NF-κB (p65) and NF-κB (p50) activities similar to that of unstimulated vehicle-control cells (1.1 ± 0.1 and 1.1 ± 0.1 fold increase, respectively; Fig. 3A and B). Remarkably, treatment with DHA alone or DHA in combination with NAC decreased both NF-κB (p65) and NF-κB (p50) activities in LPS-stimulated cells by 1.8 and 1.6 fold for DHA alone, and 2.3 and 1.9 fold for DHA + NAC, respectively, compared to LPS-stimulated cells in the absence of DHA (Fig. 3A and B).

### 3.4. DHA reduces LPS-induced MMP-9 secretion and activation

LPS-induced MMP-9 secretion and activation in NCI-H292 cells was previously demonstrated [15,16]. Here, we analyzed the effects NF-κB activation, as well as the influence of DHA treatment on the secretion and activation of MMP-9 in NCI-H292 cells stimulated with LPS. LPS-stimulated cells treated with PDTC presented a 3.8 and 5.9 fold decrease in both secretion and activation of MMP-9 (Fig. 3C and D), indicating that NF-κB activity is critical for LPS to induce MMP-9 secretion and activation. Furthermore, DHA alone or DHA in combination with PTDC reduced both secretion and activation of MMP-9 in LPS-stimulated cells by 2.8 and 4.3 fold for DHA alone, and 4.3 and 7.1 fold for DHA+PDTC, respectively, compared to LPS-stimulated cells in the absence of DHA (Fig. 3C and D).

### 3.5. LPS-stimulated MUC5AC production is downregulated by DHA

Lastly, we used specific inhibitors to examine the effects of the inhibition of EGFR, Rac1, ROS, NF-κB or MMP-9, alone or in combination with DHA treatment, on the production of MUC5AC in LPS-stimulated NCI-H292 cells. As expected, all these inhibitors, AG1478, NSC23766, NAC, PTDC and GM6001, diminished MUC5AC levels (fold increases of 1.6 ± 0.3, 1.5 ± 0.3, 1.9 ± 0.4, 1.7 ± 0.2 and 1.8 ± 0.3, respectively; Fig. 4). Additionally, DHA alone or in combination with each of the inhibitors for EGFR, Rac1, ROS, NF-κB or MMP-9, also decreased MUC5AC levels in LPS-stimulated cells (fold increases of 1.8 ± 0.4, 1.4 ± 0.3, 1.3 ± 0.3, 1.6 ± 0.4, 1.4 ± 0.3 and 1.5 ± 0.2, respectively; Fig. 4).

## 4. Discussion

We have previously reported that EGFR/Rac1/ROS/MMP-9 regulate MUC5AC production in LPS-challenged NCI-H292 cells [16]. In the present study, we showed that DHA is capable of counteracting MUC5AC overproduction in LPS-stimulated cells by abrogating both EGFR phosphorylation and its downstream signaling pathway. This signaling pathway not only includes Rac1, ROS and MMP-9, but also NF-κB, since we have found that ROS require NF-κB activity to induce MMP-9 secretion and activation.

EGFR is a transmembrane protein that is activated by EGFR ligands such as EGF, but which can also be transactivated by stimuli

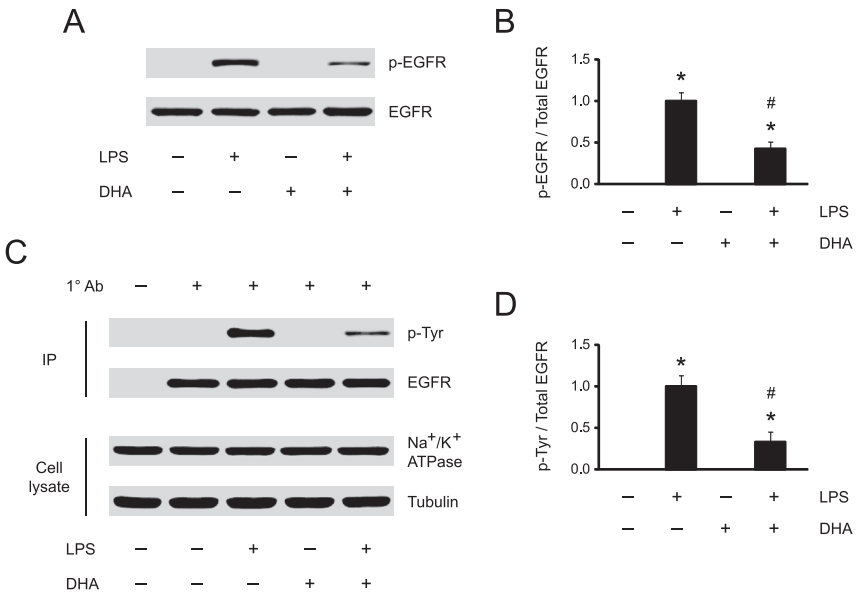


**Fig. 1.** Docosahexaenoic acid (DHA) decreases LPS-stimulated EGFR transactivation. NCI-H292 cells were untreated (control) or treated with 50 μM DHA for 48 h. For the final 24 h of this treatment and throughout the experiment, cells were incubated in serum free media with the same concentration of DHA. Cells were either unstimulated (control) or stimulated with LPS for 15 min and subsequently harvested. (A) Equal amounts of protein from whole cell lysates were Western blotted for total and phosphorylated (Tyr845) EGFR. (B) Densitometric analysis of EGFR activity from immunoblots as in (A). (C) EGFR was immunoprecipitated from total cellular lysates prior to Western blotting for EGFR and phosphorylated tyrosine residues. $Na^+/K^+$ ATPase and tubulin are loading controls for membrane and cytosolic proteins. (D) Densitometric analysis of EGFR activity from immunoblots as in (C). Blots are representative of 3 independent experiments, each of which was done in triplicate. Data are expressed as the ratio of the phosphorylated protein to total protein and normalized to control. $^*p < 0.01$ compared to unstimulated vehicle-control cells, $^\#p < 0.05$ compared to LPS-stimulated cells in the absence of DHA.

like *Helicobacter pylori* protein HP0175 [20]. Interestingly, DHA has been shown to suppress EGFR transactivation as well as activation of downstream signaling cascade including ERK/JNK and NF-κB/AP-1, in *H. pylori*-infected gastric epithelial cells [21]. In colonic cells, DHA reduces wound-induced EGFR transactivation. Moreover, under wounding conditions, the suppression of EGFR activation is associated with a reduction in downstream activation of cytoskeletal remodeling proteins such as Rac1, as well as a reduced cell migration [22]. These observations are consistent with our findings in human airway cells and suggest that inhibition of both EGFR transactivation and downstream signaling by DHA could be independent of the cell type. The EGFR is a strong candidate for both initiating and determining clinical outcomes in many respiratory diseases. Deregulation of the EGFR pathway causing aberrant EGFR signaling is associated with the early stage in the pathogenesis of lung fibrosis, cancer and numerous airway hypersecretory diseases, including asthma, cystic fibrosis and COPD [23].

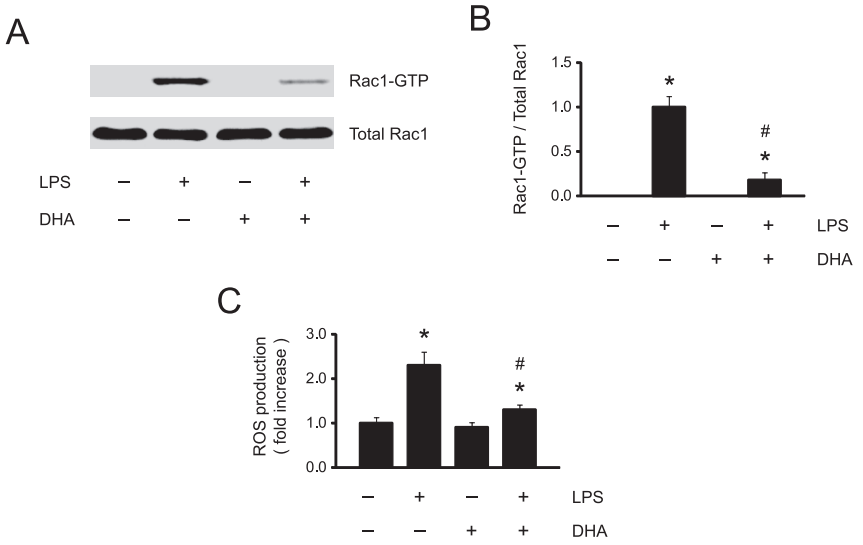


**Fig. 2.** LPS-induced Rac1 activation and downstream ROS production via EGFR are abrogated by DHA. NCI-H292 cells were untreated (control) or treated with 50 μM DHA for 48 h. For the final 24 h of this treatment and throughout the experiment, cells were incubated in serum free media with the same concentration of DHA. (A) Cells were either unstimulated (control) or stimulated with LPS for 15 min and subsequently Rac1 activation levels were assessed in cell lysates. Blots are representative of 3 independent experiments, each of which was done in triplicate. (B) Densitometric analysis of Rac1 activity from immunoblots as in (A). Data are expressed as the ratio of Rac1-GTP to Total Rac1 and normalized to control. (C) Cells were either unstimulated (control) or stimulated with LPS for 30 min and subsequently reactive oxygen species (ROS) production was assessed. ROS levels were expressed as fold increase over unstimulated vehicle-control. $^*p < 0.05$ compared to unstimulated vehicle-control cells, $^\#p < 0.05$ compared to LPS-stimulated cells in the absence of DHA.

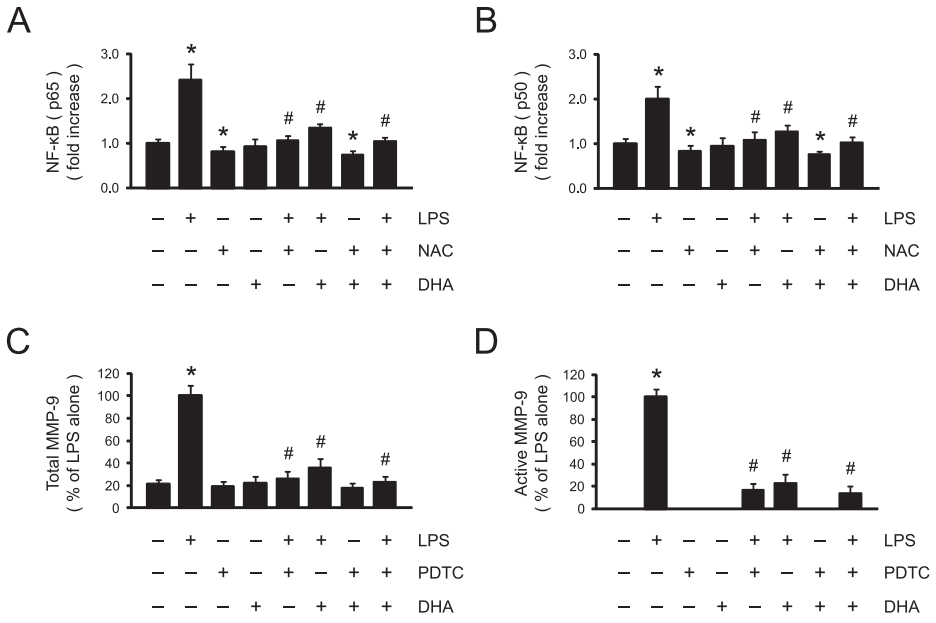


**Fig. 3.** DHA diminishes LPS-induced NF-κB activity and MMP-9 secretion and activation. NCI-H292 cells were untreated (control) or treated with 50 μM DHA for 48 h. For the final 24 h of this treatment and throughout the experiment, cells were incubated in serum free media with the same concentration of DHA. (A–B) Cells were either unstimulated (control) or stimulated with LPS for 1 h. To determine the effect of ROS inhibition, 30 mM NAC was added to the cells 1 h before LPS, and it was maintained in the media throughout the experiment. NF-κB (p65) (A) and (p50) (B) activation levels were assessed in nuclear extracts and expressed as fold increase over unstimulated vehicle-control. (C–D) Cells were either unstimulated (control) or stimulated with LPS for 12 h. To determine the effect of NF-κB inhibition, 50 μM PTDC was added to the cells 1 h before LPS, and it was maintained in the media throughout the experiment. Total (C) and active (D) levels of MMP-9 were assessed in cell supernatants and expressed as a percentage of levels in cell supernatants of LPS-stimulated cells in the absence of DHA. $^{*}p < 0.05$ compared to unstimulated vehicle-control cells, $^{\#}p < 0.05$ compared to LPS-stimulated cells in the absence of DHA.

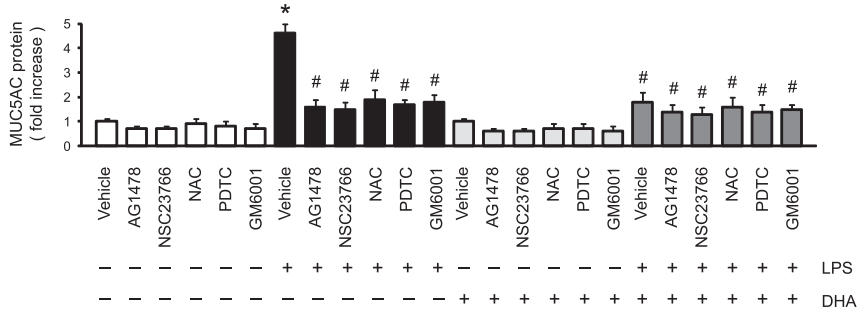


**Fig. 4.** LPS-stimulated MUC5AC production is downregulated by DHA. NCI-H292 cells were untreated (control) or treated with 50 μM DHA for 48 h. For the final 24 h of this treatment and throughout the experiment, cells were incubated in serum free media with the same concentration of DHA. Cells were either unstimulated (control) or stimulated with LPS for 12 h. To determine the effect of specific inhibitors for EGFR (10 μM AG1478), Rac1 (50 μM NSC23766), ROS (30 mM NAC), NF-κB (50 μM PTDC) and MMP (20 μM GM6001) on the production of MUC5AC, they were added to the cells 1 h before LPS, and they were maintained in the media throughout the experiment. MUC5AC levels were measured in cell culture supernatants and in cell lysates. The total production of MUC5AC protein was the sum of MUC5AC protein in cell supernatant and in cell lysate for each condition, and was expressed as fold increase over the vehicle-control. $^{*}p < 0.05$ compared to unstimulated vehicle-control cells, $^{\#}p < 0.05$ compared to LPS-stimulated cells in the absence of DHA and inhibitors.

The lung has a permanent contact with a varied array of infectious agents, foreign antigens and host-derived danger signals. To protect itself from these, both lung resident myeloid and stromal/structure cells express a full complement of toll like receptors (TLR) which recognize exogenous- and endogenous danger-associated molecular patterns. TLR play a vital role in immune host defense against bacterial, mycobacterial, fungal, and viral pathogens, while also contributing to disease pathogenesis in non-infectious pulmonary disorders, including acute lung injury, interstitial lung disease and airway disease [24]. In human airway cells, *Pseudomas* LPS transactivate EGFR via TLR4 [25]. Activation of TLR4 requires dimerization and recruitment of TLR4 into lipid rafts [26]. LPS enhance the association of TLR4 with downstream adaptor molecules into lipid rafts, leading to the activation of downstream signaling pathways and target gene expression [26]. Importantly, DHA was shown to inhibit LPS-induced dimerization and recruitment of TLR4 into lipid raft fractions [26]. Accordingly, DHA was recently shown to reduce LPS-induced MUC5AC overproduction in two *in vitro* models related to cystic fibrosis; however, the underlying mechanism was not addressed [27]. Here, we have presented evidence indicating that DHA inhibits mucin MUC5AC overproduction in airway cells challenged with LPS by altering the EGFR/Rac1/ROS/NF-κB/MMP-9 signaling.

In summary, our findings support the use of omega-3 PUFA, in particular DHA, as a potential pharmacological therapy for airway hypersecretory diseases, particularly COPD.

## Conflict of interest

No conflicts of interest to declare.

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## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.056.

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