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Dual anti-oxidant and anti-inflammatory actions of the electrophilic cyclooxygenase-2-derived 17-oxo-DHA in lipopolysaccharide- and cigarette smoke-induced inflammation

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ABSTRACT

Background: 17-Oxo-DHA is an endogenous electrophilic derivative of the omega-3 fatty acid docosahexaenoic acid (DHA) which is generated in activated macrophages by the action of cyclooxygenase-2.
Methods: The ability of 17-oxo-DHA to control inflammation and oxidative stress was tested in human macrophages (THP-1) and bronchial epithelial cell line (16HBE) stimulated with cigarette smoke extract (CSE) and lipopolysaccharide (LPS). All data were further confirmed using primary bronchial epithelial cells, alveolar macrophages and peripheral blood mononuclear cells.
Results: 17-Oxo-DHA was a strong inducer of the anti-oxidant response promoting Nrf2 nuclear accumulation, leading to the expression of heme oxygenase 1 and more than doubling glutathione levels. This resulted in suppression of CSE-induced ROS generation in macrophages. In macrophages, 17-oxo-DHA potently suppressed TNFα release in response to LPS, CSE and IL-1β acting at transcriptional level via a mechanism independent of Nrf2. Externally supplemented 17-oxo-DHA displayed the same effects in the presence of the Cox-inhibitor indomethacin. The non-electrophilic 17-oxo-DHA precursor DHA did not show any biological actions, indicating that the electrophilic moiety was required for this compound to become bioactive.
Conclusions: 17-Oxo-DHA promotes cytoprotective actions both in immune and structural cells. In immune cells, 17-oxo-DHA is effective in contrasting CSE- and LPS-induced oxidative damage and inflammation acting via multiple independent pathways.
General significance: Herein we provide insights on how the novel endogenous electrophilic DHA-derivative 17-oxo-DHA promotes anti-oxidant and anti-inflammatory actions. Data herein reported indicate that 17-oxo-DHA is an attractive lead compound for the development of new treatments for cigarette smoke-related airway inflammatory disorders.

1. Introduction

Dietary omega-3 polyunsaturated fatty acids (n−3 PUFAs) provide well established cardiovascular benefits and are beneficial in a variety of inflammatory disorders, including asthma [1–5]. Several reports have shown that docosahexaenoic acid (C22:6, DHA), docosapentaenoic acid (C22:5, DPA) and eicosapentaenoic acid (C20:5, EPA) are converted by lipoxygenases (LOs) and cyclooxygenase-2 (Cox-2) into hydroxy and ketone derivatives transducing the cytoprotective and anti-inflammatory properties typical of n−3 PUFAs [6–10]. Among the oxygenated derivatives, electrophilic species are of great interest because they covalently and reversibly react via Michael reaction with nucleophilic residues of target proteins, modulating their activity [11,12]. In particular, they activate the Nrf2-dependent anti-oxidant response by adducting the Nrf2 inhibitor Keap1, resulting in Nrf2 release and nuclear accumulation, which leads to the induction of phase II genes [12,13]. Furthermore, electrophilic n−3 PUFAs are partial agonists of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR-γ) and repress the NF-κB pathway at multiple levels, thus promoting the resolution of inflammation [6,9,14,15].

17-Oxo-DHA is a recently discovered endogenous electrophilic α,β-unsaturated ketone derivative of DHA which is generated by activated

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macrophages via two-step enzymatic synthesis involving the enzyme Cox-2 followed by a cellular dehydrogenase. Interestingly, aspirin enhanced the formation of 17-oxo-DHA by modulating the activity of Cox-2 [6]. The endogenous nature of 17-oxo-DHA together with its electrophilic reactivity conferring a wide range of beneficial anti-oxidant and anti-inflammatory properties make this compound attractive for the development of new therapeutic options for chronic disorders presenting persistent inflammation and oxidative damage such as chronic obstructive pulmonary disease (COPD) [16,17]. Cigarette smoking represents a major risk factor for COPD, causing oxidative stress and cell damage and directly activating the inflammatory cascade via the Toll-like receptor 4 (TLR4) [17–19]. The greater susceptibility to oxidative stress in COPD patients compared to healthy smokers has been related to defects in the Nrf2 pathways, in particular to reduced levels of Nrf2 and of its positive regulator DJ-1, decreased levels of glutathione and down-regulation of Nrf2 target genes [20–22]. In the upper airways resident macrophages and bronchial epithelial cells are the first responders to external insults, and in the lung of COPD patients they promote and sustain inflammatory reactions [23,24]. In COPD patients, inflammation becomes self-sustained and is further worsened by bacterial infections, which cause exacerbations and increase disease severity. A typical feature of severe COPD is the resistance to steroid therapy, which is related to oxidation-dependent inactivation of histone deacetylase 2 (HDAC2). As a consequence, no current therapy is able to stop inflammation or revert disease progression and therefore the search for new drugs is highly active [25,26].

Herein, the efficacy of the electrophilic 17-oxo-DHA in contrasting the TLR4 agonist lipopolysaccharide- (LPS) and cigarette smoke-induced oxidative stress and inflammation was evaluated in human macrophages and bronchial epithelial cell lines. Primary bronchial epithelial cells, alveolar macrophages (AM) and human peripheral blood mononuclear cells (PBMCs) were used to further validate the results. Reported findings show that 17-oxo-DHA promotes anti-oxidant and anti-inflammatory actions in immune and structural cells directly contrasting inflammatory reactions acting via multiple Nrf2-dependent and -independent mechanisms. Furthermore, the comparison with the non-electrophilic precursor DHA confirmed that oxygenation of the molecule is necessary for DHA to exert the observed biological actions.

2. Materials and methods

2.1. Reagents

17-Oxo-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid (17-oxo-DHA, ≥98%), docosahexaenoic acid (DHA, >99%) and indomethacin were purchased from Cayman Chemical (Ann Arbor, MI-USA). The following chemicals were purchased from Sigma-Aldrich (St Louis, MO-USA): phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharides from *Escherichia coli* 0111:B4 (LPS). IL-1β was purchased from R&D System (Minneapolis, MN-USA).

2.2. Cell lines and primary bronchial epithelial cells

16HBE, an immortalized normal bronchial epithelial cell line [27] and the human monocytic cell line THP-1 (ATCC TIB-202, kindly provided by Dott. Angelo Sala) were used in this study. 16HBE cells were maintained in MEM medium (Gibco, BRL, Germany), supplemented with 10% FBS (Gibco) and 0.5% gentamicin (Gibco). THP-1 cells were grown in complete RPMI medium supplemented with 10% FBS and differentiated into macrophages by treating with 80 nM PMA for 48 h. Primary bronchial/tracheal epithelial cells (normal, human) were purchased from ATCC (catalog n. PCS-300-010) and grown in complete airway epithelial cell basal medium according to ATCC Primary Cell Solutions™ system. Cell cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C.

2.3. Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from peripheral blood of healthy volunteers using Lympholyte (Cedarlane, CL5015) following the manufacturer's instruction, resuspended in complete RPMI medium supplemented with 10% FBS and stimulated for 18 h with the indicated treatment. All subjects had given their written informed consent.

2.4. Patient population and isolation of alveolar macrophages from mini-bronchoalveolar lavage (mini-BAL)

Non-smoking subjects (n = 3) with acute respiratory failure upon surgery for abdominal or thoracic aneurysm but without previous airway chronic inflammatory diseases were recruited. Patients with X-ray or clinical evidence of sepsis or pneumonia at the time of mini-BAL collection were not included. The study fulfilled the criteria of the Ethics Committee of Policlinico-Giaccone Hospital—Palermo Italy (7/2012) and was in agreement with Helsinki Declaration. Informed written consent from either the patients or closest relatives was obtained. Distal lung fluid samples (mini-BAL) were obtained using BAL Cath system (by Kimberly-Clark Health Care, Kent, ME19 4HA, United Kingdom) within 1 h from the intubation as previously described [28]. The protected catheter was blindly advanced through the endotracheal tube until it was wedged into a distal airway and two aliquots of 10 ml of sterile 0.9% NaCl were instilled and gently suctioned (recovered volume about 70% of the instilled volume). Mini-BAL samples were filtered through a sterile gauze and then centrifuged at 1300 rpm for 10 min to separate cells from supernatants. Differential count was performed after staining with Hemacolor® staining kit (Merck Millipore) showing that macrophages represented >90% of the cell population (Fig. 3A).

2.5. Preparation of cigarette smoke extract (CSE)

Cigarette smoke solution was prepared using commercial cigarettes (Marlboro) as previously described [18]. Briefly, each cigarette was smoked for 5 min in 25 ml of PBS and the obtained smoke solution was filter-sterilized through a 0.22 μm-pore filter. The concentration of this solution was checked spectrophotometrically measuring the OD at 320 nm and adjusted to give OD of 1.0. This solution was considered to be 100% CSE and was diluted to obtain the indicated concentration.

2.6. Analysis of intracellular reactive oxygen species (ROS)

Intracellular ROS were measured by flow cytometry using the ROS-sensitive probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Life Technologies, C-2938) [18]. Cells were incubated with the indicated stimuli, harvested after 18 h, washed with PBS, stained with 1 μM H2DCFDA (10 min, RT in the dark), washed and analyzed by flow cytometry.

2.7. Measurement of cellular glutathione (GSH) content

Total intracellular GSH content was assessed in cell extracts as previously reported [29,30]. Briefly, cell extracts were prepared in 0.1 M potassium phosphate extraction buffer containing 0.6% (w/v) sulfosalicylic acid, 0.1% (v/v) Triton X-100, and 5 mM EDTA. After harvesting and resuspension in extraction buffer, cells were sonicated in ice-cold water and underwent 2 cycles of freezing and thawing. Supernatants/extracts were collected by centrifugation and used for the following colorimetric assay: 10 μl of extract was incubated in the presence of 60 μl of 0.6 mg/ml 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and of 60 μl of 250 U/ml glutathione reductase for 30 s at RT; 50 μl of 0.6 mg/ml β-NADPH was added and 2-nitro-5-thiobenzoic acid formation was immediately evaluated by measuring the absorbance at 412 nm in a microplate reader. The concentration of GSH in cell extracts was calculated using a standard curve and normalized for the total protein content.

2.8. Western blot

For western blot analysis the following antibodies were used: heme oxygenase 1 (Enzo Life Sciences, SPA-896, Farmingdale, NY-USA), Nrf2 (sc-722, Santa Cruz Biotechnology, Dallas, TX-USA), Lamin B1 (#9087, Cell Signaling Technology, Danvers, MA-USA), and actin (Sigma Aldrich, A2066). All secondary antibodies were purchased from Santa Cruz Biotechnology. For preparation of nuclear extracts the commercial kit NE-PER Nuclear and Cytoplasmic Extraction Kit from Thermo Scientific (Waltham, MA-USA) was used following the manufacturer's instruction. Protein concentration in cell lysate was measured by Bradford assay.

2.9. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from PMA-differentiated THP-1 cells with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and was reverse-transcribed into cDNA, using M-MLV-RT and oligo(dT)12-18 primer (Invitrogen). Quantitative real-time PCR of TNFα transcripts was carried out on Step One Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using specific FAM-labeled probe and primers (prevalidated TaqMan Gene expression assay for TNFα, Hs99999043m1, Assays on Demand, Applied Biosystems). TNFα gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous control gene. Relative quantitation of gene expression was carried out with the comparative CT method ($2-\Delta\Delta Ct$) [3] and was plotted as relative fold-change compared to untreated cells that were chosen as the reference sample.

2.10. Nrf2 siRNA transfection

Macrophage-differentiated THP-1 cells were transfected with Nrf2 targeting siRNA or non-targeting control (Dharmacon RNA Technologies, OnTarget Plus SmartPool, L-003755 and D0018101020, Waltham, MA-USA). Cells were seeded in 12-well plates (0.75×10^6 cells/well) and incubated overnight at 37 °C and 5% CO_2 in complete growth medium without antibiotics. The transfection was performed as follows: solution A (250 nM siRNA in complete RPMI w/o FBS) and solution B (DharmaFECT 1, 1:50 dilution) were prepared. After 5 min incubation at RT, solution A was mixed with B, and incubated for 20 min at room temperature. The mix was then diluted 5 times with complete medium (RPMI with 10% FBS) and this was added to the cells. After 24 h, the transfection medium was removed and complete fresh medium was added for additional 24 h and then stimulation was performed as indicated.

2.11. Measurement of TNFα

The concentration of TNFα in cell supernatants was determined using the commercially available EIA Kit from Cayman Chemical (#589201) following the manufacturer's instructions. TNFα levels are presented as ng per mg of total cell protein.

2.12. Total and surface expression of TLR4

For total TLR4, cells were permeabilized using a commercial fix–perm cell permeabilization kit (Caltag Laboratories, Burlingame, CA), incubated in the dark for 30 min at 4 °C with specific mouse anti-human TLR4-PE antibody (eBioscence, San Diego, CA) and then analyzed by flow cytometry. For surface TLR4 expression, non-permeabilized cells were stained with specific anti-TLR4 antibody. Negative controls were performed using mouse immunoglobulins (Dako). Data are expressed as geomean fluorescence intensity.

2.13. Statistics

All experiments were done in triplicate with parallel samples being prepared in some cases. For each figure specific information is reported regarding the number of independent experiments and the number of samples (n) that were collected and used for statistics. Data are expressed as mean ± standard deviation. Kolmogorov–Smirnov Normality test was initially performed to assess whether parametric analyses of data could be performed. Comparison between different experimental conditions was done by ANOVA corrected with the Bonferroni test. Where indicated, comparison between conditions was evaluated by paired t test. $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. 17-Oxo-DHA induces the anti-oxidant response in human bronchial epithelial cells and macrophages

Progression of COPD is associated with impaired Nrf2 pathway and high oxidative stress [16]. Aiming at assessing the potential of 17-oxo-DHA in inducing the Nrf2-dependent antioxidant response in THP-1 macrophages and 16HBE bronchial epithelial cells, the expression of the Nrf2 target HO-1, as well as total glutathione levels (GSH) and nuclear Nrf2 were measured in cells stimulated with 17-oxo-DHA and cigarette smoke extract (CSE), alone or in combination (Fig. 1). The concentration of 20 μM 17-oxo-DHA was chosen based on dose–response experiments as the one giving the strongest biological actions without causing any toxicity (Supplementary Figs. S1–S4). Results were compared with those obtained stimulating the cells with the non-electrophilic 17-oxo-DHA precursor, DHA. Both cell types gave a similar response. In particular, while stimulation with 17-oxo-DHA resulted in a significant increase of total GSH and induction of HO-1 expression, stimulation with DHA alone did not induce glutathione synthesis and did not cause any increase in HO-1 expression. When 17-oxo-DHA was added in combination with CSE, the induction of HO-1 and total glutathione levels were about two-to-twenty times higher compared to the two stimuli given alone. Interestingly, when given in combination with CSE, DHA increased the levels of GSH (mainly for 16HBE cells) and induced HO-1 expression displaying the same additive effects observed in cells stimulated with 17-oxo-DHA and CSE. When looking at the nuclear accumulation of Nrf2, all the stimuli, alone or in combination (with the exception of DHA for THP-1 cells), caused Nrf2 nuclear accumulation at a similar extent (Fig. 1C, F). The induction of the anti-oxidant response by 17-oxo-DHA observed in 16HBE and THP-1 cells was confirmed in human primary bronchial epithelial cells and alveolar macrophages (Figs. 2 and 3). In particular, in primary bronchial epithelial cells 17-oxo-DHA alone caused a significant increase of HO-1 expression and more than doubled glutathione levels displaying a much stronger effect when compared to CSE alone (Fig. 2A, B). The two stimuli given in combination displayed additive effect only when looking at HO-1 expression. All the stimuli, given alone or in combination, caused nuclear accumulation of Nrf2 at a similar extent, with a small additive effect when the two stimuli were given in combination (Fig. 2C). In alveolar macrophages, 18 h stimulation with 17-oxo-DHA led to an increase of total glutathione, similar to what was observed in THP-1 macrophages (Fig. 3A, B). No additive effect was observed on GSH levels when 17-oxo-DHA was given in combination with CSE.

3.2. Induction of the anti-oxidant response by externally supplemented 17-oxo-DHA is not affected by the Cox-inhibitor indomethacin

Formation of endogenous 17-oxo-DHA was previously reported in activated macrophages by the action of Cox-2 [6]. To assess whether the endogenous amount of 17-oxo-DHA contributed to the actions

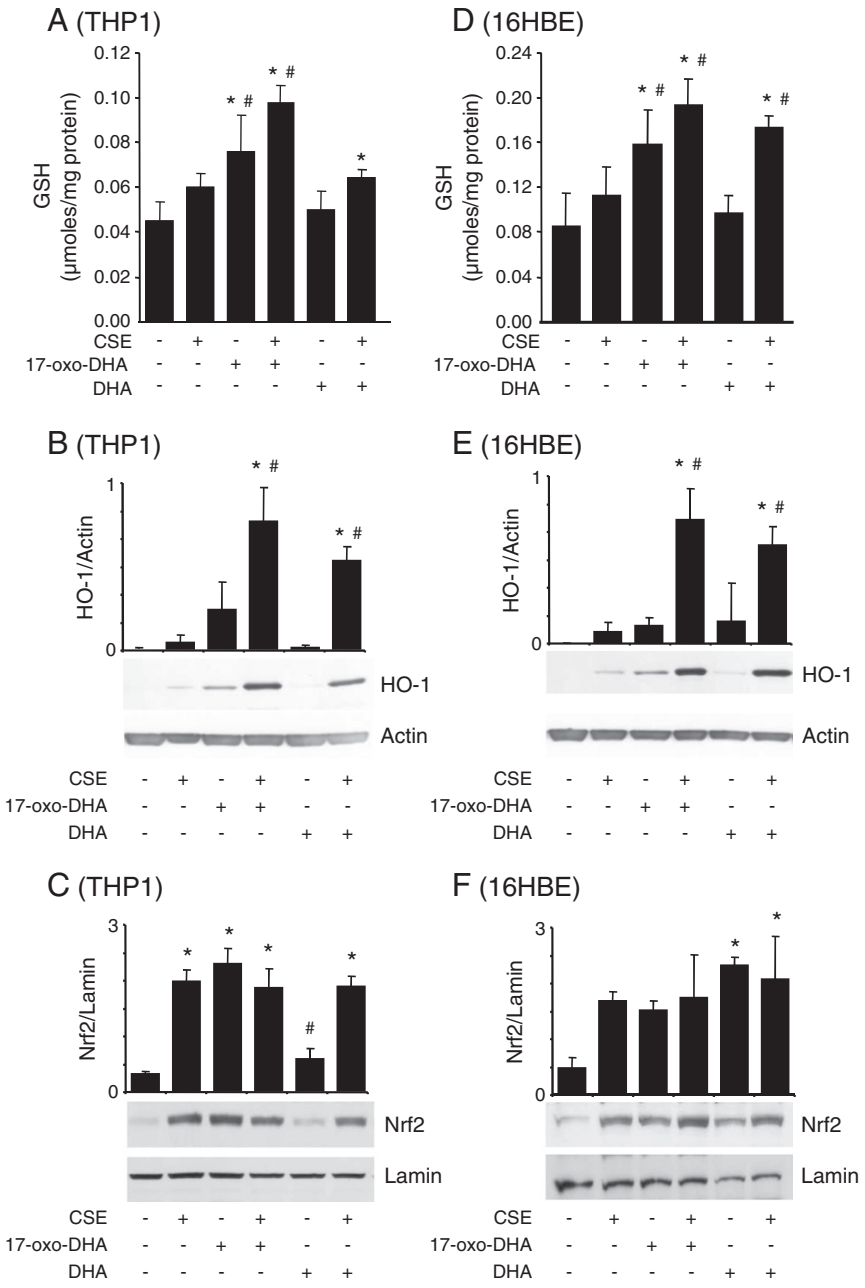

Fig. 1. 17-Oxo-DHA induces the anti-oxidant response in 16HBE and THP-1 cells. Total glutathione levels (GSH), expression of HO-1 and nuclear Nrf2 accumulation were measured in THP-1 macrophages (A, B, C) and in 16HBE bronchial epithelial cells (D, E, F) stimulated with 20 μM 17-oxo-DHA or DHA and 10% CSE, alone or in combination. THP-1 monocytes were differentiated to macrophages by treating with 80 nM PMA for 24 h. Cells were treated with the indicated stimulus for 18 h. Total GSH (A, D) and HO-1 (B, E) were measured in cell extracts by, respectively, the enzymatic recycling method as previously described [29] and western blot. Nuclear Nrf2 (C, F) was measured by western blot in nuclear extracts from cells treated for 2 h (THP-1) or 4 h (16HBE) with the indicated stimulus. Densitometric analysis is reported for western blot images where HO-1 was normalized to actin and Nrf2 to Lamin B1. For glutathione determination, experiments were performed in quadruplicate ($n = 8$). For Nrf2 and HO-1 expression experiments were performed in triplicate ($n = 3$). Densitometric analysis and representative western blot images are reported. Data are expressed as mean ± SD. The comparison between different experimental conditions was evaluated by ANOVA corrected with the Bonferroni test. *, statistically different from non-treated; #, statistically different from CSE.

that we observed when externally supplementing the cells with this electrophilic compound, induction of the anti-oxidant response by 17-oxo-DHA was evaluated also in the presence of 25 μM indomethacin, a Cox-1/Cox-2 inhibitor. It was previously shown that at this concentration indomethacin completely abolished the formation of electrophilic oxo-derivatives of DHA in activated macrophages [6]. Herein we show that both 16HBE and THP-1 cells displayed identical behavior when stimulated with 17-oxo-DHA in the presence of indomethacin. In particular, externally supplemented 17-oxo-DHA led to Nrf2 nuclear accumulation and induction of HO-1 to a level similar to what was observed in the absence of indomethacin (Fig. 4A, B). When 17-oxo-DHA was given in combination with CSE a strong additive effect was observed for HO-1 expression, but not for Nrf2 nuclear accumulation, consistent with what was observed in the absence of indomethacin. Induction of GSH levels by 17-oxo-oxo-DHA was much stronger (about three-fold change) compared to what was observed in the absence of the Cox-inhibitor and no additive effect was observed when 17-oxo-DHA and CSE were given in combination (Fig. 4C).

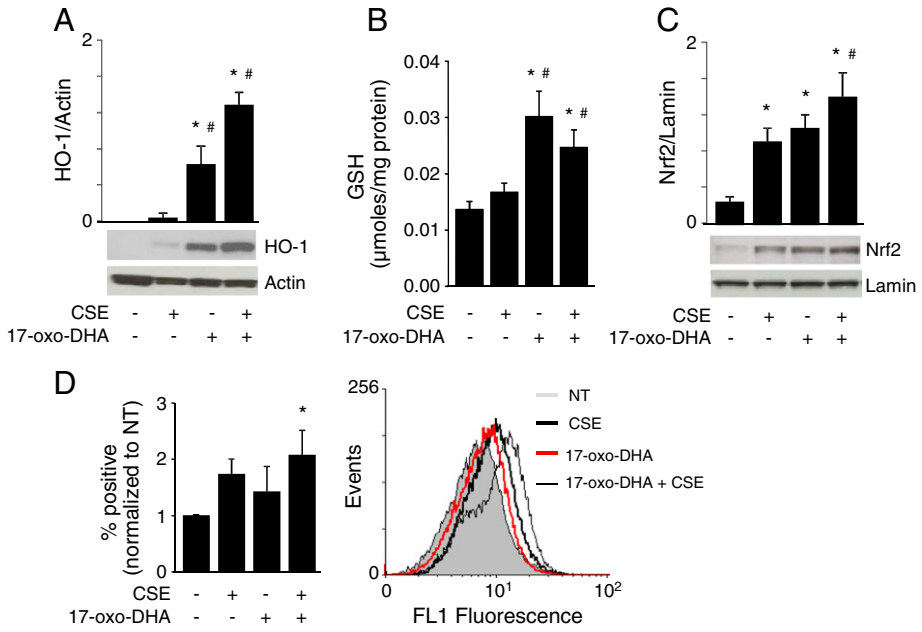

Fig. 2. Induction of the anti-oxidant response and ROS modulation by 17-oxo-DHA in primary bronchial epithelial cells. Expression of HO-1 (A), total glutathione levels (B), nuclear Nrf2 accumulation (C), and ROS modulation (D) were measured in primary bronchial epithelial cells stimulated with 20 μM 17-oxo-DHA and 10% CSE, alone or in combination. For HO-1 expression (western blot) and total GSH measurement (enzymatic recycling method) cells were treated with the indicated stimulus for 18 h. Nuclear Nrf2 was measured by western blot in nuclear extracts from cells treated for 4 h with the indicated stimulus. Densitometric analysis is reported for western blot images where HO-1 was normalized to actin and Nrf2 was normalized to Lamin B1. For ROS measurement, cells were pretreated for 6 h with 20 μM 17-oxo-DHA then 10% CSE was added for 18 h. ROS were detected by flow cytometry using 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester). Bar plots represent the percentage of positive cells normalized to non-treated cells (NT). Experiments were performed in triplicate (n = 3). Data are expressed as mean ± SD. The comparison between different experimental conditions was evaluated by ANOVA corrected with the Bonferroni test. *, statistically different from non-treated; #, statistically different from CSE.

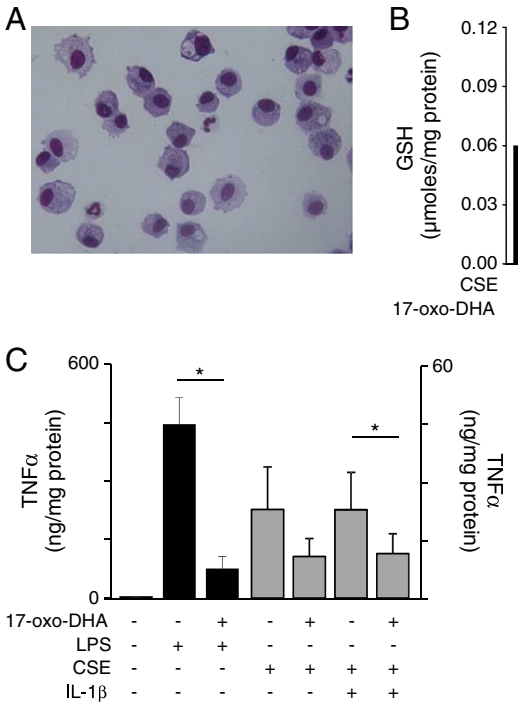

Fig. 3. Induction of total glutathione levels and TNFα suppression by 17-oxo-DHA in primary alveolar macrophages. (A) Human alveolar macrophages were isolated from mini-BAL and stained with Hemacolor® staining kit for differential count; (B) total glutathione levels were measured in alveolar macrophages treated with the indicated stimulus for 18 h (experiment was performed only once); (C) alveolar macrophages were pretreated with 20 μM 17-oxo-DHA for 6 h, then the indicated stimulus was added (LPS 1 μg/ml, CSE 10%, IL-1β 30 ng/ml) and supernatants were collected after 18 h for measurement of TNFα in the supernatants. Experiments were performed in triplicate (n = 3). Data are expressed as mean ± SD. Pair wise comparison was evaluated by paired t-test. $p < .05$ was accepted as statistically significant. Gray bars indicate those data that are represented in the secondary y-axis.

3.3. 17-Oxo-DHA suppressed CSE-induced ROS formation in THP-1 macrophages, but not in bronchial epithelial cells

It was next evaluated whether the induction of the anti-oxidant response in macrophages and bronchial epithelial cells resulted also in the modulation of CSE-induced ROS generation. As reported in Fig. 5A, 17-oxo-DHA effectively protected THP-1 macrophages from CSE-induced oxidative stress by suppressing ROS generation. Interestingly, the non-electrophilic precursor DHA reduced CSE-induced ROS generation displaying a behavior very similar to 17-oxo-DHA. Contrarily to what was observed for THP-1 macrophages, in 16HBE and primary bronchial epithelial cells 17-oxo-DHA did not reduce CSE-induced ROS generation (Figs. 2D and 5B). In fact, it caused a slight increase in ROS formation without causing cell necrosis or apoptosis as assessed by annexin V/propidium iodide assay (Fig. S1).

3.4. 17-Oxo-DHA suppressed the production of TNFα in macrophages and PBMCs

TNFα is highly expressed in the lung of COPD patients and its production is increased by several pro-inflammatory stimuli, including bacterial endotoxin (LPS) and CSE [31]. To investigate the anti-inflammatory potential of 17-oxo-DHA, its effect on TNFα formation was evaluated in cells stimulated with LPS, CSE and IL-1β + CSE or IL-1β alone. IL-1β was used because (i) it is known to enhance the pro-inflammatory effects of CSE, (ii) its levels are high in the lung of COPD patients and (iii) it has been linked to the corticosteroid-resistant phenotype typical of severe COPD [32]. While THP-1 macrophages were highly responsive to the selected stimuli by releasing high levels of TNFα, 16HBE cells did not release detectable amount of this cytokine (data not shown). In THP-1 cells, supplementation with 17-oxo-DHA resulted in significant reduction of cytokine release in response to all the tested stimuli (Fig. 6A). The non-electrophilic precursor DHA did not suppress TNFα production, with the exception of CSE-

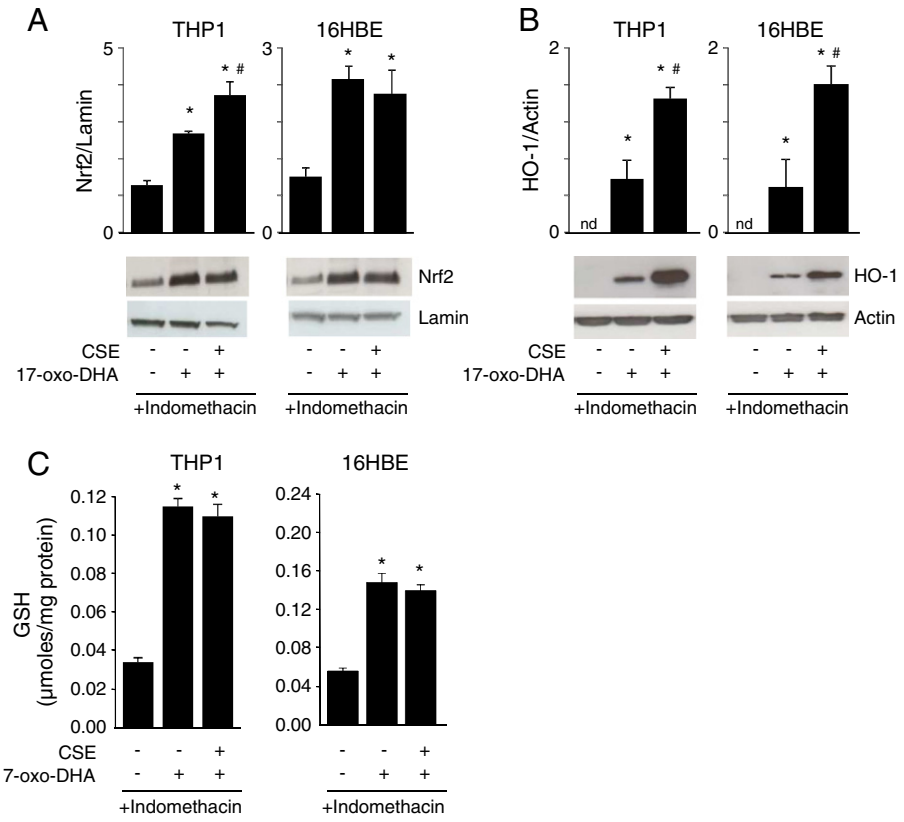

Fig. 4. Externally supplemented 17-oxo-DHA induces the anti-oxidant response also in the presence of a Cox-inhibitor. 16HBE cells and PMA-differentiated THP-1 were treated with 25 μM indomethacin for 30 min prior to the addition of 17-oxo-DHA. (A) Cells were treated with 20 μM 17-oxo-DHA for 2 h (THP-1) or 4 h (16HBE) and nuclear Nrf2 was quantified by western blot. (B, C) Cells were treated with 20 μM 17-oxo-DHA for 18 h; (B) the expression of HO-1 was quantified by WB in total cell extracts; (C) total glutathione was measured in total extracts using the enzymatic recycling method as already described [29]. Experiments were performed in triplicate (n = 3). For western blot, densitometric analysis and representative images are reported. Data are expressed as mean ± SD. The comparison between different experimental conditions was evaluated by ANOVA corrected with the Bonferroni test. *, statistically different from non-treated; #, statistically different from 17-oxo-DHA.

stimulated cells. To evaluate the anti-inflammatory action of 17-oxo-DHA in a more clinically relevant setting, the same experiments were reproduced in alveolar macrophages and PBMCs isolated from healthy subjects. As reported in Fig. 3C, 17-oxo-DHA showed potent suppression of LPS-, CSE- and IL-1β-induced TNFα production in alveolar macrophages. In PBMCs, 10 μM 17-oxo-DHA strongly suppressed LPS-induced TNFα production (Fig. 6C; PBMCs did not release detectable amount of TNFα in response to CSE or IL-1β + CSE, data not shown). Inhibition of TNFα production by 17-oxo-DHA occurred at transcriptional level as assessed by quantitative RT-PCR measurement of the TNFα mRNA (Fig. 7). Suppression of TNFα production in response to all the selected stimuli occurred to a similar extent also in the presence of the Cox-inhibitor indomethacin (Figs. 6B and 7B).

3.5. 17-Oxo-DHA slightly decreased TLR4 expression in THP-1 cells

To investigate possible mechanisms through which 17-oxo-DHA exerts anti-inflammatory actions and since the expression of TLR4 correlates with the ability of the cell to respond to pro-inflammatory stimuli [33] we evaluated whether 17-oxo-DHA was able to modulate TLR4 expression. Total and surface expression of TLR4 was measured in THP-1 macrophages stimulated with 17-oxo-DHA, LPS and CSE, alone or in combination (Fig. 8). When added alone, all the stimuli failed to induce any modulation of total (Fig. 8A) or surface (Fig. 8B) TLR4 expression. This was consistent with previous reports showing that in PMA-differentiated THP-1 macrophages this receptor has a very high basal expression that cannot be further induced [34]. When added in combination with CSE and LPS, 17-oxo-DHA slightly, but significantly, decreased the levels of both the total and the surface receptor.

3.6. 17-Oxo-DHA promotes anti-oxidant and anti-inflammatory actions via Nrf2-dependent and -independent mechanisms

It was next evaluated whether the anti-oxidant and anti-inflammatory actions of 17-oxo-DHA were both dependent on the activation of the Nrf2 pathway. To this purpose, Nrf2 was silenced using a siRNA strategy (Fig. 9A) and the extent of HO-1 induction was measured in response to 17-oxo-DHA in the presence of the Nrf2 targeting or the non-targeting control. Silencing Nrf2 significantly reduced the induction of HO-1 in response to 17-oxo-DHA (Fig. 9B). On the contrary, silencing Nrf2 expression did not affect the extent of TNFα suppression by 17-oxo-DHA in response to the pro-inflammatory stimuli used in the present study thus excluding the involvement of the Nrf2 pathway in the regulation of TNFα gene expression by 17-oxo-DHA (Fig. 9C).

4. Discussion

Herein we show that the electrophilic 17-oxo-DHA induced the anti-oxidant response in human macrophages and bronchial epithelial cells and suppressed inflammatory reactions in macrophages contrasting the oxidative and inflammatory insult of cigarette smoke and bacterial LPS. To enhance the clinical relevance of the reported findings, data obtained using the 16HBE and THP-1 cell lines were validated using human primary bronchial/tracheal epithelial cells, alveolar macrophages and PBMCs. Of note, while the induction of the anti-oxidant response by 17-oxo-DHA depended on the activity of Nrf2, suppression of LPS- and CSE-induced TNFα generation occurred via a Nrf2-independent mechanism indicating that 17-oxo-DHA contrasts inflammation and oxidative

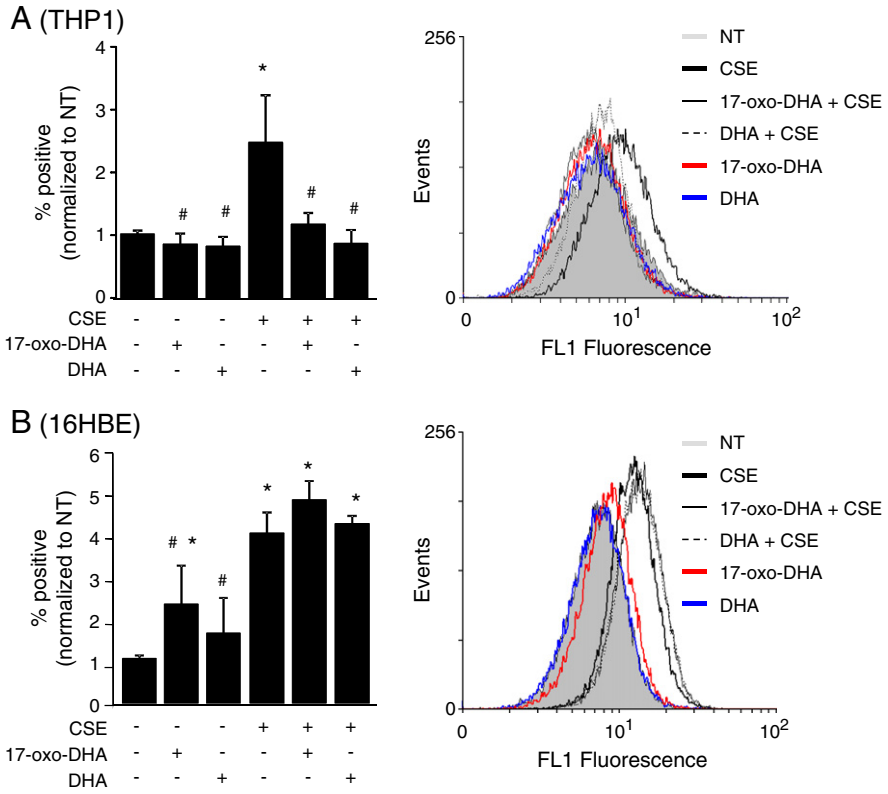

Fig. 5. 17-oxo-DHA suppresses CSE-induced ROS generation in THP-1 cells but not in 16HBE. ROS were detected by flow cytometry using 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester). Bar plots represent the percentage of positive cells normalized to non-treated cells (NT). Representative histograms are reported for each cell line. PMA-differentiated THP-1 cells and 16HBE cells were treated with 20 μM 17-oxo-DHA or DHA for 6 h, then 10% CSE was added and ROS analysis was performed after 18 h. Experiments were performed in triplicate (n = 6). Data are expressed as mean ± SD. The comparison between different experimental conditions was evaluated by ANOVA corrected with the Bonferroni test. *, statistically different from non-treated; #, statistically different from CSE.

stress by acting on multiple independent pathways. The reported findings gain further relevance when considering that our in vitro experimental model might reflect what occurs in the lung of COPD patients, where resident alveolar macrophages and bronchial epithelial cells are the primary responders to insults coming from cigarette smoke and bacterial infections [16,23].

Elevated oxidative stress is a hallmark of the COPD lung and is related to genetic susceptibility, exposure to environmental insults such as cigarette smoke, persistent inflammatory reactions and impaired oxidative defenses [16]. Reduced nuclear Nrf2, with consequent decrease of Nrf2 target gene expression and lower glutathione levels, has been associated with disease progression and inactivation of HDAC2 leading to steroid resistance [21,35,36]. As a consequence, targeting this pathway appears to be a promising strategy for the development of new therapies for COPD [26]. In in vitro experimental models, while long-term exposure to cigarette smoke down-regulates the Nrf2 pathway, 24 h treatment induces the Nrf2-dependent anti-oxidant response as a consequence of cell oxidation leading, among others, to the increase of total glutathione as confirmed by data herein reported [37,38]. Herein we show that during short-term stimulation, 17-oxo-DHA promoted Nrf2 nuclear accumulation, it was a potent inducer of HO-1 expression and doubled total glutathione levels in macrophages and bronchial epithelial cells showing an effect much stronger when compared to CSE. Notably, in THP-1 macrophages, this induction was not accompanied by any cell oxidation, contrarily to what was observed in CSE-stimulated cells, and it resulted in complete suppression of CSE-induced ROS generation. In bronchial epithelial cells 17-oxo-DHA caused a slight increase in cell oxidation and did not suppress CSE-induced ROS formation. These data showed that the two cell types display different mechanisms of redox homeostasis, with macrophages being more resistant to oxidation compared to structural epithelial cells. In this regard, ROS induction in response to electrophilic PUFAs was already reported in epithelial cells where it represented a signaling rather than a toxic event [39]. Our data further support this view as the extent of oxidation induced by 17-oxo-DHA in bronchial epithelial cells was much less compared to that induced by CSE with a much stronger increase of the anti-oxidant response, resulting in a positive balance of anti-oxidants vs oxidants.

Interestingly, when looking at the induction of HO-1 expression, 17-oxo-DHA displayed a stronger effect when supplemented in combination with CSE compared to stimulation with 17-oxo-DHA alone. In this respect, the fact that nuclear accumulation of the transcriptional factor Nrf2 did not parallel the expression of HO-1 suggests the presence of additional mechanisms leading to the upregulation of this redox-sensitive gene. The heat shock transcription factor 1 (HSF1) and the hypoxia-inducible factor 1α (HIF-1α) are likely candidates. The transcription factor HSF1 becomes activated in response to high temperature and other stressors that cause accumulation of non-native proteins [12]. In some circumstances, HSF1 upregulates the expression of HO-1 [40,41]. Interestingly, long-chain electrophilic PUFAs induce the activity of HSF1 suggesting that 17-oxo-DHA may contribute to enhance HO-1 induction in the presence of cigarette smoke via HSF1 [12,42]. The transcriptional factor HIF-1α synergizes with Nrf2 for the expression of HO-1 and it has been recently reported that the electrophilic 15d-PGJ_2 can induce its activity [43,44], once again supporting that the induction of HO-1 that we observed in response to 17-oxo-DHA may be partially mediated by this transcriptional factor.

The non-electrophilic precursor DHA when given alone did not induce the Nrf2-dependent anti-oxidant response nor suppressed LPS-induced TNFα production. This was consistent with the requirement of the keto-group to confer electrophilic reactivity to DHA converting this long chain fatty acid into a biologically active compound.

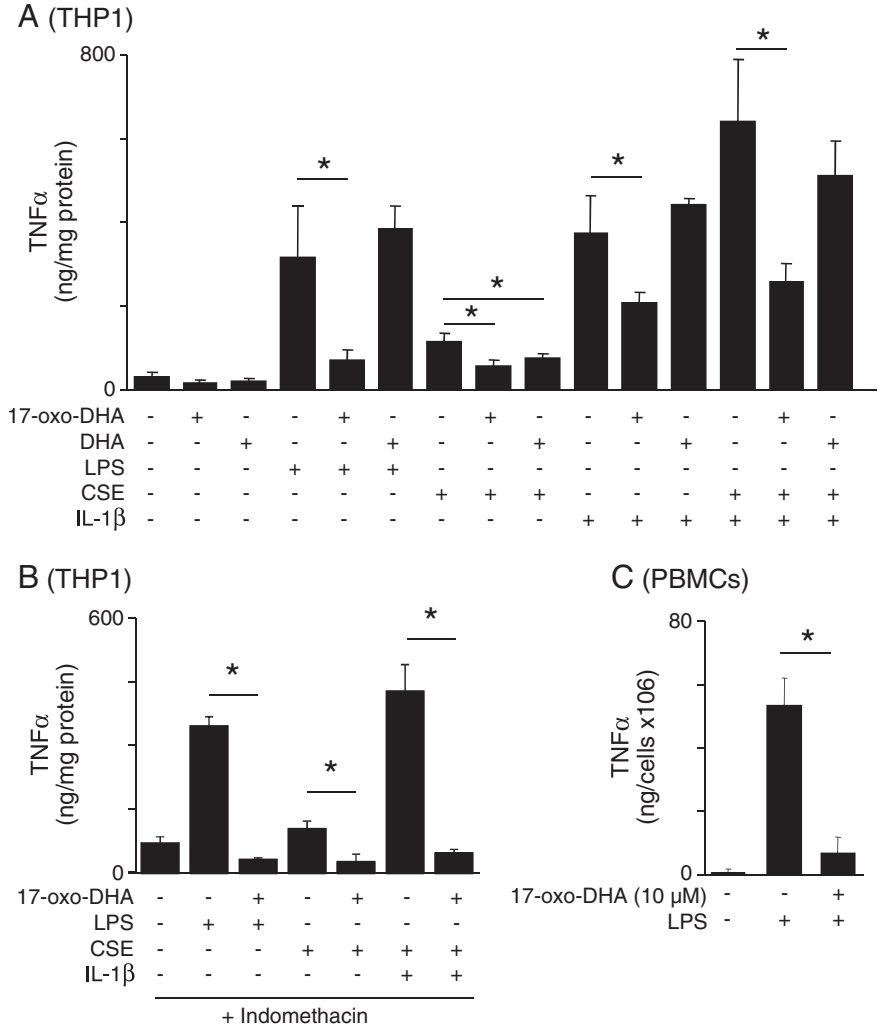

Fig. 6. 17-Oxo-DHA suppresses TNFα production in THP-1 macrophages and PBMCs. (A) PMA-differentiated THP-1 cells and (C) freshly isolated PBMCs were pretreated with 20 μM 17-oxo-DHA or DHA (THP-1 cells) or 10 μM 17-oxo-DHA (PBMCs) for 6 h, then the indicated stimulus was added (LPS 1 μg/ml, CSE 10%, IL-1β 30 ng/ml) and supernatants were collected after 18 h for measurement of TNFα. (B) PMA-differentiated THP-1 cells were treated with 25 μM indomethacin for 30 min, then 17-oxo-DHA was added and after 6 h the indicated treatments were performed. Supernatants were collected after 18 h for TNFα measurement. Experiments were performed in triplicate (n = 6 for THP-1 and n = 3 for PBMCs). Data are expressed as mean ± SD. Pair wise comparison was evaluated by paired *t*-test. $p < .05$ was accepted as statistically significant.

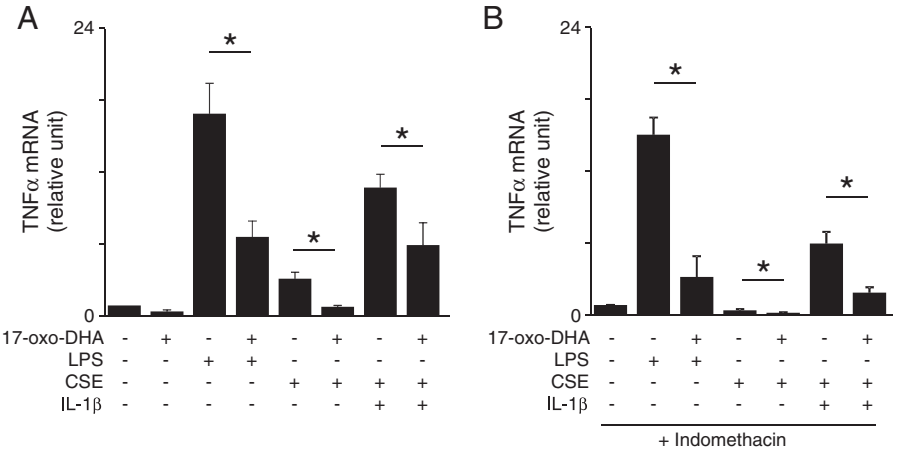

Fig. 7. 17-Oxo-DHA suppresses TNFα expression at transcriptional level. (A) PMA-differentiated THP-1 cells were treated with 20 μM 17-oxo-DHA or (B) incubated for 30 min with 25 μM indomethacin prior to the treatment with 17-oxo-DHA. 6 h after the addition of 17-oxo-DHA the indicated stimulus was added (LPS 1 μg/ml, CSE 10%, IL-1β 30 ng/ml), cells were harvested after 4 h and total RNA was extracted. TNFα mRNA was quantified by quantitative RT-PCR. Results are reported as relative unit and normalized to non-treated control. Experiments were performed in quadruplicate (n = 4). Data are expressed as mean ± SD. Pair wise comparison was evaluated by paired t-test. $p < .05$ was accepted as statistically significant.

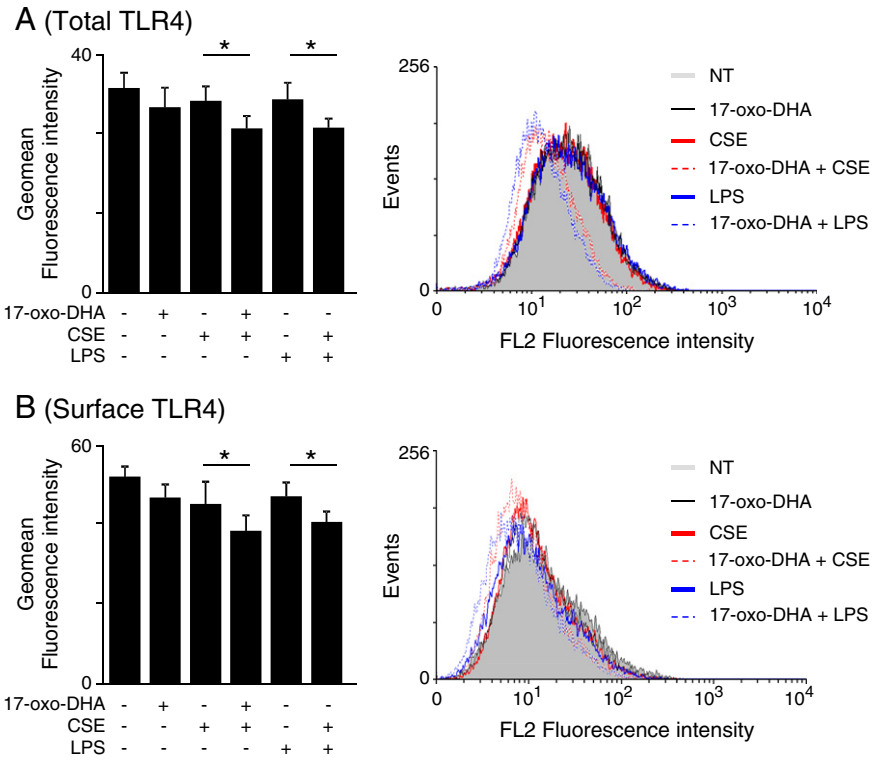

Fig. 8. Modulation of TLR4 receptor by 17-oxo-DHA. PMA-differentiated THP-1 cells were treated with 20 μM 17-oxo-DHA for 6 h, then 10% CSE or LPS 1 μg/ml was added and cells were harvested after 18 h. Total (A) and surface (B) TLR4 expression was evaluated by flow cytometry using a specific antibody. For total TLR4, cells were permeabilized prior to anti-TLR4 antibody addition. Experiments were performed in triplicate (n = 6). Data are expressed as mean ± SD. Pair wise comparison was evaluated by paired *t*-test. $p < .05$ was accepted as statistically significant.

Interestingly, when supplemented in combination with CSE, DHA contributed to the increase of glutathione levels and HO-1 expression to a similar extent compared to 17-oxo-DHA (Fig. 1). Furthermore, in THP-1 macrophages, DHA suppressed CSE-induced TNFα production and ROS generation. Overall, the fact that DHA displayed a biological activity comparable to 17-oxo-DHA only when given in combination with CSE would suggest that the oxidative environment created by cigarette smoke might favor the conversion of DHA into an oxygenated electrophilic bioactive derivative. This is in line with previously published data showing that DHA displays anti-oxidant and anti-inflammatory actions at a very high concentration (above 50 μM) which are likely to cause cell oxidation, and that this activity is inhibited by anti-oxidants [39,45–47].

Besides CSE-induced oxidative damage, chronic inflammation in the lung of COPD patients contributes to tissue damage and increases susceptibility to bacterial infections. Persistent activation of resident macrophages and bronchial epithelial cells causes a continuous release of inflammatory mediators which in turn attract circulating immune cells to the lung. TNFα is particularly elevated in the lung of COPD patients [31,48]. Although this cytokine is primarily released by cells of monocytic lineage, production by bronchial epithelial cells has also been reported [31,49–51]. In our experimental model, 16HBE cells did not release detectable amount of TNFα and therefore it was not possible to investigate the action of 17-oxo-DHA on this cell type. In macrophage-differentiated THP-1 cells and in alveolar macrophages we observed the production of TNFα in response to LPS, CSE and IL-1β, alone or in combination. In PBMCs, release of TNFα was observed only in response to LPS. 17-Oxo-DHA potently suppressed TNFα production in all the analyzed conditions acting at transcriptional level most likely through the inhibition of p65 binding to DNA as we have previously reported [6].

To evaluate whether the endogenous amount of 17-oxo-DHA contributed to the effects observed when externally supplementing this electrophilic compound, the work was repeated in the presence of the Cox-inhibitor indomethacin. It is known that this inhibitor abolishes endogenous formation of electrophilic oxo-derivatives of DHA in activated macrophages [6]. Data herein reported show that, in the presence of indomethacin, exogenous 17-oxo-DHA displays the same biological actions in terms of HO-1 and Nrf2 induction, increase of total glutathione levels and TNFα suppression at protein and mRNA level when compared to cells treated with 17-oxo-DHA in the absence of indomethacin (Figs. 4, 6 and 7). These results indicate that the biological actions of the endogenous amount of oxo-DHA become non-relevant when cells are exposed to micromolar concentration of exogenous 17-oxo-DHA.

It has been recently shown that electrophilic nitro-fatty acids suppress inflammatory reactions acting upstream of the inflammatory cascade by reducing the surface expression of the receptor TLR4 which is required for transducing the signals coming from LPS and CSE [52]. In our experimental model, 17-oxo-DHA did not show a potent activity in suppressing the expression of this receptor therefore excluding that this may be a relevant mechanism for 17-oxo-DHA-mediated suppression of TNFα expression in THP-1 cells. Of note, the TLR4 was poorly modulated also in response to LPS and CSE and this was in line with previous reports showing that macrophage-differentiated THP-1 cells express this receptor at maximal levels and therefore are poorly responsive to positive stimulations [34].

An important outcome of this work was to demonstrate that while the induction of the anti-oxidant response by 17-oxo-DHA was dependent on Nrf2, the suppression of TNFα production by 17-oxo-DHA occurred via a Nrf2-independent mechanism supporting that 17-oxo-DHA directly limits inflammatory reactions independently of its anti-oxidant properties. This becomes highly relevant when comparing 17-oxo-DHA with current anti-inflammatory drugs, such as corticosteroids, which are ineffective in contrasting oxidative stress. Although elucidating the molecular mechanism of action of 17-oxo-DHA will require further investigation, data herein presented show its efficacy

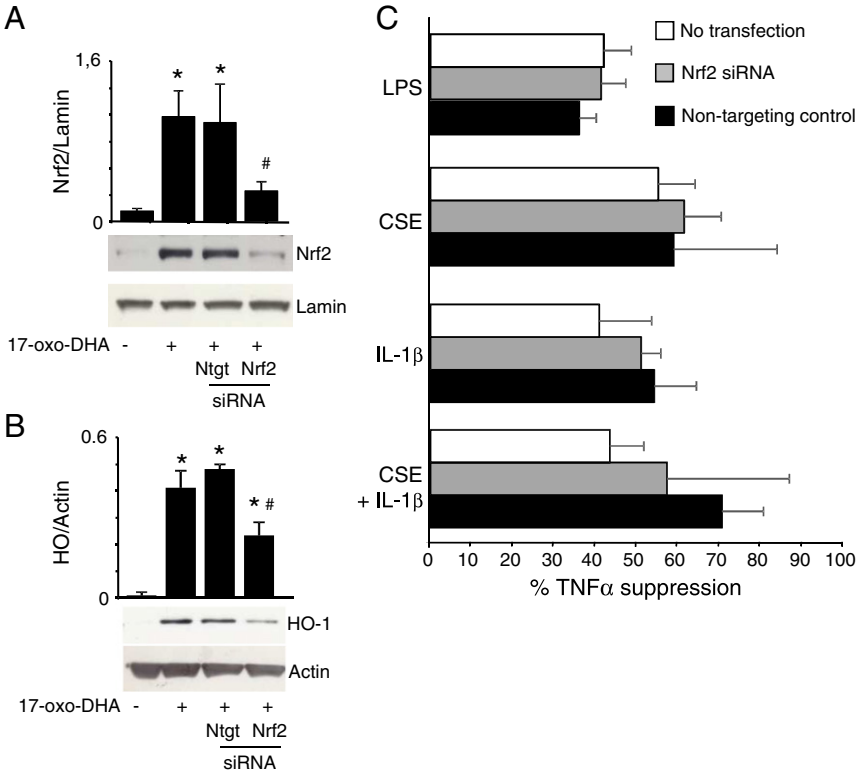

Fig. 9. TNFα suppression by 17-oxo-DHA is independent of Nrf2. PMA-differentiated THP-1 cells were transfected with Nrf2 targeting siRNA (Nrf2) or non-targeting control (Ntgt). (A) 48 h after transfection, cells were treated with 20 μM 17-oxo-DHA for 2 h and nuclear Nrf2 was measured by western blot (Ntgt, transfection with the non-targeting control; Nrf2, transfection with the Nrf2 targeting siRNA). (B) Cells were transfected and after 48 h were treated with 17-oxo-DHA for 18 h and HO-1 expression was measured in total cell extracts by western blot. (C) Cells were transfected and after 48 h were either pretreated with 17-oxo-DHA for 6 h or left in complete medium, then the indicated stimulus was added and supernatants were collected after 18 h for TNFα measurement. Data are expressed as percentage of TNFα suppression by comparing the TNFα levels in cells treated with 17-oxo-DHA plus the indicated inflammatory stimulus, with the TNFα levels produced by cells treated with the proinflammatory stimulus alone, in the presence of the indicated silencing conditions. Experiments were performed in triplicate (n = 6). The comparison between different experimental conditions was evaluated by ANOVA corrected with the Bonferroni test. *, statistically different from non-treated; #, statistically different from cells treated with 17-oxo-DHA alone.

in contrasting LPS- and CSE-induced oxidative stress and inflammation, in particular in macrophages. The efficacy of 17-oxo-DHA in suppressing TNFα production in freshly isolated human PBMCs and alveolar macrophages further highlighted the clinical relevance of data herein presented. In addition, the fact that 17-oxo-DHA is endogenously generated in activated macrophages by the action of Cox-2 supports the pro-resolving nature of this enzyme and suggests that dietary intake of the precursor DHA may represent an alternative/complementary approach together with direct administration of 17-oxo-DHA for increasing its endogenous levels. At the moment, limited and controversial data are available regarding the role of n−3 PUFA supplementation in modulating COPD occurrence and progression, and important trials are still ongoing [53–57]. Considering the volatile nature of 17-oxo-DHA, an interesting therapeutic option would be the administration via inhalation. This would allow reaching higher topical concentration with a better safety profile. In this regard, a recent work demonstrated that the DHA-derived Resolvin D1 administered by inhalation reduced cigarette smoke-induced acute lung inflammation in a murine model [58]. Most interestingly, our data support that in the presence of cigarette smoke Resolvin D1 may be further oxidized yielding an electrophilic ketone derivative.

Overall, results herein reported confirmed that 17-oxo-DHA promotes anti-oxidant and anti-inflammatory actions in experimental models relevant for COPD by contrasting the effects of cigarette smoke and LPS via multiple pathways and encourage its use for the development of new therapies for the treatment of cigarette smoke-related chronic inflammatory disorders.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.02.024.

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