Oxidative stress-induced glucocorticoid resistance is prevented by dual PDE3/PDE4 inhibition in human alveolar macrophages

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Running Head: PDE3/PDE4 inhibition prevents glucocorticoid resistance

Abstract

Background: Oxidative stress is present in airway diseases such as severe asthma or Chronic Obstructive Pulmonary Disease and contributes to the low response to glucocorticoids through the down-regulation of histone deacetylase (HDAC) activity.

Objective: to study the effects of the phosphodiesterase (PDE)-3 and 4 inhibitors and their combination versus glucocorticoids in a model of lipopolysaccharide (LPS)-induced cytokine release in alveolar macrophages under oxidative stress conditions.

Methods: differentiated U937 or human alveolar macrophages were stimulated with H_2O_2 (10-1000µM) or cigarette smoke extract (CSE, 0-15%) for 4h before LPS (0.5 µg/mL, 24h) addition. In other experiments cells were pre-treated with dexamethasone or budesonide (10^{-9}-10^{-6}M), with the PDE4 inhibitor rolipram (10^{-9}-10^{-5}M), PDE3 inhibitor motapizone (10µM), cAMP enhancer PGE_2 (10nM), or with the combination of rolipram (10^{-6}M) + PGE_2 (10 nM) + motapizone (10µM) 15 min before oxidants. IL-8 and TNF-α were measured by ELISA and HDAC activity by a colorimetric assay.

Results: Budesonide and dexamethasone produced a concentration-dependent inhibition of the LPS-induced IL-8 and TNF-α secretion with an E_{max} about 90% of inhibition which was reduced by approximately 30% in presence of H_2O_2 or CSE. Pre-treatment with rolipram, motapizone or PGE_2 only reached about 20% of inhibition but was not affected by oxidative stress. In contrast, PDE4/PDE3 combination in presence of PGE_2 effectively inhibited the LPS-induced cytokine secretion by about 90% and was not affected by oxidative stress. Combined PDE4 and PDE3 inhibition reversed glucocorticoid resistance under oxidative stress conditions. HDAC activity was reduced in presence of oxidative stress, and in contrast to glucocorticoids, pre-treatment with PDE4/PDE3 combination was able to prevent HDAC inactivity.

Conclusions & Clinical Relevance: this study shows that the combination of the PDE3/PDE4 inhibitors prevents alveolar macrophage activation in those situations of glucocorticoid resistance which may be of potential interest to develop new effective anti-inflammatory drugs in airway diseases.

Keywords: Phosphodiesterase 4, phosphodiesterase 3, glucocorticoid resistance, alveolar macrophages, oxidative stress.

Introduction

There is a growing medical need for research in respiratory diseases concerning effective anti-inflammatory therapies for conditions such as severe asthma and chronic obstructive pulmonary disease (COPD). These diseases share common characteristics such as enhanced and uncontrolled inflammatory response in the lungs, which contributes to disease progression. These diseases are poorly controlled by current anti-inflammatory therapies including glucocorticoids (GCs), which are otherwise effective in many other inflammatory conditions or in milder disease such as asthma [1].

Oxidative stress is characteristic of chronic airway inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD) [2]. Elevated intracellular reactive oxygen species (ROS) are generated under various physiological and pathological conditions, including acute and chronic inflammation. Major ROS are superoxide anions (O^{2-}), hydroxyl radicals ($^{\cdot}OH$) and H_2O_2 which can be derived from sources as diverse as activated inflammatory cells, cigarette smoke and structural cells [3]. Alveolar macrophages (AMs) originate from monocytes and are suggested to be the main orchestrators of the chronic inflammatory response and tissue destruction observed in patients with COPD [4]. Furthermore, AMs are of importance in asthma because of their key role in the innate immune response holding the capability to release a broad range of cytokines and chemokines [5]. Acute cigarette smoke exposure up-regulates the production of some AMs inflammatory mediators, including neutrophil chemotactic mediators such as IL-8, and pro-inflammatory cytokines such as TNF-α, which are increased in the sputum of patients with COPD contributing to the worsening of this disease [6].

GC insensitivity is a feature of severe asthma and COPD, and oxidative stress is an important factor in its development by inhibition of histone deacetylase 2 (HDAC-2)

activity and expression [7]. Histone deacetylation by HDACs causes rewinding of DNA and hence silences gene transcription of several pro-inflammatory genes. HDACs not only cause the inhibition of gene transcription but also directly affect the nuclear binding of transcription factors such as NF-κB preventing gene transcription [8]. In this respect, HDAC activity and expression is down-regulated in several types of cells from COPD patients such as AMs [9, 10], in which HDAC2 inactivation causes hyperacetylation of GC receptors which prevents steroids from switching off activated inflammatory genes [7]. Therefore, there is an increasing interest in discover drugs with the capability of maintaining high levels of HDAC in order to reduce GC resistance. This is the case for theophylline at low doses, which has been prove to be effective to prevent GC resistance induced by oxidative stress by means of the restoration of the HDAC2 activity through inhibition of the δ-phosphoinositide-3-kinase (PI3K) [11, 12]. Theophylline is also known to inhibit phosphodiesterases (PDEs) that partially account for their classical bronchodilator activity. However, presently, there is no evidence confirming the role of PDE on HDAC activity and GC resistance. Currently, it is known that oxidative stress activates PI3K which subsequently phosphorylates PDE4 down-regulating adenosine 3′,5′-cyclic monophosphate (cAMP) thus inducing inflammatory responses in macrophages [13]. PDE4 inhibitors showed potential anti-inflammatory effects in clinical trials and they will be soon available for the clinical management of COPD [14]. Thus, would be useful to study the role of PDE4 inhibition on HDAC activity as well as their effect on AM activation in a context of oxidative stress where GCs are ineffective.

Therefore, we hypothesized that inflammatory mediators produced by AM in a context of oxidative stress, such as H_2O_2 or cigarette smoke, change the sensitivity to GC and that the increase in cAMP levels by means of PDE4 inhibition may reverse this situation

by maintaining the activity of HDAC. Furthermore, PDE3 isoenzyme inhibitor will be studied since it has been shown that dual PDE4/PDE3 inhibition increase the reduction of inflammatory mediators emitted by human AM [15, 16].

Material and methods

Materials

Unless otherwise stated, all reagents used were obtained from Sigma (Chemical Co, UK). Both, rolipram and trichostatin A (TSA) were dissolved in dimethyl sulfoxide (DMSO) at 10 mM stock concentration. Dexamethasone and budesonide were dissolved in deionised mili-Q water (Millipore Iberica, Madrid, Spain). Several stock dilutions of the stocks were performed with cell culture medium. The final concentration of DMSO in the culture cell did not exceed 0.1 % and had no significant pharmacological activity.

Cell culture

The human monocytic cell line U937 (American Type Culture Collection, Rockville, MD, USA) was cultured in RPMI 1640 medium (Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 10% foetal bovine serum (FCS), 100 U/ mL penicillin and 100 mg/mL streptomycin at 37 ºC with 5% CO_2 in a humidified incubator. U937 cells were incubated at 37ºC with 5 nM of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich Co.). The U937 monocyte cell line was differentiated after a 72h incubation with TPA to macrophage-like cells as previously outlined [17]. Under these conditions, macrophage-like phenotype was proven based on cell surface markers such as down-regulation of CD14 as well as up-regulation of NaF-insensitive esterase and acid phosphatise [15]. In addition, the expression of different PDE isoenzymes of these macrophage-like phenotype cells closely resembles that of human lung macrophages from bronchoalveolar lavage (BAL) [18]. Cells were centrifuged and resuspended in fresh media in 24-well plates at a concentration of $500x10^3$ cells per well for 24 h before experimental use. In order to establish the oxidative stress conditions to evaluate GC resistance, cells were stimulated with H_2O_2 (10-1000µM) or cigarette smoke extract

(CSE, 0-15%) for 20 min or 4 h before lipopolysaccharide (LPS; 0.5 µg/mL for 24 h) addition, remaining together until the end of 24 h of incubation. The time point of 24 h was established based on preliminary experiments using differentiated U937; LPS-induced TNF-α and IL-8 production reached a peak value at 24 h (data not shown). In other experiments cells were pre-treated with rolipram (10^{-9}-10^{-5}M), dexamethasone (10^{-9}-10^{-6}M), budesonide (10^{-9}-10^{-6}M), PGE_2 (10nM), motapizone (10µM) or with the MIX formed by rolipram (10^{-9}-10^{-5}M) + PGE_2 (10 nM) + motapizone (10µM) 15 min before oxidants. Cell supernatants were collected after a 24h cell stimulation. In some cases, cells were pre-treated with the selective HDAC inhibitor TSA (10-1000 ng/mL) 20 min before LPS (0.5 µg/mL for 24 h). Culture media from these macrophages were collected and stored at -80°C for further analysis. The culture media was used for IL-8 and TNF-α assays.

Bronchoscopy and BAL Processing

Human AMs from BAL were obtained from 8 patients subjected to this procedure for diagnostic and/or therapeutic reasons. All subjects had diffuse interstitial lung disease, were no smokers and they did not receive bronchodilator, theophylline, antibiotic, antioxidant, and/or glucocorticoid therapy in the last week before BAL. Clinical features are showed in table 1. With the approval of the local ethics committee, informed consent was obtained. The bronchoscope (Olympus was planted into the right or left upper lobes, and a maximum of four 60 mL aliquots of pre-warmed sterile 0.9% saline solution as instilled into each lung (maximum, 480 mL). The total volume instilled depended on how well the patient tolerated the procedure. The aspirated fluid was stored on ice until filtration (100µm filter; BD Biosciences; Oxford, UK) and centrifugated at 500*g* for 10 min at 4°C. The cell pellet was washed with RPMI 1640

medium supplemented as describe above. Macrophage purity was 95.2% ± 4.9%, as assessed with May-Grunwald staining of cytospin preparations. Total viable cell count and total AM cell counts were determined by trypan blue exclusion (Neubauer chamber). The cell suspension was adjusted to $500x10^3$ cells per well in 24-well plates and incubated for 4 h before non-adherent cells were discarded and remaining cells were kept in RPMI containing 0.25% FCS for at least 6 h before stimulation. Experimental conditions were as described for differentiated U937 cells.

Preparation of CSE solutions

CSE was prepared as previously outlined [19, 20]. Briefly, the smoke of a research cigarette (2R4F; Tobacco Health Research, University of Kentucky, KY, USA) was generated by a respiratory pump (Apparatus Rodent Respirator 680; Harvard, Germany) through a puffing mechanism related to the human smoking pattern (3 puff/min; 1 puff 35 ml; each puff of 2 s duration with 0.5 cm above the filter) and was bubbled into a flask containing 25 ml of pre-warmed (37°C) RPMI 1640 culture medium supplemented as describe above. The CSE solution was sterilized by filtration through a 0.22-µm cellulose acetate sterilizing system (Corning, NY). The resultant CSE solution was considered to be 100% CSE and was used for experiments within 30 min of preparation. CSE 10% corresponds approximately to the exposure associated with smoking two packs per day. The quality of the prepared CSE solution was assessed based on the absorbance at 320 nm, which is the specific absorption wavelength of peroxynitrite. Stock solutions with an absorbance value of 3.0 ± 0.1 were used. To test for cytotoxicity from CSE, differentiated U937 and AMs were treated with CSE concentrations of up to 20% for 24. No significant difference in the lactate

dehydrogenase supernatant level (lactate dehydrogenase cytotoxicity assay; Cayman, Spain) was observed in comparison with the control group (data not shown).

ELISA

IL-8 and TNF-α levels were assayed in culture supernatant samples and were determined by using commercially available enzyme-linked immunosorbent assay kits for IL-8 and TNF-α (R&D Systems, UK) according to the manufacturer's protocol.

Nuclear protein extraction and quantification

Nuclear protein extraction was performed to measure total HDAC activity with the active motif extraction kit (Active Motif Europe, Rixensart, Belgium) in a total of $8x10^6$cells per condition according to the manufacturer's protocol. The Bio-Rad assay (Bio-Rad Laboratories Ltd., Herts, UK) was adopted (following manufacturer's instructions) to quantify the level of protein in each sample to ensure equal nuclear protein loading to measure HDAC activity.

HDAC Activity

HDAC activity was measured by use of a colorimetric assay system (AK-501, Biomol) according to the manufacturer's protocol. Essentially, the procedure involves the use of HDAC colorimetric substrate (Color de Lys substrate, 500 µM), which comprises an acetylated lysine side chain and is incubated with a sample containing nuclear extract. Deacetylation sensitizes the substrate and treatment with the lysine developer produces a chromophore which can be analyzed with a colorimetric plate reader at 405 nm. HeLa cell nuclear extract was used as a positive control. A standard curve was prepared, using

the known amount of the deacetylated standard (Boc-Lys-pNA) included in the kit. Results were expressed as μM of HDAC per mg of nuclear protein.

Statistics

Data are presented as mean ± SEM of *n* experiments. The IC_{50} values were calculated from the concentration inhibition curves by non-linear regression analysis. Statistical analysis of data was carried out by analysis of variance (ANOVA) or two-way ANOVA as appropriate followed by post-hoc Bonferroni test (GraphPad Software Inc, San Diego, CA, U.S.A). Significance was accepted when $P < 0.05$.

RESULTS

Effect of oxidative stress on LPS-induced IL-8 and TNF-α secretion in differentiated U937 cells

H_2O_2 was selected to represent ROS. The addition of H_2O_2 induced a time and concentration-dependent increase of IL-8 and TNF-α being highest at 1mM following 4 h of stimulation (basal IL8: 156.9 ± 31 ng/mL and TNF-α: 372.9 ± 52 pg/mL versus H_2O_2 1mM for 4h; IL-8: 678.2 ± 121.1 ng/mL and TNF-α: 1040.8 ± 110 pg/mL; Fig. 1A and 1B; $P<0.05$). LPS stimulation increased significantly IL8 and TNF-α supernatant levels (Fig. 1C and 1D at H_2O_2 zero concentration point) respect those values from basal levels (Fig. 1A and 1B at H_2O_2 zero concentration point).

To evaluate the effect of oxidative stress on LPS-induced cytokine release, differentiated U937 were incubated with H_2O_2 (0-1000µM) for short (20 min) or long (4 h) periods of times preceding the addition of LPS. The additive effect was observed only when H_2O_2 was added 4 h before LPS and was significant at H_2O_2 500µM (IL-8: 1094.6±140 ng/mL and TNF-α: 6582±1623 pg/mL following 20 min of H_2O_2 500µM exposure versus IL-8: 1684.5 ± 129 ng/mL and TNF-α: 20259.5 ± 8471 pg/mL following 4h of H_2O_2 500µM exposure; Fig. 1C and 1D, $P<0.05$) and highest at 1000 µM. Similar results were observed when CSE was used to promote cellular oxidative stress. CSE increased IL-8 and TNF-α supernatant levels reaching a peak value at 5% concentration with similar results following 4 h and 24 h of stimulation (Fig. 2A and 2B). Similar to H_2O_2, CSE increased the LPS-induced IL-8 and TNF-α secretion, only following 4h of exposure (Fig. 2C and 2D), suggesting that previous oxidative stress is necessary to up-regulate the inflammatory effect of LPS. In other experiments, inhibition of the HDAC activity with TSA (10-1000 ng/mL for 20 min) enhanced the

LPS-induced IL-8 and TNF-α secretion which reached a significant value at 10 ng/mL (Fig. 3A and 3B; $P<0.05$).

Oxidative stress reduces HDAC activity in differentiated U937

Cells were exposed to H_2O_2 (500 µM) or CSE 5% for 20 min, 4h or 24h and HDAC activity was monitored. Both, H_2O_2 and CSE 5% did not modify HADC activity at short periods of exposure (20 min; Fig. 4). However, when they were added for 4 h or 24 h, the activity of HDAC was reduced at levels similar to those found for TSA (100 ng/mL, 20 min; Fig. 4).

Effect of H_2O_2 on glucocorticoid repression of cytokine release by LPS in differentiated U937 macrophages

Budesonide and dexamethasone produced a concentration-dependent inhibition of the LPS-induced IL-8 secretion in differentiated U937 cells with an E_{max} of 86.8±0.79 % and 79±2.5 % respectively, and $-\log IC_{50}$ of 8.4±0.09 and 7.98±0.13 for each GC (Fig. 5A and 5B; table 2). Similar results were observed for LPS-induced TNF-α release, with an E_{max} of 90.4±2.6 % and 77.1±0.79 % for budesonide and dexamethasone respectively, and $-\log IC_{50}$ of 8.1±0.21 and 7.98±0.05 for each GC (Fig. 5A and 5B; table 2). Differentiated U937 cells exposed to H_2O_2 500µM for 4h, which was according to the inhibition of HDAC activity, resulted in lesser E_{max} and potency of GCs. In this sense, budesonide and dexamethasone attenuated the LPS-induced IL-8 secretion with an E_{max} of 79.2±1.71 % and 59.7±3.14 % respectively, and $-\log IC_{50}$ of 7.7±0.09 and 7.75±0.19 (Fig. 5A and 5B; table 2; $P<0.05$ versus cells not exposed to H_2O_2). In the same fashion, the inhibitory effect of budesonide and dexamethasone on LPS-induced TNF-α was reduced in presence of H_2O_2 with an E_{max} of 68.1±2.4 % and 62±0.29 %

respectively, and –logIC_{50} of 7.8±0.17 and 7.7±0.01 (Fig. 5A and 5B; table 2; P<0.05 versus cells not exposed to H_2O_2). For comparison, the inhibitory response produced by a selective inhibitor of the cAMP-specific PDE4 was examined in separate experiments. As previously outlined [16], the PDE4 inhibitor rolipram showed weak concentration-dependent inhibition of the LPS-induced IL-8 and TNF-α with an E_{max} of 21.3±0.07 % and 21.71±0.71 % respectively, and –logIC_{50} of 6.72±0.21 and 6.72±0.08 (Fig. 5C and 5D; table 2). In contrast to GCs, the exposure of differentiated U937 cells to H_2O_2 did not significantly modify the efficacy and potency of rolipram (Fig 5C and 5D; table 2). Previously, it has been shown that LPS-induced TNF-α up-regulation in macrophages is rather insensitive to inhibition by PDE4 inhibitors unless an additional cAMP trigger such as PGE_2 and PDE3 inhibitor are added [15]. We found that PGE_2 10nM and the PDE3 inhibitor motapizone 10μM inhibited IL-8 secretion by about 24% and 28% and TNF-α by about 30% and 32% respectively which were not influenced by the addition of H_2O_2 (Fig. 5C and 5D). However, when PGE_2 10nM and motapizone 10μM were added together with rolipram (1nM-10μM), the LPS-induced IL-8 and TNF-α were inhibited with an E_{max} of 92.9±1.2 % and 97.86±3.8 % respectively, and –logIC_{50} of 7.66±0.06 and 7.70±0.19 (Fig 5C and 5D; table 2). In contrast to GCs, this combination was not affected by oxidative stress conditions such as H_2O_2 (Fig. 5C and 5D; table 2). In other experiments we found that combination of PDE3/PDE4 inhibitors in absence of PGE_2 reached approximately 75% of IL-8 and TNF-α inhibition in macrophages stimulated with LPS in presence or absence of H_2O_2 (data not shown). Thus, confirming that oxidative stress does not affect PDE3/PDE4 % inhibition.

Effect of CSE on glucocorticoid repression of cytokine release by LPS in differentiated U937 macrophages

We next studied the effect of CSE on GC resistance since it is closer than H_2O_2 to the real oxidative stress conditions found in COPD patients. Based on the IC_{50} values obtained for GCs and rolipram for LPS-induced IL-8 and TNF-α secretion, we selected sub-maximal concentrations for budesonide (100nM), dexamethasone (1μM) and rolipram (1μM). In this context, different drugs and their combinations where added 15 min before CSE 5% which remained 4h before the stimulation with LPS for 24h. CSE treatment effectively reduced the % of inhibition of budesonide which was 42 ± 6% for IL-8 and 45 ± 10% for TNF-α versus 81 ± 16% and 83 ± 10% for those cells non exposed to CSE (Fig. 6A and 6B; $P<0.05$). Similar results were observed for dexamethasone with % of inhibition of 38 ± 9% for IL-8 and 33 ± 11% for TNF- α in those cells exposed to CSE versus 75 ± 7% for IL-8 and 74 ± 8% for TNF-α in those cells non exposed to CSE (Fig. 6A and 6B; $P<0.05$). In contrast to GCs, the % of inhibition of rolipram, PGE_2 or motapizone were not affected by CSE, even though their absolute values remained around 8-16% inhibition for TNF-α and 15-24% for IL-8. However, the combination of PGE_2 (10nM) with motapizone (10μM) and rolipram (1μM) increased the % of inhibition by 83% and 86% for TNF- α and IL-8 respectively (Fig. 6A and 6B). As was observed with H_2O_2, the addition of CSE did not affect the % inhibition of this combination. Interestingly, the addition of PDE3/PDE4 inhibitors restored completely the % of inhibition of dexamthasone under oxidative stress conditions (Fig. 6A and 6B). In order to study the direct implication of HDAC on GC resistance, we used TSA as an inhibitor of its activity. Both GCs studied, budesonide and dexamethasone were almost without effect when TSA was added to cells 20 min before LPS. In these conditions, the % of inhibition of TNF-α and IL-8 were 35% and 39% for budesonide and 41% and 32% for dexamethasone (Fig. 6C and 6D; $P<0.05$ versus untreated cells with TSA).

Cells pre-treated with rolipram, PGE_2 or motapizone were not affected by TSA. However their % inhibition values remained at low levels between 8-27% (Fig. 6C and 6D). On the other hand, TSA effectively reduced the % inhibition of the combination of PGE_2 (10nM) with motapizone (10µM) and rolipram (1µM) at 36% for TNF-α and 35% for IL-8 (Fig. 6C and 6D; $P<0.05$ versus cells none treated with TSA), suggesting that the combination of these PDE3/PDE4 inhibitors with the cAMP enhancer PGE_2 do not directly modify the HDAC activity.

Effect of H_2O_2 and CSE on glucocorticoid repression of cytokine release by LPS in human alveolar macrophages

To approximate to the *in vivo* conditions we studied GC resistance in human AMs. As occurred in differentiated U793 cells, both H_2O_2 (500µM) and CSE (5%) increased TNF-α and IL-8 secretion induced by LPS by about 3 and 2-fold respectively (Fig. 7A to 7D). Dexamethasone and budesonide were able to nearly suppress the LPS-induced TNF-α and IL-8 secretion (Fig. 7A-7D). However, in presence of H_2O_2, budesonide and dexamethasone weakly reduced the LPS-induced TNF-α and IL-8, by about 42% and 22% respectively for TNF-α and by about 44% and 27% respectively for IL-8 (Fig. 7A and 7B). Similarly, rolipram, PGE_2 or motapizone only reached a 22% of inhibition for TNF-α and 36% of inhibition for IL-8% (Fig. 7A and 7B). In contrast, the combination of rolipram, PGE_2 and motapizone nearly suppressed TNF-α release by about 93%, and to a lesser extent IL-8 by about 77% (Fig. 7A and B). Results obtained when CSE was employed as an oxidative stress promoter were similar to those obtained with H_2O_2. In this regard, the combination of PDE3/PDE4 inhibitors and PGE_2 achieved a maximum % inhibition of 72% for both TNF-α and IL-8 which were significantly higher than those found for budesonide (about 39%) and dexamethasone (about 34%) for TNF-α

and IL-8 (Fig. 7C and D). Interestingly, in presence of oxidative stress, dual PDE3/PDE4 inhibition restored the efficacy of dexamethasone enabling it to inhibit both TNF-α and IL-8 (Fig. 7A to 7D).

HDAC activity is down-regulated by oxidative stress and rescue by the combination of PD3/PDE4 inhibitors.

Under oxidative stress conditions, GCs are less effective in preventing cytokine release unlike PD3/PDE4 inhibitors which nearly abolish them. Since HDAC is down-regulated by oxidative stress contributing to the increase of LPS-induced cytokine release, we studied the effect of GCs and PDE3/PDE4 inhibitors on HDAC activity. In presence of H_2O_2 or CSE followed by LPS for 24 hours, both budesonide and dexamethasone were unable to prevent the down-regulation of HDAC activity (Fig. 8A). Likewise, neither rolipram (1μM) nor PGE_2 (10nM) or motapizone (10μM) alone, was able to prevent the fall in HDAC activity. On the contrary, PDE3/PDE4 inhibitors in presence of the cAMP enhancer PGE_2 completely prevented the inhibition of HDAC activity induced by oxidative stress (Fig. 8B).

Discussion

This study was designed to establish an *in vitro* model of GCs resistance in AMs, to evaluate new effective and potential anti-inflammatory agents in those situations in which GCs are ineffective as occurs in airway diseases such as COPD and severe asthma [21]. Here, we show for the first time that the combination of the PDE3/PDE4 inhibitors effectively prevented cytokine release induced by the bacterial *endotoxin* LPS under oxidative stress conditions, where GCs were found weakly effective. Furthermore, PDE3/PDE4 inhibitors were able to prevent HDAC inactivation induced by oxidative stress which is one of the main mechanisms of GC resistance.

Interestingly, PDE4 inhibitor rolipram as well as PDE3 inhibitor motapizone alone were poorly effective on LPS-induced cytokine release in both, oxidative stress and non-oxidative contexts. This observation requires explanation. In contrast to monocytes (where PDE4 isoenzyme is abundant), both, differentiated monocytes and AMs showed a differential pattern of PDE expression. Thus, PDE1 is abundant in AMs followed by PDE3 and lower amounts of PDE4 [15, 18]. This data explain why in monocyte-derived macrophages and in AMs, neither PDE3 nor PDE4-inhibitors alone markedly prevented cytokine release [15]. In contrast, PDE4 inhibitors, unlike PDE3 inhibitors, are effective in monocytes preventing LPS-induced cytokine release [16].

In this work, we found that neither rolipram nor motapizone were able to effectively reduce cytokine release by LPS. In contrast, the association of both PDE3/PDE4 inhibitors in presence of the cAMP enhancer PGE_2, effectively suppressed (nearly 84-98% inhibition) the LPS-induced TNF-α and IL-8 secretion which was not modified by oxidative stress. Previous reports have shown the efficacy and additive effects between the dual PDE3/PDE4 inhibition in AMs as well as in other inflammatory and structural cells relevant to asthma and COPD [22]. Rational explanation for these findings is

based on the similar low expression of PDE3 and PDE4 found in AMs. Furthermore, PDE3 isoenzyme shows a dual action hydrolyzing both cAMP and cGMP, however it seems that the additive effect between PDE4 and PDE3 inhibitors in AMs activation is mainly due to the enhanced cAMP as previously outlined [23]. The presence of PGE_2 reinforces this idea. Previous reports have shown that exogenous PGE_2 potentially increases the effect of PDE3/PDE4 inhibitors in AMs [15, 24]. There are also studies that describe autocrine PGE_2 release as a cAMP enhancer [25]. Furthermore, PGE_2 has been found at nM concentrations in inflammatory conditions such as BAL from COPD patients [26]. Therefore, these observations are in agreement with the use of PGE_2 in *in vitro* models of AM activation. It is interesting to note that under oxidative stress conditions such as H_2O_2 or CSE, the PDE3/PDE4 inhibitors almost totally suppressed the TNF-α and IL-8 secretion induced by LPS in differentiated monocytes as well as in AMs. In contrast to these findings, GCs diminished their % inhibition by approximately 50% in oxidative stress conditions.

GC resistance is a common feature in airways diseases such as severe asthma and COPD, and although the clinical relevance of this topic is largely known, the molecular bases of their establishment are now being elucidated [7]. Indeed, it is known that oxidative stress impairs the activity of the GC receptor co-repressor HDAC-2 which reduces the ability of GCs to mediate trans-repression of pro-inflammatory genes [7-9, 21]. Therefore, oxidative stress and the consequent HDAC down-regulation is a pre-requisite to establish the GC resistance phenomenon. In this sense, we observed that it is necessary to create oxidative stress conditions (H_2O_2 or CSE) for a determined period of time (4 h) before the additive LPS effect could be noted. Thus, short periods of time (20 min) did not created additive effects between oxidative stress and LPS. In contrast, long periods of time (4 h), coincided with the time necessary to observe a significant

reduction of HDAC activity in macrophages, which were once absent at 20 min. These observations are in agreement with the reduced potency and efficacy of dexamethasone and budesonide to reduce the LPS-induced cytokine release in oxidative stress conditions. Similar results were found for bronchial epithelial cells, where a 4-6 h of H_2O_2 pre-treatment was necessary in order to observe additive effects on IL-1β-induced IL-8 secretion [27]. Furthermore, AMs from smokers or COPD patients showed as well, a reduced sensitivity to GCs [11, 28]. These data are according to the low HDAC activity found in AMs and biopsies from COPD patients [10]. Therefore, oxidative stress has a clear implication in HDAC inactivity and the consequent emergence of the GC resistance. In this line, the antioxidant glutation (GSH) effectively suppressed the CSE-induced impaired activity of HDAC in macrophages [8]. Similar results were found in BEAS-2B cells where the antioxidant N-acetyl-L-cysteine significantly prevented additive IL-8 release induced by H_2O_2 and IL-1β treatment [27].

However, it is interesting to note that, in some conditions, increasing the concentration of GC may nearly overcome the inhibitory effect of oxidative stress (see fig. 5A and B at 1μM of GCs). This could be explained by two hypothesis; first: it is known that oxidative stress reduces HDAC activity which causes GC receptor hyperacetylation and failure to switch off activated inflammatory genes. This effect may be overcome by enhancing GC concentration that in turn may couple to enough free non-acetylated GC receptor to do its inhibitory action. Second: GCs have potent anti-inflammatory effects on mitogen-activated protein kinase (MAPK) signalling pathways through the induction of MAP kinase phosphatase (MKP-1), and this may inhibit the expression of multiple inflammatory genes [29]. Therefore, high GC doses could activate this mechanism that in turn may overcome the inhibitory effect of oxidative stress. However the clinical

relevance of the administration of high doses of GC to overcome GC resistance is not applicable due to GC toxicity.

In this work, we have found that the combination of PDE3/PDE4 inhibitors effectively inhibited the TNF-α and IL-8 secretion induced by LPS in presence of oxidative stress unlike GCs. These findings were in accordance with those found for PDE3/PDE4 inhibitors which effectively preventing the HDAC down-regulation induced by oxidative stress and LPS. In this context, GCs were unable to prevent HDAC down-regulation. Several mechanisms may be behind this observation. In this regard, previous results showed that oxidative stress induces PIK3 activation subsequently phosphorylating PDE4 isoenzyme and reducing intracellular cAMP levels [13]. It has long been established that cAMP levels possess anti-inflammatory and antioxidant activities. In fact, PDE1 and PDE4 inhibitors were able to inhibit oxidative stress-induced TNF-α release in macrophages and alveolar epithelial cells [30]. On the other hand, PDE3/PDE4 inhibitor association was found to be effective preventing superoxide generation in AMs [31]. Since PDE3/PDE4 inhibition reduces oxidative stress in AMs and oxidative stress reduces HDAC activity which is responsible of the GC resistance, thus mechanistic action of PDE3/PDE4 inhibition seems to be centre in their antioxidant properties. Furthermore, it is known that oxidative stress activates PIK3 which inactivates HDAC causing GC resistance [12]. Whether PDE3/PDE4 may inhibit PIK3 activity through its antioxidant activity remains to be elucidated.

Results of the *in vitro* experiments presented here, are an attempt to approximate to the *in vivo* conditions with the corresponding limitations. In this respect, *in vivo* alveolar macrophages are undergoing different stimulus such as IL-1β or TNF-α among others that may stimulate alveolar macrophages, under oxidative stress conditions, in a different fashion to that observed for the *endotoxin* LPS as occurs in the present work.

Therefore, PDE4/PDE3 inhibition could show an impaired inhibitory response compared to that observed in our experimental conditions. Furthermore, CSE is a liquid phase extract that contains aqueous soluble and volatile compounds that are retained for almost 30 minutes [32, 33]. In our CSE preparation, the particulate phase and lipophilic components of the cigarette smoke are absent, so it is almost difficult to exactly match the *in vivo* exposure from the *in vitro* conditions.

Despite the limitations of this study, PDE3/PDE4-inhibitor association has shown to inhibit several inflammatory and remodelling outputs underlying COPD [22]. In this sense, the development of inhaled agents at low doses coupled with a rapid systemic clearance could prove effective in counteracting adverse effects observed from the current PDE3 and PDE4 inhibitors.

Clinical relevance: PDE3/PDE4 inhibitors may rescue GC resistance in AM *in vitro*, in those situations of inflammation and oxidative stress. Therefore, PDE3/PDE4 inhibitors could be of potential value in those clinical situations in which chronic airway inflammation is poorly controlled by GCs such as COPD and severe asthma.

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Legend to figures

Figure 1. H_2O_2 enhances the LPS-induced IL-8 and TNF-α secretion in differentiated U937 macrophages. IL-8 and TNF-α were measured by ELISA in cell supernatants. A and B) H_2O dose-dependently increases IL-8 and TNF-α secretion. C and D) H_2O_2 pre-treatment increased the LPS-induced IL-8 and TNF-α secretion in a dose-dependent manner, being significant after H_2O_2 500µM during 4h of pre-treatment. Results are the mean±SEM of three different experiments per condition. *P<0.05 *vs*. cells exposed to H_2O_2 during 20 min.

Figure 2. CSE enhances the LPS-induced IL-8 and TNF-α secretion in differentiated U937 macrophages. A and B) CSE dose-dependently increases IL-8 and TNF-α secretion. C and D) CSE pre-treatment increased the LPS-induced IL-8 and TNF-α secretion in a dose-dependent manner, being significant after CSE 5% during 4h of pre-treatment. Results are the mean±SEM of four different experiments per condition. *P<0.05 *vs*. cells exposed to CSE during 20 min.

Figure 3. TSA pre-treatment effectively increases the LPS-induced IL-8 and TNF-α secretion in differentiated U937 macrophages.

Differentiated U937 cells where pre-treated with trichostatin A (TSA) at different doses for 20 min and then stimulated with LPS at 500 ng/mL for further 24 h. Cell culture supernatants were collected and IL-8 (panel A) and TNF-α (panel B) protein levels were measured by ELISA. Results are the mean±SEM of four different experiments per condition. *P<0.05 *vs*. cells exposed to TSA alone.

Figure 4. Oxidative stress reduces HDAC activity in differentiated U937 macrophages. Differentiated U937 cells were treated for 20 min, 4 h or 24 h with H_2O_2 500µM, CSE 5% or TSA (100ng/ml), then nuclear protein was extracted and HDAC activity was quantified by colorimetric assay system. Results are the mean±SEM of four different experiments per condition. *P<0.05 *vs*. control.

Figure 5. H_2O_2 reduces the potency of glucocorticoids but not of the PDE3/PDE4 inhibitors in a model of LPS-induced cytokine release.

Differentiated U937 cells were pre-incubated with drugs for 15 min and then exposed or not to H_2O_2 500µM for 4h. Then, LPS (0.5 µg/mL) was added for 24 h. Different drug combinations where as follow: panels A and B) dexamethasone (DEX; 10^{-9}-10^{-6}M) and budesonide (BUD; 10^{-9}-10^{-6}M) plus LPS in presence (▲ for DEX and ■ for BUD) or absence (Δ for DEX and □ for BUD) of H_2O_2. Panels C and D) rolipram (ROL; 10^{-9}-10^{-5}M) plus LPS in (■) presence or (●) absence of H_2O_2. Combination of PDE4 inhibitor rolipram (10^{-9}-10^{-5}M) with PDE3 inhibitor motapizone (10µM) in presence of PGE_2 (10 nM) plus LPS (□) without or (Δ) with H_2O_2. Effects of PGE_2 (10nM) plus LPS (▲) with or (▼) without H_2O_2, or motapizone (10µM) plus LPS in (◊) presence or (♦) absence of H_2O_2 were largely ineffective and were not modified by H_2O_2. Cell supernatants were collected after 24h of cell stimulation and were used for IL-8 and TNF-α assays. Results are the mean±SEM of % of inhibition of LPS-induced IL-8 or TNF-α from three to five different experiments per condition.

Figure 6. CSE reduces the % inhibition of glucocorticoids but not of the PDE3/PDE4 inhibitors in a model of LPS-induced cytokine release.

Panels A and B) differentiated U937 cells were pre-incubated with drugs for 15 min and then exposed or not to CSE 5% for 4h (panels A and B) or to trichostatin A (TSA) 100 ng/mL for 20 min (panels C and D). Then, LPS (0.5 µg/mL) was added for 24 h. Different drug combinations where as follow: dexamethasone (DEX; 10^{-6}M), budesonide (BUD; 10^{-7}M), rolipram (ROL; 10^{-6}M), PGE_2 (10nM), motapizone (MOTA; 10µM), MIX (composed by rolipram (10^{-6}M) + PGE_2 (10 nM) + motapizone (10µM)) or DEX (10^{-6}M) + MIX. Cell supernatants were collected after 24h of stimulation and were used for IL-8 and TNF-α assays. Results are the mean±SEM of % of inhibition of LPS-induced IL-8 or TNF-α from three to five different experiments per condition. *P<0.05 *vs*. cells not exposed to CSE or TSA.

Figure 7. Oxidative stress impairs glucocorticoid but not PDE3/PDE4 inhibitory effect on LPS-induced IL-8 and TNF-α secretion in human alveolar macrophages. Human alveolar macrophages were pre-incubated with drugs for 15 min and then exposed or not to H_2O_2 500µM (panels A and B) or CSE 5% for 4h (panels C and D). Then, LPS (0.5 µg/mL) was added for 24 h. Different drug combinations where as follow: dexamethasone (DEX; 10^{-6}M), budesonide (BUD; 10^{-7}M), rolipram (ROL; 10^{-6}M), PGE_2 (10nM), motapizone (MOTA; 10µM), MIX (composed by rolipram (10^{-6}M) + PGE_2 (10 nM) + motapizone (10µM)) or DEX (10^{-6}M) + MIX. Cell supernatants were collected after 24h of stimulation and were used for IL-8 and TNF-α assays. Results are the mean±SEM of three to five different experiments per condition. *P<0.05 *vs*. control. #P<0.05 *vs*. cells stimulated with LPS alone. ♦P<0.05 *vs*. cells treated with glucocorticoids or PDE3/PDE4 inhibitors alone in presence of LPS and H_2O_2.

Figure 8. Oxidative stress impairs glucocorticoid but not PDE3/PDE4 inhibitory effect on LPS-induced IL-8 and TNF-α secretion in human alveolar macrophages.

Differentiated U937 cells were pre-treated with drugs for 15 min and exposed to oxidants H_2O_2 500µM or CSE 5% for 4 h before LPS (0.5 µg/mL) was added for 24 h. Nuclear protein was then extracted and HDAC was quantified as defined in methods. Different drug combinations where as follow: panel A) budesonide (BUD; 10^{-7}M) or dexamethasone (DEX; 10^{-6}M). Panel B) rolipram (ROL; 10^{-6}M), PGE_2 (10nM), motapizone (MOTA; 10µM) or MIX (formed by rolipram (10^{-6}M) + PGE_2 (10 nM) + motapizone (10µM)). Results are the mean±SEM of three to five different experiments per condition. *P<0.05 *vs*. cells not exposed to oxidants.

Table 1.

Clinical features. FEV1: forced expiratory volume in one second; FVC: forced vital capacity; PaO_2: oxygen tension in arterial blood; $PaCO_2$: carbon dioxide tension in arterial blood; Pack-yr = 1 year smoking 20 cigarettes-day; TLC: total lung capacity. Data are mean ± SEM for 8 subjects.

Characteristics	**Subjects (n=8)**
Median age, years	67±7
Male/female ratio	7/1
Tobacco consumption, pack-yr	0
FEV1, % pred	90.1± 6
FVC, % pred	93.3±7
FEV1/FVC %	87.7±4
TLC %pred	89±6
PaO_2, mmHg	95±7
$PaCO_2$ mmHg	38±5

Table 2. Maximal efficacy and –log IC_{50} of dexamethasone (DEX), budesonide (BUD), rolipram (ROL), or MIX (combination of PGE_2 (10nM), motapizone (10μM) and rolipram (10^{-9}M-10^{-5}M)). Results are expressed as mean±SEM of n experiments per condition. **P*=0.001 and #*P*=0.01 *vs*. cells not treated with H_2O .

	IL-8			TNF-α		
	n	Maximal inhibition (%)	-log IC_{50}	n	Maximal inhibition (%)	-log IC_{50}
BUD + LPS	6	86.8±0.79	8.4±0.09	6	90.4± 2.6	8.1±0.21
BUD + H_2O_2 + LPS	6	79.2±1.71*	7.7±0.09*	6	68.1±2.4*	7.8±0.17*
DEX+ LPS	5	79±2.5	7.98±0.13	5	77.1±0.79	7.98±0.05
DEX + H_2O_2 + LPS	5	59.7±3.14#	7.75±0.19#	5	62±0.29#	7.7±0.01#
ROL + LPS	8	21.3±0.07	6.72±0.21	8	21.71±0.71	6.72±0.08
ROL + H_2O_2 + LPS	8	19.98±0.38	6.79±0.05	8	23.68±1.58	6.88±0.17
MIX + LPS	7	92.9±1.2	7.66±0.06	7	97.86±3.8	7.70±0.19
MIX + H_2O_2 + LPS	7	93.4±2.1	7.85±0.1	7	93.41±2.8	7.68±0.16

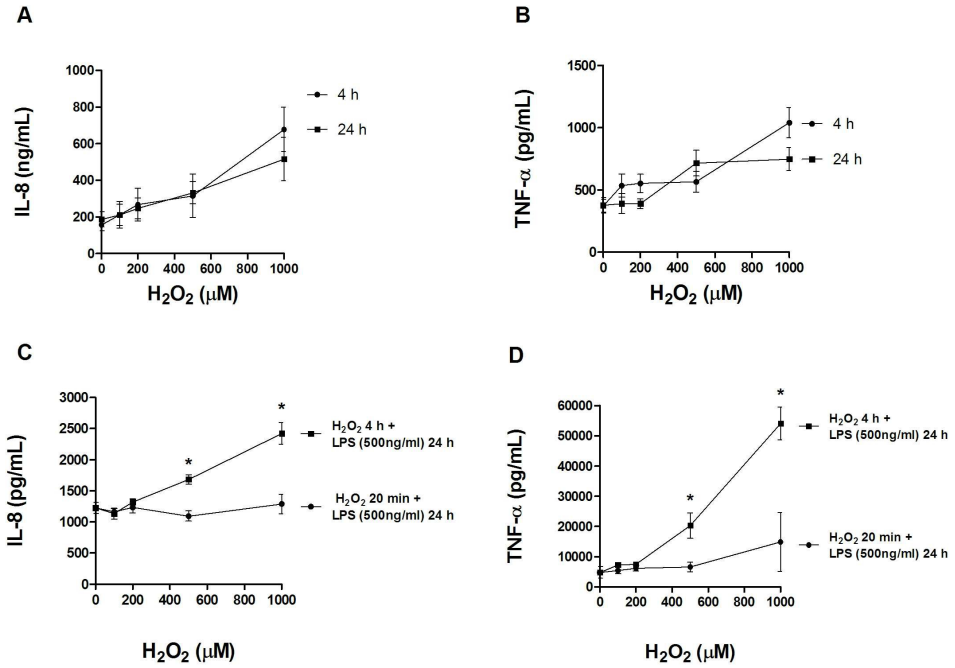

Figure 1

H2O2 enhances the LPS-induced IL-8 and TNF-α secretion in differentiated U937 macrophages. IL-8 and TNF-α were measured by ELISA in cell supernatants. A and B) H2O2 dose-dependently increases IL-8 and TNF-α secretion. C and D) H2O2 pre-treatment increased the LPS-induced IL-8 and TNF-α secretion in a dose-dependent manner, being significant after H2O2 500μM during 4h of pre-treatment. Results are the mean±SEM of three different experiments per condition. *P<0.05 vs. cells exposed to H2O2 during 20 min.
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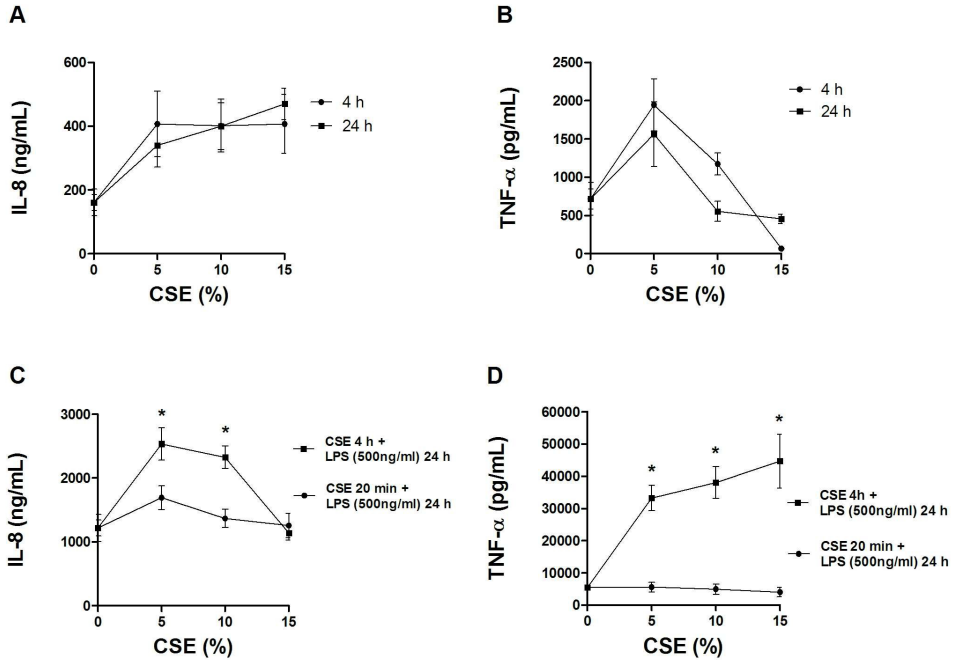

Figure 2

CSE enhances the LPS-induced IL-8 and TNF-α secretion in differentiated U937 macrophages. A and B) CSE dose-dependently increases IL-8 and TNF-α secretion. C and D) CSE pre-treatment increased the LPS-induced IL-8 and TNF-α secretion in a dose-dependent manner, being significant after CSE 5% during 4h of pre-treatment. Results are the mean±SEM of four different experiments per condition. *P<0.05 vs. cells exposed to CSE during 20 min.
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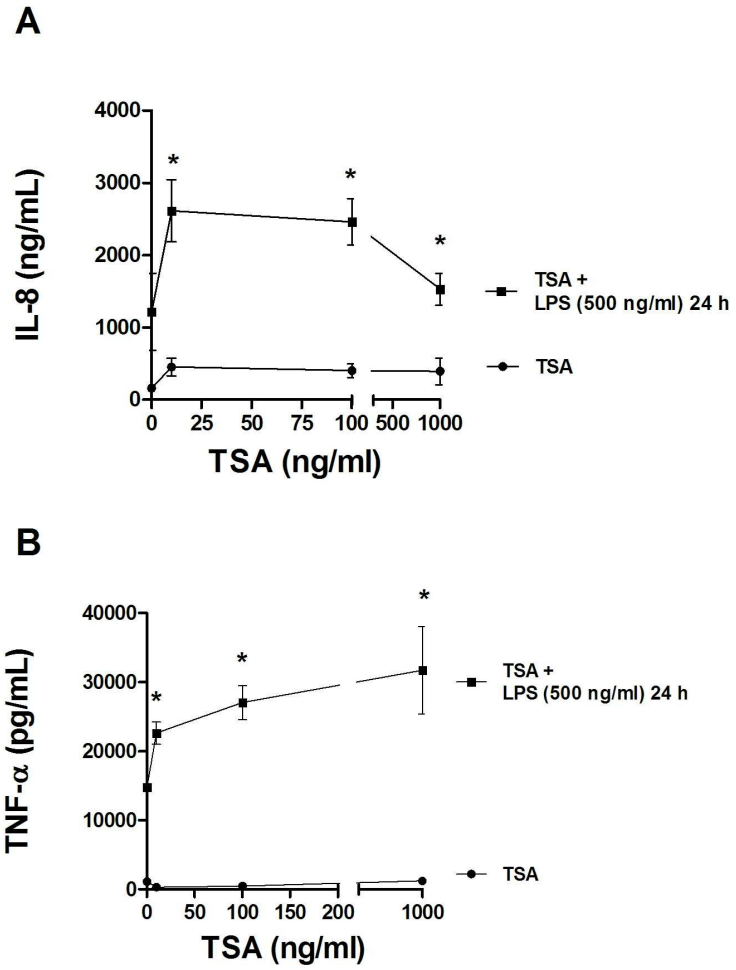

Figure 3

TSA pre-treatment effectively increases the LPS-induced IL-8 and TNF-α secretion in differentiated U937 macrophages.

Differentiated U937 cells where pre-treated with trichostatin A (TSA) at different doses for 20 min and then stimulated with LPS at 500 ng/mL for further 24 h. Cell culture supernatants were collected and IL-8 (panel A) and TNF-α (panel B) protein levels were measured by ELISA. Results are the mean±SEM of four different experiments per condition. *P<0.05 vs. cells exposed to TSA alone.

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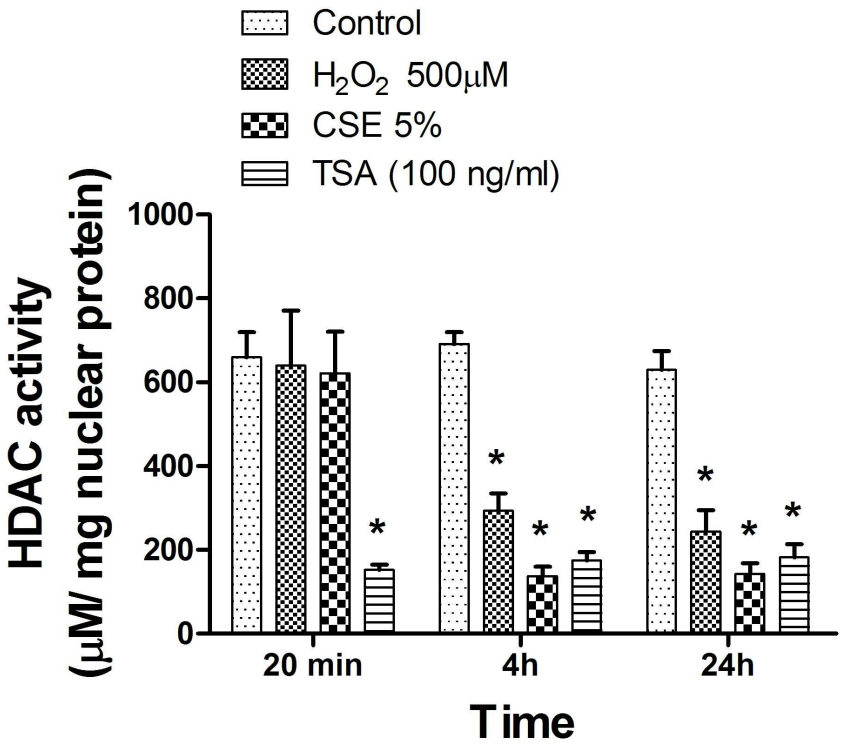

Figure 4

Oxidative stress reduces HDAC activity in differentiated U937 macrophages. Differentiated U937 cells were treated for 20 min, 4 h or 24 h with H2O2 500µM, CSE 5% or TSA (100ng/ml), then nuclear protein was extracted and HDAC activity was quantified by colorimetric assay system. Results are the mean±SEM of four different experiments per condition. *P<0.05 vs. control
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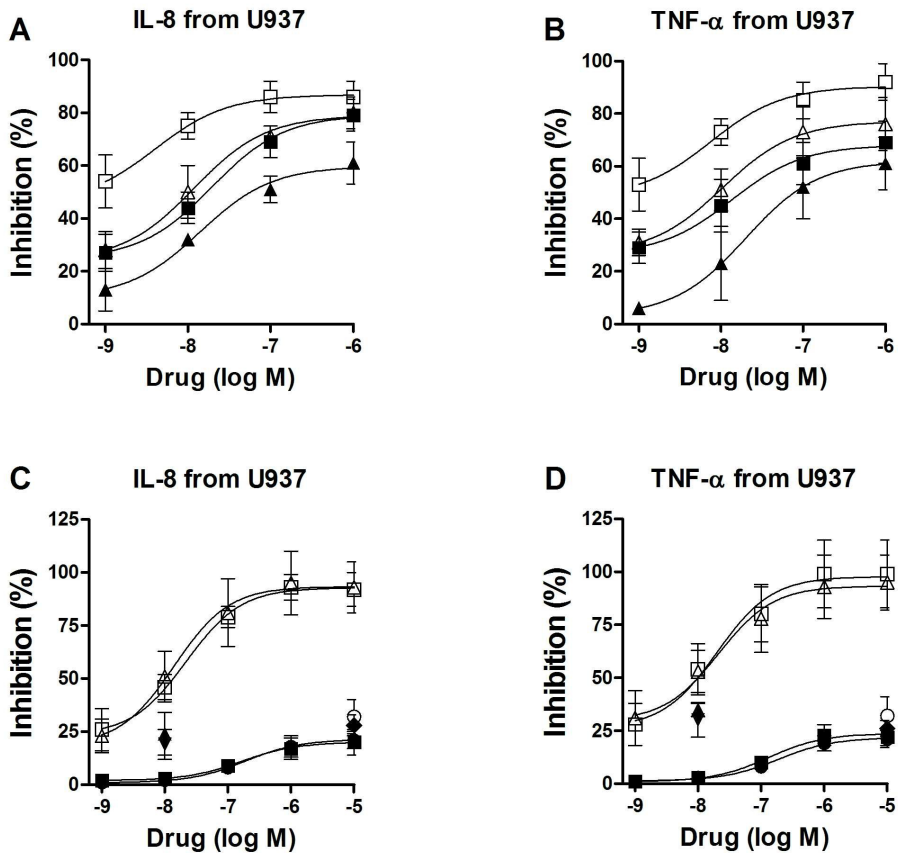

Figure 5

H2O2 reduces the potency of glucocorticoids but not of the PDE3/PDE4 inhibitors in a model of LPS-induced cytokine release.

Differentiated U937 cells were pre-incubated with drugs for 15 min and then exposed or not to H2O2 500μM for 4h. Then, LPS (0.5 μg/mL) was added for 24 h. Different drug combinations where as follow: panels A and B) dexamethasone (DEX; 10-9-10-6M) and budesonide (BUD; 10-9-10-6M) plus LPS in presence (▲ for DEX and ■ for BUD) or absence (Δ for DEX and □ for BUD) of H2O2. Panels C and D) rolipram (ROL; 10-9-10-5M) plus LPS in (■) presence or (●) absence of H2O2. Combination of PDE4 inhibitor rolipram (10-9-10-5M) with PDE3 inhibitor motapizone (10μM) in presence of PGE2 (10 nM) plus LPS (□) without or (Δ) with H2O2. Effects of PGE2 (10nM) plus LPS (▲) with or (▼) without H2O2, or motapizone (10μM) plus LPS in (○) presence or (♦) absence of H2O2 were largely ineffective and were not modified by H2O2. Cell supernatants were collected after 24h of cell stimulation and were used for IL-8 and TNF-α assays. Results are the mean±SEM of % of inhibition of LPS-induced IL-8 or TNF-α from three to five different experiments per condition.

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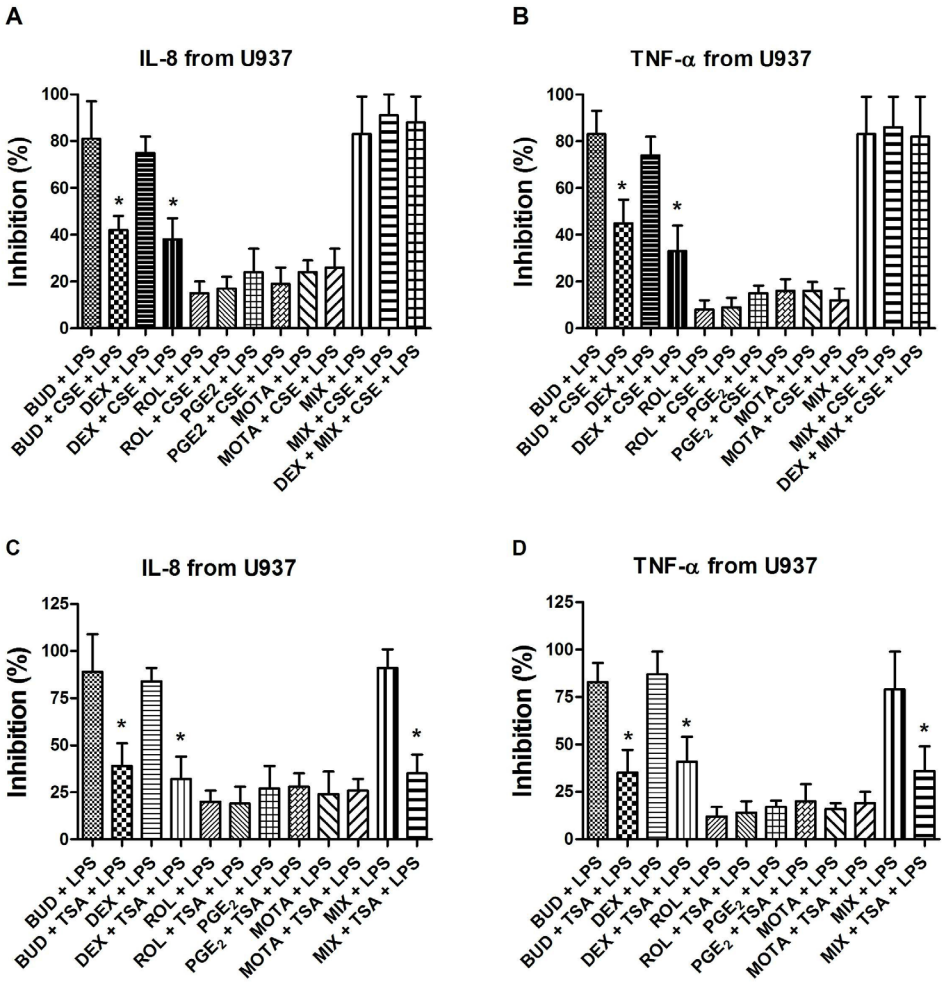

Figure 6

CSE reduces the % inhibition of glucocorticoids but not of the PDE3/PDE4 inhibitors in a model of LPS-induced cytokine release.
Panels A and B) differentiated U937 cells were pre-incubated with drugs for 15 min and then exposed or not to CSE 5% for 4h (panels A and B) or to trichostatin A (TSA) 100 ng/mL for 20 min (panels C and D). Then, LPS (0.5 µg/mL) was added for 24 h. Different drug combinations where as follow: dexamethasone (DEX; 10-6M), budesonide (BUD; 10-7M), rolipram (ROL; 10-6M), PGE2 (10nM), motapizone (MOTA; 10µM), MIX (composed by rolipram (10-6M) + PGE2 (10 nM) + motapizone (10µM)) or DEX (10-6M) + MIX. Cell supernatants were collected after 24h of stimulation and were used for IL-8 and TNF-α assays. Results are the mean±SEM of % of inhibition of LPS-induced IL-8 or TNF-α from three to five different experiments per condition. *P<0.05 vs. cells not exposed to CSE or TSA.

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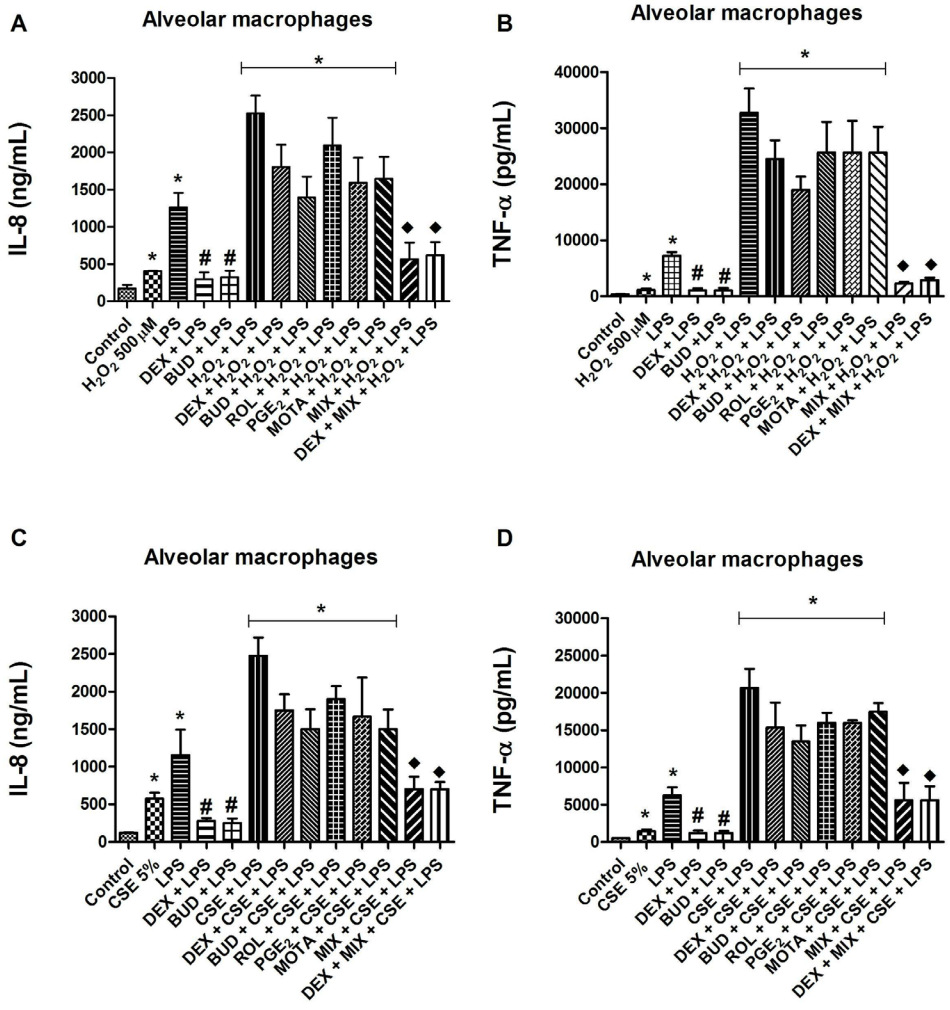

Figure 7

Oxidative stress impairs glucocorticoid but not PDE3/PDE4 inhibitory effect on LPS-induced IL-8 and TNF-α secretion in human alveolar macrophages.

Human alveolar macrophages were pre-incubated with drugs for 15 min and then exposed or not to H2O2 500μM (panels A and B) or CSE 5% for 4h (panels C and D). Then, LPS (0.5 μg/mL) was added for 24 h. Different drug combinations where as follow: dexamethasone (DEX; 10-6M), budesonide (BUD; 10-7M), rolipram (ROL; 10-6M), PGE2 (10nM), motapizone (MOTA; 10μM), MIX (composed by rolipram (10-6M) + PGE2 (10 nM) + motapizone (10μM)) or DEX (10-6M) + MIX. Cell supernatants were collected after 24h of stimulation and were used for IL-8 and TNF-α assays. Results are the mean±SEM of three to five different experiments per condition. *P<0.05 vs. control. #P<0.05 vs. cells stimulated with LPS alone. ♦P<0.05 vs. cells treated with glucocorticoids or PDE3/PDE4 inhibitors alone in presence of LPS and H2O2.

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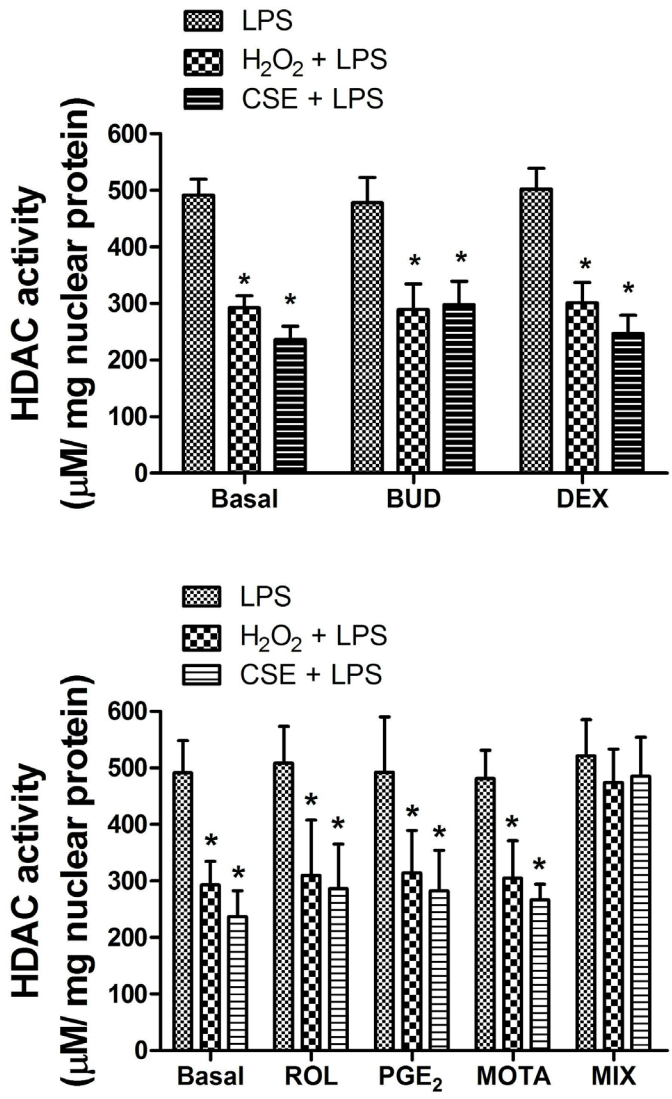

Figure 8

Oxidative stress impairs glucocorticoid but not PDE3/PDE4 inhibitory effect on LPS-induced IL-8 and TNF-α secretion in human alveolar macrophages.
Differentiated U937 cells were pre-treated with drugs for 15 min and exposed to oxidants H2O2 500μM or CSE 5% for 4 h before LPS (0.5 μg/mL) was added for 24 h. Nuclear protein was then extracted and HDAC was quantified as defined in methods. Different drug combinations where as follow: panel A) budesonide (BUD; 10-7M) or dexamethasone (DEX; 10-6M). Panel B) rolipram (ROL; 10-6M), PGE2 (10nM), motapizone (MOTA; 10μM) or MIX (formed by rolipram (10-6M) + PGE2 (10 nM) + motapizone (10μM)). Results are the mean±SEM of three to five different experiments per condition. *P<0.05 vs. cells not exposed to oxidants.

181x271mm (300 x 300 DPI)