ERJ Express. Published on May 3, 2011 as doi: 10.1183/09031936.00171110

Title:

TACE/TGF-α/EGFR regulates CXCL8 in bronchial epithelial cells exposed to PM-components

Running head:

TACE/TGF-α/EGFR regulates PM-induced CXCL8

Authors:

J. Øvrevik, M. Refsnes, A.I. Totlandsdal, J.A. Holme, P.E. Schwarze and M. Låg

Affiliation:

Department of Air Pollution and Noise, Division of Environmental Medicine, Norwegian Institute of Public Health.

Corresponding author:

Johan Øvrevik

Department of Air Pollution and Noise

Division of Environmental Medicine

Norwegian Institute of Public Health

P.O.Box 4404 Nydalen

N-0403 Oslo, Norway

E-mail: johan.ovrevik@fhi.no

Phone: +47 21076408

Fax: +47 21076686

Abstract

Airborne particulate matter (PM) may induce or exacerbate neutrophilic airway disease by triggering release of inflammatory mediators such as CXCL8 from the airway epithelium. It is still unclear which PM-components are driving CXCL8-responses as most candidates occur at low concentrations in the dusts. We therefore hypothesized that different PM-constituents may contribute through common mechanisms to induce CXCL8.

Human bronchial epithelial cells (BEAS-2B) were exposed to different PM-components (zinc/iron salts, 1-nitropyrene, lipopolysaccharide, diesel exhaust/mineral particles). Gene-expression patterns were detected by real-time PCR-array. CXCL8-responses were measured by real-time PCR and ELISA. CXCL8-regulation was assessed with a broad inhibitor-panel and neutralizing antibodies. EGFR-phosphorylation was examined by immunoprecipitation and Western-blotting.

Component-induced gene-expression was mainly linked to NF-κB, Ca^{2+}/PKC, PLC, LDL and mitogenic signalling. Many inhibitors attenuated CXCL8-release induced by all PM-components, but to varying extent. However, EGFR-inhibition strongly reduced CXCL8-release induced by all test-compounds and selected compounds increased EGFR-phosphorylation. Interference with TGF-α or TNF-α converting enzyme (TACE), which mediates TGF-α ectodomain-shedding, also attenuated CXCL8-release.

Different PM-constituents induced CXCL8 partly through similar signalling pathways but the relative importance of the different pathways varied. However, TACE/TGF-α/EGFR-signalling appears to be a convergent pathway regulating innate immune responses of airway epithelial cells upon exposure to multiple airborne pollutants.

Introduction

Airborne particulate matter (PM) may induce or exacerbate airway diseases through pulmonary inflammation-reactions. The airway epithelium plays a key role in these processes by regulating innate immune responses upon inhalation of noxious compounds [1-3]. Epithelial cells express a number of pattern-recognition receptors, including Toll-like receptors (TLRs), which recognise conserved pathogen motifs and trigger host responses mediated by various effectors such as cytokines and chemokines [4]. We have recently shown that increased levels of the neutrophil-selective chemokine CXCL8/Interleukin-8 is a dominating pro-inflammatory response to PM and PM-components of bronchial epithelial BEAS-2B cells [5-7]. Markedly elevated CXCL8-levels along with neutrophilia is also characteristic of airway diseases such as chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and severe asthma [3, 8, 9]. The importance of neutrophils in pulmonary diseases has gained increased attention as neutrophilic inflammation appears to be resistant towards corticosteroid treatment, and CXCL8 is therefore considered a potential therapeutic target [9, 10]. CXCL8 may also contribute to cancer development through its pro-inflammatory effects along with its potential role as a mitogenic, angiogenic and motogenic factor [11].

Multiple signalling pathways contribute in CXCL8-regulation. In most cell types, its transcription depends on nuclear factor (NF)-κB activation, while activator protein (AP)-1 and CCAAT/enhancer-binding protein (C/EBP) are required for maximal gene expression [12]. Maximal CXCL8-responses further require a combined activation of the three major MAPK-cascades: ERK1/2, JNK, and p38 [13, 14]. A range of studies also suggests involvement of the epidermal growth factor receptor (EGFR) in CXCL8-responses induced by different agents [15-21], and EGFR- and CXCL8-expression correlate in the airway epithelium of patients with CF and severe asthma [22, 23]. Accordingly, EGFR-signalling has been suggested as a convergent pathway for regulation of CXCL8 and other immune responses [24]. Recent studies with endogenous and microbiological ligands have shown that CXCL8-regulation by EGFR involves TGF-α ectodomain-shedding by the metalloprotease TNF-α converting enzyme (TACE) or ADAM-17 [20, 21, 25, 26]. Whether organic or inorganic PM-components may regulate CXCL8 through similar mechanisms remains to be elucidated.

Airborne PM is a complex, variable mixture containing some (by mass) minor components with considerable gene-regulatory potential. These include endotoxins, metals, organics such as polycyclic aromatic hydrocarbons (PAHs), and ultrafine combustion-derived particles [27]. Clarifying which constituents of PM are causing the adverse pulmonary effects is important to allow for more effective abatement strategies to improve outdoor air quality, while elucidating the underlying mechanisms for their effects may improve treatment of sensitive groups. A particular puzzle is that PM from different locations have showed surprisingly little variation in risk estimates, despite considerable variation in the composition of the dust [27]. We hypothesized that different PM-components contribute through common mechanisms to induce CXCL8-responses in airway epithelial cells. If several components act through similar mechanism this could explain why PM may elicit effects despite low concentrations of its individual active constituents, and also why variation in PM-composition not necessarily leads to considerable variation in effects. To assess this, we have investigated CXCL8-regulation in BEAS-2B cells. Crystalline silica, zinc and iron salts, 1-nitropyrene (1-NP) and bacterial lipopolysaccharide (LPS) were chosen to model different components of PM [5]. Comparative experiments with diesel exhaust particles (DEP) were also performed.

Materials and methods

Reagents

Crystalline silica (MIN-U-SIL® 5) was kindly provided by U.S. Silica Company (Maryland, USA). DEP was kindly provided by Dr. F.R. Cassee at the National Institute for Public Health and the Environment (RIVM; Bilthoven, The Netherlands). $ZnCl_2$, $FeSO_4$, 1-NP, LPS and BAPTA-AM were from Sigma-Aldrich (St. Louis, MO, USA). SB202190, PD98059, SP600125, PP2, AG1478, Gö6976, rottlerin, GF109203, H-8 wortmannin, AG490, verapamil, W7, curcumin, TAPI-1 and TGF-α-neutralizing antibody were all from Calbiochem (La Jolla, CA, USA). EGFR-neutralizing antibody (LA1) was from Millipore (Billerica, MA, USA). All other chemicals used were purchased from commercial sources at the highest purity available.

Cell cultures and exposures

BEAS-2B cells, a SV40 hybrid (Ad12SV40) transformed human bronchial epithelial cell line, were purchased from American Tissue Type Culture Collection (ATCC, Rockville, MD, USA) and grown to near confluence in serum-free LHC-9 medium (Biosource, CA, USA) at 37°C in a humidified atmosphere of 5% CO_2 in air, prior to exposure. Concentrations and incubation times for both the test compounds and inhibitors/neutralizing antibodies were based on previous studies with BEAS-2B or other epithelial lung cells [5, 7, 18, 28-33], or initial screenings in the BEAS-2B cells (data not shown).

Gene expression

Total RNA was isolated by use of Absolutely RNA Miniprep Kits (Stratagene, La Jolla, CA, USA), and reverse transcribed to cDNA on a PCR system 2400 (PerkinElmer) by using a High Capacity cDNA Archive Kit (Applied Biosystems). Gene expression was screened by use of the "Signal Transduction PathwayFinder" real-time PCR array, #PAHS-014 (SABioscience (QIAGEN), Frederick, MD, USA), run on an ABI 7500 fast (Applied Biosystems, Foster City, CA, USA), and normalized against average expression housekeeping genes (B2M, HPRT1, RPL13A, GAPDH, and ACTB). Results were expressed as fold change compared to untreated control. Genes were grouped under signaling pathways according to suggestions by the manufacturer (SABioscience). DEP-induced CXCL8-expression was measured by pre-designed TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix (Applied Biosystems). IL-8-expression (Hs00174103_m1) was normalized against 18S rRNA (18S, Hs99999901_s1).

CXCL8-release. CXCL8-protein levels in cell-supernatants were determined by ELISA (Biosource International, Camarillo, CA, USA.) as described elsewhere [5]. Absorbance was measured using a plate reader (TECAN Sunrise, Phoenix Research Products, Hayward, CA, USA) complete with software (Magellan V 1.10).

Immunoprecipitation (IP)

EGFR was immunoprecipitated as described elsewhere [18]. In brief, cells were washed twice in ice-cold PBS, once in ice-cold IP-buffer and, lysed in IP-buffer with 1% NP-40. Cell lysates were centrifuged at 2500x *g* for 10 min, incubated with anti-EGFR antibodies (Cell Signalling Technology, Beverly, MA, USA) for 1 h and incubated overnight with magnetic beads. The complexes were washed in IP-buffer containing 0.5% NP-40 and immunoprecipitates were released by boiling in 2x Laemmli buffer for 5 min. EGFR-phosphorylation was detected by Western blotting with anti-phospho-tyrosine antibodies (P-Tyr-100; Cell Signalling Technology).

Statistical analysis

Statistical significance was evaluated by GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA) using analysis of variance (ANOVA) with Bonferroni post-test.

Results

Component-induced gene expression profiles

To compare effects of crystalline silica particles (silica), Zn^{2+}, Fe^{2+}, 1-NP and LPS on cell signalling pathways in BEAS-2B cells, changes in expression of 84 genes were examined. As shown in Fig. 1, 26 genes were up-regulated more than 2-fold after exposure to at least one of the compounds, while 12 were down-regulated more than 2-fold compared to controls. The results revealed a considerable quantitative variation, with 1-NP and silica in general inducing the strongest responses, and some qualitative variation in the types of genes activated. In agreement with previous findings [5], CXCL8 was among the most responsive genes. In addition, all compounds induced a marked up-regulation of CCL20 (MIP-3α), TNF-α, CSF2 (granulocyte-macrophage colony stimulating factor: GM-CSF), JUN (c-Jun: a main AP-1 subunit), and the anti-apoptotic gene BCL2A1 (less reliable due to low gene expression levels in most samples). Since the results are based on examination of effects of only one concentration at one time-point, some caution should be taken when interpreting the mRNA

data. However, the relative effects on CXCL8, CCL20 and TNF-α are comparable to previous findings [5].

As a first step to identify intracellular signalling pathways induced by the test-compounds, the 84 genes were clustered according to common upstream regulation mechanisms. As shown in Table 1, the different exposures predominantly induced up-regulation of genes linked to the NF-κB, Ca^{2+}/PKC, PLC, LDL and mitogenic pathways.

Screening signalling pathways involved in CXCL8-regulation

All test-compounds also induced a significant increase in CXCL8 protein-release (Fig. 2). To further examine the mechanisms of CXCL8-regulation, BEAS-2B cells were exposed to the different test-compounds in combination with a battery of 15 inhibitors, targeting various signalling pathways (Table 2). Generally, the inhibitors did not seem to affect cell viability as judged by microscopy (decrease in cell density and increase in rounded/floating cells), except the Ca^{2+}-chelator BAPTA-AM and the JAK-inhibitor AG 490 which had some toxicity (not quantified). Furthermore, since the screening was based on single concentrations and one incubation time, the results should be interpreted with some caution.

Whereas the gene expression data indicated activation of Ca^{2+}/PKC and mitogenic signalling, the inhibitor-screen further suggested that these pathways were involved in CXCL8-regulation (Fig. 3). Overall, the results show that many of the same inhibitors reduced CXCL8-release induced by different test-compounds, but that the relative effects varied considerably (Fig. 3). Inhibitors of the three main MAPK-pathways were generally effective inhibitors of CXCL8-release. However, whereas p38-inhibition significantly attenuated CXCL8 induced by silica, Zn^{2+}, Fe^{2+} and 1-NP, only marginal effects were observed for LPS. Moreover, ERK- and JNK-inhibition significantly reduced the effects of Fe^{2+}, 1-NP and LPS, but did not affect silica and Zn^{2+}-induced responses significantly. The PKCα/β-inhibitor increased background levels of CXCL8 in controls and boosted the effects of Zn^{2+} and LPS. Similarly, the JAK2/3-inhibitor increased background levels of CXCL8, suggesting that both PKCα/β and JAK2/3 under normal conditions suppress CXCL8-release in BEAS-2B cells. However, both PKCα/β- and JAK2/3-inhibitors attenuated CXCL8-induction by 1-NP. Whether this may be due to unspecific effects of the inhibitors, or linked to the ability of 1-NP to induce CXCL8 remains to be clarified.

Despite some differences, the inhibitor-screen also revealed apparent similarities: CXCL8-release induced by all compounds was significantly attenuated by a general PKC-inhibitor. Moreover, the PKCδ/θ-inhibitor rottlerin appeared to reduce CXCL8 levels induced by all test-compounds, except LPS. Another general observation was that the intracellular Ca^{2+}-chelator BAPTA-AM more or less blocked CXCL8-release by all compounds, although only Fe^{2+}-induced CXCL8 was reduced by inhibitors of Ca^{2+}-channels and calmodulin (Fig. 3). Some caution should however be taken regarding the effect of BAPTA-AM, since it seemed to increase cytotoxicity (data not shown). The anti-inflammatory compound curcumin which affects multiple intracellular targets including NF-κB and AP-1 [12, 34, 35], also displayed a broad CXCL8-inhibitory potential. Notably, the EGFR-inhibitor AG1478 more or less completely blocked the effects of silica, Fe^{2+} and LPS on CXCL8, and strongly down-regulated the effects of Zn^{2+} and 1-NP (Fig. 3), suggesting that EGFR is a central regulator of CXCL8 in BEAS-2B cells.

Role of the TACE/ TGF-α /EGFR pathway in CXCL8-regulation

The role of EGFR in CXCL8-regulation was further assessed by use of an EGFR-neutralizing antibody and EGFR-phosphorylation was examined by immunoprecipitation (Fig. 4). Blocking EGFR ligand-binding reduced CXCL8-induction by all exposures. Moreover, selected test-compounds (silica and Zn^{2+}) also induced EGFR-phosphorylation (Fig. 4). Thus, the role of EGFR in CXCL8-regulation appeared to involve ligand-binding and receptor activation. Based on this, we hypothesized that the TACE-mediated cleavage of pro-TGF-α could be a common mechanism of CXCL8-regulation by PM-components. Accordingly, the TACE-inhibitor TAPI-1 and a TGF-α-neutralizing antibody both attenuated CXCL8-release induced by all test-compounds, except Fe^{2+} (Fig. 5). The lack of effect of TGF-α-neutralization on Fe^{2+}-induced CXCL8 is surprising given the effects of AG1478, TAPI-1 and EGFR-neutralization. It is possible that Fe^{2+} may elicit effects through TACE-mediated cleavage of other EGFR-ligands such as amphiregulin.

The above results suggest that multiple model-compounds of common PM-constituents stimulate CXCL8-release in BEAS-2B cells through activation of the TACE/TGF-α/EGFR-cascade. To explore whether more complex particles could elicit effects through similar mechanisms, cells were treated with DEP in absence or presence of AG1478 and TAPI-1. Since DEP may bind CXCL8 protein and thereby interfere with ELISA assays [36], CXCL8-responses were measured at the mRNA-level. In accordance with the effects on responses of the model-compounds, both EGFR- and TACE-inhibitors attenuated DEP-induced CXCL8-expression significantly (Fig 6).

Discussion

Innate immune responses of the airway epithelium are important in host defence against inhaled pollutants and pathogens, but inflammatory mediators from epithelial cells may also promote development and exacerbation of airway disease [1, 2, 37]. Previous exploration of cytokine/chemokine expression induced by the different PM-components in BEAS-2B cells revealed that crystalline silica, Zn^{2+}, Fe^{2+}, 1-NP and LPS induced expression of at least one of the neutrophil-specific chemokines CXCL1/-3/-5 and -8. CXCL8 was the only of these induced by all agents and among the most up-regulated on average [5]. Given its central role in neutrophilic airway disease [9, 10, 38], the present study was initiated to explore the regulation of CXCL8 in BEAS-2B cells upon exposure to PM-components.

The selected PM-components mainly induced gene expression linked to NF-κB, calcium/PKC, PLC, LDL and/or mitogenic signalling pathways. The generally strong effects on CXCL8 corroborate available data suggesting CXCL8-expression as a highly important epithelial response to inhaled air pollutants such as PM. We also found marked effects on

GM-CSF which prevents neutrophil apoptosis [39, 40]. Thus, PM-components may not only trigger expression of epithelium-derived factors activating and recruiting neutrophils, but also factors promoting sustained neutrophil survival in the lung.

By applying a panel of 15 inhibitors interfering with separate signalling pathways we found that each component most likely activates several signalling pathways which presumably act in concert on CXCL8-regulation. Different pathways may be triggered through separate mechanisms, possibly at different concentrations of the compound in question. PAHs for instance, may initiate effects through interactions with the aryl hydrocarbon receptor or formation of reactive metabolites and oxygen and nitrogen radicals (ROS/RNS) [41, 42]. Interestingly, LPS which triggers effects through TLR2/4-activation [43], a relatively specific mechanism, induced changes in the lowest number of genes. Variations in the relative effects of different inhibitors suggest that although different compounds may induce CXCL8 through similar pathways the relative importance of the different signalling pathways varies.

We have previously observed that EGFR-inhibition greatly reduces CXCL8-release induced by both fluoride and crystalline silica in A549 alveolar epithelial cells [18, 44]. In line with this, the inhibitor screen identified EGFR-signalling as a common mechanism of CXCL8-regulation in BEAS-2B cells. Use of EGFR-neutralizing antibodies further supported this and implicated EGFR–ligation in regulation of CXCL8. Silica-exposure seems to trigger an immediate phosphorylation of Src but not EGFR [18]. Here we show that the receptor is activated at later time points, strikingly similar to asbestos-effects in epithelial cells [45], suggesting that EGFR-activation at least in the case of mineral particles is a downstream event rather than a triggering mechanisms.

EGFR-ligands such as TGF-α and amphiregulin can induce CXCL8 in bronchial epithelial cells, and appear to mediate cigarette smoke-induced CXCL8 through an autocrine

loop [46-48]. These growth factors are produced as membrane-bound pro-forms and released through ectodomain-shedding by metalloproteases, primarily TACE/ADAM-17 [49]. TLR-ligands (including LPS), rhinovirus and neutrophil elastase induce CXCL8-release from airway epithelial cells through activation of TACE, cleavage of pro-TGF-α and activation of EGFR [20, 21, 26, 50]. Our present data confirm that LSP-induced CXCL8-responses involve TACE/TGF-α/EGFR-signalling also in BEAS-2B cells. Furthermore, the study extends the list of compounds utilising this pathway in CXCL8-regulation in airway epithelial cells, from endogenous and microbiological molecules to also include organic and inorganic airborne pollutants. We also observed that inhibitors of TACE and EGFR attenuated DEP-induced CXCL8-expression in BEAS-2B cells, providing a possible mechanistic link between EGFR-activation and increased CXCL8-levels in the airways of DEP-exposed human volunteers [51, 52].

Both in the case of neutrophil elastase and TLR-ligand exposure, TACE activation in airway epithelial cells appears to involve ROS production by dual oxidase (Duox1) [20, 26]. Oxidative stress is also considered a central mechanisms in cellular responses to PM [53]. Thus, ROS formation either directly by the compounds, or indirectly through Duox1-activation may explain why effects of such a diverse group of PM-compounds may converge onto the same pathway. Notably, PKCδ/θ has been implicated in activation of the Duox-1/ROS/TACE/TGF-α/EGFR-cascade [20, 54]. In accordance with this, we observed that rottlerin significantly reduced CXCL8-release induced by Zn^{2+}, Fe^{2+} and 1-NP, and partially (but not significantly) also silica. However, our data does not support a role of PKCδ/θ in LPS-induced CXCL8, in contrast to what has been proposed by others [3]. Several studies implicate that also IL-1α/β is important in regulation of CXCL8 through an autocrine signalling loop [55-57], and preliminary results from our lab suggest that the IL-1-antagonising drug anakinra/kineret (soluble IL-1RA) represses CXCL8-induction by all test-

compounds except LPS (unpublished results). IL-1 receptors and TLRs belong to the same superfamily, and utilize many identical adaptor/signalling molecules including MyD88 and IRAK [58]. It is therefore intriguing to speculate that IL-1 may activate TACE and that signalling pathways induced by several test-compounds converge already onto IL-1 regulation. Indeed, IL-1β may induce shedding of EGFR-ligands in other cell types, and even CXCL8 may induce such effects leading to a self-perpetuating loop [59].

Extrapolations from data obtained by immortalized cell lines have its obvious pit falls. However, studies with normal human bronchial epithelial cells have shown that CXCL8-regulation by TACE/TGF-α/EGFR-signalling is not restricted to cell lines [26]. Although one cannot exclude that other pathways may be predominant in epithelial cells from risk subjects, EGFR-expression is increased in the airway epithelium of asthmatics, smokers and patients with COPD and CF, and the pulmonary expression of EGFR and CXCL8 correlates in patients with severe asthma [3]. Accordingly, animal-models have revealed elevated tissue-levels of TACE in COPD [60], and that TACE-inhibitors decrease neutrophil influx in both allergic and non-allergic airway inflammation [61]. Notably, TACE does not only cleave growth factors involved in CXCL8-regulation, but also proteins involved in adhesion and transmigration of leukocytes across the endothelium, including V-CAM/ICAM-1, L-selectin and tight-junction molecules [62]. Thus, activation of TACE may trigger a number of reactions participating to promote neutrophil inflammation in the airways. In addition, TACE/TGF-α/EGFR-signalling also regulates mucin-production which contributes to exacerbation of asthma, COPD and CF along with neutrophilia [3, 25, 63-65].

Our present data confirm that CXCL8-regulation is complex and involves a number of signalling pathways which conceivably act in concert to elicit the final response. Thus, interference with just one pathway may not be sufficient to abrogate CXCL8-responses induced by air pollutants such as PM. Nevertheless, our present findings strengthen the

impression that TACE/TGF-α/EGFR is an important convergent pathway regulating innate immune responses induced by a variety of inhaled compounds in airway epithelial cells. It will be of interest to clarify any synergy between different PM-components or other inhalable irritants on TACE/EGFR-signalling and whether existing inflammation or airway disease may lower the threshold-level for TACE/EGFR-activation.

Acknowledgement

We thank E. Lilleaas and T. Skuland for expert assistance throughout the study. The work was supported by the Research Council of Norway, through the Environment, Genetics and Health-program (grants no. 165386 and 160863).

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Table 1. Real-time PCR array of 84 genes representative of 18 different signalling pathways in BEAS-2B cells exposed for 10 h to crystalline silica (40 µg/cm^2), $ZnCl_2$ (90 µM), $FeSO_4$ (250 µM), 1-NP (30 µM) or LPS (10 µg/ml).

Pathway	Genes	Silica	$ZnCl_2$	$FeSO_4$	1-NP	LPS
Mitogenic	*EGR1*	-	-	↓	↑	-
	FOS	↑	-	-	↑	-
	JUN	↑	↑	↑	↑↑	↑
Wnt	CND1C	-	-	-	-	-
	JUN	↑	↑	↑	↑↑	↑
	LEF1	-	-	-	-	-
	MYC	-	-	-	-	-
	PPARG	-	-	-	-	-
	TCF7	-	-	-	-	↓[B]
	VEGFA	-	-	-	-	-
	WISP1	-	-	-	-	-
Hedgehog	BMP2	↑	-	-	↑	-
	BMP4	-	-	-	-	-
	EN1	-	-	-	-	-
	FOXA2	-	↓	-	-	-
	PTCH1	-	-	-	-	-
	WNT1	-	-	-	-	-
	WNT2	↑[B]	-	-	↑[B]	-
TGF-β	*CDKN1A*	-	-	↑	↑	-
	CDKN1B	-	↓	-	-	-
	CDKN2A	-	-	-	-	-
	CDKN2B	-	↓	-	-	-
PI3K/AKT	*BLC2*	-	-	-	-	-
	CCND1	-	-	-	-	-
	FN1	-	-	-	-	-
	JUN	↑	↑	↑	↑↑	↑
	MMP7	-	↓[B]	-	-	↓[B]
	MYC	-	-	-	-	-
Jak/Src	*BCL2*	-	-	-	-	-
	BCL2L1	-	-	-	-	-
Jak-Stat	CXCL9	↑[B]	-	-	-	-
	IL4	-	-	-	-	-
	IL4R	-	-	-	-	-
	IRF1	-	-	-	↑	-
	MMP10	-	-	-	-	-
	NOS2A	-	-	-	-	-
Calcium/ PKC	*CSF2*	↑↑[A]	↑↑[A]	↑[A]	↑↑[A]	↑[A]
	FOS	↑	-	-	↑	-
	ICAM1	↑	-	-	↑	↑
	JUN	↑	↑	↑	↑↑	↑
	MYC	-	-	-	-	-
	ODC1	-	-	-	-	-
	PRKCA	-	-	-	-	-
	PRKCE	-	-	-	-	-
	TERT	-	-	-	-	-
PLC	*BCL2*	-	-	-	-	-
	EGR1	-	-	↓	↑	-
	FOS	↑	-	-	↑	-
	ICAM1	↑	-	-	↑	↑
	JUN	↑	↑	↑	↑↑	↑
	NOS2A	-	-	-	-	-
	PTGS2	↑	-	-	-	-
	VCAM1	-	-	-	-	-
NFAT	CD5	-	-	-	-	↓[B]
	FASLG	-	-	-	-	-
	IL2	-	-	-	-	-

Pathway	Genes	Silica	$ZnCl_2$	$FeSO_4$	1-NP	LPS
NF-κB	BCL2A1	↑↑[B]	↑↑[B]	↑↑[B]	↑↑[A]	↑↑[B]
	BIRC2	-	-	-	-	-
	BIRC3	-	-	-	↑	-
	NAIP	-	-	-	-	-
	TERT	-	-	-	-	-
	CCL20	↑↑[A]	↑[A]	↑[A]	↑↑[A]	↑[A]
	ICAM1	↑	-	-	↑	↑
	IKBKB	-	↓	-	-	-
	IL1A	-	-	-	↑	-
	IL2	-	-	-	-	-
	IL8	↑↑	↑↑	↑	↑↑	↑
	LTA	-	↑[B]	↑[B]	-	-
	NFKB1	-	-	-	↑	-
	NOS2A	-	-	-	-	-
	PECAM1	-	-	-	-	-
	TANK	↓	-	-	-	-
	TNF	↑↑[A]	↑[B]	↑[B]	↑↑[A]	↑[B]
	VCAM1	-	-	-	-	-
p53	BAX	-	-	-	-	-
	CDKN1A	-	-	↑	↑	-
	FAS	-	-	-	-	-
	GADD45A	-	-	-	-	-
	IGFBP3	-	-	-	-	-
	MDM2	-	-	-	-	-
	TP53I3	-	-	-	-	-
CREB	CYP19A1	-	-	-	-	-
	EGR1	-	-	↓	↑	-
	FOS	↑	-	-	↑	-
Estrogen	*BCL2*	-	-	-	-	-
	BRCA1	-	-	-	↓	-
	GREB1	-	-	-	-	-
	NRIP1	-	-	-	-	-
Androgen	CDK2	-	-	-	-	-
	CDKN1A	-	-	↑	↑	-
	KLK2	-	-	-	-	-
	PMEPA1	-	-	↑	-	-
Insulin	CEBP	-	-	-	↑	-
	FASN	-	↓	-	-	-
	GYS1	-	-	-	-	-
	HK2	-	-	-	-	↓
	LEP	-	↑[B]	↑[B]	-	-
LDL	CCL2	-	-	-	-	-
	CSF2	↑↑[A]	↑↑[A]	↑[A]	↑↑[A]	↑[A]
	SELE	-	-	-	↑[B]	-
	SELPG	-	↑[B]	↑[B]	-	-
	VCAM1	-	-	-	-	-
Etinoic acid	EN1	-	-	-	-	-
	HOXA1	-	-	-	-	-
	RBP1	-	-	-	-	-
Stress	ATF2	-	-	-	-	-
	FOS	↑	-	-	↑	-
	HSF1	-	-	-	-	-
	HSPB1	-	-	-	-	-
	HSP90AA2	-	↑	-	-	-
	MYC	-	-	-	-	-
	TP53	-	-	-	-	-

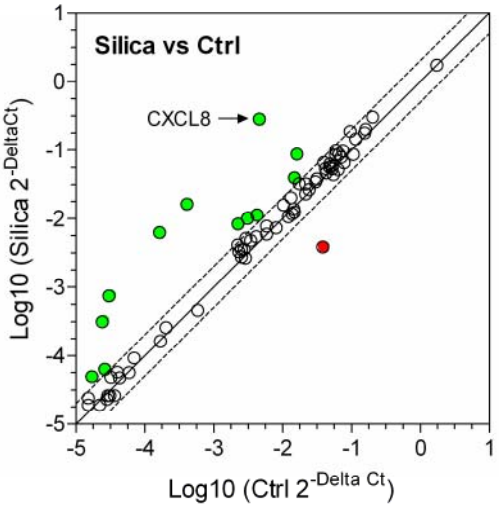
Genes written in *italic* may be regulated through several signalling pathways and are repeated in the table. ↑↑ up-regulated > 10-fold; ↑ up-regulated > 2-fold; - unchanged expression; ↓ down-regulated > 2-fold. [A]Gene expression in control is low suggesting that the actual fold-change value is at least as large as the calculated result. [B]Gene expression in control and exposed samples are low suggestion that estimated fold-change result may have greater variations.

Table 2. Panel of pharmacological inhibitors used to screen for mechanisms of CXCL8 regulation, concentrations used in the study, and their molecular targets.

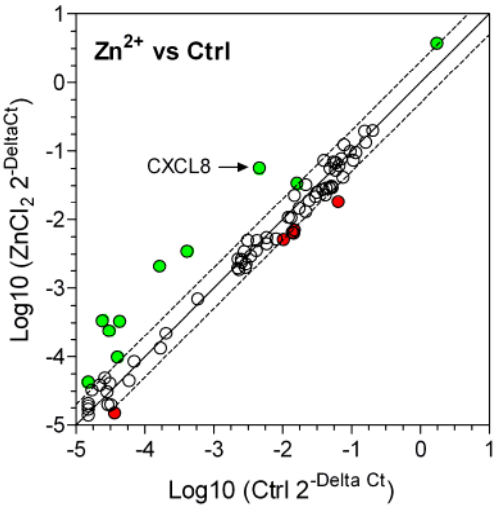
Inhibitor	*Target*	*Inhibitor*	*Target*
SB 202190 (5 μM)	p38	H-8 (50 μM)	PKA
PD 98059 (10 μM)	MEK1/2 (ERK1/2)	Wortmannin (0.1 μM)	PI3-K
SP 600125 (10 μM)	JNK	AG 490 (10 μM)	JAK2/JAK3
PP2 (5 μM)	Src-family kinases	Verapamil (100 μM)	Ca^{2+}-channels
AG 1478 (5 μM)	EGFR	BABPTA-AM (20 μM)	Ca^{2+}-chelator
Gö 6976 (3 μM)	PKCα/β_1	W-7 (25 μM)	Calmodulin (Ca^{2+})
Rottlerin (3 μM)	PKCδ/θ	Curcumin (10 μM)	Multiple targets incl. NF-κB and AP-1
GF 109203X (3 μM)	PKC (general)		

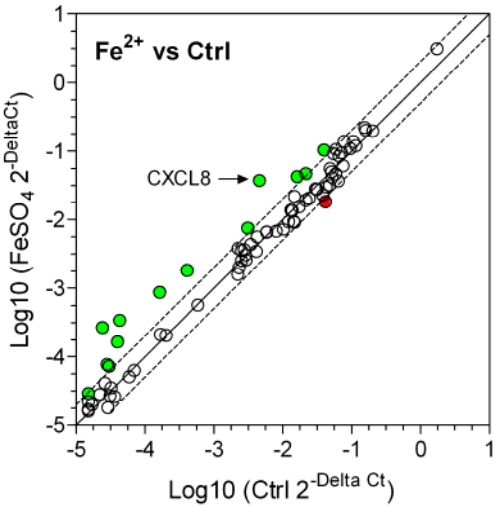
Figure legends

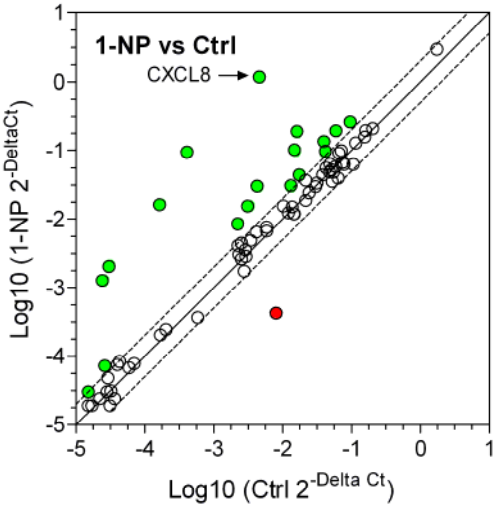
Figure 1. Scatter-plots of gene expression induced by different PM-components in human bronchial epithelial cells (BEAS-2B). The cell cultures were incubated for 10 h with crystalline silica (40 μg/cm^2), $ZnCl_2$ (90 μM), $FeSO_4$ (250 μM), 1-NP (30 μM) or LPS (10 μg/ml). Changes in mRNA levels of 84 genes were measured by real-time PCR array as described under "Materials and Methods". The results are expressed as Log10($2^{-\Delta Ct}$) where -ΔCt is the cycle time for each gene after adjustment for the average Ct of housekeeping genes, and distance from the solid line represents the Log10 value of fold-change compared to the untreated control. Green plots represent genes up-regulated more than two-fold, whereas red plots represent genes down-regulated more than two-fold. The data represents means of two independent experiments. The figure also contains a table of the genes up-regulated (green) or down-regulated (red) more than two-fold by the different test-compounds. Genes in bold letters have more than 10-fold changed expression compared to control.



Silica	Zn^{2+}	Fe^{2+}	1-NP	LPS
IL8	**BCL2A1**	**BCL2A1**	**IL8**	**BCL2A1**
CCL20	**CSF2**	IL8	**CCL20**	CCL20
CSF2	**IL8**	SELPLG	**CSF2**	IL8
TNF	CCL20	CSF2	**TNF**	TNF
BCL2A1	TNF	CCL20	**BCL2A1**	CSF2
JUN	SELPLG	LTA	**JUN**	ICAM1
PTGS2	LEP	MMP10	ICAM1	JUN
BMP2	LTA	JUN	FOS	-
CXCL9	HSP90A	CDKN1A	BMP2	HK2
FOS	A2	BMP2	BIRC3	TCF7
ICAM1	JUN	TNF	CDKN1A	MMP7
WNT2	-	PMEPA1	CEBPB	**CD5**
-	FASN	LEP	WNT2	
TANK	MMP7	-	IL1A	
	FOXA2	EGR1	IRF1	
	IKBKB		NFKB1	
	CDKN2B		EGR1	
	CDKN1B		SELE	
			-	
			BRCA1	







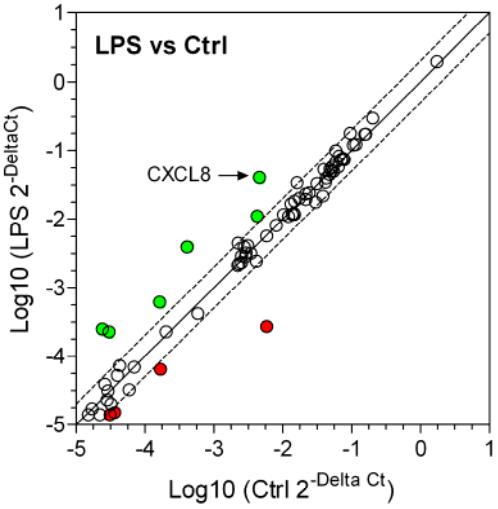


Figure 2. Induction of CXCL8-release after 10 and 20 h exposure to different PM-components in human bronchial epithelial cells (BEAS-2B). The cell cultures were incubated for 10 and 20 h with crystalline silica (40 μg/cm^2), $ZnCl_2$ (90 μM), $FeSO_4$ (250 μM), 1-NP (30 μM) or LPS (10 μg/ml). Supernatants were harvested and analyzed for CXCL8-release by ELISA, as described under "Materials and Methods". Data are presented as relative increase (fold) compared to untreated controls. Each point represents mean ± SEM of independent experiments (n ≥ 3). *Significant increase in CXCL8-release compared to unexposed control ($P < 0.05$).

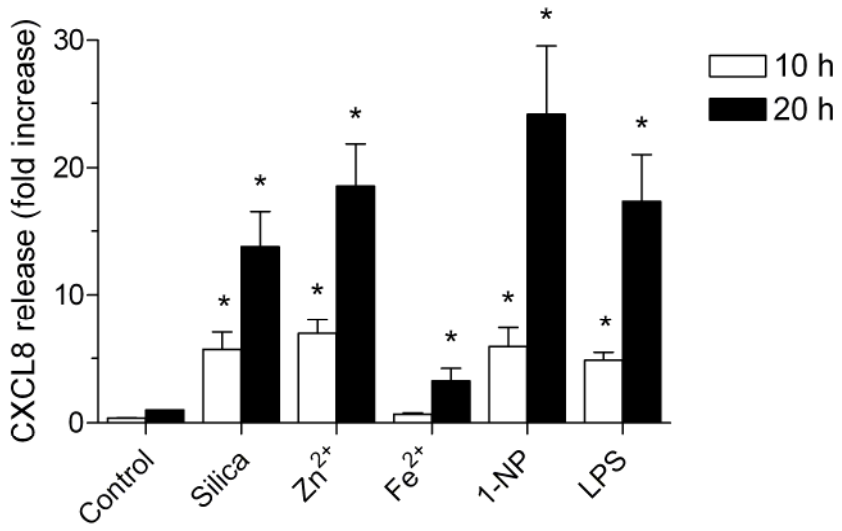


Figure 3. Inhibitor-screen to identify pathways involved in CXCL8-release induced by different PM-components in human bronchial epithelial cells (BEAS-2B). The cell cultures were pretreated with a panel of 15 different inhibitors targeting different signaling pathways for 1 h prior to 20 h incubation with crystalline silica (40 μg/cm^2), $ZnCl_2$ (90 μM), $FeSO_4$ (250 μM), 1-NP (30 μM) or LPS (10 μg/ml). The inhibitors used are given in table 2. Supernatants were harvested and analyzed for CXCL8-release by ELISA, as described under "Materials and Methods". The results are expressed as relative CXCL8-release compared to exposed cells in absence of inhibitor (100% response). $^{\#}$Curcumin targets multiple pathways. Each value represents mean ± SEM (n = 3-6). White columns = controls; black columns = exposed to test-compound. *Significant reduction of CXCL8-release by the inhibitor ($P < 0.05$).

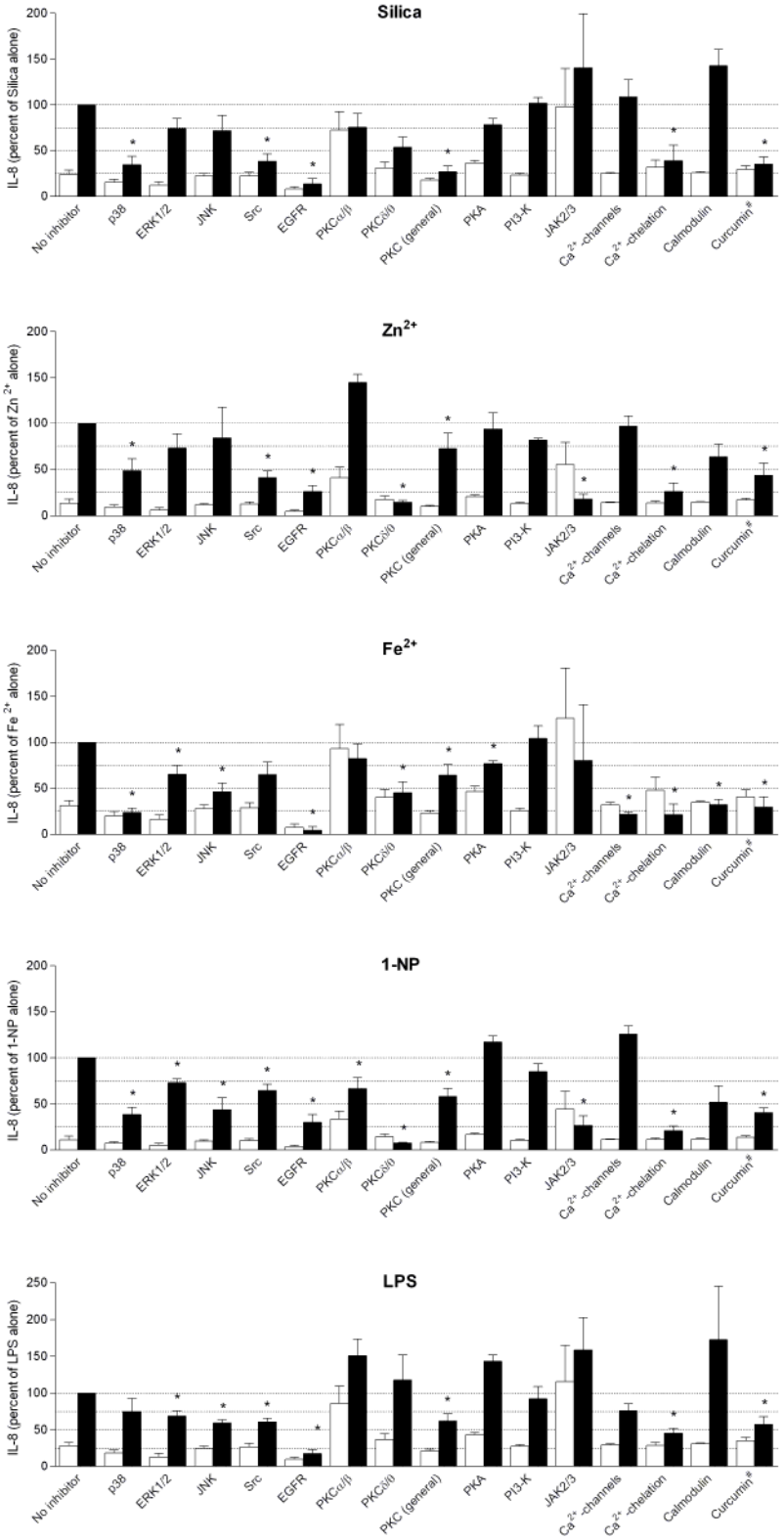

Silica
IL-8 (percent of Silica alone)
Zn2+
IL-8 (percent of Zn 2+ alone)
Fe2+
IL-8 (percent of Fe 2+ alone)
1-NP
IL-8 (percent of 1-NP alone)
LPS
IL-8 (percent of LPS alone)
No inhibitor
p38
ERK1/2
JNK
Src
EGFR
PKCα/β
PKCδ/θ
PKC (general)
PKA
PI3-K
JAK2/3
Ca2+ -channels
Ca2+ -chelation
Calmodulin
Curcumin#

Figure 4. Involvement of EGFR-ligand binding in PM-component-induced CXCL8-release from human bronchial epithelial cells (BEAS-2B). The cell cultures were pre-treated with an anti-EGFR-neutralizing antibody for 1 h prior to 20 h incubation with crystalline silica (40 μg/cm^2), $ZnCl_2$ (90 μM), $FeSO_4$ (250 μM), 1-NP (30 μM) or LPS (10 μg/ml). Supernatants were harvested and analyzed for CXCL8-release by ELISA, as described under "Materials and Methods". The results are expressed as relative CXCL8-induction compared to exposed cells in absence of neutralizing antibody (100% response). Each value represents mean ± SEM (n = 3). *Significant reduction of CXCL8-release by the inhibitor ($P < 0.05$). The figure also displays the effect of two selected test-compounds on EGFR-phosphorylation. BEAS-2B cells were incubated with crystalline silica (40 μg/cm^2) or $ZnCl_2$ (90 μM) for 2 h prior to immunoprecipitation of the EGFR, as described under "Materials and Methods". The precipitated proteins were separated by SDS-PAGE and Western blots were incubated with an antibody against phospho-tyrosine to investigate EGFR-activation (n = 2).

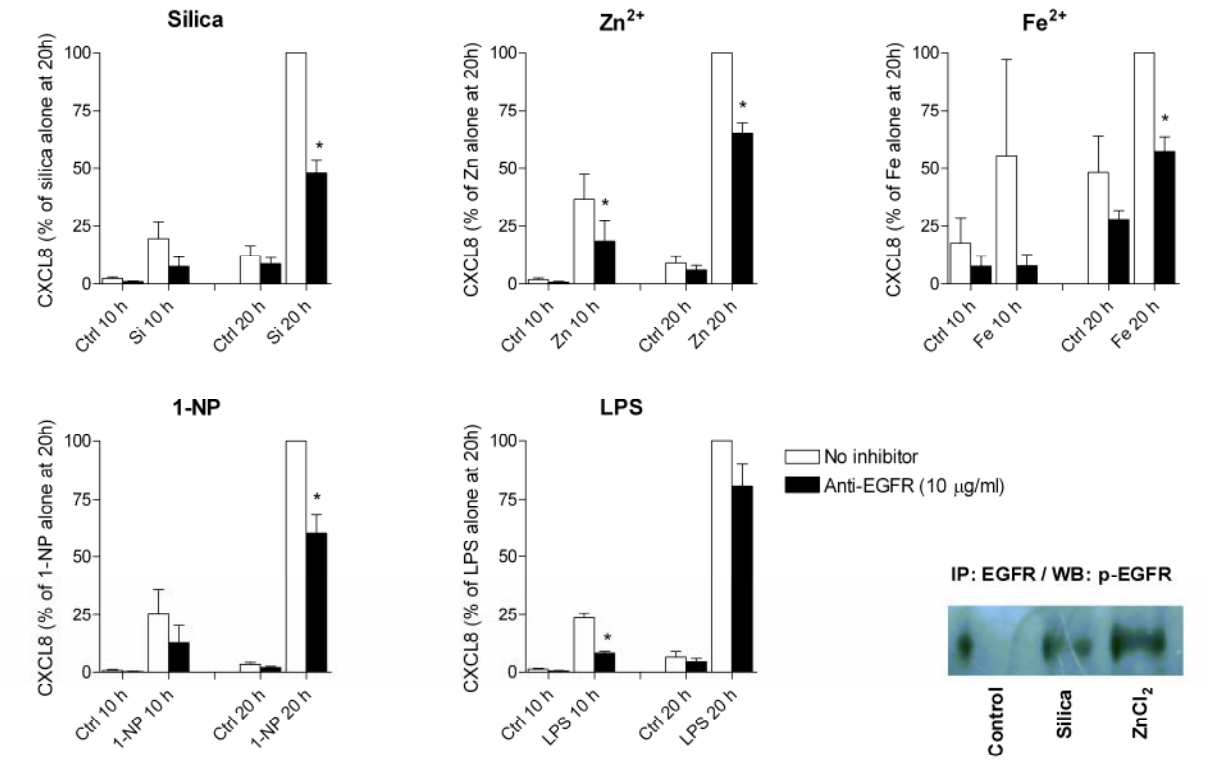


Figure 5. Involvement of TACE and TGF-α in PM-component-induced CXCL8-release from human bronchial epithelial cells (BEAS-2B). The cell cultures were pre-treated with the TACE-inhibitor TAPI-1 or a TGF-α-neutralizing antibody for 1 h prior to 20 h incubation

with crystalline silica (40 μg/cm^2), $ZnCl_2$ (90 μM), $FeSO_4$ (250 μM), 1-NP (30 μM) or LPS (10 μg/ml). Supernatants were harvested and analyzed for CXCL8-release by ELISA, as described under "Materials and Methods". The results are expressed as relative CXCL8-induction compared to exposed cells in absence of inhibitor or neutralizing antibody (100% response). Each value represents mean ± SEM (n = 3). *Significant reduction of CXCL8-release by the inhibitor ($P < 0.05$).

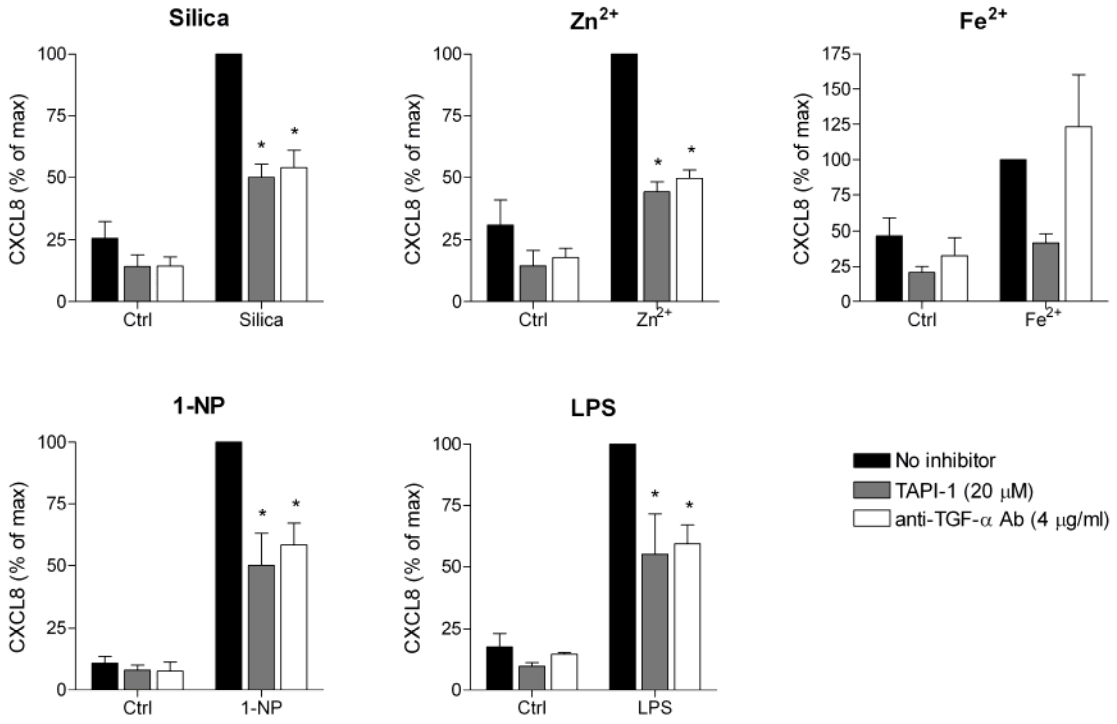
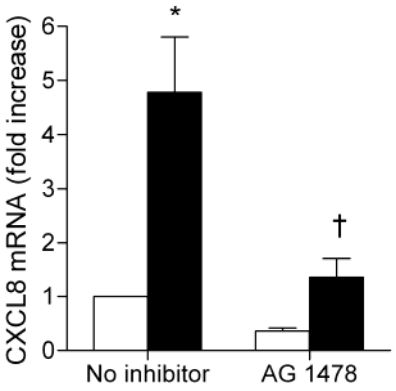
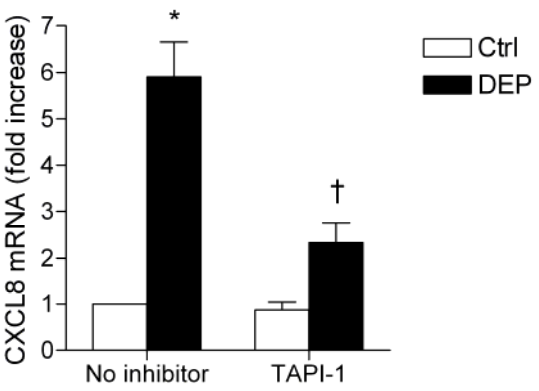


Figure 6. Involvement of TACE and EGFR in DEP-induced CXCL8-expression from the human bronchial epithelial cell line, BEAS-2B. The cell cultures were pretreated with the TACE-inhibitor TAPI-1 or the EGFR-inhibitor AG1478 for 1 h prior to 4 h incubation with DEP (100 μg/ml). Changes in CXCL8 mRNA levels were measured by real-time PCR as described under "Materials and Methods". The results are expressed as relative CXCL8-induction (fold) compared to unexposed controls after normalization against 18S rRNA. Each value represents mean ± SEM (n = 3-5). *Significant induction of CXCL8-expression compared to control; †significant reduction of CXCL8-expression by the inhibitor ($P < 0.05$).


CXCL8 mRNA (fold increase)
0
1
2
3
4
5
6
*
†
No inhibitor
AG 1478


CXCL8 mRNA (fold increase)
0
1
2
3
4
5
6
7
*
†
No inhibitor
TAPI-1
Ctrl
DEP