IκB-α glutathionylation and reduced histone H3 phosphorylation inhibit eotaxin and RANTES

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ABSTRACT

Airway smooth muscle cells (ASMC) secrete eotaxin and RANTES in response to TNF-α, which was inhibited by the NF-κB inhibitor dimethylfumarate (DMF). NF-κB/IκB glutathionylation and changes in chromatin remodelling can inhibit NF-κB activity. In this study, we determined whether NF-κB/IκB glutathionylation and reduced histone H3 phosphorylation might underlie DMF’s inhibitory effect on NF-κB activity and on eotaxin and RANTES secretion. Primary human ASMC were treated with DMF, diamide and/or GSH-OEt (glutathione-ethylester) prior to TNF-α stimulation and were subsequently analysed by ELISA, EMSA, immunofluorescence, co-immunoprecipitation, or immunoblotting. DMF reduced intracellular GSH and induced IκB-α glutathionylation (IvB-α-SSG), which inhibited IκB-α degradation, NF-κB p65 nuclear entry and NF-κB/DNA binding. In addition, DMF inhibited the phosphorylation of histone H3, which was possibly mediated by DMF’s inhibitory effect on MSK-1. However, the p38 MAPK, ERK MAPK or MKP-1, up-stream of MSK-1, were not inhibited by DMF. Importantly, DMF-mediated effects on NF-κB, histone H3, eotaxin and RANTES were reversed by addition of GSH-OEt. Our data suggest that DMF inhibits NF-κB dependent eotaxin and RANTES secretion, by reduction of GSH with subsequent induction of IκB-α -SSG and inhibition of histone H3 phosphorylation. Our findings offer new potential drug targets to reduce airway inflammation in asthma.

KEYWORDS: asthma, glutathionylation, histone H3, inhibitor of NF-κB (IκB)-α, mitogen- and stress-activated protein kinase (MSK)-1.
INTRODUCTION

Asthma is a chronic inflammatory disease of the airways. The airways of asthmatic patients are characterised by an increased number of eosinophils and increased sputum eosinophil counts correlated with asthma severity [1]. The chemokines eotaxin and RANTES are centrally involved in the recruitment of eosinophils into the inflamed airway tissue and both chemokines are elevated in bronchial biopsies of asthmatic donors [2].

Both chemokines were detected in airway smooth muscle in vivo [3, 4] and TNF-α increased human airway responsiveness [5]. Local cytokine secretion may cause eosinophil infiltration along a gradient into the submucosa in asthma [6]. In cultured human airway smooth muscle cells (ASMC), TNF-α activated NF-κB and induced the expression of cytokines [7]. In vitro studies have shown that tumour necrosis factor (TNF)-α induced eotaxin and RANTES secretion by ASMC is down regulated by inhibition of the transcription factor NF-κB [7, 8].

NF-κB forms dimers, consisting of the different subunits p50, p52, p65 (RelA), RelB, and c-Rel. In un-stimulated cells, NF-κB is retained in the cytosol in a complex formed with NF-κB inhibitory proteins (IκB). Cell stimulation induces degradation of IκB, leaving free and active NF-κB to migrate into the nucleus where it binds to the DNA to initiate gene transcription [9].

The IκB protein IκB-α contains cysteine thiols, which are susceptible to redox-regulated post-translational modifications [10]. One such reversible modification is protein glutathionylation (protein-SSG), whereby a cysteine-thiol of a protein (protein-SH), forms a disulfide bond with the cysteine-thiol of glutathione (GSH). Protein-SSG is
inducible by reduction of intracellular GSH level [11] and was shown to result in an inhibition of NF-κB nuclear entry and binding to the DNA [12, 13]. Dimethylfumarate (DMF) reduced cellular glutathione in many different cell types [14-16] and was also shown to inhibit the nuclear entry of NF-κB and subsequent binding to the DNA in ASMC [7]. Therefore, IκB-α-SSG is a possible mechanism by which DMF inhibits NF-κB activity.

Histone H3 phosphorylation is crucial for chromatin relaxation and for optimal binding of transcription factors to the corresponding DNA sequences [17]. Histone H3 phosphorylation at serine (Ser)10 occurs in promoters of NF-κB-regulated cytokines such as RANTES [18] and was shown to increase the accessibility of the NF-κB binding sites [19]. Mitogen- and stress-activated protein kinase (MSK)-1 phosphorylates histone H3 at Ser10 [20] and MSK-1 was inhibited by DMF in ASMC and keratinocytes [7, 21]. Therefore, inhibition of MSK-1 mediated histone H3 phosphorylation by DMF may contribute to its inhibitory effect on NF-κB dependent chemokine secretion.

In this study in human ASMC we aimed to determine whether glutathionylation of NF-κB/IκB-α and reduced histone H3 phosphorylation underlie DMF’s inhibitory effect on NF-κB dependent eotaxin and RANTES secretion.

**MATERIAL AND METHODS**

**Material**

All chemicals were purchased from Sigma, St. Louis, MO unless specified differently.

**Isolation and culture of human ASMC**
Human ASMC were isolated from bronchi of patients undergoing surgical resection for carcinoma or lung transplantation as approved by the South West Sydney Area Health Service and the Human Ethics Committee; University of Sydney, Australia and by the local ethics committee of the University Hospital Basel, Switzerland. Primary ASMC lines were isolated and characterised as previously described [7, 22]. ASMC were used between passages 5-8 and experiments were performed in ASMC of at least three different donors; the demographic data of the patients are summarized in Table I.

**Table I.** Demographic data of the patients.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Sex</th>
<th>Age</th>
<th>Diagnosis</th>
<th>Smoker</th>
<th>FEV1 (% predicted)</th>
<th>FVC</th>
</tr>
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<tr>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>2.93 (82%)</td>
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</tr>
<tr>
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<td>NA</td>
</tr>
<tr>
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<tr>
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<td>smoker</td>
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<td>1.98 (70%)</td>
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<tr>
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<td>COPD*</td>
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<td>NSCLC</td>
<td>ex-smoker</td>
<td>2.75 (65%)</td>
<td>2.96</td>
</tr>
</tbody>
</table>
*COPD: chronic obstructive pulmonary disease. NSCLC: non-small cell lung carcinoma. N/A: not available

None of the patients had received any therapy before lung tissue sampling.

**Intracellular GSH level**

Confluent ASMC were deprived of serum (24 h) and were then treated with DMF (50 µM) or the drug vehicle DMSO (0.05%) for 1 or 3 h. The glutathione assay was performed according to the manufacturers’ protocol (Glutathione Assay Kit, BioVision, San Diego, CA).

**IkB-α-SSG**

Confluent ASMC were deprived of serum (24 h) and incubated with diamide (100-500 µM, 30 min), or with DMF (50 µM, 1 h) and/or GSH-OEt (1 mM, 90 min) before TNF-α stimulation (10 ng/ml, R&D Systems, Minneapolis, MN, 30 min). The Catch and Release version 2.0 Reversible Immunoprecipitation System (Millipore, Billerica, MA, USA) was used to perform co-immunoprecipitation (IP) of the NF-κB/IκB complex. Briefly, ASMC were lysed (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% NP-40, and 1x protease inhibitor cocktail). The samples were incubated with antibodies against NF-κB p65 (4 µg, C-20), or NF-κB p50 (4 µg, H-119) or the normal rabbit IgG negative control (4 µg, all SantaCruz Biotechnology, Santa Cruz, CA, USA) in spin columns containing 0.5 ml of IP capture resin. IP-eluates were mixed with non-reducing SDS sample buffer supplemented with 5 mM N-ethylmaleimide to block unreacted thiol groups, size-fractionated by SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes. Membranes were incubated with antibodies against NF-κB p65 (C-20), NF-κB p50 (H-119), IκB-α (C-21)
(all SantaCruz Biotech.), or GSH (ViroGen, Watertown, MA). Primary antibodies were
detected by horseradish peroxidase-conjugated IgG antibodies (Cell Signalling
Technology, Danvers, MA) and protein bands were visualised by enhanced
chemiluminescence (PerkinElmer, Wellesley, MA).

**NF-κB/DNA binding and NF-κB p65 nuclear entry**

Confluent ASMC were serum deprived (24 h) and then pre-incubated with DMF (50 μM,
1 h) and/or GSH-OEt (1 mM, 90 min) before TNF-α stimulation (10 ng/ml, 30 min). NF-
κB/DNA binding was analysed by electrophoretic mobility shift assay (EMSA) as
previously described [7]. To determine NF-κB p65 nuclear entry, cells were trypsinised,
and cell pellets were resuspended in low salt buffer (10 mM HEPES, 10 mM KCl, 0.1
mM EDTA, 0.1 mM EGTA, 0.25% Nonidet P-40, 1x protease inhibitor cocktail) for 20
min. Nuclei were incubated in high salt buffer (20 mM HEPES, 400 mM NaCl, 1 mM
EDTA, 1 mM EGTA, 1x protease inhibitor cocktail) for 40 min, size-fractionated by
SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes. The
membranes were then incubated with antibodies against NF-κB p65 (C-20, SantaCruz
Biotech.) or lamin A/C (Cell Signaling Technol.) to confirm equal protein loading.

For immunofluorescence analysis ASMC were plated on cover slips and treated as
described above. ASMC were fixed with ice-cold 100 % methanol (5 min, -20°C),
blocked with 10 % donkey serum (20 min), and incubated with an antibody against NF-
κB p65 (C-20,) or normal rabbit IgG (both SantaCruz Biotech.) for 1 h. The primary
antibody was detected by addition of an Alexa488 conjugated donkey anti-rabbit IgG
antibody (Invitrogen Corporation, Carlsbad, CA) for 1h. To visualise the nuclei 4,6-
diamido-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) was added (15 min) and
the cover slips were subsequently examined on a laser scan microscope.

**MAPK/MSK-1/histone H3 pathway activation**

Confluent ASMC were serum deprived for 24 h and then incubated with DMF (50 μM, 1 h) and /or GSH-OEt (1 mM, 90 min) prior to stimulation with TNF-α (10 ng/ml) for 0, 5, 10, 15, 30, 60 or 120 min. Total cell lysates were collected, size-fractionated by SDS-PAGE electrophoresis and transferred onto nitrocellulose membranes. Membranes were then incubated with antibodies against phospho-MSK-1 (Ser360), phospho-MSK-1 (Ser376), phospho-MSK-1 (Thr581), MSK-1, phospho-histone H3 (Ser10) (D2C8), histone H3, phospho-p38 MAPK, p38 MAPK, phospho-ERK, ERK MAPK, (all Cell Signaling Technol.), MKP-1 or α-Tubulin (DM1A, SantaCruz Biotech.).

**Chemokine secretion**

Confluent ASMC were serum deprived for 24 h, before a single drug (DMF 1 h, GSH-OEt 90 min), or the drugs in combination, were added prior to stimulation with TNF-α (10 ng/ml, 24 h). Supernatants were collected and eotaxin and RANTES were measured by enzyme-linked immunosorbent assay (ELISA Duo Set, R&D Systems).

**Data analysis**

The statistical analysis was performed using Mann Whitney U-test, p-values ≤ 0.05 were considered significant.

**RESULTS**

**Cell characterisation:**
In figure 1a we provide proof that the cells isolated from human bronchi express at least two ASMC markers (α-smooth muscle cell actin: α-SMA; smooth muscle myosin heavy chain: SMMHC), and are negative for fibronectin expression. This expression pattern of the ASMC markers is maintained until passage 10 without significant changes, and is dependent by the addition of vitamins to the cell culture medium (data will be published elsewhere). In contrast human bronchial myo-fibroblasts express significant levels of fibronectin, but no SMMHC and a low level of α-SMA (figure 1a).

**DMF reduces ASMC GSH level**

DMF reduced intracellular GSH in other cell types [14-16]. In this study, incubation of ASMC with DMF (50 μM) for 1 and 3 h reduced the level of GSH by ~50 %, when compared to the vehicle treated cells (fig. 1a).

**Diamide induces IκB-α-SSG in ASMC**

It is unknown whether glutathionylation occurs in cultured ASMC. Therefore, we first determined the effect of the well-known glutathionylation inducer diamide [11] on NF-κB/IκB. After ASMC treatment with diamide (100-500 μM, 30 min), NF-κB/IκB was immunoprecipitated with antibodies against NF-κB p65 or NF-κB p50 (fig. 1b). Diamide did not induce NF-κB p65-SSG or NF-κB p50-SSG (data not shown). Importantly, IκB-α-SSG level increased after diamide (500 μM) treatment when compared to untreated cells (fig. 1b). To confirm that the NF-κB/IκB complex was successfully pulled-down after co-IP and to prove equal protein loading, the membranes were incubated with antibodies against NF-κB p65, NF-κB p50 or IκB-α (fig. 1b). NF-κB/IκB was not precipitated by normal rabbit IgG, demonstrating the specificity of the antibodies (fig. 1b).
**DMF induces I\(\kappa\)B-\(\alpha\) -SSG and inhibits I\(\kappa\)B-\(\alpha\) degradation**

In order to determine whether I\(\kappa\)B-\(\alpha\) -SSG mediates NF-\(\kappa\)B inhibition by DMF [7], we assessed DMF effects on I\(\kappa\)B-\(\alpha\) -SSG and I\(\kappa\)B-\(\alpha\) degradation. NF-\(\kappa\)B/I\(\kappa\)B was immuno-precipitated with antibodies against NF-\(\kappa\)B p65 or NF-\(\kappa\)B p50 (fig. 1c). In un-stimulated ASMC I\(\kappa\)B-\(\alpha\) levels were high, but I\(\kappa\)B-\(\alpha\) -SSG was not detectable (fig. 1c). In TNF-\(\alpha\) treated ASMC I\(\kappa\)B-\(\alpha\) was fully degraded and therefore no I\(\kappa\)B-\(\alpha\) -SSG was detectable (fig. 1c). Importantly, when DMF was added to TNF-\(\alpha\) stimulated ASMC, I\(\kappa\)B-\(\alpha\) -SSG was induced and I\(\kappa\)B-\(\alpha\) degradation was inhibited (fig. 1c). In the presence of GSH-OEt, DMF did not induce I\(\kappa\)B-\(\alpha\) -SSG (fig. 1c), suggesting that a reduction of ASMC GSH mediates DMF-induced I\(\kappa\)B-\(\alpha\) -SSG. Most importantly, addition of GSH-OEt also reversed DMF’s inhibitory effect on I\(\kappa\)B-\(\alpha\) degradation (fig. 1c), suggesting that I\(\kappa\)B-\(\alpha\) -SSG mediates the inhibition on I\(\kappa\)B-\(\alpha\) degradation. TNF-\(\alpha\) induced I\(\kappa\)B-\(\alpha\) degradation or the level of I\(\kappa\)B-\(\alpha\) -SSG was not altered in the presence of GSH-OEt alone (fig. 1c). Equal protein loading was confirmed by incubating the membranes with antibodies against NF-\(\kappa\)B p65 or NF-\(\kappa\)B p50 (fig. 1c).

**DMF-mediated inhibition of NF-\(\kappa\)B is reversed by the addition of GSH-OEt**

Next we assessed whether the reversal of DMF-induced I\(\kappa\)B-\(\alpha\) -SSG by GSH-OEt also reverses inhibition of NF-\(\kappa\)B p65 nuclear entry and NF-\(\kappa\)B/DNA binding by DMF [7]. Immuno-blotting and immuno-fluorescence analysis revealed that the amount of nuclear NF-\(\kappa\)B p65 increased after stimulation with TNF-\(\alpha\), when compared to the vehicle control (fig, 2a, 2b). The TNF-\(\alpha\) induced NF-\(\kappa\)B p65 nuclear accumulation (NF-\(\kappa\)B p65/Lamin A/C = 1.15 ± 0.09) was significantly inhibited by DMF to 0.73 ± 0.06 (NF-\(\kappa\)B p65/Lamin A/C; p ≤ 0.05; fig. 2a, 2b), and this inhibitory effect was reversed by the addition of
GSH-OEt (fig. 2a, 2b). EMSA analysis showed that NF-κB/DNA binding was not detectable in un-stimulated cells (fig. 2c). TNF-α induced binding of NF-κB to the DNA and this was not altered by GSH-OEt (fig. 2c). Treatment with DMF inhibited TNF-α induced NF-κB/DNA binding and importantly, this was reversed by GSH-OEt (fig. 2c).

**DMF inhibits histone H3 phosphorylation, which was reversed by GSH-OEt**

DMF inhibited MSK-1 activity in ASMC [7], up-stream of histone H3 [20], which is important for optimal NF-κB/DNA binding [19]. Here we show that TNF-α induces histone H3 phosphorylation between 15-60 min, with a maximal effect at 30 min (Figure), which was inhibited by DMF (fig. 3a). Since inhibition of NF-κB/DNA binding by DMF was fully reversed by addition by GSH-OEt, we were curious whether DMF’s effect on histone H3 is also dependent on GSH. Interestingly, DMF’s inhibitory effect on TNF-α induced histone H3 phosphorylation at 30 min was fully reversed by addition of GSH-OEt, whereas GSH-OEt alone had no effect on TNF-α induced histone H3 phosphorylation (fig. 3b). TNF-α, GSH-OEt, DMF alone or in combination had no effect on total histone H3 protein expression (fig. 3a, 3b).

**DMF inhibits MSK-1 independently of up-stream molecules p38 and ERK MAPK**

DMF inhibited MSK-1 in ASMC [7] and this was confirmed in this study, where DMF inhibited the phosphorylation of TNF-α induced MSK-1 at Ser376 between 10-30 min (fig. 4a). In contrast, TNF-α induced phosphorylation of MSK-1 at Ser360 and threonine (Thr)581 between 10-30 min was not affected by DMF treatment (fig. 4a). TNF-α or DMF had no effect on total MSK-1 protein expression (fig. 4a). p38 and ERK MAPKs can phosphorylate and thereby activate MSK-1 [20]. Here we show that DMF-induced
MSK-1 inhibition is not mediated by an inhibition of p38 or ERK MAPK. TNF-α activated p38 MAPK phosphorylation between 5-30 min, which was even enhanced and prolonged by DMF treatment (fig. 4b). ERK phosphorylation was induced by TNF-α between 10-30 min and this was not altered by DMF (fig. 4b). Total p38 and ERK MAPK protein expression was not affected by either TNF-α or DMF between 0-120 min (fig. 4b). TNF-α up-regulated the endogenous MAPK inhibitor MKP-1 after 60 min, as previously described [23] and this was not affected by DMF (fig. 4b). Equal protein loading was confirmed by incubation of the membrane with α-Tubulin (fig. 4b).

**GSH-OEt reverses DMF’s inhibitory effect on eotaxin and RANTES**

Next we assessed whether GSH-OEt can also reverse DMF’s inhibitory effect on eotaxin and RANTES. TNF-α significantly induced eotaxin and RANTES secretion when compared to the vehicle control and this was inhibited by DMF (fig. 5a, 5b) as previously reported [7]. Importantly, the addition of GSH-OEt significantly reversed DMF’s inhibitory effect on eotaxin and RANTES (fig. 5a, 5b). GSH-OEt alone had no effects on TNF-α induced eotaxin or RANTES or the baseline levels of the two chemokines (fig. 5a, 5b).

**DISCUSSION**

In this study we showed that IκB-α -SSG and reduced histone H3 phosphorylation contribute to DMF’s inhibitory effect on NF-κB dependent eotaxin and RANTES secretion. DMF reduced intracellular GSH in ASMC and this has previously been shown to induce protein glutathionylation [11]. In this study, we showed for the first time that
I\(_{\kappa}B-\alpha\) -SSG is inducible in cultured ASMC. Furthermore, we showed that DMF inhibited the degradation of I\(_{\kappa}B-\alpha\) and subsequent NF-\(_{\kappa}\)B p65 nuclear entry, which was mediated by I\(_{\kappa}B-\alpha\) -SSG. In addition, we found that histone H3 phosphorylation, which enhances accessibility of NF-\(_{\kappa}\)B binding sides within promoters [19], was inhibited by DMF. This effect was most likely mediated by DMF-induced inhibition of MSK-1, upstream of histone H3. Importantly, not only DMF-mediated inhibition of NF-\(_{\kappa}\)B and histone H3 were reversed by addition of GSH-OEt, but also its inhibitory effect on eotaxin and RANTES, suggesting that DMF inhibits these factors by altering ASMC GSH level. Taken together, our study reveals new mechanisms by which eotaxin and RANTES secretion can be inhibited in ASMC. Development of new drugs, targeting the here-described pathways may help to limit eosinophilic inflammation in asthma.

NF-\(_{\kappa}\)B is considered to be one of the most important regulators of immune responses and was shown to play a critical role in inflammatory airways diseases [24]. NF-\(_{\kappa}\)B regulates the expression of many pro-inflammatory factors and its inhibition was shown to be anti-inflammatory in ASMC [7]. Earlier studies have shown that reduced level of intracellular GSH can inhibit NF-\(_{\kappa}\)B activity [25, 26]. This is in agreement with our study, showing that DMF mediates its inhibitory effect on NF-\(_{\kappa}\)B via a reduction of intracellular GSH. Glutathionylation, which is inducible by reduced intracellular GSH level [11], is a reversible redox-regulated mechanism and a number of studies have shown that the NF-\(_{\kappa}\)B pathway was inhibited by glutathionylation on multiple levels. In tracheal epithelial cells, glutathionylation of I\(_{\kappa}B\) kinase complex resulted in an inhibition of NF-\(_{\kappa}\)B p65 nuclear entry and NF-\(_{\kappa}\)B DNA binding [27]. In HeLa cells, diamide induced the I\(_{\kappa}B-\alpha\) -SSG, which decreased phosphorylation and ubiquitinylation of I\(_{\kappa}B-\alpha\) in vitro [10].
Glutathionylation of the NF-κB p65 subunit was induced in cinnamaldehyde treated endothelial cells, leading to an inhibition of NF-κB p65 nuclear translocation [12]. NF-κB p50-SSG in vitro inhibited the capacity of NF-κB to bind to the DNA [13]. The here reported induction of IκB-α -SSG by DMF is therefore likely to contribute to its inhibitory effect on NF-κB nuclear entry and NF-κB/DNA binding.

Previously it was shown that DMF inhibits the phosphorylation of MSK-1 at Ser376, resulting in reduced phosphorylation of down-stream CREB and NF-κB p65 [7, 21]. MSK-1 also phosphorylates histone H3 at Ser10 [20], which increases the accessibility of NF-κB binding sides within different promoters [19]. In this study, we showed that DMF inhibits the phosphorylation of histone H3, which was most likely mediated by its inhibition of MSK-1. Interestingly, DMFs inhibitory effect on MSK-1 was not mediated by reduced activation of p38 or ERK MAPK, up-stream of MSK-1. Furthermore, MSK-1 phosphorylation at Ser360 and Thr581, phosphorylation sides, which are directly targeted by p38 or ERK MAPK [28], were not affected by DMF treatment. These findings are in agreement with another study in keratinocytes, showing that DMF inhibited MSK-1 phosphorylation at Ser376 independently of p38 or ERK MAPK [21]. MKP-1, an endogenous inhibitor of MAPK, can be up regulated by reduced GSH level [29] and was reported, besides inhibiting MAPK p38 and ERK in ASMC [23], to dephosphorylate histone H3 at Ser10 [30]. However, DMF treatment did not affect MKP-1 level in our study, suggesting it does not mediate DMFs inhibitory effect on histone H3 phosphorylation. Importantly, the DMF effect on histone H3 phosphorylation was reversed by addition of GSH-OEt, suggesting that DMF-induced changes in intracellular GSH also mediate DMFs effect on the MSK-1/histone H3 pathway. However, the exact
mechanisms how DMF inhibits the MSK-1/histone H3 pathway remains to be determined in future studies.

The DMF-dependent down-regulation of eotaxin and RANTES confirmed our previous study [7] and is of particular importance as these chemokines attract eosinophils towards the inflamed airway, which in turn alter ASMC function [1]. Earlier we found that DMF inhibits eotaxin and RANTES by inhibiting NF-κB [7]. Here we show that both effects dependent on DMF-induced reduction of intracellular GSH. Similarly, DMF inhibited IL-1β, TNF-α and IL-6 secretion in human peripheral blood mononuclear cells, which was rescued by GSH supplementation [16]. Furthermore, DMF reduced glutathione level and inhibited LPS-induced IL-1β, TNF-α and IL-6 secretion in glial cells [15].

In conclusion, new molecular mechanisms by which TNF-α induced chemokine secretion can be inhibited in ASMC have been demonstrated in this study. Targeting the here-described pathways might help to reduce eosinophilic inflammation in asthma.

SUPPORT STATEMENT

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REFERENCES


**FIGURE LEGENDS**

Figure 1: a) Cell characterization for one ASMC at passage 3 and 10. Human lung fibroblasts were used at passage 3: SMMHC: smooth muscle myosin heavy chain, α-SMA: α-smooth muscle cell actin. b-d) DMF reduces intracellular GSH and induces IκB-α glutathionylation. b) ASMC of three different donors were treated with DMF (50 μM) or vehicle DMSO (0.05%) and intracellular glutathione was measured after 1 and 3 h. Data represent means ± SE from 5 replicates (Mann Whitney U-test). c) ASMC were treated with diamide or d) were left unstimulated (US) for 30 min (left panel) or c) were pre-treated with GSH-OEt (1mM; 90 min) and/or DMF (50 μM; 1 h) followed by stimulation with TNF-α (10 ng/ml) for 30 min. (right panel). Cells were lysed and the NF-κB/IκB complex was immunoprecipitated (IP) with an antibody against either NF-κB p65 or NF-κB p50 or normal rabbit IgG (IgG). Following immunoblotting under non-reducing conditions the membranes were incubated with antibodies against glutathione (IκB-α-SSG), or total IκB-α, NF-κB p65, or p50.
a) 

![Image of fibronectin, SMMHC, and α-SMA expression in cells at different passages.]

b) 

![Graph showing glutathione (GSH) levels over time with vehicle and DMF treatments.]

![Bar chart showing p-Ser65 and p-Ser535 levels at 1 and 3 hours.]

![Western blot images showing IKK-α and NF-κB p65 protein levels at different concentrations of diamide.]
Figure 2: Effects of DMF and its reversal by GSH-OEt on NF-κB p65 nuclear entry and NF-κB to DNA binding. 

a) Nuclear entry of NF-κB p65 is depicted by a representative fluorescence immunostaining of ASMC treated with GSH-OEt (1mM; 90 min) and/or DMF (50 μM; 1 h) prior to TNF-α stimulation (10 ng/ml) for 30 min. b) Nuclear entry of NF-κB p65 at 30 min after stimulation was confirmed by immunoblotting using nuclear protein extracts. The bar chart below summarized the densitometric analysis of NF-κB p65 nuclear entry at 30 min normalized to lamin A/C. Bars represent mean ± S.E.M. of experiments performed in four different cell lines. Mann Whitney U-test, * indicates p ≤ 0.05 for the comparison: TNF-α vs. TNF-α&DMF. c) shows a representative NF-κB specific EMSA; three additional independent experiments showed comparable results.
Figure 3: Effects of DMF and its reversal by GSH-OEt on histone H3 phosphorylation. a) ASMC (n = 3) were treated with GSH-OEt (1mM; 90 min) and/or DMF (50 μM; 1 h) prior to stimulation with TNF-α (10 ng/ml) for the indicated time points. A representative immunoblot of the kinetic of histone H3 phosphorylation at Ser10 induced by TNF-α and its inhibition by DMF. b) a representative immunoblot of the reversing effect of exogenous GSH-OEt on DMF-dependent histone H3 (Ser10) inhibition at 30 min.

Figure 4: DMF effects on MSK-1 and MAPK phosphorylation and on MKP-1 expression. a) ASMC lines (n = 3) were treated with DMF 1 h prior to stimulation with TNF-α for 0-120 min. Representative immunoblots of the kinetics of MSK-1 phosphorylation at Thr581, Ser360, Ser376 and of total MSK-1 expression. b) a representative immunoblot
of the p38 MAPK phosphorylation kinetics and of total p38 MAPK expression; of ERK 1/2 MAPK phosphorylation, and of total ERK 1/2 MAPK and MKP-1 expression and the loading control α-tubulin. Similar results were obtained in two additional cell lines.
Figure 5: GSH-OEt reverses the effects of DMF on eotaxin and RANTES secretion by ASMC. ASMC lines of four different donors were treated with GSH-OEt (1mM; 90 min) and/or DMF (50 μM; 1 h) prior to stimulation with TNF-α for 24 h and a) eotaxin and b) RANTES secretion was measured by ELISA. Bars represent means ± SE from 6-7 replicates and were analyzed using Mann Whitney U-test. * indicates p ≤ 0.05 TNF-α vs. TNF-α&drugs.