Dimethylfumarate protects against TNF-α-induced secretion of inflammatory cytokines in human endothelial cells

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Abstract

Background: Inflammation, angiogenesis and oxidative stress have been implicated in the pathogenesis of various vascular diseases. Recent evidence suggests that dimethylfumarate (DMF), an antipsoriatic and anti-multiple sclerosis agent, possesses anti-inflammatory, anti-oxidative and anti-angiogenic properties. Here, we analyze the influence of DMF on TNF-α-induced expression of the important pro-inflammatory and pro-atherogenic chemokine MCP-1 and investigate the underlying mechanisms of this expression.

Findings: We analyzed constitutive and TNF-α-induced expression of MCP-1 in human umbilical vascular endothelial cells (HUVEC) +/- DMF treatment via enzyme-linked immunosorbent assay (ELISA). DMF significantly inhibited the protein expression levels in a time- and concentration-dependent manner. Furthermore, MCP-1 mRNA expression was also reduced in response to DMF, as demonstrated by RT-PCR. Thus, the regulation occurs at the transcriptional level. Interestingly, DMF prolonged the TNF-α-induced p38 and JNK phosphorylation in HUVEC, as demonstrated by Western blot analysis; however, the p38 and JNK inhibitor SB203580 did not affect the DMF-conveyed suppression of TNF-α-induced MCP-1 expression. DMF suppressed the TNF-α-induced nuclear translocation and phosphorylation (Serine 536) of p65 in these cells. These results were additionally approved by p65 luciferase promoter assays. Furthermore, we found that DMF slightly inhibited the early degradation of IκBα. In addition, we verified our results using other important inflammatory cytokines such as CCL-5, PDGF-BB, GM-CSF and IL-6.

Conclusion: DMF suppresses various TNF-α-induced pro-inflammatory and pro-atherogenic cytokines/chemokines in human endothelial cells. This action is regulated by reduced p65 activity and nuclear translocation, which can be explained in part by the reduced early degradation of IκBα and more important the reduced phosphorylation of p65 at Serine 536. These effects were independent of the p38, PI3K and p42/44 signaling pathways. As a result, DMF might be suitable for treating patients with vascular diseases.

Keywords: Dimethylfumarate, p65, MCP-1, TNF-α, IκBα

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Dimethylfumarate (DMF) has been successfully used in the treatment of psoriasis, a chronic inflammatory disease, for more than 40 years [5], and several clinical trials have proven its efficacy. Last year, DMF was approved for the treatment of relapsing-remitting multiple sclerosis (MS) [6]. There are a number of mechanisms by which DMF mediates its anti-inflammatory action. Stoof et al. showed that IL-8, Gro alpha, IP-10 and Mig are downregulated during DMF treatment [7]. In addition, DMF treatment has been shown to result in leukocytopenia and a reduction in the number of CD8+ and CD4+ T cells in psoriasis lesions, primarily due to apoptosis [8, 9]. Recent research has revealed the anti-oxidant properties of DMF in various conditions induced in part by the expression of anti-oxidant molecules such as HO-1 and the activation of Nrf-2 [10]. Vandermeeren et al. demonstrated that DMF is an effective inhibitor of cytokine-induced VCAM-1, ICAM-1 and E-selectin expression in human endothelial cells [11]. Additional investigations revealed that DMF inhibits nuclear translocation of activated NF-κB in endothelial cells and suppresses the activation of NF-κB in human dendritic cells [12, 13]. Recently, we demonstrated that DMF inhibits angiogenesis via the downregulation of VEGFR-2 expression [14]. These results were the first hint that DMF also possesses distinct anti-angiogenic actions [15]. Recently, it was demonstrated that DMF might be a therapeutic drug for patients with vascular diseases. Oh et al. demonstrated that DMF attenuates restenosis in a rat carotid artery balloon injury model [16]. Silhavy et al. also showed that DMF blocks the pro-inflammatory actions of human CRP in transgenic spontaneously hypertensive rats [17]. Ashafrian et al. demonstrated the cardioprotective action of DMF in an in vivo model of myocardial infarction [18]. In addition, Milenkovic et al. demonstrated that DMF has beneficial effects in autoimmune myocarditis [19].

Here, we investigated whether DMF suppresses the constitutive and TNF-α-induced expression of important pro-inflammatory chemokines and cytokines in human endothelial cells. We also analyzed the underlying mechanisms of this expression.

Material and methods

Reagents

Recombinant human TNFα was purchased from R&D Systems (Minneapolis, MN, USA). Dimethylfumarate, SB203580, PD98059 and Wortmannin were obtained from Sigma-Aldrich (Hamburg, Germany).

Cell culture

Human umbilical vascular endothelial cells (HUVECs) were purchased from PromoCell (Heidelberg, Germany) and were cultured until the fifth passage at 37 °C and 5 % CO2 in Endothelial Cell Growth Medium (Lonza, East Rutherford, NJ, USA).

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of MCP-1, PDGF-BB, GM-CSF, CCL-5 and IL-6 in cell culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA). Commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) were used for the quantification as described in the manufacturer’s instructions.

Western blot analysis

The protein extracts were prepared as described previously [14]. Following SDS-PAGE and electroblotting, the membranes were incubated with the following primary antibodies: p65, phospho-p65 (Ser536), IκBα p38, p42/44, JNK, phospho-p38, phospho-p42/44, phospho-JNK, Phospho-Akt, Akt and tubulin (Cell Signaling, Danvers, MA, USA); Laminin A/B (Santa Cruz, Dallas, Texas, USA); SP1 (Sigma-Aldrich, St. Louis, MO, USA). Primary antibody application was followed by incubation with horseradish peroxidase-conjugated secondary antibodies (anti-mouse and anti-rabbit IgG, Amersham, Uppsala, Sweden; anti-goat, Dako, Glostrup, Denmark). The blots were visualized using an enhanced chemiluminescence detection system (ECL) (Amersham, Freiburg, Germany) according to the manufacturer’s instructions. Densitometry was used to quantify band intensities using ImageJ (v1.29 s). Optical densities of the bands were corrected for loading differences based on corresponding control bands.

RNA extraction and RT-PCR

The RT-PCR analyses were performed using total RNA (150 ng) extracted from sub-confluent cell cultures. The total cellular mRNA was isolated using the RNeasy Mini Procedure (Qiagen, Hilden, Germany) after DNase digestion. The RT-PCR analyses for MCP-1 and GAPDH were performed with a One Step RT-PCR Kit (Qiagen, Hilden, Germany). The PCR products were resolved by gel electrophoresis in a 1-2 % agarose gel, and the ethidium bromide-stained bands were visualized using an ultraviolet transilluminator. The primer sets for MCP-1 and GAPDH were previously published [14].

Transient transfection and analysis of reporter gene expression

HUVECs (1.0 × 10^4 cells/well in 12-well plates) were transfected with 0.5 μg of the appropriate firefly luciferase construct and 0.1 μg phRG-TK vector (Promega, Madison, WI, USA) using the SuperFect transfection reagent (Qiagen). The P TransLucent NFκB promoter vector was purchased from Panomics (Affymetrix, Santa Clara, CA, USA).
USA). Twenty-four hours after transfection, the cells were treated with vehicle (0.3 % DMSO) or with DMF for 24 h. The luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

**Fluorescence microscopy**
HUVECs were plated onto 8-well chamber slides (Lab-Tek, Christchurch, New Zealand) at a density of 80 %/well. The cells were grown in endothelial cell basal medium with 5 % FCS for 24 h, fixed in 4 % paraformaldehyde at room temperature for 10 min, and permeabilized with 0.1 % Triton X-100 for 5 min at room temperature. Permeabilized cells were rinsed three times with PBS and incubated in blocking solution (1 % bovine serum albumin/PBS) for 30 min at room temperature to remove nonspecific binding of the antibody. All subsequent steps were carried out at room temperature, and cells were rinsed three times in 1 % bovine serum albumin/PBS between each of the steps. p65 was detected using a rabbit polyclonal anti-NkB p65 antibody (D14E12; Cell Signaling) and an Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody (Alexa). The cytoskeleton was stained by phalloidin (Alexa-Fluor 488). The slides were mounted in Vectashield immuno-fluorescence mounting medium (Vector Laboratories) and viewed with fluorescence microscopy. Cells were counterstained for nuclei with Hoechst staining.

**Statistical analysis**
The data are expressed as the mean ± SEM of at least three independent experiments. The statistical analyses were performed using Student’s *t*-test.

**Results**

**Dimethylfumarate suppresses constitutive and TNF-α-induced MCP-1 protein expression**
First, we analyzed the effect of DMF on constitutive MCP-1 expression in HUVECs. DMF inhibited MCP-1 expression significantly in a time- and concentration-dependent manner (Fig. 1a,b). The concentrations and incubation times that we adopted have been published recently and have been proven to have no cytotoxic effects in HUVEC [14]. DMF in concentrations less than 20 μM reduced the MCP-1 expression level by almost 50 %; within 3 h of treatment, the MCP-1 expression was significantly reduced compared with that of the matching controls. To determine whether DMF was also able to reduce TNF-α-induced MCP-1 expression, we analyzed the effects of the combinational treatment with DMF and TNF-α (20 ng/ml) in a time- and concentration-dependent manner. We found that 80 μM DMF reduced the TNF-α-induced MCP-1 expression by more than 50 % (Fig. 1c). A first significant reduction of MCP-1 expression could be observed as early as 6 h after treatment with 80 μM DMF and TNF-α (Fig. 1d).

**Dimethylfumarate inhibits constitutive and TNF-α-induced MCP-1 mRNA expression**
To determine whether a transcriptional mechanism was responsible for the reduced MCP-1 expression observed in response to DMF, we performed RT-PCR analysis. As observed in our protein expression analysis, DMF inhibited the constitutive as well as the TNF-α-induced MCP-1 mRNA expression after 24 h (Fig. 2a,b). To exclude that this effect did not only occur at later time points, we also performed RT-PCR analysis after 3 h of treatment (Fig. 2c). We were able to demonstrate that DMF supresses TNF-α-induced MCP-1 mRNA expression effectively.

**Dimethylfumarate prolongs p38 and JNK phosphorylation in TNF-α-treated HUVECs**
The p38/JNK, PI3K/Akt and p42/44 signaling pathways are known to be important in TNF-α-induced MCP-1 expression. Therefore, we analyzed the phosphorylation status of p38, JNK, Akt and p42/44 in HUVEC over time (0–60 min.) in response to TNF-α (20 ng/ml) or TNF-α plus DMF (80 μM, 3 h pre-treatment). Surprisingly, we found that DMF prolonged the TNF-α-induced phosphorylation of p38 and JNK in HUVECs significantly based on Western blot and densitometry analyses (Fig. 3a,b). For Akt phosphorylation, we observed an early significant (0 and 5 min.) superinduction but no prolonged phosphorylation compared with TNF-α treatment. The phosphorylation of p42/44 was not influenced by DMF treatment. To determine whether these signaling pathways were responsible for the effects of DMF on MCP-1 expression, we performed an enzyme-linked immunosorbent assay (ELISA) with the p38/JNK inhibitor SB203580, the PI3K/Akt inhibitor Wortmannin and the p42/44 inhibitor PD98059. Interestingly, blocking these signaling pathways did not affect the DMF-induced suppression of MCP-1 expression (Fig. 3c).

**Dimethylfumarate reduces TNF-α-induced p65 nuclear entry, phosphorylation and promoter activity and inhibits IκBα degradation**
It is well known that TNF-α mediates its effects, such as increased MCP-1 expression, mainly by nuclear translocation and phosphorylation of p65 after the degradation of IκBα. Recently, Loewe et al. demonstrated the reduced nuclear translocation of p65 in response to DMF treatment in HUVEC, partially reversing the TNF-α effects. In our experiments, we observed reduced p65 translocation but
additionally recorded reduced nuclear p65 phosphorylation at Serine 536 during DMF treatment (Fig. 4a). Furthermore, we found a significant reduction in nuclear translocation of p65 by immunofluorescence staining of NFκB p65 in HUVECs treated with a combination of DMF and TNF-α compared with a treatment of TNF-α alone (Fig. 4b). To exclude a reduced overall expression of p65 during the treatment with DMF, we analyzed p65 expression by Western blot analysis (Additional file 1); no reduction of p65 expression was observed.

In contrast with Loewe et al., we did observe a slightly reduced degradation of IkBa in response to treatment with DMF, which might in part explain the early (5-30 min) decreased p65 nuclear translocation (Fig. 4c). In HUVECs transiently transfected with an NFkB-luciferase promoter construct, DMF significantly reduced both the basal transcriptional activity as well as the TNF-α-dependent NFkB promoter activity after 24 h of treatment (Fig. 4d).

**Discussion**

The endothelial cell is one of the central components of various inflammatory diseases such as psoriasis, diabetes, cancer and rheumatoid arthritis [20]. Recent evidence has shown that chronic inflammation is associated with
accelerated atherosclerosis and increased cardiovascular mortality and morbidity [21]. It is believed that the therapy targeting the inflammatory processes shared in psoriasis and atherosclerosis may reduce cardiovascular risk in psoriatic patients [22]. In our study, we analyzed the influence of DMF, a potent anti-psoriatic and anti-MS drug, on the constitutive and TNF-α-induced expression of MCP-1 and other important pro-inflammatory and pro-atherogenic cyto- and chemokines. We additionally examined the underlying mechanisms of this expression. We demonstrated that DMF significantly suppressed the constitutive and TNF-α-induced expression of MCP-1 and other important pro-inflammatory and pro-atherogenic cyto- and chemokines. The TNF-α-induced expression of these pro-inflammatory cyto- and chemokines is known to be mainly transcriptionally regulated by NfκB [24]. We used MCP-1 as a model chemokine and demonstrated a transcriptional way of regulation. To determine whether this regulation was controlled by the p38/JNK, PI3K/Akt or p42/44 signaling pathways, we performed Western blot analysis. We detected prolonged and superinduced phosphorylation of p38 and JNK in response to DMF compared with TNF-α alone. Interestingly, Seidel et al. demonstrated an enhancement of PDGF-BB-induced p38 phosphorylation by DMF in smooth muscle cells [25]. On the other hand, Gesser et al. found that DMF did not influence IL-1β-induced p38 phosphorylation in human keratinocytes [26]. Recently, Xie et al. demonstrated that
Fig. 3 (See legend on next page.)
DMF induces p38, JNK and p42/44 phosphorylation in colon carcinoma cell lines and induces necroptosis [27]. In our experiments, we did not find any significant influence of DMF on p42/44 phosphorylation. Therefore, this effect might be cytokine- and/or cell-type specific. To determine whether the signaling pathways were related to the inhibition of TNF-α-induced MCP-1 expression, we blocked them using specific inhibitors. Interestingly, blocking the p38, JNK, Akt and p42/44 pathways did not affect the DMF-induced MCP-1 suppression. Therefore, we showed that the prolonged activity of p38 and JNK by DMF treatment was independent of its effect on MCP-1 expression.

It is well known that TNF-α is a potent NfkB inductor and that MCP-1 and the other analyzed cyto- and chemokines are regulated by NfκB nuclear translocation and phosphorylation [28]. Therefore, we studied the TNF-α-induced nuclear translocation and phosphorylation of p65 to determine whether DMF has the ability to reduce these effects. We found that DMF clearly inhibits the TNF-α-induced nuclear p65 translocation. This phenomenon has also been described by Loewe et al. [13]. We extended this observation concerning the phosphorylation status of p65 by demonstrating that the TNF-α-induced p65 phosphorylation was almost...
completely eliminated by DMF. In addition, we found that treatments with DMF resulted in slightly reduced early degradation of IκBα. This observation only explains in part the early decreased p65 nuclear translocation (5-30 min). The later inhibition of p65 translocation by DMF seems to be independent of IκBα degradation, as demonstrated by Peng et al. in dendritic cells treated with DMF [29]. Sasaki et al. demonstrated that phosphorylation of p65 on Serine 536 defines an IκBα-independent NF-κB pathway [30]. This group suggests that there are two mutually exclusive populations of p65 that are distinguished in their phosphorylation status at serine 536. They postulate that during activation, the populations translocate independently and induce transcription of different genes. These results are in accordance with our experiments demonstrating a significant reduction of p65 phosphorylation at Serine 536, which now might explain the reduced p65 translocation mainly independent of IκBα in human endothelial cells. Thus, our results not only confirm but also broaden and explain the results demonstrated by Loewe et al. concerning the nuclear entry of p65 [13].

To prove that the DMF-conveyed reduced nuclear translocation and phosphorylation of p65 is functionally relevant, we performed NFκB luciferase promoter assays demonstrating significant inhibition of constitutive and TNF-α-induced promoter activity by DMF.

In summary, we demonstrated that DMF inhibits constitutive and TNF-α-induced expression of pro-inflammatory and pro-atherogenic cytokines in human endothelial cells. Our findings are consistent with the inhibition of nuclear translocation, phosphorylation and activity of p65, which can be explained in part by the reduced early degradation of IκBα and more important the reduced phosphorylation of p65 at Serine 536. These results, which may help to explain the cardioprotective and anti-inflammatory effects of DMF, highlight its potential importance in the future treatment of vascular diseases.
Additional file

Additional file 1: Figure S1. Analysis of p65 expression during treatment with DMF, TNF-α and DMF + TNF-α in HUVECs. Western blot analysis of whole lysate proteins of HUVECs treated with vehicle (solvent only), TNF-α (20 ng/ml) or DMF (80 μM) + TNF-α for the indicated times. The experiments were performed with comparable results at least 3 times. (PPTX 1310 kb)

Abbreviations
HUVEC: human umbilical vascular endothelial cells; DMF: Dimethylfumarate; DMSO: Dimethylsulfoxide; TNF: Tumor necrosis factor; MCP: Monocyte chemotactant protein; IL: Interleukin; CCL: Chemokine ligand.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
MM designed the study, performed the data analysis, interpreted the data, and wrote the manuscript. HG acquired the data. TH performed with comparable results at least 3 times. RK interpretated the data. SK interpretated the data. All authors have read and approved the final manuscript.

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