

Dimethylfumarate Inhibits TNF-Induced Nuclear Entry of NF- κ B/p65 in Human Endothelial Cells¹

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Fumaric acid esters, mainly dimethylfumarate (DMF), have been successfully used to treat psoriasis. Based on previous observations that DMF inhibited expression of several TNF-induced genes in endothelial cells, we wished to explore the molecular basis of DMF function in greater detail. In first experiments we analyzed DMF effects on tissue factor expression in human endothelial cells in culture, because tissue factor is expressed by two independent sets of transcription factors, by NF- κ B via TNF and by early gene response-1 transcription factor via vascular endothelial growth factor (VEGF). We show that DMF inhibits TNF-induced tissue factor mRNA and protein expression as well as TNF-induced DNA binding of NF- κ B proteins, but not VEGF-induced tissue factor protein, mRNA expression, or VEGF-induced early gene response-1 transcription factor/DNA binding. To determine where DMF interferes with the TNF/NF- κ B signaling cascade, we next analyzed DMF effects on I κ B and on the subcellular distribution of NF- κ B. DMF does not inhibit TNF-induced I κ B α phosphorylation and I κ B degradation; thus, NF- κ B is properly released from I κ B complexes even in the presence of DMF. Importantly, DMF inhibits the TNF-induced nuclear entry of NF- κ B proteins, and this effect appears selective for NF- κ B after the release from I κ B, because the constitutive shuttling of inactive NF- κ B/I κ B complexes into and out from the nucleus is not blocked by DMF. Moreover, DMF does not block NF- κ B/DNA binding. In conclusion, DMF appears to selectively prevent the nuclear entry of activated NF- κ B, and this may be the basis of its beneficial effect in psoriasis. *The Journal of Immunology*, 2002, 168: 4781–4787.

Activation of NF- κ B by proinflammatory stimuli leads to the expression of genes inducing and maintaining inflammation. The NF- κ B family consists of several different proteins such as p50, p52, p65 (RelA), RelB, and c-Rel, which all share a conserved Rel homology domain responsible for dimerization, nuclear localization, and DNA binding of NF- κ B proteins. The biological activity of these proteins is regulated by the concentration, the nucleocytoplasmic distribution, and the DNA binding activity. All those parameters are controlled by a family of inhibitory proteins, termed I κ B, to which NF- κ B proteins are bound under unstimulated conditions. The best-characterized protein in this family is I κ B α , which binds p65/p50 heterodimers, the most ubiquitous and biologically active NF- κ B dimer and p65/c-Rel heterodimers. I κ B α shifts the subcellular distribution of NF- κ B predominantly toward the cytoplasm and prevents DNA binding by occupying the Rel domain (1, 2). Upon stimulation by, e.g., TNF, I κ B α and I κ B ϵ undergo rapid phosphorylation, ubiquitinylation, and subsequent degradation by the 26S proteasome (3), resulting in the release, nuclear entry, and DNA binding of NF- κ B (4). Other known I κ B proteins are I κ B β and I κ B ζ , which have been more recently identified. I κ B β degra-

tion is more delayed and does not occur before 30–60 min following TNF stimulation, and I κ B ζ appears not to be involved in retaining NF- κ B in the cytosol (5–7).

Because of the well-established biological significance of NF- κ B in inflammation, many efforts have been undertaken to block NF- κ B-induced gene transcription. Most pharmacological inhibitors are not specific for NF- κ B. Some of them block the NF- κ B pathway upstream of I κ B as curcumin (8), thiol-reactive metal ions (9), or aspirin (10). Others seem to exhibit their function more downstream in the NF- κ B pathway, as, for example, mesalamine, which interferes with the phosphorylation of NF- κ B (11) and glucocorticoids, or cAMP, which appear to directly prevent DNA binding of p65 (12, 13). There are some specific inhibitors of NF- κ B described (all of which have been used in experimental settings only and are not registered for their use in humans), e.g., helenalin, which directly and irreversibly alkylates p65 (14); caffeic acid phenethyl ester, which prevents nuclear translocation of NF- κ B (15); and BAY 11-7082, which inhibits cytokine-mediated I κ B α phosphorylation (16).

Fumaric acid (FA)³ esters have been used empirically in the treatment of psoriatic patients for many years. In 1989 the first controlled study was performed showing the efficacy of dimethylfumarate (DMF) (17). Subsequently, several open and controlled clinical trials have confirmed the efficacy of FA esters in psoriasis. In vitro studies have only partially resolved the molecular basis of their function. For example, in lymphocytes, DMF and its hydrolysis product, methylhydrogen fumarate (MHF), modulated cytokine expression toward a Th2 cytokine profile (18, 19). Both substances inhibited keratinocyte proliferation (20) and monocyte differentiation into dendritic cells (21).

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³ Abbreviations used in this paper: FA, fumaric acid; DMF, dimethylfumarate; MHF, methylhydrogen fumarate; VEGF, vascular endothelial growth factor; EGR-1, early gene response-1 transcription factor.

Several lines of evidence suggest that in endothelial cells DMF inhibits TNF-induced gene expression by a mechanism involving NF- κ B (22, 23). In endothelial cells *in vivo*, DMF inhibits E-selectin (CD62E) expression when given as a therapeutic agent for psoriasis. In endothelial cells in culture, DMF inhibits TNF-induced expression of E-selectin and NF- κ B but not of AP-1 promoter constructs (22). In contrast to other cell types, in endothelial cells this effect is specific for DMF and not seen with other FA esters (22, 24).

In this study we wished to determine the site where DMF interferes with the TNF/NF- κ B signaling cascade. We analyzed tissue factor expression in endothelial cells, because tissue factor is rapidly induced by two unrelated pathways. The first is induced by TNF through binding of p65/c-Rel heterodimers and/or through binding of p65/p50 heterodimers to a single NF- κ B site within the promoter. The second pathway is induced by vascular endothelial growth factor (VEGF) through Sp1/murine early gene response-1 transcription factor (EGR-1) sites within the promoter (25–27). Thus, VEGF-induced tissue factor expression can be used as an internal control. In this work we show that DMF inhibits TNF-induced gene transcription of tissue factor at the level of the nuclear entry of NF- κ B (after the release from I κ B), whereas VEGF-induced tissue factor expression remains unaffected.

Materials and Methods

Cells

HUVECs were isolated and cultured as described previously (28). Cells were used between passages 3 and 6. Before HUVEC were subjected to experimental procedures, standard culture medium was switched to RPMI 1640 and supplemented with 10% FBS (both from Life Technologies, Gaithersburg, MD), without any further additions.

Abs and reagents

Mouse anti-human tissue factor mAb (clone TFE, IgG1) was from Enzyme Research Laboratories (Minneapolis, MN). CD31 mAb (clone 7E4) was a gift from Dr. O. Majdic (Institute of Immunology, University of Vienna, Vienna, Austria). Rabbit anti-p65 (clone C-20), anti-p50 (clone H-119), anti-c-Rel (clone N), anti-Sp1 (clone PEP 2), anti-EGR-1 (clone C-19), anti-I κ B α (clone C-21), anti-I κ B β (clone C-20), and anti-I κ B ϵ (clone M-121) Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-phospho-I κ B α (Ser³²) Ab was from Cell Signaling Technology (Beverly, MA). The mouse IgG1 isotype control (clone MOPC 31c) and the FITC-conjugated second-step anti-mouse IgG were from Sigma-Aldrich (Steinheim, Germany). HRP-labeled goat anti-rabbit and HRP-labeled goat anti-mouse Abs were from Bio-Rad (Hercules, CA). Alexa 488 goat anti-rabbit Ab was from Molecular Probes (Leiden, The Netherlands). Human rTNF- α and VEGF₁₆₅ were purchased from Strathmann Biotech (Hamburg, Germany) and used at concentrations of 10 and 50 ng/ml, respectively.

DMF (Fumapharm, Muri, Switzerland) was solubilized in methanol as a 70 mM stock solution and diluted in RPMI 1640 for final concentrations. FA (Fumapharm) was used as a control in most experiments and prepared as a 70 mM stock solution in RPMI 1640. The solvent, methanol, routinely used as a control in all experiments, did not alter results in any of the assays (Ref. 22 and data not shown). In selected experiments, MHF (Fumapharm), the hydrolysis product of DMF, was used. All stock solutions were used within 12 h.

Flow cytometry of surface protein expression

Confluent HUVEC were stimulated with TNF, VEGF, or both for 6–18 h in the presence or absence of indicated FA derivatives. Stimulated cells were detached with trypsin/EDTA (Life Technologies) and incubated with anti-tissue factor mAbs or mouse IgG1 isotype control Abs (each 1 μ g/ml), followed by a FITC-conjugated anti-mouse IgG. Surface-bound fluorescence was analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA). Geometric mean fluorescence values were calculated using CellQuest software (BD Biosciences) and corrected for the geometric mean fluorescence values of the respective isotype control.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed using LightCycler technology (Roche Molecular Biochemicals, Vienna, Austria) with SYBR Green I detection. Primers for tissue factor were designed using the PRIMER3 software from Whitehead Institute for Biomedical Research (Cambridge, MA) (29). The tissue factor primers were planned around a 1.7-kb intron to prevent accidental amplification of genomic DNA (GenBank accession number of tissue factor cDNA is NM_001993), and amplification products were analyzed on an agarose gel and sequenced. The primer pair 5'-CCGAA CAGTTAACCGGAAGA-3' (804 forward) and reverse 5'-TCAGTGGG GAGTTCTCCTTC-3' (1000 reverse) produced a single band with a defined melting point and a defined sequence and was used in this study. The primers for β_2 -microglobulin were 5'-GATGAGTATGCCTGCCGTGTG-3' and reverse 5'-CAATCCAAATGCGGCATCT-3' (30). mRNA from confluent HUVECs and stimulated as indicated was isolated using oligo(dT)₂₅ beads (DynaL Biotech, Oslo, Norway) and reverse transcribed using the first cDNA synthesis kit for RT-PCR from Boehringer Mannheim (Mannheim, Germany). Each LightCycler capillary was loaded with 2 μ l DNA master mix, 2.4 μ l MgCl₂, 13.5 μ l H₂O, and 0.5 μ l of each primer (10 μ M; final concentration, 250 nM). cDNA was amplified using a standardized program with a 10-min denaturing step following 55 cycles of 5 s at 95°C, 15 s at 65°C, and 15 s at 72°C, melting point analysis in 0.1°C steps, and a final cooling step. Relative quantification of target gene expression was calculated according to Pfaffl et al. (31). The PCR efficiencies for β_2 -microglobulin and tissue factor (amplicons) were calculated using the Relative Quantification Software from Roche Molecular Biochemicals (version 1.0). Tissue factor mRNA was normalized according to the relative content of β_2 -microglobulin under each treatment condition. Results are shown as fold inductions of tissue factor mRNA compared with the unstimulated control.

Immunoblotting

After indicated treatments, 10⁶ HUVEC were detached using trypsin/EDTA and centrifuged. Pellets were resuspended in 300 μ l of cytoplasmic lysis buffer (modified buffer A, containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 0.6% Nonidet P-40) and centrifuged. The supernatants (cytoplasmic fractions) were snap-frozen in liquid nitrogen and stored at -70°C. Pellets containing the nuclei were washed in buffer A without Nonidet P-40 and resuspended in 60 μ l nuclear lysis buffer (buffer C, containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 2 mM EDTA, 1 mM DTT, and 1 mM PMSF). After a 30-min incubation at 4°C, supernatants (nuclear fractions) were snap-frozen in liquid nitrogen and stored at -70°C. Protein concentration of each sample was adjusted by determining protein concentrations with the DC protein assay kit (Bio-Rad). Cytoplasmic or nuclear lysates were then loaded onto 7–12% polyacrylamide gels, electrophoresed, and blotted on polyvinylidene difluoride membranes as described previously (32). After blocking with 1% low-fat milk (Bio-Rad) for 12 h, incubation with first-step Abs as indicated diluted in 0.5% Tween/TBS for 1 h, rinsing, and incubation with the respective HRP-labeled second-step Abs, bound Abs were visualized by ECL (ECL plus system; Amersham Pharmacia, Little Chalfont, U.K.) and exposed on Hyperfilm MP (Amersham Pharmacia). To confirm equal protein content of samples, Coomassie stainings and immunoblots using CD31 or anti-Sp1 mAbs were performed in parallel.

EMSA

Oligonucleotides containing the NF- κ B consensus site (5'-CAGAGG GACTTTCGGAGA-3') and EGR-1 consensus site (5'-GCGGCGGGG GCGGGCGG-3') were radiolabeled with ³²P using T4 polynucleotide kinase (Roche Molecular Biochemicals; the underlined nucleotides depict the respective consensus sites). The labeled oligonucleotides were purified on Microspin G-50 columns (Amersham Pharmacia) and hybridized to the complementary strand. For the protein binding reaction, 5 μ g of nuclear or cytoplasmic extracts (prepared as described above) were incubated in a 15- μ l reaction mix containing 20 mM HEPES (pH 7.9), 1 mM MgCl₂, 10% glycerol, 40 mM KCl, 0.1 mM EDTA, 1 mM NaF, 10 mM DTT, 5 mM aprotinin, 1 mM benzamidine, and 50 ng salmon sperm DNA (for NF- κ B) or 1 μ g poly(dI:dC) (for EGR-1; Amersham Pharmacia). Where indicated, Abs or a 100-fold molar excess of unlabeled oligonucleotides were added to the reaction mixture and incubated for 10 min on ice. Thereafter, 1 ng of ³²P-labeled oligonucleotide (~100,000 cpm) was added to each reaction and further incubated for 20 min at room temperature. DNA/protein complexes were resolved on 6% nondenaturing polyacrylamide gels in 0.3 \times TBE buffer, dried, and visualized by autoradiography.

Immunofluorescence

A construct of p65 cDNA cloned into pEGFP-C1 (Clontech Laboratories, Palo Alto, CA) was used, which resulted in the expression of a green fluorescent p65 fusion protein (33). HUVEC at 80% confluency were transfected using the standard Ca^{2+} phosphate precipitation method as described elsewhere (34). Briefly, cells were conditioned with DMEM (Life Technologies) and overlaid with a precipitate containing 1 μg DNA per well (10^5 cells). After washing, full medium was added and cells were grown for another 18 h. Thereafter, cells were stimulated with TNF plus FA or DMF (84 μM each) for 2 h.

In a first set of experiments, nuclei were counterstained with propidium iodide and the subcellular distribution of GFP-p65 was analyzed by laser scan microscopy (Zeiss, Oberkochen, Germany).

In a second set of experiments, nuclei of transfected cells were collected using Dounce buffer containing 10 mM Tris-HCl (pH 7.6), 0.5 mM MgCl_2 , 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, 1 mM PMSF, and 1.8 mg/ml iodoacetamide. Nuclei were then subjected to FACS analysis to quantify the relative content of eGFP-p65.

In a third set of experiments, the distribution of p65 in the presence or absence of a nuclear export inhibitor, leptomycin B (Sigma-Aldrich), was analyzed in nontransfected HUVEC by direct immunofluorescence using anti-p65 Abs. Cells were cultured in the presence or absence of 84 μM DMF for 120 min. Thereafter, 20 nM leptomycin B was added for indicated times. After washing, cells were fixed with acetone/methanol 1:1 for 10 min at -20°C , rinsed, stained with anti-p65 Ab for 60 min at 4°C , and, following rinsing, stained with an Alexa 488-labeled second-step Ab for 60 min at 4°C . Cells were examined on a laser scan microscope.

Results

DMF inhibits TNF-induced tissue factor expression

Following TNF or VEGF stimulation, HUVEC expressed moderate amounts of tissue factor protein and mRNA (Fig. 1). The combination of TNF plus VEGF was synergistic, which has been reported previously (35). DMF significantly reduced TNF-induced tissue factor protein and mRNA expression in a dose-dependent fashion, as shown by FACS analysis ($p < 0.05$) and quantitative real-time PCR, but had only insignificant effects on VEGF-induced tissue factor expression. Moreover, DMF inhibited dose-dependently the synergistic effect of TNF plus VEGF (Fig. 1).

DMF reduces NF- κB /DNA complex formation

To analyze the effect of DMF on DNA binding of transcription factors, EMSAs were performed. Using nuclear extracts, TNF induced the binding of proteins to an oligonucleotide harboring a NF- κB consensus site, which was markedly reduced by DMF (Fig. 2A). Using cytoplasmic instead of nuclear extracts for EMSAs, we could also detect a TNF-inducible NF- κB /DNA complex. In contrast to the situation seen in the nucleus, DMF did not block the formation of these cytoplasmic NF- κB /DNA complexes, which allows the conclusion that DMF did not exert its effect by blocking binding of NF- κB proteins to DNA. Moreover, the addition of DMF or of its hydrolysis product, MHF, directly into the binding reaction of nuclear extracts of TNF-stimulated HUVEC did not alter NF- κB /DNA binding, confirming that DMF had no negative effect on the ability of NF- κB to bind DNA (data not shown).

A 100-fold molar excess of unlabeled wild-type oligonucleotide competed off binding of radioactive probes. Abs to p50, p65 (Fig. 2B), and c-Rel (data not shown) supershifted DNA/protein complexes, confirming the specificity of the bands.

The VEGF-induced protein binding to the Sp1/EGR-1 composite site analyzed for control purposes was not affected by DMF. The addition of anti-Sp1 and anti-EGR-1 Abs into the binding reaction inhibited specific complex formation (Fig. 2C). Destruction of the complex instead of supershifting with these Abs has been shown previously (27).

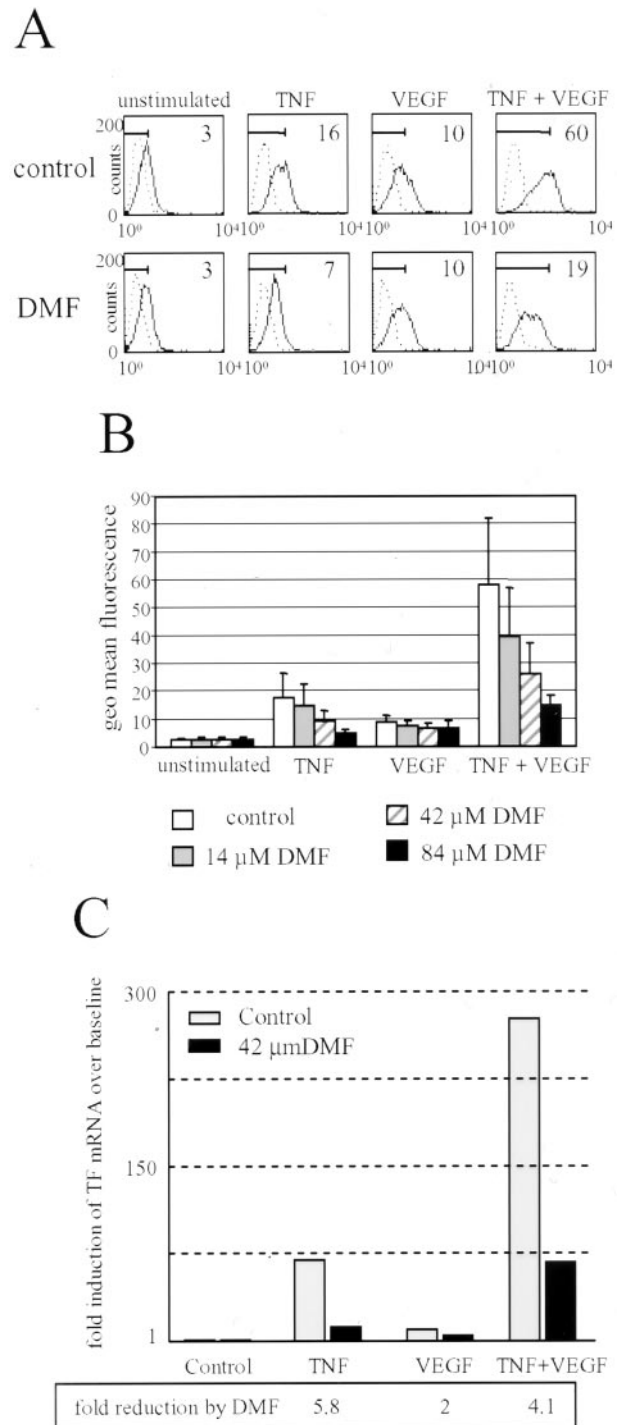


FIGURE 1. DMF inhibits TNF-induced tissue factor protein and mRNA expression. *A*, FACS analysis of tissue factor protein expression after stimulation of HUVEC with TNF (10 ng/ml), VEGF (50 ng/ml), or TNF plus VEGF for 6 h. *Upper panels*, Medium control; *lower panels*, medium supplemented with 84 μM DMF. Dotted lines show isotype control stainings; solid lines represent the specific staining. Values inserted into each histogram represent geometric mean fluorescence of the respective experiment. *B*, Summary of four independent FACS experiments analyzing tissue factor surface expression in the presence of indicated concentrations of DMF. Means \pm SD of the geometric mean fluorescence are shown. *C*, Quantitative real-time RT-PCR of tissue factor mRNA expression. HUVEC were incubated in medium containing 42 μM FA or 42 μM DMF and stimulated as indicated. Shown is the fold increase above baseline tissue factor expression; data are from one of two experiments.

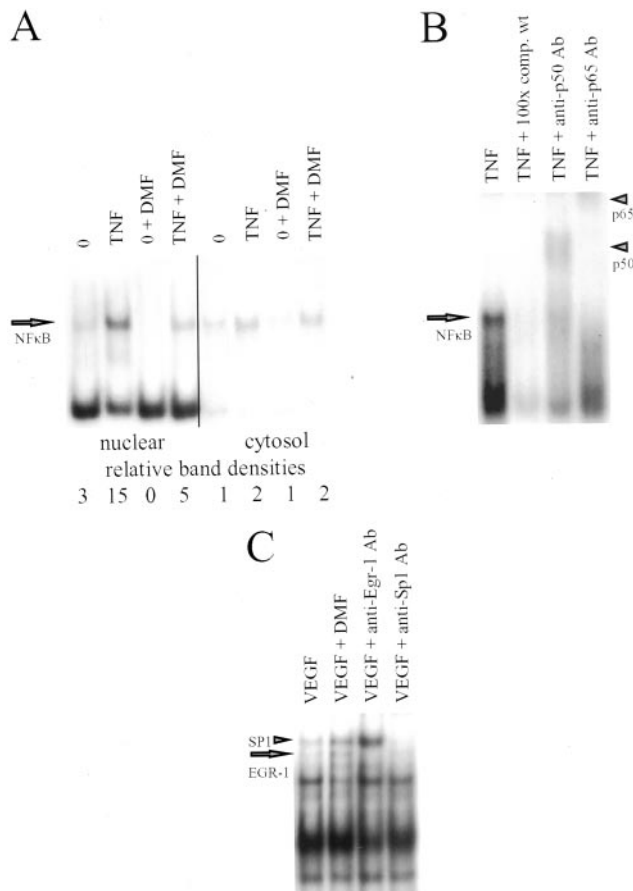


FIGURE 2. DMF reduces NF- κ B/DNA complex formation. HUVEC were incubated with 84 μ M DMF and stimulated with TNF or left untreated as indicated. Nuclear and cytoplasmic extracts were prepared as described and incubated with oligonucleotides harboring the consensus binding sites for NF- κ B (A and B) or EGR-1 (C), and the binding reactions were separated on 6% acrylamide gels. Preincubation of extracts with unlabeled oligonucleotides competed off all bands. Specific bands were identified with indicated Abs. With anti-p65 Abs a complete supershift was seen, and with anti-p50 Abs a partial supershift was seen (B). Anti-Sp1 and anti-EGR-1 Abs destroyed the specific complexes (C).

DMF impairs nuclear entry of NF- κ B

To analyze whether DMF alters the subcellular distribution of NF- κ B proteins, we prepared cytoplasmic and nuclear extracts and subjected them to immunoblotting. The TNF-induced increase in nuclear NF- κ B was markedly inhibited by DMF (Fig. 3). For p50 and p65, inhibition was by mean 65% (as determined by densitometric analysis of three independent experiments) and nuclear c-Rel was undetectable in DMF-treated cells. In the cytoplasm, p50, p65, and c-Rel were abundant at baseline, and their relative amounts were not visibly affected by DMF (Fig. 3). Equal protein loading of cytoplasmic and nuclear extracts was shown by blotting with CD31 and anti-Sp1 immunoblots, respectively (Fig. 3).

We next analyzed the subcellular distribution of p65 by transfecting HUVEC with a p65/eGFP plasmid. Using confocal laser scanning microscopy, the TNF-induced translocation p65/eGFP fusion protein into the nucleus was strongly inhibited by DMF. A representative image is shown in Fig. 4A. In unstimulated endothelial cells, the relative amount of cytoplasmic p65/eGFP was not affected by DMF. Because results obtained by laser scanning microscopy are difficult to quantify, we collected the nuclei as described in *Materials and Methods* and subjected them to FACS analysis. Following TNF stimulation, there was a mean 2- to 3-fold

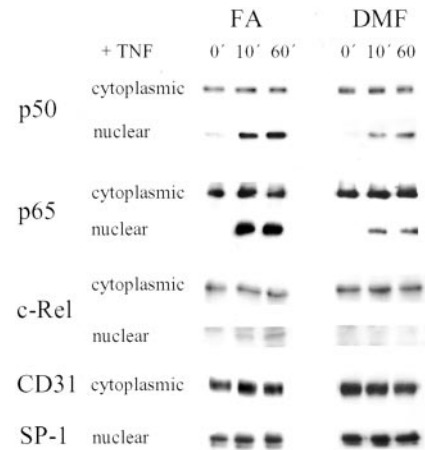


FIGURE 3. DMF inhibits TNF-induced nuclear accumulation of NF- κ B. HUVEC were treated with FA or DMF (84 μ M each) and stimulated with TNF for indicated times. Cytoplasmic and nuclear extracts were prepared as described and immunoblotted using anti-p50, -p65, and -c-Rel Abs, respectively. One representative experiment of four is shown. CD31 and Sp1 were used as loading controls for cytoplasmic and nuclear extracts, respectively.

increase in fluorescence emitted from these nuclei, which reflected nuclear translocation of p65/eGFP. DMF inhibited nuclear translocation of p65/eGFP down to baseline (Fig. 4, B and C). We conclude that DMF prevented the nuclear entry of p65.

DMF does not inhibit TNF-induced I κ B phosphorylation and degradation

To determine whether DMF inhibits nuclear entry of NF- κ B by preventing its release from the cytoplasmic I κ B complex, we analyzed TNF-induced degradation of I κ B proteins by using immunoblotting. TNF-induced I κ B α degradation was seen after 10 min, and this was not altered by DMF (Fig. 5A). I κ B α levels returned to baseline 60 min after TNF stimulation, and again this was not altered by DMF. Moreover, DMF did not alter baseline levels of I κ B α in unstimulated HUVEC, as determined in time kinetics for up to 3 h following the addition of DMF (data not shown). Also, degradation of I κ B ϵ was observable 10 min following TNF stimulation, and this was not altered by DMF. With regard to I κ B β , over a time period of 60 min no TNF-induced degradation was seen, and this was also not changed by DMF (data not shown). This finding is in line with a previous observation that I κ B β degradation is delayed and that I κ B β is involved in delayed and not in early TNF effects (5). Equal protein loading of cytoplasmic extracts was shown by blotting with CD31.

Also, I κ B α phosphorylation seen after 2 min of TNF stimulation was unaffected by DMF (Fig. 5B), confirming that TNF signaling to I κ B is not inhibited by DMF.

DMF does not inhibit leptomycin B-induced nuclear accumulation of p65

It has been reported previously that leptomycin B traps NF- κ B/I κ B complexes within the nucleus by inhibiting the nuclear export signaling receptor CRM1 (36). To analyze whether DMF alters the constitutive nucleocytoplasmic shuttling of NF- κ B/I κ B complexes (1), HUVEC were treated with 20 nM leptomycin B. In unstimulated HUVEC, leptomycin B treatment resulted in a strong nuclear p65 staining, as determined by immunofluorescence techniques using anti-p65 Abs. Cytoplasmic p65 staining was nearly absent, which indicates a nuclear accumulation of p65/I κ B complexes. This was not altered by DMF (Fig. 6A). It can thus be assumed that

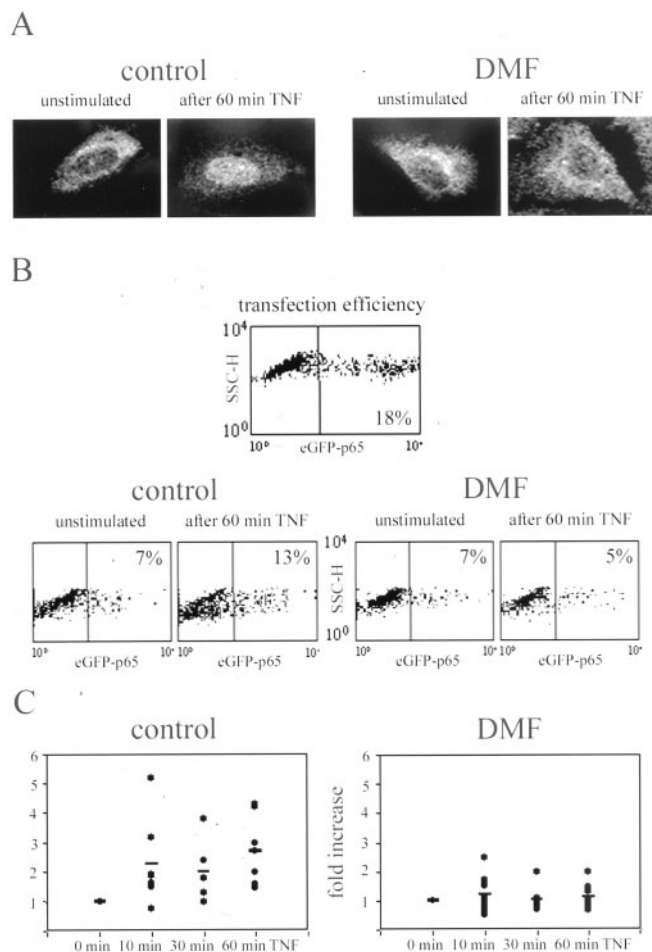


FIGURE 4. DMF blocks the nuclear entry of a p65/eGFP fusion protein. *A*, Laser scanning microscopy of HUVEC transfected with a p65/eGFP fusion protein. Cells were incubated with or without 84 μ M DMF and stimulated with TNF for 60 min. In DMF-treated cells, the fluorescence remains largely cytoplasmic even after TNF stimulation. One of three independent experiments is shown. *B*, HUVEC were transfected with p65/eGFP as described. Transfection efficiency is shown in the *upper panel*, where endothelial cell suspensions were subjected to FACS analysis. In parallel, cells were incubated with or without 84 μ M DMF and stimulated with TNF for indicated times. Then nuclei were isolated as described and subjected to FACS analysis. One representative result of five independent experiments is shown. p65/eGFP-positive nuclei appear to the *right* of the vertical lines. *C*, Summary of five independent experiments. Each dot represents the fold increase of nuclear fluorescence above the corresponding baseline value. Horizontal bars represent mean values.

the constitutive shuttling of p65/I κ B complexes was not affected by DMF.

We next performed EMSA using nuclear extracts from endothelial cells preincubated with or without leptomycin B. In unstimulated cells, NF- κ B binding to NF- κ B consensus oligonucleotides did not occur even in the presence of leptomycin B (Fig. 6*B*), indicating that leptomycin B induced the nuclear accumulation of p65/I κ B complexes, which are functionally inactive. Importantly, following TNF stimulation, using nuclear extracts from controls or DMF-treated cells, we found equal amounts of NF- κ B binding to NF- κ B oligonucleotides (Fig. 6*B*). It can thus be concluded that leptomycin B-induced accumulation of NF- κ B is accessible to TNF signals even in the presence of DMF. This experiment excludes the possibility that DMF inhibits transcription by inhibiting NF- κ B binding to the DNA (as also shown in Fig. 2*A*,

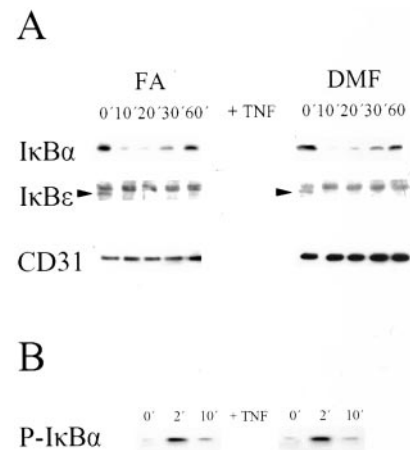


FIGURE 5. DMF does not inhibit I κ B degradation and resynthesis. HUVEC were incubated with FA or DMF (84 μ M each) and stimulated with TNF for indicated times. Cells were lysed and immunoblotted as described using anti-I κ B α , anti-I κ B ϵ (*A*), or anti-phospho-Ser³²-I κ B α (*B*) Abs. One of four experiments is shown. The arrowhead denotes I κ B ϵ , which is detectable in unstimulated cells only; the *upper band* is considered unspecific. A representative CD31 immunoblot is shown as loading control.

where cytoplasmic extracts were used: NF- κ B/DNA binding is not prevented by DMF).

Discussion

We show that DMF impairs TNF-induced tissue factor expression by blocking NF- κ B-driven transcription but has no significant effect on VEGF-induced tissue factor expression. DMF exerts this effect by impairing the nuclear entry of NF- κ B following its dissociation from I κ B.

Inhibition of nuclear entry of NF- κ B proteins by DMF is herein shown for p65 by using a green fluorescent p65 fusion protein. However, the TNF-induced p50 and c-Rel translocation was also impaired by DMF, as determined by immunoblotting of nuclear extracts. All these members of the NF- κ B family share a highly conserved sequence of \sim 100 amino acids, the so-called Rel domain, which carries the nuclear localization sequence and which is required for DNA binding. Importantly, DMF does not collectively inhibit nuclear transport mechanisms of transcription factors, because the function of transcription factors like EGR-1, Sp1 (Fig. 2), or AP-1 (22) is not affected by DMF. Moreover, the site of DMF action appears to be specifically located at the level of the nuclear entry of NF- κ B, because upstream events like I κ B phosphorylation and degradation, as well as downstream events like the ability of NF- κ B to bind to the cognate DNA, were not impaired by DMF.

How can DMF prevent nuclear entry of NF- κ B after its release from I κ B? DMF may alter functions of proteins transporting NF- κ B into the nucleus. Because shuttle proteins for NF- κ B are yet not identified (37), we cannot address this question. Alternatively, DMF may block nuclear translocation by altering phosphorylation patterns of NF- κ B. Several different kinases and putative phosphorylation sites exist, which regulate the activity of NF- κ B (11, 38, 39). Serine residues have been shown to be responsible for DNA binding, but phosphorylation patterns of NF- κ B responsible for nuclear entry are yet not well defined (40). Glutathione S-transferase might be a possible link between DMF and NF- κ B. It was previously shown that DMF induces glutathione S-transferase expression and activity in various cell lines (41–43). Glutathione S-transferase not only controls the intracellular redox status but

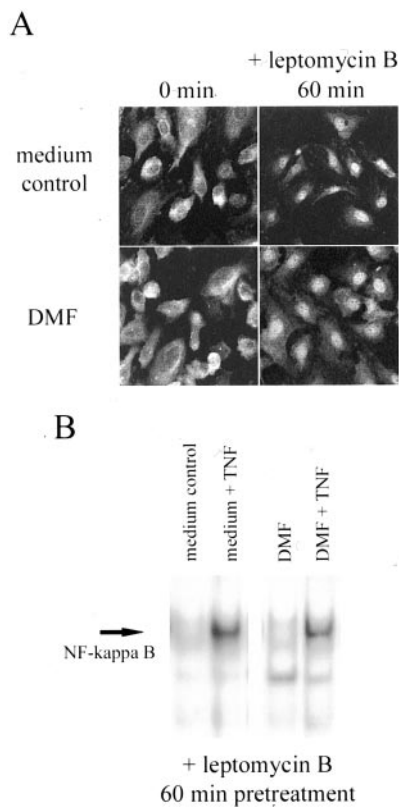


FIGURE 6. DMF does not inhibit leptomyacin B-induced nuclear accumulation of p65. **A**, Laser scan images of HUVEC grown on tissue culture plastic and treated with and without 84 μ M DMF. After 2 h, 20 nM leptomyacin B was added. Thereafter, at indicated times, cells were fixed and stained with an anti-p65 Ab followed by an Alexa 488-labeled anti-rabbit Ab. **B**, EMSA using nuclear extracts from HUVEC treated with or without 84 μ M DMF for 2 h followed by a 1-h incubation with 20 nM leptomyacin B. Thereafter, cells were treated with TNF for 1 h and nuclear extracts were prepared as described in *Materials and Methods*. In the presence of leptomyacin B, the amount of TNF-induced NF- κ B/DNA binding is equal in DMF-treated cells and controls.

also down-regulates p21^{ras} expression (44). p21^{ras} is known to increase phosphorylation and transcriptional activity of p65 in endothelial cells (45). Thus, by increasing the activity of glutathione S-transferase and thereby decreasing p21^{ras} activity, DMF might alter phosphorylation patterns of p65 and thereby inhibit its nuclear entry, but this speculation is only one possibility among many others.

In certain aspects, our results are in conflict with a recent publication, where DMF was shown to inhibit nuclear translocation of p50, but not of p65 or c-Rel (23). One reason may be the cell type used. Whereas we used endothelial cells, fibroblasts have been used in Vandermeeren's study (23), and it is well documented that there are cell type-specific reaction patterns in response to DMF (18–20). The selective DMF-induced inhibition of nuclear translocation of p50 in fibroblasts as seen by Vandermeeren et al. (23) cannot explain the effects of DMF seen in endothelial cells. In endothelium and many other cell types, p50/p50 homodimers are transcriptionally inactive and exhibit an inhibitory function on p65/p50 and/or p65/c-Rel-dependent gene transcription (46, 47). Because CD62E and tissue factor expression depend on p65/p50 and/or p65/c-Rel heterodimers, it is highly unlikely that DMF blocks expression of these genes by a selective inhibition of p50 only.

In conclusion, we show that DMF prevents NF- κ B-driven gene activation in endothelial cells by inhibiting the nuclear entry of Rel proteins. Indirect evidence would support the assumption that this function of DMF is not restricted to endothelial cells. In T cells, FA esters induce a switch toward a Th2 cytokine profile (18, 19). Because a Th2 switch is also found in mice that have an impaired NF- κ B signaling pathway (48, 49), by analogy it may well be that the Th2 switch induced by DMF is mediated by preventing nuclear entry of NF- κ B. Finally, inhibition of NF- κ B signaling may be the basis of the beneficial effect of FA esters in psoriatic patients. In addition, diseases aggravated or caused by NF- κ B-mediated cell activation might also benefit from a treatment with DMF.

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References

- Johnson, C., D. Van Antwerp, and T. J. Hope. 1999. An N-terminal nuclear export signal is required for the nucleocytoplasmic shuttling of I κ B α . *EMBO J.* 18:6682.
- Harhaj, E. W., and S. C. Sun. 1999. Regulation of RelA subcellular localization by a putative nuclear export signal and p50. *Mol. Cell. Biol.* 19:7088.
- Baeuerle, P. A., and T. Henkel. 1994. Function and activation of NF- κ B in the immune system. *Annu. Rev. Immunol.* 12:141.
- Ghosh, S., M. J. May, and E. B. Kopp. 1998. NF- κ B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* 16:225.
- Spiecker, M., H. Darius, and J. K. Liao. 2000. A functional role of I κ B ϵ in endothelial cell activation. *J. Immunol.* 164:3316.
- Thompson, J. E., R. J. Phillips, H. Erdjument-Bromage, P. Tempst, and S. Ghosh. 1995. I κ B β regulates the persistent response in a biphasic activation of NF- κ B. *Cell* 80:573.
- Yamazaki, S., T. Muta, and K. Takeshige. 2001. A novel I κ B protein, I κ B ζ , induced by proinflammatory stimuli, negatively regulates nuclear factor- κ B in the nuclei. *J. Biol. Chem.* 276:27657.
- Jobin, C., C. A. Bradham, M. P. Russo, B. Juma, A. S. Narula, D. A. Brenner, and R. B. Sartor. 1999. Curcumin blocks cytokine-mediated NF- κ B activation and proinflammatory gene expression by inhibiting inhibitory factor I- κ B kinase activity. *J. Immunol.* 163:3474.
- Jeon, K. I., J. Y. Jeong, and D. M. Jue. 2000. Thiol-reactive metal compounds inhibit NF- κ B activation by blocking I κ B kinase. *J. Immunol.* 164:5981.
- Yin, M. J., Y. Yamamoto, and R. B. Gaynor. 1998. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I κ B kinase- β . *Nature* 396:77.
- Egan, L. J., D. C. Mays, C. J. Huntoon, M. P. Bell, M. G. Pike, W. J. Sandborn, J. J. Lipsky, and D. J. McKean. 1999. Inhibition of interleukin-1-stimulated NF- κ B RelA/p65 phosphorylation by mesalamine is accompanied by decreased transcriptional activity. *J. Biol. Chem.* 274:26448.
- De Bosscher, K., M. L. Schmitz, W. Vanden Berghe, S. Plaisance, W. Fiers, and G. Haegeman. 1997. Glucocorticoid-mediated repression of nuclear factor- κ B-dependent transcription involves direct interference with transactivation. *Proc. Natl. Acad. Sci. USA* 94:13504.
- Ollivier, V., G. C. Parry, R. R. Cobb, D. de Prost, and N. Mackman. 1996. Elevated cyclic AMP inhibits NF- κ B-mediated transcription in human monocytic cells and endothelial cells. *J. Biol. Chem.* 271:20828.
- Lyss, G., A. Knorre, T. J. Schmidt, H. L. Pahl, and I. Merfort. 1998. The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF- κ B by directly targeting p65. *J. Biol. Chem.* 273:33508.
- Natarajan, K., S. Singh, T. R. Burke, Jr., D. Grunberger, and B. B. Aggarwal. 1996. Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF- κ B. *Proc. Natl. Acad. Sci. USA* 93:9090.
- Pierce, J. W., R. Schoenleber, G. Jesmok, J. Best, S. A. Moore, T. Collins, and M. E. Gerritsen. 1997. Novel inhibitors of cytokine-induced I κ B α phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. *J. Biol. Chem.* 272:21096.
- Nieboer, C., D. de Hoop, A. C. van Loenen, P. N. Langendijk, and E. van Dijk. 1989. Systemic therapy with fumaric acid derivatives: new possibilities in the treatment of psoriasis. *J. Am. Acad. Dermatol.* 20:601.
- de Jong, R., A. C. Bezemer, T. P. Zomerdijk, T. Pouw-Kraan, T. H. Ottenhoff, and P. H. Nibbering. 1996. Selective stimulation of T helper 2 cytokine responses by the anti-psoriasis agent monomethylfumarate. *Eur. J. Immunol.* 26:2067.
- Ockenfels, H. M., T. Schultewolter, G. Ockenfels, R. Funk, and M. Goos. 1998. The antipsoriatic agent dimethylfumarate immunomodulates T-cell cytokine secretion and inhibits cytokines of the psoriatic cytokine network. *Br. J. Dermatol.* 139:390.
- Thio, H. B., T. P. Zomerdijk, C. Oudshoorn, J. Kempenaar, P. H. Nibbering, J. G. van der Schroeff, and M. Ponc. 1994. Fumaric acid derivatives evoke a transient increase in intracellular free calcium concentration and inhibit the proliferation of human keratinocytes. *Br. J. Dermatol.* 131:856.
- Zhu, K., and U. Mrowietz. 2001. Inhibition of dendritic cell differentiation by fumaric acid esters. *J. Invest. Dermatol.* 116:203.

22. Loewe, R., M. Pillinger, R. de Martin, U. Mrowietz, M. Gröger, W. Holthöner, K. Wolff, W. Wiegrebe, D. Jirovsky, and P. Petzelbauer. 2001. Dimethylfumarate inhibits TNF-induced CD62E expression in an NF- κ B-dependent manner. *J. Invest. Dermatol.* 117:1363.
23. Vandermeeren, M., S. Janssens, H. Wouters, I. Borghmans, M. Borgers, R. Beyaert, and J. Geysen. 2001. Dimethylfumarate is an inhibitor of cytokine-induced nuclear translocation of NF- κ B1, but not RelA in normal human dermal fibroblast cells. *J. Invest. Dermatol.* 116:124.
24. Vandermeeren, M., S. Janssens, M. Borgers, and J. Geysen. 1997. Dimethylfumarate is an inhibitor of cytokine-induced E-selectin, VCAM-1, and ICAM-1 expression in human endothelial cells. *Biochem. Biophys. Res. Commun.* 234:19.
25. Parry, G. C., and N. Mackman. 1995. Transcriptional regulation of tissue factor expression in human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 15:612.
26. Moll, T., M. Czyz, H. Holzmüller, R. Hofer-Warbinek, E. Wagner, H. Winkler, F. H. Bach, and E. Hofer. 1995. Regulation of the tissue factor promoter in endothelial cells: binding of NF κ B-, AP-1-, and Sp1-like transcription factors. *J. Biol. Chem.* 270:3849.
27. Mechtcheriakova, D., A. Wlachs, H. Holzmüller, B. R. Binder, and E. Hofer. 1999. Vascular endothelial cell growth factor-induced tissue factor expression in endothelial cells is mediated by EGR-1. *Blood* 93:3811.
28. Petzelbauer, P., J. R. Bender, J. Wilson, and J. S. Pober. 1993. Heterogeneity of dermal microvascular endothelial cell antigen expression and cytokine responsiveness in situ and in cell culture. *J. Immunol.* 151:5062.
29. Rozen, S., and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* 132:365.
30. Wellmann, S., T. Taube, K. Paal, V. E. Graf, W. Geilen, G. Seifert, C. Eckert, G. Henze, and K. Seeger. 2001. Specific reverse transcription-PCR quantification of vascular endothelial growth factor (VEGF) splice variants by LightCycler technology. *Clin. Chem.* 47:654.
31. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:E45.
32. Groger, M., G. Sarmay, E. Fiebiger, K. Wolff, and P. Petzelbauer. 1996. Dermal microvascular endothelial cells express CD32 receptors in vivo and in vitro. *J. Immunol.* 156:1549.
33. Schmid, J. A., A. Birbach, R. Hofer-Warbinek, M. Pengg, U. Burner, P. G. Furtmüller, B. R. Binder, and R. de Martin. 2000. Dynamics of NF κ B and I κ B α studied with green fluorescent protein (GFP) fusion proteins: investigation of GFP-p65 binding to DNA by fluorescence resonance energy transfer. *J. Biol. Chem.* 275:17035.
34. Gille, J., R. A. Swerlick, T. J. Lawley, and S. W. Caughman. 1996. Differential regulation of vascular cell adhesion molecule-1 gene transcription by tumor necrosis factor α and interleukin-1 α in dermal microvascular endothelial cells. *Blood* 87:211.
35. Camera, M., P. L. Giesen, J. Fallon, B. M. Aufiero, M. Taubman, E. Tremoli, and Y. Nemerson. 1999. Cooperation between VEGF and TNF- α is necessary for exposure of active tissue factor on the surface of human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* 19:531.
36. Fukuda, M., S. Asano, T. Nakamura, M. Adachi, M. Yoshida, M. Yanagida, and E. Nishida. 1997. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* 390:308.
37. Conti, E., and E. Izaurralde. 2001. Nucleocytoplasmic transport enters the atomic age. *Curr. Opin. Cell Biol.* 13:310.
38. True, A. L., A. Rahman, and A. B. Malik. 2000. Activation of NF- κ B induced by H₂O₂ and TNF- α and its effects on ICAM-1 expression in endothelial cells. *Am. J. Physiol.* 279:L302.
39. Wesselborg, S., M. K. A. Bauer, M. Vogt, M. L. Schmitz, and O. K. Schulze. 2000. Activation of transcription factor NF- κ B and p38 mitogen-activated protein kinase is mediated by distinct and separate stress effector pathways. *J. Biol. Chem.* 272:12422.
40. Jans, D. A., and S. Hubner. 1996. Regulation of protein transport to the nucleus: central role of phosphorylation. *Physiol. Rev.* 76:651.
41. Orta, T., J. J. Eady, J. H. Peacock, and G. G. Steel. 1995. Glutathione manipulation and the radiosensitivity of human tumour and fibroblast cell lines. *Int. J. Radiat. Biol.* 68:413.
42. Spencer, S. R., C. A. Wilczak, and P. Talalay. 1990. Induction of glutathione transferases and NAD(P)H:quinone reductase by fumaric acid derivatives in rodent cells and tissues. *Cancer Res.* 50:7871.
43. Pereira, M. A., L. H. Barnes, V. L. Rassman, G. V. Kelloff, and V. E. Steele. 1994. Use of azoxymethane-induced foci of aberrant crypts in rat colon to identify potential cancer chemopreventive agents. *Carcinogenesis* 15:1049.
44. Miller, A. C., J. Gafner, E. P. Clark, and D. Samid. 1993. Posttranscriptional down-regulation of *ras* oncogene expression by inhibitors of cellular glutathione. *Mol. Cell. Biol.* 13:4416.
45. Anrather, J., V. Csizmadia, M. P. Soares, and H. Winkler. 1999. Regulation of NF- κ B RelA phosphorylation and transcriptional activity by p21^{ras} and protein kinase C ζ in primary endothelial cells. *J. Biol. Chem.* 274:13594.
46. Baer, M., A. Dillner, R. C. Schwartz, C. Sedon, S. Nedospasov, and P. F. Johnson. 1998. Tumor necrosis factor α transcription in macrophages is attenuated by an autocrine factor that preferentially induces NF- κ B p50. *Mol. Cell. Biol.* 18:5678.
47. Sheppard, K. A., D. W. Rose, Z. K. Haque, R. Kurokawa, E. McInerney, S. Westin, D. Thanos, M. G. Rosenfeld, C. K. Glass, and T. Collins. 1999. Transcriptional activation by NF- κ B requires multiple coactivators. *Mol. Cell. Biol.* 19:6367.
48. Aronica, M. A., A. L. Mora, D. B. Mitchell, P. W. Finn, J. E. Johnson, J. R. Sheller, and M. R. Boothby. 1999. Preferential role for NF- κ B/Rel signaling in the type 1 but not type 2 T cell-dependent immune response in vivo. *J. Immunol.* 163:5116.
49. Das, J., C. H. Chen, L. Yang, L. Cohn, P. Ray, and A. Ray. 2001. A critical role for NF- κ B in GATA3 expression and TH2 differentiation in allergic airway inflammation. *Nat. Immunol.* 2:45.