0026-895X/02/6204-856–863$7.00
MOLECULAR PHARMACOLOGY
Vol. 62, No. 4
1672/1010924
Mol Pharmacol 62:856–863, 2002
Printed in U.S.A.

Tranilast Inhibits Cytokine-Induced Nuclear Factor κB Activation in Vascular Endothelial Cells

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Received February 12, 2002; accepted June 26, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Tranilast [*N*-(3,4-dimethoxycinnamoyl)anthranilic acid] inhibits vascular inflammation. However, the relevant anti-inflammatory mechanisms are not completely understood. We studied the effects of tranilast on nuclear factor-κB (NF-κB)-dependent endothelial cell adhesion molecule expression and transcriptional regulation. Cultured human umbilical vein endothelial cells were preincubated with 12.5 to 100 μg/ml tranilast. Tumor necrosis factor-α (TNF-α)-induced endothelial VCAM-1, ICAM-1, and E-selectin surface expression was inhibited dose dependently. Maximal inhibition achieved with 100 μg/ml tranilast was 38 ± 6.9, 31.8 ± 1.5, and 31.9 ± 1.9%, respectively (mean ± S.E.M., $p < 0.001$, $n = 5$). Secretion of interleukin 6, which is also NF-κB–sensitive, was significantly inhibited by tranilast. Endothelial MHC-I expression, which is independent of NF-κB, was not inhibited. Although cytokine-induced degradation of NF-κB inhibitor proteins (IκB-α, -β, and -ϵ), nuclear translocation of NF-κB, and binding of NF-κB to κB *cis*-acting elements in the adhesion molecule promoters were not affected by tranilast, ICAM-1-κB and E-selectin-κB reporter gene activity was inhibited by 53% ($n = 5$, $p < 0.01$) and 51% ($n = 5$, $p < 0.001$), respectively. In contrast, using SP-1 and C/EBP constructs, reporter gene activity was not altered. Expression of the transcriptional coactivator cAMP response element binding protein binding protein (CBP) was inhibited by tranilast, resulting in a loss of interaction between NF-κB and CBP. Therefore, in therapeutically relevant concentrations (50 μg/ml), tranilast inhibits NF-κB-dependent transcriptional activation by interfering with the NF-κB/CBP association. We propose that inhibition of NF-κB dependent gene transcription contributes to the anti-inflammatory effects of tranilast.

N-(3,4-dimethoxycinnamoyl)anthranilic acid (tranilast) is an anti-inflammatory drug with several mechanisms of action. Initially, it was found to inhibit the antigen-induced release of histamine from mast cells (Azuma et al., 1976). Therefore, it was developed for the treatment of allergic diseases such as bronchial asthma and allergic rhinitis. Subsequently, other biological effects of tranilast have been suggested, such as inhibition of oxygen radicals, cytokines, leukotrienes, prostaglandins, and cyclooxygenase-2 expression (Isaji et al., 1998). In several models of inflammatory disease, tranilast is antiproliferative and inhibits collagen deposition (Tanaka et al., 1994; Miyazawa et al., 1996; Nie et al., 1996). On the basis of these findings, tranilast has been used to inhibit keloid formation. More recently, it has been proposed that tranilast inhibits restenosis after percutaneous transluminal coronary angioplasty. The beneficial effect in animal models was confirmed in smaller clinical studies (Fukuyama et al., 1996; Kosuga et al., 1997; Tamai et al., 1999), but not in a clinical megatrial involving 11,488 patients (SoRelle, 2001). Inhibition of transplant-associated atherosclerosis by tranilast was reported in an animal model of cardiac transplantation (Saiura et al., 2001).

In contrast to other substances, tranilast has a broad range of anti-inflammatory actions. Inhibition of vascular chymase, of platelet-derived growth factor (PDGF)- and transforming growth factor β1 (TGF-β1)-induced smooth muscle cell proliferation and migration are mechanisms that may explain, at least in part, inhibition of vascular inflammation. Although several effects of tranilast have been described, the relevant mechanisms inhibiting vascular inflammation are not completely understood. Therefore, we investigated the effects of tranilast on the proinflammatory transcription factor nuclear factor-κB (NF-κB). NF-κB is essential for the induction of numerous proinflammatory genes in the vascular wall, such as endothelial cell adhesion molecules (ICAM-1, VCAM-1, and E-selectin), cytokines [i.e., tumor necrosis factor (TNF)-α and -β, interleukin (IL)-2, -6, and -8,

This work was supported by Deutsche Forschungsgemeinschaft Grant DFG SP537/2-1.

ABBREVIATIONS: PDGF, platelet-derived growth factor; TGF, transforming growth factor; NF-κB, nuclear factor κB; DMSO, dimethyl sulfoxide; TNF-α, tumor necrosis factor-α; MHC, major histocompatibility complex; HUVEC, human saphenous vein endothelial cell; BAEC, bovine aortic endothelial cells; CBP, cAMP response element binding protein binding protein; MG-132, *Z*-Leu-Leu-Leu-aldehyde; ROS, reactive oxygen species; VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; IL, interleukin.

interferon β, viral enhancers, immunologic mediators, and other transcription factors] (Collins et al., 1995; Verma et al., 1995; May and Ghosh, 1998). We have found that tranilast inhibits NF-κB dependent transcriptional activation of endothelial cell adhesion molecules without affecting the cytoplasmic NF-κB inhibitory proteins IκB-α, -β, and -ε. Accordingly, inhibition by tranilast seems to be independent of nuclear translocation and DNA binding activity of NF-κB. Instead, our findings suggest a role for tranilast more downstream as a modulator of transcriptional activation of NF-κB once bound to its corresponding promoter sequence.

Materials and Methods

Enzyme Immunoassays. Enzyme immunoassays using specific monoclonal antibodies were performed to determine the cell-surface expression of ICAM-1, E-selectin, and VCAM-1, on confluent human umbilical vein endothelial cell monolayers. After incubation with tranilast or its solvent, DMSO (0.05%), for 1 h and stimulation with TNF-α (500 U/ml) for 4 h, endothelial cells were incubated with the indicated monoclonal primary antibodies (Immunotech, Marseilles, France), with biotinylated horse anti-mouse IgG secondary antibody (Vector Labs, Burlingame, CA), followed by incubation with streptavidin-alkaline phosphatase (Zymed, South San Francisco, CA). Anti-MHC-class I antibody was purchased from Ancell (Bayport, MN). Cells were treated with *p*-nitrophenylphosphate for 30 min at 22°C and absorbance was measured at 410 nm. Integrity of the monolayers was checked before analysis. IL-6 immunoassays (R & D Systems, Minneapolis, MN) were performed using supernatants of confluent endothelial cell monolayers as recommended by the manufacturer. In the IL-6 studies, preincubation with tranilast was started 1 h before incubation with TNF-α for 16 h. At least four independent experiments were performed, each experiment in quadruplicate.

Immunofluorescence. Endothelial cells were grown on gelatin-coated coverslips. After incubation with TNF-α (2 h) and tranilast (1 h), cells were fixed and permeabilized with acetone at −20°C for 2 min. Blocking was performed with 3% normal goat serum for 20 min. Cells were incubated with a rabbit polyclonal antibody directed against the NF-κB subunit RelA (p65) for 1 h at room temperature. A biotinylated goat anti-rabbit antibody (Vector Labs, Burlingame, CA) was used as secondary antibody. After 45 min of incubation with the secondary antibody, streptavidin-fluorescein isothiocyanate (Vector Labs, Burlingame, CA) was added for 45 min. Immunofluorescence was visualized using a Nikon Diaphot microscope (Nikon, Tokyo, Japan). Photographic images were taken from four random fields.

Western Blotting. Cell lysis and Western blots was performed as described previously (Spiecker et al., 1997). Briefly, proteins were separated by SDS-polyacrylamide gel electrophoresis (12% running for IκB, 5% running for CBP, 4% stacking). The separated proteins were electrophoretically transferred to polyvinylidene fluoride membranes with a semidry transfer system (Bio-Rad, Hercules, CA). The blots were incubated with rabbit polyclonal IκB antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and with a horseradish peroxidase-coupled antibody (Amersham Biosciences, Piscataway, NJ). Immunodetection was accomplished using the enhanced chemiluminescence kit (Amersham Biosciences).

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared as described previously (Peng et al., 1995). Oligonucleotides corresponding to the κB sequences in the human VCAM-1 promoter (5′-CCTGGGTTTCCCCT TGAAGGGATTTCCCTCC-3′), ICAM-1 promoter (5′-TTAGCTTGGAAATTCCGGAGC-3′), and E-selectin promoter (5′-AGCTTAGAGGGGATTTCCGAGAGGA-3′) were synthesized (Roth, Karlsruhe, Germany), annealed, and 3′ end-labeled with digoxigenin (Roche Diagnostics, Mannheim, Germany). Nuclear extracts (5–10 μg) were added to digoxigenin-labeled oligonucleotides in a buffer containing poly[dI-dC] and poly(L-lysine). DNA-protein complexes were resolved on 6% nondenaturing polyacrylamide gel electrophoresed at 12 V/cm for 3 h in low ionic strength buffer (0.25× Tris/borate/EDTA) at 4°C. The digoxigenin-labeled probes were detected after blotting by an enzyme immunoassay using anti digoxigenin-AP and the chemiluminescent substrate disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenylphosphate (Roche Diagnostics, Mannheim, Germany). For supershift assays, the indicated antibody (15 μg/ml) was added to the nuclear extracts 10 min before the addition of labeled probe. To determine the specificity of shifted bands, excess unlabeled oligonucleotide (50-fold excess) was added directly to the nuclear extracts for 10 min before addition of the digoxigenin-labeled probe.

Immunoprecipitation. Immunoprecipitations were performed as described previously (Spiecker et al., 1997).

Plasmids and Transient Transfection. Construction of the ICAM-1 κB reporter plasmid has been described previously (Spiecker et al., 2000). A luciferase reporter plasmid with three κB sites from the E-selectin promoter and a noninducible control plasmid were described previously and kindly provided by J. Anrather (New England Deaconess Hospital, Boston, MA) (Brostjan et al., 1997). A 1014-base pair ICAM-1 promoter construct in a luciferase reporter plasmid was kindly provided by Dr. J. Johnson (Institute of Immunology, University of Munich, Munich, Germany).

The following oligonucleotides corresponding to the human ICAM-1 promoter were synthesized with *Kpn*I and *Xho*I restriction sites (Roth): I-001 (SP1+C/EBP binding site): 5-ACCGCCGCCCGATTGCTTT-3′ (sense-strang); I-002 (SP1 binding site): 5′-CCCGCCGCCCGAT-3′. The oligonucleotides were subcloned into the luciferase reporter plasmid pGL$_2$ enhancer (Promega, Madison, WI) after digestion of the polylinker region with *Kpn*I and *Xho*I. Reporter gene constructs were confirmed by DNA sequencing. The empty pGL$_2$ enhancer vector was used for control studies. Mouse CBP was cloned into a pRc/RSV vector (Invitrogen, Carlsbad, CA).

Transient transfection was performed as described previously (Spiecker et al., 2000). Luciferase and β-galactosidase activity were measured in a Berthold luminometer using a kit (Tropix, Bedford, MA). Each experiment was performed in duplicate.

Statistics. Readings from enzyme immunoassays and luciferase reporter gene studies are expressed as mean ± S.E.M. Multiple comparisons were done by analysis of variance (ANOVA). Differences were tested by Scheffé's F-test. A confidence level of $P < 0.05$ was taken to represent a significant difference between two means.

Results

Effect of Tranilast on Endothelial Cell Adhesion Molecule Expression. In cultured unstimulated HUVECs, expression of VCAM-1 and E-selectin was near baseline and not affected by incubation with tranilast. Expression of ICAM-1 in unstimulated HUVECs was 28% of the absorbance observed with TNF-α–treated cells, preincubation with 50 μg/ml tranilast reduced the basal ICAM-1 expression to 22% (Fig. 1). Treatment with tranilast for 1 h reduced TNF-α–(500 U/ml) stimulated endothelial ICAM-1, VCAM-1, and E-selectin expression dose-dependently (Fig. 1A). Using 100 μg/ml tranilast, TNF-α–induced expression was reduced by 38 (VCAM-1), 32 (E-selectin), and 32% (ICAM-1). In contrast, preincubation with the solvent for tranilast, 0.05% DMSO, did not inhibit TNF-α–induced expression of endothelial ICAM-1, VCAM-1, and E-selectin. Induction of these adhesion molecules requires the transcription factor NF-κB. Therefore, we investigated the effect of tranilast on the expression of another NF-κB–sensitive gene. TNF-α–induced interleukin-6 secretion by endothelial cells was dose-dependently inhibited by tranilast (Fig. 1B). Using 100 μg/ml tra-

nilast, TNF-α–induced secretion of IL-6 was reduced by 67%. To exclude an unspecific inhibitory effect, we studied endothelial MHC class I expression as an NF-κB independent marker. MHC I is constitutively expressed in vascular endothelial cells and additionally induced by TNF-α. Expression of endothelial MHC class I was not prevented by tranilast (Fig. 1C).

Tranilast Does Not Inhibit Cytokine Induced Degradation of NF-κB Inhibitor Proteins IκB. Transcriptional induction of ICAM-1, VCAM-1, E-selectin and IL-6 requires the transcription factor NF-κB. Nuclear translocation and activation of NF-κB is crucially regulated by phosphorylation and degradation of cytoplasmic IκB proteins. We therefore examined the effect of tranilast on TNF-α–induced degradation of endothelial IκB-α, IκB-β, and IκB-ϵ. According to the different kinetics of IκB protein degradation and resynthesis in endothelial cells (Spiecker et al., 2000), expression of IκB-α was investigated at 30 min and of IκB-β and -ϵ at 60 min after TNF-α stimulation. As shown in Fig. 2, preincubation with 50 μg/ml tranilast did not inhibit TNF-α–induced degradation of IκB-α, -β, and -ϵ proteins. Similarly, higher concentrations of tranilast (100 μg/ml) had no effect on protein degradation, and no induction of IκB synthesis was observed by Western blot (data not shown).

TNF-α–Induced Nuclear Translocation of RelA Is Not Affected by Tranilast. To confirm the results of IκB Western blotting, we analyzed the intracellular distribution of the NF-κB subunit RelA by immunofluorescence. The inability of tranilast to inhibit TNF-α–induced IκB protein degradation should result in nuclear translocation of RelA in stimulated endothelial cells preincubated with tranilast. In unstimulated endothelial cells, RelA is predominantly located in the cytoplasm (Fig. 3, A). Upon stimulation with TNF-α, RelA translocates to the nucleus (Fig. 3, B). Preincubation with 50 μg/ml tranilast was unable to prevent TNF-α–induced nuclear translocation of RelA (Fig. 3C). Similarly, DMSO (0.05%) had no effect on the intracellular distribution of RelA (Fig. 3D).

DNA Binding Activity of Rel Protein Dimers Is Not Inhibited by Tranilast. Because nuclear translocation of NF-κB was not inhibited, we analyzed the effect of tranilast on the following step in the NF-κB activation cascade: the interaction between NF-κB and the specific κB *cis*-acting elements in adhesion molecule promoters. Using oligonucleotides corresponding to the specific κB sites of the human ICAM-1 and VCAM-1 promoter, electrophoretic mobility shift assays were performed. The ICAM-1 κB oligonucleotide forms a complex with nuclear extracts of TNF-α–stimulated cells (Fig. 4A). This complex is supershifted by antibodies directed against RelA and p50, indicating a specific binding to RelA/p50 heterodimers. Additionally, an excess of unlabeled oligonucleotide specifically abolishes this cytokine-induced complex, but not the unspecific band below this complex. TNF-α–induced binding of the ICAM-1 κB oligonucleotide to NF-κB was not affected by preincubation of endothelial cells with 50 or 100 μg/ml tranilast. Similar results were obtained using a VCAM-1 κB oligonucleotide (Fig. 4 B). We also tested binding to an E-Selectin κB oligonucleotide. Again, TNF-α–induced binding was not inhibited by tranilast (data not shown).

Tranilast Inhibits NF-κB–Dependent Adhesion Molecule Gene Transcription. To determine a potential effect of tranilast on NF-κB–dependent gene transcription, we transiently transfected bovine aortic endothelial cells (BAEC) with a 1014-base pair ICAM-1 promoter linked to a luciferase reporter. Basal ICAM-1 reporter gene activity was

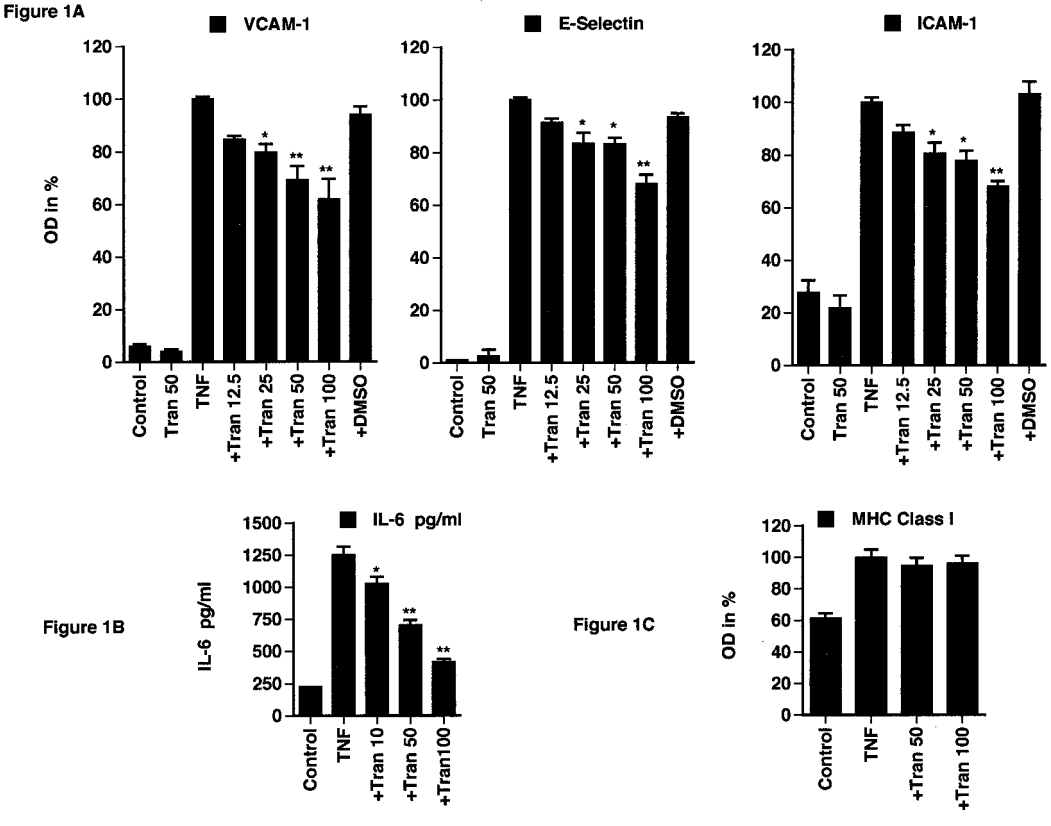

Fig. 1. Effect of tranilast on cytokine induced endothelial cells. A, cell surface enzyme immunoassays showing the effect of tranilast on endothelial VCAM-1, E-Selectin, and ICAM-1 expression. Results from four independent experiments are expressed as percentage relative to TNF-α stimulation. TNF indicates 4-h incubation with TNF-α, 500 U/ml; Tran, 1 h preincubation with tranilast (Tran) in micrograms per milliliter; DMSO, 1 h preincubation with 0.05%. B, interleukin-6 concentration in endothelial cell culture supernatant after stimulation with TNF-α for 16 h and preincubation with tranilast (Tran) indicated in micrograms per milliliter for 1 h. C, endothelial cell surface expression of MHC I, TNF-α incubation 24 h, preincubation with 50 (Tran 50) or 100 μg/ml tranilast (Tran 100) for 1 h. *, $p < 0.05$; **, $p < 0.001$ compared with TNF-α group.

significantly reduced by tranilast (Fig. 5). In addition, preincubation with 50 μg/ml tranilast resulted in a 55% reduction of TNF-α–induced ICAM-1 promoter activity. To further characterize the effect of tranilast on ICAM-1 transcription, we used fragments of the cytokine inducible ICAM-1 promoter region linked to a luciferase reporter. TNF-α induced a 4.7-fold increase in ICAM-1 κB reporter gene activity. Preincubating with tranilast, the cytokine-induced increase was 2.2 fold, corresponding to an inhibition of more than 50%. In contrast, DMSO did not inhibit luciferase activation by TNF-α (Fig. 5). Using promoter constructs with the ICAM-1, SP-1, or C/EBP sites (SP-1 alone and SP1+C/EBP), TNF-α was unable to stimulate promoter activity and tranilast had no effect (data not shown). To confirm the κB-site dependent effect, we transfected BAECs with a promoter construct corresponding to three κB sites of the E-selectin promoter. Tranilast inhibited TNF-α–induced E-selectin-κB reporter gene activity by 51% (Fig. 5).

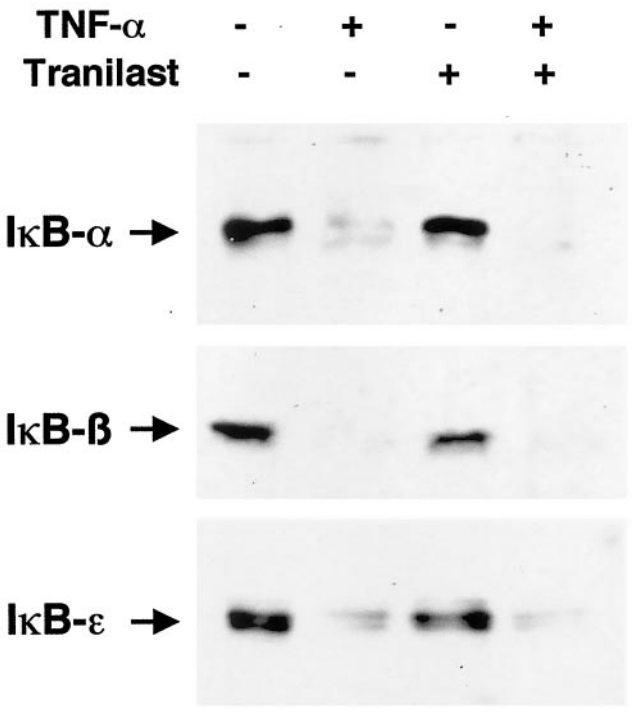

Fig. 2. Tranilast- and cytokine-induced degradation of IκB proteins. Western blot of total cell lysates from HUVECs with antibodies directed against IκB-α, -β, and -ε. Stimulation with 500 U/ml TNF-α for 30 min (IκB-α) or 60 min (IκB-β and -ε). Tranilast 50 μg/ml. Three separate experiments yielded similar results.

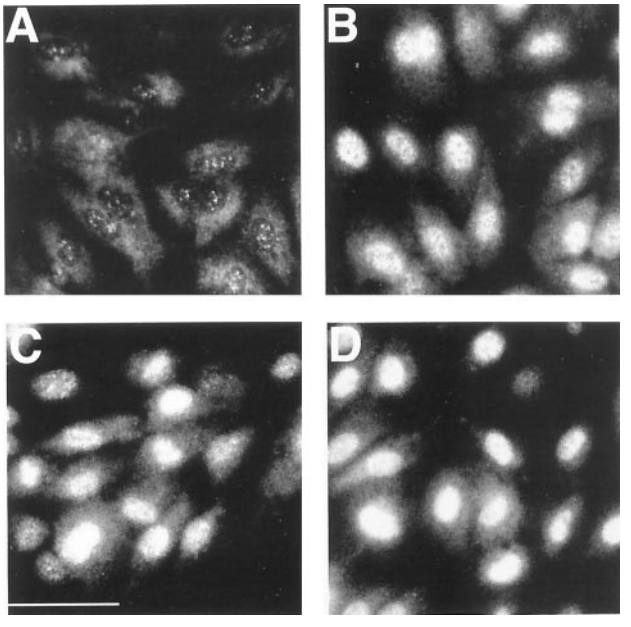

Fig. 3. Tranilast and nuclear translocation of NF-κB. Immunofluorescence staining of the NF-κB subunit Rel A in HUVECs. A, resting cells; B, TNF-α stimulation 2 h; C, preincubation with tranilast for 1h, TNF-α stimulation; D, preincubation DMSO 1 h, TNF-α stimulation. Scale bar, 20 μm. Results are representative of three separate experiments.

Inhibition of TNF-α–Induced RelA/CBP Interaction by Tranilast. Because tranilast inhibits transcriptional activity of NF-κB–dependent genes without interfering with DNA binding of the transcription factor, a further step in transcriptional activation of NF-κB was investigated by immunoprecipitation and Western blotting: interaction with the transcriptional coactivator cAMP response element-binding protein binding protein (CBP). Lysates from HUVECs were immunoprecipitated with anti-CBP, and immunoblotting was performed with anti-RelA. TNF-α stimulation induced a band at 65 kDa, indicating an association between RelA and CBP (Fig. 6). This band was absent in TNF-α–stimulated cells pretreated with 50 μg/ml tranilast. In unstimulated endothelial cells, association between RelA and CBP was barely detectable. Control experiments with RelA immunoprecipitation followed by RelA Western blotting confirmed a band with identical size compared with the lysates of TNF-α–stimulated cells immunoprecipitated and immunoblotted with CBP/RelA (data not shown). In contrast, a 65-kDa band was not detected after control immunoprecipitation with preimmune IgG instead of anti-CBP (data not shown). This studies demonstrated the ability of tranilast to inhibit TNF-α–induced interaction between the NF-κB subunit RelA and its transcriptional coactivator CBP.

CBP Induced Increase in NF-κB–Dependent Transcriptional Activation Is Inhibited by Tranilast. To further analyze the effect of tranilast on transcriptional coactivation by CBP, BAEC were cotransfected with an E-selectin-κB promoter-reporter construct and a CBP expression plasmid. Whereas CBP was unable to significantly increase NF-κB dependent reporter gene activity in unstimulated endothelial cells, TNF-α–induced promoter activity was significantly increased by CBP expression (Fig. 7). Preincubation with 50 μg/ml tranilast abolished this additional increase in promoter activity.

Stability of Tranilast Inhibitory Effect. To analyze the mechanisms of tranilast interference with RelA/CBP association, reversibility of the tranilast inhibitory effect was tested. Interestingly, inhibition of endothelial VCAM-1 expression persisted after a 1-h preincubation period with tranilast followed by washing with tranilast-free medium and another 24- or 48-h period before cytokine stimulation (Fig. 8). At 48 h, inhibition was more effective compared with the 1-h period ($p < 0.05$), suggesting a high stability of the tranilast effect. Enzyme immunoassay plates were checked for cell confluence during the last immunoassay steps and no cell loss was detected up to 48 h after preincubation with tranilast.

Tranilast Inhibits CBP Expression. One of the possible mechanisms for decreased binding of CBP to NF-κB is a

decreased protein expression of CBP after preincubation with tranilast. We therefore analyzed the effect of tranilast on CBP levels by Western blotting. Preincubation with tranilast resulted in a reduced expression of CBP (Fig. 9), which might explain, at least in part, the inhibitory effect of the substance.

Discussion

Tranilast is an anti-inflammatory and antiproliferative drug with a broad range of actions. Accordingly, this substance has several applications, reaching from antiallergic therapy to inhibition of keloid formation and inhibition of transplant-associated atherosclerosis. In addition to the mechanisms of action described so far, tranilast significantly inhibits IL-6 secretion and expression of vascular endothelial cell adhesion molecules ICAM-1, VCAM-1, and E-selectin. Expression of these adhesion molecules mediates adhesion of monocytes, lymphocytes, and granulocytes to the vascular endothelium (Springer, 1990; Cybulsky and Gimbrone, 1991). The mechanisms of adhesion molecule inhibition by tranilast were investigated in more detail in our study. Because all three endothelial adhesion molecules and IL-6 are transcriptionally induced by cytokines such as TNF-α and interleukin-1 (Collins et al., 1995), we investigated transcriptional effects of tranilast. Although several transcription factors are involved in the regulation of gene expression of each endothelial adhesion molecule studied, only NF-κB is essentially required for the cytokine induced up-regulation of all three of them (Collins et al., 1995). Inhibition of IL-6 secretion further supported the assumption that tranilast acts via inhibition of NF-κB. In contrast, endothelial MHC class I expression, which is not regulated by NF-κB, remains unaffected by tranilast. Compared with VCAM-1, ICAM-1 and E-selectin, TNF-α–induced MHC class I expression was relatively low in our studies. This is probably related to differences in the time course of cytokine-induced activation. Whereas VCAM-1, ICAM-1 and E-selectin are up-regulated within hours of TNF-α stimulation, maximal stimulation of endothelial MHC I requires up to 4 days (Collins et al., 1986).

Given the inhibition of several NF-κB–dependent genes, we investigated a potential interference of tranilast with the NF-κB activation cascade. Several inhibitors of endothelial cell adhesion molecule expression interfere with the activation cascade of NF-κB. Salicylates, pyrrolidinedithiocarbamate, and *N*-acetylcysteine inhibit cytokine-induced phosphor-

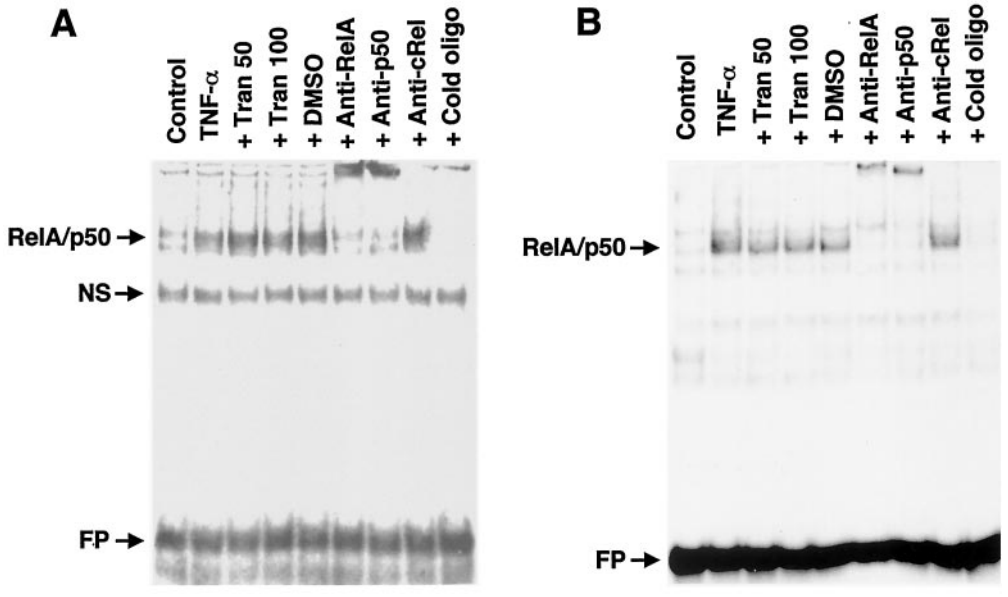

Fig. 4. Tranilast– and TNF-α–induced NF-κB/DNA-binding activity. Electrophoretic mobility shift assay using specific oligonucleotides corresponding to the κB-sites from the ICAM-1 (A) and VCAM-1 promoter (B). Nuclear extracts were prepared from HUVECs and incubated with digoxigenin-labeled oligonucleotides. For supershift analysis, 15 μg/ml of specific Ab directed against the indicated NF-κB subunit (anti-RelA, anti-p50, anti-c-Rel) was added 15 min before the oligonucleotide. TNF-α indicates incubation with 500 U/ml TNF-α for 2 h; Tran50 or 100, tranilast 50 or 100 μg/ml preincubation for 1 h; DMSO, preincubation with 0.05% DMSO for 1 h; cold oligo, 50-fold excess of unlabeled oligonucleotide; FP, free probe; NS, nonspecific band.

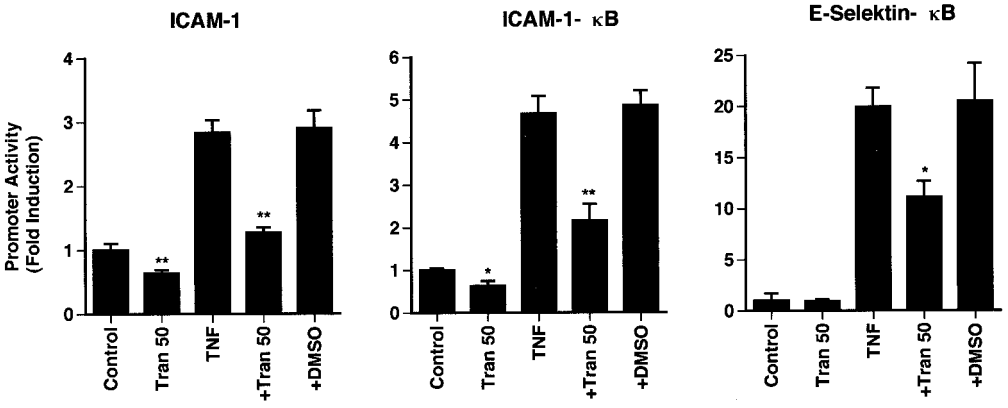

Fig. 5. Effect of tranilast on NF-κB–dependent transcriptional activation. Transient transfection of the ICAM-1 promoter and heterologous promoter κB sites (ICAM-1 and E-selectin promoter) linked to a luciferase reporter gene. Endothelial cells were transfected with the indicated luciferase reporter plasmid (0.7 μg) and RSV.β-Gal expression plasmid (internal control, 0.3 μg) before stimulation with TNF-α (500 U/ml, 8h) $\pm$ tranilast (50 μg/ml) or DMSO (0.05%). Promoter activity was standardized to β-galactosidase activity and expressed as multiples of basal activity (-fold induction). *, $p < 0.05$ (TNF-α + Tran50 compared with TNF-α); **, $p <$ 0.001 (Tran50 compared with control).

ylation of IκB-α, 26S proteasome inhibitors (i.e., MG-132) inhibit proteolytic degradation of IκB-α after phosphorylation and ubiquitination, and nitric oxide induces IκB-α and enhances its nuclear translocation (Marui et al., 1993; Kopp and Ghosh, 1994; Traenckner et al., 1994; Spiecker et al., 1997, 1998). Because most NF-κB inhibitors are IκB-dependent, we studied degradation of IκB-α, -β, and -ϵ after preincubation with tranilast and stimulation with TNF-α. In contrast to the IκB-dependent inhibitors mentioned above, tranilast was unable to prevent cytokine-induced degradation and induction of IκB-α. Additionally, other IκB proteins with a functional role in endothelial cell activation, such as IκB-β and -ϵ, are neither inhibited nor induced by tranilast. Because reactive oxygen species (ROS) are inhibited by tranilast and ROS are known as activators of NF-κB after cytokine stimulation, we considered inhibition of ROS by tranilast a possible mechanism of NF-κB-inhibition. However, antioxidants, which abolish the NF-κB stimulating effect of ROS, inhibit phosphorylation and degradation of IκB-α, in contrast to tranilast. Therefore, it is very unlikely that tranilast inhibits NF-κB via ROS-inhibition. We found that cytokine-induced nuclear translocation of RelA is not inhib-

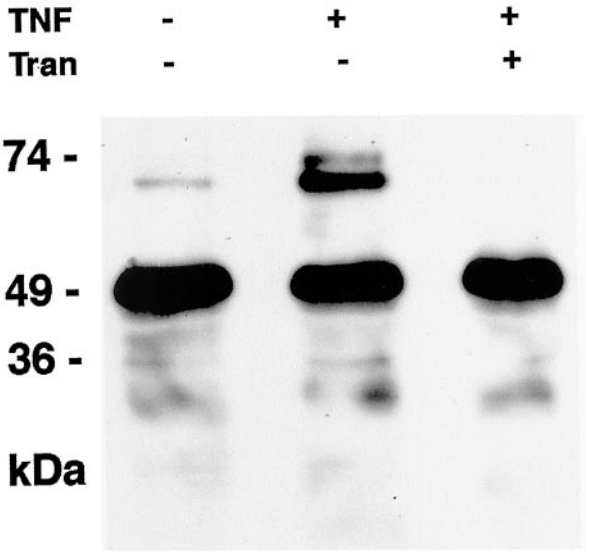

Fig. 6. Effect of tranilast on association between NF-κB (Rel A) and CBP in HUVECs. Coimmunoprecipitation of CBP and Rel A in HUVECs. Whole cell lysates were immunoprecipitated with anti-CBP followed by Western blotting with an antibody directed against RelA. The ~50 kDa band corresponds to IgG and the ~65 kDa band corresponds to RelA. TNF, TNF-α 500 U/ml, 2-h incubation; Tran, tranilast 50 μg/ml, 1-h preincubation.

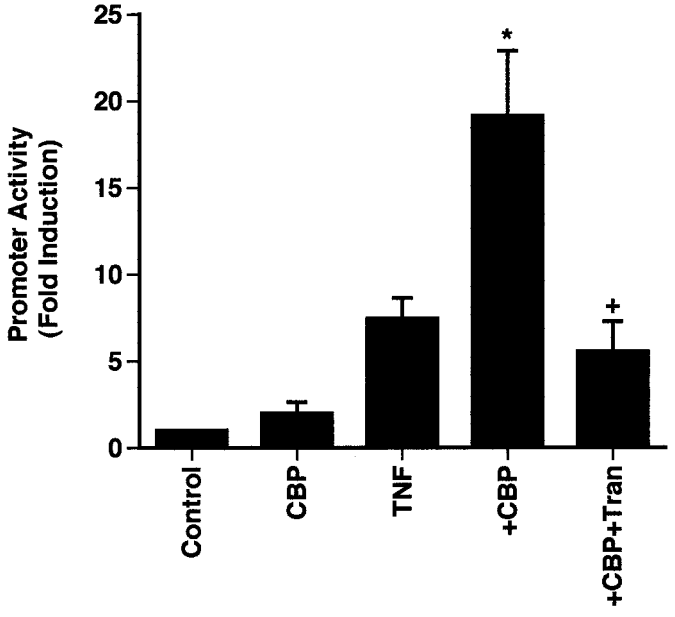

Fig. 7. Effect of tranilast on transcriptional coactivator-induced increase in NF-κB–dependent transcriptional activation. Transient transfection of BAECs with 0.7 μg of E-selectin-κB reporter plasmid (0.7 μg), 0.5 μg of CBP expression plasmid, and RSV.β-Gal expression plasmid (internal control, 0.3 μg). Promoter activity was standardized to β-galactosidase activity and expressed as multiples of basal activity (fold induction). TNF, TNF-α (500 U/ml, 8h); Tran, tranilast 50 μg/ml; *, $p < 0.05$ for TNF-α versus TNF-α + CBP; +, $p < 0.05$ for TNF-α + CBP versus TNF-α + CBP + tranilast. $n = 3$ experiments.

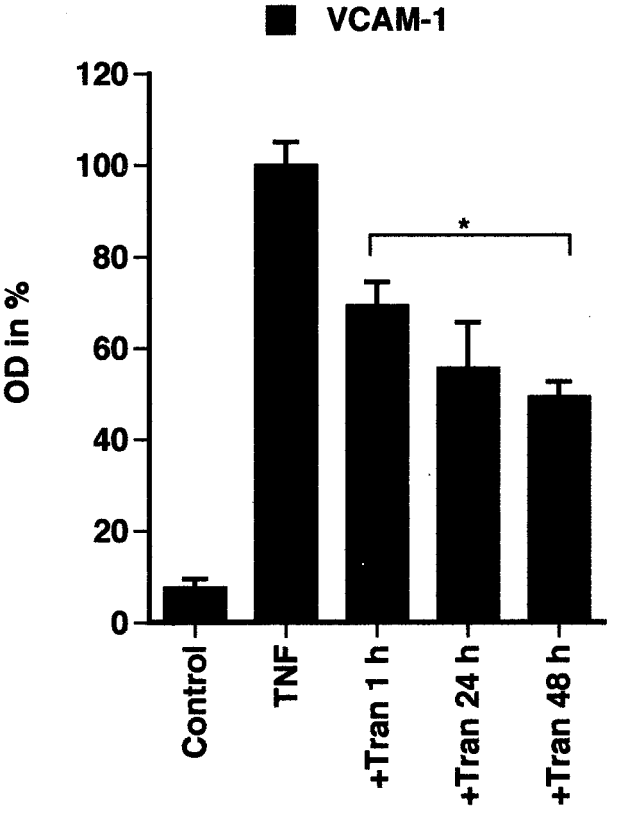

Fig. 8. Stability of tranilast inhibition. Cell surface enzyme immunoassay, preincubation with 50 μg/ml tranilast for 1 h, followed by 4-h TNF-α incubation immediately (+Tran 1 h) or after washing endothelial cells with tranilast-free medium for 24 h (+Tran24 h) or 48 h (+Tran 48 h). Results from three independent experiments are expressed as percentage relative to TNF-α stimulation. *, $p < 0.05$.

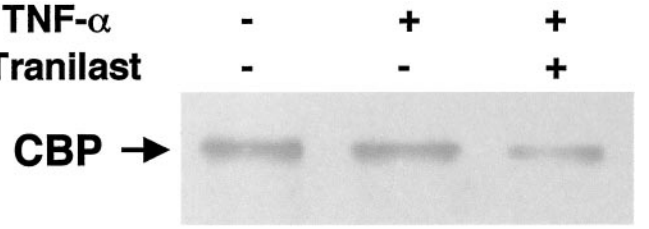

Fig. 9. Tranilast reduces CBP expression. Western blot of total cell lysates from human vascular endothelial cells, antibodies directed against CBP. Stimulation with 500 U/ml TNF-α for 60 min, tranilast 50 μg/ml.

ited by tranilast. Furthermore, binding of NF-κB to the *cis*-acting elements was not altered by tranilast. These experiments confirmed the inability of tranilast to interfere with the proximal events in the NF-κB activation cascade, which was already suggested by the IκB protein studies.

Several other studies suggested a regulation of NF-κB *trans*-activation independent of nuclear translocation and DNA binding. Possible mechanisms responsible for a second, IκB-independent level of NF-κB regulation include phosphorylation of RelA (Naumann and Scheidereit, 1994; Zhong et al., 1997; Anrather et al., 1999), transcriptional coactivators (Gerritsen et al., 1997), transcriptional repressors (Kannabiran et al., 1997), phosphatidylcholine-specific phospholipase C, and p38 mitogen-activated protein kinase-dependent pathways (Van den Berghe et al., 1998). IκB-independent regulation could contribute to a more differential regulation of NF-κB–dependent genes. Inhibition of NF-κB by tranilast is IκB-independent. Our data suggest inhibition of interaction between the NF-κB subunit RelA and the transcriptional coactivator CBP by tranilast. CBP is an important modulator of NF-κB–dependent transcription in endothelial cells. Cotransfection with RelA and CBP expression plasmids in COS cells increases NF-κB–dependent promoter activity 3- to 5-fold (Gerritsen et al., 1997). In untransfected cells, CBP is available in limited amounts. Because overexpression of CBP is not a physiological situation, our transfection studies do not necessarily indicate an inhibitory function of tranilast on CBP/RelA interaction under conditions of limited CBP availability. However, this is suggested by the immunoprecipitation studies, in which the physical interaction between CBP and RelA was inhibited by tranilast in nontransfected endothelial cells.

Although the mechanisms by which CBP regulates transcriptional activation are not completely understood (Vo and Goodman, 2001), the data published so far offer many potential explanations of the mechanisms by which tranilast may inhibit interaction between NF-κB and CBP: inhibition of NF-κB/CBP association, disruption of already associated molecules, inhibition of CBP interaction with RNA polymerase II, basal transcription factor TFIIB, or p300/CBP-associated factor (P/CAF). The first of these possibilities, inhibition of NF-κB/CBP association, could be achieved by many different mechanisms: reduction of CBP levels, changes in CBP phosphorylation, interaction with repressor proteins, or preferential binding of CBP to other transcription factors. Additional possibilities include interaction with the mediator complex, a complex that leads to the recruitment of the general transcription machinery, or disturbance of the enhanceosome complex, a stable multiprotein complex that promotes the cooperative recruitment of coactivator and RNA polymerase II complex to active sites of transcription.

Our immunoprecipitation and Western blotting studies indicate that inhibition of NF-κB/CBP association by tranilast is related to reduced CBP expression. On the other hand, the long-lasting effect of tranilast after washing with tranilast-free medium and the transfection studies with CBP overexpression suggest that mechanisms other than reduced CBP expression might play a role in NF-κB inhibition by tranilast. Given the numerous possible mechanisms mentioned above, further studies should address these questions. To do this, a better understanding of CBP transcriptional regulation is required.

Tranilast inhibited endothelial adhesion molecules and IL-6 secretion to a different extent in our studies. This is probably related to the time periods between preincubation and TNF-α stimulation. A 48-h preincubation with tranilast (with elimination of extracellular tranilast by washing with tranilast-free medium after 1 h) inhibited VCAM-1 expression more effectively than a 1-h preincubation. Additionally, promoter regions of the IL-6 and adhesion molecule genes have different transcription factor binding sites adjacent to NF-κB binding sites. Interaction of these transcription factors with NF-κB allows a differential transcriptional regulation of NF-κB–dependent genes.

NF-κB activation pathways involving stimuli other than TNF-α have been described. In vascular endothelial cells after stimulation with TNF-α, the pathway involving IκB degradation and nuclear translocation of RelA is probably the only relevant pathway of NF-κB activation. Specific inhibitors in the proximal cascade of NF-κB activation, such as the proteasome inhibitor MG-132, completely inhibit NF-κB–dependent transcriptional activation after TNF-α stimulation.

TNF-α activates not only the antiapoptotic NF-κB signaling cascade but also a caspase-dependent proapoptotic pathway. Therefore, complete inhibition of RelA nuclear translocation sensitizes endothelial and other cell lines to TNF-α–induced apoptosis (Beg and Baltimore, 1996; Wang et al., 1996; Soares et al., 1998). This is a major obstacle for therapeutical applications of substances inhibiting nuclear translocation of NF-κB. In contrast, NF-κB inhibitors, which do not inhibit nuclear translocation of RelA (i.e., dominant negative mutant of RelA) are not necessarily proapoptotic (Soares et al., 1998). By interfering with the distal steps in the NF-κB activation cascade, tranilast is unable to completely inhibit NF-κB but has a potentially favorable mechanism of inhibiting NF-κB. Further studies should address this point, which is of interest for the development of other therapeutically relevant NF-κB inhibitors.

The concentrations of tranilast we used are comparable with other studies showing inhibition of TGF-β– and PDGF-dependent effects on vascular smooth muscle cells and collagen synthesis by smooth muscle cells (Suzawa et al., 1992; Tanaka et al., 1994; Miyazawa et al., 1995). Plasma concentrations in dogs 2 and 12 h after oral administration of approximately 5 mg/kg tranilast were 297 and 55 μM (100 μg/ml = 305 μM) (Shiota et al., 1999). Based on these data, 50 μg/ml tranilast is a therapeutically relevant concentration.

In conclusion, tranilast in therapeutically effective concentrations inhibits vascular endothelial adhesion molecules via inhibition of the interaction between NF-κB and its transcriptional coactivator CBP. The mechanism of NF-κB inhibition is independent of IκB proteins. Inhibition of endothelial ICAM-1, VCAM-1, E-selectin expression and IL-6 secretion probably contributes to the antiinflammatory properties of tranilast.

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