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4-trifluoromethyl derivatives of salicylate, triflusal and its main metabolite 2-hydroxy-4-trifluoromethylbenzoic acid, are potent inhibitors of nuclear factor κB activation

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1 The effect of two derivatives of salicylate, 2-hydroxy-4-trifluoromethylbenzoic acid (HTB) and 2-acetoxy-4-trifluoromethylbenzoic acid (triflusal), on the activation of NF-κB elicited by tumour necrosis factor-α (TNF-α) on human umbilical vein endothelial cells (HUVEC) was tested.

2 The expression of the mRNA of vascular cell adhesion molecule-1 (VCAM-1) was studied as an example of a gene the expression of which is regulated by NF-κB. To extend these findings to other systems, the induction of nitric oxide synthase in rat adherent peritoneal macrophages was studied.

3 Both HTB and triflusal were more potent than aspirin or salicylate as inhibitors of the nuclear translocation of NF-κB. The calculation of the IC_{50} values showed $\approx$2 mM for HTB, 4 mM for aspirin and $>$4 mM for salicylate.

4 Comparison of the potency of these compounds on VCAM-1 mRNA expression showed complete inhibition by both triflusal and HTB at a concentration of 4 mM whereas aspirin and salicylate produced only 36–43% inhibition at the same concentration.

5 Inhibition of NF-κB activation was also observed in rat peritoneal macrophages stimulated *via* their receptors for the Fc portion of the antibody molecule with IgG/ovalbumin immune complexes. This was accompanied by a dose-dependent inhibition of nitrite production by the L-arginine pathway *via* iNOS. IC_{50} values for this effect were 1.13 ± 0.12 mM (triflusal), 1.84 ± 0.34 (HTB), 6.08 ± 1.53 mM (aspirin) and 9.16 ± 1.9 mM (salicylate).

6 These data indicate that the incorporation of a 4-trifluoromethyl group to the salicylate molecule strongly enhances its inhibitory effect on NF-κB activation, VCAM-1 mRNA expression and iNOS induction, irrespective of the presence of the acetyl moiety involved in the inhibition of cyclo-oxygenase.

Keywords: Adhesion molecules; atherogenesis; cyclo-oxygenase; Fcγreceptors; human umbilical vein endothelial cells; immune complex; nitric oxide; nuclear factor κB; salicylates; thrombosis; triflusal

Abbreviations: COX-2, cyclo-oxygenase-2; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FcγR, receptor for the Fc portion of the immunoglobulin G molecule; HTB, 2-hydroxy-4-trifluoromethylbenzoic acid; HUVEC, human umbilical vein endothelial cells; IC, immune complexes; NO, nitric oxide; iNOS, inducible isoform of nitric oxide synthase; NF-κB, Nuclear factor κB; PCR, polymerase chain reaction; RT, reverse transcriptase; TNF-α, tumour necrosis factor-α; Triflusal, 2-acetoxy-4-trifluoromethylbenzoic acid; VCAM-1, vascular cell adhesion molecule-1

Introduction

Salicylates or aspirin-like drugs are some of the most commonly used anti-inflammatory agents. For more than two decades, the anti-inflammatory properties of aspirin have been almost exclusively attributed to blockade of prostaglandin synthesis *via* inhibition of cyclo-oxygenase activity (Vane, 1971; Ferreira *et al*., 1971) and assigned to the acetylation of an essential serine at the active site of the enzyme (DeWitt *et al*., 1990). A major challenge to this view stemmed from the fact that salicylic acid lacks an acetyl group. Therefore, this mechanism could not fully explain the anti-inflammatory properties of salicylates. This has prompted new studies directed to identify additional effects of these drugs which could help to explain their pharmacological properties. Some of these studies focussed on possible targets for salicylates in the prostaglandin pathway. For instance, a recent report has showed that sodium salicylate is an effective inhibitor of COX-2 activity by competing with arachidonic acid for the active site of the enzyme (Mitchell *et al*., 1997) and another study has shown inhibition by aspirin of the expression of type IIA phospholipase A_2 (Vervoordeldonk *et al*., 1996) an enzyme implicated in the generation of unesterified arachidonic acid available for cyclo-oxygenase enzymes.

Irrespective of their interference on arachidonate release and prostanoid production, aspirin and sodium salicylate have been found to inhibit the activation of the transcription factor NF-κB without affecting other transcription factors (Kopp & Ghosh, 1994). This finding could help to explain the antithrombotic effects of aspirin-like drugs on endothelial cells, for instance, the blockade of the expression of COX-2 induced by interleukin-1 (Wu *et al*., 1991), the blunting of the expression of vascular cell adhesion molecule-1 (VCAM-1) and E-selectin in response to TNF-α, and the inhibition of the adhesion of monocytes to endothelial cells (Weber *et al*., 1995). Other pharmacological effects of salicylates that could be related to the aforementioned mechanisms include the blockade of the activation of the extracellular signal-regulated kinase (ERK) subgroup of mitogen-activated protein kinases in response to TNF-α (Schwenger *et al*., 1996), and the

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transcriptional inhibition of iNOS (Kepka-Lenhart *et al*., 1996; Sakitani *et al*., 1997). Taken together, these findings indicate that the mechanism of action of salicylates in both the inflammatory response and the progression of atherothrombotic processes is not straightforward. On this basis, achieving a complete appraisal of the pharmacological properties of different aspirin-like drugs could help to optimize their therapeutic properties, since their mechanism of action has been almost exclusively explained on the basis of attributing unique COX-inhibitory effects to this family of compounds.

Triflusal (2-acetoxy-4-trifluoromethylbenzoic acid) is a new antiplatelet drug that, despite its structural analogy to acetylsalicylic acid (Figure 1), exhibits different pharmacological and pharmacokinetic properties (Rabasseda & García-Rafanell, 1993). The deacetylated and main metabolite of triflusal, 2-hydroxy-4-trifluoromethylbenzoic acid (HTB), retains significant antiplatelet activity, an effect not shown by salicylic acid, the deacetylated metabolite of acetylsalicylic acid (De La Cruz *et al*., 1988). The purpose of this study has been to address the effect of 4-trifluoromethyl derivatives of salicylate and aspirin on the activation of the transcription factor NF-κB elicited by agonists acting on HUVEC and rat peritoneal macrophages, and the consequences for the expression of some genes regulated by this transcription factor.

Methods

Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained by the procedure of Dejana *et al*. (1987) by treatment with 0.2% w v^{-1} collagenase P from *C. histolyticum* (Boehringer Manheim GmbH, Manheim, Germany) for 20 min at 37°C, and cultured in M199 medium (Flow Lab, Herts, U.K.) containing 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2.5 μg ml^{-1} amphotericin B, and 20% v v^{-1} foetal calf serum. Primary cultures were plated in 25 cm^2 plastic flasks, washed 24 h thereafter to remove non-adhered cells and refed with the same medium containing 10% v v^{-1} foetal calf serum, 50 μg ml^{-1} endothelial growth supplement, and 100 μg ml^{-1} heparin. After 5–7 days, the cultures reached confluence and HUVEC were detached with 0.05% v v^{-1} trypsin and 0.02% w v^{-1} EDTA (Flow Lab), grown to confluence in gelatin coated flasks and treated with TNF-α or thrombin either in the presence or absence of salicylates. Cells were used for experiments from passages 2–7.

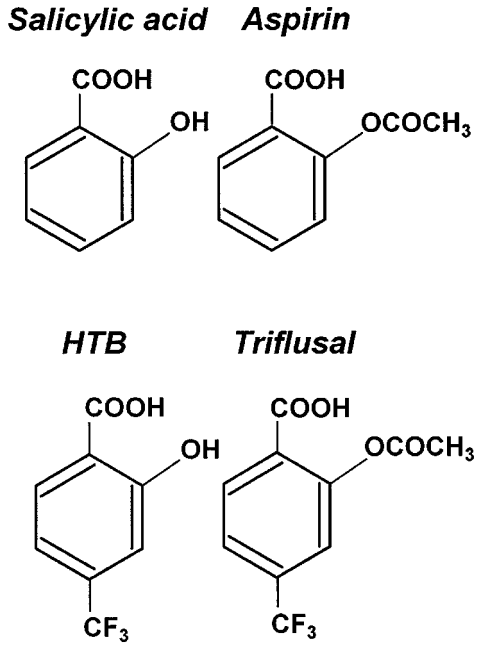

Figure 1 Chemical structures of salicylic acid, aspirin, triflusal and its main metabolite 2-hydroxy-4-trifluoromethylbenzoic acid (HTB).

Electrophoretic mobility shift assay

HUVEC were washed with ice-cold hypotonic lysis buffer (HEPES-KOH 10 mM, pH 7.9, KCl 10 mM, $MgCl_2$ 1.5 mM, dithiothreitol 0.5 mM, phenylmethylsulphonyl fluoride 0.5 mM, aprotinin 5 μg ml^{-1}, leupeptin 5 μg ml^{-1}, and Nonidet P-40 0.6% v v^{-1}). The cells were allowed to swell on ice for 10 min and vortexed vigorously for 10 s. Unbroken cells were eliminated by centrifugation at $1000 \times g$ for 10 min, and the nuclei were collected by centrifugation at $15{,}000 \times g$ for 1 min in a microcentrifuge. The nuclear pellet was resuspended in high salt extraction buffer containing 25% v v^{-1} glycerol and KCl 0.5 M, and the nuclear extract was obtained by pelleting for 30 min at $105{,}000 \times g$ in an Optima TL ultracentrifuge (Beckmann) using a TLA 100.2 rotor. 22-mer double-stranded oligonucleotide probes containing NF-κB sequence were end-labelled with [γ-^{32}P]-ATP using T4 polynucleotide kinase and separated from the unincorporated label by minicolumn chromatography. The κB sequence used was, sense 5′-AGTTCAGGGGAATTTCCCAGGC-3′ and the complement 5′-GCCTGGGAAATTCCCCTGAACT-3′. 10 μg of nuclear protein was incubated for 20 min on ice with radiolabelled oligonucleotide probes ($2-6 \times 10^4$ c.p.m.) in a 25 μl reaction buffer containing (in mM) poly(dI-dC) 2 μg, Tris HCl 10, pH 7.5, NaCl 100, EDTA 1, DTT 1, Ficoll 8%, and glycerol 4%. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis in a 4% nondenaturing polyacrylamide gel in Tris-borate/EDTA buffer at 175 V for 3 h at 4°C. The gel was dried and autoradiographed with an intensifying screen at −80°C for 2–12 h. The specificity of the DNA-protein complex was confirmed by competition with a 100 fold molar excess of unlabelled nucleotide containing the consensus sequences. Quantitation of the DNA-protein complex containing the NF-κB sequence was carried out by densitometric scanning using software of the series Discovery 3.1 from pdi-Pharmacia.

Synthesis of first strand cDNA and PCR of VCAM-1

Total cellular RNA was extracted from culture plates according to the guanidium isothiocyanate method (Chomczynski & Sacchi, 1987). cDNA first strand was synthesized from total RNA by reverse transcription reaction. The reaction mixture containing 0.2 mg ml^{-1} total RNA (pre-heated at 68°C for 10 min), H_2O 2.5 μl, RNasin ribonuclease inhibitor 20 u, buffer 5×4 μl, 0.1 M 2 μl DTT , 2.5 mM 4 μl dNTP, 0.1 mM 1 μl hexanucleotide, and 200 u of Moloney-murine leukaemia virus reverse transcriptase. The reaction was carried out at 37°C for 60 min in a volume of 20 μl. The cDNA was amplified by PCR in a reaction mixture containing DNA template 2 μl, H_2O 10 μl, buffer 10×2.5 μl, 50 mM 0.75 μl $MgCl_2$, 2.5 mM 1.0 μl dNTP, 1.25 μl of each sense and antisense primers and 0.25 μl of Taq DNA polymerase 5 u ml^{-1}. Negative control using water (instead of DNA template) was included in each PCR reaction. The amplification profile included: 1 cycle of initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, primer

annealing at 59°C for 30 s, and extension at 72°C for 1 min; 1 cycle of final extension at 72°C for 7 min. This PCR protocol yielded a unique PCR product, which was purified and cloned into a pBluescript II SK(±) vector (Stratagene, San Diego, CA, U.S.A.) for sequencing on the two strands by the dideoxynucleotide technique. The relative amounts of each amplified cDNA were determined by measuring the density of the bands stained by ethidium bromide using the Gel Doc video gel documentation system and the Molecular Analyst software from Bio-Rad Laboratories, Hercules, CA, U.S.A. The expression of β-actin was used as control for the assay of a constitutively expressed gene.

Assay of NO production by rat peritoneal macrophages

Resident rat peritoneal cells were resuspended in DMEM supplemented with 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 50 μg ml^{-1} gentamicin, 2 mM glutamine, and 0.5 mM L-arginine in the absence of serum. Non-adherent cells were removed 2 h after adherence at 37°C by washing three times with fresh medium. More than 95% of the adherent cells were macrophages, as assessed by their ability to engulf zymosan particles and nonspecific esterase staining. The production of NO was assessed after different times of incubation at 37°C in an atmosphere containing 5% CO_2. Cell viability was determined by trypan blue exclusion.

Determination of NO and nitrite

NO released from macrophage cultures was determined by the accumulation of nitrite (Green *et al*., 1982). The cell culture media were collected and pelleted to eliminate cell components, and then supplemented with 100 μl of a solution of 1 mM sulphanilic acid and 100 mM HCl (final concentration). After incubation for 5 min the medium was centrifuged in an Eppendorf microcentrifuge and then supplemented with naphthylenediamine to reach a 1 mM final concentration. The reaction was completed after 15 min of incubation. The absorbance at 548 nm was compared with a standard of $NaNO_2$, and the production of NO was expressed as nmol of NO_2^-mg $protein^{-1}$.

Statistical analysis

Results are expressed as mean ± s.e.mean. For comparison of two groups of samples normally distributed, Student's two-tailed *t*-test was used. The calculation of IC_{50} values was carried out with Prism software version 2.0, from Graph Pad Software Inc., San Diego, CA, U.S.A.

Reagents

Recombinant TNF-α was from Genzyme Diagnostics, Cambridge, MA, U.S.A. Thrombin was from Sigma, Saint Louis, MO, U.S.A. Sodium salicylate and acetylsalicylic acid were from Fluka Chemika-BioChemika, Buchs, Switzerland. 2-Acetoxy-4-trifluoromethylbenzoic acid and 2-hydroxy-4-trifluoromethylbenzoic acid were from URIACH Laboratories, Barcelona, Spain. Sodium salicylate was dissolved in dimethylsulphoxide and diluted in phosphate-buffered saline solution to prepare a 1 M stock solution. The stock solutions of the remaining drugs were directly made in dimethylsulphoxide. Control cells were treated with the vehicle solution used to convey the drugs. IgG/ovalbumin equivalence immune complexes were made from rabbit antiserum as described (Bayon *et al*., 1997). Oligonucleotide primers for the detection of VCAM-1 mRNA by RT–PCR were designed from human gene sequence (EMBL/Gen Bank AC: M30257), using the Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin and were 5′-TGTCACTGTAAGCTGCAAG-3′ and 5′-TTCCAGCCTGGTTAATTC-3′, corresponding to nucleotides 1090-1108 and 1589-1572

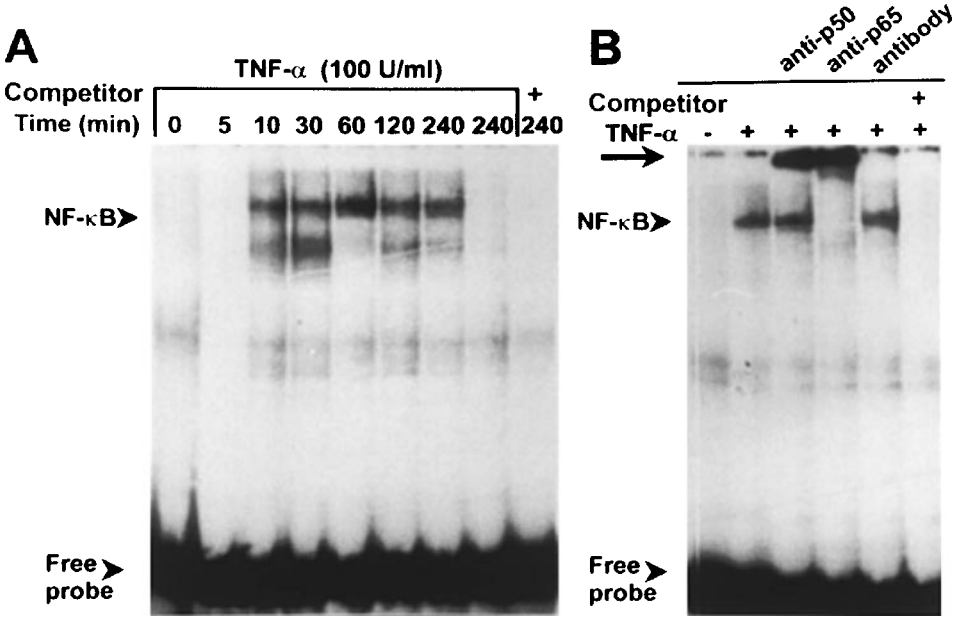

Figure 2 HUVEC were incubated in the presence of 100 u ml^{-1} of TNF-α for the times indicated. At the end of these periods nuclear extracts were collected for the assay of κB-binding activity. A control of cells incubated for 240 min in the absence of TNF-α was also included (A). The experiment shown in (B) was carried out with nuclear extracts of cells stimulated with TNF-α for 90 min. The nuclear extract protein was incubated for 15 min at 4°C with a 1:40 dilution of the indicated polyclonal rabbit antibodies prior to the addition of the ^{32}P-labelled oligonucleotide probe. The lane marked *antibody* indicates an experiment where the nuclear extract was incubated with the same dilution of a rabbit anti-ovalbumin antibody. The protein-oligonucleotide complexes supershifted by the antibodies are noted by an arrow. The lanes marked competitor indicate that the nuclear extracts were incubated with the ^{32}P-labelled probe in the presence of a 100 fold excess of unlabelled probe.

(Osborn *et al*., 1989). 5′-ATCATGTTTGAGACCTTCAA-3′ and 5′-TTGCGCTCAGGAGGAGCAAT-3′, corresponding to nucleotides 405-424 and 1029-1048 were used as primers for the detection of human β-actin mRNA. Polyclonal rabbit antisera against p65 and p50 members of the NF-κB/Rel family were a kind gift of Dr Nancy Rice, National Cancer Institute, Frederick, MD. Protein was assayed by the method of Bradford (1976).

Results

Aspirin-like drugs inhibit NF-κB activation in HUVEC stimulated by TNF-α and thrombin

Since aspirin has been reported to inhibit NF-κB activation in different cell systems with a higher potency than sodium salicylate, we compared the effect of different concentrations of aspirin, salicylate, triflusal and its deacetylated metabolite HTB on the activation of NF-κB in HUVEC, which is a sensitive and pathophysiologically relevant model to study transcriptional regulation (Collins *et al*., 1995). As shown in Figure 2A, TNF-α at the concentration of 100 u ml^{-1}, produced rapid activation of NF-κB, since κB-binding activity was already present in nuclear extracts 10 min after addition of the stimulus and could be detected without a significant decline for up to 4 h thereafter. In general, two κB-binding complexes of different sizes were observed, although their temporal pattern showed some differences and at some times only one complex was observed. Attempts to characterize the complexes were carried out by supershift assays with specific antibodies. As shown in Figure 2B, antibody reacting with p-50 protein of the NF-κB/Rel protein family induced a partial shift of the complexes, whereas anti-p65 antibody produced a complete supershift. A non-related control antibody lacked any effect in these experiments. Further evidence on the specificity of the complexes was provided by the reversal of the binding complexes with a 100 fold excess of the unlabelled probe. On the basis of the time-course experiments, a period of 90 min of incubation of HUVEC with TNF-α was selected for fixed-time experiments intended to assess differences of potency of the pharmacological compounds. Initial experiments using aspirin, triflusal and HTB showed that both 4-trifluoromethyl derivatives were more potent than aspirin (Figure 3), thus suggesting that the presence of the acetyl moiety in 4-trifluoromethylbenzoic acid is not a key requirement for the inhibition of NF-κB activation. Attempts to compare the potency of these compounds were carried out by densitometric scanning of the complexes. In seven independent experiments, HTB inhibited κB-binding complexes by 81±7,5% (4 mM HTB) versus 52±12% by the same concentration of aspirin (Figure 4). Comparison of sodium salicylate and HTB showed a more marked difference between both compounds, since sodium salicylate (4 mM) produced only a marginal inhibition of κB-binding activity, i.e., 21±11% ($n=8$) (Figure 5). Calculation of the IC_{50} values obtained from all experiments showed ≈2 mM for HTB and triflusal, 4 mM for aspirin and >4 mM for sodium salicylate. HUVEC viability was not significantly affected by the drugs as judged by the trypan blue exclusion test. In fact, the number of cells excluding the dye 24 h after addition of HTB and triflusal was 75±8% of total cells in the presence of 4 mM triflusal, 78±11% in the presence of 4 mM HTB, and 83±13% in control cells ($n=3$). However, a careful appraisal of these data should take into account that the experimental protocol entails removal of foetal calf serum containing medium to provide fresh medium with the

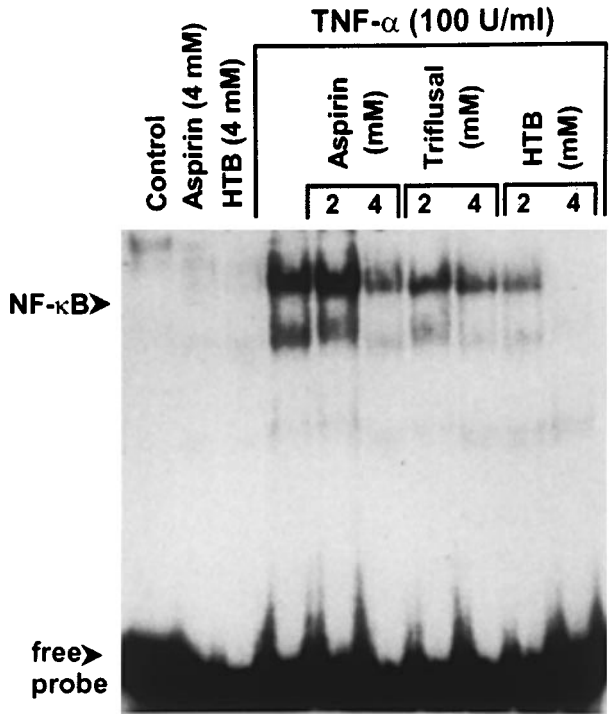

Figure 3 Effect of different concentrations of aspirin, triflusal and HTB on the activation of NF-κB elicited by TNF-α. HUVEC were incubated with TNF-α for 90 min in the presence of the indicated additions, and at the end of this period, the nuclear extract was collected and used for the assay of κB-binding activity.

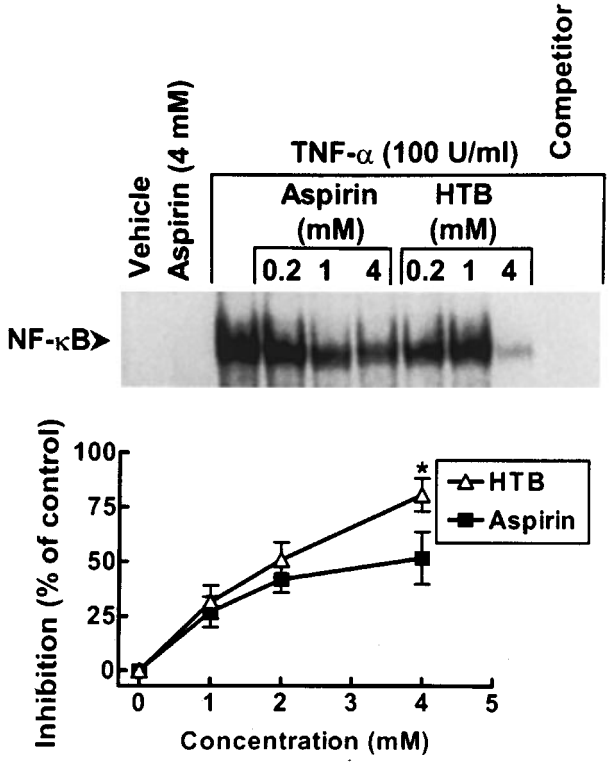

Figure 4 Effect of aspirin and HTB on the activation of NF-κB produced by TNF-α. Aspirin and HTB were added 10 min before TNF-α. Nuclear extracts were collected 90 min after addition of TNF-α. The panel represents a typical experiment of seven with identical trend. The graph shows the results expressed as mean± s.e.mean of seven blots quantitated by densitometric scanning. *Indicates $P<0.05$.

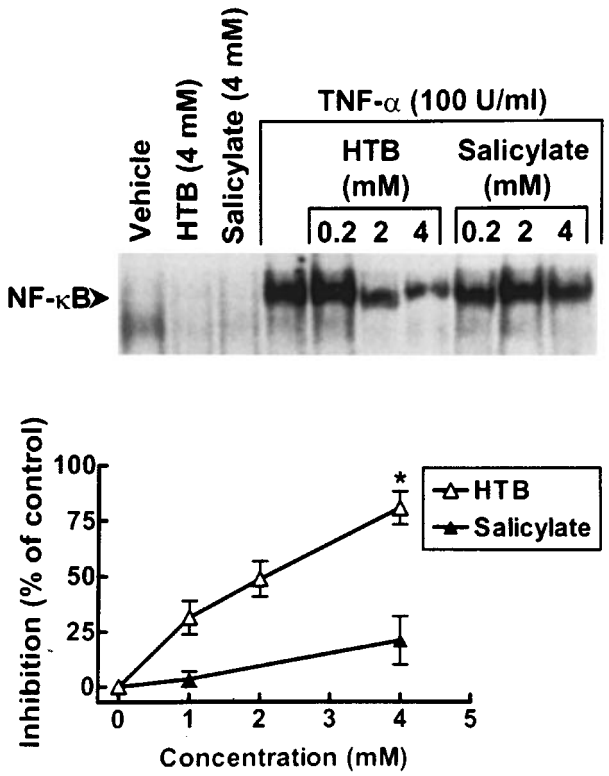

Figure 5 Effect of HTB and sodium salicylate on the activation of NF-κB elicited by TNF-α. Sodium salicylate and HTB were added 10 min before TNF-α. Nuclear extracts were collected 90 min after addition of TNF-α. The panel represents a typical experiment from eight similar experiments quantitated by densitometric scanning.

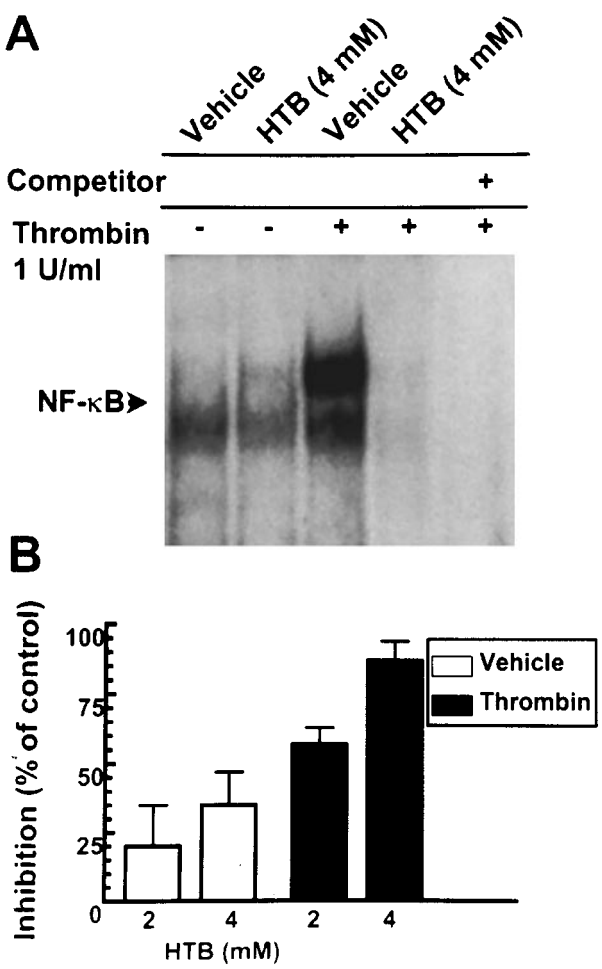

Figure 6 Effect of HTB on the activation of NF-κB produced by thrombin. HUVEC were stimulated with 1 u ml^{-1} thrombin for 90 min prior to the collection of the nuclear extract. A typical experiment is shown in (A). (B) shows the results of the densitometric scanning of the protein/oligonucleotide complexes observed in three independent experiments with two concentrations of compounds.

additions, thus leading to a reduction of foetal calf serum which can affect HUVEC viability.

Since endothelial cells may show different patterns of response to agonists, we also tested whether aspirin-like compounds influenced the activation of NF-κB induced by thrombin. As shown in Figure 6, incubation of HUVEC with 4 mM HTB produced a $87 \pm 7\%$ ($n=3$) reduction in the amount of κB-binding activity in the nuclear extract observed in cells incubated with 1 u ml^{-1} thrombin for 90 min. This suggests that HTB blunts NF-κB activation in HUVEC irrespective of the type of agonist. In order to determine whether the inhibitory effect of HTB on NF-κB activation might have functional consequences with respect to the expression of a gene which is under the control of this transcription factor and plays a role in the adhesion of monocytes to stimulated human endothelial cells, we looked at the effect of aspirin-like drugs on the expression of vascular cell adhesion molecule-1 (previously shown to be inhibited by aspirin in HUVEC, Weber *et al.*, 1995). As shown in Figure 7A, incubation of HUVEC with 2 mM triflusal and 2 mM HTB prior to TNF-α addition, produced a near complete inhibition of the expression of VCAM-1, whereas the inhibition produced by aspirin and salicylate at the same concentration was below 20% and reached 36–43% at 4 mM (Figure 7B).

Aspirin-like drugs inhibit NF-κB activation and NO production in rat peritoneal macrophages

We have previously shown that stimulation of receptors for the Fc portion of the antibody molecule leads to both NF-κB activation and induction of iNOS (Bayon *et al.*, 1997) with a potency similar to that of LPS. As shown in Figure 8A and B, both triflusal and HTB inhibited activation of NF-κB under these conditions of treatment. In contrast, aspirin and salicylate were less effective, since 4 mM aspirin caused a $42 \pm 12\%$ ($n=3$) inhibition and 4 mM salicylate was without a significant effect (Figure 8C). Since iNOS induction and NO production are events strictly controlled at the transcriptional level by NF-κB (Lowenstein *et al.*, 1993; Xie, 1997), we assessed whether the effect of aspirin-like compounds on nitrite production paralleled those observed on NF-κB activation. Incubation of adherent peritoneal macrophages with 100 μg ml^{-1} of IgG/ovalbumin IC produced a time-dependent accumulation of nitrite in the medium which reached 135 ± 27 nmol mg $protein^{-1}$ ($n=30$) 24 h after addition of the stimulus, and was abrogated in the presence of the NOS inhibitor L-N^G-monomethylargine thus indicating the dependence of this production on the L-arginine pathway. To assess the potency of the compounds, several concentrations of drugs were tested. Calculation of the IC_{50} concentrations was carried out from the data plotted in Figure 9, and showed the following values: 1.130 ± 12 mM (triflusal), 1.840 ± 34 (HTB), 6.081 ± 53 mM (aspirin) and 9.16 ± 1.9 mM (salicylate). When peritoneal macrophages were stimulated with 10 μg ml^{-1} of bacterial lipopolysaccharide the same inhibitory effect was observed (not shown), thus suggesting that similar salicylate-sensitive biochemical pathways are involved in the induction of iNOS

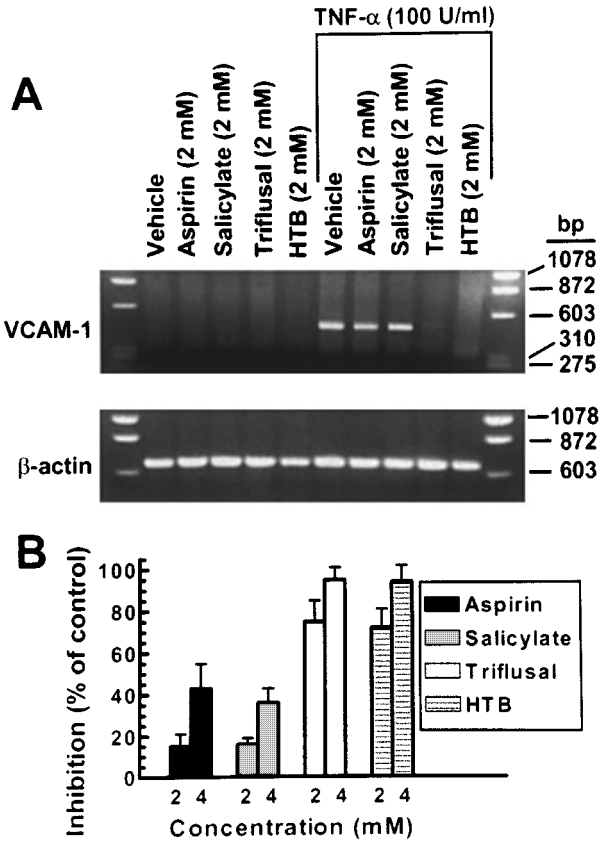

Figure 7 Effect of salicylates on the expression of VCAM-1 mRNA induced by TNF-α in HUVEC. Cells were incubated with 100 u ml^{-1} TNF-α in the presence and absence of the indicated compounds. One hour thereafter, total RNA was extracted and used for RT and PCR amplification with primers designed from the sequences of VCAM-1 and human β-actin. PCR products were separated by electrophoresis in 1.8% agarose gel and quantitated by measuring the density of ethidium bromide stained bands using the Gel Doc video gel documentation system and the Molecular Analyst software from Bio-Rad Laboratories. The molecular size of the amplification products is inferred from the electrophoretic migration of the DNA markers. A typical experiment of three similar experiments is shown in (A). The histogram shows the result of the quantitation of four independent experiments with two concentrations of compounds (B).

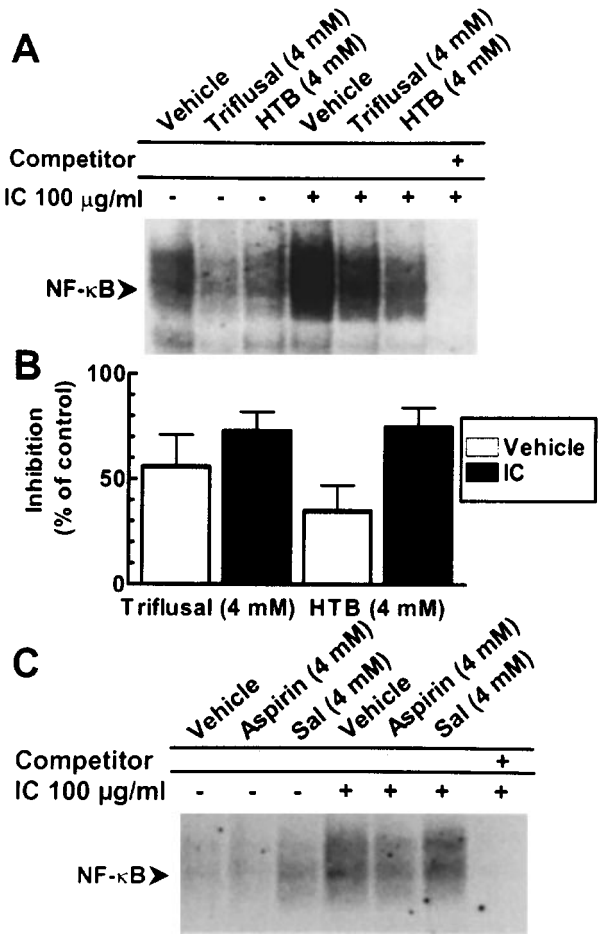

Figure 8 Effect of triflusal, HTB, aspirin and sodium salicylate on the activation of NF-κB elicited by insoluble IgG/ovalbumin immune complexes in rat peritoneal adherent cells. Adherent peritoneal macrophages were incubated in the presence of 100 μg ml^{-1} IgG/ovalbumin immune complexes for 2 h in the presence or absence of the additions indicated. A characteristic experiment of three with identical trend is shown in (A). (B) shows the results of the densitometric scanning of these experiments. (C) shows a representative experiment of three carried out with aspirin and salicylate (Sal).

by immune complexes and lipopolysaccharide. Viability of the cells at the end of the incubation period was 95% in control cells and ≈90% in the presence of 5 mM HTB, thus suggesting that the inhibition observed was not simply due to drug toxicity.

Discussion

The recent description of the inhibitory effect of aspirin and sodium salicylate on the activation of the transcription factor NF-κB (Weber *et al*., 1995; Grilli *et al*., 1996; Pierce *et al*., 1996), on the activation of the MAP-kinase cascade (Schwenger *et al*., 1996), as well as on the induction of iNOS (Aeberhard *et al*., 1995; Kwon *et al*., 1997; Amin *et al*., 1995; Brouet & Ohshima, 1995; Farivar & Brecher, 1996) has opened new avenues to explain the therapeutic properties of salicylate derivatives. Taking these reports into account, we studied 4-trifluoromethyl derivatives of 2-hydroxybenzoic acid, the antithrombotic properties of which have previously been explained on the basis of their ability to inhibit COX and to interfere with cyclic AMP-mediated signalling events (Rabasseda & García-Rafanell, 1993). Since NF-κB plays a central role in the interactions between cells of the immune system and endothelial cells (Collins *et al*., 1995; Bach *et al*., 1997) and since these interactions are important in the initiation of the atherosclerotic lesion (Collins, 1993) we focused on the effect of aspirin-like drugs on both activation of NF-κB and expression of VCAM-1 elicited by TNF-α. Moreover, VCAM-1 is an inducible molecule whose mRNA is only expressed after stimulation by TNF-α, thus making the assay of its inhibition by pharmacological agents easily detectable by PCR assays.

Our data agree with the reported functional relevance of κB sites in cytokine-mediated transcriptional response accounted for by two tandem κB sites in the VCAM-1 promoter (Neish *et al*., 1992; Shu *et al*., 1993; Read *et al*., 1996), since the inhibitory effects of aspirin, triflusal and HTB on NF-κB activation and VCAM-1 mRNA expression are similar. However, as the inhibition of VCAM-1 expression was bigger than the inhibition of NF-κB translocation, an additional ill-defined effect of these drugs which contributes to the down regulation of VCAM-1 cannot be ruled out. It has recently been reported, using sensitive transfection experiments, that the expression of other NF-κB regulated genes (e.g., P-selectin) is inhibited by sodium salicylate without a detectable

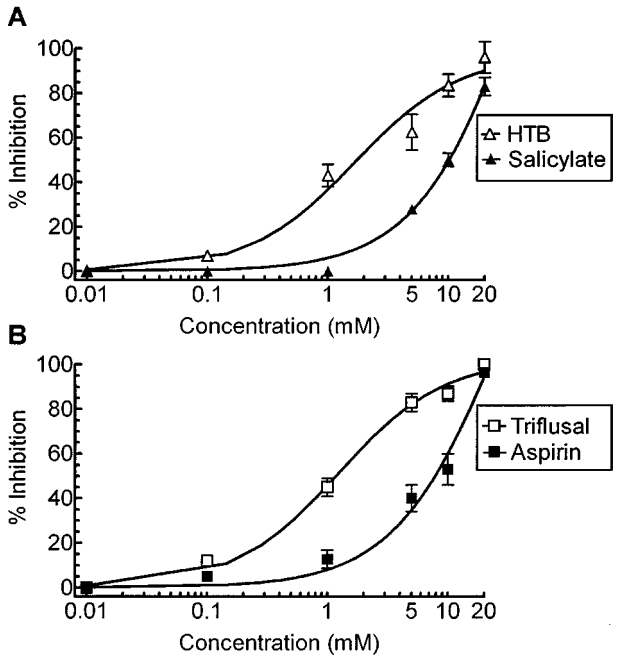

Figure 9 Effect of triflusal, HTB, aspirin and sodium salicylate on the production of nitrite elicited by IgG/ovalbumin complexes. The drugs were added 10 min prior to the addition of 100 μg ml^{-1} immune complexes and NO production was assayed as nitrite after 24 h. (A) shows the effect of sodium salicylate and HTB. (B) shows the effect of aspirin and triflusal. Results are expressed as mean$\pm$ s.e.mean of 7–9 experiments in duplicate.

requirement for an intact κB element in the P-selectin gene (Xia *et al*., 1998).

Our findings provide some information about the requirements involved in the inhibition of NF-κB activation by aspirin-like drugs. First, whereas the acetyl moiety could explain the higher inhibitory effect on NF-κB activation of aspirin compared with salicylate, this group is not an absolute requirement, since 4-fluoromethyl derivatives, triflusal and HTB, show similar properties, even though HTB lacks the acetyl moiety. A corollary to these findings is that 2-hydroxybenzoic acid structure of salicylate could be modulated by substitutions at different positions to yield compounds with NF-κB inhibitory activity at concentrations near the high end of therapeutic concentrations (Cotty *et al*., 1977; Insel, 1991). The pharmacological effect of HTB is not restricted to a unique stimulus, as it was observed in response to agonists engaging structurally distinct receptors, e.g., thrombin and TNF-α. This is an important issue, since endothelial cells show overlapping programs of activation (Mantovani *et al*., 1997), and thrombin enhances endothelial cell activation by TNF-α, thus providing a mechanism by which products of the coagulation cascade enhance cytokine-mediated inflammatory responses (Anrather *et al*., 1997). In contrast to our findings, some of the newly described effects of salicylates have been reported as stimulus-dependent. For instance, the effect of salicylate on the p42 and p44 isoforms of the MAP/ERK-kinase subgroup was observed in cells activated by TNF-α, but not in response to epidermal growth factor (Schwenger *et al*., 1996).

To extend these findings to other cell types and to other genes the transcriptional regulation of which is under the control of NF-κB, we looked at the effect of aspirin-like compounds on the production of NO elicited by immune complexes. Our data indicate that both HTB and triflusal inhibit with similar potency the activation of NF-κB elicited by immune complexes on rat peritoneal macrophages. Again, this agrees with the similar potency of these compounds on NF-κB activation. In contrast, even though aspirin appears to be more potent than salicylate, we have been unable to show unambiguous differences in the potency of these compounds as judged by their IC_{50} concentrations. This agrees with a previous study, where it was reported a similar inhibitory effect on NO production of aspirin and sodium salicylate (Aeberhard *et al*., 1995), thus suggesting that the acetyl moiety is not essential for some effects of salicylate, and also that a portion of the effect of salicylates on iNOS induction could be explained by a mechanism of transcriptional inhibition independent of NF-κB activation (Farivar & Brecher, 1996).

Irrespective of the molecular mechanisms underlying the biological effects discussed above, the present findings may have implications for the therapeutic and pharmacokinetic properties of salicylates. Aspirin loses its acetyl moiety shortly after intestinal absorption, whereas salicylate, the main metabolite that accumulates in tissues and which accounts for most of the therapeutic effect, shows a half-life of 2.4 h at therapeutic doses in studies addressed to assess its antiplatelet effects (Insel, 1991). In contrast, the $t_{1/2}$ of HTB can reach 50 h after repeated doses (Ramis *et al*., 1991). In view of the prominent effects of both triflusal and HTB on VCAM-1 expression, coupling a 4-trifluoromethyl group to 2-hydroxybenzoic acid seems a suitable approach for enhancing the pharmacological effects and maintaining a therapeutic concentration able to inhibit the induction of adhesion molecules on the endothelial cells.

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