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Effect of 4-trifluoromethyl derivatives of salicylate on nuclear factor κB-dependent transcription in human astrocytoma cells

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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 expression of several proteins displaying pro-inflammatory activities the regulation of which is associated to the transcription factor NF-κB, was assayed in the human astrocytoma cell line 1321N1.

2 Tumour necrosis factor-α (TNF-α) activated NF-κB as judged from both the appearance of κB-binding activity in the nuclear extracts, the degradation of IκB proteins in the cell lysates, and the activation of IκB kinases using an immunocomplex kinase assay with glutathione S-transferase (GST)-IκB proteins as substrates.

3 HTB up to 3 mM did not inhibit the nuclear translocation of NK-κB/Rel proteins as judged from electrophoretic mobility-shift assays; however, HTB inhibited the degradation of IκBβ without significantly affecting the degradation of both IκBα and IκBε.

4 In keeping with their inhibitory effect on IκBβ degradation in the cell lysates, both HTB and triflusal inhibited the phosphorylation of GST-IκBβ elicited by TNF-α, without affecting the phosphorylation of GST-IκBα.

5 The effect of both HTB and triflusal on κB-dependent *trans*-activation was studied by assaying the expression of both cyclo-oxygenase-2 (COX-2) and vascular cell adhesion molecule-1 (VCAM-1). HTB and triflusal inhibited in a dose-dependent manner the expression of COX-2 and VCAM-1 mRNA and the induction of COX-2 protein at therapeutically relevant concentrations.

6 These findings show the complexity of the biochemical mechanisms underlying the activation of NF-κB in the different cell types and extend the anti-inflammatory effects of HTB and triflusal to neural cells.

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Keywords: Adhesion molecules; Alzheimer disease; aspirin; astrocytes; cyclo-oxygenase; gliosis; inflammation; nuclear factor κB; triflusal; tumour necrosis factor

Abbreviations: ALLN, *N*-acetyl-leucinyl-leucinyl-norleucinal; COX-2, cyclo-oxygenase-2; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; HTB, 2-hydroxy-4-trifluoromethylbenzoic acid; IκB, inhibitor of NF-κB activity; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; iNOS, inducible isoform of nitric oxide synthase; NF-κB, nuclear factor κB; NSAIDs, non-steroidal anti-inflammatory drugs; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptors; RT, reverse transcriptase; TNF-α, tumour necrosis factor-α; Triflusal, 2-acetoxy-4-trifluoromethylbenzoic acid; VCAM-1, vascular cell adhesion molecule-1

Introduction

Salicylates are some of the most commonly used anti-inflammatory agents the action of which has been related to blockade of prostaglandin synthesis *via* inhibition of cyclooxygenase activity (Vane, 1971; Ferreira *et al*., 1971), and more recently linked to their ability to inhibit the activation of the transcription factor NF-κB (Kopp & Ghosh, 1994; Grilli *et al*., 1996; Oeth & Mackman, 1995; Pierce *et al*., 1996). The association of both pharmacological effects into the same molecule can be a significant advantage for therapeutics, since the inducible isoform of the cyclo-oxygenase enzyme (COX-2) contains two κB sites in its promoter/enhancer (Appleby *et al*., 1994), thus explaining that inhibition of κB-binding activity might block the expression of COX-2 and the ensuing production of prostanoids at inflammatory sites. The effect of some derivatives of salicylate, namely, the compounds 2-acetoxy-4-trifluoromethylbenzoic acid (triflusal) and 4-trifluoromethyl-benzoic acid (HTB) (De la Cruz *et al*., 1992; Rabasseda & Garcia-Rafanell, 1993) has been studied as to their ability to inhibit COX-2 protein expression and prostaglandin E_2 (PGE_2) production, on the one hand (Fernández de Arriba *et al*., 1999), and κB-dependent *trans*-activation of some genes such as the chemokine MCP-1 (Alonso *et al*., 2000), the inducible isoform of nitric oxide syntase (iNOS) and vascular cell adhesion molecule-1 (VCAM-1) (Bayón *et al*., 1999), on the other. NF-κB/Rel plays an important role in gene

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regulation during inflammatory reactions in a variety of disease settings, its activation being mediated by a network of kinases leading to the phosphorylation of IκB, which is subsequently degraded by the proteasome, a multicatalytic high molecular weight protease system. The possibility that salicylates might interfere with intracellular kinases has been suggested by the description of 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 by the involvement of p38 MAP kinase in the inhibitory effect of sodium salicylate on NF-κB activation (Alpert *et al*., 1999). Interestingly, it has recently been shown that salicylate behaves as a competitive inhibitor of IκB kinase-β (IKKβ) by inhibiting the binding of ATP (Yin *et al*., 1998), which seems to be the main biochemical mechanism explaining its pharmacological effect on NF-κB activation.

Interestingly, the biochemical mechanisms leading to the activation of IKK and the sensitivity to pharmacological inhibitors show significant differences related to the cell type, which might influence the therapeutic applications of anti-inflammatory drugs. Unlike hemopoietic cells, the proinflammatory cytokines IL-1α and TNF-α induce NF-κB-regulated adhesion molecules in astrocytes by a mechanism insensitive to inhibition by the antioxidants pyrrolidine dithiocarbamate and *N*-acetyl-cysteine (Moynagh *et al*., 1994). In addition, glucocorticoids, the anti-inflammatory effect of which has been related to their ability to inhibit NF-κB activation (Scheinman *et al*., 1995; Auphan *et al*., 1995), seem to produce this effect in brain cells by a mechanism different from the proposed effect on NF-κB (Bourke & Moynagh, 1999). Moreover, in a study addressing the activation of NF-κB by amiloid Aβ peptide, an event likely involved in the Alzheimer's pathogenic pathway, sodium salicylate only inhibited NF-κB activation at concentrations beyond its therapeutic level (>10 mM), which might indicate the resistance of NF-κB to salicylate inhibition in glial cells (Dodel *et al*., 1999). On this basis, we have addressed the effect of both triflusal and HTB on the expression of the products of several genes containing κB sites in their promoters, taking into account that the proteins encoded by these genes are involved in inflammatory responses in astrocytoma cells. Our data indicate that both triflusal and HTB show an inhibitory effect on the phosphorylation and degradation of IκBβ, without influencing the outcome of both IκBα and IκBε, as well as a definite inhibitory effect on the expression of proinflammatory proteins at therapeutically significant concentrations (McNeely & Goa, 1998). These findings extend the inhibitory effect of triflusal and HTB on the expression of proinflammatory proteins to astrocytoma cells and add further to the complexity of the biochemical mechanisms underlying this effect by disclosing significant differences between neural cells on the one hand, and endothelial and monocytic cells on the other hand.

Methods

Cells and reagents

1321N1 astrocytoma cells were cultured in DMEM containing 5% fetal calf serum at 37°C in an atmosphere containing 5% CO_2. Recombinant TNF-α was from Genzyme Diagnostics (Cambridge, MA, U.S.A.). Sodium salicylate was 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. *N*-acetyl-leucinyl-leucinyl-norleucinal (ALLN, calpain I inhibitor, MG-101) was purchased from Sigma (Saint-Louis, MO, U.S.A.). Oligonucleotide primers for the detection of VCAM-1 mRNA by RT–PCR were designed from human gene sequence (EMBL/Gen Bank AC: M30257), and were 5′-TGTCACTGTAAGCTGCAAG-3′ and 5′-TTCCAGCCTGGTTAATTC-3′, corresponding to nucleotides 1090–1108 and 1589–1572 (Osborn *et al*., 1989). Oligonucleotide primers for the detection of COX-2 mRNA by RT–PCR were designed from human gene sequence (EMBL/GenBank AC: M90100), and were 5′-TTCAAAT-GAGATTGTGGGAAAATTGCT-3′ (sense) and 5′-AGAT-CATCTCTGCCTGAGTATCTT-3′ (antisense) corresponding to nucleotides 574–600 and 855–878 of human COX-2 encoding sequence (Hla & Neilson, 1992). 5′-ATCATGTTT-GAGACCTTCAA-3′ and 5′-TTGCGCTCAGGAGGAG-CAAT-3′, corresponding to nucleotides 405–424 and 1029–1048 were used as primers for the detection of human β-actin mRNA. Rabbit polyclonal antiserum for the detection of human COX-2 was from Cayman Chemical Co., (Ann Arbor, MI, U.S.A.). Antibodies against IκBα, IκBβ, IκBε, IKKα, IKKβ and p38-MAP kinase were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). GST-IκBα and IκBβ fusion proteins were a kind gift of Dr Michel Karin, Dept. of Pharmacology, University of California, (San Diego, CA, U.S.A.).

Western blot analysis of IκB proteins and COX-2

Cells were washed with ice-cold phosphate-buffered saline, and lysed in 0.1 ml of ice-cold extraction buffer containing 10 mM HEPES, 2 mM EGTA, 10% glycerol, 10 μg ml^{-1} leupeptin, and 1 mM phenylmethylsulphonyl fluoride, pH 7.4. Cell lysate was mixed with Laemmli's buffer and boiled for 5 min. The amount of protein in each sample was assayed using the Bradford reagent and equal amounts of protein were loaded on each lane of a 10% SDS–PAGE gel. Proteins were transferred to nitrocellulose membranes using a semi-dry transfer module. The membranes were blocked with BSA for 2 h, washed with Tris-buffered saline containing Tween 20, and used for immunoblotting using anti-IκB or anti-COX-2 antibodies followed by donkey anti-rabbit IgG-horseradish peroxidase antibody. Detection was performed using the Amersham ECL system.

Assay of IκB kinase activity

Anti-IKKα and anti-IKKβ antibodies were used for the incubation with the cell lysates, followed by the addition of GammaBind G-Sepharose to trap the antibody/kinase complex according to standard protocols (Hernández *et al*., 1997; 1999). The kinase reaction was carried out with 500 ng

of either GST-IκBα or IκBβ fusion protein as substrate and 20 mM ATP and 5 μCi [γ-^{32}P]-ATP in a volume of 30 μl. The reaction was diluted in buffer and centrifuged to discard supernatant and then boiled in Laemmli SDS sample buffer and DTT. Phosphorylated GST-IκB was resolved by 10% SDS–PAGE and detected with the Bio-Rad Molecular Imager FX system. c-Jun N-terminal kinase was assayed using GST-c-Jun fusion protein as the substrate. Phosphorylated GST-c-Jun was resolved by 10% SDS–PAGE and immunodetected by Western blot using rabbit phosphospecific c-Jun (Ser^{63}) antibody (Hernández *et al*., 1998). The activation of p38-MAP kinase was assessed by immunoprecipitation of the kinase with anti-p38-MAP kinase antibody, and assay of the extent of tyrosine phosphorylation with anti-phosphotyrosine specific antibody.

Electrophoretic mobility shift assay

This was carried out according to previously described protocols (Bayón *et al*., 1997). Briefly, 1321N1 cells were washed with ice-cold hypotonic lysis buffer, and allowed to swell on ice for 10 min. Unbroken cells were eliminated by centrifugation and the nuclei were collected by centrifugation at 15,000 × *g* for 1 min in a microcentrifuge. 22-mer double-stranded oligonucleotide probes containing NF-κB were end-labelled with [γ-^{32}P]-ATP using T4 polynucleotide kinase. The κB sequence used was, sense 5′-AGTTCAGGG-GAATTTCCCAGGC-3′ and the complement 5′-GCCTGGGAAATTCCCCTGAACT-3′. Nucleoprotein-oligonucleotide complexes were resolved by electrophoresis in a 4% nondenaturing polyacrylamide gel in Tris borate/EDTA electrophoresis buffer at 175 V for 3 h at 4°C. The specificity of the DNA-protein complex was confirmed by competition with a 100 fold molar excess of unlabelled nucleotide containing the consensus sequence.

Synthesis of first strand cDNA and PCR of VCAM-1 and COX-2

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, 2.5 μl H_2O, 20 u of RNasin ribonuclease inhibitor, 4 μl buffer 5×, 2 μl DTT 0.1 M, 4 μl dNTP 2.5 mM, 1 μl hexanucleotide 0.1 mM, and 200 u of Moloney-murine leukemia virus reverse transcriptase. The cDNA was amplified by PCR in a reaction mixture containing 2 μl of DNA template, 10 μl H_2O, 2.5 μl buffer 10×, 0.75 μl $MgCl_2$ 50 mM, 1.0 μl dNTP 2.5 mM, 1.25 μl of each sense and antisense primers and 0.25 μl of Taq DNA polymerase 5 u ml^{-1}. The amplification profile included: one 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; one cycle of final extension at 72°C for 7 min. 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.

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.

Results

HTB does not produce inhibition of NF-κB activity in EMSA assays, but decreases the degradation of IκBβ

Stimulation of 1321N1 cells with concentrations of TNF-α as low as 25 u ml^{-1}, induces a rapid and long-lasting activation of NF-κB (at least 6 h), as judged from the presence of κB-binding protein containing p65 and p50 proteins of the Rel family in the nuclear extract (Hernández *et al*., 1999). Attempts to ascertain the possible inhibitory effect of HTB on the activation of NF-κB was first addressed by looking at the effect of this compound on the nuclear translocation of NF-κB/Rel proteins. As shown in Figure 1A, concentrations of both sodium salicylate and HTB up to 3 mM, did not

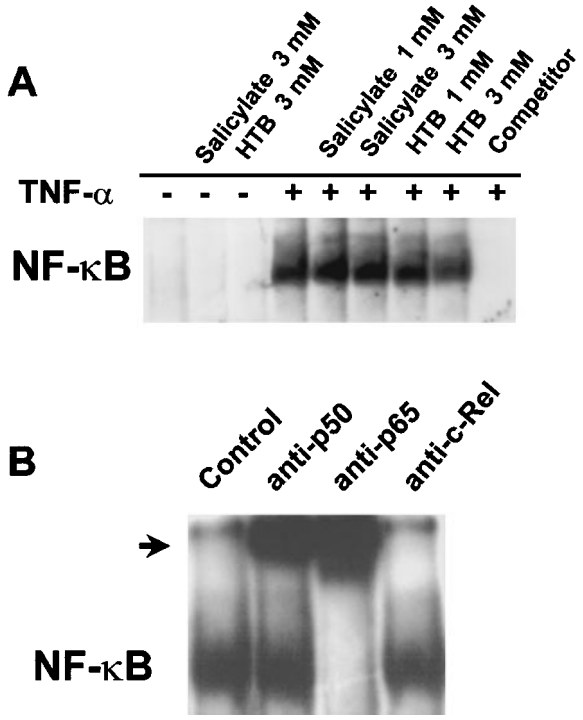

Figure 1 Electrophoretic mobility shift assays showing κB-binding activity in nuclear extracts from 1321N1 cells stimulated with TNF-α. Cells were stimulated with 100 u ml^{-1} TNF-α for 15 min in the presence of the additions indicated and at the end of this time, cell lysates were processed for the assay of κB-binding activity in the nuclear extracts. The lane marked competitor indicates that the binding reaction was carried out with a 100 fold molar excess of unlabelled nucleotide containing the consensus sequence. This is an experiment of four identical ones (A). In the experiment shown in (B), nuclear extracts from cells stimulated with 100 u ml^{-1} TNF-α for 15 min in the presence of 3 mM HTB were incubated for 15 min at 4°C with a 1 : 40 dilution of polyclonal rabbit antibodies prior to the addition of the ^{32}P-labelled oligonucleotide probe. The protein-oligonucleotide complexes supershifted by the antibodies are noted by an arrow.

influence nuclear translocation of proteins displaying κB-binding activity, thus agreeing with previous observations in astrocytoma cells which suggest insensitivity of NF-κB activation to compounds active on other cell types, for example, pyrrolidine dithiocarbamate and *N*-acetyl-cysteine (Bourke & Moynagh, 1999; Hernández *et al*., 1999; Dodel *et al*., 1999), or alternatively, that the effect of these drugs on NF-κB activation is exerted at defined molecular steps not disclosed by the EMSA approach. To obtain further insight into these mechanisms, attempts to disclose the composition of the κB-binding complexes with specific antibodies were carried out. As shown in Figure 1B, anti-p50 antibody produced a partial shift of the protein-oligonucleotide complexes, whereas anti p65 antibody produced a complete supershift, thus indicating that at least at the time of the experiments, the complexes showed an overall composition similar to that disclosed in the absence of HTB (Hernández *et al*., 1999). TNF-α induced a marked degradation of IκB proteins showing distinct kinetics for the different proteins (Figure 2A). IκBα showed the earliest degradation, undetectable levels being found as soon as 5 min after addition of the stimulus and reaching pre-stimulation levels after 60 min. IκBβ decreased after 10 min and was almost undetectable for at least 6 h after challenge (Figure 3). IκBε decreased after 15 min and reappeared after 2 h. These data suggests that IκBα-dependent release of NF-κB may not fully account for the long-lasting induction of IκB-binding activity involved in the regulation of NF-κB-dependent transcriptional regulation, thus agreeing with findings in U937 cells where TNF-α and IL-1β produce a similar activation of κB-binding activity, whereas TNF-α produces a complete loss of IκBα and IL-1β only produces a 40% reduction (Nasuhara *et al*., 1999). Interestingly, preincubation with HTB at concentrations 0.5–1 mM inhibited the degradation of IκBβ (Figure 2B) at a similar extent to that produced by 100 μM ALLN (Figure 2C), i.e., a proteasome inhibitor which blocks the nuclear translocation of NF-κB and affects the expression of adhesion molecules and COX-2 (Read *et al*., 1995; Gallois *et al*., 1998). However, the levels of both IκBα (Figure 2B) and IκBε (not shown) were not influenced. Quantitation by densitometric scanning of the IκBβ blots from samples obtained 15 min after the addition of TNF-α showed a $39 \pm 7\%$ inhibition for 0.5 mM HTB, and a $61 \pm 9\%$ inhibition at 1 mM ($n=5$, $P<0.05$), thus confirming the anticipated paradigm that using only the EMSA approach, inhibitions of NF-κB activation exerted by agents only acting on some elements of the IκB family might be overlooked. Noteworthy, the viability of the cells was not affected by the concentrations of drugs used, which in fact were selected on the basis of the pharmacological concentrations of these agents that can be obtained in the serum of patients (McNeely & Goa, 1998).

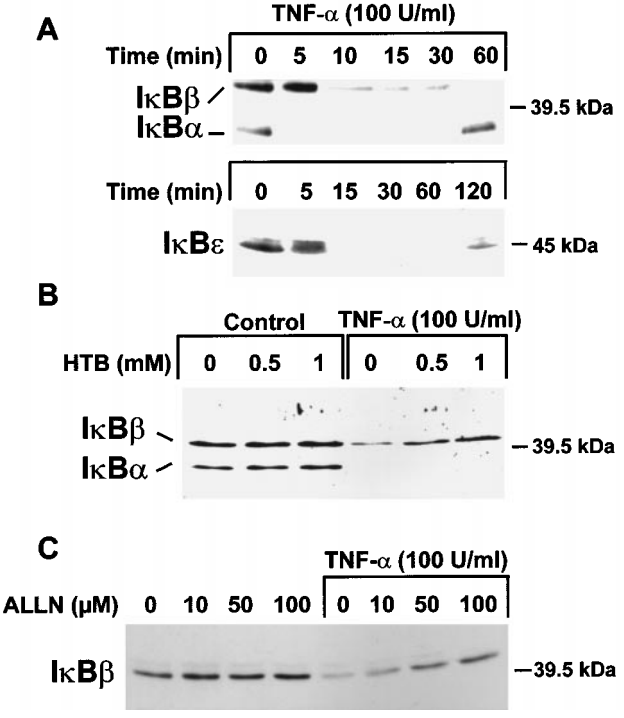

Figure 2 Immunodetection of IκB proteins in 1321N1 cells stimulated with 100 u ml^{-1} TNF-α. Cells were stimulated with TNF-α for the times indicated and at the end of these periods the cell lysate was used for SDS–PAGE and immunodetection of IκBα, IκBβ, and IκBε (A). The same membrane was used for the sequential immunodetection of IκBα and IκBβ with their cognate antibodies, whereas IκBε was immunoblotted in a separate membrane. (B) Represents the effect of different concentrations of HTB on the degradation of IκBβ. (C) Shows the effect of different concentrations of ALLN. The assay of IκBβ in (B) and (C) was conducted on cell lysates collected 15 min after the addition of TNF-α.

HTB and triflusal inhibit the phosphorylation of IκBβ by IKK

Since it has been described a selective inhibitory effect of sodium salicylate on IKKβ from both COS and HeLa cells (Yin *et al*., 1998), the effect of triflusal and HTB on IKK activity was assessed. As shown in Figure 4, TNF-α induced a rapid and transient activation of IKK in 1321N1 cells, as judged from the presence of IKK activity in immunoprecipitates obtained with each of the antibodies, thus agreeing with current views stressing the association of both IKK in the signalsome. Maximal activity was observed at 5–10 min, and decreased to reach prestimulation values by 15–30 min.

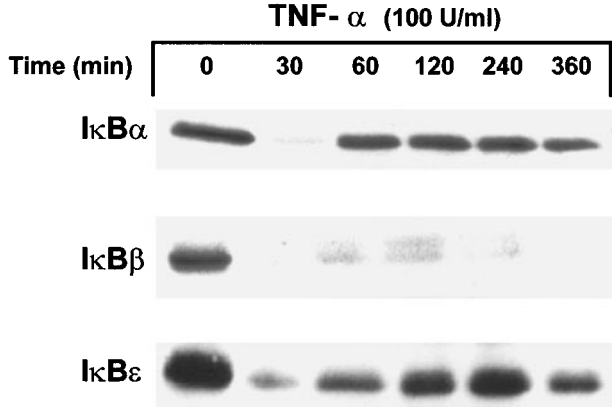

Figure 3 Resynthesis of IκBα and IκBε in 1321N1 cells after stimulation with TNF−α. The conditions of the assay were as those in Figure 2, but the cell lysates were collected at the time indicated to assess the resynthesis at later times.

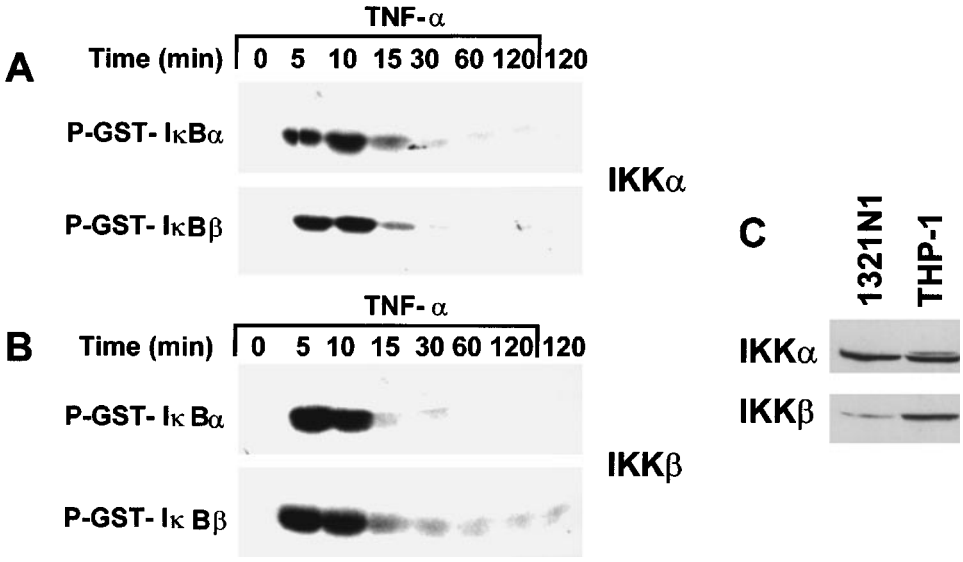

Figure 4 Activation of IKK in 1321N1 cells stimulated with TNF-α. 1311N1 cells were incubated in the presence of 100 u ml^{-1} of TNF-α for the times indicated. Cell lysates were collected for immunoprecipitation of the signalsome with anti-IKK and the assay of IKK activity with the immunoprecipitated kinase and either GST-IκBα or GST-IκBβ. Cells incubated for 120 min in the absence of TNF-α were also included as controls. The experiment shown in (A) corresponds to an experiment carried out with anti-IKKα, whereas the experiment shown in (B) was carried out using anti-IKKβ antibody for the immunoprecipitation. (C) Compares the amount of both IKKα and IKKβ in cell lysates from 1321N1 and THP-1 cells. P-, phosphorylated.

Under the assay conditions, no differences of enzyme activity towards each of the substrates were observed, which agrees with the aforementioned ability of TNF-α to induce degradation of both IκBα and IκBβ. The relative amount of each IKK present in 1321N1 cells was addressed by immunodetection in the cell lysates and compared to that detected in THP-1 cells. As shown in Figure 4C, THP-1 cells showed a significantly higher amount of IKKβ than 1321N1 cells. Preincubation of 1321N1 cells with both HTB and triflusal at the concentration of 1 mM prior to the addition of TNF-α diminished the IKK activity associated to the immunoprecipates when GST-IκBβ was used as a substrate, whereas no inhibition was observed when the assay was conducted with GST-IκBα (Figure 5), thus agreeing with the diminished degradation of IκBβ in whole cells incubated with HTB. Attempts to address the effect of HTB on other kinases that are activated by TNF-α focused on JNK, since unlike the ERK module of MAP kinases, activation of the stress module of MAP kinases, i.e., JNK and at lower extent p38-MAP kinase, is a hallmark of the response to TNF-α in astrocytoma cells (Hernández *et al*., 1999). As shown in Figure 6, HTB at the highest concentration used throughout these experiments did not significantly affect JNK activity nor the tyrosine phosphorylation of p38-MAP kinase, thus suggesting that the effects herein reported can not be explained by inhibition of the stress module of MAP kinases.

HTB and triflusal inhibit the expression of COX-2 and VCAM-1 induced by TNF-α in astrocytoma cells

Attempts to address whether the aforementioned effects of triflusal and HTB on IκBβ phosphorylation and degradation might have consequences on the regulation of IκB-regulated genes, experiments were carried out to assess the effect of these compounds on VCAM-1 and COX-2 mRNA as prototypic inducible genes expressed in astrocytoma cells the expression of which has been found to be inhibited in other cell types (Bayón *et al*., 1999; Fernández de Arriba *et al*., 1999). As shown in Figure 7A, incubation with HTB in the range 1–3 mM, induced a significant inhibition of the expression of COX-2 mRNA induced by TNF-α as judged from RT–PCR reactions, whereas sodium salicylate at the same doses failed to produce inhibitions higher than 20%. This was accompanied by a reduction of COX-2 protein expression as judged from Western blot assays carried out under identical conditions. As shown in Figure 7B, 1 mM HTB produced a $58 \pm 12\%$ inhibition of COX-2 expression induced by TNF-α, whereas the inhibition with 3 mM HTB was $92 \pm 6\%$. The expression of VCAM-1 mRNA, which shows a long-lasting induction maintained for at least 6 h after stimulation with TNF-α (Figure 8A), was also inhibited by HTB in the range 1–5 mM (Figure 8B).

Discussion

The initial description of the inhibitory effect of aspirin and sodium salicylate on the activation of the transcription factor NF-κB (Kopp & Ghosh, 1994; Weber *et al*., 1995; Grilli *et al*., 1996; Pierce *et al*., 1996), has opened new avenues to explain the therapeutic properties of salicylate derivatives, and has been followed by the description of analogous properties of some derivatives including sulfasalazine (Wahl *et al*., 1998) and 2-hydroxy-4-trifluoromethylbenzoic acid (Bayón *et al*., 1999; Fernández de Arriba *et al*., 1999), and by the description of new mechanisms of action for other non-steroidal anti-inflammatory agents, for instance sulindac (Yamamoto *et al*., 1999), *pari passu* with the unveiling of

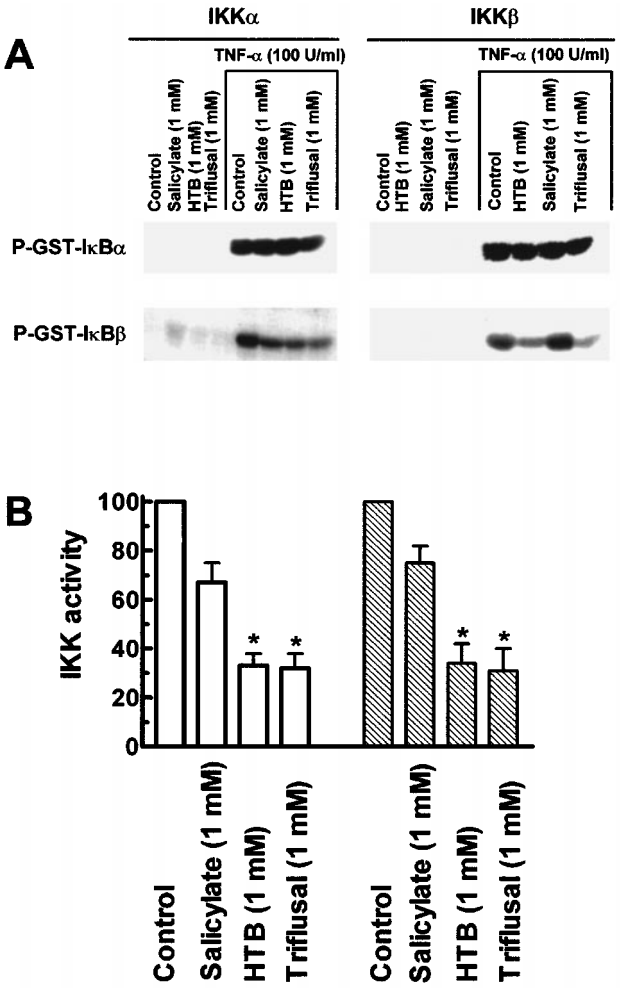

Figure 5 Effect of salicylate, HTB and triflusal on IKK activity. 1321N1 cells were stimulated with 100 u ml^{-1} of TNF-α for 5 min in the presence of the indicated additions. At the end of this period, cell lysates were used for immunoprecipitation with anti-IKKα and anti-IKKβ antibodies and used for *in vitro* kinase assay with both GST-IκBα and GST-IκBβ as substrates (A). The histogram (B) shows the quantitation of five independent experiments carried out with the immunoprecipitates obtained using both anti-IKKα (open bars) and anti-IKKβ (striped bars) antibodies and GST-IκBβ as substrate. $^*P<0.05$.

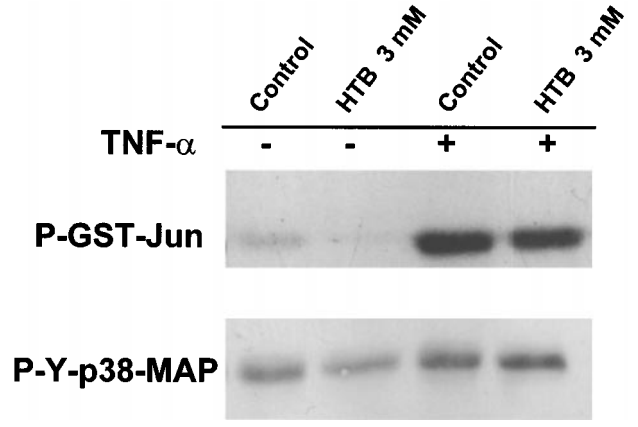

Figure 6 Effect of HTB on the activation of JNK and p38-MAP kinase tyrosine phosphorylation. 1321N1 cells were incubated with 100 u ml^{-1} TNF-α for 15 min, in the presence and absence of 3 mM HTB. At the end of this period cell lysates were collected for the assay of P-GST-c-Jun phosphorylation with phosphospecific anti-c-Jun antibody. This is a representative experiment of three identical ones (upper panel). The lower panel shows a typical experiment carried out under identical conditions, in which cell lysates were used for immunoprecipitation with anti-p38-antibody, SDS–PAGE separation of the immunoprecipitate, and blotting with anti-phosphotyrosine antibody. P-Y, phosphotyrosine.

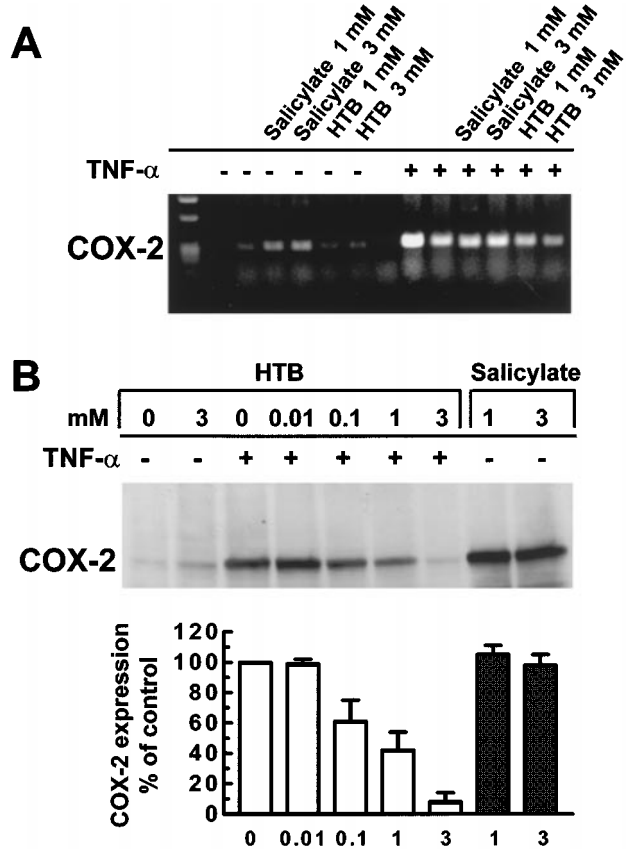

Figure 7 Effect of the incubation with sodium salicylate and HTB on the expression of COX-2 mRNA in cells incubated for 6 h in the presence of 100 u ml^{-1} TNF-α (A). This is a representative experiment of five identical ones. Effect of salicylate and HTB on the expression of COX-2 protein induced by TNF-α. Cells were incubated with 100 u ml^{-1} TNF-α for 4 h and then the cell lysates were collected for the immunodetection of COX-2 protein (B). The histogram in panel (B) shows mean$\pm$s.e.mean of the densitometric scanning of four independent experiments.

the mechanism of activation of NF-κB. Namely, the role for salicylate as a specific inhibitor of IKKβ (Yin *et al*., 1998), and the description of cell-specific mechanisms of activation of NF-κB, which might explain distinct properties of pharmacological agents on some cell types. Taking these reports into account, we have extended previous studies in human umbilical vein endothelial cells (Bayón *et al*., 1999) and THP-1 monocytic cells (Alonso *et al*., 2000) to human astrocytoma cells in view of the role for astrocytes in inflammatory and degenerative neural diseases, and the reported evidence of unique pharmacological resistance of these cells to inhibitors of the activation of NF-κB that are operative in other cell types (Moynagh *et al*., 1994; Bourke & Moynagh, 1999).

Our data agree with these reports (Moynagh *et al*., 1994; Bourke & Moynagh, 1999) by showing that unlike umbilical

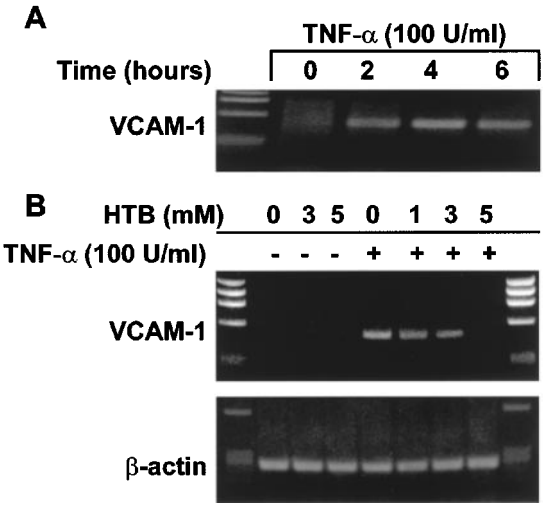

Figure 8 Effect of HTB on VCAM-1 expression. Cells were incubated with 100 u ml^{-1} TNF-α for the times indicated and then processed for the detection of VCAM-1 mRNA by RT–PCR (A). (B) Shows an experiment of three carried out with cells stimulated 4 h with TNF-α in the presence of different concentrations of salicylate and HTB. The expression of β-actin is shown for comparison.

vein endothelial cells and THP-1 cells, HTB is not able to block the nuclear translocation of NF-κB, as judged from EMSA assays, at pharmacologically relevant concentrations. However, it blocked the degradation of IκBβ in cell lysates at a similar extent to that found with the proteasome inhibitor *N*-acetyl-leucinyl-leucinyl-norleucinal, which in contrast to the antioxidants pyrrolidine dithiocarbamate and *N*-acetyl-cysteine was active in 1321N1 cells. These data stress the complexity of the NF-κB/Rel system and the requirement for different experimental approaches addressing the involvement of the different elements of the family before ruling out its involvement in some settings. For instance, NF-κB/Rel p50 homodimers, which lack κB-*trans*-activating activity can be present in nuclear extracts from RBL5 cells incubated for several hours in the presence of sulfasalazine under conditions where other NF-κB/Rel heterodimers are blocked (Liptay *et al*., 1999). Our data agree with the reported effect of salicylate as an almost irreversible inhibitor of IKK the effect of which is still observed in the cell-free system (Yin *et al*., 1998). However, our data do not allow to state whether the effect of HTB is exerted on either IKKα or IKKβ since both isoforms are associated to the proteasome and are immunoprecipitated in our system irrespective of the specificity of the antibody used. Attempts to characterize the isoforms expressed in 1321N1 cells have disclosed a lower amount of IKKβ than in THP-1 monocytic cells. This seems of interest, since activation of THP-1 cells by lipopolysaccharide and TNF-α produces different patterns of activation of IKKs for each stimulus. In fact, lipopolysaccharide predominantly produces a long-lasting activation of IKKβ activity, whereas TNF-α produces a rapid and short-lived predominant activation of IKKα (O'Connell *et al*., 1998; Fischer *et al*., 1999). On this basis, the increased expression of IKKβ in THP-1 cells and monocytes might explain a privileged functional role for this IKK in these cells, and might also favour the chance of obtaining selective immunoprecipitation of each IKK by disrupting the signalsome by adding Triton X-100 to the immunoprecipitation buffer, as described by Fischer *et al*. (1999). It should be noted, however, that this approach was unsuccessful in our hands. The functional significance of IκBβ degradation should also be discussed on the basis of previous studies in 1321N1 cells. For instance, glucocorticoids inhibit the expression of IL-8, VCAM-1 and ICAM-1 in the absence of inhibition of both nuclear translocation of NF-κB proteins and degradation of IκBα (Bourke & Moynagh, 1999). However, since the degradation of IκBβ was not assessed in this report, the chance that glucocorticoids do affect the phosphorylation and degradation of IκBβ can not be ruled out. Alternatively, the effect of some anti-inflammatory agents previously described as inhibitors of NF-κB activation should be explained on the basis of other pharmacological properties. In fact, it has 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 requirement for an intact κB element in the P-selectin gene (Xia *et al*., 1998). In addition, inhibition by both aspirin and sodium salicylate of nitric oxide production exerted at a translational step have also been reported in hepatocytes (Sakitani *et al*., 1997), thus agreeing with earlier suggestions that a portion of the effect of salicylates on iNOS induction could be explained by mechanisms other than inhibition of κB-dependent *trans*-activation (Farivar & Brecher, 1996). These cautions seem particularly adequate for the effects observed on COX-2, since in addition to κB sites a role for peroxisome proliferator-activated receptors (PPAR) in the *trans*-activation of COX-2 has been reported, thus indicating that PPAR activators, e.g. non-steroidal anti-inflammatory drugs (NSAIDs), might under some conditions enhance the expression of COX-2 in epithelial cells (Meade *et al*., 1999). However, the net effect of PPAR agonists on COX-2 expression induced by pro-inflammatory cytokines is inhibitory, since the prototypical compounds sulindac disulfide and flufenamic acid only enhance COX-2 expression at concentrations below 200 μM, whereas *via* their inhibitory effect on NF-κB activation, they inhibit the induction elicited by pro-inflammatory cytokines at concentrations above 200 μM (Paik *et al*., 2000).

Irrespective of the biochemical mechanism that might explain the distinct effect of HTB on the phosphorylation and degradation of IκBβ, our results indicate a significant effect of HTB on κB-dependent *trans*-activation by showing a decreased expression of mRNA of both COX-2 and VCAM-1, both molecules being of relevance for the functional involvement of astrocytes in immunoinflammatory conditions. However, as the inhibition of COX-2 protein expression was bigger than the inhibition of its mRNA expression, an additional post-translational effect of HTB contributing to the down regulation of COX-2 seems likely (Mitchell *et al*., 1997). These findings may have implications for the therapeutic applications of trifluoromethyl derivatives of salicylates in view of the prominent effects of HTB on both VCAM-1 and COX-2 expression in neural cells, and the pharmacokinetics of HTB which shows a $t_{1/2}$ of 35 h after repeated doses in humans (McNeely & Goa, 1998), as compared to a half-life of 2.4 h of salicylate at therapeutic doses (Insel, 1991).

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