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Modulation of transcription factor NF-κB by enantiomers of the nonsteroidal drug ibuprofen

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1 The nonsteroidal drug ibuprofen exists as an **R**(−)- and **S**(+)-enantiomer. Only the **S**(+)-enantiomer is an effective cyclo-oxygenase inhibitor, while the **R**(−)-enantiomer is inactive in this respect. Thus the molecular mechanism by which **R**(−)-ibuprofen exerts its anti-inflammatory and antinociceptive effects remains unknown.

2 In this study the effects of the enantiomers of ibuprofen on modulation of transcription factors have been examined with electrophoretic mobility-shift assay (EMSA), transient transfection experiments, confocal immunofluorescence and nuclear import experiments, to determine their selectivity and potency as inhibitors of the activation of transcription factor nuclear factor-κB (NF-κB).

3 **R**(−)-ibuprofen (IC_{50}: 121.8 μM) as well as the **S**(+)-enantiomer (IC_{50}: 61.7 μM) inhibited the activation of NF-κB in response to T-cell stimulation. The effect of ibuprofen was specific because, at concentrations up to 10 mM, ibuprofen did not affect the heat shock transcription factor (HSF) and the activation of NF-κB by prostaglandin E_2 (PGE_2). Very high concentrations of ibuprofen (20 mM) did not prevent NF-κB binding to DNA *in vitro*. Immunofluorescence and nuclear import experiments indicate that the site of ibuprofen action appeared to be upstream of the dissociation of the NF-κB-IκB-complex.

4 Our data raise the possibility that **R**(−)-ibuprofen exerts some of its effects by inhibition of NF-κB activation.

Keywords: **R**-Ibuprofen; NF-κB; heat shock transcription factor; prostaglandin E_2; electrophoretic mobility-shift assay; transient transfection; immunofluorescence; nuclear import experiments

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are, as a group, the most frequently consumed drugs worldwide in the treatment of pain and inflammation. Traditionally, the effects of aspirin and other NSAIDs have been explained on the basis of their inhibition of cyclo-oxygenases that synthesize prostaglandins (Meade *et al*., 1993; Vane, 1994; Allen, 1995). However, over the last few years evidence has been accumulating that the mechanism of some of the actions of NSAIDs is not related to their ability to inhibit prostaglandin synthesis (Brooks & Day, 1991; McCormack & Brune, 1991). Weak inhibitors of prostaglandin synthesis, e.g. salicylic acid, exert a potent antinociceptive effect and are able to reduce inflammation at comparable doses to aspirin (Weissmann, 1991). However, neither the cellular target nor cell signalling pathways possibly involved in the effect of weak prostaglandin inhibitors have been identified yet. Hence, additional mechanisms unrelated to inhibition of prostaglandin synthesis are currently being investigated.

A mechanism unrelated to inhibition of prostaglandin synthesis has been recently found for aspirin and sodium salicylate (Kopp & Ghosh, 1994; Frantz & O'Neill, 1995; Grilli *et al*., 1996). Both NSAIDs affect the transcription factors nuclear factor-κB (NF-κB) and heat shock factor (HSF) (Jurivich *et al*., 1992; Kopp & Ghosh, 1994; Frantz & O'Neill, 1995; Lee *et al*., 1995; Grilli *et al*., 1996).

NF-κB was originally described as a constitutive transcription factor in mature B-cell lines (Sen & Baltimore, 1986) but has subsequently been found in an inducible form in many other cell types. NF-κB is a heterodimeric transcription factor composed of p50 and p65 subunits that belong to the protein family NF-κB/Rel/Dorsal (Liou & Baltimore, 1993; Baeuerle & Henkel, 1994; Siebenlist *et al*., 1994). In unstimulated cells the NF-κB proteins, unlike other transcription factors, are held in the cytoplasm in an inactive state by an inhibitory subunit IκB. The phosphorylation of IκB and its degradation in stimulated cells uncovers nuclear localization signals on the Rel proteins which results in translocation of NF-κB to the nucleus (Sun *et al*., 1994; Traenckner *et al*., 1994; Verma *et al*., 1995). The biological functions of NF-κB comprise communication between cells, embryonic development, neurodegeneration, the response to stress, inflammation and viral infection, and the maintenance of cell type specific expression of genes (Baeuerle & Henkel, 1994). Most of the genes known to be activated by NF-κB are involved in immune and inflammatory responses. These include cytokines such as interleukin 1 (IL-1), IL-6, IL-8, interferon-β, and tumour necrosis factor-α, and the cell adhesion molecules vascular cell adhesion molecule-1, intercellular adhesion molecule-1 and endothelial leukocyte adhesion molecule-1 (Degitz *et al*., 1991; Neish *et al*., 1992; Grilli *et al*., 1993). Some of the cellular processes can be modulated by aspirin and salicylate at doses much higher than those required to inhibit prostaglandin synthesis (Kopp & Ghosh, 1994; Frantz & O'Neill, 1995; Grilli *et al*., 1996).

In the present study we used the two enantiomers of ibuprofen, a 2-arylpropionic acid, to distinguish between inhibition of prostaglandin synthesis and additional molecular activities. So far it is assumed that the mode of action of ibuprofen is via inhibition of cyclo-oxygenases (COX) 1 and 2 predominantly by **S**-ibuprofen. **S**-Ibuprofen inhibited the activity of COX 1 and COX 2 equally in human platelets

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and blood monocytes at therapeutically relevant concentrations. **R**-Ibuprofen inhibited COX 1 less potently than **S**-ibuprofen and showed no inhibition of COX 2 (Boneberg *et al*., 1996; Neupert *et al*., 1997). Thus **S**-ibuprofen represents the active entity in the racemate with respect to cyclooxygenase activity. However, experimental and clinical data employing racemic and pure **S**-ibuprofen indicate that the anti-inflammatory and analgesic effects cannot be attributed solely to **S**-ibuprofen (Brune *et al*., 1991; Geisslinger *et al*., 1994). Such observations, together with the well documented effects of salicylate, question the conventional concept that NSAIDs act exclusively by inhibiting prostaglandin synthesis (Abramson & Weissmann, 1989; Kopp & Ghosh, 1994; Frantz & O'Neill, 1995; Grilli *et al*., 1996).

With electrophoretic mobility-shift assays, transient transfection experiments, confocal immunofluorescence and nuclear import experiments, we found that pure enantiomers of ibuprofen are specific inhibitors of the activation of transcription factor NF-κB.

Methods

Cell culture

Human Jurkat T-lymphocytes (DSZM, Braunschweig, Germany) were cultured in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% foetal calf serum (FCS) and 1% glutamine in a humidified atmosphere (5% CO_2/95% air) at 37°C. Cells were preincubated at 37°C with nonsteroidal anti-inflammatory drugs added directly to the medium in various concentrations for 2 h. After exposure to test agents cells were stimulated with the phorbolester tetradecanoylphorbol-13-acetate (TPA, 50 ng ml^{-1}, Sigma, Germany) for 1 h or prostaglandin E_2 (PGE_2, 10^{-6} M, Sigma, Germany) for 1 h.

Electrophoretic mobility-shift experiments

Nuclear extracts were prepared from Jurkat T-cells as previously described (Baeuerle & Baltimore, 1988). Briefly, cells were harvested by centrifugation and resuspended in four cell volumes of buffer A (composition in mM: HEPES-buffer (pH 7.5) 25, NaCl 25, $MgCl_2$ 5, EDTA 1, $CaCl_2$ 2, NaF 10, PMSF 0.8, β-mercaptoethanol 0.1%, sucrose 5%, glycerol 30% and NP 40 0.16%). The cells were lyzed on ice in a Teflon homogenizer and centrifuged through a sucrose bed (4000 r.p.m., 15 min, 4°C). Nuclear fractions were resuspended in nuclear extraction buffer (composition in mM: HEPES-buffer (pH 7.5) 25, NaCl 500, $MgCl_2$ 5, EDTA 1, NaF 10, PMSF 0.8, β-mercaptoethanol 0.1%, glycerol 10% and NP 40 0.2%). Binding reactions were performed by using equal amounts of nuclear extracts (5–10 μg) in binding buffer (composition in mM: HEPES-buffer 100, EDTA 5, $(NH_4)_2SO_4$ 50, DTT 5, KCl 150 and Tween 20 1%) containing 1 gl^{-1} poly dI-dC, 0.1 gl^{-1} poly L-lysine and digoxygenine-labelled oligonucleotide for NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′, TIB MOLBIOL, Berlin, Germany) in a total volume of 20 μl for 15 min at room temperature.

The oligonucleotide used for detection of HSF DNA binding was 5′-Dig-GCCTCGAATGTTCGCGAAGT (TIB MOLBIOL, Berlin, Germany). Samples were analysed on a 6% nondenaturating polyacrylamide gel. After electrophoretical transfer to a positively charged nylon membrane (Boehringer Mannheim, Germany) complexes were visualized with a chemiluminescence detection system (Boehringer Mannheim, Germany). To ascertain the specificity of the binding reaction, competition assays were performed in the presence of excess unlabelled corresponding oligonucleotide.

Transient transfection of Jurkat T-cells

Jurkat cells were transiently transfected with DMRIE-C reagent (Gibco BRL) according to the manufacturer's instructions; 5×10^5 cells were transfected with 2 μg 6×NF-κBtk-Luc plasmid, containing NF-κB-driven luciferase gene (Bachelerie *et al*., 1991). The cells were cultured for 20 h after transfection and then stimulated with TPA (20 ng ml^{-1}) in the presence or absence of test drugs. After a further 20 h incubation, cells were lyzed for measurement of luciferase activity with reagents from Packard (LucLite) and normalized for protein recovery.

Immunofluorescence microscopy

Jurkat cells, grown on coverslips, were washed twice in PBS and then fixed in freshly prepared 4% paraformaldehyde in PBS for 10 min at room temperature. After being washed the cells were permeabilized with 50% acetone/methanol at −20°C for 10 min. The fixed and permeabilized cells were first blocked with 10% FCS in PBS for 30 min. Cells were then incubated overnight at 4°C with polyclonal anti-NF-κB-p65 antiserum (Santa Cruz Biotechnology, California). After being washed, FITC-conjugated goat anti-rabbit IgG in PBS was applied to the cells for 2 h. Finally, coverslips were washed, mounted in a solution of 10% (v/v) glycerol in PBS, sealed, and observed under a confocal laser scanning microscope (MRC-600, Bio-Rad Laboratories, Palo Alto) mounted on an Optiphot II Nikon microscope equipped with a 100× objective. An argon laser adjusted to 488 nm wavelength was used for the analysis of fluorescein, and a helium-neon ion laser adjusted to 543 nm was used for the analysis of rhodamine. The electronic train of the microscope was adjusted to obtain the maximum dynamic range (0–255) for each sample observed.

Assay for nuclear import

Synthetic p65 nuclear localization signal (NLS) peptide sequence was prepared by ABIMED (Langenfeld, Germany) corresponding to the amino acid sequence of p65 H-CDTDDRHRIEEKRKRT-OH as previously described (Zabel *et al*., 1993). The extra cysteine residue was added for coupling of peptide to bovine serum albumin carrier. The NLS-peptide was coupled to fluorescein-conjugated bovine serum albumin (BSA) (Sigma) by use of the heterobifunctional crosslinker sulphsuccinimidyl 4-[N-maleimidomethyl]-cyclohexane-1-carboxylate (sulpho-SMCC) (PIERCE, Netherlands) according to the manufacturer's procedure. As a control conjugate, TRITC-labelled BSA was used. The p65 NLS-peptide conjugate was analysed for crosslink efficiency and specificity by SDS gel electrophoresis.

The microinjection import assay was modified from previously published work (Adams & Pringle, 1991; Harootunian *et al*., 1993). Jurkat T-cells were plated on glass coverslips coated first with bovine serum albumin and then with poly-L-lysine (Guse *et al*., 1997). By use of Eppendorf Femtotips, the cells were microinjected with an Eppendorf 5170 micromanipulator and microinjector 5242 (Eppendorf Co., Germany) equipped with a Zeiss Axiovert 100 microscope (Zeiss, Germany). In each experiment more than 100 cells were microinjected within 20 min. Each experiment was repeated at

least five times. After microinjection of the conjugate p65 NLS-peptide-BSA, the cells were incubated for 30 min at 37°C in RPMI-1640 medium and then directly observed or fixed with 4% paraformaldehyde. For observations a epifluorescence microscope with a confocal lens was used.

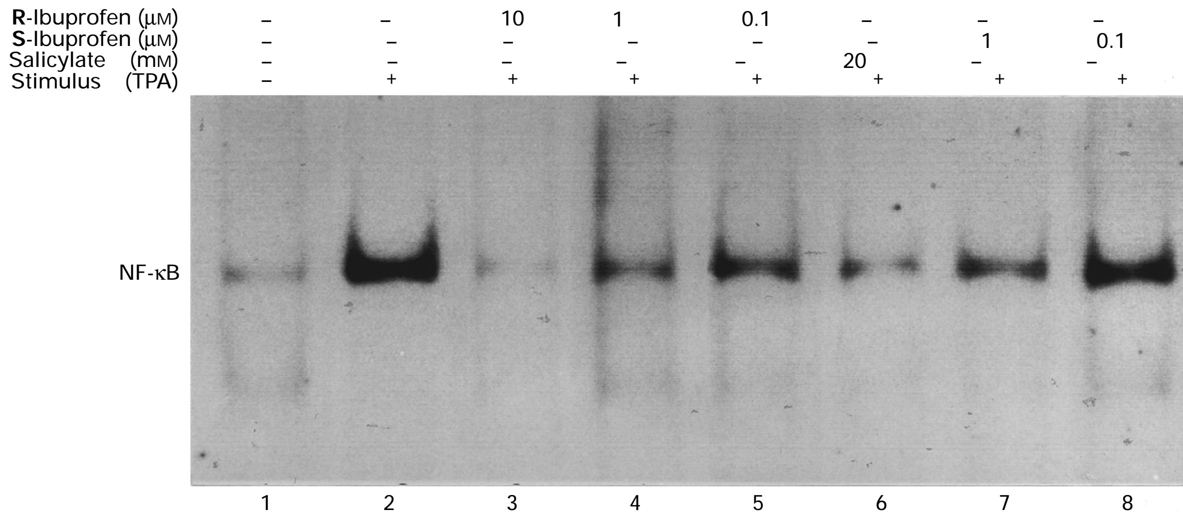
Drugs

S- and **R**-ibuprofen were used as the sodium-salt (Pharma Trans Sanaq AG, Basel, Switzerland).

Results

Effect of ibuprofen-enantiomers on NF-κB activation

Jurkat T-cells were incubated with various concentrations of ibuprofen-enantiomers for 2 h. Subsequently, cells were stimulated for 1 h with phorbolester (TPA), after which nuclear extracts were prepared. The effect of **R**(−)- and **S**(+)-ibuprofen on the stimulation of NF-κB binding to its DNA response elements was investigated with electrophoretic



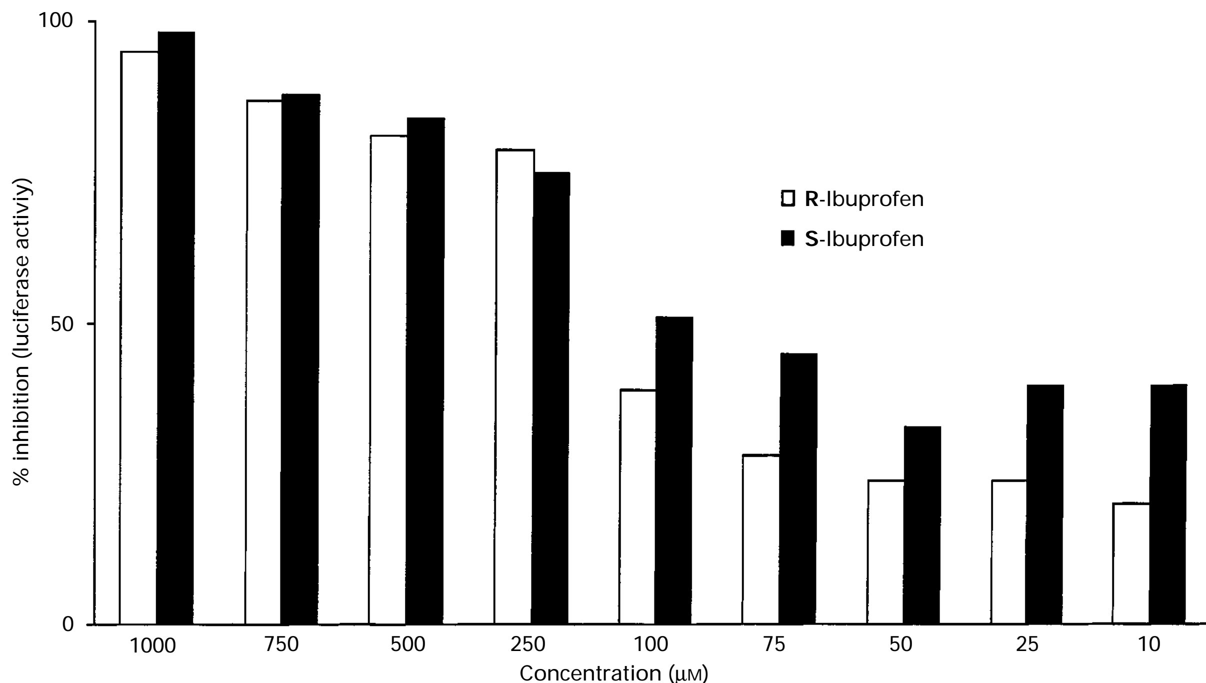


Figure 1 (a) Effect of antinociceptive concentrations of ibuprofen-enantiomers and salicylate on the binding of NF-κB to DNA. Nuclear extracts were prepared and equal amounts of protein were assayed for NF-κB. Jurkat T-cells were preincubated for 2 h with indicated concentrations of ibuprofen or salicylate followed by 1 h stimulation with 50 ng ml^{-1} phorbol ester (lanes 2–8). Lane 1 shows the unstimulated control cells. (b) Effect of **R**(−)- and **S**(+)-ibuprofen treatment on NF-κB-dependent gene expression as demonstrated by reporter assay. Jurkat T-cells were transiently transfected with an NF-κB-enhancer-driven luciferase reporter gene. Twenty hours after transfection, the cells were preincubated for 2 h with indicated concentrations of **R**(−)- and **S**(+)-ibuprofen and then stimulated for another 20 h with 20 ng ml^{-1} phorbol ester. Cells were lyzed and assayed for luciferase activity. Data shown are representative of three independent experiments.

mobility-shift assays (EMSA). As shown in Figure 1a (lane 1), nuclear extracts of resting Jurkat T-cells did not contain active NF-κB for binding to the DNA-κB probe. Stimulation with TPA induced a slow migrating complex (Figure 1a, lane 2). Competition and super shift assays identified this complex as an NF-κB p50/p65 heterodimer (data not shown). NF-κB activation was significantly reduced by treatment of Jurkat T-cells with 1 μM **R**(−)-ibuprofen, whereas 10 μM completely prevented NF-κB induction (Figure 1a, lanes 3, 4). A similar dose-response was observed for **S**(+)-ibuprofen (Figure 1a, lanes 7, 8).

A comparison of ibuprofen with salicylate in the same EMSA experiment demonstrated that the effects of salicylate on the induction of NF-κB and HSF binding activity in Jurkat T-cells were prevented only at the high concentration of 20 mM (Figure 1a, lane 6; Figure 2, lane 3).

Effect of ibuprofen-enantiomers on NF-κB mediated transactivation

In order to quantify the suppressive effect of ibuprofen on NF-κB stimulation and see whether it can inhibit transcription of an NF-κB-dependent gene, we examined the effects of **R**(−)- and **S**(+)-ibuprofen on NF-κB mediated gene transactivation. Plasmid constructs bearing a luciferase reporter gene under the control of the NF-κB enhancer were transiently transfected into Jurkat T-cells. Treatment of transfected Jurkat cells with TPA caused a 15–20 fold induction of NF-κB dependent promotor activity. The NF-κB-mediated transactivation was suppressed by **R**(−)-ibuprofen as well as the **S**(+)-enantiomer in a dose-dependent manner (Figure 1b). The IC_{50} of **R**(−)- and **S**(+)-ibuprofen for the specific inhibition of NF-κB-luciferase activity were calculated to be 121.8 μM and 61.7 μM respectively.

Specificity of ibuprofen effects

To investigate whether the inhibition of a transcription factor by ibuprofen is a general phenomenon or a specific effect on NF-κB, we therefore analysed the Jurkat cell extracts shown in Figure 1a in EMSAs using DNA probes containing consensus motifs for the inducible heat shock transcription factor HSF. Treatment with both enantiomers of ibuprofen up to 10 mM did not apparently affect DNA binding of HSF (Figure 2, lanes 4, 5).

Ibuprofen as a 2-arylpropionic acid has the potential to modify proteins by lowering pH. Since preincubation of Jurkat T-cells with 20 mM benzoic acid did not alter NF-κB activation and binding, any nonspecific effects of an organic acid with a comparable pK_a value could be excluded (Figure 2, lane 7). The specificity of ibuprofen was further assessed by measuring effects of ibuprofen on preformed activated NF-κB. The addition of 10 mM ibuprofen to nuclear extracts of TPA-stimulated Jurkat cells did not influence the DNA binding of NF-κB (Figure 2, lane 6).

NF-κB can be activated by many agents, such as inflammatory cytokines, mitogens, bacterial products, oxidative stress, ultraviolet light and phorbol ester (Baeuerle & Henkel, 1994). Recently, prostaglandin E_2 (PGE_2) receptors have been identified on Jurkat T-cells (Blaschke *et al*., 1996). To determine the influence of PGE_2 on NF-κB in Jurkat T-cells we performed EMSA experiments with PGE_2 stimulation. Like TPA, NF-κB could be induced with PGE_2 (Figure 2, lane 9). But preincubation of Jurkat T-cells with 10 mM **R**(−)-ibuprofen did not prevent PGE_2 stimulated NF-κB activation (Figure 2, lane 10), indicating that ibuprofen does not interfere with a common event of different inducers.

Cellular localization studies with confocal immunofluorescence

To explore further the mechanism of ibuprofen-induced inhibition of NF-κB activation we analysed the release of the cytosolic NF-κB from inactive complexes by immunofluorescence microscopy. Nuclear translocation of NF-κB is controlled by the association of Rel/NF-κB proteins with inhibitory cytosolic proteins, called IκBs (Beg & Baldwin, 1993). Phosphorylation of IκB leads to release of NF-κB from inactive complexes and consequently to translocation of active NF-κB into the nucleus (Ghosh & Baltimore, 1990; Beg *et al*., 1993; Miyamoto *et al*., 1994). By use of p65 antibodies, in

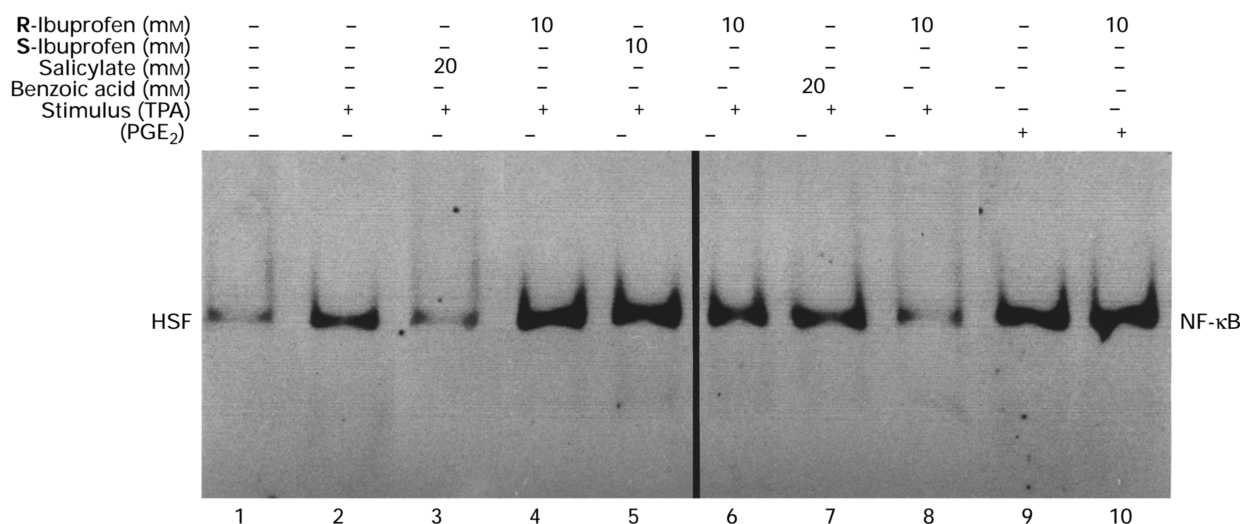


Figure 2 Effect of ibuprofen on different transcription factors and in response to distinct stimuli. The nuclear extracts from Jurkat T-cells used in Figure 1a were analysed for binding of the heat shock transcription factor (lanes 1–5). The effect of *in vitro* addition of **R**(−)-ibuprofen (lane 6) and a pK_a comparable organic acid on NF-κB DNA binding was analysed (lane 7). Lane 8 shows the effect of **R**(−)-ibuprofen on phorbol ester stimulated binding of NF-κB to DNA. **R**(−)-ibuprofen had no effect on prostglandin E_2 stimulated NF-κB DNA binding (lanes 9–10).

resting cells NF-κB was found in the cytoplasm (Figure 3a). In TPA treated cells NF-κB was colocated in the cytoplasm and the nucleus (Figure 3b). Concentrations of 10 μM **R**(−)-ibuprofen, comparable to inhibitory doses for NF-κB in EMSA, completely prevented the translocation of NF-κB after TPA stimulation (Figure 3c).

Nuclear import experiments

Protein migration into nuclei involves at least two steps: nuclear pore binding and an energy dependent translocation (Rexach & Blobel, 1995; Pante & Aebi, 1996). To exclude the possibility that ibuprofen prevents nuclear uptake of NF-κB by interacting with either the nuclear location signal (NLS) of p65 NF-κB or nonspecific energy depletion of cells, we performed nuclear import experiments with Jurkat T-cells. p65 NLS-peptide linked to BSA-FITC import were obtained by fluorescence microscopic monitoring after microinjection in the cytoplasm. Figure 4b shows that the p65 NF-κB NLS-peptide was transported efficiently into T-cell nuclei after 20 min, whereas the control conjugate BSA-TRITC (missing a NLS sequence) was excluded from the nuclei (Figure 4a). The observed import kinetics are comparable to published results in which a green-fluorescent-NLS construct was used (Pante & Aebi, 1996; Shulga *et al*., 1996). When we preincubated T-cells with 100 μM **R**(−)-ibuprofen the accumulation in the nuclei of the NLS peptide-FITC conjugate after 20 min was not affected (Figure 4c). These results suggest that **R**(−)-ibuprofen does not act as an inhibitor of protein transport into nuclei nor does it reduce cell viability.

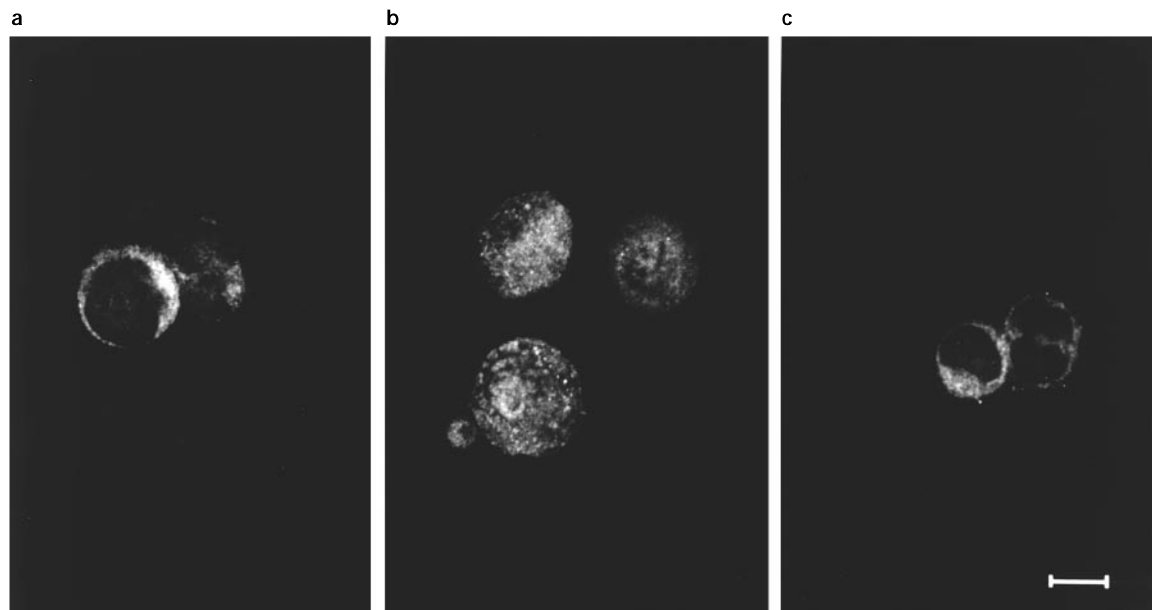


Figure 3 Ibuprofen modulated translocation of NF-κB in Jurkat T-cells. Jurkat T-cells were treated with 10 μM **R**(−)-ibuprofen for 2 h and then stimulated with phorbol ester. Shown are indirect immunofluorescence micrographs of distribution of NF-κB in either quiescent (a), in stimulated (b) or stimulated and **R**(−)-ibuprofen treated cells (c). Note the predominant cytoplasmic staining of ibuprofen treated cells (c). The intracellular localization of NF-κB was determined by an anti-p65 antibody. Bar = 10 μM

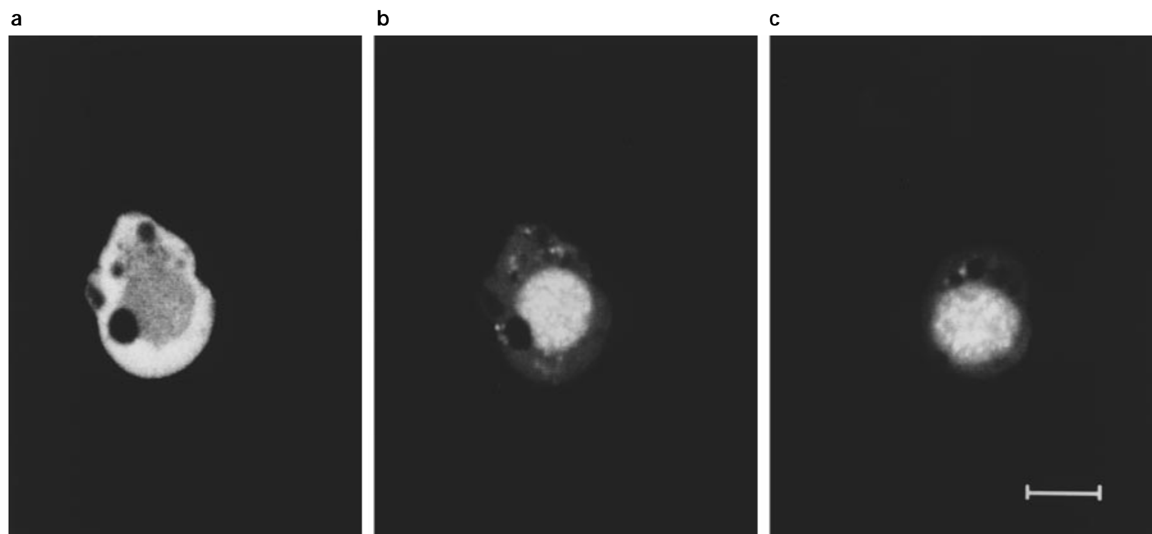


Figure 4 Ibuprofen did not influence the nuclear accumulation of the synthetic NF-κB nuclear localization sequence. Jurkat T-cells were microinjected into the cytoplasm with a NLS-NF-κB peptide conjugated to FITC-BSA. The synthetic NF-κB peptide was able to direct BSA from the cytoplasm to the nucleus (b). The nuclear distribution of the NF-κB peptide conjugate was not affected by pretreatment with 100 μM **R**(−)-ibuprofen (c). Note the absence of nuclear accumulation of the TRITC-BSA control conjugate (a). Bar = 10 μM

Discussion

Annually about 100 million prescriptions of NSAIDs are filled across the United States to treat arthritis, related musculoskeletal problems, headaches and fever (Brooks & Day, 1991). The 2-arylpropionic acids ibuprofen, ketoprofen and flurbiprofen are the most frequently prescribed class of NSAIDs. They are administered as racemic mixtures, although only the **S**-enantiomer inhibits the cyclo-oxygenases (Geisslinger *et al.*, 1994). In contrast, experimental and clinical data employing racemic and pure **S**-ibuprofen indicate that the anti-inflammatory and analgesic effects cannot be attributed solely to **S**-ibuprofen (Brune *et al.*, 1991; Geisslinger *et al.*, 1994). Here, we demonstrated that **R**(−)-ibuprofen inhibits the activation of transcription factor NF-κB, as evidenced by analysis of DNA-binding of various transcription factors after different stimulations, transient transfection experiments and characterization of NF-κB transport processes. To our knowledge, this is the first time that a specific interaction with a cellular event is detected for **R**(−)-ibuprofen.

Previously, salicylate and aspirin are the only NSAIDs characterized for their ability to modify transcription factor activity (Kopp & Ghosh, 1994). However, the mechanism of action by which the different NSAIDs (salicylate, aspirin and ibuprofen) exert their effects seems to be distinctive. Activation of NF-κB DNA binding induced by many agents is inhibited only at high doses of salicylate (20 mM), which appears to result from the ability of salicylate to nonspecifically inhibit cellular kinases or acidify the cytoplasm (Feuillard *et al.*, 1991; Frantz & O'Neill, 1995). In contrast, **R**(−)-ibuprofen inhibition was specific for phorbol ester and effective at a 100 to 1000 fold lower concentration. Quantification by reporter-gene assays revealed that **R**(−)- ibuprofen (IC_{50}: 121.8 μM) was as effective as **S**(+)-ibuprofen (IC_{50}: 61.7 μM), despite its lack of COX inhibition. Since the concentrations of our test compounds are in the μM range, our results may also be of clinical relevance. Further evidence for the specificity of the ibuprofen action in contrast to aspirin is supported by the lack of inhibition of TPA-induced HSF binding to DNA in the same extracts.

The mechanism of action by which the **S**(+)- enantiomer exerts its effect on NF-κB activation could be a matter of debate. It has been shown that phorbol ester induces prostaglandin synthesis in several cell types (Phipps *et al.*, 1991). Furthermore, we have shown that PGE_2 activates NF-κB. Theoretically, inhibition of NF-κB activation by **S**(+)-ibuprofen could result from inhibition of prostaglandin synthesis via cyclo-oxygenase modification. Two lines of evidence excluded this explanation. Western blot analysis and RT-PCR revealed that stimulated T-cells showed no cyclo-oxygenase expression and did not produce any PGE_2 (Pahl, unpublished results). How **R**(−)-ibuprofen inhibits the activation of NF-κB is not clear. It does not chemically modify NF-κB, unlike herbimycin A and protease inhibitors which can modulate the DNA binding sequence present in NF-κB proteins (Kumar *et al.*, 1992; Finco *et al.*, 1994; Mahon & O'Neill, 1995). In principle, inhibition of NF-κB DNA binding activity may be due to a block in nuclear translocation of NF-κB complexes or a suppression of NF-κB binding to its consensus DNA elements. With confocal immunofluorescence experiments we showed that ibuprofen inhibited the nuclear translocation of NF-κB. Nuclear translocation of NF-κB is accompained by rapid degradation of IκBα. It has been shown that activation of Jurkat T-cells leads to rapid decay of IκBα and the nuclear appearance of p65 (Beg *et al.*, 1993). IκB proteins are usually located in the cytoplasm. They are characterized by so called ankyrin repeats. Ankyrin repeats are structural motifs of ∼ 33 amino acids which are thought to mediate protein complex formation. Through these motifs IκBα seems to retain NF-κB in the cytosol by masking the nuclear location signal (Beg & Baldwin, 1993). In a recent study (Miyamoto *et al.*, 1994) it was convincingly demonstrated that even small changes in cytoplasmic IκB concentration result in profound changes in nuclear NF-κB activity. Although the actual mechanism of ibuprofen-induced inhibition of NF-κB remains unclear, our results suggest that ibuprofen may inhibit activation of NF-κB by interfering with a pathway that leads to changes in cytoplasmic IκB concentration. Additionally, the results obtained with microinjection experiments imply that ibuprofen did not interact with either the positively charged nuclear localization sequence of NF-κB or the energy-dependent nuclear import processes.

Immunofluorescence and microinjection experiments indicate that the ibuprofen influenced process must be located upstream of the dissociation of the NF-κB-IκB complex. Although the molecular events leading to an active nuclear NF-κB are poorly understood, it is very likely that one of the essential steps involves protein phosphorylation (Baeuerle & Baltimore, 1988; Ghosh & Baltimore, 1990). The major pathways used by a variety of stimuli to activate NF-κB involves the phosphorylation of IκBα at its regulatory N-terminus on serines 32 and 36, an event leading to subsequent conjugation with ubiquitin and proteasome mediated degradation of the inhibitor (Verma *et al.*, 1995). It has been shown that activation of protein kinase C (PKC) via phorbol ester induces phosphorylation and inactivation of IκB *in vitro* suggesting PKC is a regulator of NF-κB activation (Ghosh & Baltimore, 1990; Verma *et al.*, 1995). In contrast to phorbol ester mediated NF-κB stimulation, ibuprofen had no effect on PGE_2 mediated NF-κB activation. Since PGE_2 is an activator of the protein kinase A (PKA) signalling pathway, it is interesting to speculate that ibuprofen may specifically interfere with PKC mediated NF-κB activation (Phipps *et al.*, 1991). However, the involvement of PKA, a cyclic AMP-dependent kinase, in NF-κB activation remains unclear and is controversial (Shirakawa & Mizel, 1989; Bomsztyk *et al.*, 1990; Ghosh & Baltimore, 1990; Meichle *et al.*, 1990; Feuillard *et al.*, 1991). To our knowledge ibuprofen is the first inhibitor of NF-κB activation which is specific for some activation pathways. Identification of the target of ibuprofen may lead to the identification of novel signalling pathways essential in inflammatory processes. A precedent for such research was the case of the immunosuppressive drug cyclosporin A, which facilitated the elucidation of the signalling cascade downstream from the T-cell receptor (Crabtree & Clipstone, 1994).

Taken together, analysis of the effects of **R**(−)-ibuprofen, a so far neglected enantiomer of an important arylpropionic acid, has led to new insights about the molecular mechanisms of this drug. Weak inhibitors of cyclo-oxygenases may exert their effects by modulation of transcription factors. The finding of a second mode of action of ibuprofen may lead to new formulations of ibuprofen either in racemic mixtures or in the single enantiomer form designed for specific indications.

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