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# Ibuprofen: New Explanation for an Old Phenomenon

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**ABSTRACT.** Nuclear factor-κB (NF-κB) translocation from the cytoplasm into the nucleus and the subsequent DNA binding is an essential prerequisite in the up-regulation of many pro-inflammatory genes, e.g. tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). The anti-inflammatory drug ibuprofen, thought to exert its beneficial effects mainly by suppressing the production of eicosanoids, inhibited the up-regulation of the pro-inflammatory cytokines IL-1β and TNF-α. This effect was independent of the described potential of ibuprofen as a cyclooxygenase inhibitor. Ibuprofen inhibited the activation and translocation of the key transcription factor NF-κB by blocking the degradation of inhibitor-κBα, a protein that forms a complex with NF-κB, thereby preventing the release and subsequent translocation of NF-κB into the nucleus and the expression of inflammatory cytokines. The presented data offer a new explanation for the anti-inflammatory effect of ibuprofen. BIOCHEM PHARMACOL **57**;3:313–320, 1999. © 1998 Elsevier Science Inc.

**KEY WORDS.** ibuprofen; nuclear factor-κB; inhibitor-κB

Until recently, there has been no firm evidence that suggested that NSAID§ act through any other mechanism than their ability to block cyclooxygenase, lipoxygenase, or both, therefore reducing the levels of pro-inflammatory AA metabolites [1]. However, there is mounting evidence that lowering levels of eicosanoids is not the sole mechanism by which NSAID exert their beneficial effects [2, 3].

Out of several NSAID, ibuprofen is one of the most widely used, with antipyretic, analgesic, and antibacterial activity [4]. This drug has a wide therapeutic window and becomes toxic only at very high levels, e.g. ibuprofen, given to patients with cystic fibrosis and mild lung disease for 4 consecutive years at plasma concentrations of up to 100 μg/mL, had no serious adverse effects while significantly slowing progression of the lung disease [5]. Interestingly, ibuprofen appears not only to act through inhibition of cyclooxygenase but also to involve other mechanisms independent of this well-known effect, as doses higher than those necessary to block eicosanoid syntheses have to be given to make use of the full anti-inflammatory potential of this drug.

The transcription factor NF-κB is central to pro-inflammatory processes, leading to the up-regulation of many gene products usually associated with inflammation, e.g. cytokines and adhesion molecules [6, 7]. In resting cells, NF-κB is located in the cytoplasm of most cells in an inactive form where it is bound to a protein termed IκBα. After stimulation of mononuclear cells by cytokines such as TNF-α, IL-1, or other pro-inflammatory substances, e.g. LPS, IκBα is phosphorylated and proteolytically degraded within minutes; this results in the release and translocation of NF-κB into the nucleus. NF-κB induces and interacts with other transcription factors to up-regulate genes involved in immune and inflammatory responses through binding to a specific sequence termed κB binding element in the enhancer region of gene promoters [6, 8]. We were interested in investigating whether ibuprofen influences very early pro-inflammatory events by interfering with the critically important transcription factor NF-κB.

## MATERIALS AND METHODS

### *Preparation of Cells*

For cytoplasm and nuclear extracts, either THP-1, U937, or freshly isolated human mononuclear cells were prepared according to published methods [9] and cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Gibco BRL), as well as 50 U/mL of penicillin/streptomycin. The cells were cultured and stimulated in 96-well plates (Nunc) purchased from Fisher Scientific. Following treatment of cells with the indicated stimuli for indicated periods of time, the supernatants were collected and centrifuged for 10 min at 3000 *g* and stored at −80° or used immediately for cytokine measurement by ELISA. For EMSA experiments, cells were preincubated

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§*Abbreviations*: AA, arachidonic acid; CRE, cyclic AMP-responsive element EMSA, electrophoretic mobility shift assay; IκBα, inhibitor-κB; IL, interleukin; LPS, lipopolysaccharide; MIP-1α, macrophage inflammatory protein-1α; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; NSAID, nonsteroidal anti-inflammatory drugs; TLCK, *N*α-*p*-tosyl-L-lysine chloromethyl ketone; TNF-α, tumor necrosis factor-α; and TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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with the indicated amounts of ibuprofen for 45 min followed by stimulation with TNF-α (5 ng/mL) for 90 min.

### *ELISA for Detection of IL-1β and TNF-α*

Human mononuclear cells were preincubated with increasing concentrations of ibuprofen for 45 min. Concentrations ranging from 0 to 3 mM were used in our experiments (ELISA, EMSA). LPS (100 ng/mL) or TNF-α (5 ng/mL) was used to stimulate the discharge of cytokines. Cells were stimulated for 5 hr before the supernatant was harvested by centrifugation. The ELISAs for the cytokine measurement in cell supernatants were performed according to the manufacturer's instructions. The ELISA kits were purchased from R&D Systems.

### *Stimulants and Reagents*

The following reagents were purchased from the Sigma Chemical Co.: ibuprofen, leupeptin, antipain, aprotinin, benzamidine, chymostatin, pepstatin, TLCK, TPCK, phenylmethylsulfonyl fluoride, and LPS serotype 055:B5; TNF-α was purchased from R&D. The transcription factor consensus oligonucleotides for NF-κB, TFIID, and CRE, as well as the polynucleotide kinase for labeling of 5′-OH blunt-ended probes, were purchased from the Promega Corp. The anti-IκBα antibody C-21 was from Santa Cruz Biotechnology. The anti-p65 and anti-p50 antibodies, used in supershift experiments, were purchased from Santa Cruz Biotechnology and from Boehringer Mannheim.

### *Viability Assay*

Viability of cells exposed to ibuprofen was evaluated using an MTT assay. The MTT assay was carried out as described elsewhere [10]. In brief, the assay was carried out in 96-well plates where RPMI-1640 had been replaced with phenol-red free medium at 80 μL/well. Two hours before termination of the assay, 20 μL of MTT stock solution (5 mg/mL in PBS) was added. At the end of the incubation period, the cells were washed twice with PBS, and the formazan was solubilized by adding ethanol (50 μL/well). The optical density of each well was measured using an automatic plate reader with a 560 nm test wavelength and a 690 nm reference wavelength.

### *EMSA*

Nuclear extracts were prepared as described [11]. The double-stranded oligonucleotides used in all experiments were end-labeled using T4 polynucleotide kinase and [γ-$^{32}$P]dATP. After labeling, 5 μg of nuclear extract was incubated with 100,000 cpm of labeled probe in the presence of 2 μg poly(dI-dC) at room temperature for 30 min followed by separation of this mixture on a 6% polyacrylamide gel in Tris/glycine/EDTA buffer at pH 8.5. Control experiments for the specificity of EMSA were performed as shown in Fig. 4, e.g. for specific competition, 7 pmol of unlabeled consensus NF-κB oligonucleotides was included; and for nonspecific competition, 7 pmol of double-stranded mutant κB oligonucleotides (5′-AGC TTA GAT TTT ACT TTC CGA GAG GA-3′) and 7 pmol of CRE (5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′) were used. For supershift assays, 1 μL of the monoclonal anti-NF-κB p56 (A)X (sc-109X), anti-NF-κB p50 (sc-1190) (Santa Cruz Biotechnology), or irrelevant antibodies (P-selectin, ELAM) was added to the nuclear extract simultaneously with the labeled probe.

### *Western Blot Analysis*

Cytosolic extracts were prepared as described [12] except that further protease inhibitors (TPCK, TLCK, leupeptin, antipain, aprotinin, benzamidine, chymostatin, and pepstatin) were added. Equal amounts of protein were separated by SDS/PAGE (12%) transferred to an Immobilon P PVDF membrane using a semi-dry transfer cell (Bio-Rad Laboratories), and probed with the rabbit polyclonal antibody C21 (Santa Cruz) directed against IκBα as described [11]. Bands were visualized using horseradish peroxidase conjugated donkey anti-rabbit IgG and the Enhanced ChemiLuminescence assay (Amersham Life Science Inc.), according to the manufacturer's instructions.

### *Statistics*

Cytokine measurements by ELISA were done in quadruplicate. All assays were done at least two times, and representative experiments are shown.

## RESULTS

To account for the anti-inflammatory effects seen with ibuprofen, when used at higher doses, we first investigated the effect of ibuprofen on the release of the pro-inflammatory cytokines TNF-α and IL-1β from human peripheral mononuclear cells, as well as the pre-mononuclear cell lines U937 and THP-1. These studies were followed by further investigation of the effect of ibuprofen on TNF-α- and/or LPS-induced activation of the key transcription factor NF-κB.

Treatment of mononuclear cells with either TNF-α or LPS leads to the up-regulation of biosyntheses and the release of the pro-inflammatory cytokines IL-1β and TNF-α [13–16]. We isolated peripheral mononuclear cells and pretreated these cells ($10^7$ cells/well) with increasing amounts of ibuprofen for 45 min. Afterwards, cells were left untreated or stimulated with LPS (100 ng/mL) or TNF-α (5 ng/mL) for an additional 5 hr. Following the harvesting of the supernatant, the levels of cytokines released into the culture medium by mononuclear cells were measured by ELISA. Pretreatment of these cells with ibuprofen for 45 min resulted in the suppression of LPS- and of TNF-α-induced cytokines (TNF-α and IL-1β were measured)

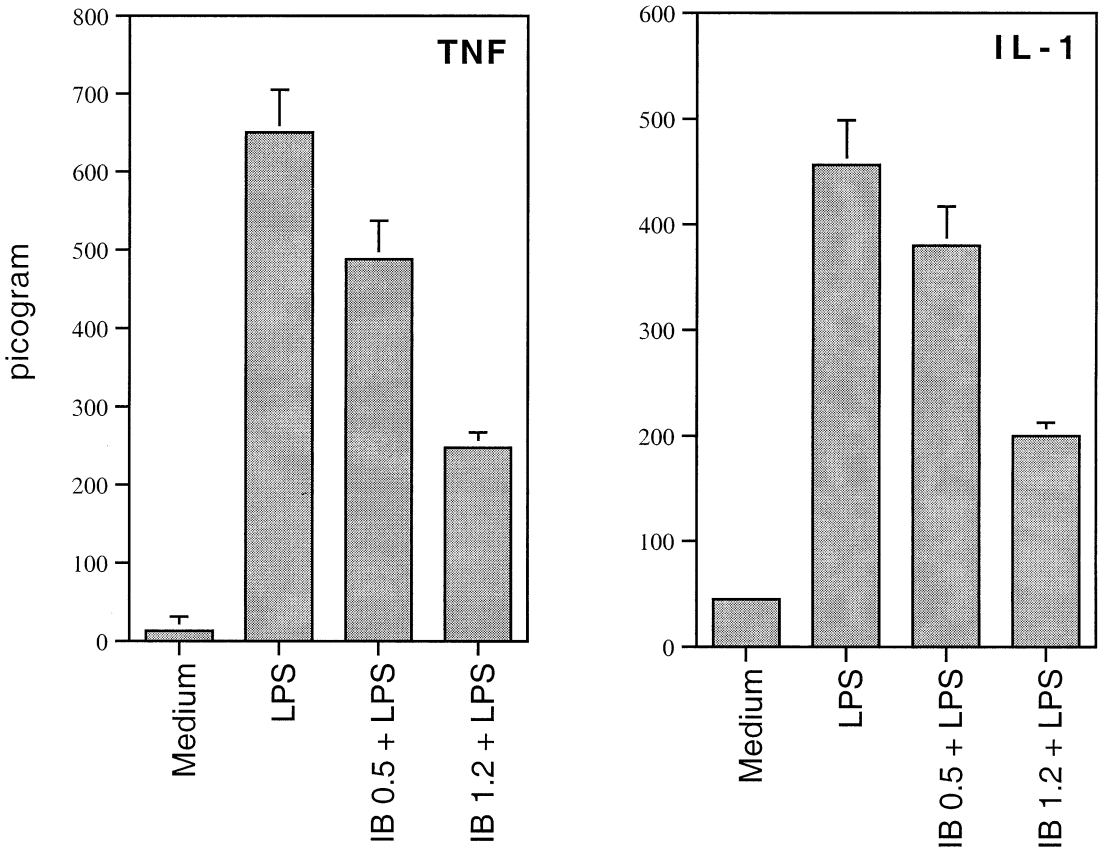


**FIG. 1. Ibuprofen inhibition of the release of IL-1β and TNF-α in human mononuclear cells. Cells were left untreated or were pretreated with the indicated amounts of ibuprofen (IB 0.5, IB 1.2) for 45 min. Subsequently, where indicated, LPS (100 ng/mL) was added, and the cells were incubated for an additional 5 hr. Levels of cytokines (TNF, IL-1) were measured in the supernatant and compared with levels in the medium (lane Medium) and in cells treated with LPS only (lane LPS). Shown are the means ± SD of one representative experiment performed in quadruplicate.**

released into the culture medium. The LPS-stimulated TNF-α release was suppressed by 62% (±8%) when 1.2 mM ibuprofen was used to treat mononuclear cells prior to stimulation with LPS, and, similarly, the level of IL-1β released by mononuclear cells after such treatment was decreased by 44% (±6%). Higher amounts of ibuprofen (3.0 mM) suppressed the LPS-induced release of TNF-α, as well as the TNF-α-induced IL-1β release by mononuclear cells, to amounts detected in untreated cells. Figure 1 shows the results of one representative experiment in which cells were left untreated or were pretreated (45 min) with 0.5 or 1.2 mM ibuprofen, followed by stimulation with LPS (100 ng/mL) for an additional 5 hr. Levels of cytokines (TNF-α, IL-1β) were measured in the supernatant and compared with levels in the medium (lane Medium) and with cells treated with LPS only (lane LPS). Plotted on the ordinate are the cytokine levels in picograms. At the end of the 5-hr incubation period, cells were checked routinely for viability; such tests demonstrated that the viability of these cells was not altered significantly by this treatment (data not shown).

Considering the central role of NF-κB in pro-inflammatory events, and because LPS and TNF-α are among the most potent inducers of NF-κB activity [8], we examined the effect of ibuprofen treatment on the translocation of NF-κB. In the first set of experiments (Fig. 2), U937 or THP-1 cells were incubated with increasing concentrations of ibuprofen for 45 min followed by TNF-α treatment for 90 min, after which the nuclear extracts were isolated. EMSAs, in which a synthetic DNA fragment representing the consensus NF-κB element was used to test NF-κB translocation and DNA binding, revealed that ibuprofen putatively could also inhibit gene regulation at the transcriptional level, as increasing levels of this substance led to decreased NF-κB-DNA interactions. Furthermore, we demonstrated (Fig. 3) that freshly isolated human mononuclear cells behave like the pre-mononuclear U937/THP-1 cells, as similar treatment of these cells with ibuprofen (3 mM) completely blocked the TNF-α-induced NF-κB translocation. In addition, we examined the effect of ibuprofen treatment on LPS-induced NF-κB activation in mononuclear cells. The EMSA data (not shown) indicated that ibuprofen treatment of mononuclear cells also inhibited the LPS-induced binding of NF-κB to its consensus NF-κB element in a concentration-dependent manner, similar to the ibuprofen effect on TNF-α-induced NF-κB binding (Fig. 2). To investigate whether the suppression of transcription factors by ibuprofen is a general phenomenon or

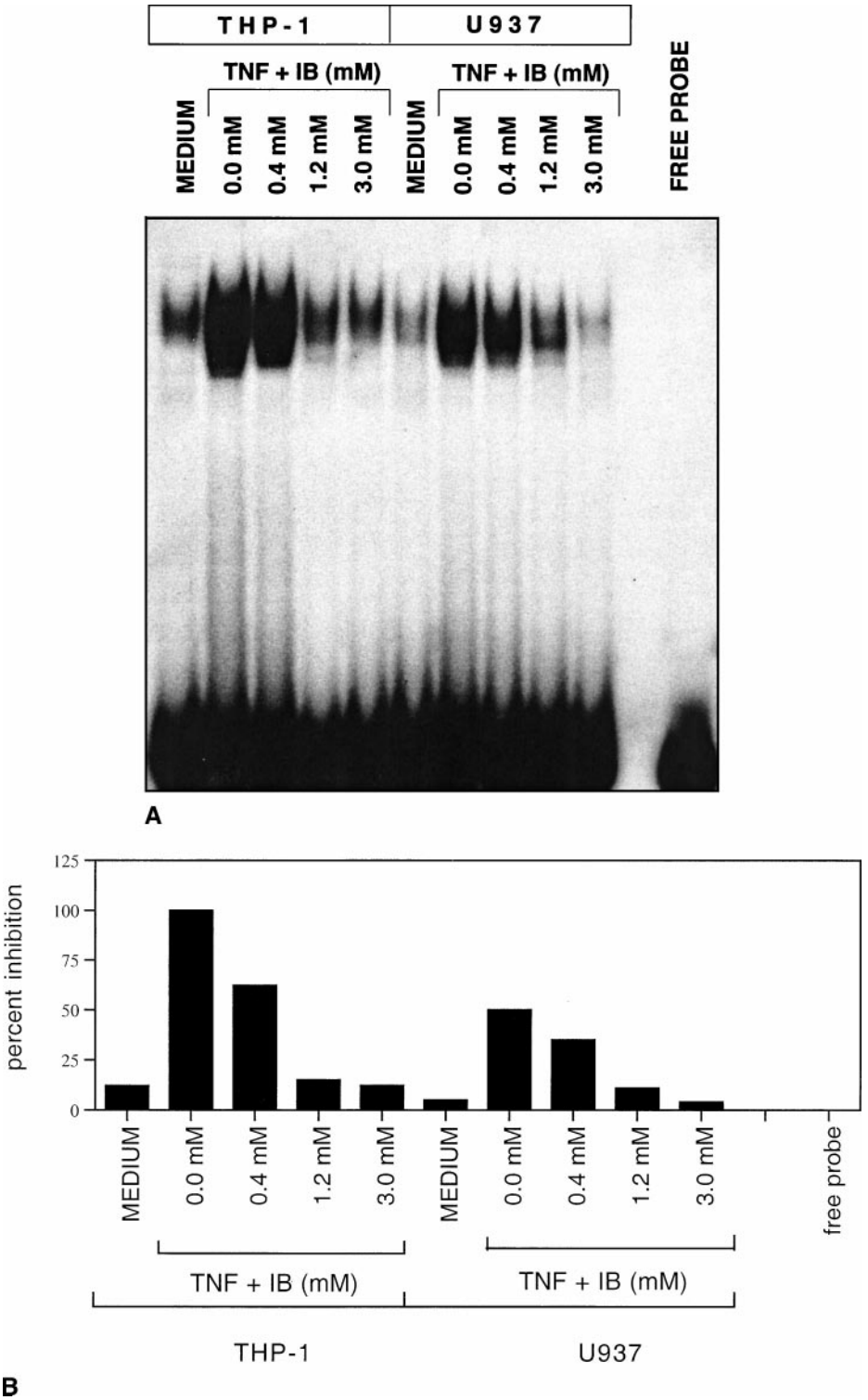


**FIG. 2. Top panel: Effect of ibuprofen on TNF-α-induced DNA binding activity of NF-κB in a concentration-dependent manner. Pre-monocyte cells (U937 and THP-1) were left untreated (lane MEDIUM) or were pretreated with the indicated amounts of ibuprofen for 45 min prior to stimulation with TNF-α (5 ng/mL) for an additional 90 min. Nuclear extracts were isolated, and an EMSA was performed as described. The extracts (5 μg/lane) were incubated for 30 min with the consensus NF-κB element prior to electrophoresis on a 6% native gel. This experiment is representative of three experiments. Bottom panel: Quantification of the EMSA experiment (top panel) by densitometry.**

specific for certain factors such as NF-κB, control experiments were performed. Using a consensus CRE instead of the κB element, under otherwise identical conditions, we found that ibuprofen treatment had no influence on the protein-CRE binding pattern. Figure 4 shows such a control experiment in which an unlabeled NF-κB, CRE, and a

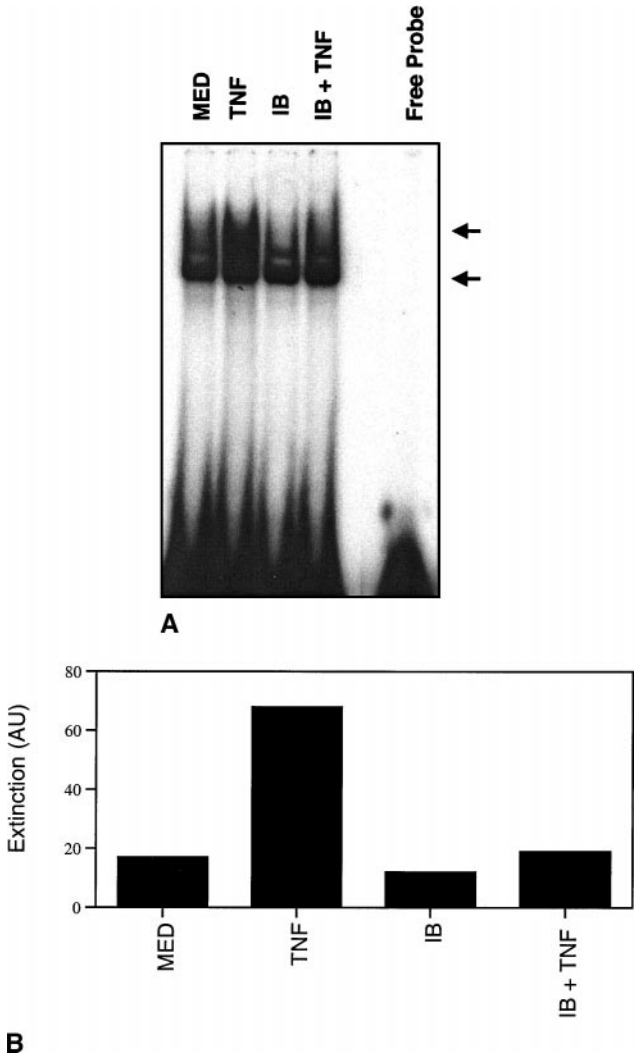


**FIG. 3. Top panel: Ibuprofen inhibition of TNF-α-induced DNA binding in human mononuclear cells. Freshly isolated peripheral mononuclear cells were treated as described in Materials and Methods. The results of one of several experiments are shown; cells were treated with 3 mM ibuprofen for 135 min (IB), pretreated with 3 mM ibuprofen for 45 min prior to stimulation with TNF-α (5 ng/mL) for 90 min (IB+TNF), left untreated (MED), or stimulated with TNF-α (5 ng/mL) for 90 min (TNF). Subsequently, nuclear extracts were isolated and an EMSA was performed. The upper arrow on the right side of the figure indicates the position of NF-κB; the lower one shows the non-specific binding to the κB element. Bottom panel: Quantification of the top panel by densitometry.**

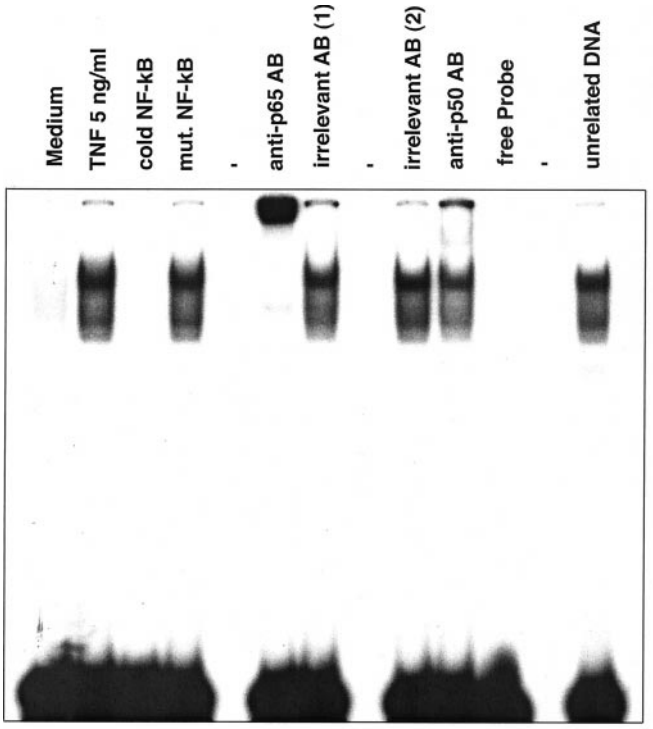


**FIG. 4. Control experiments demonstrating the specificity of the EMSA for NF-κB. Nuclear extract from unstimulated cells was loaded in the first lane. The second lane demonstrates the accumulation induced by TNF-α (5 ng/mL for 45 min) of NF-κB in the nucleus. This nuclear extract was also used for further EMSA control experiments. The specificity of binding to the κB element is demonstrated by competition for κB binding by an excess of unlabeled NF-κB (cold NF-κB) and the failure to compete by two non-κB oligonucleotides [mut. NF-κB, unrelated DNA (CRE)]. The specificity of the EMSA for NF-κB is demonstrated further by the presence of p65 (anti-p65 AB) and, to a lesser degree, of p50 (anti-p50 AB) in the complex bound to the κB element, as shown by the supershift experiments. Further controls (irrelevant AB 1 and 2) demonstrate the failure of unrelated antibodies to supershift the DNA–protein complex.**

mutated κB element as well as a supershift experiment with specific (anti-p65, anti-p50) antibodies and unspecific antibodies demonstrated the specificity of the EMSA.

Although several mechanisms might account for these findings, since a prerequisite for NF-κB-DNA interactions is the phosphorylation and degradation of IκB and the subsequent translocation of NF-κB into the nucleus [17], we investigated the effect of ibuprofen on TNF-α-induced degradation of IκBα. U937 and THP-1 cells were pretreated with increasing amounts of ibuprofen (0, 0.5, 1.5, and 3.5 mM) followed by stimulation with TNF-α (5 ng/mL) for 10 additional min. The TNF-α-induced degradation of IκBα was monitored by western blot analysis (Fig. 5). Identical results were obtained when THP-1 cells were used (data not shown). The levels of IκBα in the cytoplasmic extracts of untreated cells were compared with those of cells treated with TNF-α only, or with those of cells treated with ibuprofen prior to stimulation with TNF-α. As shown in Fig. 5, treatment with TNF-α only led to the expected degradation of IκBα, but treatment of monocyte-like cells with increasing amounts of ibuprofen prior to stimulation with TNF-α led to concentration-dependent stabilization of IκBα. The detection of IκBα in the cytoplasm of cells treated with 3.5 mM ibuprofen plus TNF-α is also a further indicator of the unchanged physiological integrity of these cells. Our western blot experiments demonstrated that ibuprofen can inhibit the translocation of NF-κB into the nucleus by stabilizing the NF-κB/IκB complex in the cytoplasm.

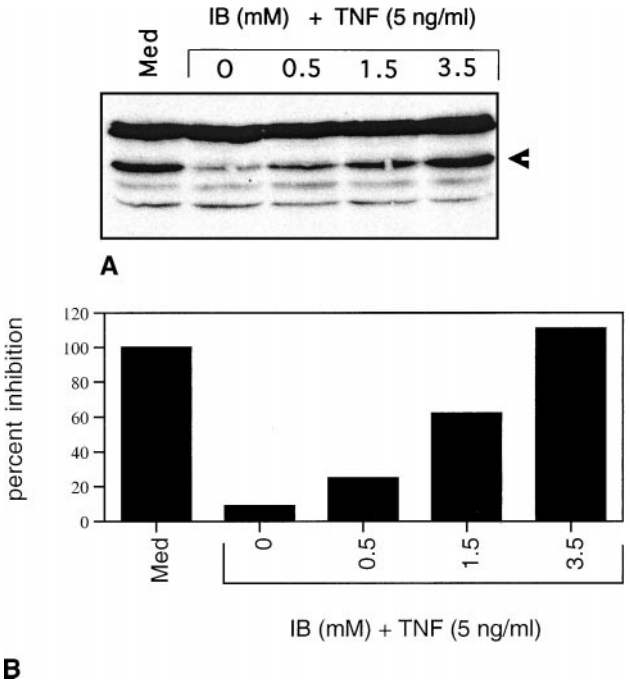


**FIG. 5. Top panel: Western blot demonstrating that treatment with ibuprofen leads to a concentration-dependent retention of NF-κB in the cytoplasm as ibuprofen treatment results in stabilization of IκBα. U937 cells were treated with increasing amounts of ibuprofen for 45 min prior to stimulation with TNF-α (5 ng/mL). After 10 min of TNF-α treatment, cytoplasmic proteins were extracted; subsequently, equal amounts of protein extract were separated by PAGE and transferred to PVDF membranes, followed by staining with an antibody recognizing IκBα. The arrow on the right site indicates the position of IκBα. Bottom panel: Quantification of the western blot (top panel) by densitometry.**

## DISCUSSION

It was in 1971 that Vane showed that NSAID block the biosynthesis of prostaglandins and proposed this as their mechanism of action [18]. Much work has been done since to confirm and extend this finding and to provide a better understanding of the underlying mechanism of NSAID [19–21]. Significant advances have been made recently in understanding the mechanisms of NSAID; nevertheless, recent studies indicated that there might be other, additional mechanisms involved in the anti-inflammatory effects of NSAID. Foremost in these studies are experiments demonstrating that salicylic acid can inhibit the translocation of NF-κB, a critically important transcription factor involved in up-regulation of most (if not all) pro-inflammatory genes [2, 3, 22], and the findings that non-acetylated salicylates, which do not interfere with prostaglandin synthesis, are effective anti-inflammatory drugs [23, 24]. Also of importance in the context of these studies are the findings that tepoxalin, a dual inhibitor of cyclooxygenase and 5-lipoxygenase, suppresses the activation of NF-κB and the expression of NF-κB-dependent genes [25].

Our data support the concept that the beneficial effects of ibuprofen cannot be attributed solely to the inhibition of eicosanoids and provide new insight into the mechanism by which ibuprofen exerts its anti-inflammatory effects. We demonstrated that ibuprofen can inhibit the translocation of NF-κB into the nucleus by stabilizing the NF-κB/IκBα complex in the cytoplasm. It is tempting to hypothesize on possible common mechanisms by which NSAID exert additional anti-inflammatory effects beyond the known effects on eicosanoid formation. We showed earlier that, contrary to most of its NF-κB activating metabolites, free AA as well as a stable AA analog (eicosatetraynoic acid) can block the activation of NF-κB in endothelial cells [11, 26]. All NSAID block to a certain degree AA metabolism, but, with the exception of a few, they do not interfere with the release of AA by phospholipase $A_2$ and other enzymes. Inflammation is associated with the release of free AA. This, combined with our earlier findings, led to our hypothesis that free AA might be involved in turning off activating events in a negative feedback-loop; however, various questions remain unresolved. Our provocative assumption that NSAID may act through temporarily elevated levels of free AA is the goal of current investigations.

The presented data might also help to better understand and explain the findings of others, such as the reported suppression of the expression of several adhesion molecules by ibuprofen [27] as most adhesion molecules seem to depend on the activation of NF-κB [8, 28, 29]. It should be noted that similar results have been reported for another widely used NSAID, aspirin, which for a long time was thought to act mainly through inhibition of cyclooxygenase and has now been found to exert anti-inflammatory effects by interfering with the translocation of the critically important transcription factor NF-κB [2, 3]. In terms of its potential as an inhibitor of NF-κB, ibuprofen is more potent than aspirin or sodium salicylate, as 20 mM sodium salicylate had to be used to get inhibition comparable to that which we got using 3 mM ibuprofen. Therapeutic approaches must account for these variables if an effective treatment for inflammation is to be developed further in order to be of maximal benefit.

However, more substantial evidence is derived from a number of studies. One previous report investigated the effects of several NSAID on NF-κB activation and found that ibuprofen does not influence LPS-induced up-regulation of tissue factor, a gene thought to be regulated by NF-κB [30]. This study showed that, contrary to the results with ibuprofen, sodium aspirin, salicylate as well as salsalate, blocked the LPS-induced NF-κB activation. One explanation might be that in this comparative experiment 50 times higher concentrations of aspirin (10 mM) than ibuprofen (0.2 mM) were used. Although very likely, it is open to question as to whether increasing the amount of ibuprofen to levels used in our studies, or to the mole levels of aspirin used in their study, could have led to suppression of LPS-induced tissue factor as well. Nevertheless, other studies support and confirm our findings that ibuprofen can indeed suppress the expression of genes in which NF-κB activation seems to be essential. Two studies reported

decreased mRNA levels for IL-1β, TNF-α, and MIP-1α in LPS-stimulated rat lungs following treatment with ibuprofen and dexamethasone [31, 32]. Also, during the review process of this manuscript, another study was published showing that, identical to our findings, ibuprofen can inhibit the translocation of NF-κB into the nucleus [33]. This group demonstrated by transient transfection experiments and EMSA that high concentrations of ibuprofen selectively inhibit the activation of this transcription factor. The reported inhibition of ibuprofen was considered to be specific as concentrations of up to 10 mM ibuprofen did not affect activation of heat shock transcription factor.

In summary, we have demonstrated that ibuprofen inhibits the activation and translocation of NF-κB. We also included experiments demonstrating the stabilizing effect of ibuprofen on the NF-κB/IκBα complex. IκBα, a molecule when bound to the subunits of NF-κB, keeps these molecules from being translocated into the nucleus [34, 35]. It is evident that the degradation of IκB is a prerequisite for NF-κB activation [36], and many reports show that stabilization of IκB is one way of suppressing NF-κB activation [17, 37, 38]. Therefore, our study confirms and extends previous findings and points to future experiments investigating the means by which ibuprofen blocks the degradation of IκBα. As with aspirin, total inhibition of NF-κB translocation can only be achieved by using relatively high concentrations of ibuprofen. Nevertheless, even partial cytoplasmic retention of NF-κB, which can be achieved readily at clinically relevant doses, could contribute considerably to the anti-inflammatory effects of this drug.

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