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Anti-inflammatory drugs and tumor necrosis factor-α production from monocytes: role of transcription factor NF-κB and implication for rheumatoid arthritis therapy

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Abstract

Inhibition of tumor necrosis factor-α (TNF-α) represents a relevant target in rheumatoid arthritis therapy. Besides inhibiting cyclooxygenase, anti-inflammatory drugs can affect the activation of transcription factors. We investigated the ability of dexamethasone, indomethacin, and rofecoxib to modulate nuclear factor-κB (NF-κB) activation and TNF-α release from human monocytes challenged with lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA). Both stimuli induced NF-κB nuclear translocation and TNF-α secretion. Dexamethasone potently inhibited TNF-α release, indomethacin inhibited only PMA-evoked release, while rofecoxib had no effect. In the electrophoretic mobility shift assay, dexamethasone and rofecoxib dose-dependently inhibited the DNA binding activity of NF-κB in stimulated monocytes, whereas indomethacin failed to inhibit the LPS-evoked one. These results were further confirmed by evaluating the drugs' ability to reduce nuclear NF-κB subunits, as well as the amount of phosphorylated IκBα in cytosolic fractions. In conclusion, these results indicate that anti-inflammatory drugs differ largely in their ability to inhibit NF-κB activity and/or TNF-α release from human monocytes. These effects can be relevant to rheumatoid arthritis therapy.

Keywords: Human monocyte; Tumor necrosis factor-α; Nuclear factor-κB; Rofecoxib; Indomethacin; Dexamethasone

1. Introduction

Rheumatoid arthritis is a common autoimmune disease of unknown aetiology characterized by symmetric erosive synovitis and infiltration of immunocompetent cells (Feldmann et al., 1996). Evidence suggests that there is an imbalance between pro-inflammatory and anti-inflammatory mediators, and much effort has been focused on the identification of cytokines that play a crucial role in joint damage (Feldmann et al., 1996; Choy and Panayi, 2001). Tumor necrosis factor-α (TNF-α) and interleukin-1 have been shown to have a pivotal role in the pathogenesis and development of rheumatoid arthritis, with high synovial and serum concentrations for both cytokines being detected in patients (Choy and Panayi, 2001). TNF-α stimulates the production of other inflammatory cytokines, promotes cartilage degradation and bone resorption in vitro, induces prostaglandin E_2 and collagenase release from synovial cells, and up-regulates the expression of vascular adhesion molecules, leading to tissue infiltration by neutrophils (Choy and Panayi, 2001). TNF-α can be produced by several cell types relevant to the rheumatoid joint, monocytes being the most important, and can be targeted to cure

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disease, as demonstrated in recent years with the clinical application of infliximab and etanercept (American College of Rheumatology Subcommittee, 2002).

It is generally accepted that some anti-inflammatory drugs regulate inflammation via the inhibition of the enzyme cyclooxygenase-2 (COX-2), but some novel mechanisms of action, notably activation of peroxisome proliferator-activated receptor-γ (PPAR-γ) and inhibition of transcription factors such as nuclear factor-κB (NF-κB) and/or activator protein-1 (AP-1), have gained considerable attention (Jiang et al., 1998; Tegeder et al., 2001). The transcription factor NF-κB regulates the expression of many pro-inflammatory genes, including that of TNF-α, and, in turn, inflammatory cytokines, such as TNF-α and interleukin-1, are potent inducers of NF-κB activation (Baldwin, 1996; Tak and Firestein, 2001). This raises the question of whether NF-κB activation could play a pathogenic role in rheumatoid arthritis; this would be supported by the observation that NF-κB is overexpressed in the inflamed synovium of patients and experimental models of arthritis (Han et al., 1998; Tak and Firestein, 2001). Five related mammalian gene products participate in NF-κB functions (p50/NF-κB1, p52/NF-κB2, p65/Rel A, c-Rel, and RelB); although different homodimeric and heterodimeric forms of this factor have been described, the predominant species in many cell types is a p50–p65 heterodimer. In resting cells, NF-κB is retained in the cytoplasm through an association with inhibitory proteins of the IκB family (including the best characterized IκBα and IκBβ), which masks the nuclear localization signal (Baldwin, 1996; Tak and Firestein, 2001). IκBα is a key molecular target involved in the regulation of NF-κB activity during the inflammatory process. Upon stimulation, IκBα is phosphorylated by IκB kinases (IKKs), ubiquitinylated, and degraded by a proteasome complex, thus allowing NF-κB to translocate to the nucleus and bind DNA. Once NF-κB enters the nucleus, it binds to the promoter region of various genes involved in the inflammatory and immune response [e.g., COX-2, TNF-α, and inducible nitric oxide synthase (iNOS)] and induces their transcription. It also causes the transcriptional activation of the IκBα gene, thus allowing a negative feedback mechanism (Baldwin, 1996; Ghosh et al., 1998). Two IKKs, namely IKK-1 (IKKα) and IKK-2 (IKKβ), have been cloned, which mediate transient or persistent NF-κB activation in response to stimuli, respectively (Baldwin, 1996).

Several anti-inflammatory drugs have been shown to inhibit NF-κB activation at different stages: aspirin and salicylate specifically inhibit IKK-2 activity (Yin et al., 1998), while corticosteroids can directly interact with and inhibit NF-κB subunits and/or up-regulate the expression of the inhibitor IκBα, in addition to inhibiting cAMP response element-binding protein (CREB) and AP-1 (Almawi and Melemedjian, 2002; De Bosscher et al., 2003).

The initial pharmacological treatment of rheumatoid arthritis usually involves the use of low-dose oral glucocorticoids, nonsteroidal anti-inflammatory drugs, or a selective COX-2 inhibitor to reduce joint pain and swelling and to improve joint function (American College of Rheumatology Subcommittee, 2002). Therefore, we decided to evaluate the ability of indomethacin (a nonselective cyclooxygenase inhibitor), rofecoxib (a selective COX-2 inhibitor), and dexamethasone (as an example of glucocorticoid drug and also as a positive control, due to its described effects on transcription factors) to modulate NF-κB activation and/or TNF-α release from human monocytes.

2. Materials and methods

2.1. Isolation of peripheral blood monocytes

Peripheral monocytes were isolated from heparinized venous blood (30–40 ml) of healthy donors (aged 22–55 years) by standard techniques of dextran sedimentation and Ficoll-Paque (density=1.077 g/cm^3) gradient centrifugation (400×*g*, 30 min, room temperature), and recovered by thin suction at the interface (Brunelleschi et al., 2001). Cells were washed twice with phosphate-buffered saline (PBS) and resuspended at 1–2×10^7 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 10 mM HEPES, 50 μg/ml streptomycin, and 5 U/ml penicillin (Brunelleschi et al., 2001). Cell viability, as assessed by Trypan blue dye exclusion, was >98%. Cell suspensions (100 μl) were plated in six-well tissue culture plates (35 mm diameter; Costar, UK) and allowed to adhere for 90 min at 37 °C in a humidified atmosphere containing 5% CO_2 to isolate purified monocytes. Nonadherent cells (mainly lymphocytes) were discarded. The purity of adherent monocytes was assessed by flow cytometry with monoclonal antibodies, anti-CD14 (a marker for the LPS receptor) and anti-CD3, with monocyte populations routinely consisting of >90% CD14$^+$ cells and <3% CD3$^+$ cells, as described (Brunelleschi et al., 2001).

2.2. Stimulation of monocytes

Monocytes (0.5–1×10^6 cells) were challenged, in the absence or presence of anti-inflammatory drugs (see below), with appropriate concentrations of phorbol 12-myristate 13-acetate (PMA) or lipopolysaccharide (LPS; from *Salmonella typhimurium*) to evaluate TNF-α release and NF-κB activation. To verify the effects of anti-inflammatory drugs on TNF-α release, monocytes were preincubated with dexamethasone (10^{-11}–10^{-5} M) for 24 h according to Van der Goes et al. (2000), indomethacin (10^{-7}–10^{-4} M) for 2 h according to Jiang et al. (1998), rofecoxib (10^{-7}–10^{-5} M) for 2 h according to Chan et al. (1999), or solvent, and then challenged with 10^{-7} M PMA or 10 ng/ml LPS. Preliminary dose–response curves (10^{-9}–10^{-6} M PMA; 0.1–1000 ng/ml LPS) and time course experiments (30 min–48 h) demonstrated that these concentrations, combined with a 24-h

stimulation period, produced the maximum cytokine production, although TNF-α release was observed also at shorter or longer stimulation times. Supernatants were harvested and stored at −20 °C until assay. Dexamethasone and indomethacin were dissolved in ethanol, and rofecoxib was dissolved in dimethyl sulfoxide (DMSO); no major effect of solvents was observed under our conditions. To evaluate NF-κB activation, monocytes were challenged with anti-inflammatory drugs as above and then stimulated by 10^{-6} M PMA or 500 ng/ml LPS for different periods (see Results). For these experiments, a greater amount of cells was needed, approximately $5–10\times10^6$ monocytes. Electrophoretic mobility shift assay (EMSA; see below) and quantitative ELISA assays for translocated p50 and p65/RelA subunits or cytosolic phosho-IκBα were performed with 5 μg of nuclear extract or 100 μg of cytosolic extract, respectively.

2.3. Measurement of TNF-α release

TNF-α in the samples was measured using enzyme-linked immunoassay kit (Pelikine Compact™ human TNF-α ELISA kit), according to the manufacturer's protocol. The minimum detectable concentration of human TNF-α was <1.4 pg/ml. No cross-reactivity was observed with any other known cytokine. Control values (e.g., cytokine release from untreated, unstimulated cells) were subtracted from all determinations. Results are mean±S.E.M. of duplicate determinations of *n* independent experiments and are expressed in picograms per milliliter.

2.4. Preparations of nuclear and cytosolic cellular fractions

After treatment, cells were washed with ice-cold PBS, scraped, and centrifuged at 1000×*g* for 5 min at 4 °C. The cell pellet was resuspended in 300 μl of lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol) and incubated on ice for 15 min. At the end of this incubation, 20 μl of 10% NP-40 was added and the tube was vortexed for 10 s. After centrifugation at 13,000×*g* for 1 min at 4 °C, supernatants (cytosolic fractions) were collected and stored at −80 °C, whereas the pellets were further processed to obtain nuclear extracts. The pellets were resuspended in extraction buffer (5 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, 0.5 mM dithiothreitol, and glycerol 25% vol/vol) and incubated for 30 min at 4 °C. Nuclear proteins were isolated by centrifugation at 13,000×*g* for 15 min. The supernatant was aliquoted and stored at −80 °C until used for EMSA or p50/p60 ELISA assays. Protein concentrations were determined by using a protein assay (Bio-Rad, USA).

2.5. EMSA

Nuclear extracts (5 μg) were incubated with 2 μg of poly(dI–dC) and the γ-[^{32}P]ATP-labeled oligonucleotide probe (100,000–150,000 cpm; Promega, St. Louis, CA, USA) in binding buffer (50% glycerol, 10 mM Tris–HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, and 1 mM dithiothreitol) in a final volume of 20 μl for 30 min at room temperature. The NF-κB consensus oligonucleotide (5′ -AGTTGAGGGGACTTTCCCAGGC-3′) was obtained from Promega. The nucleotide–protein complex was separated on a 5% polyacrylamide gel in 0.5× TBE buffer (100 mM Tris–HCl, 100 μM boric acid, and 2 mM EDTA) at 150 V on ice. The gel was dried and radioactive bands were detected by autoradiography.

2.6. p50 and p65/RelA assays

Nuclear extracts were prepared as described above and evaluated for the presence of p50 and p65/RelA subunits using Trans-AM™ NF-κB p50 and NF-κB p65 Transcription Factor Assay kits (Active Motif Europe, Belgium), according to the manufacturer's instructions. Briefly, an equal amount (5 μg) of nuclear lysate was added to incubation wells precoated with an oligonucleotide containing the NF-κB consensus site (5′ -GGGACTTTCC-3′) sequence, the active form of NF-κB contained in the cell extract specifically binding to this oligonucleotide. These assay kits specifically detected bound NF-κB p65 or p50 subunits in human extracts; activities of p50 and p65 were measured using a microplate spectrophotometer at 450 nm, and results are expressed as OD.

2.7. Assay of phospho-IκBα in cytosolic extracts

Cytosolic extracts (100 μg) were prepared as described above and evaluated for the presence of phosphorylated IκBα using Phospho-IκBα-ActivELISA™ kit (Alexis, Switzerland), according to manufacturer's protocol. This procedure allows quantitative measurement of IKK-induced phosphorylation of IκBα in response to external stimuli.

2.8. Data and statistical analysis

Data are expressed as mean±S.E.M. of *n* independent determinations. Concentration–response curves for anti-inflammatory drugs were constructed and logarithmically transformed. IC_{50} values were interpolated from curves of best fit using Graph-Pad Software (Graph-Pad, San Diego, CA, USA). When required, statistical analysis was performed by Student's *t* test for paired or unpaired samples. In all cases, significance was set at a P value <0.05.

2.9. Reagents

Dextran T-500, Ficoll-Paque, and poly(dI–dC) were obtained from Pharmacia (Uppsala, Sweden). Fluorochrome-conjugated anti-CD14 and anti-CD3 were purchased from Becton Dickinson (UK). PBS, RPMI 1640, L-glutamine, HEPES, streptomycin, penicillin, PMA, LPS from *S.*

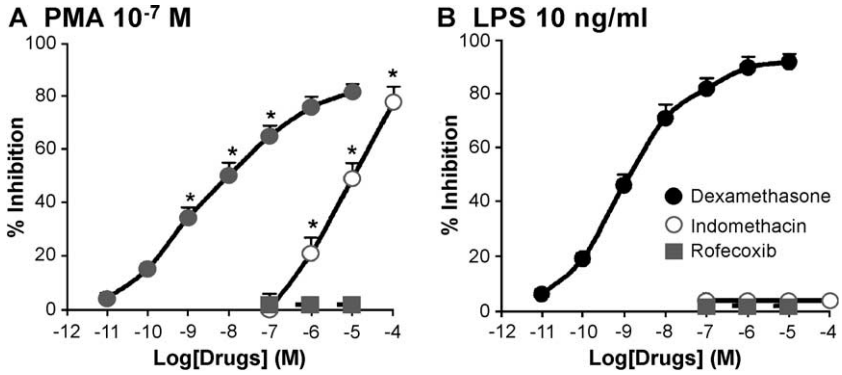

Fig. 1. Effects of anti-inflammatory drugs on TNF-α release from PMA- or LPS-stimulated monocytes. Adherent monocytes ($0.5–1\times10^6$ cells/plate) were preincubated at 37 °C in the absence or presence of dexamethasone (●; 24 h), indomethacin (○; 2 h), or rofecoxib (■; 2 h) and then treated with PMA 10^{-7} M (A) or 10 ng/ml LPS (B) for a further 24 h. The cytokine release was measured by ELISA. Results are mean±S.E.M. of four to six experiments in duplicate. Asterisk denotes a statistically significant difference ($P<0.05$) in the inhibitory effect of PMA-stimulated cells vs. LPS-stimulated cells.

typhimurium, DMSO, indomethacin, and ethanol were from Sigma (St. Louis, MO, USA). All cell culture reagents, with the exception of fetal bovine serum, were endotoxin-free according to details provided by the manufacturer. Fetal bovine serum (lot 40G3410K, containing <10 EU/ml) was from Life Technologies (Rockville, MD, USA). Dexamethasone was purchased from Alexis. Rofecoxib was a kind gift from Merck and Co. (Rahway, NJ, USA). TNF-α immunoassay kit was obtained from CBL, Central Laboratory of The Netherlands Red Cross (The Netherlands). Gel shift assay core system and all the reagents for NF-κB EMSA were from Promega. Trans-AM™ NF-κB p65 and NF-κB p50 Transcription Factor Assay kits were from Active Motif Europe, and Phospho-IκBα-ActivELISA™ kit was from Alexis.

3. Results

3.1. TNF-α release from human monocytes

Isolated adherent monocytes released TNF-α spontaneously or upon challenge with LPS or PMA. Basal TNF-α release in monocytes from healthy donors was low yet detectable (7.6±3 pg/ml; $n=10$). When challenged with stimuli at concentrations affording maximal effects, monocytes released 1039±160 pg/ml TNF-α ($n=10$; 10^6 monocytes/plate) after 10^{-7} M PMA, and 1367±178 pg/ml ($n=10$; not significant vs. PMA) after 10 ng/ml LPS. TNF-α release was unaffected by ethanol or DMSO, the solvents in which anti-inflammatory drugs were dissolved: a slight, but insignificant, increase was observed only at the highest (0.1% vol/vol) DMSO concentration (data not shown).

3.2. Effects of anti-inflammatory drugs on TNF-α release

In the concentration range 10^{-11}–10^{-5} M, dexamethasone inhibited TNF-α release from monocytes, the maximal effects being observed at 1–10 μM and the IC_{50} values being 3.8 nM and 1.2 nM following PMA or LPS challenge, respectively ($n=6$; Fig. 1A and B).

At intermediate concentrations (10^{-10}–10^{-7} M), the inhibition afforded by dexamethasone was higher ($P<0.05$) in LPS-challenged monocytes as compared to PMA-stimulated ones (Fig. 1). The nonselective cyclo-

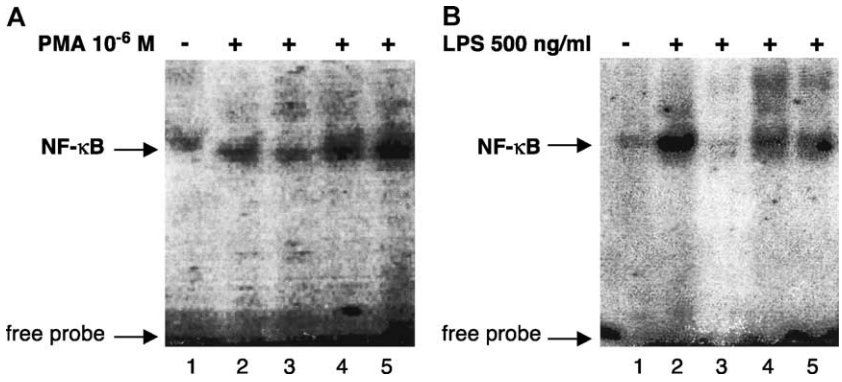

Fig. 2. PMA and LPS induce NF-κB activation in human monocyte in a time-dependent manner. Human monocytes were stimulated by 10^{-6} M PMA (A) or 500 ng/ml LPS (B) for different periods; then nuclear extracts (5 μg) were prepared and assayed for NF-κB activity by EMSA (see Materials and methods for further details). (A) PMA-induced NF-κB nuclear translocation. Unstimulated cells (lane 1) and PMA-stimulated cells (lanes 2–5); monocytes were challenged with PMA for 0.5 h (lane 2), 1 h (lane 3), 2 h (lane 4), or 4 h (lane 5). (B) LPS-induced NF-κB nuclear translocation. Unstimulated cells (lane 1) and LPS-stimulated cells (lanes 2–5); monocytes were challenged with 500 ng/ml LPS for 1 h (lane 2), 2 h (lane 3), 6 h (lane 4), or 8 h (lane 5).

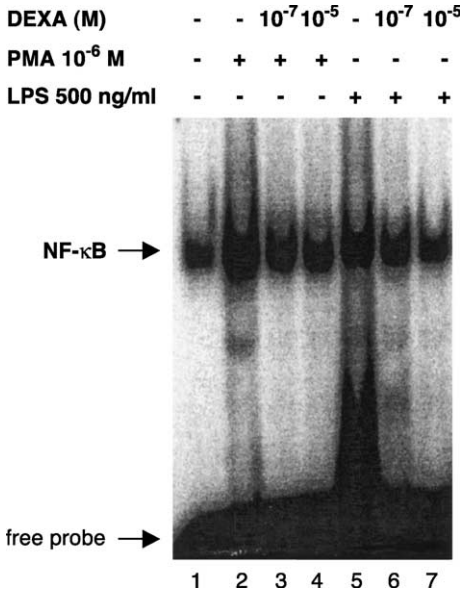

Fig. 3. Effects of dexamethasone on NF-κB activity in human monocytes. Cells were incubated in the absence (lanes 1, 2, and 5) or presence of dexamethasone, 10^{-7} M (lanes 3 and 6) or 10^{-5} M (lanes 4 and 7), for 24 h and then stimulated by 10^{-6} M PMA (lanes 2– 4) for 4 h or 500 ng/ml LPS (lanes 5–7) for 1 h. Nuclear extracts (5 μg) were subjected to EMSA as described (see text for further details). A representative blot of three independent experiments is shown.

oxygenase inhibitor, indomethacin, inhibited PMA-evoked TNF-α release in a concentration-dependent (10^{-7}–10^{-4} M) manner, the maximal effect being 78±3% inhibition (n=5; IC_{50}=4.6 μM), but it did not affect LPS-evoked TNF-α release (Fig. 1A and B). Unlike the two other drugs, the COX-2-selective inhibitor rofecoxib (10^{-7}–10^{-5} M) had no effect on either LPS- or PMA-induced TNF-α release from monocytes (Fig. 1).

3.3. LPS- and PMA-induced NF-κB activation

In unstimulated control monocytes, DNA binding of NF-κB was minimal, although detectable, whereas treatment with PMA or LPS considerably increased the DNA binding activity. The specificity of the NF-κB DNA binding was confirmed by the reversal of the binding by a 100-fold molar excess of unlabeled probe (data not shown). Nuclear translocation of the transcription factor was maximal (the intensity of the complex was threefold above the basal level) when monocytes were stimulated by PMA 10^{-6} M for 4 h, with some activation being present also at shorter time points (0.5–4 h; Fig. 2A). LPS enhanced NF-κB nuclear migration in a time-dependent manner, with maximal activation when monocytes were challenged with 500 ng/ml LPS for 1 h (Fig. 2B). LPS-induced nuclear translocation was negligible at 2 h, but present again when monocytes were stimulated for longer periods (6–8 h; Fig. 2B). Therefore, in the experiments aimed to evaluate the effects of anti-inflammatory drugs on NF-κB activation, monocytes were challenged with LPS for 1 h or PMA for 4 h.

3.4. Effects of anti-inflammatory drugs on NF-κB activation

We first assessed the effects of anti-inflammatory drugs on the DNA binding activity of NF-κB by EMSA. None of the drugs used here affected per se DNA binding activity (not shown). Dexamethasone, evaluated at two different concentrations of 10^{-7} M (Fig. 3, lanes 3 and 6) and 10^{-5} M (Fig. 3, lanes 4 and 7), strongly inhibited NF-κB nuclear translocation and DNA binding activity in both LPS- and PMA-challenged monocytes: maximal inhibition was observed at 10^{-5} M dexamethasone and reached approximately 50% for both stimuli (Fig. 3).

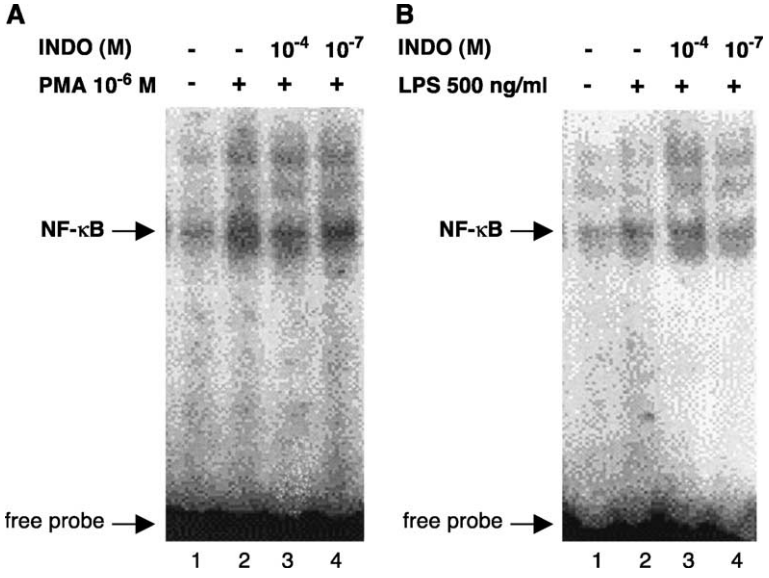

Fig. 4. Effects of indomethacin on NF-κB activity in human monocytes. Cells were incubated in the absence (lanes 1 and 2) or presence of indomethacin, 10^{-7} M (lane 4) or 10^{-4} M (lane 3), for 2 h and then stimulated by 10^{-6} M PMA (A; lanes 2–4) for 4 h or 500 ng/ml LPS (B; lanes 2–4) for 1 h. Nuclear extracts (5 μg) were subjected to EMSA as described (see text for further details). A representative blot of three independent experiments is shown.

As reported in Fig. 4, indomethacin did not affect LPS-induced NF-κB nuclear translocation (Fig. 4B), whilst causing minimal inhibition (about 15%, as evaluated by laser scanning densitometry) of PMA-induced translocation at the high 10^{-4} M concentration only (Fig. 4A, lane 4). The selective COX-2 inhibitor, rofecoxib, while devoid of effects on TNF-α production, inhibited NF-κB DNA binding activity in nuclear extracts (Fig. 5). At 10^{-5} M (Fig. 5A, lane 3), rofecoxib potently blocked PMA-induced NF-κB nuclear translocation (densitometric analysis revealed that the decrease was >60%) and exerted a 35% inhibition at 10^{-7} M (Fig. 5A, lane 4). When monocytes were stimulated by LPS, rofecoxib still inhibited NF-κB activation, although to a lesser extent (40% inhibition at 10^{-5} M and about 10% inhibition at 10^{-7} M) (Fig. 5B).

To ensure a better quantitative evaluation of the drugs' effect on NF-κB activation, we assessed the translocation of p50 and p65 subunits in nuclear extracts from PMA- or LPS-stimulated monocytes by using a commercially available ELISA kit. A significant increase (threefold to fivefold over unstimulated monocytes) in p65 and p50 content was detected in LPS- or PMA-challenged cells, with binding of p50 to DNA being higher in LPS-treated monocytes as compared to PMA-challenged cells (Table 1). Dexamethasone and rofecoxib inhibited the nuclear translocation of p50 and p65 subunits, in a concentration-dependent manner, in both PMA- or LPS-challenged monocytes, whereas indomethacin exerted a modest inhibition in PMA-stimulated monocytes only (Table 1), largely supporting the results obtained in EMSA assays. By subtracting the OD value of unstimulated control monocytes from all determinations, the inhibition afforded by dexamethasone was around 50% at 0.1 μM and about 80% at 10 μM in both PMA- or LPS-stimulated human monocytes (Table 1).

Table 1
Effect of anti-inflammatory drugs on the translocation of p50 and p65 subunits in PMA- or LPS-stimulated monocytes

Drug		% Inhibition			
		PMA (10^{-6} M)		LPS (500 ng/ml)	
		p50	p65	p50	p65
DEXA	10^{-7}	51±3	44±3	49±2	50±2
	10^{-5}	71±4	90±2	77±3	75±3
ROFE	10^{-7}	45±4	31±5	32±4	25±1
	10^{-5}	80±4	51±7	54±5	50±6
INDO	10^{-7}	13±4	7±4	7±5	12±7
	10^{-4}	25±2	35±4	7±5	18±8

Nuclear extracts were prepared as described in Materials and methods. The OD values of control monocytes (0.4±0.02 for p50; 0.5±0.03 for p65) were subtracted from all determinations. PMA (10^{-6} M) induces a threefold increase over control for p50 (OD=1.32±0.2) and p65 (OD=1.2±0.05). LPS (500 ng/ml) induces a fivefold increase over control for p50 (OD=1.8±0.1) and a threefold increase for p65 (OD=1.28±0.03). Values are mean±S.E.M. of four determinations.

Rofecoxib, too, dose-dependently inhibited p50 and p65 nuclear translocation and was particularly effective in inhibiting p50 subunit in PMA-challenged monocytes, whereas indomethacin had no effect in LPS-stimulated monocytes (Table 1). As previously demonstrated, activation of NF-κB requires phosphorylation by IKK of IκB proteins at specific protein residues (Ser 32 and Ser 36). We used a commercially available ELISA kit to measure the amount of phoshorylated IκBα in cytosolic extracts, and so indirectly evaluated whether or not anti-inflammatory drugs could affect PMA- or LPS-induced activation of IKK. In the experiments reported in Table 2, monocytes were stimulated in the absence or presence of anti-inflammatory drugs, with 500 ng/ml LPS or 10^{-6} M PMA, for 1 and 4 h, respectively, as for EMSA studies. These stimulation times were selected

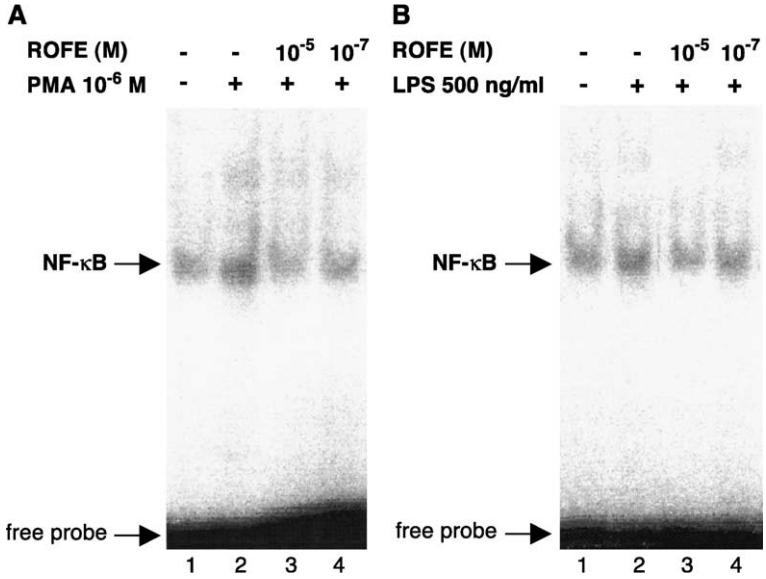

Fig. 5. Effects of rofecoxib on NF-κB activity in human monocytes. Cells were incubated in the absence (lanes 1 and 2) or presence of rofecoxib, 10^{-5} M (lane 3) or 10^{-7} M (lane 4), for 2 h and then stimulated by 10^{-6} M PMA (A; lanes 2–4) for 4 h or 500 ng/ml LPS (B; lanes 2–4) for 1 h. Nuclear extracts (5 μg) were subjected to EMSA as described (see text for further details). A representative blot of three independent experiments is shown.

Table 2
Effect of anti-inflammatory drugs on cytosolic phosphorylation IκBa in PMA- or LPS-stimulated monocytes

Drug		% Inhibition	
		PMA (10^{-6} M)	LPS (500 ng/ml)
DEXA	10^{-7}	34±8	31±6
	10^{-5}	62±4	56±6
ROFE	10^{-7}	21±9	28±4
	10^{-5}	55±7	71±10
INDO	10^{-7}	4±2	0
	10^{-4}	31±2	0

Cytosolic extract (100 μg) was prepared as described in Materials and methods. Values are mean±S.E.M. of four determinations (see Materials and methods for further details).

to give a snapshot of the moment in which DNA binding activity of NF-κB is maximal. In our hands, 10^{-6} M PMA induced the phoshorylation of IκBα (1092±220 RLU—relative chemiluminescence units; mean±S.E.M; n=4), a threefold increase over control unstimulated monocytes (323±72 RLU) being observed after 4 h and a fourfold increase after 1-h challenge (not shown). The amount of cytosolic phosphorylated IκBα was more than doubled after a 1-h challenge with 500 ng/ml LPS (806±180 RLU; mean±S.E.M; n=4) and fourfold higher when monocytes where challenged with LPS for 30 min. Dexamethasone and rofecoxib reduced LPS- or PMA-induced IKK activity, as shown by the reduced amount of phosphorylated IκBα in the cytosolic fractions, whereas indomethacin exerted some inhibition at the highest 10^{-4} M concentration only on PMA-challenged monocytes (Table 2).

4. Discussion

In this study, representative anti-inflammatory drugs differed markedly in their ability to affect TNF-α release from PMA- or LPS-stimulated monocytes. Dexamethasone, which was selected as an example of glucocorticoid drug and also as a positive control, due to its well-known ability to affect cytokine production in various cell types (Almawi and Melemedjian, 2002), potently inhibited TNF-α release from human monocytes challenged with both stimuli, thus confirming different previous observations (Adcock et al., 1995; Steer et al., 2000). The nonselective cyclooxygenase inhibitor indomethacin inhibited PMA-evoked TNF-α release, but failed to affect the LPS-induced one, whereas the selective COX-2 inhibitor, rofecoxib, was devoid of effect on TNF-α release from monocytes. Inhibition of cytokine release does not represent a common feature of nonsteroidal anti-inflammatory drugs and is regarded as an "extra" effect independent of cyclooxygenase inhibition. In this regard, indomethacin, ibuprofen, and fenoprofen have been described to possess PPAR-γ agonistic activity at high concentrations and, for this reason, to block the production of inflammatory cytokines (Jiang et al., 1998; Lehmann et al., 1997). Moreover, in human monocytes, LPS-induced cytokine secretion is largely refractory to the effects of PPAR-γ agonists, whereas PMA- and okadaic acid-induced cytokine release is susceptible to their action (Jiang et al., 1998). Although TNF-α production is largely regulated by the transcription factor NF-κB, evidence exists that TNF-α and other cytokines can also be induced through NF-κB-independent pathways. For example, LPS-induced TNFα production in human macrophages is largely controlled by NF-κB, whereas the zymosan-induced production is largely NF-κB-independent (Bondeson et al., 1999). For the PMA-induced activation of monocytic cells, the results are controversial: some studies indicate that NF-κB is involved (Bondeson et al., 1999; Kaufman et al., 1992) and others do not (Makarov et al., 1997; Tran-Thi et al., 1995). In THP-1 cells modified through stable retroviral gene transfer of IκB, PMA-induced cytokine release was independent of NF-κB activation (Makarov et al., 1997). Thus, the ability of a single gene to be controlled by several signal transduction pathways implies a level of complexity that allows its varied expression, depending either on the nature, duration, and intensity of the external stimuli and/or the particular cell type. Interestingly, a recent paper indicated the existence of two distinct pathways of LPS-induced NF-κB activation and cytokine production in human cells, with regulation being cell type-specific (Andreakos et al., 2004). In fact, myeloid differentiation protein 88 (MyD88), MYD88 adaptor-like/TIR domain-containing adaptor protein (Mal/TIRAP), and IKK2 were essential for LPS-induced IκBα phosphorylation, NF-κB activation, and cytokine production in human synovial fibroblasts and endothelial cells; on the contrary, neither MyD88, Mal/TIRAP, nor IKK2 was required for NF-κB activation or TNF-α secretion in human macrophages (Andreakos et al., 2004). So, the cytokine secretion by LPS- or PMA-stimulated monocytes could be regulated, cooperatively or not, by different signalling pathways [e.g., extracellular signal-regulated kinase (ERK)-1, ERK-2, p38 mitogen-activated protein (MAP) kinase, MAP kinase kinase (MEK), c-jun N-terminal kinase (JNK), and phosphatidylinositol 3 kinase] independently of NF-κB activation. This was the case for GM-CSF production in LPS-challenged human monocytes, which was dependent on both p38 MAP kinase and MEK, but not on NF-κB (Meja et al., 2000).

In our experiments, LPS and PMA were both able to activate NF-κB and to induce TNF-α secretion from monocytes (a more detailed evaluation of TNF-α/NF-κB mutual interactions being beyond the scope of this article).

Consistent with previous reports in which quiescent cells of the monocyte lineage were used (Frankenberger et al., 1994; Meja et al., 2000), a constitutive expression of NF-κB in the nucleus of unstimulated monocytes was always observed, probably due to the adhesion procedure. As previously demonstrated in a number of cells, nuclear NF-κB, besides inducing the transcription of different pro-inflammatory genes, also causes the transcriptional activation of the IκBα gene and a rapid reaccumulation of cytoplasmatic IκBα (Baldwin, 1996; Ghosh et al., 1998). By

using cDNA microarray analysis in a murine macrophage cell line, Nemeth et al. (2003) recently reported that stimulation with LPS induced a sevenfold induction of IκBα gene and a threefold induction of NF-κB1 gene after 3 h. Therefore, at a given time point, NF-κB can simultaneously affect the transcription of pro-inflammatory and anti-inflammatory genes. To gain some insights into the inhibitory effects of anti-inflammatory drugs on TNF-α release, we investigated NF-κB activity by EMSA (as well as p50, p65/RelA, and phospho-IκBα assays) using nuclear (cytosolic for the phospho-IκBα assay) extracts from PMA- or LPS-stimulated monocytes. Cells were stimulated by 500 ng/ml LPS for 1 h or 10^{-6} M PMA for 4 h, which is the moment in which DNA binding activity of NF-κB is maximal (Fig. 2).

As expected, dexamethasone dose-dependently inhibited, with a maximum of about 50% at 10^{-5} M, NF-κB nuclear translocation in PMA- or LPS-challenged human monocytes. This observation is in good agreement with previous reports, indicating the steroid's ability to decrease by 40–50% NF-κB and AP-1 binding in PMA-stimulated monocytes and to suppress transcription of TNF-α in LPS-stimulated THP-1 cells (Adcock et al., 1995; Steer et al., 2000). Moreover, the suppression of NF-κB activity was observed at the concentration range required for dexamethasone inhibition of TNF-α production. The fact that anti-inflammatory and immunosuppressive actions of glucocorticoids mostly depend on inhibition of cytokine expression and/or cytokine release is widely accepted, as well as the appraisal that these effects are mainly achieved by antagonizing the activity of transcription factors, through direct and indirect molecular mechanisms (e.g., protein–protein interactions between the transcription factor and the activated glucocorticoid receptors or up-regulation of IκB) (see De Bosscher et al., 2003 for a review). The capacity of glucocorticoid receptor to associate with the transactivating domain of p65/RelA subunit of NF-κB (De Bosscher et al., 2000) and the fact that dexamethasone predominantly inhibited (at 50 μM, a concentration higher than the ones here used) p65 subunit expression in rat striatal neurones (Simpson and Morris, 1999) prompted us to evaluate if it could be the case in our system, too. Analysis of p65 content in nuclear extracts (as evaluated by ELISA kits) in PMA- or LPS-stimulated monocytes indicated that dexamethasone potently and dose-dependently inhibited the translocation of this NF-κB subunit, with maximal inhibition being achieved at 10 μM and reaching about 80%, with no significant variation between stimuli. However, p50 translocation was equally inhibited, so we can exclude a dexamethasone-selective effect on p65 in human monocytes. Dexamethasone also inhibited IKK kinase activity (as evaluated by measuring the amount of phosphorylated IκBα in cytosol) in both PMA- or LPS-stimulated monocytes. Therefore, we provided further experimental evidence that, in human monocytes, inhibition of p65/RelA and p50 binding to DNA, as well as inhibition of IKK, represent chief molecular mechanisms for the anti-inflammatory actions of dexamethasone.

Cyclooxygenase-independent actions of nonsteroidal anti-inflammatory drugs have gained a considerable attention and some of them (e.g., indomethacin, fenoprofen, and ibuprofen) have been claimed to activate PPARs (Jiang et al., 1998; Oates et al., 2002). In human monocytes and other cell types, activation of PPAR-γ receptors has been repetitively demonstrated to inhibit the production of inflammatory mediators (including TNF-α, COX-2, nitric oxide, interleukin-1, interleukin-2, and interleukin-6), with transrepression of the transcription factors NF-κB and AP-1 being involved (Oates et al., 2002).

In this study, indomethacin had no effect on LPS stimulation, but it dose-dependently inhibited TNF-α release in PMA-stimulated human monocytes, in keeping with observations by Jiang et al. (1998). Maximal inhibition was approximately 80% and the IC_{50} value (4.6 μM) measured in this condition could be of clinical relevance, as a high-dose therapy is expected to produce an indomethacin plasma concentration of about 10 μM (Jiang et al., 1998). In our hands, indomethacin failed to inhibit LPS-evoked NF-κB activation, thus confirming previous results in murine macrophages (Callejas et al., 2003), but exerted a modest inhibition on PMA-evoked one, in both EMSA and p50/p65 assays, but only at the high supratherapeutic (100 μM) concentration. Accordingly, at this high concentration, indomethacin also reduced the amount of phosphorylated IκBα induced by PMA in cytosolic extracts. Furthermore, indomethacin effects are significantly smaller than those afforded by refecoxib or dexamethasone. In other cellular models, indomethacin did not modify NF-κB activity (Tegeder et al., 2001; Yamamoto et al., 1999), but the experimental conditions (cell type, indomethacin concentrations, stimuli, etc.) varied largely from the ones we used.

In contrast to indomethacin, the selective COX-2 inhibitor, rofecoxib, inhibited PMA- or LPS-induced NF-κB activation in human monocytes, but had no effect on PMA- or LPS-induced release of TNF-α. Similar results have been recently published by others (Niederberger et al., 2003; Callejas et al., 2003). In the rat, rofecoxib administration at doses of 1, 10, and 50 mg/kg has been shown to inconsistently affect zymosan-induced TNF-α expression in lumbar spinal cord, with some reduction being observed at the intermediate 10 mg/kg dose only (Niederberger et al., 2003). In elicited peritoneal murine macrophage, the release of TNF-α by LPS-stimulated macrophages was inhibited by 25% after treatment with 10 μM rofecoxib (Callejas et al., 2003). However, the experimental conditions used (i.e., LPS concentration; 200 ng/ml, 20-fold higher than the 10 ng/ml we used), time of stimulation (8 h, instead of the 24 h we used), and the cell type (a mouse-activated inflammatory phagocyte) are quite different from our experimental conditions. Previous data indicate that LPS promotes the phosphorylation of ERK-1, ERK-2, p38 MAP kinase, and JNK, and activates the transcription factor NF-κB in human

monocytes (Meja et al., 2000); however, the extent to which any of these signalling molecules contribute to the generation of TNF-α is not fully elucidated.

Callejas et al. (2003) reported that the phosphorylation of p44 ERK, p38 MAP kinase, and p46 JNK, which are rapidly and transiently activated in response to LPS, was completely blocked in murine macrophages treated with 10 μM rofecoxib. At 2 μM, rofecoxib potently inhibited (about 80%) p46 JNK phosphorylation, but exerted a 30% reduction only vs. p44 ERK (Callejas et al., 2003).

The mechanisms by which rofecoxib affects NF-κB activation in human monocytes are currently under investigation. Results here reported indicate that at 10 μM, a concentration sufficient to completely block COX-2 (Chan et al., 1999), rofecoxib inhibited more than 60% NF-κB activity in PMA-stimulated monocytes, as evaluated by EMSA, and was just active at 0.1 μM. Interestingly, a peak plasma concentration around 10 μM has been reported in human volunteers given a high rofecoxib dose (375 mg), whereas a 3-μM plasma concentration has been achieved after the administration of 100 mg of rofecoxib (Depré et al., 2000). In the EMSA assays here reported, rofecoxib dose-dependently reduced, although to a lesser extent, LPS-induced NF-κB activity. To our knowledge, this is the first demonstration of such an effect for rofecoxib on human monocytes and represents a somewhat expected result, since COX-2 activity is largely regulated by the transcription factor NF-κB. Similar results have been obtained in mouse peritoneal macrophages and RAW 264.7 cells, a murine macrophage cell line (Callejas et al., 2003; Niederberger et al., 2003).

In RAW 264.7 macrophages, rofecoxib inhibited the DNA binding capacity of NF-κB at 10–100 μM, whereas the binding activity of the transcription factor AP-1 was significantly enhanced at the 100 μM in vitro concentration (Niederberger et al., 2003). In elicited peritoneal macrophages, 10 μM rofecoxib reduced the LPS-dependent COX-2 and iNOS expression, as well as prostaglandin E_2 and nitric oxide release, and also delayed the LPS-induced NF-κB activation (Callejas et al., 2003). The COX-2 inhibitor impaired IκBα phosphorylation and significantly inhibited IKK activity, thus suggesting IKK as a direct target of rofecoxib (Callejas et al., 2003). Our results are in keeping with and further extend these observations by indicating the PMA-induced NF-κB activity as another target for rofecoxib anti-inflammatory effects. By measuring the nuclear amounts of p50 and p65 subunits, which represent the main NF-κB complexes present in the nucleus of monocyte/macrophages in response to stimulation by LPS and inflammatory cytokines (Ghosh et al., 1998), we found that rofecoxib dose-dependently inhibited them. Our observation that, in cytosolic extracts, rofecoxib strongly inhibits the phosphorylation of IκBα evoked by LPS or PMA indirectly confirms the results by Callejas et al. (2003) and further indicates IKK inhibition as an important mechanism for rofecoxib-mediated anti-inflammatory effects. Experiments are ongoing to further elucidate this point, as well as the role, if any, of signal transduction inhibitors.

In conclusion, this study demonstrates that anti-inflammatory drugs differ largely in their ability to inhibit NF-κB activity and/or TNF-α release from monocytes. Inhibition of TNF-α release from monocytes could represent a relevant adjunctive feature for an anti-inflammatory drug to be clinically effective in rheumatoid arthritis, due to the role played by TNF-α in the "cytokine cascade" in inflammatory sites and in the activation of transcription factors, including NF-κB.

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