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Selective Inhibitors of Cyclooxygenase-2 Delay the Activation of Nuclear Factor κB and Attenuate the Expression of Inflammatory Genes in Murine Macrophages Treated with Lipopolysaccharide

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ABSTRACT

The effect of rofecoxib, a selective cyclooxygenase-2 inhibitor, on inflammatory signaling has been investigated in elicited murine peritoneal macrophages. Macrophages treated with 10 μM rofecoxib exhibited an important inhibition in the early activation of nuclear factor κB (NF-κB) and the mitogen-activated protein kinase p38, the extracellular-regulated kinase p44, and the c-Jun N-terminal kinase. Moreover, this drug decreased the protein levels of nitric-oxide synthase-2 and cyclooxygenase-2 in lipopolysaccharide (LPS)-treated macrophages. Rofecoxib delayed and attenuated NF-κB activation, which impaired significantly the expression of κB-dependent genes. This drug and related coxibs did not affect cell viability and protected against LPS-induced apoptosis through the impairment of the inflammatory response. These data show an additional anti-inflammatory mechanism of selective cyclooxygenase-2 inhibitors through the attenuation of macrophage activation.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of acute and chronic inflammatory diseases. Studies in a variety of animal models of colon cancer and in human colon cancer cell lines have shown a significant reduction in tumor multiplicity and metastatic potential by treatment with NSAIDs (Thun et al., 1991; Williams et al., 1999). The main analgesic and anti-inflammatory effects of NSAIDs are derived from the inhibition of COX enzymes, which catalyze the synthesis of prostaglandin H_2 from arachidonate (DeWitt, 1991; Smith et al., 1996; Williams et al., 1999). The two isoenzymes, COX-1 and COX-2, are encoded by different genes and have distinct physiological functions (DeWitt, 1991; Pilbeam et al., 1993). COX-1 is constitutively expressed in many tissues and is involved in the homeostatic function of prostaglandins (Pilbeam et al., 1993). COX-2 is induced by a variety of stimuli and plays an important role in ovulation, fertilization, and inflammation (Feng et al., 1995; Langenbach et al., 1999). Overexpression of COX-2 has been shown to mediate cell-cycle progression and to contribute to angiogenesis (Tsujii et al., 1997) and tissue invasion (Tsujii et al., 1998). Previous work from our group demonstrated that COX-2 expression and PG synthesis are key components in the secretion of matrix metalloproteinase-2 and -9 and, therefore, in the remodeling of the extracellular matrix that occurs under pathological circumstances in liver (Kim et al., 2000; Callejas et al., 2001). However, there is evidence suggesting that some of the NSAID effects are independent of COX-2 inhibition because the concentration of these drugs required to inhibit cell growth and to induce apoptosis are two or three orders of magnitude higher than those necessary to inhibit PG synthesis (Tegeder et al., 2001). Therefore, alternative mechanisms to explain the anti-inflammatory effects of NSAIDs include the inhibition of MAPKs and IKKs, which impairs the transcription of genes dependent on NF-κB and AP-1, and cyclin-dependent kinases (Tegeder et al., 2001). These COX-inde-

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; NF-κB, LPS, lipopolysaccharide; nuclear factor κB; COX, cyclooxygenase; PG, prostaglandin; IKK, IκB kinase; AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2(5*H*)-furanone; PG, prostaglandin; JNK, c-Jun NH_2-terminal kinase; FCS, fetal calf serum; DTT, dithiothreitol; NOS, nitric-oxide synthase 2; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; PPAR, peroxisomal proliferator-activated receptor; IP, immunoprecipitate; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; Ab, antibody; TNF-α, tumor necrosis factor α; IL, interleukin; GST, glutathione *S*-transferase.

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pendent effects vary among NSAIDs. For example, aspirin, salicylate, and sulindac inhibit IKK-2, thereby preventing activation by NF-κB of genes involved in the pathogenesis of the inflammatory and proliferative response (Yin et al., 1998; Jones et al., 1999). ERK-1 and -2 can be inhibited by sodium salicylate and aspirin (Pillinger et al., 1998), whereas p38 MAPK is activated by sodium salicylate in human fibroblasts (Schwenger et al., 1998).

Previous work has shown that rat hepatocytes and HepG2 cells, a human hepatoma cell line, are not sensitive to NF-κB inhibition by DFU, a fluorinated derivative of the selective COX-2 inhibitor rofecoxib (Riendeau et al., 1997; Chan et al., 1999; Callejas et al., 2002). Celecoxib, rofecoxib, and derivatives belong chemically to the group of diarylheterocycles and were the first COX-2–selective inhibitors to be used in therapeutics. In this study, we investigated the anti-inflammatory properties of rofecoxib, a new selective and potent COX-2 inhibitor, in activated murine macrophages. We found that rofecoxib that is assayed at concentrations greater than the therapeutic dose, in addition to inhibiting efficiently PGE_2 synthesis as expected, impairs the NF-κB signaling pathway by inhibiting IKK activity in vivo and in vitro. Moreover, rofecoxib inhibits the phosphorylation of ERK p38 and JNK in response to LPS challenge and protects macrophages from proinflammatory stimulation-induced apoptosis.

Materials and Methods

Chemicals. LPS from *Salmonella typhimurium*, growth factors, and cytokines were from Sigma Chemical Co. (St. Louis, MO) and Roche Diagnostics (Mannheim, Germany). The COX-2 inhibitors DFU and rofecoxib were Merck (Whitehouse Station, NJ). Antibodies were obtained from Santa Cruz Biochemicals (Santa Cruz, CA) and Calbiochem (San Diego, CA). Tissue culture dishes were from Falcon (Lincoln Park, NJ). Tissue culture media were from BioWhittaker (Walkersville, MD). Reagents for electrophoresis were obtained from Bio-Rad (Hercules, CA).

Preparation of Macrophages. Elicited peritoneal macrophages were prepared from male mice 4 days after intraperitoneal administration of 1 ml of 10% thioglycollate broth (Terenzi et al., 1995). Cells were seeded at 2×10^6 in 6-cm plates or 5×10^5 in 24-multiwell plates and cultured with RPMI 1640 medium supplemented with 10% heat-inactivated FCS and antibiotics (50 μg each of penicillin, streptomycin, and gentamicin per ml) at 37°C in an atmosphere of humidified 5% CO_2. After incubation for 4 h, nonadherent cells were removed, and remnant cells were cultured for the indicated time. RAW 264.7 cells were seeded in RPMI 1640 medium supplemented with 2 mM glutamine, 10% FCS, and antibiotics. Twenty-four hours before stimulation, the culture medium was replaced with fresh medium containing 0.5% FCS. Unless otherwise specified, NSAIDs were added 30 min before activation of macrophages with proinflammatory stimuli.

Determination of Apoptotic Cells. Macrophages were treated with propidium iodide (50 μg/ml), and total DNA content was analyzed using a BD Biosciences (San Jose, CA) model LSR flow cytometer, as described previously (Callejas et al., 2002). The resulting histogram was analyzed using ModFit software (Verity Software House, Topsham, ME). Apoptosis was quantified by determining the percentage of cells with hypodiploid DNA content, followed by cell sorting and analysis of the DNA integrity in agarose gels (data not shown) (Terenzi et al., 1995). Cell viability was assayed with use of trypan blue exclusion up to 72 h.

Preparation of Cytosolic and Nuclear Extracts. Cells were washed with phosphate-buffered saline and homogenized in 200 μl of buffer A (10 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin, 40 μg/ml aprotinin, 2 μg/ml tosyl-lysyl-chloromethane, 5 mM NaF, 1 mM $NaVO_4$, and 10 mM Na_2MoO_4), and Nonidet P-40 was added to reach 0.5% (v/v). After 15 min at 4°C, the tubes were gently vortexed for 15 s, and nuclei were collected by centrifugation at 8000*g* for 15 min. The supernatants were stored at −80°C (cytosolic extracts), and the pellets were resuspended in 50 μl of buffer A supplemented with 20% (v/v) glycerol and 0.4 M KCl, then mixed for 30 min at 4°C. Nuclear proteins were obtained by centrifugation at 13000*g* for 15 min, and aliquots of the supernatant were stored at −80°C.

Electrophoretic Mobility Shift Assays. The sequence 5′-TGCTAGGGGGATT-TTCCCTCTCTCTGT-3′ corresponding to the consensus NF-κB binding site (nucleotides 978 to 952) of the murine NOS-2 promoter (Diaz-Guerra et al., 1999) was used. The oligonucleotide was annealed with the complementary sequence by incubation for 5 min at 85°C in 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. Aliquots (50 ng) of annealed oligonucleotide were end-labeled with Klenow enzyme in the presence of 50 μCi of [α-^{32}P]dCTP and the other unlabeled deoxynucleotides in a final volume of 50 μl. A total of 5×10^4 dpm of the DNA probe was used for each binding assay of nuclear proteins: 5 μg of protein was incubated for 20 min at 4°C with the DNA and 2 μg of poly(dI-dC), 5% glycerol, 1 mM EDTA, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, and 10 mM Tris-HCl, pH 7.8, in a final volume of 20 μl. The DNA protein complexes were separated onto native 6% PAGE in 0.5% Tris-borate-EDTA buffer. Supershift assays were carried out after incubation of the nuclear proteins with 2 μg of Ab (anti-p50, anti–c-Rel, and anti-p65) for 20 min at 4°C followed by electrophoretic mobility shift assay (EMSA), and the retained complexes contained p50/p50 and p50/p65 dimers, respectively (data not shown).

Transfection of RAW 264.7 Cells and Assay of Luciferase Activity. Plasmids were purified with use of the Endo-free plasmid kit (QIAGEN, Izasa, Spain). The cells were washed twice with phosphate-buffered saline and incubated with 0.5 ml of RPMI 1640 medium without FCS in a 24-multiwell plate. Cells were transfected overnight with 1 μg of $(\kappa B)_3$ConA.Luc (Castrillo et al., 2000), and 0.5 μg of pCMV-β-Gal was used as internal control (BD Biosciences Clontech, Palo Alto, CA) by lipofection with FuGENE, as instructed by the supplier (Roche). After transfection, the cells were maintained for 4 h in RPMI 1640 medium with 10% FCS before stimulation. The luciferase and β-galactosidase activities were determined after 18 h of stimulation of the cells, as described previously (Callejas et al., 2000).

Western Blot Analysis. The levels of COX-2, NOS-2, peroxisomal proliferators-activated receptor-α (PPARα), IκBα, p-(Ser^{32})IκBα, ERK, p-ERK, p38, p-p38, JNK and p-JNK were determined in soluble extracts. Equal amounts of protein (20–30 μg) were size-fractionated in 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Amersham Biosciences Inc., Piscataway, NJ), and after blocking with 5% nonfat dry milk, incubated with the corresponding Abs. Different exposition times of each blot were performed to ensure the linearity of the band intensities. Densitometric analysis of the bands was performed using a laser scanner, and the results were expressed in arbitrary units.

Measurement of IKK Activity. Cytosolic extracts from ~4×10^6 cells were centrifuged and homogenized in buffer A. protein extract (250 μg) was immunoprecipitated (IP) with 1 μg of anti–IKK-2 Ab (DiDonato et al., 1997; Castrillo et al., 2000). After extensive washing of the immunoprecipitant with buffer A, the pellet was resuspended in kinase buffer (20 mM HEPES, pH 7.4, 0.1 M EDTA, 100 mM NaCl, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml *N*-tosyl-L-lysine chloromethyl ketone, 5 mM NaF, 1 mM $NaVO_4$, 10 mM Na_2MoO_4, and 10 nM okadaic acid). The kinase activity of the IKK complex was assayed in 100 μl of buffer A containing 100 ng of IP protein, 1 mM MgATP, 5 mM $MgCl_2$, and, using as substrate, 100 ng of GST-IκBα (1–54). In some experiments, 50 μM [γ-^{32}P]ATP and MBP were used

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instead of cold ATP and GST-IκBα as substrates. GST-IκBα was purified by glutathione-agarose chromatography and analyzed in 10% SDS-PAGE. The linearity of the reaction was confirmed over a period of 30 min.

Determination of Metabolites. To determine the amount of NO released to the culture medium, nitrate was reduced to nitrite, and this was measured spectrophotometrically using Griess reagent, as described previously (Castrillo et al., 2000). TNF-α and PGE_2 levels were determined in the culture medium using a specific enzyme-immunoassay system, following the instructions of the manufacturer (Amersham).

Data Analysis. The number of experiments is indicated in the figures. Statistical differences ($P < 0.05$) between mean values were determined by one-way analysis of the variance followed by Student's t test.

Results

Inhibition of COX-2 and NOS-2 Expression by Supra-pharmacological Concentrations of Rofecoxib. Treatment of peritoneal macrophages with 10 μM rofecoxib decreased notably the protein levels of COX-2 and NOS-2 that were induced after 18 h of activation with LPS. This process was dependent on the dose of LPS used, and the inhibition was more potent at concentrations of LPS lower than 100 ng/ml (Fig. 1, A and B). Moreover, when the accumulation of nitrate plus nitrite in the culture medium was determined, a significant delay and attenuation in the synthesis of NO was observed (Fig. 1C). The synthesis of PGE_2 was completely abolished in the presence of rofecoxib. Analysis of NF-κB activity by EMSAs showed an important inhibition by 10 μM rofecoxib after activation of the macrophages for 30 min with LPS (Fig. 1D). The effect of rofecoxib on NF-κB activity was compared with that of other NSAIDs, and as Fig. 1E shows, the coxibs DFU and rofecoxib as well as salicylate at high concentrations notably inhibited this activation process. However, the NSAID indomethacin failed to influence NF-κB activation. To determine the step at which rofecoxib was interfering with early NF-κB activation, the cytosolic levels and phosphorylation state of IκBα were determined by Western blot using specific antibodies. As Fig. 2 shows, IκBα phosphorylation in S^{32} was observed after treatment of the cells with LPS, TNF-α, IL-1β, or a combination of these proinflammatory stimuli. Incubation of the cells with rofecoxib inhibited the specific phosphorylation and degradation of IκBα, and a parallel result was obtained in terms of the activation of NF-κB; IL-1β was the least potent stimulus among those assayed.

Rofecoxib Inhibits LPS-Dependent IKK Activation. To determine the range of concentrations of rofecoxib that inhibited NF-κB activity, the dose-dependence was analyzed. As Fig. 3A shows, 500 nM rofecoxib, a dose in the pharmacological range, attenuated significantly NF-κB activation at 30 min, as reflected by the decrease in the phosphorylation state and targeting of IκBα and the binding of NF-κB to the κB motif by EMSAs. However, rofecoxib alone failed to influence the binding of NF-κB (from LPS-activated cells) to the DNA probe in an in vitro assay (data not shown).

The impairment of IκBα phosphorylation by rofecoxib pointed to an inhibition of IKK activity. In agreement with this suggestion, when IKK was IP from cell cultures treated with LPS and rofecoxib, a clear inhibition in the capacity to phosphorylate in vitro MBP (Fig. 3B) and GST-IκBα (data not shown) was observed. Moreover, experiments in which activated IKK was treated in vitro with 1 to 100 μM rofecoxib showed a significant inhibition of the activity at 10 μM concentrations or higher, which suggests that IKK is a direct target of this drug, at least in vitro (Fig. 3C). In addition to the inhibition of the IKK/NF-κB pathway by rofecoxib, the effects of this drug on other early signaling pathways acti-

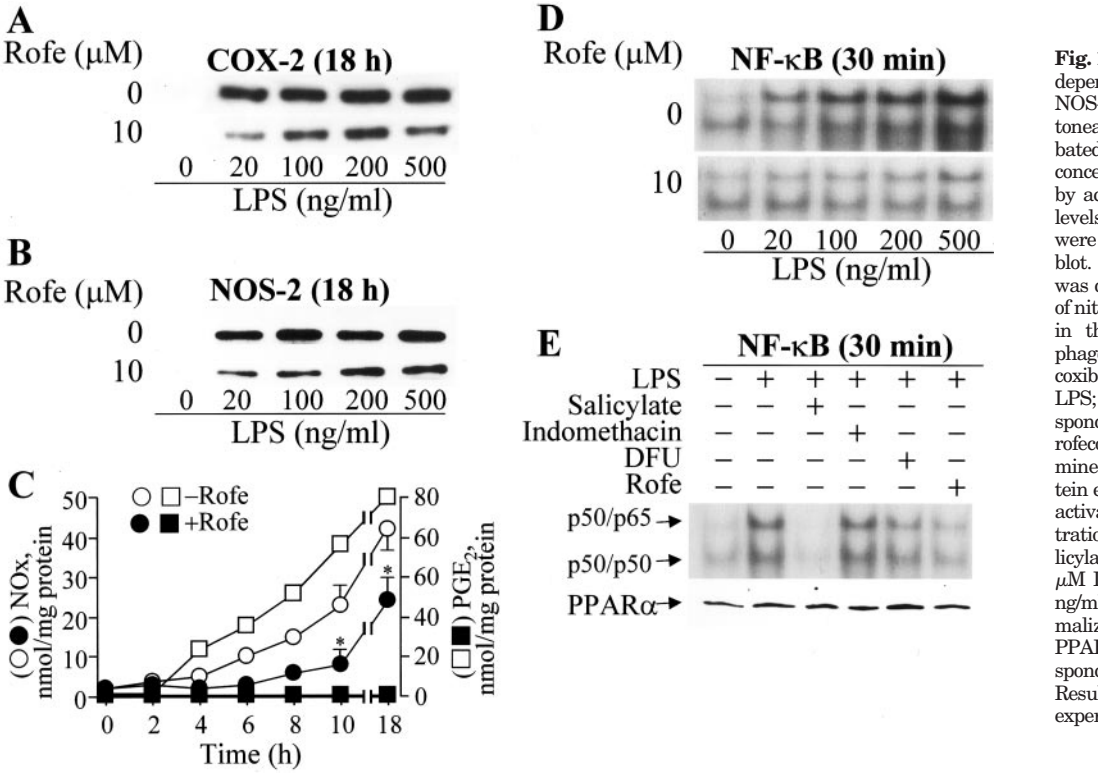

Fig. 1. Rofecoxib attenuates the LPS-dependent expression of COX-2 and NOS-2 in macrophages. Elicited peritoneal macrophages were preincubated for 30 min with the indicated concentrations of rofecoxib followed by activation with LPS. The protein levels of COX-2 (A) and NOS-2 (B) were determined at 18 h by Western blot. C, the synthesis of NO and PGs was determined by the accumulation of nitrate and nitrite (NOx) and PGE_2 in the culture medium of macrophages pretreated with 10 μM rofecoxib and activated with 200 ng/ml of LPS; *, $P < 0.01$ versus the corresponding condition in the absence of rofecoxib. NF-κB activity was determined by EMSAs using nuclear protein extracts prepared after 30 min of activation with the indicated concentrations of LPS (D) or with 1 mM salicylate, 100 μM indomethacin, 10 μM DFU, 10 μM rofecoxib, and 200 ng/ml of LPS (E). EMSAs were normalized by measuring the amount of PPARα by Western blot in the corresponding nuclear protein extracts. Results show a representative of five experiments.

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vated by LPS were investigated. As Fig. 4A shows, the phosphorylation of p44 ERK, p38 MAPK, and p46 JNK, which are rapid and transiently activated in response to LPS, was almost completely inhibited in cells treated with 10 μM rofecoxib, even at LPS concentrations of up to 500 ng/ml. The dose-dependent effect of rofecoxib on the phosphorylation of p44 ERK, p38 MAPK, and p46 JNK in LPS-activated macrophages is shown in Fig. 4B.

NF-κB Activation Is Delayed in Macrophages Treated with Rofecoxib. The results obtained in Fig. 1, A and B, indicate that rofecoxib attenuates significantly the expression of NOS-2 and COX-2 (measured after 18 h of treatment), although the inhibition of NF-κB was complete at least in samples obtained 30 min after LPS challenge. In addition to this, transfection of the macrophage cell line RAW 264.7 with a κB reporter gene revealed a 50% reduction in the luciferase activity when cells were incubated with 10 μM rofecoxib, indicating that NF-κB was active during the 18-h period of stimulation with LPS (Fig. 5A). Also, the release of TNF-α by LPS-activated macrophages measured in the cul-

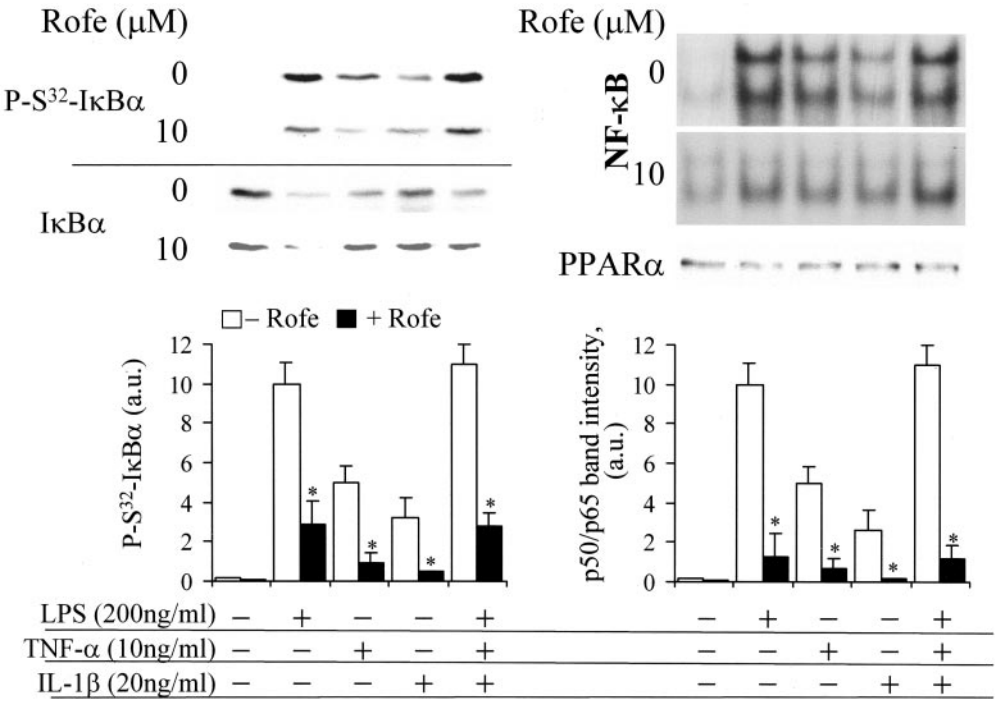

Fig. 2. Effect of rofecoxib on LPS, TNF-α, and IL-1β–dependent NF-κB activation. Macrophages were preincubated for 30 min with rofecoxib and activated for 30 min with the indicated concentrations of proinflammatory stimuli. The levels of phospho–S^{32}-IκBα and total IκBα were determined by Western blot using specific Abs. NF-κB activity was analyzed by EMSAs, and the measurement of the amount of PPARα by Western blot in the corresponding nuclear protein extracts was used to assess equal lane charge. Results show a representative blot from four experiments and the mean ± S.D. of the densitometry of the bands of the indicated conditions. *, $P < 0.01$ versus the corresponding condition in the absence of rofecoxib.

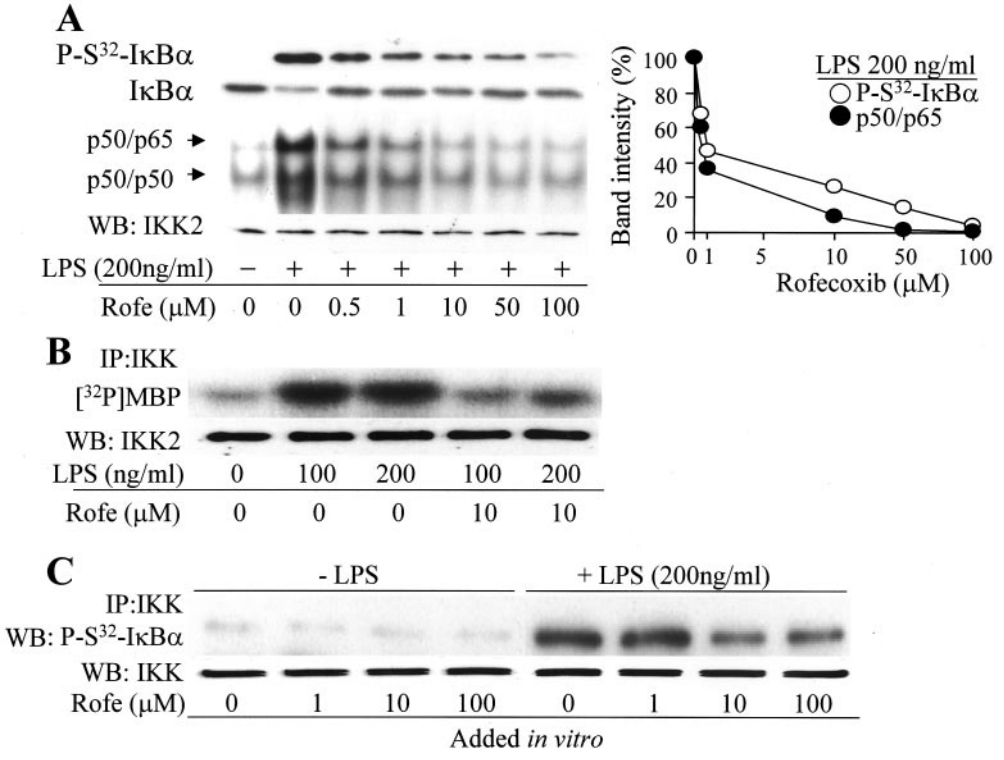

Fig. 3. Dose-dependent effect of rofecoxib on the IKK/NF-κB pathway. A, cells were pretreated for 30 min with the indicated concentrations of rofecoxib and activated for 30 min with 200 ng/ml of LPS. The levels of phospho–S^{32}-IκBα, IκBα, and NF-κB were determined as described in Fig. 2. B, IKK was immunoprecipitated with anti–IKK-2 Ab from extracts of cells treated for 20 min with the indicated stimuli, and the kinase activity was measured using MBP as substrate. A blot of IKK was used to evaluate the kinase content in the immunoprecipitate. C, the effect of rofecoxib on IKK activity in vitro was determined after immunoprecipitation of the complex from control and LPS-treated cells and using GST-IκBα as substrate. Results show a representative of four experiments, and the mean ± S.D. of the band intensities corresponding to the levels of phospho-IκBα and p50/p65 (100% corresponds to the absence of rofecoxib).

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ture medium at 8 h was inhibited 25% and 53% after treatment with 10 μM and 100 μM rofecoxib, respectively (Fig. 5B). Moreover, incubation of cells for 24 h with up to 100 μM rofecoxib or DFU did not affect cell viability and reduced significantly the LPS-induced apoptosis, which suggests that the inhibitory effects of rofecoxib on the expression of some genes cannot be attributed to a reduction in cell viability (Fig. 5C). Taking into account these data, one possible explanation for the expression of κB-dependent genes in the presence of rofecoxib is to consider a rapid degradation of this molecule in the culture medium. However, this was not the case; when the effect of this drug on NOS-2 expression and activity was investigated, similar results were obtained in terms of nitrite and nitrate synthesis in cells pretreated for 24 h with rofecoxib, before activation with LPS, or when rofecoxib was added again after 2 h of activation (Fig. 5D). To gain insight on the effect of rofecoxib on NF-κB activation, a time-course analysis was performed. As Fig. 6 shows, in the presence of 10 μM rofecoxib, NF-κB activation was delayed, and only after 2 h of LPS stimulation was a moderate NF-κB activity measured. This activity still persisted after 6 h, whereas in untreated cells the effect was overcome. The levels of IκBα and IκBβ were measured under these conditions, and although IκBα rapidly recovered after LPS challenge, the degradation and resynthesis of IκBβ paralleled the kinetics of the binding of nuclear extracts to the κB sequence.

Discussion

The clinical use of classic NSAIDs is often limited by side effects such as bleeding, gastrointestinal ulcers, and salt retention (DeWitt, 1999; McCarthy, 1999; Gupta and DuBois, 2000). These actions are believed to be caused by COX-1 inhibition (Williams and DuBois, 1996); therefore, selective inhibition of inducible COX-2 would provide more specific and beneficial anti-inflammatory and analgesic effects (Masferrer et al., 1994; Seibert et al., 1994). Accordingly, all selective COX-2 inhibitors cause significantly less gastrointestinal toxicity than general NSAIDs (Simon et al., 1998), and its anti-inflammatory and analgesic efficacies are comparable with that of nonselective NSAIDs such as naproxen and diclofenac (Maini et al., 1999). There is some controversy about the COX-2–independent effects of NSAIDs. Most of the studies use high concentrations of NSAIDs and selective COX-2 inhibitors (100–1000 μM) that are difficult to attain in humans without severe toxic effects. For example, it has been described that 50 mg/kg celecoxib significantly reduced the inflammatory paw edema, but doses of 100 to 200 mg/kg abolished this anti-inflammatory effect; however, in cultured rat renal mesangial cells, high concentrations of celecoxib activated NF-κB (Niederberger et al., 2001). Therapeutic administration of rofecoxib in adult human achieves a peak plasma concentration of 0.6 to 0.8 μM within 4 h after a single dose of 25 mg, and the half-life is between 10 and 17 h; the major route of elimination is via urine (Depre et al., 2000).

The current view from these studies is that NSAIDs cause anti-inflammatory and antiproliferative effects independent of COX-2 activity and prostaglandin synthesis through the inhibition of certain transcription factors such NF-κB and AP-1 (Niederberger et al., 2001). In this regard, it has been described that aspirin, salicylate, and other NSAIDs specifically inhibit IKK-2 activity in vitro and in vivo in several cancer cell lines. The mechanism implicates the interaction of these compounds with the ATP binding site of the enzyme (Yin et al., 1998). Moreover, preliminary results indicate that salicylate, in addition to inhibiting IKK activity, increases the degradation of IκBα by a mechanism independent of the 26S proteasome, whereas indomethacin and ibuprofen did not alter NF-κB–dependent expression (Rahman et al., 2000). In addition to this, it has been described that some NSAIDs, such as indomethacin and ibuprofen, are activators of PPARγ, a known repressor of the expression of genes that are dependent on NF-κB and AP-1 activation (Tegeder et al., 2001); however, there are no available reports about the effects of the selective inhibitors of COX-2 on this nuclear receptor.

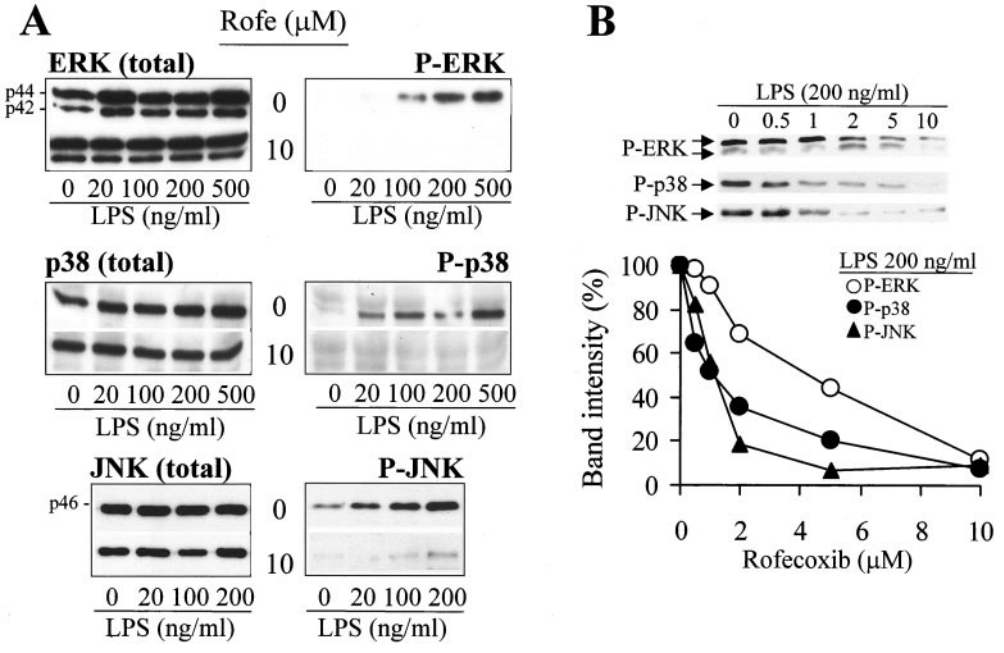

Fig. 4. Effect of rofecoxib on LPS-dependent MAPKs activation. Macrophages were treated for 20 min with the indicated stimuli, and the activation of p42 and p44 ERKs, p38 MAPK, and p46 JNK was determined by Western blot using specific anti–phospho-MAPKs Abs and anti–total-MAPKs Abs. A, results show a representative experiment from three experiments. B, the dose-dependent effect of rofecoxib on the activation of p44 ERK, p38 MAPK, and p46 JNK was determined, and the mean of the band intensities (n = 3) was plotted by considering 100% the band in the absence of rofecoxib.

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Our results demonstrate that rofecoxib assayed at 10 μM, or even at lower concentrations, inhibits IKK activity in peritoneal macrophages both in vivo and in vitro. This inhibition occurs when cells are challenged with either LPS or TNF-α, which suggests that rofecoxib interferes with a common point in the signaling pathways activated by these stimuli. This inhibition of IKK impairs NF-κB activity, but it does so through a mechanism that delays the response by ~2 h, in addition to requiring a lower degree of activation. To our knowledge, this is the first description of an attenuated and delayed response in terms of NF-κB activation mediated by rofecoxib and opens new perspectives for the use of these drugs. The levels of IκBα seem to be quite elevated at the times of sampling considered (up to 6 h), and the possibility exists that IκBβ might mediate, at least in part, this response, as described for NF-κB activation, in other cells (Velasco et al., 1997; Ghosh et al., 1998). Moreover, this alteration in the pattern of temporal events might contribute to the attenuation in the expression of genes that mediate the inflammatory response, such as NOS-2 and COX-2. Rofecoxib, in contrast to other NSAIDs such as NS398 and indomethacin, did not up-regulate but actually decreased COX-2 protein levels when cells were treated with pro-inflammatory stimuli, therefore preventing an overproduction of prostaglandins when the drug is cleared and contributing to its anti-inflammatory effects. In addition, it seems that the actions of the coxibs are cell-specific, because in hepatocytes, these drugs failed to influence NF-κB activity, and in mesangial cells, at higher concentrations, they increased this response (Callejas et al., 2001; Niederberger et al., 2001). In macrophages, the NF-κB complexes present in the nucleus in response to cell stimulation with LPS and pro-inflammatory cytokines are composed mainly of p50 and p65 subunits (Thanos and Maniatis, 1995; Ghosh et al., 1998), and this has been confirmed consistently by supershift assays (data not shown). In this regard, it has been shown that the complexes that mediate the transcriptional activity of NF-κB may vary along the time, affecting the rate of transcription of the target genes (Saccani et al., 2001). This activation of NF-κB requires phosphorylation by IKK of IκB proteins in specific serine residues (S^{32} and S^{36}) that target these proteins for ubiquitin conjugation and degradation by the 26S proteasome (May and Ghosh, 1998). Two IKKs (1 and 2) and a third essential component (NEMO) constitute the IKK complex. Despite the sequence similarity, they have different functions (Zandi et al., 1997; Karin, 1999): IKK-1 participates in differentiation of various cell types, whereas IKK-2 is involved in the response to proinflammatory stimuli.

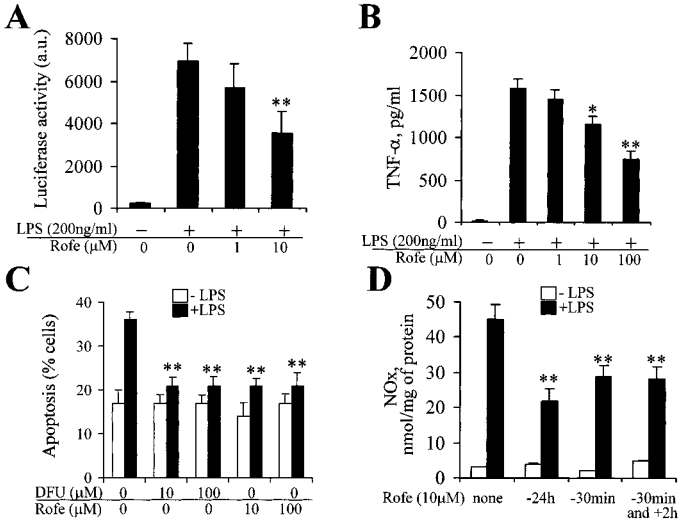

Fig. 5. Effect of rofecoxib on the activation of NF-κB, release of TNF-α and NO, and apoptosis in macrophages. A, RAW 264.7 cells were transfected with a κB-dependent reporter gene, and the corresponding luciferase activity was determined after 18 h of treatment with the indicated stimuli. B, peritoneal macrophages were treated as indicated, and the accumulation of TNF-α was determined in the culture medium after 8 h of activation. C, the percentage of apoptotic cells was determined 24 h postactivation with LPS. D, the release of nitrate plus nitrite was determined in cells pretreated or with additional treatment of rofecoxib at the indicated times and activated with LPS for 18 h (0 time corresponds to LPS challenge). Results show the mean ± S.D. of four experiments. *, $P < 0.05$, **, $P < 0.01$ versus the condition in the absence of rofecoxib.

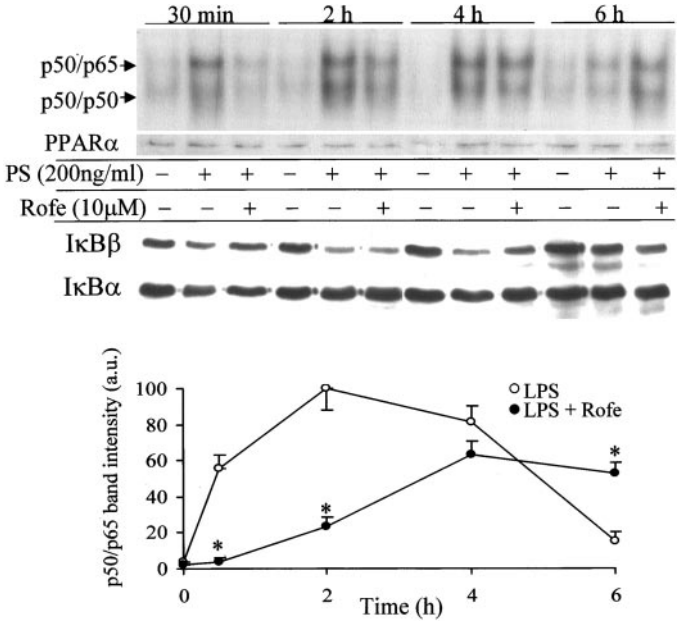

Fig. 6. Delayed NF-κB activation in macrophages treated with rofecoxib. Cells were treated with rofecoxib and LPS, and at the indicated times soluble and nuclear protein extracts were prepared. The activation of NF-κB was determined by EMSAs, and the levels of IκBα and IκBβ were determined by Western blotting the soluble extracts. The band intensities corresponding to the p50/p65 heterodimers were quantified, and the mean value ± S.D. ($n = 3$) was plotted. *, $P < 0.05$ versus the condition in the absence of rofecoxib.

Our data also show that rofecoxib inhibits the LPS-dependent activation of MAPKs. The phosphorylation of p44 ERK and p38 is completely abolished after treatment with 10 μM rofecoxib, and the same occurs for JNK. It is noteworthy that some of the effects of rofecoxib are observed at quite low concentrations (2–10 μM), whereas the amounts of other NSAIDs (e.g., salicylate) required to reach similar results are at least two orders of magnitude higher (Pillinger et al., 1998; Schwenger et al., 1998). The mechanism by which rofecoxib impaired MAPK activation in LPS-activated macrophages deserves further study, but previous work demonstrated that ERK activation in epithelial cells in response to endothelial growth factor and hepatocyte growth factor was inhibited by coxibs (Jones et al., 1999; Baatar et al., 2002).

Finally, the delayed activation of NF-κB in macrophages treated with rofecoxib cannot be attributed to a rapid degradation of the drug, but it is probably caused by the action on different targets, including IKK and MAPKs, which act as early pacemakers of the LPS-dependent signaling pathway.

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In summary, our data indicate that rofecoxib assayed at 10 μM, in addition to the inhibition of COX-2–dependent PG synthesis, exerts a moderate but significant attenuation of macrophage activation in response to various proinflammatory stimuli, and it does so through an unusual mechanism that involves a delayed and attenuated NF-κB activation and impairment of MAPKs stimulation. These data describe an additional anti-inflammatory mechanism of selective cyclooxygenase-2 inhibitors through the attenuation of macrophage activation.

References

Baatar D, Jones MK, Pai R, Kawanaka H, Szabo IL, Moon WS, Kitano S, and Tarnawski AS (2002) Selective cyclooxygenase-2 blocker delays healing of esophageal ulcers in rats and inhibits ulceration-triggered C-met/hepatocyte growth factor receptor induction and extracellular signal-regulated kinase 2 activation. *Am J Pathol* **160:**963–972.

Callejas NA, Bosca L, Williams CS, DuBois RN, and Martin-Sanz P (2000) Regulation of cyclooxygenase-2 expression in hepatocytes by CCAAT-enhancer binding proteins. *Gastroenterology* **119:**493–501.

Callejas NA, Casado M, Bosca L, and Martin-Sanz P (2002) Absence of nuclear factor κB inhibition by NSAIDs in hepatocytes. *Hepatology* **35:**341–348.

Callejas NA, Casado M, Diaz-Guerra MJ, Bosca L, and Martin-Sanz P (2001) Expression of cyclooxygenase-2 promotes the release of matrix metalloproteinase-2 and -9 in fetal rat hepatocytes. *Hepatology* **33:**860–867.

Castrillo A, Diaz-Guerra MJ, Hortelano S, Martin-Sanz P, and Bosca L (2000) Inhibition of IκB kinase and IκB phosphorylation by 15-deoxy-$\Delta^{12,\ 14}$-prostaglandin J_2 in activated murine macrophages. *Mol Cell Biol* **20:**1692–1698.

Chan CC, Boyce S, Brideau C, Charleson S, Cromlish W, Ethier D, Evans J, Ford-Hutchinson AW, Forrest MJ, Gauthier JY, et al. (1999) Rofecoxib [Vioxx, MK-0966; 4-(4′-methylsulfonylphenyl)-3-phenyl-2-(5H)-furanone]: a potent and orally active cyclooxygenase-2 inhibitor. Pharmacological and biochemical profiles. *J Pharmacol Exp Ther* **290:**551–560.

Depre M, Ehrich E, Van Hecken A, De L, I, Dallob A, Wong P, Porras A, Gertz BJ, and De Schepper PJ (2000) Pharmacokinetics, COX-2 specificity and tolerability of supratherapeutic doses of rofecoxib in humans. *Eur J Clin Pharmacol* **56:**167–174.

DeWitt DL (1991) Prostaglandin endoperoxide synthase: regulation of enzyme expression. *Biochim Biophys Acta* **1083:**121–134.

DeWitt DL (1999) Cox-2-selective inhibitors: the new super aspirins. *Mol Pharmacol* **55:**625–631.

Diaz-Guerra MJM, Castrillo A, Martin-Sanz P, and Bosca L (1999) Negative regulation by protein tyrosine phosphatase of IFN-γ-dependent expression of inducible nitric oxide synthase. *J Immunol* **162:**6776–6783.

DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, and Karin M (1997) A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. *Nature (Lond)* **388:**548–554.

Feng L, Xia Y, Garcia GE, Hwang D, and Wilson CB (1995) Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-α and lipopolysaccharide. *J Clin Investig* **95:**1669–1675.

Ghosh S, May MJ, and Kopp EB (1998) NF-κB and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* **16:**225–260.

Gupta RA and DuBois RN (2000) Combinations for cancer prevention. *Nat Med* **6:**974–975.

Jones MK, Wang H, Peskar BM, Levin E, Itani RM, Sarfeh IJ, and Tarnawski AS (1999) Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. *Nat Med* **5:**1418–1423.

Karin M (1999) The beginning of the end: IκB kinase (IKK) and NF-κB activation. *J Biol Chem* **274:**27339–27342.

Kim TH, Mars WM, Stolz DB, and Michalopoulos GK (2000) Expression and activation of pro-MMP-2 and pro-MMP-9 during rat liver regeneration. *Hepatology* **31:**75–82.

Langenbach R, Loftin CD, Lee C, and Tiano H (1999) Cyclooxygenase-deficient mice. A summary of their characteristics and susceptibilities to inflammation and carcinogenesis. *Ann NY Acad Sci* **889:**52–61.

Maini R, St. Clair EW, Breedveld F, Furst D, Kalden J, Weisman M, Smolen J, Emery P, Harriman G, Feldmann M, et al. (1999) Infliximab (chimeric anti-tumour necrosis factor alpha monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. ATTRACT study group. *Lancet* **354:**1932–1939.

Masferrer JL, Zweifel BS, Manning PT, Hauser SD, Leahy KM, Smith WG, Isakson PC, and Seibert K (1994) Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. *Proc Natl Acad Sci USA* **91:**3228–3232.

May MJ and Ghosh S (1998) Signal transduction through NF-κB. *Immunol Today* **19:**80–88.

McCarthy DM (1999) Comparative toxicity of nonsteroidal anti-inflammatory drugs. *Am J Med* **107:**37S–46S.

Niederberger E, Tegeder I, Vetter G, Schmidtko A, Schmidt H, Euchenhofer C, Bräutigam L, Grosch S, and Geisslinger G (2001) Celecoxib loses its anti-inflammatory efficacy at high doses through activation of NF-κB. *FASEB J* **15:**1622–1624.

Pilbeam CC, Kawaguchi H, Hakeda Y, Voznesensky O, Alander CB, and Raisz LG (1993) Differential regulation of inducible and constitutive prostaglandin endoperoxide synthase in osteoblastic MC3T3–E1 Cells. *J Biol Chem* **268:**25643–25649.

Pillinger MH, Capodici C, Rosenthal P, Kheterpal N, Hanft S, Philips MR, and Weissmann G (1998) Modes of action of aspirin-like drugs: salicylates inhibit Erk activation and integrin-dependent neutrophil adhesion. *Proc Natl Acad Sci USA* **95:**14540–14545.

Rahman MA, Dhar DK, Masunaga R, Yamanoi A, Kohno H, and Nagasue N (2000) Sulindac and exisulind exhibit a significant antiproliferative effect and induce apoptosis in human hepatocellular carcinoma cell lines. *Cancer Res* **60:**2085–2089.

Riendeau D, Percival MD, Boyce S, Brideau C, Charleson S, Cromlish W, Ethier D, Evans J, Falgueyret JP, Ford-Hutchinson AW, et al. (1997) Biochemical and pharmacological profile of a tetrasubstituted furanone as a highly selective COX-2 inhibitor. *Br J Pharmacol* **121:**105–117.

Saccani S, Pantano S, and Natoli G (2001) Two waves of nuclear factor κB recruitment to target promoters. *J Exp Med* **193:**1351–1359.

Schwenger P, Alpert D, Skolnik EY, and Vilcek J (1998) Activation of P38 mitogen-activated protein kinase by sodium salicylate leads to inhibition of tumor necrosis factor-induced IκB alpha phosphorylation and degradation. *Mol Cell Biol* **18:**78–84.

Seibert K, Zhang Y, Leahy K, Hauser S, Masferrer J, Perkins W, Lee L, and Isakson P (1994) Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc Natl Acad Sci USA* **91:**12013–12017.

Simon LS, Lanza FL, Lipsky PE, Hubbard RC, Talwalker S, Schwartz BD, Isakson PC, and Geis GS (1998) Preliminary study of the safety and efficacy of SC-58635, a novel cyclooxygenase 2 inhibitor: efficacy and safety in two placebo-controlled trials in osteoarthritis and rheumatoid arthritis and studies of gastrointestinal and platelet effects. *Arthritis Rheum* **41:**1591–1602.

Smith WL, Garavito RM, and DeWitt DL (1996) Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J Biol Chem* **271:**33157–33160.

Tegeder I, Pfeilschifter J, and Geisslinger G (2001) Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J* **15:**2057–2072.

Terenzi F, Diaz-Guerra MJ, Casado M, Hortelano S, Leoni S, and Bosca L (1995) Bacterial lipopeptides induce nitric oxide synthase and promote apoptosis through nitric oxide-independent pathways in rat macrophages. *J Biol Chem* **270:**6017–6021.

Thanos D and Maniatis T (1995) NF-KB: a lesson in family values. *Cell* **80:**529–532.

Thun MJ, Namboodiri MM, and Heath CWJ (1991) Aspirin use and reduced risk of fatal colon cancer. *N Engl J Med* **325:**1593–1596.

Tsujii M, Kawano S, and DuBois RN (1997) Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc Natl Acad Sci USA* **94:**3336–3340.

Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, and DuBois RN (1998) Cyclooxygenase regulates angiogenesis induced by colon cancer cells. *Cell* **93:**705–716.

Velasco M, Diaz-Guerra MJ, Martin-Sanz P, Alvarez A, and Bosca L (1997) Rapid up-regulation of IκBβ and abrogation of NF-KB activity in peritoneal macrophages stimulated with lipopolysaccharide. *J Biol Chem* **272:**23025–23030.

Williams CS and DuBois RN (1996) Prostaglandin endoperoxide synthase: why two isoforms? *Am J Physiol* **270:**G393–G400.

Williams CS, Mann M, and DuBois RN (1999) The role of cyclooxygenases in inflammation, cancer and development. *Oncogene* **18:**7908–7916.

Yin MJ, Yamamoto Y, and Gaynor RB (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-β. *Nature (Lond)* **396:**77–80.

Zandi E, Rothwarf DM, Delhase M, Hayakawa M, and Karin M (1997) The IκB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IκB phosphorylation and NF-KB activation. *Cell* **91:**243–252.

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