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Original Research Communication

Inhibition of Nuclear Factor-κB by a Nitro-Derivative of Flurbiprofen: A Possible Mechanism for Antiinflammatory and Antiproliferative Effect

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ABSTRACT

Nonsteroidal antiinflammatory drugs (NSAIDs) are widely used in the treatment of chronic inflammatory states. In addition, they show promise for the prevention and therapy of colon cancers and of Alzheimer's disease, although their gastrointestinal toxicity is of concern for these indications. Nitric oxide-releasing NSAIDs are reported to be safer than their parent compounds. We report here that flurbiprofen nitroxybutyl ester inhibits nuclear factor-κB (NF-κB) activity and cell growth in L929 cells at a concentration of 100 μ*M*, whereas flurbiprofen is inactive. Inhibition of cell growth is not due to the induction of apoptosis, but to a retardation of all phases of the cell cycle. NF-κB is implicated both in the control of immune and inflammatory response and in the control of cell proliferation and apoptosis. Therefore, its inhibition at low concentrations by an NSAID with low gastrointestinal toxicity could be important for all the above-mentioned therapeutic indications. *Antioxid. Redox Signal.* 5, 229–235.

INTRODUCTION

Nonsteroidal antiinflammatory drugs (NSAIDs) are widely used in the therapy of various inflammatory diseases. Based on several observations in experimental animal models and from epidemiological studies, their use has also been suggested for chemoprevention of colorectal cancer (11, 12, 22, 25).

The current theory is that the mechanism for the suppressor effect on carcinogenesis is cyclooxygenase (COX)-2 inhibition. However, other targets are likely to be involved. For instance, the nuclear factor-κB (NF-κB) pathway, in addition to activating the expression of genes involved in the control of the immune and inflammatory response, is also a key mediator in the control of cellular proliferation and apoptosis (3). Constitutive NF-κB activation is likely involved in the enhanced growth properties seen in a variety of cancers (2). Moreover, both COX-2 and the inducible isoform of nitric oxide synthase (iNOS) are overexpressed in colonic tumors (1, 6), and both these proteins are induced by the activation of NF-κB (10, 16, 29).

Actually, aspirin, sodium salicylate, and other NSAIDs have been shown to inhibit NF-κB, independently of their ability to block COXs (21, 26, 30, 31), although at concentrations that would show, *in vivo*, significant gastrointestinal toxicity.

We report here that a nitroderivative of flurbiprofen [flurbiprofen nitroxybutyl ester (NO-flurbiprofen)] inhibits NF-κB activation and cell growth in murine fibroblasts (L929 cells), whereas the parent compound is inactive.

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MATERIALS AND METHODS

Drugs and reagents

NO-flurbiprofen (Nicox, Sophia Antipolis, France) and flurbiprofen were dissolved in dimethyl sulfoxide (DMSO) at the concentration of 27.6 m*M*, and the following dilutions were made in medium. In all the experiments, parallel controls containing the same amount of DMSO were run.

(Hydroxynitrosohydrazino)*bis*-ethanamine (DETA/NO) was from Calbiochem (Darmstadt, Germany). All other reagents, unless otherwise indicated, were from Sigma (St. Louis, MO, U.S.A.).

Derivation of an NF-κB-CAT reporter gene cell line

The plasmid J16 containing two copies of the NF-κB binding site GGGACTTTCC upstream of a truncated c-fos promotor, including only 56 bp upstream from the transcription start site and linked to the chloramphenicol acetyltransferase (CAT) gene, has been described previously (15, 20). Stable co-transfection of L929 cells with the selection plasmid pSV2neo and a 10-fold excess of the reporter plasmid J16 was performed by the calcium phosphate procedure. Transfected cells were selected in 400 μg/ml G418 for 2 weeks, after which individual colonies were isolated and expanded. These clones were then characterized for CAT expression and tumor necrosis factor (TNF) inducibility in a CAT enzymatic assay (see below). A clone with the highest TNF response and a relatively high basal expression of CAT was chosen for further work (J16 clone).

Cell culture

L929 cells, wild type (from ATCC) and J16 clone (L929/J16), were maintained in a humidified incubator under an atmosphere of 5% CO_2 in air, in RPMI 1640 (Seromed, Berlin, Germany) supplemented with 2 m*M* L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate (all from GIBCO, Paisley, Scotland, U.K.), 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT, U.S.A.), 1.5 g/L $NaHCO_3$, and 12 m*M* HEPES buffer (Carlo Erba, Milan, Italy). L929 cells were plated at a density of 1.5×10^5/ml in 96-well plates, 100 ml/well for the cell growth experiments, or in T75 flasks (Falcon Becton Dickinson, Lincoln Park, NJ, U.S.A.), 15 ml/flask for NF-κB activation experiments. Twenty-four hours later, they were washed with fresh medium and incubated with the indicated drugs or vehicle for 30 min, then with human recombinant TNFα (100 ng/ml) or saline. Cell viability was determined by trypan blue exclusion of trypsin-detached cells or by measuring optical density at 540 nm on an automated microplate reader (Perkin Elmer, Wilton, CT, U.S.A.) after crystal violet staining of adhering cells.

CAT assay

L929/J16 cells were used for the NF-κB directed gene synthesis, they were plated at the density of 1.5×10^5/ml in 96-well plates, 100 ml/well, and treated as for cell growth experiments. At the end of the stimulation period, cells were lysed by subjecting to three cycles of freeze-thawing in their incubation medium. CAT activity was then measured by adding 40 μl of cell extract to 40 μl of Tris-HCl buffer (250 m*M*, pH 7.5), and D-threo-[dichloroacetyl1,2-^{14}C) chloramphenicol (10 μl, 8.3×10^{-2} mCi/sample; Amersham, Little Chalfont, U.K.). The reaction was started by the addition of *n*-butyryl coenzyme A (10 μl, 2.5 m*M*) as a substrate instead of acetyl coenzyme A, for improved phase separation (32). After 2 h at 37°C, the reaction was stopped by extraction with hexane/xylene (2:1). The upper organic phase containing the reaction product was then recovered, and radioactivity was counted on a β-counter. Intraassay variability was extremely low (<5%). Therefore, we pooled samples from triplicate wells and tested them in single determinations.

Electrophoretic mobility shift assay (EMSA)

L929 cells were treated as indicated above. Two hours after TNF addition cells ($2–5 \times 10^6$/flask) were washed twice with phosphate-buffered saline (PBS) and resuspended in 100 μl of ELB buffer (50 m*M* HEPES, 250 m*M* NaCl, 5 m*M* EDTA, and an antiprotease cocktail—Complete; Boehringer Mannheim, Mannheim, Germany). After addition of NP40 to a final concentration of 0.1%, they were incubated on ice for 5 min and centrifuged at 13,000 rpm for 5 min (Heraeus, Handu, Germany). The supernatant was then recovered, aliquoted, and stored at −80°C. The oligonucleotide used contained tandemly repeated NF-κB sites identical to those of the human immunodeficiency virus type 1 long terminal repeat (5′-GCTTGCTACAAGGGACTTTCCGCTGGGGACTTTCC-3′ underlined are the NF-κB binding sequences) and was end-labeled with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Promega, Madison, WI, U.S.A.) and purified by Sephadex G-50 Fine column (Boehringer Mannheim). Cell lysate proteins (30 mg) were added to ^{32}P-labeled NF-κB oligonucleotide and 1 mg of poly[dI-dC] (Pharmacia Biotech, Uppsala, Sweden) in 15 ml of binding reaction buffer (40 m*M* Tris, pH 7.5, 120 m*M* KCl, 8% Ficoll, 4 m*M* EDTA, 1 m*M* dithiothreitol, and 10% glycerol) for 20 min at room temperature. The specificity of binding was also checked by competition (1,000-fold molar excess) with two unlabeled oligonucleotides [the same used for binding and the 23-mer corresponding to the NF-κB site at position −96 of the PTX3 promoter and its flanking sequences 5′-AATTCAGGGGAACTCCCGTTACC-3′ (4)], and by supershift of the band with an antibody against p65 protein (kindly provided by Dr. Nancy Rice, Frederick, MD, U.S.A.). The resulting DNA/protein complexes were separated from the free probe by electrophoresis in 5% native polyacrylamide gel in 0.5% Tris-buffered EDTA at 150 V for 2 h. The gels were dried and autoradiographed with intensifying screen at −80°C for 18 h.

Nuclear morphology

L929 cells were plated at the density of 1.5×10^5/ml in six-well plates, 3 ml/well; 24 h later, the indicated drugs were added. Nuclear morphology was examined after 8 and 24 h of incubation, as previously described (9). In brief, cells (detached with trypsin treatment and resuspended at the density of 2×10^5/ml) were seeded on microscope slides (100 μl) by cytospin centrifugation (Heraeus) at 500 rpm for 10 min, fixed in Carnoy solution (methanol/acetic acid, 3:1) for 1 h, stained with Hoechst 33258 (0.1 μg/ml in PBS) for 1 h at 37°C, washed with tap water for 1 h, air-dried, and mounted.

Slides were observed with a fluorescence microscope (Zeiss, Oberkochen, Germany) at an excitation wavelength of 365 nm.

Cell cycle: monoparametric DNA analysis

Exponentially growing L929 cellswere treated for 6, 24, 48, and 72 h with 100 m*M* NO-flurbiprofen. At the end of the incubation, the number of cells was evaluated by counting on a hemocytometer, and the cells were fixed in 70% ethanol and kept at 4°C before DNA staining . The cells ($1–2 \times 10^6$) were washed with PBS and resuspended in 2 ml of PBS containing 25 µg/ml propidium iodide plus 25 µl of RNase 10,000 units (1 mg/ml) for at least 4 h. DNA histogram analysis was done on at least 20,000 cells for each sample by using a FacsCalibur instrument (Becton Dickinson, Sunnyvale, CA, U.S.A.). The percentages of the cell cycle phase distribution were calculated by the ModFit analysis (Becton Dickinson) (7).

RESULTS

NO-flurbiprofen inhibits NF-κB directed gene synthesis

The effect of NO-flurbiprofen on NF-κB activation was first tested in murine fibroblast cells (L929 cells) that were transfected with CAT reporter constructs, driven by a truncated c-fos promoter under the control of two NF-κB binding sites. Transfected cells (L929/J16 cells) were treated with NO-flurbiprofen or flurbiprofen, in the presence or in the absence of human recombinant TNF, and transactivation was determined by CAT enzymatic determination. Figure 1A shows the dose–response curve of NO-flurbiprofen inhibition of basal and TNF-stimulated CAT synthesis. Maximal inhibition was afforded at the concentration of 100 µ*M*, whereas higher NO-flurbiprofen concentrations were less effective. The percent inhibition of basal and TNF-stimulated CAT synthesis was comparable. The results of several experiments with 100 µ*M* NO-flurbiprofen, compared with the same concentration of the parent compound, flurbiprofen, are summarized in Fig. 1B. NO-flurbiprofen inhibited TNF-stimulated CAT synthesis by 40%, whereas flurbiprofen did not. In some experiments, flurbiprofen was also tested at higher concentrations (up to 300 µ*M*), and it never affected either basal or TNF-stimulated CAT synthesis (data not shown).

In J32 cells, which were transfected with a mutated, inactive NF-κB site, basal and TNF-stimulated CAT activities were identical. Moreover, no changes were seen in the presence of NO-flurbiprofen or flurbiprofen (data not shown), demonstrating that NO-flurbiprofen inhibition of CAT synthesis in J16 cells is not due to the inhibition of protein synthesis. This also suggests that CAT activity observed in L929/J16 cells in the absence of TNF, which was inhibited by NO-flurbiprofen, is due at least in part to a basal NF-κB activation.

Inhibition of NF-κB activation by NO-flurbiprofen

To relate the results from the NF-κB directed gene synthesis experiments to the effect of NO-flurbiprofen on NF-κB activation and binding, we ran EMSA. Consistent with the results from the reporter assays, the κB binding activity was inhibited in the cells treated with NO-flurbiprofen, but not in those treated with flurbiprofen (Fig. 2A). Neither NO-flurbiprofen nor flurbiprofen, at the concentration of 100 µ*M*, inhibited the formation of NF-κB–DNA complex, when added directly during the binding reaction of TNF-treated cell lysates to DNA (data not shown). The EMSA experiment demonstrates that the inhibition of NF-κB occurs before the binding to DNA of the κB activating complex.

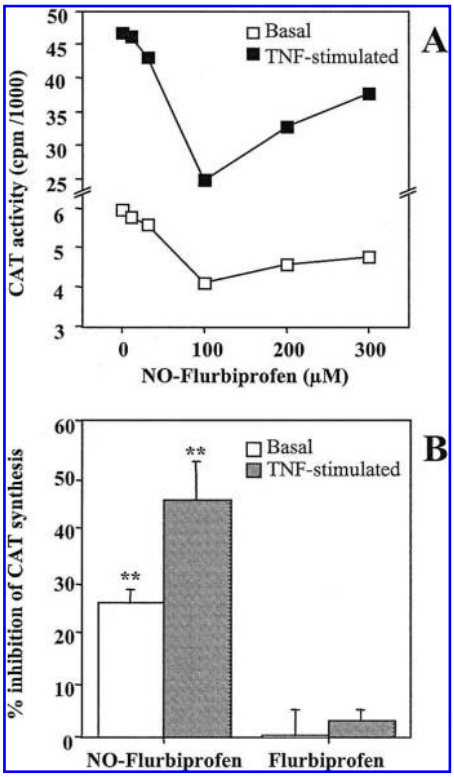

FIG. 1. Inhibition of NF-κB activity by NO-flurbiprofen, as assessed by a reporter gene synthesis. (**A**) L929/J16 cells were incubated with different concentrations of NO-flurbiprofen, either in the presence of 100 ng/ml TNF (Black squares) or with vehicle (Open squares) for 18 h at 37°C; then CAT activity was measured in the cell lysates with a radiometric assay. DMSO concentration was 0.36% in samples with up to 100 µ*M* NO-flurbiprofen, and 0.72 and 1.09% in samples with 200 µ*M* and 300 µ*M* NO-flurbiprofen, respectively. Basal and TNF-stimulated CAT activity in the absence of DMSO was 5,172 and 42,377 cpm, respectively. The effect of the different DMSO concentrations (0.36–1.09%) differed by <6%. Flurbiprofen was tested at concentrations up to 300 µ*M*, and never changed CAT activity (data not shown). (**B**) Inhibition of TNF-stimulated CAT synthesis by NO-flurbiprofen, but not by flurbiprofen. Shown are the means ± SEM of the results of seven independent experiments. Both compounds were used at 100 µ*M* concentration. **Significantly different from zero, $p < 0.001$, Student's *t* test for the comparison of a mean to a constant value.

NO-flurbiprofen inhibits L929 cell growth

On the same cells that were used for the NF-κB activation experiments, we studied the effect on cell proliferation and death. NO-flurbiprofen, but not flurbiprofen, dose-dependently inhibited the growth of L929 cells, when they were

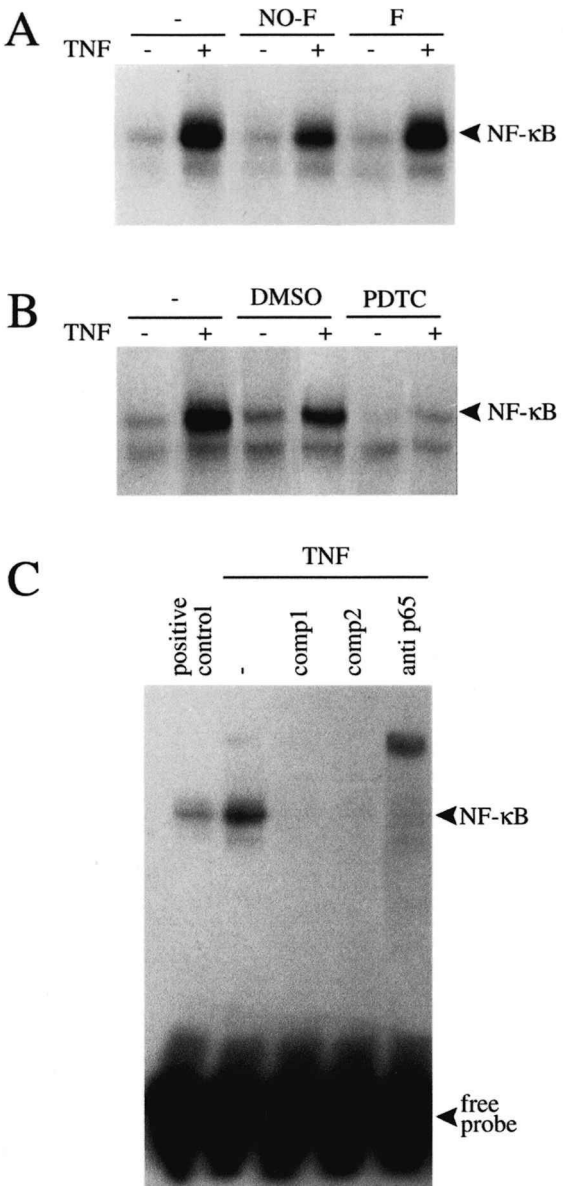

FIG. 2. Inhibition of the activation of NF-κB upon TNF treatment. Cell extracts were prepared from L929 cells treated for 4 h either with or without 100 ng/ml TNF, in the presence of the following substances: (**A**) 100 m*M* NO-flurbiprofen (NO-F) or 100 μ*M* flurbiprofen (F); (**B**) 100 μ*M* PDTC or 10% DMSO. (**C**) Specificity controls: competition and supershift analysis. Lane 1: fibrosarcoma cells (8387) stimulated with TNF (positive control) (4); lanes 2–5: lysate from TNF-stimulated L929 cells, incubated with the labeled NF-κB oligonucleotide, either alone (lane 2) or in the presence of 1,000-fold molar excess of the same unlabeled specific oligonucleotide (comp1), or another oligo corresponding to the NF-κB site at position −96 of the PTX3 promoter (see Materials and Methods; comp2), and anti-p65 antibody (anti p65).

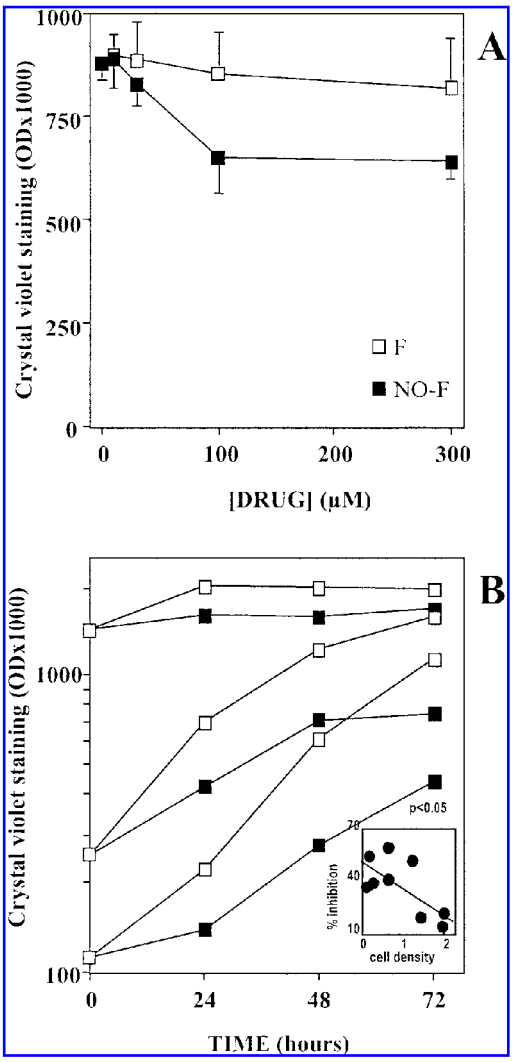

FIG. 3. Inhibition of cell growth. L929 cells were incubated with different concentrations of NO-flurbiprofen (NO-F) or flurbiprofen (F) for 24 h (**A**) or with 100 μ*M* NO-flurbiprofen (black squares) or vehicle (open squares) for different times, at different cell densities (**B**). Cells were seeded at the concentration of 15×10^4 cells/well in A, or at 8×10^4, 15×10^4, and 80×10^4 cells/well in B, and NO-flurbiprofen was added 4 h later, when all cells were adhering to the substrate (time 0). In all the experiments, parallel samples with the same concentrations of flurbiprofen were run, and never showed any effect of this drug on cell growth. The **inset** in B shows the correlation between cell growth inhibition and initial cell density. The line of best fit ($r = 0.699$) was obtained by linear regression analysis.

plated at an intermediate density (Fig. 3A). Inhibition was maximal at the concentration of 100 μ*M*. Cell growth inhibition was higher when the cells were exponentially growing, and virtually absent when they were almost confluent (Fig. 3B). The inset shows a significant correlation between cell density at a given time and percent growth inhibition observed 24 h later.

NO-flurbiprofen does not induce cell death and retards growth by slowing all cell cycle phases

In some experiments, cell number and viability were determined by trypan blue exclusion. This confirmed the results reported in Fig. 3, and showed that cell viability was >95% up to 3 days after plating, with any of the compounds tested. Moreover, nuclear morphology as assessed with the nuclear

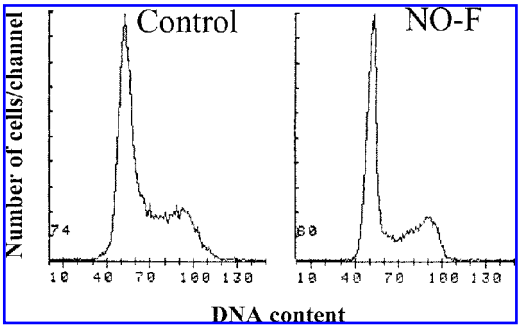

FIG. 4. Cell cycle analysis of L929 cells following a 24-h NO-flurbiprofen incubation. Phase percentages derived with the S rectangle method were: 50.5 G_1, 41.5 S, 8.0 G_2M for control, and 56.0 G_1, 31.0 S, 13.0 G2M for NO-flurbiprofen-treatedcells.

dye Hoechst 33258, never revealed any sign of apoptosis after up to 3 days of incubation with 100 μ*M* NO-flurbiprofen (data not shown).

We also did cell cycle analysis (Fig. 4). No signs of cells with hypodiploid DNA content were seen after 24 hours of treatment (Fig. 4), nor at longer times (up to 3 days; data not shown), demonstrating that no dying cells or debris was present. Additionally, we showed no significant changes in the distribution of cells in the different phases of cell cycle, except a little decrease in S phase. We suggest therefore that NO-flurbiprofen inhibits cell growth by retarding all phases of the cell cycle.

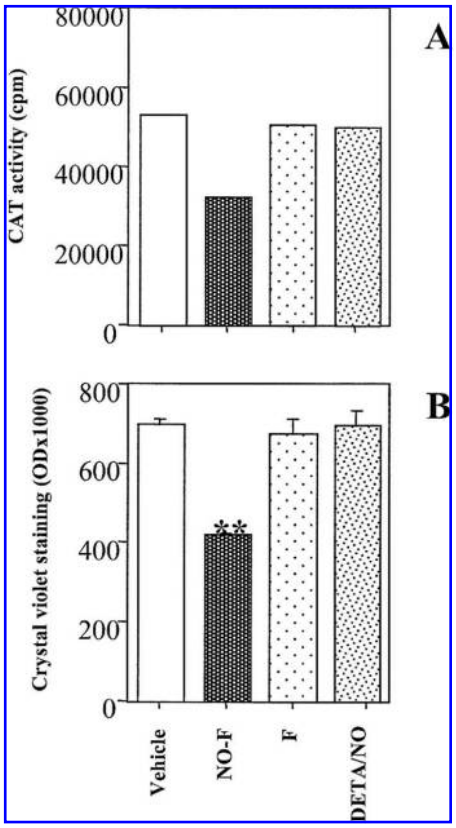

FIG. 5. Effect of a NO donor on NF-κB activation and cell growth. DETA/NO (10 μ*M*), NO-flurbiprofen (NO-F; 100 μ*M*), flurbiprofen (F; 100 μ*M*), and vehicle (DMSO, 0.37% final concentration) were incubated either with L929 cells for 24 h followed by crystal violet staining (**B**), or with L929/J16 cells in the presence of 100 ng/ml TNF for 18 h, followed by CAT activity determination (**A**; single determination of pooled triplicate samples). $^{**}p < 0.01$, Dunnett's test.

A nitric oxide (NO) donor does not cause NF-κB inhibition nor cell growth retardation

To see whether the observed effect of NO-flurbiprofen was due to the NO donating moiety, we used a NO donor (DETA/NO) with a half-life of 20 h at physiological pH and 37°C (19). This release kinetics and the concentration we used (10 μ*M*) were chosen to give slightly higher concentrations of NO than the ones expected to be released by 100 μ*M* NO-flurbiprofen. In these conditions, NO had no effect either on NF-κB activation or on L929 cell growth (Fig. 5).

Other NF-κB inhibitors also inhibit cell growth

To support the idea that NF-κB inhibition and cell growth reduction are linked phenomena, we studied the effect of two NF-κB inhibitors on the growth of L929 cells. The results are shown in Fig. 6. Both pyrrolidine thiocarbamate (PDTC; left panels) and DMSO (right panels) inhibited CAT synthesis (upper panels) and cell growth (lower panels), with parallel dose–response curves. PDTC did not induce cell death at all the concentrations tested, after 24 h of incubation, although

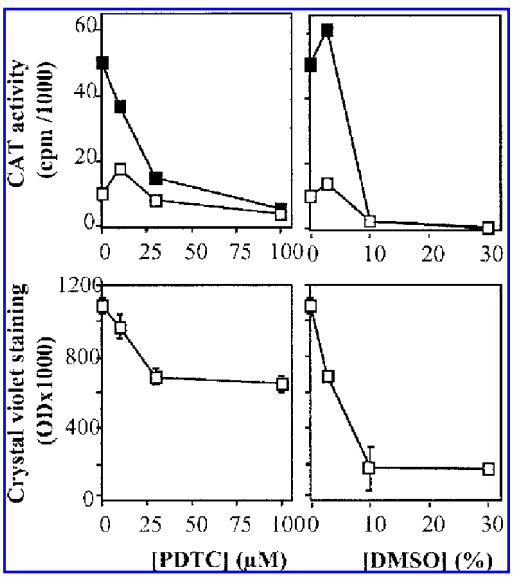

FIG. 6. Inhibition of cell growth by NF-κB inhibitors. (**Lower panels**) PDTC (**left panel**) and DMSO (**right panel**) at the indicated concentrations were incubated for 24 h with L929 cells. Then the number of cells was measured by crystal violet staining. (**Upper panels**) The same substances were incubated for 30 min with L929/J16 cells, followed by 18-h incubation in the presence of 100 ng/ml TNF (black squares) or saline (open squares). NF-κB activation was then assessed by measuring CAT activity. The data shown in the figure are from a single experiment, representative of two that gave similar results.

cell death, as assessed by trypan blue dye exclusion, was apparent after 3 days at the highest concentrations (70 and 40% viability, at 30 and 100 m*M*, respectively). DMSO already induced some cell death at 24 h (80 and 50% cell viability at 10 and 30% concentration, respectively). This cell death could be due to the highest extent of inhibition of NF-κB with respect to NO-flurbiprofen, as well as to other effects of these molecules, nonspecific for NF-κB.

The inhibition of NF-κB activation by these molecules was also demonstrated in EMSA (Fig. 2B), at times when no cell death had occurred in any condition.

DISCUSSION

We demonstrate here that NO-flurbiprofen inhibits NF-κB activity. Evidence for this inhibition was obtained in two ways: by measuring NF-κB directed synthesis of a reporter gene and by assessing NF-κB DNA binding in gel-shift experiments. The inhibitory effect of NF-κB activation by NO-flurbiprofen does not depend exclusively on the NO release. This was shown by the absence of an effect of the NO donor DETA/NO and by the fact that NO-flurbiprofen, when added after NF-κB activation, does not inhibit the formation of NF-κB–DNA complexes, as has been demonstrated for other NO donors (17, 18). The observed inhibition may clearly result from a synergy between several actions of NO and the NSAID, including inhibition of COX, and inhibition of cytokine signaling mechanism, for instance through the *S*-nitrosylation of caspases (8).

We have also shown that NO-flurbiprofen, at the same concentrations that inhibit NF-κB activation, inhibits cell growth. Several observations suggest that cell growth reduction was due to inhibition of cell proliferation and not to the induction of cell death: (a) cell growth inhibition was dependent on cell density and no inhibition was seen in confluent cells, where proliferation was virtually absent; (b) in all the conditions tested (up to 300 μ*M* NO-flurbiprofen, up to 72 h of incubation), cell viability as assessed by trypan blue exclusion was always >95%; and (c) no signs of apoptosis as assessed by nuclear morphology or by FACS analysis were detectable with 100 μ*M* NO-flurbiprofen.

A basal NF-κB activity, also in the absence of TNF, was evident both in the reporter gene and in the EMSA assays. NO-flurbiprofen and the two antioxidants (DMSO and PDTC) inhibited this activity as much as they inhibited the TNF-dependent activation, in the reporter gene experiments. Due to the less quantitative nature of EMSA assays, only PDTC was shown to inhibit basal activity in these experiments. Both DMSO and PDTC were shown to inhibit also cell growth, with comparable dose–response curves. On the other hand, hydrocortisone, which inhibits TNF-stimulated, but not basal, NF-κB activation in L929/J16 cells (unpublished results), does not inhibit L929 cell growth.

On the basis of these correlations, we hypothesize that the basal NF-κB activation could be responsible for survival/proliferation of L929 cells, and its inhibition by NO-flurbiprofen, also in the absence of TNF, could explain its cytostatic effect. This is possible because, in addition to its antiapoptotic action, NF-κB can promote cell growth by interacting with the cyclin kinases system (for review, see 13). Accordingly, we have shown that NO-flurbiprofen retards cell growth by slowing all the phases of cell cycle.

Of course, several other actions, including the above-mentioned inhibition of COX, and inhibition of cytokine signaling mechanism, may be relevant to the inhibition of cell growth.

Recently, some nitro-NSAIDs, other than NO-flurbiprofen, have been shown to reduce cancer cell growth more effectively than their parent compounds (28). We propose that a similarly enhanced potency in inhibiting NF-κB, conferred by the NO donating moiety of the molecules, could explain their advantage.

The therapeutic potential of NF-κB inhibition is really wide, going from the reduction of inflammatory response (in inflammatory diseases such as asthma, rheumatoid arthritis, inflammatory bowel disease and including Alzheimer's disease) to the antiproliferative effect (for cancer prevention and potentiation of chemotherapy for cancer treatment). Therefore, an NSAID provided with an NF-κB inhibitory action and a low gastrointestinal toxicity (5, 14, 23, 24, 27) could prove useful in many conditions.

ACKNOWLEDGMENT

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ABBREVIATIONS

CAT, chloramphenicol acetyltransferase; COX, cyclooxygenase; DETA/NO, (hydroxynitrosohydrazino)*bis*-ethanamine; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor-κB; NO, nitric oxide; NO-flurbiprofen, flurbiprofen nitroxybutyl ester; NSAID, nonsteroidal antiinflammatory drug; PBS, phosphate-buffered saline; PDTC, pyrrolidine thiocarbamate; TNF, tumor necrosis factor.

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