Anti-Inflammatory Effects of Moxifloxacin on Activated Human Monocytic Cells: Inhibition of NF-κB and Mitogen-Activated Protein Kinase Activation and of Synthesis of Proinflammatory Cytokines

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We previously showed that moxifloxacin (MXF) exerts protective anti-inflammatory effects in immunosuppressed mice infected with *Candida albicans* by inhibiting interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α) production in the lung. Immunohistochemistry demonstrated inhibition of nuclear factor (NF)-κB translocation in lung epithelium and macrophages in MXF-treated mice. In the present study we investigated the effects of MXF on the production of proinflammatory cytokines (i.e., IL-8, TNF-α, and IL-1β) by activated human peripheral blood monocytes and THP-1 cells and analyzed the effects of the drug on the major signal transduction pathways associated with inflammation: NF-κB and the mitogen-activated protein kinases ERK and c-Jun N-terminal kinase (JNK). The levels of IL-8, TNF-α, and IL-1β secretion rose 20- and 6.7-fold in lipopolysaccharide (LPS)-activated monocytes and THP-1 cells, respectively. MXF (5 to 20 μg/ml) significantly inhibited cytokine production by 14 to 80% and 15 to 73% in monocytes and THP-1 cells, respectively. In THP-1 cells, the level of NF-κB nuclear translocation increased fourfold following stimulation with LPS-phorbol myristate acetate (PMA), and this was inhibited (38%) by 10 μg of MXF per ml. We then assayed the degradation of inhibitor (I)κB by Western blotting. LPS-PMA induced degradation of I-κB by 73%, while addition of MXF (5 μg/ml) inhibited I-κB degradation by 49%. Activation of ERK1/2 and the 46-kDa p-JNK protein was enhanced by LPS and LPS-PMA and was significantly inhibited by MXF (54 and 42%, respectively, with MXF at 10 μg/ml). We conclude that MXF suppresses the secretion of proinflammatory cytokines in human monocytes and THP-1 cells and that it exerts its anti-inflammatory effects in THP-1 cells by inhibiting NF-κB, ERK, and JNK activation. Its anti-inflammatory properties should be further assessed in clinical settings.

Fluoroquinolones are synthetic, broad-spectrum antimicrobial agents in common use for a variety of infections including systemic infections in immune-compromised hosts (11, 17). In addition to their antimicrobial properties, certain quinolones were shown to have immune-modulating activities in animal models and humans (for a review, see reference 10). Moxifloxacin (MXF) is a fluoroquinolone with activities against both gram-positive and gram-negative bacteria. It has been suggested that MXF has inhibitory and stimulatory effects on the immune system, primarily by studies that have shown that the production of several cytokines by human and murine leukocytes can be affected by this drug. It has previously been shown (34) that MXF enhances the production of granulocyte-macrophage colony-stimulating factor and interleukin-6 (IL-6) by various organs of cyclophosphamide-injected mice. Other investigators have shown that MXF significantly inhibits IL-8 production by human neutrophils (45) and tumor necrosis factor alpha (TNF-α) and IL-1α production by human monocytes stimulated in vitro with lipopolysaccharide (LPS) (3).

Recently, we performed in vivo studies and investigated the protective effect of MXF in cyclophosphamide-injected mice inoculated intratracheally with *Candida albicans*. In that study, control and ceftazidime-treated mice developed bronchopneumonia with significant neutrophilic infiltration in association with increased levels of TNF-α and IL-8 production in the lungs. By contrast, mice pretreated with MXF showed no bronchopneumonia and the levels of TNF-α and IL-8 secretion were significantly inhibited compared to those in the controls (35; H. Blau, I. Fabian, Y. Kletter, L. Horev, N. Kariv, I. Shechtman, H. Alteraz, Y. Ben-Neriah, and I. Shalit. Abstract, Am. J. Respir. Crit. Care Med. 165:A782, 2002). Immunohistochemistry of the lung tissue showed that nuclear translocation of nuclear factor (NF)-κB occurred in more than 50% of airway epithelial cells as well as in inflammatory cells within the lungs of both saline- and ceftazidime-treated mice. In contrast, there was a marked decrease in the levels of NF-κB activation in the lungs of MXF-treated mice (Blau et al., Abstract, Am. J. Respir. Crit. Care Med. 165:A782, 2002). The chemokine IL-8 is known to have a critical role in the recruitment of neutrophils, T lymphocytes, and monocytes to regions of tissue injury (4, 13). It has been reported that IL-8 is the chemokine responsible for the maintenance of various chronic inflammatory diseases, such as gastritis caused by *Helicobacter*...
pH of 7.4) containing 0.1% Tween (TBST) for 1 h. The membranes were subjected to centrifugation (20,000 x g) at 4°C for 5 min, and the supernatant was stored at −20°C until use.

The oligonucleotide described below was radiolabeled with T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.) (1:20,000 and 1:1,000 dilutions, respectively) and electroblotted to the nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.) before storage at −20°C. Aliquots of the cytosol containing 50 μg of protein for ERK1/2 and JNK1/2 and 20 μg of protein for IκBα were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10, 12, and 15% polyacrylamide gels, respectively. After electrophoresis and electrotransfer of the proteins to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.), the membranes were blocked with 3% nonfat milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween (TBST) for 1 h. The membranes were then rinsed three times in TBST and incubated at room temperature (RT) with mouse monoclonal anti-MAPK antibody and with activated, diphosphorylated, ERK1/2, phospho-p42/p44 MAPK antibody (Sigma Chemical Co.), and anti-phospho-JNK1/2, phospho-p46/p54 JNK antibody (New England Biolabs, Beverly, Mass.) (1:20,000 and 1:1,000 dilutions, respectively) and anti-ERK1/2 antibody and anti-JNK1/2 antibody (1:1,000 dilution) or rabbit polyclonal antibody against IκBα (1:1,000 dilution) (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.) for 1 h. Activin levels were also assessed as a loading control by using an antibody (Santa Cruz Biotechnology) that reacts with a broad range of actin isoforms. The blots were then incubated with a secondary antibody, horseradish peroxidase-linked anti-mouse immunoglobulin G (Santa Cruz Biotechnology) or rabbit polyclonal antibody against IκBα (1:1,000 dilution) (Santa Cruz Biotechnology) for 1 h. After 1 h at RT and three washes in TBST, the blots were incubated in enhanced chemiluminescence reagent (Amersham Pharmacia Biotech). The relative densities of ERK1/2, JNK1/2, and IκBα were determined by densitometric analysis, followed by photography of the specific bands (Kodak XLS-1 film).

Electrophoretic mobility shift assay (EMSA). THP-1 cells were preincubated in serum-free medium at 37°C for 24 h in the absence or presence of 5 or 10 μg of MFX per ml prior to the addition of LPS (1 μg/ml) or LPS-PMMA (1 μg/ml and 100 nM, respectively) for 60 or 120 min. The cells were collected on ice before isolation of nuclear extracts by the protocol reported by Akawka et al. (1). Briefly, the cells (10^6/ml) were washed with ice-cold PBS, suspended in 200 μl of lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT) and allowed to swell on ice for 15 min, after which 12.5 μl of 10% Nonidet P-40 was added. The tube was then mixed thoroughly with a Vortex mixer for 10 s prior to centrifugation (20,000 x g) at 4°C for 5 min. The nuclear pellets thus obtained were resuspended in 25 μl of ice-cold nuclear extraction buffer (20 mM HEPES [pH 7.9], 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) and kept on ice for 15 min with intermittent agitation. The samples were subjected to centrifugation for 5 min at 4°C, and the supernatant was stored at −70°C after measurement of its protein content with the Bio-Rad protein assay kit, as described above.

The ATP oligonucleotide described below was radiolabeled with T4 polynucleotide kinase (Epience Technologies) and [γ-32P]ATP (Sigma Chemical Co.) before being annealed to oligonucleotides whose sequences were complementary. An
NF-κB consensus sequence is GGGAGGGGACCTTTGGAGG (Santa Cruz Biotechnology). A binding reaction buffer (10 mM Tris HCl [pH 7.9], 60 mM KCl, 0.4 mM DTT, 10% glycerol, 2 μg of bovine serum albumin) (44) was used to optimize NF-κB binding. Volumes of 20 μl of the binding reaction mixtures were prepared and contained 8 μg of nuclear extract, 0.5 ng of 32P-labeled double-stranded oligonucleotide, and 1 μg of poly(dI-dC). The reaction mixtures were incubated at room temperature before electrophoresis through 5% polyacrylamide gels with 0.5% TGA (1 × TGA is 25 mM Tris, 190 mM glycine, and 10 mM EDTA). For competition assays, preincubation at RT for 30 min with a 100-fold molar excess of competitor was carried out before the addition of the labeled oligonucleotides. The gels were dried under vacuum and exposed to intensifying screens at −70°C. The relative densities of NF-κB were determined by densitometric analysis followed by photography of the specific bands (Kodak XLS-1 film).

WB analysis of NF-κB. The nuclear extracts of THP-1 cells used in the WB analyses were prepared as described above. An aliquot of the nuclear fraction containing 50 μg of protein for NF-κB was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% polyacrylamide gels as described above. NF-κB was detected by incubating the blots with anti-NF-κB p65 rabbit polyclonal antibody (1:500 dilution; Santa Cruz Biotechnology). The blots were then incubated with a secondary antibody, horseradish peroxidase-linked anti-rabbit immunoglobulin G (Santa Cruz Biotechnology). After 1 h at 37°C and three washes in TBST, the blots were incubated in enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

Statistical significance was determined by Student’s t test.

RESULTS

Effects of MXF on IL-8, TNF-α, and IL-1β secretion induced by exposure of monocytes and THP-1 cells to LPS. To determine the effects of MXF on the production of proinflammatory cytokines, LPS was added to the cells with or without MXF. Exposure of monocytes to 10 ng of LPS per ml for a short period (4 h) induced a marked increase in the levels of IL-8 production by the cells. MXF at concentrations of 5, 10, and 20 μg/ml inhibited the induction of IL-8 secretion by 26, 35, and 40%, respectively (P < 0.05) (Fig. 1). Similarly, preincubation of THP-1 cells with MXF suppressed the enhanced IL-8 secretion induced by exposure to 100 ng of LPS per ml (47 to 57% inhibition at 5 to 20 μg/ml) (P < 0.05) (Fig. 1). Similar results were obtained when MXF was given concomitantly with LPS (7 to 42% inhibition at 5 to 20 μg/ml).

From our previous in vivo research, which indicated that MXF exerts pronounced anti-inflammatory effects when it is administered as a pretreatment to immune-suppressed animals, we conducted further in vitro studies using preincubation with MXF to elucidate the cellular mechanisms underlying the in vivo anti-inflammatory effects of the drug.

Marked increases in the levels of secretion of TNF-α and IL-1β by monocytes and THP-1 cells were observed following exposure of the cells to LPS. MXF inhibited the induction of TNF-α secretion in monocytes by 14 to 51% and that of IL-1β by 50 to 83%. Similarly, the drug inhibited the induction of TNF-α and IL-1β secretion in THP-1 cells by 15 to 73% (P < 0.05) (Fig. 1).

These data indicate that MXF suppresses the signaling that transmits LPS stimuli into the cell and suppresses proinflammatory cytokine production by monocytes and THP-1 cells. To further explore the mechanism of action of MXF, additional experiments were performed with THP-1 cells.

Effect of MXF on ERK1/2 activation. MAPKs are required for IL-8 induction mediated by LPS-PMA in THP-1 cells (25, 26, 36). On the basis of our observation that MXF inhibits the release of IL-8 induced by LPS alone, we investigated the effect of MXF on the activation of ERK1/2 in THP-1 cells exposed to LPS alone or LPS in combination with PMA (Fig. 2). Activation of ERK1/2 was examined by detecting the appearance of the 42-kDa p-ERK protein and the 44-kDa p-ERK protein.

Initially, we performed time-dependent studies. LPS-PMA ligation resulted in the rapid and time-dependent activation of ERK1/2, which was detected 5 min after LPS-PMA ligation and which was maximal at 30 min (Fig. 2A). ERK1/2 activation decreased after longer periods of stimulation. On the basis of the results of the time-dependent studies, all further experiments were performed following exposure of the cells to LPS or LPS-PMA for 30 min. Exposure to LPS resulted in the activation of the 42-kDa p-ERK protein (2.2-fold increase relative to the level of activation for unstimulated cells) (Fig. 2B, second lane). When cells were pretreated with 5 μg of MXF per ml before incubation with LPS, activation of the 42-kDa p-ERK protein was completely attenuated (Fig. 2B, third lane), while preincubation with 10 and 20 μg of MXF per ml resulted in 22 and 25% inhibition, respectively (Fig. 2B, fourth and fifth lanes, respectively). Treatment of the cells with LPS-PMA markedly induced the activation of the 42-kDa p-ERK protein and the 44-kDa p-ERK protein (threefold increase in phosphorylation compared to that with LPS alone, as determined by densitometric analysis) (Fig. 2B, sixth and second lanes, respectively). Preincubation with 5 and 10 μg of MXF per ml before exposure to LPS-PMA induced 33 and 54% reductions in the levels of activation of ERK1/2 (Fig. 2B, seventh and eighth lanes, respectively; compare the results with those in the sixth lane). No reduction in LPS-PMA-induced ERK1/2 was observed in the presence of 20 μg of MXF per ml (Fig. 2B, ninth lane). Immunoblot analysis with an antibody that detected total ERK showed equivalent levels of ERK1/2 expression in all samples (Fig. 2B, lower blot).

Effects of MXF on JNK activation. Activation of p-JNK1/2 was assessed after treatment of THP-1 cells with LPS-PMA. At various times after exposure to LPS-PMA, cell lysates were subjected to immunoblot analysis with an antibody that detected dually phosphorylated JNK. Figure 3A indicates that phosphorylation of JNK1 was observed at 5 min and was maximal at 60 min. High levels of activated JNK were observed up to 120 min after exposure to LPS-PMA. Subsequent experiments were performed following exposure of the cells to LPS-PMA for 60 min. We examined the effect of MXF on LPS-PMA-induced activation of the 54-kDa p-JNK protein and the 46-kDa p-JNK protein. Figure 3B indicates that exposure of the cells to LPS-PMA induced a 3.6-fold increase in the level of activation of the 46-kDa p-JNK protein (Fig. 3B, second lane); and preincubation with MXF at concentrations of 5, 10, and 20 μg/ml for 24 h inhibited the activation of the 46-kDa p-JNK protein by 37, 42, and 50%, respectively (Fig. 3B, third to fifth lanes, respectively). Immunoblot analysis with an antibody that detected total JNK confirmed that equivalent amounts of the 54- and 46-kDa JNK proteins were present.

MXF inhibits LPS-PMA-induced degradation of IκBα. We studied the effect of MXF on LPS-PMA-induced IκBα degradation (Fig. 4). As shown in Fig. 4A, LPS-PMA stimulation induced degradation of IκBα. The band markedly decreased within 15 min of incubation (Fig. 4A, second lane), while by 30 and 60 min it gradually reappeared (Fig. 4A, third and fourth
FIG. 1. Effects of MXF on the production of IL-8, TNF-α, and IL-1β by LPS-stimulated monocytes and THP-1 cells. Monocytes and THP-1 cells were preincubated in the presence or absence of MXF (5 to 20 μg/ml). LPS was added to the cells at 10 and 100 ng/ml, respectively, and the cells were further incubated for 4 and 24 h, respectively. The concentrations of IL-8, TNF-α, and IL-1β in the culture supernatants were measured by ELISA, and the values are the means ± standard errors of three independent experiments. Asterisks denote a statistically significant difference compared with the results for the LPS-stimulated cells without MXF. (P < 0.05).
lanes, respectively). Figure 4B (third lane) indicates that pre-incubation with MXF at a concentration of 5 μg/ml for 24 h before the addition of LPS-PMA for 15 min markedly inhibited I-κB degradation. Slight inhibition was observed with 10 μg/ml, while no inhibition was observed in the presence of 20 μg of MXF per ml (Fig. 4B, fourth and fifth lanes, respectively). Actin was also immunoblotted as an internal loading control (Fig. 4A and B). It is known that NF-κB is present in an inactive form in the cytoplasms of resting cells, where it is bound to the inhibitor I-κB. It is also well established that phosphorylation and degradation of I-κB are crucial steps in NF-κB translocation to the nucleus. Thus, our observation that MXF inhibits the degradation of I-κB indicates that the drug inhibits the release of NF-κB from the inhibitor and prevents NF-κB activation and translocation to the nucleus.

**Effects of MXF on LPS- and LPS-PMA-induced NF-κB activation (EMSA and WB studies).** THP1 cells were preincubated for 24 h in serum-free medium in the absence or presence of MXF (5 or 10 μg/ml) and then treated with LPS (1 μg/ml) for 1 h. Cells alone showed no NF-κB activity. Growth in serum-free medium induced a slight increase in the level of NF-κB activity (Fig. 5A; compare the second and first lanes). Addition of LPS significantly induced NF-κB nuclear translocation (Fig. 5A; compare the fourth and second lanes). MXF at 5 μg/ml inhibited serum-free-induced NF-κB activity (Fig. 5A, third lane), while both concentrations of the drug (5 and 10 μg/ml) inhibited LPS-induced NF-κB activation (Fig. 5A, fifth and sixth lanes, respectively). Densitometric scans of the films revealed that the decreases induced by MXF were 60% at 5 μg/ml and 72% at 10 μg/ml compared with the levels for the LPS-stimulated samples (Fig. 5B, fifth and sixth lanes, respectively). WB analysis was performed to detect whether MXF also affects the expression of the p65 NF-κB protein within the nucleus. Nuclear extracts isolated from cells pretreated with MXF or medium and exposed to LPS were analyzed for the p65 NF-κB protein. Figure 5C shows that LPS enhances protein expression, while pretreatment with MXF decreases protein expression in a dose-dependent manner. The effect of MXF on LPS-PMA-induced activation of NF-κB was also evaluated (Fig. 6A). Cells incubated in serum-free medium showed basal levels of NF-κB activity, as shown in the first lane of Fig. 6A. LPS-PMA induced enhanced NF-κB binding activity (approximately fourfold; Fig. 6A, second lane). Densitometric scans of the films revealed that MXF at a concentration of 10 μg/ml inhibited LPS-PMA-induced NF-κB activation by about 35% compared with the level of activation in LPS-PMA-stimulated samples (Fig. 6B). Figure 6C shows that MXF also inhibited the expression of the p65 NF-κB protein under these conditions.

**DISCUSSION**

The present study demonstrates that MXF inhibits the production of IL-8, TNF-α, and IL-1β in LPS-stimulated human peripheral blood monocytes and in the THP-1 monocytic cell line in a dose-dependent manner. This finding confirms those presented in previous reports showing that MXF inhibits the accumulation of cytokines from activated human leukocytes (3, 9, 45).

The inhibitory effects described above confirm the results of previous in vivo studies showing that pretreatment with MXF of mice injected with cyclophosphamide and inoculated with C.
albicans significantly inhibited neutrophilic infiltration and TNF-α and IL-8 production in the lungs (35).

A wide range of antimicrobial agents, including quinolones, have been reported to modify immune and inflammatory responses both in vivo and in vitro (for a review, see reference 10). To further characterize the nature of the inhibitory effect of MXF on cytokine production, we examined the effects of MXF on the activation of the MAPKs ERK and JNK, which are known to be involved in the regulation of these cytokines, and on the activation of the transcription factor NF-κB, which regulates the expression of many immune and inflammatory genes. Activation of ERK is an early biochemical event that follows inflammatory responses, which is required for the regulation of many immune cells (43). We report here that MXF inhibits activation of the ERK induced by LPS and LPS-PMA in THP-1 cells. The inhibition of ERK activation by MXF could explain at least in part the inhibitory effects of MXF on IL-8 secretion by LPS-stimulated cells.

Other members of the MAPK group of kinases, such as JNK, have also been implicated in the control of cytokine production in different cell types (2). In the present study we show that MXF inhibits the nuclear translocation of NF-κB, which undergoes rapid translocation to the nucleus for DNA binding. MXF suppressed NF-κB binding to the DNA, as demonstrated by EMSA with the nuclear extracts of stimulated THP-1 cells (Fig. 5). The suppression was observed over the concentration range required for the inhibition of IL-8 production.

To ascertain whether MXF inhibited the nuclear translocation of NF-κB due to an effect on IκB degradation, we assayed...
I-κB degradation after stimulation with LPS-PMA in the presence of increasing concentrations of MXF. WB analysis of the I-κBα protein in the cytoplasmic extracts revealed that stimulation of THP-1 cells with LPS-PMA caused rapid depletion of I-κBα, followed by its gradual reappearance, presumably owing to a feedback mechanism which regulated its resynthesis. Treatment with 5 or 10 μg/ml of MXF per ml inhibited I-κB degradation. It is of note that additional anti-inflammatory agents, such as sodium salicylate, acetylsalicylic acid, and sanguinarine, were also found to inhibit I-κB degradation (8, 22).

The findings from previous in vivo studies (35) and the present in vitro data, which demonstrate and expand our understanding of the anti-inflammatory effects of MXF, provide further support for other observations relating to modulation of the inflammatory response by other quinoline compounds. In vitro studies with LPS-stimulated human monocytes have shown that ciprofloxacin, trovafloxacin, moxifloxacin, grepafloxacin, and levofloxacin have inhibitory effects on various proinflammatory cytokines, mainly IL-1α, IL-1β, TNF-α, IL-6, and IL-8 (3, 5, 9, 21, 30, 45, 47). Reduction of IL-8 levels was also found in stimulated human airway cells after treatment with grepafloxacin (15). In vivo studies have demonstrated that trovafloxacin, rufloxacin, and ciprofloxacin have significant anti-inflammatory activities in animal models of intra-abdominal infections caused by anaerobic organisms (with the drugs being devoid of activity against anaerobic organisms) or abscesses induced by heat-killed bacteria. It was demonstrated in those studies that the drugs have significant anti-TNF-α effects (14, 39, 40). In clinical settings it is worthwhile to mention the effect of ciprofloxacin on Crohn’s disease, which is partly attributed to TNF-α inhibition by the drug (36). Sparfloxacin was also found to reduce TNF-α, IL-1, and IL-6 levels in patients with nonbacterial prostatitis. The cytokine reduction was also associated with the disappearance of symptoms (46). In some of the studies cited above it was shown that the synthesis of the mRNAs of the relevant cytokines was indeed inhibited following exposure to the quinolones.

Nonetheless, none of these studies explored the signal transduction pathways preceding the synthesis of the mRNAs of the proinflammatory cytokines. The present study offers a more comprehensive explanation and basic mechanisms for the observed anti-inflammatory effects of quinolones, based on the inhibition of the two major signal transduction pathways associated with many of the inflammatory processes studied, as described above.

Future studies should focus on basic mechanisms on the one hand and on the clinical relevance of our studies on the other. It is not clear at present how MXF inhibits the NF-κB and MAPKs in stimulated cells, and upstream events up to the Toll-like receptor level should be investigated. The known effects of quinolones on nuclear topoisomerases should also be explored in relationship to the present findings due to their
possible effects on the delicate nucleic-acid balance of NF-κB and various kinases.

From a clinical standpoint, the anti-inflammatory effects of MXF could be of significant importance when these effects are linked to its antimicrobial properties. The effects of MXF on lung infection, inflammation, and tissue injury should be further studied both in animal models and in the clinical setting.

REFERENCES


