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Inhibition of Mitogen-Activated Protein Kinase Signaling by Chloroquine


Previously, we demonstrated that the anti-inflammatory drug chloroquine (CQ) inhibited LPS-induced TNF-α transcription. To define further the mechanism of CQ, we studied the effect of this drug on mitogen-activated protein kinase signaling pathways involved in regulation of TNF production. CQ interfered with phosphorylation of extracellular signal-regulated kinases (ERK)1/2 and the ERK-activating kinases mitogen-activating protein/ERK kinase (MEK)1/2. Both CQ and PD98059, a MEK1 inhibitor, reduced luciferase reporter activity driven by human TNF promoter sequences. However, CQ appeared to mediate these effects by deactivating Raf, the upstream activator of MEK. These findings were supported by functional data demonstrating that CQ and PD98059 interfered with TNF expression in several human and murine cell types while neither inhibitor blocked TNF production in murine RAW264.7 macrophages, a cell line that does not require MEK-ERK signaling for TNF production. Finally, we evaluated whether CQ could sensitize HeLa cells to undergo anti-Fas-mediated apoptosis, an effect observed when ERK production in murine RAW264.7 macrophages, a cell line that does not require MEK-ERK signaling for TNF production. Taken together, these data argue that therapeutic concentrations of CQ interfere with ERK activation by a novel mechanism, an effect that could be responsible, at least in part, for the potent anti-inflammatory effects of this drug. The Journal of Immunology, 2002, 168: 5303–5309.

The diprotic weak base chloroquine (CQ) has been used for over 60 years in the treatment of malaria and inflammatory disorders. Despite its numerous clinical applications, the molecular mechanisms responsible for the anti-inflammatory effects of CQ remain poorly understood. Its lipophilicity allows CQ to readily enter host cells (1). Due to its weak base properties and highly lipid soluble nature, CQ was thought to act by accumulating within and alkalizing acidic compartments such as endosomes and lysosomes (2). Indeed, in low pH environments such as acidic organelles, CQ becomes protonated (1), resulting in intracellular accumulation to millimolar levels (3).

Recently, we demonstrated that CQ inhibits human TNF-α gene expression in human PBMC stimulated with LPS (4). In these studies, CQ-mediated reduction of TNF mRNA levels occurred outside of acidic compartments. Moreover, CQ did not interfere with TNF message stability, suggesting that this drug might block signal transduction events triggered by LPS. However, nuclear trafficking of the transcription factor NF-κB was unaffected by CQ (4). Taken together, these data suggested that NF-κB-independent signaling pathways might be inhibited by CQ.

In addition to the activation of NF-κB, signaling by mitogen-activated protein (MAP) kinases (for review, see Ref. 5) is required for optimal TNF production. Recent studies have demonstrated that the extracellular signal-regulated kinases (ERK)1/2 are stringently required for TNF transcription (6, 7) in certain human and murine macrophage populations, while p38 and the c-Jun N-terminal kinase (JNK) play a major role in posttranscriptional regulation of TNF synthesis (8). ERK is activated by a serine-threonine kinase cascade initiated by Raf phosphorylation of the ERK-activating kinases MAP/ERK kinases (MEK)1/2. Raf activation is triggered by recruitment of this protein to the membrane by the protooncogene Ras, leading to Raf phosphorylation (9). Alternatively, it has recently been demonstrated that phosphorylation of Raf at Ser259 results in inactivation of this enzyme (10). Thus, phosphorylation at distinct sites can result in up- or down-regulation of this signaling pathway.

Mechanistic studies of this pathway were significantly augmented by the discovery of PD98059 (11), an inhibitor that binds to unactivated MEK1 and prevents its phosphorylation by Raf. Pretreatment with PD98059 has been shown to potently reduce phosphorylation (activation) and function of ERK (11). Similar results have been observed using an inhibitor that blocks the activation of both MEK isoforms, U0126 (12). Use of these soluble inhibitors has implicated MEK-ERK signaling in a variety of human and murine macrophage effector functions, including TNF (6), IL-1β (7) and IL-6 production (13), protection from Fas-mediated apoptosis (14), and inducible NO synthesis (15). Thus, it appears that MAP kinase function, in particular Raf-MEK-ERK signaling, plays a critical role in a variety of macrophage inflammatory processes.

A previously published report suggested that CQ concentrations exceeding attainable pharmacological levels (approximated, in vitro, by ≤100 μM pretreatment) by 2-fold did not affect TNF transcription in the murine peritoneal macrophage cell line.
RAW264.7 (16). These results differed significantly from our previous findings using human PBMC (4) and several human monocytic-like cell lines. Furthermore, a recent publication demonstrated that murine peritoneal macrophages and the peritoneal macrophage cell line RAW264.7 produce normal levels of TNF in the presence of PD98059 (7). Together, these observations suggested that CQ and PD98059 might interfere with the same signaling pathway. Thus, the current study set out to further characterize the mechanism of the action of CQ to determine whether this anti-inflammatory drug inhibits TNF release from certain mononuclear cell populations by interfering with the ERK-MAP kinase signaling cascade.

We found that CQ specifically interfered with the activation of ERK-MAP kinase proteins, which are required for optimal LPS-induced TNF expression in human mononuclear phagocytes (13), as well as the murine macrophage cell line AMJ2C-8 (7). Furthermore, the mechanism by which CQ inhibited was distinct from that of PD98059, as CQ treatment resulted in deactivation of Raf, an early member of the ERK signaling pathway. Both CQ and PD98059 reduced TNF promoter activity in a dose-dependent fashion, and their site of action was within 182 bp of the transcription start site on the human TNF promoter. CQ interfered with ERK activation in a variety of cell lines activated by diverse stimuli. The functional significance of these data was demonstrated by evaluating the effect of CQ on ERK-dependent processes in two unrelated cellular systems; TNF production in murine cell lines differentially sensitive to PD98059, and ERK-dependent resistance to apoptosis in HeLa cells. These data strongly suggest that CQ abrogates TNF transcription in human mononuclear cells by blocking ERK activation. Furthermore, it appears that the inability of CQ to block TNF production in RAW264.7 cells results from the fact that this murine peritoneal macrophage cell line expresses TNF independent of MEK-ERK signaling. In contrast, the other human and murine cell types evaluated in these studies and others (human PBMC, THP-1 and AMJ2C-8 cell lines) require MEK-ERK pathways for TNF production.

Materials and Methods

Cell culture

Reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Experiments were designed to minimize endotoxin contamination. All reagents were obtained prepacked and endotoxin-free. RPMI 1640, DMEM, and PBS were obtained from BioWhittaker (Walkersville, MD) and contained <0.005 U/ml endotoxin. The human monocyte-like cell line THP-1, the murine macrophage line RAW264.7, and the human cervical carcinoma cell line HeLa were obtained from the American Type Culture Collection (Manassas, VA). The murine alveolar macrophage line AMJ2C-8 was a gift of Dr. A. Palleroni (Hoffman LaRoche, Nutley, NJ) and has been described previously (17). THP-1, AMJ2C-8, and RAW264.7 were grown in RPMI 1640 supplemented with 10% FBS, L-glutamine, and ciprofloxacin (Bayer, Westhaven, CT), while HeLa cells were maintained in DMEM supplemented with 10% FBS, L-glutamine, and penicillin, and streptomycin.

PBMC were isolated from healthy volunteers using standard methods (19). Briefly, after obtaining informed consent from the donors, blood was collected by venipuncture, anticoagulated with 5 U pyrogen-free heparin (Fujisawa, Deerfield, IL) per milliliter of blood and centrifuged at 500 × g for 15 min. Leukocyte-rich buffy coats were then subjected to Ficoll-Hypaque density gradient centrifugation followed by collection of PBMC from the light density fraction. Cells were washed three times with ice-cold PBS before counting by hemocytometer and resuspension in RPMI 1640 at the desired density. PBMC were maintained in RPMI 1640 supplemented with 1-glutamine, penicillin, and streptomycin.

Western blotting for MAP kinase

PBMC (5 × 10⁶) were pretreated in 12-well polystyrene culture plates for 2 h in the absence or presence of CQ (10–100 μM) or PD98059 (20 μM). Cells were then stimulated with LPS (100 ng/ml) for the indicated period of time (10–30 min). Cells were lysed and Western immunoblotting was performed as in our previous studies (20) using Abs and the protocol supplied by New England Biolabs (Beverly, MA).

Plasmids

Reporter plasmids containing point mutations and truncations of the human TNF promoter (see Fig. 3) regulating the expression of the luciferase reporter gene were generously provided by Dr. N. Muckman (The Scripps Research Institute, La Jolla, CA) and have been described previously (21). Plasmids were transformed into E. coli, strain DH5α, and purified using commercial affinity columns that exclude endotoxin from the purified DNA (Qiagen, Valencia, CA). No endotoxin was detected in the purified plasmid DNA as measured by the Limulus Amoeocyte Lysate method (BioWhittaker).

Transient transfection assays

THP-1 cells were transiently transfected using the DEAE-dextran method (22). Briefly, 5–10 × 10⁶ THP-1 cells were washed once in PBS and once in transfection buffer (150 mM NaCl, 5 mM KCl, 0.7 mM NaH₂PO₄·H₂O, 25 mM Tris base, 1 mM MgCl₂, and 0.7 mM CaCl₂). Cells were then resuspended in 1 ml of transfection buffer containing 20 μg DEAE-dextran and 1 μg plasmid DNA per 1 × 10⁶ THP-1 cells for 15 min at 25°C. Transfection was halted by adding 9 ml of complete medium to the transfection mixture for 30 min at 37°C. Cells were then washed and resuspended in complete medium. Approximately 18 h posttransfection, THP-1 cells (1 × 10⁶/well) were treated with CQ (10, 30, and 75 μM) or PD98059 (20 μM) for 2 h at 37°C. Cells were then stimulated for 4 h with LPS (100 ng/ml) in the presence of monensin (2 μM), an inhibitor of protein secretion (23). Cells were washed twice with cold PBS, fixed with 2% paraformaldehyde, permeabilized with saponin, and stained with FITC-conjugated anti-murine TNF, which recognizes intracellular forms of TNF (R&D Systems). Cells (1 × 10⁶) were acquired by a FACScan flow cytometer (BD Biosciences, San Jose, CA) and analyzed using CellQuest software (BD Biosciences).

Flow cytometry

Intracellular TNF was quantitated as in prior studies (4) for the manufacturer’s protocol (R&D Systems, Minneapolis, MN). Murine macrophage cell lines AMJ2C-8 or RAW264.7 (1 × 10⁶) were incubated in the absence or presence of CQ (10, 30, or 100 μM) or PD98059 (20 μM) for 2 h at 37°C in 12-well polystyrene plates. Cells were then stimulated for 4 h with LPS (100 ng/ml) in the presence of monensin (2 μM), an inhibitor of protein secretion (23). Cells were washed twice with cold PBS, fixed with 2% paraformaldehyde, permeabilized with saponin, and stained with FITC-conjugated anti-murine TNF, which recognizes intracellular forms of TNF (R&D Systems). Cells (1 × 10⁶) were acquired by a FACScan flow cytometer (BD Biosciences, San Jose, CA) and analyzed using CellQuest software (BD Biosciences).

In situ detection of apoptosis by the TUNEL staining method

HeLa cells (1 × 10⁶) were pretreated with CQ (75 μM) or PD98059 (30 μM) for 2 h at 37°C before the addition of 100 ng/ml anti-human Fas Ab (Upstate Biotechnology, Lake Placid, NY) for 4 h. The TUNEL assay was performed according to the manufacturer’s protocol (R&D Systems). In brief, cells were washed in PBS, fixed with formaldehyde, and dried on a glass slide. Slides were rehydrated, permeabilized, and treated with terminal deoxynucleotidyl transferase and bromodeoxyuridine, a modified deoxyribonucleotide incorporated into DNA strand breaks by terminal deoxynucleotidyl transferase. DNA ends were detected by the addition of anti-bromodeoxyuridine Abs and cells were counterstained with methyl green. Slides were then washed extensively and evaluated by light microscopy in a blinded fashion.
Statistics and presentation of data

Data are expressed as mean ± SE. Data sets were compared by the Student two-tailed, paired t test using a software package (Microsoft Excel; Microsoft, Redmond, WA). The Bonferroni correction was used for multiple comparisons. Significance was considered achieved when the p value multiplied by the number of comparisons was <0.05.

Results

CQ blocks phosphorylation of ERK but not the activation of other MAP kinase proteins

As MAP kinase signaling is required for optimal TNF production, we sought to determine the effect of CQ treatment on LPS-induced activation of ERK, p38, and JNK. PBMC were pretreated with CQ (10 or 100 μM) or, for comparison, the MEK inhibitor, PD98059 (20 μM), for 2 h before stimulation with LPS (100 ng/ml) for 15 min. This time point was shown in preliminary experiments to correspond with peak levels of activated MAP kinase proteins (data not shown). CQ pretreatment blocked ERK activation by LPS (Fig. 1) as measured by Western blotting with Abs that recognize only the phosphorylated form of the given MAP kinase protein. In five independent experiments, preincubation with CQ (100 μM) reduced the levels of LPS-activated ERK to 43.9 ± 26.5% of control levels (p = 0.04) as determined by densitometry of the Western blots. A comparable result was observed following pretreatment with the well-characterized inhibitor of ERK activation, PD98059. Similar effects were observed following use of U0126, which inhibits activation of both MEK isoforms (data not shown). However, activation of neither p38 nor JNK (108.5 and 136.6 μM), for 2 h and then stimulated with LPS (100 ng/ml) for 10 min. Cells were lysed and Western blotting was performed with phosphospecific antisera as described in Materials and Methods. Pan-ERK refers to an Ab that recognizes all forms of ERK. Gel shown is representative of at least three independent experiments.

CQ reduces ERK phosphorylation by blocking activation of Raf and MEK kinases

Having demonstrated that CQ blocked ERK activation, we sought to identify the specific point in the Raf-MEK-ERK signaling cascade affected by CQ. Using a similar Western blotting approach with phosphospecific Abs, we found that unstimulated PBMC contained low levels of phosphorylated Raf, MEK, and ERK proteins (Fig. 2, lane 1). It is important to note that the phosphospecific Abs used in the immunoblots shown in Fig. 2 detected activated ERK and MEK but deactivated Raf (10). Interestingly, CQ (100 μM) pretreatment of human PBMC appeared to trigger Raf deactivation both in the absence and in the presence of LPS stimulation (Fig. 2, lane 2). Compared with unstimulated PBMC, LPS potently induced phosphorylation of both MEK and ERK (Fig. 2, lane 3). While CQ pretreatment down-regulated levels of both activated MEK and ERK, this inhibitory effect was most pronounced on the activation of ERK, significantly reducing its levels. These effects were observed in the presence of significantly elevated levels of inactivated Raf, suggesting that the downstream effects were mediated by an initial phosphorylation and deactivation of Raf. CQ did not inhibit total cellular levels of ERK as measured by Western blotting with an Ab, pan-ERK, that recognizes both phosphorylated and unactivated forms of the protein. These results argue that CQ blocks activation of MEK, thus reducing the levels of phosphorylated ERK.

CQ and PD98059 block LPS-induced human TNF promoter activity

Recent studies have argued that ERK signaling is required for optimal transcription of the TNF gene in human mononuclear cells (6, 13, 24). Consistent with these findings, our recent studies argued that CQ blocked TNF production at the level of transcription (4). Thus, to determine whether CQ and PD98059 blocked TNF promoter activation by a shared mechanism of action, we sought to identify which element of the human TNF promoter was targeted by these drugs. THP-1 cells, a human monocyte-like cell line, were transiently transfected with various forms of the human TNF promoter encompassing successive truncations (Fig. 3A) or point mutations (Fig. 3B), which eliminated transcription factor binding sites previously shown to be important for human TNF expression (21). The largest construct evaluated, pTNF(−1135)Luc, contains human TNF promoter sequences from −1135 bp to the transcription start site driving expression of the luciferase reporter gene. This construct exhibits TNF promoter activity comparable to the full-length human TNF promoter (21). Additional constructs, pTNF(−479)/Luc and pTNF(−82)/Luc, contain TNF promoter sequences from −479 and −182 bp to the transcription start site that successively eliminate the κB1 site at −587 bp and the κB2 site at −212 bp, respectively. Constructs truncated beyond −182 bp did not show significant induction following LPS activation (data not shown). Thus, to evaluate this region of the human TNF promoter, we used three constructs encompassing point mutations of consensus binding sites downstream of −182 bp. These constructs, Egr-1mut, CREmut, and κB3mut contain point mutations of the early growth response (Egr)-1 gene, cAMP response element (CRE), and NF-κB binding sites at −172, −106, and −97 bp,
respectively. In a previous study, these binding sites were shown to be the most important regulators of human TNF expression within 182 bp of the start site of transcription (21). However, in contrast to our data, these investigators found a minimal role of the H9260/H11002 B1 site at H11002/H587 bp in human TNF regulation (21).

THP-1 cells transiently transfected with the indicated luciferase reporter constructs encompassing either truncations (A) or point mutations (B) of the human TNF promoter. Transfected cells were preincubated with the indicated micromolar concentrations of CQ or PD98059 (PD) for 2 h and then stimulated with LPS (1 μg/ml) for 6 h. Cell lysates were prepared and assays were performed to detect luciferase activity as described in Materials and Methods. Unstimulated THP-1 cells transfected with pTNF(-1135)Luc, (-479)Luc, (-182)Luc, CRE mut , Egr-1 mut , and kB3 mut produced 8,379 ± 3,938, 19,380 ± 11,798, 12,413 ± 10,152, 10,503 ± 6,616, 11,119 ± 7,018, and 7,185 ± 5,026 relative light units, respectively. Data are expressed as fold induction relative to unstimulated luciferase levels. Data represent mean ± SE of at least five independent experiments performed in duplicate.

CQ and PD98059 reduce transcriptional activity of the human TNF promoter. THP-1 cells were transiently transfected with the indicated luciferase reporter constructs encompassing either truncations (A) or point mutations (B) of the human TNF promoter. Transfected cells were preincubated with the indicated micromolar concentrations of CQ or PD98059 (PD) for 2 h and then stimulated with LPS (1 μg/ml) for 6 h. Cell lysates were prepared and assays were performed to detect luciferase activity as described in Materials and Methods. Unstimulated THP-1 cells transfected with pTNF(-1135)Luc, (-479)Luc, (-182)Luc, CRE mut , Egr-1 mut , and kB3 mut produced 8,379 ± 3,938, 19,380 ± 11,798, 12,413 ± 10,152, 10,503 ± 6,616, 11,119 ± 7,018, and 7,185 ± 5,026 relative light units, respectively. Data are expressed as fold induction relative to unstimulated luciferase levels. Data represent mean ± SE of at least five independent experiments performed in duplicate.

A recent publication suggested that murine macrophage cell lines exhibit differential requirements for MEK-ERK signaling in TNF gene expression (7). Specifically, it was demonstrated that the murine peritoneal macrophage line RAW264.7 and primary peritoneal macrophages express TNF normally in the presence of PD98059, while the alveolar macrophage line AMJ2C-8 was exceedingly sensitive to inhibitors of MEK signaling. Thus, we sought to assess the functional significance of our Western blot findings by determining whether these cell lines were differentially sensitive to the effects of CQ as well as PD98059. As has been previously demonstrated by our laboratory and others (4, 16, 26), CQ can interfere with posttranslational processing and secretion of TNF. Thus, we...
used flow cytometry to evaluate intracellular levels of TNF. Cell lines were incubated in the absence or presence of a micromolar concentration range of CQ (100 μM shown) or PD98059 (50 μM) before stimulation with LPS for 4 h in the presence of monensin, an inhibitor of secretion. Thus, inclusion of monensin allowed an estimation of total TNF production over the 4-h period of stimulation. TNF production was determined by flow cytometric analysis of AMJ2C-8 and RAW264.7 cells stained intracellularly for TNF. In the absence of CQ, LPS vigorously stimulated TNF synthesis in both cell lines (Fig. 4). TNF staining in LPS-stimulated RAW264.7 cells was not affected by pretreatment with CQ or PD98059 (86.5 ± 18.6 and 92.6 ± 20.1% of control TNF levels; \( p = 0.36 \) and \( p = 0.48 \), respectively; \( n = 4 \)). However, CQ treatment of AMJ2C-8 cells resulted in background levels of TNF staining following LPS stimulation. These data provide further evidence that CQ and PD98059 block TNF production by interfering with a shared signaling pathway.

**CQ and PD98059 sensitize the normally resistant cell line, HeLa, to anti-Fas-mediated apoptosis**

HeLa cells are characteristically resistant to apoptosis following Fas ligation by either anti-Fas Ab or recombinant Fas ligand. However, this cell line undergoes programmed cell death in response to these triggers when MEK-ERK signaling is interrupted (14). In this set of experiments, we sought to evaluate the effect of CQ on MEK-ERK function in a system unrelated to macrophage cytokine production. Thus, we assessed whether CQ could sensitize HeLa cells to anti-Fas-mediated apoptosis as measured by TUNEL staining (Fig. 5), which measures DNA strand breaks, a proxy for apoptotic cell death. Following a 2-h incubation in the absence of CQ (75 μM) or PD98059 (30 μM), HeLa cells were treated with an Ab (clone CH-11, 100 ng/ml) that activates the Fas receptor for 4 h. Cells left untreated or subjected to anti-Fas treatment alone exhibited infrequent apoptotic cells, 9.3 ± 0.9% and 17.7 ± 4.9%, respectively (\( p = 0.11 \), \( n = 3 \), Fig. 5). Pretreatment of HeLa cells with either CQ or PD98059 resulted in a slight increase in TUNEL-positive HeLa cells (23.3 ± 5.6 and 26.7 ± 7.8%, respectively), consistent with the observation that basal MEK-ERK signaling confers a survival signal in diverse tumor cell lines (27, 28). However, both CQ and PD98059 sensitized HeLa cells to anti-Fas-mediated apoptosis (86.7 ± 3.1 and 75.3 ± 1.8% apoptosis, respectively). Thus, CQ and PD98059 sensitize HeLa cells to Fas-mediated programmed cell death. Moreover, functional inhibition of MEK-ERK signaling by CQ is not limited to cells of myelomonocytic origin.

**Discussion**

The data presented in this work demonstrate that CQ interferes with the activation of ERK-MAP kinase. While this is likely not the only cellular function affected by CQ, these data define a new molecular mechanism of action of this complex drug. CQ interfered with the phosphorylation of ERK in several different cellular systems without reducing cellular levels of ERK. Rather, CQ appeared to increase total ERK levels as measured by Western blotting, consistent with previous studies showing that this drug can reduce proteolysis (29, 30). Furthermore, these findings were not recapitated by treatment with another weak base, ammonium chloride. Our results argue that, although this drug is highly concentrated within acidic compartments, its site of action is extracellular, consistent with the data demonstrating that CQ blocks MEK-ERK signaling, a cytoplasmic kinase cascade.

Previous findings from this laboratory (4) suggested that CQ interfered with specific aspects of LPS signaling in human PBMC while not blocking other effector pathways, such as those leading to NF-κB mobilization. LPS binds to a receptor complex that includes the glycosyl-phosphatidylinositol-linked protein CD14 and Toll-like receptor (TLR)4 (31). The cytoplasmic domain of TLR4 interacts with MyD88 (32), which then binds to and activates the IL-1R-associated kinase (33). IL-1R-associated kinase, via interaction with TNFR-associated factor 6, activates the NF-κB-inducing kinase to phosphorylate two IκB kinases (33, 34), resulting in the liberation of the transcription factor NF-κB. Although NF-κB is an important transcriptional regulator, previous studies have called into question the role it plays in human TNF expression (35). Furthermore, recent findings indicate that LPS induction of the human TNF gene requires mobilization of additional transcription factors such as CREB and Egr-1 (36).

In addition to DNA-binding transactivators, additional signaling molecules, such as the MAP kinase family, play a critical role in LPS-induced TNF expression. There is some controversy surrounding the specific point at which TLR signaling initiates MAP
kinase activation (32). However, it is clear that ERK signaling is particularly important for TNF transcription in human mononuclear cells (6), while the other MAP kinase family members participate at the translational level (JNK) or at both levels of TNF production (p38). Interestingly, it appears that several human and murine macrophage populations have a strict requirement for ERK signaling in TNF production, while murine peritoneal macrophages and the RAW264.7 murine peritoneal macrophage cell line do not. These findings suggest that diverse macrophage populations differentially regulate their respective inflammatory responses. The intracellular cytokine staining data presented in this work confirm that both CQ and PD98059 do not interfere with TNF production in RAW264.7 macrophages, consistent with the possibility that these drugs inhibit ERK activation. Furthermore, these results provide for the first time an explanation for why human and most murine cells and cell lines appear to differ from the RAW264.7 cell lines in terms of their sensitivity to CQ.

Our transient transfection studies using luciferase reporter constructs driven by human TNF promoter sequences showed that both CQ and PD98059 act upon a region of the promoter within 182 bp of the start site of transcription. These studies did not identify the specific binding site on the promoter at which the effects of these drugs was localized. However, the finding that CQ and PD98059 regulate this region of the TNF promoter without blocking the function of the CRE, Egr-1, or NF-kB (xB3) binding sites is consistent with inhibition of ERK activation. Accordingly, a recent publication identified the presence of three Elk-1 consensus binding sites within this region (37). As this transcriptional regulator is phosphorylated and activated by ERK, it is possible that Elk-1 function is ultimately disrupted secondary to the effects of CQ on ERK activation. Alternatively, it has been shown that MAP kinases act in the region of the TATA box (25) possibly by phosphorylating a member of the transcription initiation complex, such as TATA-binding protein (38). The fact that each plasmid construct contained this region of the human TNF promoter could explain why the activity of each promoter construct was reduced by both CQ and PD98059.

Optimal TNF production requires a virtual symphony of signaling inputs, of which MAP kinase signaling is but a single instrument. Significant inhibition of TNF mRNA accumulation is seen with CQ concentrations in the range of 30–100 μM (4). This correlates well with the present data suggesting that CQ, at similar levels, reduces ERK activation. Thus, it appears that much of the pretranslational inhibition of TNF expression could result from down-regulation of MEK-ERK signaling. However, it is likely that inhibition of ERK signaling is not the sole mechanism by which CQ interferes with TNF synthesis. In addition to its effects on TNF gene regulation, CQ appears to interfere with posttranslational processing and release of TNF protein (16). Taken together, these data suggest that CQ is active at multiple levels in the biogenesis of TNF.

Although high concentrations of CQ can cause cell death, the effects of CQ reported in this study were not a result of nonspecific cytotoxicity. The highest concentration of CQ, 100 μM, used in the experiments reported herein results in intracellular levels approximating those seen in people undergoing CQ therapy (3). In previous studies, we demonstrated that PBMC viability and nuclear trafficking of NF-kB were unaffected by pretreatment with similar concentrations of CQ (4). Moreover, CQ affects neither the transcriptional activity of NF-kB nor the constitutive expression of an SV40-driven β-galactosidase construct (S. M. Weber and S. M. Levtiz, unpublished data). Furthermore, the finding that CQ (100 μM) pretreatment abrogated ERK activation without affecting p38 or JNK phosphorylation suggests that the mechanism of this drug is specific for the MEK-ERK pathway. Last, at all concentrations tested, CQ did not induce cell death as measured by Live/Dead staining.

These data demonstrated that CQ interferes with ERK activation triggered by diverse stimuli in multiple cellular systems. The finding that CQ and PD98059 sensitized HeLa cells to anti-Fas-mediated apoptosis argued that the inhibition of ERK signaling by CQ was not limited to the stimulus LPS or to mononuclear phagocytes. Furthermore, these experiments showed that CQ abrogated the effector function of activated ERK in a context other than TNF production, suggesting that these effects are functionally relevant. Thus, it appears that CQ acts in a fashion similar to PD98059, in that this drug prevents the activation of MEK, the upstream activators of ERK. However, our data suggest that CQ and PD98059 target different portions of the ERK cascade. Specifically, CQ appears to result in deactivation of the most upstream member of this MAP kinase signaling pathway, while PD98059 binds to and prevents the activation of MEK. Despite these mechanistic differences, both inhibitors target the final common pathway, preventing ERK phosphorylation and activation. It has been demonstrated previously that Akt, a member of a distinct kinase family, binds to and phosphorylates Raf, resulting in functional inhibition of this signaling pathway in vitro (10). As it appears that CQ triggers phosphorylation of Raf at this specific residue, it is possible that this drug blocks ERK activation by inducing Akt activity in human and certain murine mononuclear cells. This possibility will be addressed in future studies.

These data suggest a novel mechanism for the myriad cellular effects of CQ. It will be interesting to determine whether inhibition of MEK-ERK signaling plays a role in other contexts where CQ is an efficacious therapy. Our findings suggest that the drug discovery process could be used to synthesize CQ congeners that minimize toxicity to the host while maximizing inhibition of ERK-MAP kinase activation. As CQ and a closely related congener, hydroxychloroquine, are well-tolerated therapies, maximizing their pharmacologic potency while maintaining their excellent safety profile could be a significant step forward in the therapy of autoimmune and infectious diseases. As these data and our recent reports suggest that at least some of the mechanism of action of CQ is independent of its weak base properties, it is possible that synthesis of a neutral congener might further enhance efficacy while reducing side effects. These data do provide the first identification of a cellular target in the anti-inflammatory armamentarium of CQ. The speculative value of these data will have to be put to the test with synthetic modification of this drug and subsequent evaluation of clinical efficacy.

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References


