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Cloricromene, a semi-synthetic coumarin derivative, inhibits tumor necrosis factor-α production at a pre-transcriptional level

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Abstract

Cloricromene decreases myocardial infarct size after ischemic-reperfusion injury in vivo, and it has been suggested that this is due to inhibition of tumor necrosis factor-α (TNF-α). The purpose of this work was to characterize the mechanism of cloricromene-induced inhibition of TNF-α in rat macrophages. Cloricromene inhibited lipopolysaccharide-induced TNF-α release in a dose-dependent manner ($IC_{50} = 5.9 \pm 0.8$ μM). This was not due to cytotoxicity, as cloricromene was well tolerated up to 500 μM. Cloricromene inhibited lipopolysaccharide-induced expression of TNF-α mRNA, which suggests a pre-transcriptional effect. We then investigated the early signal transduction pathway triggered by lipopolysaccharide. The binding of lipopolysaccharide to its receptor CD14 activates protein kinase C and nuclear factor-κB (NF-κB). Cloricromene inhibited NF-κB activation in a dose-dependent manner, but affected protein kinase C translocation only slightly. We then established that cloricromene inhibited lipopolysaccharide-induced cellular oxidative activity, which is important for NF-κB activation. Our results show that cloricromene interferes with the early signal transduction pathway triggered by lipopolysaccharide.

Keywords: Macrophage; Anti-inflammatory; NF-κB (nuclear factor-κB); Lipopolysaccharide

1. Introduction

Cloricromene (8-monochloro-3-β-diethylaminoethyl-4-methyl-7-ethoxy-carbonylmethoxy coumarin) is a semi-synthetic non-anticoagulant coumarin derivative with anti-platelet and anti-leukocyte properties, and has beneficial effects in several experimental models of ischemia and shock (Milei et al., 1992a,b; Squadrito et al., 1992; Lidbury et al., 1993; Ribaldi et al., 1992; Groban et al., 1998). Previous work has shown that cloricromene modifies several granulocyte as well as monocyte/macrophage functions, which explains at least in part its protective action in ischemia. Cloricromene decreases superoxide anion production, inhibits the chemotaxis of polymorphonuclear cells, reduces levels of platelet-activating factor, and leukotriene B_4 and decreases the release of arachidonic acid by interfering with phospholipase A_2 activation (Lidbury et al., 1993; Zatta and Bevilacqua, 1999). In monocyte/macrophages, cloricromene inhibits adhesion to endothelial cells (Tranchina et al., 1994), the expression of nitric oxide synthase (Zingarelli et al., 1993) and the release of tumor necrosis factor-α (TNF-α) in vivo (Squadrito et al., 1992, 1993).

Despite extensive research efforts, the molecular mechanism of action of cloricromene is still poorly understood at the cellular level. It has been shown that it can interfere with phospholipase A_2 activation, reducing arachidonic acid release from membrane phospholipids (Gresele et al., 1993), and that it can inhibit cyclic GMP phosphodiesterase (Hakim et al., 1988). The purpose of this work was to investigate the mechanism of action of cloricromene at the molecular level. In particular, we studied the effect of cloricromene on lipopolysaccharide-induced TNF-α production in rat macrophages. We focused attention on TNF-α because this pro-inflammatory cytokine plays a fundamental role in the pathogenesis of ischemia/reperfusion damage and endotoxic shock (Beutler and Cerami, 1986; Paul and Ruddle, 1988; Fiers, 1991). Furthermore, cloricromene administration has been shown to have a protective effect against lipopolysaccharide-induced

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death, to reverse lipopolysaccharide-induced hypotension and leukopenia, and to diminish the lipopolysaccharide-induced rise in serum TNF-α levels (Squadrito et al., 1992, who suggested that cloricromene increased the survival rate by inhibiting TNF-α production).

We found that cloricromene acts at the pre-transcriptional level, inhibiting lipopolysaccharide-induced TNF-α transcription and nuclear factor-κB (NF-κB) activation by interfering with lipopolysaccharide-induced cellular oxidative activity.

2. Materials and methods

2.1. Animals

The experiments were performed with alveolar macrophages obtained from young adult male Sprague–Dawley rats (Charles River, Calco, Italy). All animal care procedures were in accordance with the strictures of the local Animal Care Committee, and no deaths were observed after receipt of rats in our animal facility. Before being used, the rats were quarantined for 2 weeks and were acclimatized to a 12-h light–dark cycle.

2.2. Chemicals

Cloricromene (8-monochloro-3-β-diethylaminoethyl-4-methyl-7-ethoxy-carbonylmethoxy coumarin) was obtained from Fidia Research Laboratories (Abano Terme, Italy), lipopolysaccharide from *Escherichia coli* serotype 0127:B12 was obtained from Sigma (St. Louis, MO, USA), and dichlorodihydrofluorescein diacetate di(acetomethyl ester) (DCFH) was from Molecular Probes (Eugene, OR, USA). Recombinant murine TNF-α (specific activity, 4×10^7 U/mg) was obtained from Genzyme (Cambridge, MA, USA). Salts were purchased from Sigma, and electrophoresis reagents were from Bio-Rad (Richmond, CA, USA). All reagents purchased were of the highest purity available.

2.3. Cells

Alveolar macrophages were collected by lavaging the lungs as described previously (Corsini et al., 1992). Recovery ranged from 10×10^6 to 15×10^6 cells/animal, of which > 98% were macrophages as determined by Giemsa staining. Once washed and resuspended to 10^6 viable alveolar macrophages/ml, for functional assays cells were allowed to adhere to plastic plates in RPMI 1640 (Sigma) containing 2 mM L-glutamine, 0.1 mg/ml streptomycin, 100 IU/ml penicillin, and 50 ng/ml gentamicin (medium) for 1 h at 37°C in 5% CO_2. For TNF-α release 0.5×10^6 cells were plated in 24-well plates (2 cm^2), while for reverse transcriptase-polymerase chain reaction (RT-PCR), Western blot analysis and electrophoretic mobility shift assay (EMSA) $4–5 \times 10^6$ cells were plated in 60 mm Petri dishes (28.3 cm^2). The plates were washed once with warm medium to remove nonadherent cells. Cells were then exposed to medium with 10% fetal calf serum (Sigma) and incubated with or without lipopolysaccharide in the presence or absence of cloricromene, or dimethyl sulfoxyde (DMSO) as vehicle control, at the times and concentrations indicated in the figure legends. The viability of cells following cloricromene treatment was measured as lactate dehydrogenase leakage in conditioned medium, using a commercial available kit (Sigma).

2.4. Assay for TNF-α

The TNF-α concentration in culture supernatants was assayed by determining the cytotoxicity against sensitive L929 cells, as previously described (Rosenthal and Corsini, 1995). The results are expressed in pg/ml, calculated against a standard curve with known amounts of recombinant murine TNF-α. In preliminary experiments, rabbit anti-mouse TNF-α was used to demonstrate that the cytolytic activity was due to the presence of TNF-α in the conditioned media of lipopolysaccharide-treated macrophages, and cloricromene at the concentrations used in the experiments was tested in the bioassay to exclude interference and, it did not affect L929 cells viability (data not shown).

2.5. Reverse transcriptase-polymerase chain reaction

For determination of TNF-α mRNA levels, 4×10^6 macrophages were first incubated overnight in medium with 10% fetal calf serum to avoid the induction of TNF-α gene expression due to cell adherence (Haskill et al., 1988). Then, fresh medium with cloricromene (10 μM) or DMSO as vehicle control was added for 10 min and finally lipopolysaccharide (100 ng/ml) for 2 h. Total RNA was isolated by guanidinium thiocyanate-phenol chloroform extraction (Chomcznski and Sacchi, 1987). RT-PCR reactions were performed as previously described (Corsini et al., 1996). Commercially available PCR primers for TNF-α and β-actin were purchased from Clontech Laboratories (Palo Alto, CA, USA). In preliminary experiments, RNA concentrations and PCR cycles were titrated to establish standard curves to document linearity and to permit semi-quantitative analysis of signal strength (5 and 50 ng for β-actin and TNF-α, respectively). Gels were photographed with type 55 film (Polaroid, Cambridge, MA, USA) and the image of the PCR products was acquired with a Nikon CCD video camera module (Nikon, Nelville, NY, USA). The optical density of the bands was calculated, and the peak area of a given band was analyzed by means of the Image 1.62 program for digital image processing (Wayne Rasband, Research Service Branch, National Institute of Mental Health, NIH, Bethesda, MA, USA).

2.6. Protein kinase C-βII translocation assay

5×10^6 alveolar macrophages were first incubated in a water bath at 37°C for 30 min in medium with 10% fetal calf serum in 15-ml polypropylene tubes. Then, cloricromene (10 μM), or DMSO as vehicle control, was added for 10 min and finally lipopolysaccharide (100 ng/ml) was added. After 5 min, cells were recovered by centrifugation for 5 min at $800 \times g$ at 4°C. The pellets were resuspended in 500 μl of homogenization buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA (pH 7.5), 50 μM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupetin) using a Teflon/glass potter. Cytosolic fractions were separated by centrifugation at $100{,}000 \times g$ for 60 min. The pellet was resuspended in the same volume of homogenization buffer by sonication, twice for 15 s; this constituted the membrane plus cytoskeleton fraction. Western blot analysis of protein kinase C-βII immunoreactivity in cytosolic and membrane fractions was performed as previously described (Corsini et al., 1999).

2.7. Preparation of nuclear extracts and electrophoretic mobility shift assay

Nuclear extracts were prepared from 4×10^6 macrophages, preincubated overnight in medium with 10% fetal calf serum to avoid the induction of NF-κB activation caused by cell adherence. After this time, fresh medium with cloricromene (10 μM) or DMSO as vehicle control was added for 10 min and then lipopolysaccharide (100 ng/ml) for 15 min. The medium was removed, the monolayer was washed with cold PBS and then 1 ml of a cold hypotonic lysis buffer (buffer A: 10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) was added. After 15 min, 75 μl of a 10% Nonidet P-40 solution was added and mixed for 15 s and the whole was then centrifuged for 30 s at 12,000 rpm. The pelleted nuclei were washed once with 400 μl of buffer A plus 25 μl of 10% NP-40, centrifuged and then suspended in 50 μl of buffer C (50 mM HEPES pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% (v/v) glycerol), mixed for 20 min, and centrifuged for 5 min at 12,000 rpm. The supernatant containing nuclear proteins was harvested, the protein concentration was determined, and then the supernatant was stored at −80°C until used in EMSA as follows. Binding reaction mixtures (20 μl) containing 5 μg protein of nuclear extract, 0.5 μg poly(dI–dC)·poly(dI–dC) (Sigma), and 10,000 cpm ^{32}P-labeled probe in binding buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, 1% Ficoll and 0.2 μg/ml albumin) were incubated for 30 min at room temperature before separation on a 7% acrylamide gel in 1× Tris borate EDTA (TBE) followed by autoradiography. A double-stranded oligonucleotide containing the binding site for NF-κB (5′-GTCTCGCAATTCCC-CTCTCTCAG-3′) was labeled with [γ-^{32}P]dATP (Amersham, Buckinghamshire, UK) using T4 polynucleotide kinase (Amersham). The NF-κB binding activity could be competitively inhibited by the non-labeled NF-κB DNA probe (Corsini et al., 1997)

2.8. Oxidative activity in living cells

0.5×10^6 macrophages kept in suspension were loaded with 10 μM DCFH (stained) or DMSO (unstained) for 1 h at 37°C in Hank's balanced salt solution without phenol red (HBSS) containing 2% bovine serum albumin. Then the cells were washed once with HBSS and oxidative activity was assessed as follows. The cells were resuspended in 2 ml of HBSS in quartz cuvettes and treated for 10 min with cloricromene (10 μM) or DMSO as vehicle control, then lipopolysaccharide (100 ng/ml) or vehicle was added. Reactive oxygen species production was measured as DCFH oxidation by the intensity of the fluores-

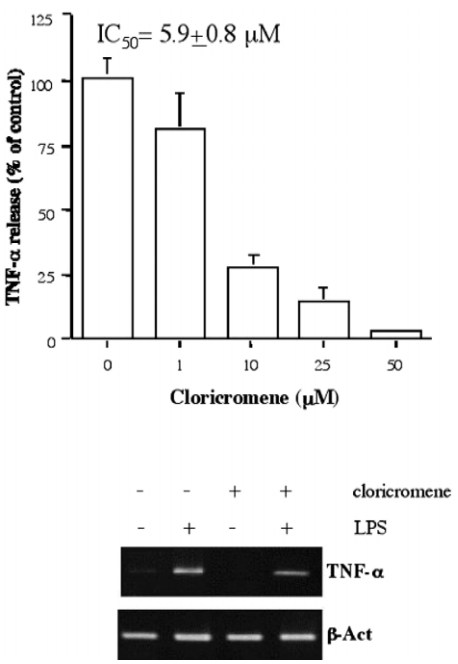

Fig. 1. Cloricromene inhibits lipopolysaccharide-induced TNF-α production at a pre-transcriptional level. Upper panel, dose-related inhibition by cloricromene of 10 ng/ml lipopolysaccharide-induced TNF-α release. Macrophages were treated for 10 min with increasing concentrations of cloricromene (0–50 μM) or DMSO as vehicle control and then lipopolysaccharide (LPS, 10 ng/ml) was added for 24 h. Each value is the mean ± S.D. of three to four determinations. All experiments were performed three times, with representative results shown. The IC_{50} value was calculated by linear regression analysis of the data from three independent experiments. Lower panel, cloricromene diminished expression of TNF-α mRNA induced by lipopolysaccharide. Macrophages were treated for 10 min with cloricromene (10 μM) or DMSO as vehicle control and then with (+) or without (−) lipopolysaccharide (100 ng/ml) for 2 h. The expression of β-actin was used as a control. Results obtained by RT-PCR.

cence emitted at 525 nm with excitation at 503 nm (Perkin Elmer LS 50B), at times 0 and 5, 15 and 30 min following treatment both in stained and unstained cells. Results are expressed as the change in fluorescence (in arbitrary units, AU) calculated as follows:

$$AU = [I_{stained} - I_{unstained}]_{tx} - [I_{stained} - I_{unstained}]_{t0}$$

where I represents the intensity of fluorescence.

2.9. Statistical analysis

All experiments were performed at least three times; representative results are shown. Statistical significance was determined by Student's t test or Dunnett's multiple comparison test as indicated, after analysis of variance.

3. Results

3.1. Cloricromene inhibits lipopolysaccharide-induced TNF-α release

Freshly isolated resident alveolar macrophages were chosen as the experimental model since they can be easily obtained at high purity (> 98%) by bronchoalveolar lavage. As shown in Fig. 1 upper panel, cloricromene inhibited lipopolysaccharide-induced TNF-α release in a dose-dependent manner, with an IC_{50} value of 5.9 ± 0.8 μM. The inhibitory effect was not due to cytotoxicity as assessed by lactate dehydrogenase leakage: cloricromene was indeed well tolerated up to 500 μM (30 ± 5 vs. 25 ± 5 U/l in control cells vs. cells treated with cloricromene 500 μM for 24 h). Cloricromene 10 μM was effective over a wide range of lipopolysaccharide concentrations (10–1000 ng/ml, Fig. 2).

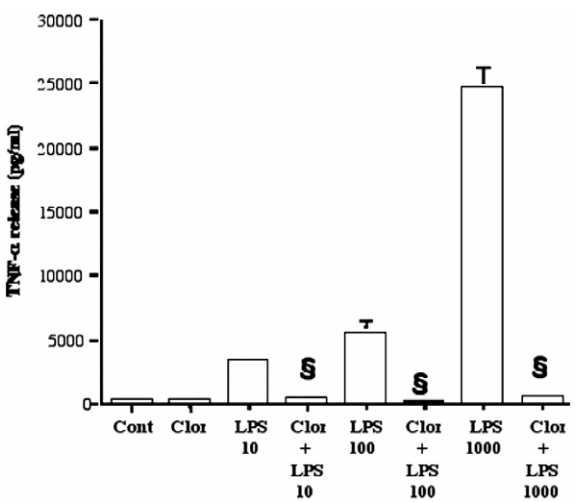

Fig. 2. Cloricromene inhibits TNF-α release over a wide range of lipopolysaccharide concentrations. Macrophages were treated for 10 min with cloricromene (Clor, 10 μM) or DMSO as vehicle control and then increasing concentrations of lipopolysaccharide (LPS, 0–1000 ng/ml) were added. TNF-α released in the conditioned media was evaluated 24 h later. Each value is the mean ± S.D. of three to four determinations. All experiments were performed three times, with representative results shown. Statistical analysis was performed by Dunnett's t test, § $P < 0.01$ vs. relevant control.

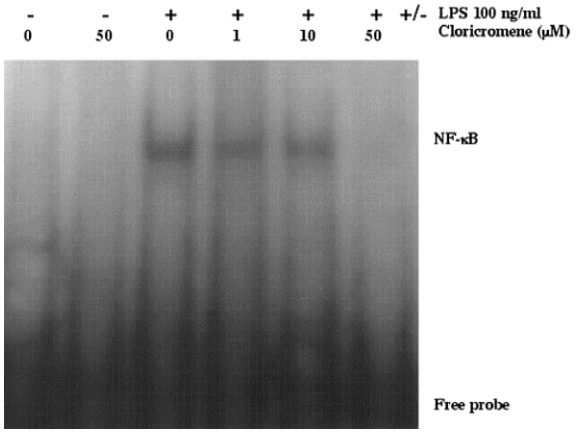

Fig. 3. Cloricromene inhibits lipopolysaccharide-induced NF-κB. Macrophages were treated for 10 min with increasing concentrations of cloricromene (0–50 μM) or DMSO as vehicle control and then with (+) or without (−) lipopolysaccharide (LPS, 100 ng/ml) for 15 min. Equal amounts (3 μg) of nuclear extract were analyzed by EMSA, with a [^{32}P]-labeled DNA probe to detect the binding activity of NF-κB.

3.2. Cloricromene inhibits TNF-α production at a pre-transcriptional level

To investigate if cloricromene exerted its inhibitory effect by acting before or after transcription, we measured by semiquantitative RT-PCR the effect of cloricromene on lipopolysaccharide-induced TNF-α mRNA. 10 μM cloricromene inhibited lipopolysaccharide-induced TNF-α mRNA expression by approximately 60% (Fig. 1, lower panel), a decrease similar to that observed at the protein level (Fig. 1, upper panel), suggesting a pre-transcriptional effect. Following densitometric analysis of the gel shown in Fig. 1, the ratio TNF-α/β actin was 4.6 and 1.9 for lipopolysaccharide- and cloricromene plus lipopolysaccharide-treated cells, respectively.

This led us to investigate the effects of cloricromene on the early signal transduction pathway triggered by lipopolysaccharide involved in TNF-α production.

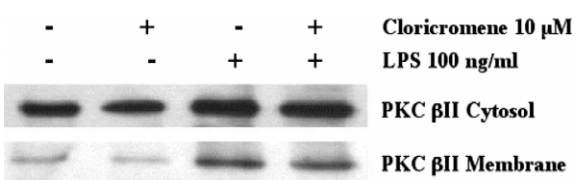

Fig. 4. Representative Western blot analysis of protein kinase C βII (PKC βII) immunoreactivity in macrophages treated with (+) cloricromene (10 μM) or DMSO as vehicle control (−) for 10 min and then treated with (+) or without (−) lipopolysaccharide (LPS, 100 ng/ml) for 5 min. Proteins from soluble (cytosol, 20 μg) and membrane (10 μg) fractions were loaded and electrophoresed on 12% SDS-PAGE.

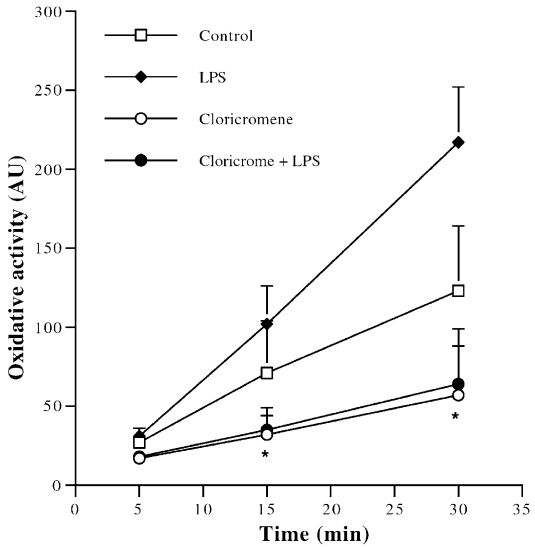

Fig. 5. Cloricromene inhibits lipopolysaccharide-induced cellular oxidative activity in live cells as measured by DCFH oxidation. Macrophages were loaded for 1 h with 10 μM DCFH and then treated for 10 min with cloricromene (10 μM) or DMSO as vehicle control and then lipopolysaccharide (LPS, 100 ng/ml) was added. Each value represents the mean ± S.D. of three determinations. Statistical analysis was performed by Student's t test; $^{*}P < 0.05$ vs. cells treated with lipopolysaccharide.

Lipopolysaccharide binding to its receptor CD14 activates protein kinase C and NF-κB. Cloricromene inhibited NF-κB activation in a dose-dependent manner (Fig. 3), only slightly affecting protein kinase C translocation (Fig. 4), indicating that cloricromene interferes with the early signal transduction pathway triggered by lipopolysaccharide. In preliminary experiments, by adding cloricromene directly to a nuclear extract, we excluded the possibility that cloricromene prevents the binding of NF-κB to the double-stranded oligonucleotide containing the binding site for NF-κB; instead, we deduced that cloricromene inhibits the activation of NF-κB.

In the activation of NF-κB, the intracellular generation of reactive oxygen species is a key event. We then investigated, using a fluorimetric assay, the effect of cloricromene on lipopolysaccharide-induced cellular oxidative activity. We found that cloricromene (10 μM) inhibited lipopolysaccharide-induced cellular oxidative activity (Fig. 5), which suggests that cloricromene, by scavenging reactive oxygen species, inhibits NF-κB activation and subsequent TNF-α synthesis.

4. Discussion

The purpose of this paper was to investigate the molecular mechanism of action of cloricromene, a non-anticoagulant coumarin derivative with antithrombotic, antiplatelet actions, and with beneficial effects in various models of ischemia and shock. In particular, we investigated in rat macrophages the mechanisms underlying the inhibition of lipopolysaccharide-induced TNF-α production.

TNF-α, a pleiotropic cytokine produced by macrophages and other cells, commonly mediates inflammatory reactions and endotoxin-induced shock. It plays an important role in the defense against viral, bacterial and parasitic infections. Overproduction of this cytokine is, however, detrimental and even lethal to the host (for review, see Fiers, 1991). Thus, the ability to modulate TNF-α production pharmacologically might have beneficial effects in several diseases. The anti-inflammatory effects of drugs such as corticosteroids (Martinet et al., 1992; Brattsand and Linden, 1996), pentoxifylline (Mandi et al., 1995) and pentamidine (Corsini et al., 1992, 1997) are indeed partially related to their ability to prevent endogenous TNF-α synthesis. In this paper, we confirmed the ability of cloricromene to inhibit lipopolysaccharide-induced TNF-α production in a dose-dependent manner, as was previously demonstrated both in vitro in peritoneal macrophages and in vivo (Squadrito et al., 1992, 1993; Ioculano et al., 1995).

The concentrations of cloricromene used in this study were physiologically relevant. It has been estimated, in experimental models, that the anti-ischemic activity of cloricromene is observed at peak plasma concentrations higher that 10 μM (Zatta and Bevilacqua, 1999), a concentration at which cloricromene is able to inhibit lipopolysaccharide-induced TNF-α production in vitro. We calculated an IC_{50} value of 5.9 ± 0.8 μM, confirming the relevance of our finding to the in vivo situation.

This paper highlights the mechanism of action of cloricromene, which, by scavenging reactive oxygen species, inhibits NF-κB activation and subsequent TNF-α neosynthesis. In macrophages, reactive oxygen species are directly or indirectly involved in lipopolysaccharide-induced NF-κB activation. The molecular mechanisms by which reactive oxygen species are involved in downstream signaling events and NF-κB activation remain, however, unclear and are a matter of controversy. One suggested mechanism is the inactivation of protein tyrosine phosphatase by oxidation of a conserved cysteine residue within the catalytic domain, leading to increased protein tyrosine phosphorylation and subsequent ubiquitination and degradation by proteosomes of I-κB, allowing nuclear translocation of NF-κB (Suzuki et al., 1997; Bours et al., 2000). However, it is likely that reactive oxygen species are cell type-specific second messengers for NF-κB activation, and thus the challenge will be to show which particular kinases or adaptor molecules are red-ox modulated (Bowie and O'Neill, 2000; Li and Karin, 1999; Bonizzi et al., 2000). Nevertheless, the ability of cloricromene to modulate NF-κB activation is likely to be relevant to other proinflammatory products of genes whose up-regulation is strongly dependent on activation of NF-κB. Most genes encoding adhesion molecules, cytokines, and other proinflammatory

proteins have functional NF-κB binding elements in their promoter regions (for review, see Baldwin, 1996; Beauerle and Henkel, 1994). Thus, the inhibition of NF-κB activation can help to explain the beneficial effect of cloricromene in several experimental models of ischemia and shock.

Protein kinase C activation, presumably via phosphorylation of the binding protein IκB and in this way releasing NF-κB subunits, is involved in the activation of NF-κB (Baeuerle and Henkel, 1994). Since cloricromene interacts with phosphoinositols, it has been postulated that it can interfere with protein kinase C activity. Under our experimental conditions, however, cloricromene only slightly affected lipopolysaccharide-induced protein kinase C βII translocation, indicating that the antioxidant effect and inhibition of NF-κB activation are the main mechanisms underlying the cloricromene-induced inhibition of TNF-α production. Protein kinase C β, protein kinase C ε (Shapira et al., 1997) and protein kinase C ζ (Herrera-Velit et al., 1997) are protein kinase C isoforms activated by lipopolysaccharide (Shinji et al., 1994). Even though unlikely, we cannot exclude an effect of cloricromene on the lipopolysaccharide-induced translocation of these other protein kinase C isoforms. The slight inhibition of protein kinase C βII translocation by cloricromene can be explained by the ability of this drug to reduce cytosolic calcium movements (Del Maschio et al., 1990): calcium is necessary for the translocation of protein kinase C βII, a calcium-dependent protein kinase C isoform.

The inhibitory activity of cloricromene on platelet-activating factor (PAF) synthesis (Ribaldi et al., 1992) may in part contribute to the inhibition of TNF-α release. However, the IC_{50} value for inhibition of A23187-stimulated PAF synthesis in neutrophils by cloricromene was 85 μM, 10 times the IC_{50} value for inhibition of TNF-α, which makes the involvement of PAF in the latter unlikely.

Finally, we cannot rule out the possibility that cloricromene may suppress the TNF message by enhancing degradation of the RNA message. However, the scavenger effect of cloricromene has been previously reported (Zatta and Bevilacqua, 1999) for neutrophil free-radical generation, with calculated IC_{50} values below 36 μM. A reduction in free-radical production by cloricromene has also been reported in vivo following myocardial ischemia and reperfusion (Milei et al., 1992a,b), which further supports the scavenger effect of this drug. These findings may also be extended to other members of the more than 1300 coumarins identified from natural sources, especially green plants. Many of these, such as coumarin itself, scoparone, osthole, warfarin, psoralenes, etc., display potent pharmacological activity and occur in various herbal remedies in Chinese medicine. Many coumarins are strong inhibitors of lipid peroxidation, scavengers of reactive oxygen species and inhibitors of eicosanoid generation, all anti-inflammatory properties that could be beneficial in several disorders in which eicosanoids and the production of reactive oxygen species are involved (for review, see Hoult and Payà, 1996).

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