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Tyloxapol Inhibits NF- κ B and Cytokine Release, Scavenges HOCl, and Reduces Viscosity of Cystic Fibrosis Sputum

ANDREW J. GHIO, BRUCE C. MARSHALL, JOSE L. DIAZ, TAKASHI HASEGAWA, WAYNE SAMUELSON, DANIEL POVIA, THOMAS P. KENNEDY, and CLAUDE A. PIANTADOSI

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Cystic fibrosis (CF) patients develop progressive cytokine-mediated inflammatory lung disease, with abundant production of thick, tenacious, protease- and oxidant-rich purulent airway secretions that are difficult to clear even with physiotherapy. In the search for a potential treatment, we have tested tyloxapol, an alkylaryl polyether alcohol polymer detergent previously used as a mucolytic agent in adult chronic bronchitis. Tyloxapol inhibits activation of the transcription factor nuclear factor-kappa B (NF- κ B), reduces resting secretion of the cytokine interleukin-8 (IL-8) in cultured human monocytes, and inhibits lipopolysaccharide (LPS)-stimulated release of tumor necrosis factor- α (TNF- α), IL-1 β , IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and the eicosanoids thromboxane A₂ and leukotriene B₄ (LTB₄). We have previously shown that tyloxapol is a potent antioxidant for hydroxyl radicals (\cdot OH). Tyloxapol (0.05 to 0.1% wt/vol) effectively scavenges the oxidant hypochlorous acid (HOCl; 1 to 7.5 mM) *in vitro*, and protects from HOCl-mediated lung injury in rats. Tyloxapol also reduces the viscosity of CF sputum (from 463 \pm 133 to 128 \pm 52 centipoise). We conclude that tyloxapol is potentially useful as a new antiinflammatory therapy for CF lung disease, and could possibly promote clearance of secretions in the CF airway. **Ghio AJ, Marshall BC, Diaz JL, Hasegawa T, Samuelson W, Povia D, Kennedy TP, Piantadosi CA. Tyloxapol inhibits NF- κ B and cytokine release, scavenges HOCl, and reduces viscosity of cystic fibrosis sputum.**

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The major cause of mortality and morbidity in patients with cystic fibrosis (CF) is lung disease from progressive pulmonary inflammation (1). Several factors contribute to this process, including production of inflammatory cytokines by alveolar macrophages (2) and airway epithelium (3, 4), and airway epithelial cytotoxicity from leukocyte myeloperoxidase-derived hypochlorous acid (HOCl) (5, 6). Also important is the abundant production of thick, tenacious, purulent airway secretions that obstruct airways and are difficult to clear even with physiotherapy (7-9).

In the search for a new treatment for CF lung disease, we have recently tested tyloxapol, a mucolytic agent used years ago in adult chronic bronchitis (10, 11). We have previously shown tyloxapol to be an antioxidant that protects against Fenton generation of hydroxyl radicals (\cdot OH) *in vitro* and the toxic effects of 100% O₂ in rats (12). A number of antioxidants prevent activation of nuclear factor-kappa B (NF- κ B) (13-15) and secretion

of inflammatory cytokines under the regulation of this nuclear transcription factor (15). We therefore hypothesized that tyloxapol might also inhibit NF- κ B activation and cytokine secretion, and scavenge HOCl. In this study, we report that tyloxapol reduces deoxyribonucleic acid (DNA) binding of NF- κ B induced by interleukin-1 β (IL-1 β) or H₂O₂, inhibits secretion of inflammatory cytokines such as IL-8 and tumor necrosis factor- α (TNF- α), is a potent scavenger of HOCl both *in vitro* and *in vivo*, and significantly reduces *in vitro* the viscosity of CF sputum.

METHODS

Materials

All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise specified.

Monocyte Release of Cytokines and Growth Factors

Human peripheral blood monocytes were prepared by centrifugal elutriation from leukopaks obtained from healthy donors. Purified monocytes were suspended at 2 \times 10⁶ cells/ml in RPMI-1640 supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% minimal essential medium (MEM) non-essential amino acids, 25 mM N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid (HEPES; GIBCO, Gaithersburg, MD), 196 Nutridoma (Boehringer Mannheim, Indianapolis, IN), and 5% pooled, heat-inactivated (56 $^{\circ}$ C, 30 min) human AB serum (Pel-freeze, Brown Deer, WI). One-half milliliter of this cell suspension was added to each well of a 48-well flat-bottomed tissue culture plate. Test materials (diluted in complete medium at 4 \times the desired final concentration) were added in 250 μ l volumes to each well. Control wells received 250 μ l of either

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This paper does not necessarily reflect the views or policies of the U.S. E.P.A. Drs. Ghio, Piantadosi, and Kennedy are the inventors of U.S. Patent 5,474,760, "Method of Inhibiting Oxidants Using Alkylaryl Polyether Alcohol Polymers," issued December 12, 1995, and owned jointly by Carolinas Medical Center and Duke University. This patent covers in part the findings disclosed in this publication.

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centration of 50 µg/ml). Tyloxapol was tested in triplicate at four concentrations in either the presence or the absence of 100 ng/ml *Salmonella typhosa* lipopolysaccharide (LPS; 250 µl of 4× desired final concentration added) and incubated at 37° C in humidified 5% CO₂ for 16 h. At this time, culture supernatants are aspirated off and the unattached cells and cell debris were removed by filtration. The release of TNF-α; IL-1β, IL-6, and IL-8; and the growth factor GM-CSF was determined in the cell-free supernatants using enzyme linked immunosorbent (ELISA) capture assays.

Monocyte Release of LTB₄, Platelet Activating Factor, and Thromboxane A₂

Monocytes were washed and resuspended in RPMI containing 5 mg/ml bovine serum albumin (BSA) at 2 × 10⁶ cells/ml, and were added to wells of a 48-well plate. The cells were allowed to adhere for 2 h and then washed in Hanks' balanced salt solution (HBSS)-BSA-HEPES buffer. Tyloxapol was added at four concentrations for 60 min and the monocytes were then stimulated by addition of 300 mg/ml zymosan A (175 µl of 2× desired final concentration). Supernatant medium was collected from the wells after 90 min incubation and stored at -20° C until assayed. Supernatants were assayed for leukotriene B₄ (LTB₄), platelet activating factor (PAF), or thromboxane A₂ (TXA₂) using specific scintillation proximity assays. Experiments were performed in triplicate for each concentration of tyloxapol.

Electrophoretic Mobility Shift Assays

A549 human pulmonary epithelial cells were cultured in Ham's F-12 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (250 ng/ml). Confluent cells were stimulated with 10 U/ml IL-1β or 100 µM H₂O₂. In some cultures 100 µg/ml tyloxapol was added at the same time as the stimulators. After 2 h of incubation, nuclear extracts were isolated as described by Dignam and colleagues (16), with minor modifications (17). In brief, after removal of the supernatant, cells were scraped gently in 20 to 30 ml of PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). The cell suspensions were centrifuged and the pellets were resuspended and incubated for 15 min in 1 ml buffer A containing 10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 1 mM PMSF, 1 mM DTT, 10 mM β-glycerolphosphate, 2.5 mM benzamidine, 1 mM NaF, 1 mM NaVO₄, 1 mg/ml leupeptin, and 1 mg/ml pepstatin A, and were then sheared by five passages of the suspensions through a 25-gauge needle. After centrifugation, the pellets were suspended and stirred for 30 min in buffer C containing 25% vol/vol glycerol, 0.25 M NaCl, 1.5 mM MgCl₂, 0.2 mM ethylenediamine tetraacetic acid (EDTA), 1 mM PMSF, 1 mM DTT, 10 mM β-glycerolphosphate, 2.5 mM benzamidine, 1 mM NaF, 1 mM NaVO₄, 1 mg/ml leupeptin, and 1 mg/ml pepstatin A. After centrifugation, nuclear extracts were obtained by dialysis of the supernatants in buffer D containing 20 mM HEPES, 20% vol/vol glycerol, 100 mM KCl, 0.2 mM EDTA, 1 mM PMSF, and 1 mM DTT. Utilizing the wild-type consensus sequences for AP-1 (18) and NF-κB (19) loci, the following oligonucleotides were synthesized (binding sites underlined):

AP-1: 5'-TTCCGGCTGACTCATCAAGCG-3'
3'-AAGGCCGACTGAGTAGTTCGC-5'
NF-κB: 5'-AGTTGAGGGGACTTTCCAGGC-3'
3'-TCAACTCCCCTGAAAGGGTCCG-5'

The oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis (PAGE) followed by passage over Sep-Pak C18 columns. Each complementary strand was end-labeled by phosphorylation with [³²P]adenosine triphosphate ([γ³²P]-ATP) and T4 polynucleotide kinase. Double-stranded DNA probes were generated by annealing the complementary end-labeled oligonucleotide strands, boiling for 3 min, and slow-cooling to room temperature in a water bath. Unincorporated radionucleotides were removed by Sephadex G-25 column chromatography. Binding reactions were performed for 20 min on ice with 5 to 10 µg total protein in a 20-µl volume containing 300 ng BSA, 1 to 2 µg poly deoxyinosine-deoxycytosine (di-dC), 50 mM DTT, 0.5 mM PMSF, and 1 to 2 × 10⁴ cpm of ³²P-labeled probes. In addition, a concentration of 6 mM MgCl₂ was used for AP-1 binding reactions. In selected samples, a 100-fold molar excess of unlabeled DNA probe was included in

interactions. DNA-protein complexes were separated from unbound DNA probe on 4.5% polyacrylamide gels under high-ionic-strength conditions in 50 mM tris-(hydroxymethyl) aminomethane (Tris), 0.4 M glycine, 2 mM EDTA, and 2.5% vol/vol glycerol, pH 8.5. Electrophoresis was done at 4° C at a constant current of 20 mA. Gels were dried under vacuum and exposed to film at -70° C for 6 to 24 h with an intensifier screen.

Investigation of Tyloxapol as an HOCl Scavenger *In Vitro*

The activity of tyloxapol as an HOCl scavenger was tested by its ability to prevent HOCl-mediated conversion of diethanolamine to its corresponding chloramine, diethanolchloramide (20). The reaction mixture (total volume: 1.2 ml) comprised 10.0 mM diethanolamine in 0.1 N sodium acetate buffer. To this were added varying concentrations (wt/vol%) of tyloxapol (0, 0.05, 0.1%) in 0.1 M NaCl, and the baseline absorbance was read at 280 nm. NaOCl (1.0 to 7.5 mM) was then added, the reaction mixture was incubated for 15 min at 37° C, and the absorbance was again measured. The difference in A₂₈₀ before and after addition of NaOCl was used as a measure of concentration of the stable chloramine. Experiments were performed in triplicate at each concentration of NaOCl and tyloxapol.

Measurement of Acute Lung Inflammation

The ability of tyloxapol to protect against lung injury from HOCl was studied in 60-d-old male Sprague-Dawley rats (n = 6 per treatment group) weighing 250 to 300 g (Charles River Breeding Labs, Wilmington, MA). After anesthesia with halothane (2 to 5%), rats were injected intratracheally with either 0.3 ml of 2.0 mM NaOCl in normal saline (buffered to pH 6.0), or with normal saline alone. The rats were allowed to recover, and 1 h later were dosed intratracheally with either 6.0 mg tyloxapol in normal saline or with normal saline. Twenty-four hours after NaOCl instillation, all rats were euthanized with sodium pentobarbital. The tracheas were cannulated and the lungs were lavaged with normal saline (35 ml/kg body weight). After staining of the lavage fluid with a modified Wright's stain (Diff-Quick stain; ASP, McGaw Park, IL), the cell differentials were determined on 500 cells/sample. Values were expressed as the percentage of total cells recovered. Lavage protein was measured using the Bio-Rad method for total protein determination as modified for use on the centrifugal analyzer.

Collection of Sputum

CF sputum was collected from hospitalized young adult CF patients of the Duke Adult Cystic Fibrosis Center and frozen at -20° C until used. The diagnosis of CF had been previously made in all subjects by a positive sweat chloride test and compatible clinical course of disease. No patient had received recombinant deoxyribonuclease (rhDNase) for at least 2 wk prior to the time of sputum collection.

Measurement of Sputum Viscosity

Sputum viscosity was studied with a model LVT Brookfield cone/plate viscometer (Brookfield Engineering Laboratories, Inc., Stoughton, MA) with a calibrated torque of 673.7 dynes-cm at full scale. Sputum (750 µl) was mixed 3:1 with 0.9% saline or 0.125% tyloxapol (wt/vol) in saline (250 µl), vortexed for 30 s, and then incubated for 15 min at 37° C. Viscosity of the 1-ml preparation was measured at 0.3 rpm (shear rate of 2.25/s) and 37° C, using a CP-40 cone spindle (cone angle of 0.8 degrees and radius of 2.4 cm), and was expressed as milli-Poiseville (mPa). Control measurements of saline or saline and tyloxapol alone showed that they behaved similarly to non-Newtonian pseudoplastic fluids, with respective viscosities of 20.6 and 10.3 centipoises, respectively, at a shear rate of 2.25/sec, and 0.78 and 0.83 centipoises, respectively, at a shear rate of 225/sec. Because the viscosity of saline-treated sputum from Patients 1 and 9 was too high for measurement, sputum of these individuals was diluted 1:3 and 1:1, respectively, with saline or tyloxapol in saline.

Statistics

Data are expressed as mean values ± SEM. The difference between tyloxapol and saline-treated sputum was analyzed with the paired *t* test (21). Differences among groups in animal experiments and human monocyte incubations were analyzed with analysis of variance (ANOVA) and

RESULTS

Inhibitory Activity on Cytokine and Mediator Secretion

The concentration of endotoxin in all buffers and tyloxapol was below the level of detection (25 pg/ml). Incubations of monocytes in concentrations of tyloxapol of 100 µg/ml or below were not associated with significant elevations in the concentration of lactate dehydrogenase (LDH) in the supernatant, supporting a lack of cytotoxicity and suggesting that the inhibition of cytokine secretion by tyloxapol was not due to an injurious detergent effect on monocytes.

Tyloxapol had no effect on the baseline release of any mediator except for IL-8, but significantly decreased secretion of this cytokine in unstimulated cells (Figure 1). However, release of several mediators by LPS-stimulated monocytes was significantly diminished at low concentrations of tyloxapol. Secretion of TNF-α, IL-1β, IL-6, and IL-8 (Figure 2) was significantly (p < 0.01) decreased by tyloxapol in a dose-dependent manner, with effective concentrations for 50% inhibition (EC₅₀) ranging from 30 to 70 µg/ml (Table 1). As in the case of the cytokines, release of GM-CSF (Figure 2) by stimulated monocytes decreased with increasing concentrations of tyloxapol in the cell suspension. Finally, secretion of the eicosanoids LTB₄ and TXA₂ (Figure 3) was also significantly (p < 0.01) reduced by tyloxapol. However, tyloxapol did not change PAF release from LPS-stimulated monocytes (data not shown).

An effect of a second detergent, Triton X, on mediator release by human monocytes was also examined. Triton X was cytotoxic at lower concentrations than tyloxapol, with significant elevations in supernatant [LDH] after 1.0 µg/ml. Therefore, the concentrations of Triton X required to inhibit 50% of the mediator release by stimulated human monocytes probably reflected the cytotoxicity of this detergent.

Inhibitory Activity on Activation of Nuclear Transcription Factors

IL-1β or H₂O₂ treatment of A549 human pulmonary epithelial cells increased DNA binding of both NF-κB and AP-1, as measured by gel shift assays (arrows, Lanes 2 and 4, Figure 4). Concomitant treatment of cells with 100 µg/ml tyloxapol substantially blocked the increase in NF-κB-binding activity induced by IL-1β and H₂O₂ (Lanes 3 and 5, Figure 4), but had no significant effect on AP-1 binding activity.

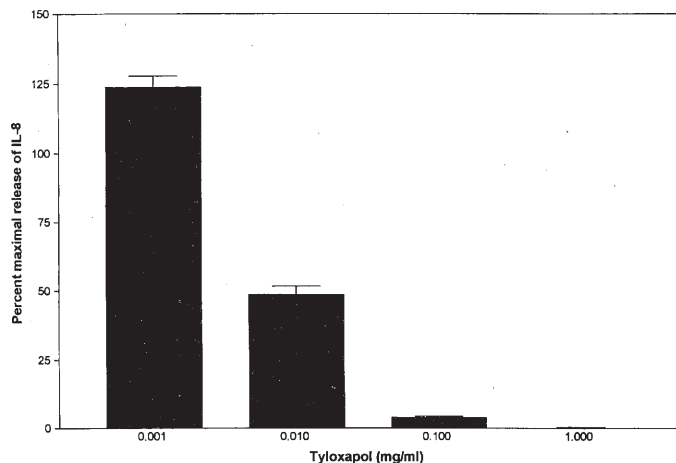


Figure 1. Tyloxapol reduces baseline secretion of IL-8 by unstimulated human monocytes. IL-8 release was significantly (p < 0.01) reduced by tyloxapol concentrations greater than 0.01 mg/ml. Methods

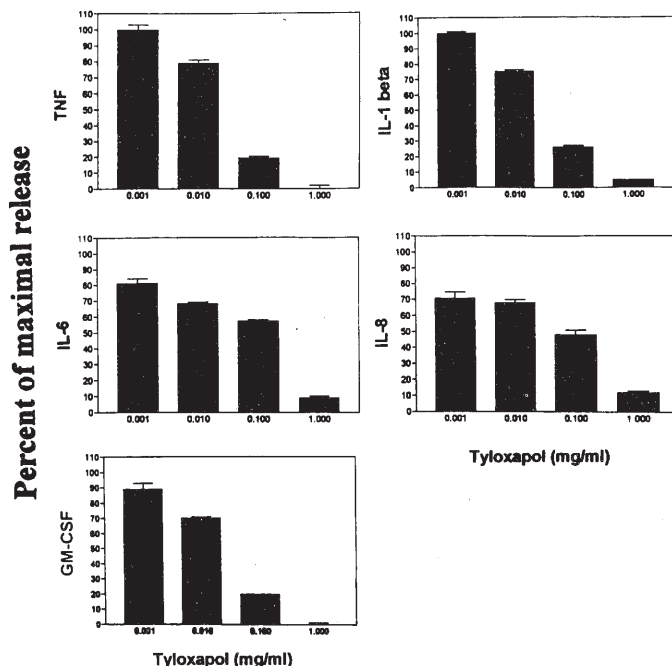


Figure 2. Tyloxapol significantly (p < 0.01) reduces secretion of the cytokines TNF-α, IL-1β, IL-6, IL-8, and of the growth factor GM-CSF by LPS-stimulated human monocytes in a dose-dependent manner. Methods are detailed in text.

In Vitro Scavenging of HOCl

NaOCl oxidized diethanolamine to its corresponding chloramine in a dose-dependent fashion (Table 2). Tyloxapol dramatically reduced chloramine formation (Table 2), suggesting that tyloxapol is an avid scavenger of HOCl.

Acute Lung Inflammation from HOCl

Intratracheal instillation of NaOCl caused acute lung injury, as demonstrated by a marked increase in protein concentration and the percentage of PMNs in lung lavage fluid (Figure 5). Postexposure treatment with tyloxapol significantly reduced lavage protein concentration (p < 0.001) and the percentage of PMNs (p < 0.01), demonstrating that tyloxapol also protects against HOCl-mediated cytotoxicity *in vivo*. Although not significantly different from saline control, tyloxapol alone increased the percentage of PMNs in lavage fluid, a finding that will require additional work to confirm.

TABLE 1
EFFECTIVE CONCENTRATIONS OF TYLOXAPOL FOR 50% INHIBITION (EC₅₀) OF MONOCYTE CYTOKINE RELEASE

Cytokine	EC ₅₀ (µg/ml)
TNF-α	30
IL-1β	60
IL-6	30
IL-8	70

Cytokine secretion was studied in cultured human monocytes stimulated with *Salmonella typhosa* lipopolysaccharide (LPS). Levels were measured with ELISA capture as-

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