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Inhibition of human neutrophil responses by α-cyano-3,4-dihydroxythiocinnamamide; a protein-tyrosine kinase inhibitor

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1 Activation of neutrophils results in increased tyrosine phosphorylation of several proteins that may have important roles in receptor/effector coupling. In this study, the effect of a protein tyrosine kinase inhibitor on receptor-mediated neutrophil activation by platelet-activating factor (PAF), leukotriene, B_4 (LTB_4) and N-formylmethionylleucylphenylalanine (FMLP) is investigated.

2 α-Cyano-3,4-dihydroxythiocinnamamide dose-dependently inhibited intracellular calcium release and superoxide generation from human neutrophils activated by 1 μM LTB_4, PAF, and FMLP.

3 In the presence of cytochalasin B, FMLP stimulated elastase release from neutrophils was also inhibited to unstimulated levels by 5 min pretreatment with α-cyano,3,4-dihydroxythiocinnamamide.

4 The inhibitory action of α-cyano-3,4-dihydroxythiocinnamamide was found to be at or upstream of phospholipase C activation, blocking both phosphatidylinositol hydrolysis and protein kinase C activation. α-Cyano-3,4-dihydroxythiocinnamamide did not affect agonist receptor binding sites or receptor affinity in neutrophils.

5 Immunoblot analysis demonstrated the tyrosine phosphorylation of proteins of 41, 56, 66, and 104 kDa in neutrophils treated with agonists. Treatment of neutrophils with α-cyano-3,4-dihydroxythiocinnamamide prior to stimulation with chemoattractants reduced tyrosine phosphorylation of the above phosphoproteins.

6 These results indicate that α-cyano-3,4-dihydroxythiocinnamamide might be a useful agent in characterizing the essential proteins and biochemical pathways that regulate neutrophil activation.

Keywords: Neutrophil; protein-tyrosine kinase; tryphostins; protein kinase C; inositol phosphates; elastase; superoxide anion

Introduction

Neutrophils respond to various chemotactic factors such as N-formylmethionylleucylphenylalanine (FMLP), leukotriene B_4 (LTB_4) and platelet-activating factor (PAF) by releasing their granule contents, generating oxygen free radicals, and undergoing directional migration towards the stimuli (Sha'afi & Molski, 1988). Activation of neutrophils by these agonists involves a pertussis toxin-sensitive G-protein (G_i) that activates phospholipase C (PLC) (Snyderman *et al.*, 1986; Sha'afi & Molsi, 1988). An initial step in the activation of neutrophils is the PLC catalyzed hydrolysis of phosphatidylinositol-4,5-bisphophate (PIP_2), producing two second messengers, inositol trisphosphate (IP_3) and 1,2-dioleoyl-sn-glycerol (DAG). IP_3 causes calcium release from intracellular stores (Berridge & Irvine, 1984) and acts synergistically with DAG to activate protein kinase C (PKC) (Nishizuka, 1988).

The signal transduction events involved in neutrophil activation are largely unknown. It has been reported that activation of neutrophils by various agonists involves phosphorylation on tyrosine residues of several proteins. Gomez-Cambronero *et al.* (1989a) reported tyrosine phosphorylation of five proteins with molecular masses of 118, 92, 78, 54, and 40 kDa in neutrophils stimulated with granulocyte-macrophage colony stimulating factor (GM-CSF). Cytosolic and particulate substrates have been shown to be tyrosine phosphorylated in neutrophils stimulated with FMLP, LTB_4, a phorbol ester, and a calcium ionophore suggesting receptor-dependent and receptor-independent tyrosine kinase activation (Berkow & Dodson, 1990). Proteins of similar molecular weight have also been shown to be tyrosine phosphorylated in neutrophils stimulated with chemotactic peptide FMLP (Gomez-Cambronero *et al.*, 1989b). In addition, similarities in the patterns of protein tyrosine phosphorylation are also evident in neutrophils activated with PAF and LTB_4 (Huang *et al.*, 1990; Gomez-Cambronero *et al.*, 1991). Attempts have been made to identify these phosphoproteins and to characterize their roles in neutrophil activation. In contrast to some growth factors such as epidermal growth factor and insulin (Ullrich *et al.*, 1984; Shechter *et al.*, 1989), the receptors for inflammatory agonists such as PAF, LTB_4, and FMLP have not been shown to have a kinase domain. In fact, the PAF receptor that was recently cloned (Honda *et al.*, 1991) contains seven transmembrane domains and guanine nucleotide binding protein (G-protein) binding sites, but no indication of how a tyrosine kinase might be activated. This suggests that the stimulation of tyrosine kinases by receptors for inflammatory mediators might be occurring as a consequence of other second messenger systems downstream of G-proteins.

Stimulation of protein tyrosine phosphorylation in electroporated human neutrophils treated with GTPγS suggests that the activated G-proteins can stimulate tyrosine kinases (Nasmith *et al.*, 1989). Similarily, receptor-mediated tyrosine phosphorylation has been shown to be mediated in part by a pertussis toxin-sensitive guanine nucleotide regulatory protein (Gomez-Cambronero *et al.*, 1989a). Various studies have demonstrated tyrosine phosphorylation of PLCγ- following interaction with ligand-activated PDGF or EGF receptors (Wahl *et al.*, 1989; Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989). Unlike PLC α, β, and δ, which are coupled to a G-protein, PLCγ activity increases following tyrosine phosphorylation (Taylor *et al.*, 1991). Therefore, the precise relationship between tyrosine kinase activation and activation via G-proteins remains unclear.

In neutrophils, the role of tyrosine phosphorylation in response to agonist stimulation has not been clearly defined. When neutrophils were treated with erbstatin, a protein-

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tyrosine kinase inhibitor, the superoxide generation elicited by FMLP was dose-dependently inhibited (Naccache *et al.*, 1990). Erbstatin also blocked the change in cytosolic pH caused by PAF and LTB_4, but not by FMLP. However, erbstatin was unable to inhibit the lysosomal enzyme release or elastase release in response to stimulation with FMLP in the presence of cytochalasin B (Naccache *et al.*, 1990). Due to the partial inhibitory activity of erbstatin, the effect of α-cyano,-3,4-dihydroxythiocinnamamide on neutrophil biochemical and physiological responses to chemoattractants were studied. The results of this study suggest that tyrosine phosphorylation is required for neutrophil activation in response to chemotactic factors. Although the precise role of tyrosine phosphorylation in neutrophil activation is unclear, inhibition of tyrosine kinase activity was associated with decreases in Ca^{2+} release, PLC activity, and PKC activity. In addition, we demonstrate that physiologically and pathologically related responses including superoxide anion production and elastase release are diminished. Thus, tyrosine kinase activity plays a major role in signal transduction and stimulus response coupling in neutrophils and inhibition of this class of enzymes decreases cellular responses.

Methods

Preparation of neutrophils

Human blood from normal volunteers was obtained in heparin. Blood was diluted with Tyrode buffer (pH 6.5) (5:1, v/v) and red cells were sedimented for 30 min after mixing blood with Dextran as described by Salari *et al.* (1985). Neutrophils were isolated following Ficoll-hypaque gradient centrifugation and the remaining erythrocytes were removed by Tris-NH_4Cl haemolysis (Boyle, 1968). Neutrophils at greater than 97% purity and greater than 95% viability as assessed by trypan blue dye exclusion were collected in an appropriate buffer.

Fura-2 loading and measurement of Ca^{2+}

Neutrophils were suspended in PBS (no Ca^{2+} or Mg^{2+}) with 1 μM Fura-2AM for 60 min, at 37°C. Free Fura-2AM was removed by washing the cells three times with PBS. Cells (5×10^6 cells ml^{-1}) were warmed to 37°C in a 1 cm^2 quartz cuvette in a volume of 2.5 ml. Changes in Ca^{2+}-dependent Fura-2 fluorescence were measured with a Perkin-Elmer (Norwalk, CT) LS-50 luminescence spectrophotometer with excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The baseline level of fluorescence before addition of agonists was subtracted from agonist-induced fluorescence in the presence and absence of drug. Values described are arbitrary units taken from ratios between the absorbances at 340 nm and 380 nm.

Polyphosphoinositide hydrolysis

Neutrophils (2.5×10^7 cells ml^{-1}) were incubated with 100 μCi ml^{-1} of *myo*-[2-^{3}H]-inositol in Tyrode buffer (no Ca^{2+}), for 90 min at 37°C. Cells were washed twice and resuspended in Tyrode buffer containing 12 mM LiCl, 1.3 mM $CaCl_2$ and 0.6 mM $MgCl_2$. Cells in 0.5 ml volumes were incubated with or without α-cyano-3,4-dihydroxythiocinnamamide for 5 min and then treated with agonists for a further 5 min. The reaction was terminated by addition of 1.5 ml methanol/chloroform/HCl (200:100:2) as described by Watson *et al.* (1984). Following overnight storage at 4°C, water (0.6 ml) was added to each sample to extract water-soluble inositol phosphates; 1.8 ml of the upper phase was diluted with 2.5 ml of water and layered onto 1 ml Dowex anion exchange resin (Bio-Rad) columns, pre-equilibrated with water. Inositol phosphates were isolated and quantified as described earlier (Salari *et al.*, 1990b).

Protein kinase C assay

Neutrophils (10^7 cells ml^{-1}) in Tyrode buffer containing 1.4 mM $CaCl_2$ were stimulated with or without pretreatment with α-cyano-3,4-dihydroxythiocinnamamide and chemotactic factors at 37°C. Treatments were terminated by quick centrifugation in a microfuge. Pellets were resuspended on ice in a homogenizing buffer and sonicated for 30 s as reported earlier (Pelech *et al.*, 1990). The particulate and cytosolic fractions were isolated by centrifugation at 200,000 *g* in a Beckman TL-100 ultracentrifuge; 500 μg of cytosolic and NP-40 solubilized particulate extracts were fractionated on a 1 ml anion exchange Mono Q column coupled to a FPLC system (Pharmacia). Fractions were eluted at a flow rate of 0.8 ml min^{-1} using a linear gradient of 0–0.8 M NaCl. PKC activity of column fractions were assayed as described previously (Pelech *et al.*, 1990). Reactions were carried out at 30°C in a volume of 25 μl with 50 μM [γ-^{32}P]-ATP (1500 c.p.m. $pmol^{-1}$), 25 mM β-glycerophosphate, 10 mM MOPS (pH 7.2), 15 mM $MgCl_2$, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 500 nM PKIP, and 1 mM sodium orthovanadate, with 1 mg ml^{-1} histone H1 or protamine chloride as the substrate. Where stated, incubations included 4.5 mM $CaCl_2$ 60 μg^{-1} ml^{-1} phosphatidylserine and 6 μg ml DAG. Reactions were terminated by spotting 20 μl aliquots on 2 cm^2 pieces of Whatman P81 phosphocellulose paper, dried 30 s, and washed with several changes of 1% (v/v) phosphoric acid. Filter papers were counted for radioactivity in a Beckman model LS5000 scintillation counter.

Tyrosine phosphorylation

Neutrophils (10^7 cells ml^{-1}) in Tyrode solution containing 1.4 mM $CaCl_2$ were treated at 37°C for 1 min with each agonist or pretreated with α-cyano-3,4-dihydroxythiocinnamamide prior to stimulation for 5 min at 37°C. After stimulation, cells were rapidly pelleted by microcentrifugation (14,000 r.p.m. for 5 s), pellets were solubilized with 3% Triton or sonicated in buffer composed of 50 mM Tris-HCl, pH 7.7, 5 mM β-methylaspartate, 150 mM NaCl, 0.2 mM Na_3VO_4, 10 mM NaF, 1 mM $NaMoO_4$, 5 mM EDTA, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} soybean trypsin inhibitor, 1 μM pepstatin, 1 mM PMSF, and 1 mM diisopropylfluorophosphate. Insoluble cellular debris were removed by microcentrifugation (14,000 r.p.m. for 1 min) and soluble cell supernatants were combined with SDS-PAGE sample buffer, boiled, and electrophoresed by discontinuous SDS-PAGE. Separated proteins were transferred to nitrocellulose sheets and blocked overnight in Tris-buffered saline (TBS) (0.02 M Tris-HCl, pH 7.5, 0.05 M NaCl) containing 5% BSA and 1% ovalbumin. Blots were washed with TBS and probed with antiphosphotyrosine specific antibody PY-20 in TBS with 1% BSA and 0.02% sodium azide. After washing, blots were incubated with alkaline phosphatase conjugated goat anti-mouse IgG in TBS + 0.05% NP-40 for 2 h at room temperature before colour development with 5-bromo-4-chloro-3-indolyphosphate (BCIP) and nitro blue tetrazolium (NBT). The specificity of antibody PY-20 has been demonstrated by competition with phosphotyrosine or phenylphosphate but not phosphoserine, phosphothreonine, or free phosphate (Glenny *et al.*, 1988).

Elastase release

[^{3}H]-elastin substrate was prepared as previously described with [^{3}H]-$NaBH_4$ used as the radioactive source (Takahashi *et al.*, 1973). The [^{3}H]-elastin suspension (specific activity = 3168 c.p.m. μg^{-1}) was sonicated, agitated, and 20 μl suspensions were evenly spread on 16 mm wells (Costar plates). The plates were dried at 45°C overnight, washed with phosphate buffered saline (PBS), and stored at 4°C until use (usually 1–7 weeks). 10^6 cells in 500 μl DMEM were added to each well, stimuli or inhibitors diluted in DMEM were added, and

plates were incubated at 37°C in an atmosphere of 5% CO_2. To monitor elastase release by the cells into the media, after 1 h incubation, 100 µl of media was removed, microcentrifuged (14,000 r.p.m. for 2 min) to remove contaminating cells, and the soluble phase was counted for radioactivity. Tritium counts from control wells incubated with DMEM in the absence of cells were subtracted from wells with cells and µg elastin degraded by 10^6 cells in 1 h was calculated.

Superoxide anion generation

Continuous spectrophotometric measurement of a superoxide dismutase inhibitable reduction of ferricytochrome C at 549 nm was measured in cuvettes as described (Robinson *et al.*, 1985). Reaction mixtures containing 500 units ml^{-1} catalase and 1.24 mg ml^{-1} cytochrome C in HBSS supplemented with 5.5 mM glucose, 0.1% gelatin and $1.5-2.5 \times 10^6$ cells ml^{-1}. Prior to stimulation, mixtures were warmed to 37°C, agonists diluted in HBSS + 0.1% gelatin were added, and absorbance at 549 nm was scanned every 10 s with a Beckman DU-65 spectrophotometer and plotted manually.

PAF binding

Competition binding studies were performed at room temperature as described previously (Duronio *et al.*, 1990). Neutrophils and reagents were suspended in binding buffer (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, and 2.5 mg ml^{-1} BSA) and 5×10^6 cells were added to start the reaction in tubes containing 0.25 nM [^{3}H]-PAF and 0 to 500 fold excess of unlabelled PAF for 30 min at room temperature. Reactions were terminated by filtration through prewetted GF/C filters and washed with 5 ml binding buffer and counted for radioactivity. Nonspecific binding was referred to as the radioactivity associated with cells in the presence of a 500 fold excess of unlabelled ligand.

Chemicals and drugs

Histone H1, bovine serum albumin (BSA), ovalbumin, L-α-phosphatidyl-L-serine, DAG, phenylmethyl sulphonyl fluoride (PMSF), leupeptin, pepstatin, β-methylaspartate, diisopropylfluorophosphate (DFP), pepstatin, PAF, LTB_4, FMLP, Ficoll-Hypaque, cytochalasin B, cyclic AMP-dependent protein kinase inhibitor peptide (amino acids 5-24) (PKIP), and other chemicals, unless stated, were purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A.). Fura-2AM was purchased from Molecular Probes (Eugene, OR, U.S.A.). Dextran T500 was purchased from Pharmacia (Montreal, Canada). Electrophoresis reagents, nitrocellulose sheets, and alkaline phosphatase conjugated goat anti-mouse antibody were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). [γ-^{32}P]-ATP (~3000 Ci $mmol^{-1}$), *myo*-[2-^{3}H]-inositol (18.3 Ci $mmol^{-1}$), and [^{3}H]-PAF (80 Ci $mmol^{-1}$) were purchased from Amersham (Arlington Heights, IL, U.S.A.). Scintillation fluid, 3-(N-morpholino) propanesulphonic acid (MOPS), and antibody PY-20 were purchased from ICN Biomedicals, Inc (Costa Mesa, CA, U.S.A.). GF/C filters and P81 phosphocellulose paper were purchased from Whatman (Mandel Scientific, Toronto, Canada). Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (Grand Island, NY, U.S.A.). α-Cyano-3,4-dihydroxythiocinnamamide was synthesized as described by Gazit *et al.* (1989) and stored in a stock solution of dimethylsulphoxide (DMSO) before serial dilution in an appropriate buffer for each experiment. DMSO concentrations were below 0.2% in all experiments.

Statistical analysis

Data are expressed as mean ± s.d. Elastase data were analysed by a two-way analysis of variance using the Systat programme version 5.1 (Evanston, IL, U.S.A.). Cellular inositol phosphate measurements were compared by Student's *t* test with correction for multiple comparisons.

Results

The effect of the α-cyano-3,4-dihydroxythiocinnamamide on ligand-induced increases in cytosolic free Ca^{2+} concentration

Since a rapid increase in intracellular free Ca^{2+} is required for neutrophil activation, the effect of this tyrosine kinase inhibitor on Ca^{2+} release was analysed. In all cases, a calcium ionophore was used as a positive control and addition of buffer alone served as a baseline. An increase in intracellular Ca^{2+} levels were observed in Fura-2 AM loaded cells stimulated with PAF, FMLP, or LTB_4. Maximum release of calcium with the three agonists were in the order $LTB_4 > FMLP > PAF$ at a concentration of 1 µM. Pretreatment of neutrophils for 5 min with α-cyano-3,4-dihydroxythiocinnamamide caused a dose-dependent decrease in Ca^{2+} mobilization in response to PAF, LTB_4 and FMLP by agonist stimulation of 10^{-5}, 10^{-7}, and 10^{-6} M, respectively (Figure 1b). α-Cyano-3,4-dihydroxythiocinnamamide was effective when added as little as 1 min before the stimulus (data not shown). α-Cyano-3,4-dihydroxythiocinnamamide did not cause neutrophil toxicity as neutrophils washed with buffer after incubation with α-cyano-3,4-dihydroxythiocinnamamide for 5 min were fully responsive to stimulation with the three agonists (data not shown).

Inhibition of human neutrophil physiological responses

It has been reported that FMLP (Lehmeyer *et al.*, 1979) and PAF (Dewald & Baggiolini, 1986) are capable of increasing superoxide production in human neutrophils. We evaluated the effect of α-cyano-3,4-dihdyroxythiocinnamamide on the generation of superoxide anion by neutrophils treated with 1 µM FMLP or PAF. When neutrophils were treated with FMLP and cytochalasin B (1 µM) a rapid generation of superoxide (O_2^-) was observed. The O_2^- generation reached a plateau at about 5 min after agonist treatment. Similarly, PAF in the presence of cytochalasin B caused a rapid generation of O_2^- from neutrophils. FMLP was a more potent stimulus for O_2^- production (Figure 2). FMLP and PAF alone caused O_2^- release, although the presence of cytochalasin B potentiated O_2^- release as reported (Dewald & Baggiolini, 1986). When cells were pretreated with 0.2 mM α-cyano-3,4-dihydroxythiocinnamamide (50 µg ml^{-1}) 5 min before the addition of FMLP or PAF, a potent inhibition of O_2^- generation was observed. The drug was able to inhibit O_2^- release by PAF or FMLP by more than 70% in several independent experiments (Figure 2).

Neutrophil degranulation leads to the production of lysosomal enzymes and oxygen radicals. One component of lysosomal enzyme contents is elastase, a serine protease that has been implicated in various disease states (Sandberg *et al.*, 1981). In the present investigation, the effect of α-cyano-3,4-dihydroxythiocinnamamide on the release of elastase from neutrophils was evaluated. As has been reported (Styrt *et al.*, 1987), cytochalasin B appreciably augments neutrophil degranulation. In the absence of cytochalasin B, no significant increases in elastase release was detected in cells stimulated with FMLP, PAF, or LTB_4 (data not shown). In the presence of 1 µM cytochalasin B, a concentration previously shown to stimulate human polymorphonuclear leukocyte functions (Salari *et al.*, 1985), 1 µM FMLP significantly stimulated elastase release from neutrophils (Figure 3a). Although cytochalasin B potentiated FMLP-induced elastase release, the presence of cytochalasin B did not significantly enhance PAF or LTB_4-induced elastase release. Pretreatment of neutrophils with α-cyano-3,4-dihydroxythiocinnamamide for 5 min before the addition of FMLP inhibited FMLP-

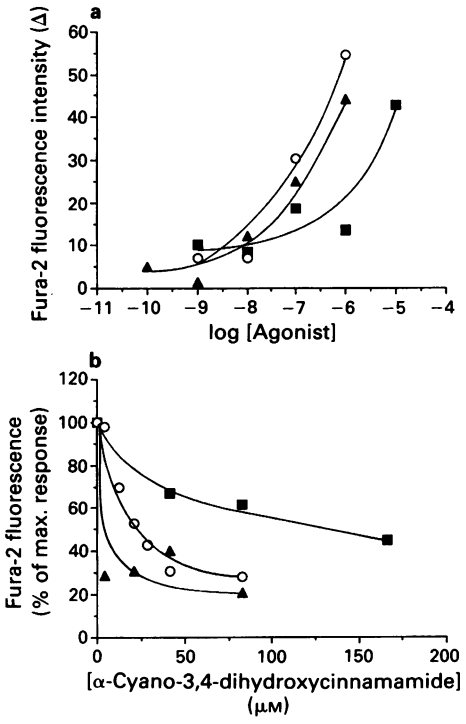

Figure 1 Effect of α-cyano-3,4-dihydroxythiocinnamamide on intracellular calcium release. (a) Effect of agonist concentration on intracellular Ca^{2+} release. Neutrophils were loaded with Fura-2AM (1 μM) and free dye was washed from cells with PBS. Cells were warmed to 37°C and stimulated with various concentrations of PAF (■), leukotriene B_4 (LTB_4) (○), and fMet-Leu-Phe (▲) and the change in fluorescence is plotted. Changes in fluorescence are reported as the difference in fluorescence before stimulation and at maximal fluorescence after stimulation. (b) Concentration-dependent dose-response curve of inhibition of intracellular Ca^{2+} release in human neutrophils by α-cyano-3,4-dihydroxythiocinnamamide in response to 10 μM PAF (■), 0.1 μM LTB_4 (○), and 1 μM FMLP (▲). Fluorescence is reported as the ratio between the absorbances at 340 nm and 380 nm. Results are illustrated by drawing curves by visual inspection and a representative of three similar experiments is illustrated.

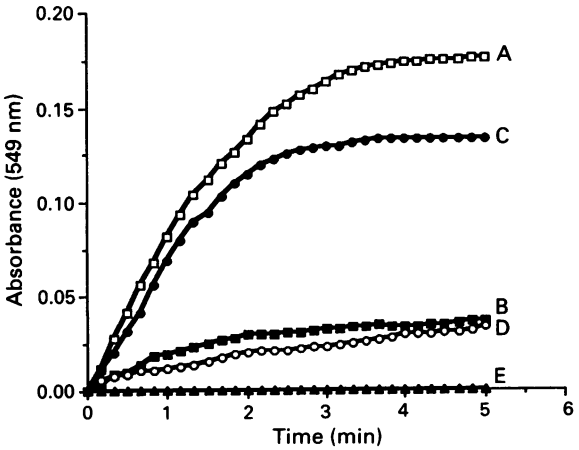

Figure 2 Effect of α-cyano-3,4-dihydroxythiocinnamamide on stimulation of superoxide generation from human neutrophils. Spectrophotometric analysis of agonist-induced reduction of ferricytochrome C was monitored at 549 nm. Cells were either stimulated with 1 μM PAF or fMet-Leu-Phe in the presence of 1 μM cytochalasin B or cells were exposed to 0.2 mM α-cyano-3,4-dihydroxythiocinnamamide for 5 min before addition of agonists: (A) FMLP, (B) α-cyano-3,4-dihydroxythiocinnamamide and FMLP, (C) PAF, (D) α-cyano-3,4-dihydroxythiocinnamamide and PAF, and (E) untreated cells or cells stimulated in the presence of superoxide dismutase. Representative data of 3 independent experiments are shown.

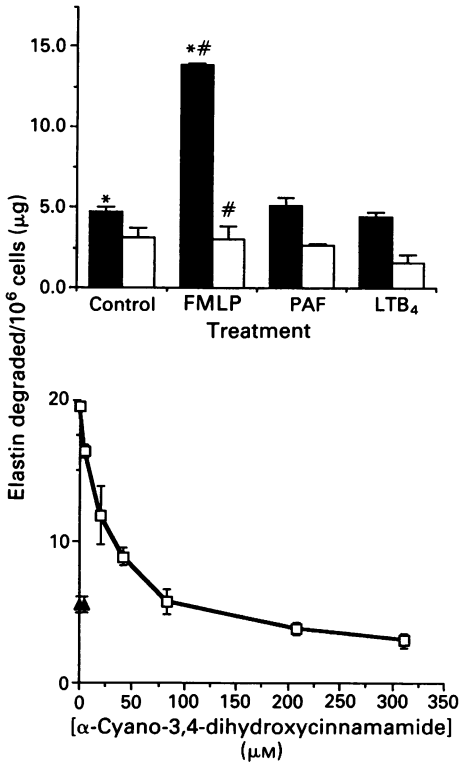

Figure 3 Inhibition of elastase release in human neutrophils by α-cyano-3,4-dihydroxythiocinnamamide. (a) Neutrophils were preincubated with buffer alone (solid columns) or 0.2 mM α-cyano-3,4-dihydroxythiocinnamamide (open columns) for 5 min at 37°C. Elastase release in the presence of 1 μM cytochalasin B was stimulated by 1 μM of each agonist used. Vertical bars span the standard deviation. The values obtained indicate that fMet-Leu-Phe (FMLP) significantly stimulates elastase release from neutrophils in the presence of cytochalasin B ($*P \leq .001$, $n = 3$) and that α-cyano-3,4-dihydroxythiocinnamamide significantly inhibits FMLP-stimulated elastase release ($\#P \leq 0.001$, $n = 3$). (b) Dose-response curve of inhibition of FMLP-stimulated elastase release by α-cyano-3,4-dihydroxythiocinnamamide. Increasing amounts of α-cyano-3,4-dihydroxythiocinnamamide were incubated for 5 min at 37°C with 10^5–10^6 neutrophils prior to 1 mM FMLP stimulation (□) or untreated cells (△). Solubilized [^{3}H]elastin released into the culture medium was counted for radioactivity after a 1 h incubation.

stimulated elastase release. The effect of α-cyano-3,4-dihydroxythiocinnamamide was dose-dependent (Figure 3b) and total inhibition of FMLP-induced elastase release to control levels was seen at a drug concentration 83 μM. The IC_{50} for inhibition of elastase release was calculated to be about 35 μM. It was observed that higher concentrations of α-cyano-3,4-dihydroxythiocinnamamide reduced non-specific elastase release, presumably due to stabilization of cellular activation processes. The basal release of elastase and the minimal effects of PAF and LTB_4 on elastase secretion were also reduced below unstimulated levels by α-cyano-3,4-dihydroxythiocinnamamide (Figure 3a). Cells pretreated with the drug were viable after 1 h incubation as determined by a trypan blue exclusion dye assay.

Effect of α-cyano-3,4-dihydroxythiocinnamamide on ligand-receptor interactions

Studies with calcium release and O_2^- generation indicated that the action of α-cyano-3,4-dihydroxythiocinnamamide is directed against receptor-mediated responses in neutrophils, perhaps due to inhibition of agonist-receptor interactions. To investigate this possibility, we tested the effect of α-cyano-3,4-dihydroxythiocinnamamide on one of the agonists that binds to neutrophil membranes to initiate cellular responses. PAF

was selected for this study as a result of our previous experience with PAF receptor binding (Duronio *et al.*, 1990). Specific PAF receptor binding was unaffected by the presence of 0.2 mM α-cyano-3,4-dihydroxythiocinnamamide (510 ± 119 d.p.m., 511 ± 105 d.p.m. ($n = 3$); α-cyano-3,4-dihydroxythiocinnamamide absent, α-cyano-3,4-dihydroxythiocinnamamide present). In a single experiment, Scatchard plot analysis of data showed that human neutrophils possess ~6000 receptors/cell with a K_d of ~2.3 nM when neutrophils were incubated with 0.2 mM α-cyano-3,4-dihydroxythiocinnamamide for 5 min before PAF binding. In the absence of the drug, the K_d remained 2.5 ± 0.2 nM with a B_{max} of about 6000 receptors/cell (data not shown). The affinity constant and number of receptor sites per neutrophil reported in this study are comparable to previously determined values for high affinity PAF binding sites on peripheral blood neutrophils (O'Flaherty *et al.*, 1986). These results suggest that α-cyano,3,4-dihydroxythiocinnamamide is not inhibiting PAF-induced responses at the receptor. Previous studies have demonstrated that erbstatin does not inhibit FMLP binding (Naccache *et al.*, 1990).

Inhibition of protein kinase C activation

PKC has been shown to play a major role in neutrophil activation as demonstrated by the inhibition of neutrophil oxidative burst and degranulation by PKC inhibitors (Berkow *et al.*, 1987). In an attempt to characterize the site of action of α-cyano-3,4-dihydroxythiocinnamamide in neutrophils, the effect of this drug on PKC activity was investigated. The effect of 5 min pretreatment of cells with α-cyano-3,4-dihydroxythiocinnamamide on agonist-induced PKC activity was analysed. A rapid extraction of kinase activity was required to prevent proteolytic degradation of PKC during enzyme preparations. Neutrophils were treated with PAF (1 μM) for 1 min and extracts were isolated and separated by Mono Q FPLC chromatography. This fractionation was required since PKC inhibitors have been found in cytosolic sources that may be removed by anion exchange chromatography (Lang & Vallotton, 1987). Fractions were assayed for PKC activity by monitoring Ca^{2+} and phospholipid-dependent phosphorylation of histone H1 as well as the Ca^{2+}/DAG/PS-independent phosphorylation of protamine, a PKC substrate in the absence of activators (Bazzi & Nelsestuen, 1987). Most of the Ca^{2+}/PS/DAG-dependent PKC activity (~60%) was present in the cytosolic fraction and PKC was only slightly translocated to the microsomal fraction by PAF stimulation (data not shown). The phosphorylation of protamine chloride was observed in the same fractions as those that phosphorylated histone H1. Basal levels of PKC activity were associated with both cytosolic and microsomal fractions of control cells (Table 1). Microsomal fractions contained two peaks of PKC activity eluting at 0.33 M and 0.5 M NaCl. Ca^{2+}/PS/DAG-dependent PKC activity in particulate derived fractions eluting at 0.33 M was stimulated ~1.5 fold by PAF. After α-cyano-3,4-dihydroxythiocinnamamide pretreatment, PKC activity stimulated by PAF treatment was reduced below control levels (Table 1). α-Cyano-3,4-dihydroxythiocinnamamide entirely abolished the activity associated with the 0.5 M NaCl peak in the particulate fraction, which was the major peak of PAF-induced activation. Cytosolic extracts of untreated neutrophils contained a single peak of PKC activity eluting at 0.33 M NaCl as separated by MonoQ chromatography. PAF also stimulated the cytosolic PKC activity by approximately 1.5 fold. As with microsomal PKC activity, α-cyano-3,4-dihydroxythiocinnamamide also inhibited the cytosolic PKC activity to levels below baseline. The Ca^{2+} and phospholipid-dependence of phosphorylating activity eluting at 0.33 M NaCl in both cytosolic and microsomal fractions is exemplified by its inability to phosphorylate histone H1 in the absence of Ca^{2+}, DAG, and PS. Similar results were observed with cells stimulated with FMLP and LTB_4 (data not shown). To confirm further the inhibitory action of α-cyano-3,4-dihydroxythiocinnamamide on PKC, neutrophils were labelled with ^{32}Pi. Cells were then stimulated with PAF, FMLP, and LTB_4. Cytosolic fractions were analysed by SDS-PAGE and phosphorylated proteins were detected by autoradiography. All agonists stimulated phosphorylation of a 47 kD protein, previously identified as a major PKC substrate in neutrophils (Kramer *et al.*, 1988). Phosphorylation of this 47 kD protein was decreased when neutrophils were treated with 0.2 mM α-cyano-3,4-dihydroxythiocinnamamide (data not shown), correlating with the PKC *in vitro* assay results.

Effect of α-cyano-3,4-dihydroxythiocinnamamide on polyphosphoinositide hydrolysis

Receptor-induced activation of PLC catalyzes the hydrolysis of PIP_2, leading to an accumulation of inositol trisphospate (IP_3), inositol bisphosphate (IP_2) and inositol monophosphate (IP). As an index of PLC activity, PLC products including inositol mono, di, and triphosphates were monitored. Addition of 1 μM PAF and 1 μM FMLP were effective stimuli, increasing the production of all inositol phosphates above control levels (Figure 4; I, II, and III, treatments c and g). Although PAF was a more potent stimulus, the effects of both agonists were inhibited by pretreatment of cells with α-cyano-3,4-dihydroxythiocinnamamide (Figure 4; I, II, and III, d and h). LTB_4 (1 μM) slightly stimulated PIP_2 hydrolysis with IP_1 the only metabolite appreciably detectable above control whereas the levels of IP_2 and IP_3 were similar to unstimulated controls (Figure 4; I, II, III, e). PI hydrolysis in

Table 1 Effect of α-cyano-3,4-dihydroxythiocinnamamide (DHC) on PAF-induced protein kinase C (PKC) activation in human neutrophils

Treatment (fraction)	*Protein phosphorylation* (pmol min^{-1} ml^{-1})		
	Histone		*Protamine*
	No addition	+ Ca^{2+}, + ,PS, + DG	
Control (cytosol)	67 ± 12	1534 ± 401	1231 ± 317
Control (particulate)	726 ± 110	1073 ± 87	273 ± 46
PAF (cytosol)	91 ± 38	2524 ± 305	2419 ± 392
PAF (particulate)	1073 ± 186	1714 ± 162	294 ± 63
PAF + DHC (cytosol)	65 ± 44	1511 ± 287	1078 ± 401
PAF + DHC (particulate)	463 ± 94	563 ± 106	264 ± 71

Histone H1 phosphorylation was assayed in the presence and absence of activators including Ca^{2+}, phosphatidylserine (PS), and diacylglycerol (DG). Cells were untreated, exposed to 1 μM PAF for 1 min or preincubated with 0.2 mM α-cyano-3,4- dihydroxythiocinnamamide for 5 min before 1 μM PAF stimulation for 1 min. Mean and s.d. are given from three independent experiments.

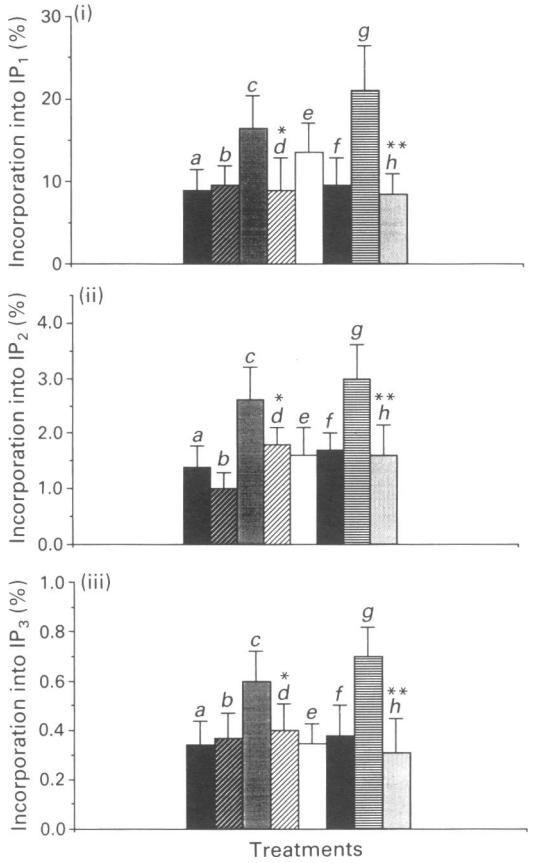

Figure 4 Effect of α-cyano-3,4-dihydroxythiocinnamamide on agonist-induced polyphosphoinositide hydrolysis. Human neutrophils prelabelled with *myo*-[2-^{3}H]-inositol were stimulated for 5 min with 1 μM leukotriene B_4 (LTB_4), PAF, or fMet-Leu-Phe (FMLP) and the effect of a 5 min preincubation with 0.2 mM α-cyano-3,4-dihydroxythiocinnamamide was analysed for stimulation of inositol phosphate incorporation into (i) IP_1 (ii) IP_2 and (iii) IP_3. Prior to harvesting and chromatography of inositol phosphates, cells were treated as follows: (a) untreated, (b) α-cyano-3,4-dihydroxythiocinnamamide; (c) FMLP; (d) α-cyano-3,4-dihydroxythiocinnamamide + FMLP; (e) LTB_4; (f) α-cyano-3,4-dihydroxythiocinnamamide + LTB_4; (g) PAF; (h) α-cyano-3,4-dihydroxythiocinnamamide + PAF. The incorporation of radioactivity into the various polyphosphatidylinositide metabolites was expressed as a percentage of the total radioactivity associated with free inositol, IP, IP_2, and IP_3 together. Results are mean of 6 samples with s.d. shown by vertical bars. Significantly inhibited compared to agonist-stimulated at $^{**}P < 0.05$; $^{*}P < 0.1$

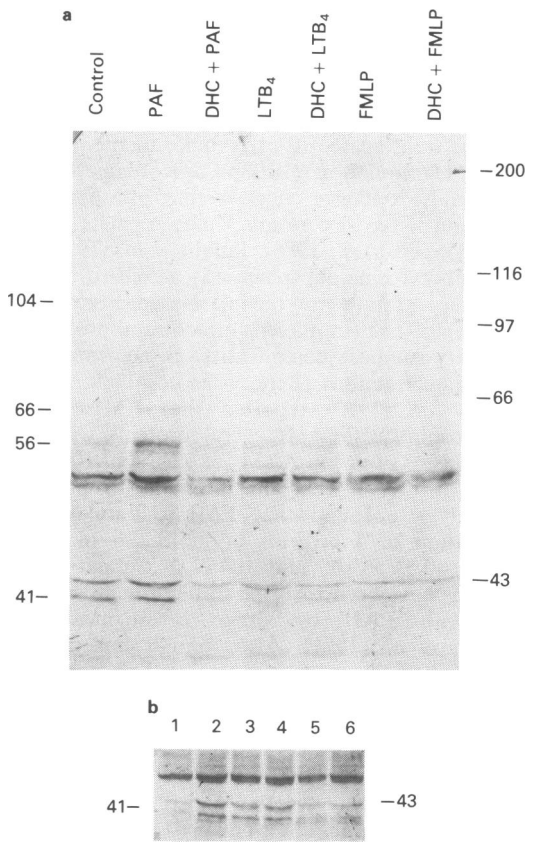

Figure 5 Inhibition of tyrosine phosphorylation in neutrophils stimulated with PAF, leukotriene B_4 (LTB_4) and fMet-Leu-Phe (FMLP) by α-cyano-3,4-dihydroxythiocinnamamide. Human neutrophils (10^7 cells ml^{-1}) in buffer containing Ca^{2+} were preincubated with 0.2 μM α-cyano-3,4-dihydroxythiocinnamamide (DHC) for 5 min prior to the addition of agonists (1 μM) at 37°C for 1 min. Cell extracts were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antiphosphotyrosine antibodies. (a) Effect of various agonists and inhibition by α-cyano-3,4-dihydroxythiocinnamamide treated as indicated. (b) Dose response of inhibition of PAF-induced protein-tyrosine phosphorylation of p41 by α-cyano-3,4-dihydroxythiocinnamamide. Cells were untreated (lane 1), treated with 1 μM PAF (lane 2), or pretreated with 4.16 μM, 20.8 μM, 41.6 μM and 104 μM α-cyano-3,4-dihydroxythiocinnamamide (lanes 3–6) prior to 1 μM PAF treatment. Molecular mass markers (in kDa) are shown to the right and molecular masses of tyrosine phosphorylated substrates are shown on the left of the figure.

all cases was reduced to levels obtained with untreated cells by drug treatment. Incorporation of [^{3}H]-inositol into these water soluble phosphates was not significantly affected by treatment with α-cyano-3,4-dihydroxythiocinnamamide (Figure 4; I, II, and III, b) as compared with untreated control cells.

Inhibition of human neutrophil protein-tyrosine phosphorylation

The ability of PAF, LTB_4 and FMLP to stimulate tyrosine phosphorylation in human neutrophils was investigated. As seen by visual inspection of Figure 5 (a), unstimulated cells contained tyrosine phosphorylated proteins, although agonist stimulation caused an increase in the tyrosine phosphorylation of proteins of 41, 56, 66, and 104 kDa. Preincubation of cells for 5 min with 0.2 mM α-cyano-3,4-dihydroxythiocinnamamide inhibited ligand-stimulated tyrosine phosphorylation of the proteins that were tyrosine phosphorylated in response to the agonists used. Tyrosine phosphorylation was reduced by drug treatment to the base line level seen in control, unstimulated neutrophils. A dose-dependent inhibition of PAF-induced tyrosine phosphorylation was observed from 0–0.1 mM α-cyano-3,4-dihydroxythiocinnamamide (Figure 5b). Complete inhibition of PAF-induced tyrosine phosphorylation was observed at 10 μg ml^{-1} (42 μM) in comparison with unstimulated neutrophils.

Discussion

Tyrosine kinases and their substrates have been associated primarily with growth factors and are believed to play important roles in the onset of oncogenes (Heldin *et al.*, 1987). We have demonstrated protein tyrosine phosphorylation in platelets in response to PAF and showed that a protein tyrosine kinase inhibitor (erbstatin) can inhibit platelet activation (Salari *et al.*, 1990a). The effect of this inhibitor was also investigated in neutrophils (Naccache *et al.*, 1990). Another tyrosine kinase inhibitor, genistein, was found to inhibit PAF-induced rabbit platelet aggregation and PLC activation at doses greater than 0.5 mM (Dhar *et al.*, 1990). Genistein was also found to inhibit human platelet responses to stimulation with thromboxane A_2 and collagen, but not thrombin (Nakashima *et al.*, 1991). In leukocytes, various

forms of protein tyrosine kinases such as c-*fgr* (Gutkind & Robbins, 1989), c-*fes/fps* (Smithgall *et al.*, 1988), c-*hck* (Ziegler *et al.*, 1988) and c-*src* (Gee *et al.*, 1986) have been identified. The mechanism of stimulation of these kinases is unknown. Recent work suggests that c-*fgr* in human neutrophils may be translocated from granules to the plasma membrane upon degranulation (Gutkind & Robbins, 1989). In the *src* gene family, evidence suggests that phosphorylation of carboxy-terminal tyrosine residues may regulate their kinase activities (Cooper *et al.*, 1986). Pulido *et al.* (1989) reported that a phosphotyrosine phosphatase is mobilized from specific and/or tertiary granules to the plasma membrane fractions when neutrophils are stimulated. In addition, protein tyrosine kinase activity has been demonstrated in neutrophil cytosolic and particulate fractions (Berkow *et al.*, 1989; 1990).

Huang *et al.* (1990) reported that FMLP stimulated tyrosine phosphorylation of several proteins with apparent molecular masses of group A (54–58 kDa and 100–125 kDa) and group B (36–41 kDa) in rabbit neutrophils. In the present study, we found that FMLP stimulated tyrosine phosphorylation of 4 proteins of molecular masses of approximately 40.5, 56, 66 and 104 kDa. Similar phosphorylated proteins have been reported to be present in neutrophils activated with PAF by other investigators (Gomez-Cambronero *et al.*, 1991). Also, similar but not identical proteins were found to be phosporylated in human neutrophils stimulated with GM-CSF (Gomez-Cambronero *et al.*, 1989a) although GM-CSF also stimulates tyrosine phosphorylation of proteins of 78 and 92 kD that may be the GM-CSF receptor (Gomez-Cambronero *et al.*, 1989b). The major human neutrophil tyrosine phosphorylated proteins as a result of PAF stimulation were pp41, pp66, pp54, pp104 and pp116 (Gomez-Cambronero *et al.*, 1991).

By using inhibitors of protein tyrosine kinases or phosphatases, several investigators have suggested that tyrosine phosphorylation may be a central process controlling neutrophil activation through receptor-mediated processes (Kraft & Berkow, 1987; Grinstein *et al.*, 1989). Although erbstatin was able to inhibit superoxide production in human neutrophils stimulated by FMLP (Naccache *et al.*, 1990), this inhibitor failed to inhibit elastase release and intracellular calcium release. Since the inhibitor used in this study is more potent than erbstatin, this may account for its ability to inhibit Ca^{2+} release. In addition, for erbstatin to inhibit O_2^- production, it required at least 1 h of exposure for effective inhibition. These data suggest that the action of erbstatin on O_2^- production may not be specific and it is possible that erbstatin was interfering with some other components of the superoxide generating system as well as tyrosine phosphorylation. Several analogues of erbstatin have been synthesized (Isshiki *et al.*, 1987; Gazit *et al.*, 1989) in an attempt to produce selective, non-toxic and potent inhibitors of tyrosine kinases. Another class of protein-tyrosine kinase inhibitors having a cinnamamide structure was tested for their ability to inhibit tyrosine kinase activity in response to growth factors. The most potent of this class of compounds was reported to inhibit EGF-dependent cell proliferation (Lyall *et al.*, 1989) with a K_i of 0.85 μM (Yaish *et al.*, 1988), about six times more potent than erbstatin (Imoto *et al.*, 1987). In the present study, the effect of α-cyano-3,4-dihydroxythiocinnamamide on human neutrophil responses was investigated. This work stems from the observation that this inhibitor was the most potent tyrosine kinase inhibitor as reported in a previous study (Yaish *et al.*, 1988).

We demonstrated in this paper that α-cyano-3,4-dihydroxythiocinnamamide is capable of inhibiting agonist-induced neutrophil activation. Responses such as O_2^- generation and elastase release as well as components of intracellular signalling pathways were inhibited. α-Cyano-3,4-dihydroxythiocinnamamide partially inhibited TPA and zymosan-induced O_2^- generation when used at 0.2 mM (data not shown). To elucidate the site of action of α-cyano-3,4-dihydroxythiocinnamamide, a series of biochemical assays was used to investigate the effect on calcium mobilization, polyphosphoinositide hydrolysis and PKC activation. It is well documented that the increase in intracellular free calcium is associated with agonist-induced neutrophil activation (Lew *et al.*, 1984; Korchak *et al.*, 1984). The ability of α-cyano-3,4-dihydroxythiocinnamamide to inhibit agonist-induced intracellular Ca^{2+} accumulation suggests that the drug is acting by inhibiting PLC, possibly the substrate for an activated tyrosine kinase. The inhibition of Ca^{2+} release was inhibited by short (5 min) preincubation with the drug and inhibition was reversible. When the drug was washed from the neutrophils, agonists were capable of inducing Ca^{2+} release from the neutrophils suggesting that the drug did not cause cell toxicity. Since the agonist-induced rise in cytosolic free Ca^{2+} was inhibited by α-cyano,3,4-dihydroxythiocinnamamide, other parameters of neutrophil activation were analysed.

We discovered that the activities of PKC and PLC were decreased by this drug suggesting that α-cyano-3,4-dihydroxythiocinnamamide was probably acting at a signal transduction process above or at the level of PLC. Therefore, the inhibition of PKC is the consequence of inhibition of DAG and IP_3-induced Ca^{2+} generation from PIP_2 hydrolysis, and may not be the direct effect of the drug on this kinase. In this study, PKC was activated in both the particulate and cytosolic fractions, although a significant translocation of PKC to the membrane fraction did not accompany PKC stimulation. Since cytochalasin B was not present during stimulation, this correlates with reports that cytochalasin B is necessary for induction of particulate protein kinase activity (Pike *et al.*, 1986). PKC inhibition below baseline levels may be due to a direct inhibitory effect on PKC as well as inhibiting upstream signals. A nonspecific inhibition of signal transduction processes may account for inhibition of all responses tested. A previous study correlates tyrosine phosphorylation and G-protein activation with the respiratory burst, indicating that kinases other than PKC are involved in this agonist-stimulated response (Nasmith *et al.*, 1989). Our results support the claim that tyrosine phosphorylation may play an important role in neutrophil activation. The ability of α-cyano-3,4-dihydroxythiocinnamamide to decrease PI hydrolysis to baseline levels indicates that this inhibitor is capable of decreasing neutrophil stimulated activity initiated via PLC. α-Cyano-3,4-dihydroxythiocinnamamide pretreatment of cells does not decrease PI hydrolysis below baseline levels, suggesting that metabolism of unstimulated cells are not affected by this inhibitor.

In conclusion, the present study demonstrates that an increase in protein tyrosine phosphorylation in neutrophils in response to chemotactic factors has an important role in neutrophil activation and α-cyano-3,4-dihydroxythiocinnamamide can specifically inhibit neutrophil activation with relevance to inflammatory reactions. In addition, we show that α-cyano-3,4-dihydroxythiocinnamamide can inhibit neutrophil activation by interrupting tyrosine phosphorylation of proteins that may have a key role in neutrophil function.

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