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Investigation of the inhibition by acetylshikonin of the respiratory burst in rat neutrophils

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1 The ability of acetylshikonin to inhibit the respiratory burst in rat neutrophils was characterized and the underlying mechanism of action was also assessed in the present study.

2 Acetylshikonin caused an irreversible and a concentration-dependent inhibition of formylmethionyl-leucyl-phenylalanine (fMLP) plus dihydrocytochalasin B (CB)- and phorbol 12-myristate 13-acetate (PMA)-induced superoxide anion ($O_2^{\cdot-}$) generation with IC_{50} values of 0.48 ± 0.03 and 0.39 ± 0.03 μM, respectively. Acetylshikonin also inhibited the O_2 consumption in neutrophils in response to fMLP/CB as well as to PMA.

3 Acetylshikonin did not scavenge the generated $O_2^{\cdot-}$ in the xanthine-xanthine oxidase system or during dihydroxyfumaric acid (DHF) autoxidation but, on the contrary, acetylshikonin enhanced the $O_2^{\cdot-}$ generation in these cell-free oxygen radical generating systems.

4 Acetylshikonin inhibited the formation of inositol trisphosphate (IP_3) ($39.0 \pm 7.8\%$ inhibition at 10 μM, $P<0.05$) in neutrophils in response to fMLP.

5 Both the neutrophil cytosolic protein kinase C (PKC) activity and the PMA-induced PKC associated with the membrane were unaffected by acetylshikonin.

6 Acetylshikonin did not affect the porcine heart protein kinase A (PKA) activity. Upon exposure to acetylshikonin, the cellular cyclic AMP level was decreased in neutrophils in response to fMLP.

7 The cellular formation of phosphatidic acid (PA) and, in the presence of ethanol, phosphatidylethanol (PEt) induced by fMLP/CB were inhibited by acetylshikonin (60.1 ± 7.3 and $63.2 \pm 10.5\%$ inhibition, respectively, at 10 μM, both $P<0.05$). Moreover, acetylshikonin attenuated the fMLP/CB-induced protein tyrosine phosphorylation (about 90% inhibition at 1 μM).

8 In PMA-activated neutrophil particulate NADPH oxidase preparations, acetylshikonin did not inhibit, but enhanced, the $O_2^{\cdot-}$ generation in the presence of NADPH. However, acetylshikonin decreased the membrane associated $p47^{phox}$ in PMA-activated neutrophils (about 60% inhibition at 1 μM).

9 Collectively, these results suggest that the attenuation of protein tyrosine phosphorylation and a failure in the assembly of a functional NADPH oxidase complex probably contribute predominantly to the inhibition of respiratory burst in neutrophils by acetylshikonin. In contrast, the blockade of phospholipase C (PLC) and phospholipase D (PLD) pathways play only a minor role in this respect.

Keywords: Acetylshikonin; rat neutrophil; superoxide anion; inositol phosphate; protein kinase C; cyclic AMP; phospholipase D; tyrosine phosphorylation; NADPH oxidase; translocation

Introduction

Neutrophils play a central role in the body's defence against bacterial infection. Upon exposure to chemoattractants, they phagocytose microorganisms or damaged tissue, increase their O_2 uptake from the surrounding medium and concomitantly generate large amounts of superoxide anion ($O_2^{\cdot-}$), which subsequently leads to the formation of other toxic O_2 metabolites (Badwey & Karnovsky, 1980). This non-mitochondrial O_2 consumption process is known as the respiratory burst. Reactive O_2 species produced during the respiratory burst are believed to serve as bactericidal agents, as evidenced by the susceptibility of patients with chronic granulomatous disease to suffer recurrent bacterial infections (Smith & Curnutte, 1991). Under certain circumstances, the excessive or inappropriate release of these highly reactive O_2 species can result in undesirable tissue damage. This is probably involved in the pathogenesis of many diseases (Halliwell & Gutteridge, 1990). Therefore, a drug that would inhibit the generation of toxic O_2 metabolites could terminate this tissue damage.

Intracellular signalling events used in the response of the neutrophil to external stimuli are complicated and not clearly defined. Stimulation of neutrophils by receptor-binding ligands results in an intracellular signalling cascade including the activation of phospholipase C (PLC) that releases inositol trisphosphate (IP_3) and diacylglycerol, which in turn, increase intracellular Ca^{2+} concentration and activate protein kinase C (PKC), respectively (Berridge, 1987). The two pathways function synergistically for $O_2^{\cdot-}$ generation. Phospholipase D (PLD) activation is also functionally linked to $O_2^{\cdot-}$ generation in neutrophils (Yasui *et al*., 1994). PLD acts upon phosphatidylcholine to release phosphatidic acid (PA), which is then converted into diradylglycerol (Billah *et al*., 1989). In addition, tyrosine phosphorylation has been shown to be involved in the production of $O_2^{\cdot-}$ (Torres *et al*., 1993). On neutrophil activation, the cytosolic components of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (mainly $p47^{phox}$ and $p67^{phox}$) translocate from the cytosol to cell membranes where they associate with a flavocytochrome b_{558}, composed of $gp91^{phox}$ and $p22^{phox}$, forming a functional complex responsible for the production of oxygen radicals in neutrophils (Segal & Abo, 1993).

Acetylshikonin, a naphthoquinone isolated from the Chinese herb, tzu ts'ao, the dried purple roots of *Lithospermum*

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erythrorhizon Sieb. et Zucc., *Arnebia euchroma* (Royle) Johnst, or *Macrotomia euchroma* (Royle) Pauls (*Boraginaceae*) (Majima & Kuroda, 1922), has been found to possess antibacterial, antipyretic and analgesic activity (Motohide, 1977). Recently, acetylshikonin was shown to inhibit rabbit platelet aggregation (Chang *et al*., 1993), and inhibit DNA topoisomerase I activity (Ahn *et al*., 1995). In addition, we demonstrated that acetylshikonin inhibits the release of mediators from rat peritoneal mast cells *in vitro* and suppresses the mouse cutaneous plasma extravasation caused by inflammatory mediators *in vivo* (Wang *et al*., 1995a). In this study, we have examined the inhibitory effect of acetylshikonin on the respiratory burst in rat peripheral neutrophils and investigated the underlying mechanism.

Methods

Isolation of neutrophils

Rat blood was collected from the abdominal aorta and the neutrophils were purified by dextran sedimentation, hypotonic lysis of erythrocytes and centrifugation through Ficoll-Hypaque (Wang *et al*., 1995b). Purified neutrophils containing >95% viable cell were normally resuspended in Hank's balanced salt solution (HBSS) containing 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid] (HEPES), pH 7.4,and 4 mM $NaHCO_3$ and kept on ice before use.

Measurement of $O_2^{\cdot-}$ generation and O_2 consumption

The $O_2^{\cdot-}$ generation in the neutrophil suspension or in the xanthine (0.15 mM)-xanthine oxidase (2.5 mu ml^{-1}) system was determined by superoxide dismutase (SOD)-inhibitable ferricytochrome *c* reduction as previously described (Wang *et al*., 1994; 1995b). The $O_2^{\cdot-}$ generation during dihydroxyfumaric acid (DHF) autoxidation was determined by the reduction of nitroblue tetrazolium (NBT) as previously described (Goldberg & Stern, 1977). Absorbance changes of the reduction of ferricytochrome *c* and NBT were continuously monitored in a double-beam spectrophotometer. Whole cell O_2 consumption was continuously measured with a Clark-type oxygen electrode connected to a YSI biological oxygen monitor (Ingraham *et al*., 1982).

Determination of inositol phosphate levels

Neutrophils (3×10^7 cells ml^{-1}) were loaded with *myo*-[^{3}H]inositol (83 Ci $mmol^{-1}$, Amersham) at 37°C for 2 h (Wang *et al*., 1994). Ten seconds after the stimulation with formylmethionyl-leucyl-phenylalanine (fMLP), reactions were stopped by adding $CHCl_3$:CH_3OH (1:1, v/v) mixture and 2.4 M HCl. The aqueous phase was removed and neutralized by 0.4 M NaOH, and then applied to AG 1-X8 resin (formate) column (Bio-Rad). Inositol monophosphate (IP), inositol bisphosphate (IP_2) and IP_3 were eluted sequentially by using 0.2, 0.4, and 1.0 M ammonium formate, respectively, in 0.1 M formic acid as eluents, and then counted as previously described (Wang *et al*., 1994).

PKC activity and membrane translocation

For the preparation of cytosolic PKC, neutrophils were disrupted by sonication. After centrifugation, the supernatant was subjected to DE-52 cellulose column (Whatman) to obtain partially purified PKC (Wang *et al*., 1995b). Enzyme activity of neutrophil cytosolic PKC was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]-ATP (Amersham) into peptide substrate by use of a PKC assay kit (Amersham), based on the mixed micelle method as previously described (Hannun *et al*., 1986). For the analysis of the subcellular distribution of PKC, neutrophils (4×10^7 cells ml^{-1}) were stimulated with 0.2 μM phorbol 12-myristate 13-acetate (PMA) for 5 min at 37°C. Reactions were stopped by the addition of 4 volume of ice-cold HBSS, and then resuspended in disruption solution (0.34 M sucrose, 10 mM Tris-HCl, pH 7.0, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM EGTA, 10 mM benzamidine, 10 μg ml^{-1} leupeptin and antipain). After sonication, lysate was centrifuged to remove the unbroken cells, and then further centrifuged at $100{,}000 \times g$ for 30 min at 4°C. Pellet (as membrane fraction) and supernatant (as cytosol fraction) were boiled in Laemmli sample buffer, subjected to SDS–PAGE, transferred to polyvinylidene difluoride membrane (Millipore) and blocked with 5% skimmed milk in TST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween 20). The proteins were then probed with mouse monoclonal antibody to PKC-β (Transduction) (1:500 dilution in TST buffer with 0.5% skimmed milk). Detection was performed by use of the enhanced chemiluminescence reagent (Amersham).

PKA activity and cellular cyclic AMP level

Porcine heart PKA activity was assayed by measuring the incorporation of ^{32}P into kemptide in the presence of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) by use of a PKA assay kit (Life Technologies), based on a method previously described (Roskoski, 1983). Cyclic AMP was determined as described by Simchowitz *et al*. (1980). Neutrophils were preincubated with test drugs for 9.5 min at 37°C. Thirty seconds after addition of fMLP, the reaction mixture was immediately added to 1.0 ml of 0.05 M acetate buffer, pH 6.2, containing 0.05 mM 3-isobutyl-1-methylxanthine (IBMX). After being boiled for 5 min, the suspension was kept in ice, sonicated and followed by sedimentation. The supernatant was acetylated by the addition of 0.025 volume of triethylamine:acetic anhydride (2:1, v/v). The cyclic AMP content in aliquots of the acetylated samples were assayed by using an enzyme immunoassay kit (Amersham).

Measurement of PLD activity

Neutrophils (5×10^7 cells ml^{-1}) were suspended in HEPES buffer A (composition (mM): NaCl 124, KCl 4, Na_2HPO_4 0.64, KH_2PO_4 0.66, $NaHCO_3$ 15.2, dextrose 5.56 and HEPES 10, pH 7.4) and loaded with 10 μCi 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine (150 Ci $mmol^{-1}$, Amersham) at 37°C for 75 min, then washed and resuspended in HEPES buffer A with 0.05% bovine serum albumin. The assay mixture containing test drugs, 1 mM $CaCl_2$, with or without 0.5% ethanol, were incubated for 3 min at 37°C before initiating the reaction by adding fMLP plus dihydrocytochalasin B (CB). Thirty seconds later, the lipids in the reaction mixture were extracted, dried and separated on silica gel 60 plate (Billah *et al*., 1989) with certain modifications. The plates were developed halfway by using the solvent system consisting of hexane:diethyl ether:methanol:acetic acid (90:20:3:2, v/v/v/v). The plates were dried and developed again to the top using the upper phase of the solvent system consisting of ethylacetate:isooctane:acetic acid:water (110:50:20:100, v/v/v/v). The lipids were located by staining with iodine vapour. The radioactivity of ^{3}H products was directly quantified with a PhosphorImager (Molecular Dynamics 445 SI) by use of ImageQuaNT software.

Detection of protein tyrosine phosphorylation

Neutrophils (1×10^7 cells ml^{-1}) in HBSS were preincubated with dimethylsulphoxide (DMSO) or test drugs at 37°C for 5 min before the reaction was initiated by adding fMLP/CB. One minute later, reactions were quenched by adding stop solution (20% trichloroacetic acid, 1 mM PMSF, 7 μg ml^{-1} aprotinin and pepstatin, 2 mM *N*-ethylmaleimide, 100 mM NaF, 5 mM diisopropylfluorophosphate) (Berkow, 1992). Protein pellets were washed with ice-cold acetone and boiled in Laemmli sample buffer. Proteins were separated through SDS–PAGE as described above and then probed with mouse monoclonal anti-phosphotyrosine antibody (PY-20, Trans-

duction) (1 : 1000 dilution in TST buffer with 0.5% skimmed milk). The approximate molecular mass of the phosphotyrosine-containing proteins was interpolated from a standard curve constructed with standard proteins of known molecular weight.

NADPH oxidase activity and p47^{phox} membrane translocation

Particulate NADPH oxidase was isolated and the oxidase activity was measured spectrophotometrically at 28°C by detecting the SOD-inhibitable ferricytochrome *c* reduction as described by Wang *et al.* (1994). For the analysis of the subcellular distribution of p47^{phox}, neutrophils (2×10^7 cells ml^{-1}) in HBSS were preincubated with DMSO or test drugs at 37°C for 5 min before stimulation with 0.2 μM PMA. Five minutes later, four volumes of ice-cold HBSS were added. Cells were disrupted by sonication, layed on discontinuous sucrose gradients and centrifuged at $100{,}000\times g$ for 60 min (Bellavite *et al.*, 1988). The band between 20 and 50% sucrose was collected, washed with 2 volumes of 0.45 M NaCl and centrifuged at $10{,}000\times g$ for 10 min. The supernatant was further centrifuged at $100{,}000\times g$ for 60 min. The NaCl-washed membrane pellet was then boiled in Laemmli sample buffer. Proteins were separated through SDS-PAGE as described above and then probed with rabbit polyclonal anti-p47^{phox} antibody (Transduction) (1 : 500 dilution in TST buffer with 0.5% skimmed milk).

Drugs

Acetylshikonin (M_r 330) was isolated and purified from *Lithospermum erythrorhizon* Sieb. et Zucc. (*Boraginaceae*) as previously described (Majima & Kuroda, 1922). The purity of acetylshikonin was identified by h.p.l.c. (>98% purity) and n.m.r. (without impurity signals). All chemicals were purchased from Sigma Chemical Co. (St. Louis, U.S.A.) except for the following: dextran T-500 (Pharmacia Biotech Ltd., Uppsala, Sweden); Hank's balanced salt solution (Life Technologies Gibco BRL Co., Gaithersburg, U.S.A.); 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122) and (8**R**,9**S**,11**S**)-(−)-9-hydroxy-9-hexoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[a,g]cycloocta[cde]trinden-1-one (KT5720) (Biomol Research Lab. Inc., Plymouth Meeting, U.S.A.). DMSO was the solvent used for the inhibitors and the final concentration of DMSO in the reaction mixture was less then 0.5%.

Statistical analysis

Statistical analyses were performed by the Bonferroni *t* test method after analysis of variance. A *P* value less than 0.05 was considered significant for all tests. Analysis of the regression line was used to calculate IC_{50} values. Data are presented as mean ± s.e.mean.

Results

Effect of acetylshikonin on $O_2^{\cdot-}$ generation and O_2 consumption by neutrophils

Addition of fMLP/CB to neutrophils induced a rapid and transient $O_2^{\cdot-}$ generation (5.0 ± 0.3 nmol per 10 min per 1×10^6 cells) and O_2 consumption (14.6 ± 1.9 nmol per 5 min per 2×10^6 cells). In contrast, PMA evoked a slow and long lasting $O_2^{\cdot-}$ generation (20.9 ± 2.1 nmol per 10 min per 1×10^6 cells) and O_2 consumption (49.6 ± 2.3 nmol per 5 min per 2×10^6 cells). In cells pretreated with acetylshikonin for 3 min before activation, a concentration dependent inhibition of both the $O_2^{\cdot-}$ generation and the O_2 consumption induced by fMLP/CB and by PMA was observed (Figure 1a, b). The IC_{50} values for

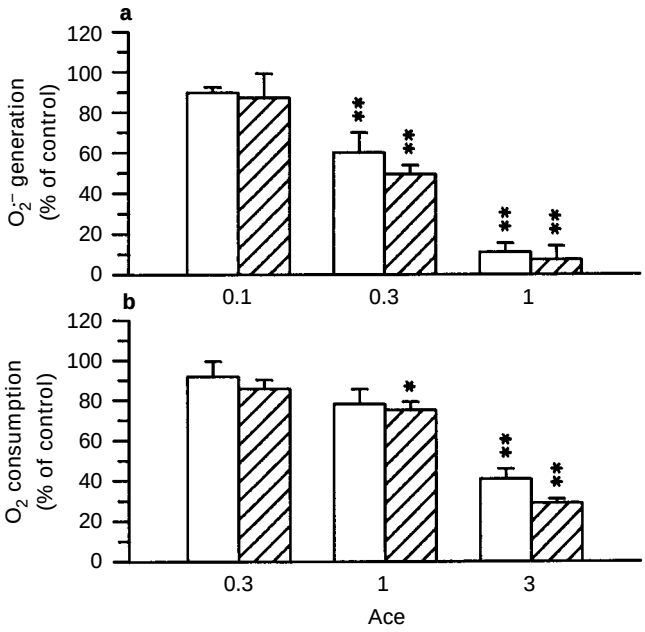

Figure 1 Concentration-dependence of acetylshikonin (Ace) inhibition of $O_2^{\cdot-}$ generation and O_2 consumption in formylmethionyl-leucyl-phenylalanine (fMLP)- and phorbol 12-myristate 13-acetate (PMA)-activated neutrophils. Neutrophils ($1-2\times10^6$ cells ml^{-1}, at 37°C) were preincubated with DMSO (as control) or 0.1−3 μM acetylshikonin for 3 min before the addition of (a) 0.3 μM fMLP plus 5 μg ml^{-1} dihydrocytochalasin (CB) (open columns) and 3 nM PMA (hatched columns) for $O_2^{\cdot-}$ generation, or (b) 0.1 μM fMLP plus 5 μg ml^{-1} of CB (open columns) and 10 nM PMA (hatched columns) for O_2 consumption. Results are expressed as mean ± s.e.mean of 4 separate experiments. $^*P<0.05$, $^{**}P<0.01$ compared to the corresponding control values.

inhibition of fMLP/CB- and PMA-induced $O_2^{\cdot-}$ generation were 0.48 ± 0.03 and 0.39 ± 0.03 μM, respectively. After the acetylshikonin (3 μM)-pretreated neutrophils had been washed twice with HBSS, a marked attenuation of fMLP/CB-induced $O_2^{\cdot-}$ generation was still observed (to 3.8 ± 1.3% of untreated cells). More than 95% viability was observed with trypan blue exclusion in cells treated with 3 μM acetylshikonin for 3 min. The scavenging ability of the drug was assessed in cell-free oxygen radical generating systems. SOD significantly reduced the $O_2^{\cdot-}$ generation in xanthine-xanthine oxidase system (1.5 ± 0.1 vs 3.1 ± 0.1 nmol min^{-1}) and during DHF autoxidation (ΔA_{560}, 0.063 ± 0.005 vs 0.114 ± 0.006). Acetylshikonin at concentrations less than 1 μM did not affect the responses. However, at concentrations $\geqslant$3 μM acetylshikonin enhanced $O_2^{\cdot-}$ generation from both systems (Figure 2a, b).

Effect of acetylshikonin on inositol phosphate formation

Addition of fMLP to the *myo*-[^{3}H]inositol-loaded neutrophil suspension resulted in a significant increase in IP_2 and IP_3 (both $P<0.01$), but not IP levels (Figure 3a, b, c). Exposure to U73122, a PLC inhibitor (Smith *et al.*, 1990), greatly reduced the IP_2 and IP_3 formation in neutrophils in response to fMLP. Formation of IP_2 and IP_3 in response to fMLP was inhibited by acetylshikonin in a concentration-dependent manner (39.0 ± 7.8 and 70.0 ± 8.9% of inhibition of IP_3 formation by acetylshikonin 10 and 30 μM, respectively).

Effect of acetylshikonin on protein kinases and cellular cyclic AMP level

To evaluate whether acetylshikonin had inhibitory effects on PKC, neutrophil cytosolic PKC activity was measured by studying the incorporation of ^{32}P from [γ-^{32}P]-ATP into pep-

tide substrate in the presence of Ca^{2+}, phosphatidylserine (PS) and PMA. Staurosporine, a protein kinase inhibitor, effectively attenuated PKC activity (0.13 ± 0.01 *vs* 0.55 ± 0.01 nmol ^{32}P min^{-1} mg^{-1} protein). Acetylshikonin (up to 100 μM) had no effect on PKC activity (Figure 4a). In the presence of cyclic AMP, porcine heart PKA was activated as evidence by the increased incorporation of ^{32}P from [γ-^{32}P]-ATP into kemptide substrate (44.2 ± 3.5 pmol ^{32}P min^{-1} μg^{-1} protein). Staurosporine as well as KT 5720, a PKA inhibitor (Kase *et al.*, 1987), inhibited the PKA activity. By contrast, acetylshikonin had a negligible effect in this respect (Figure 4b). Moreover, acetylshikonin alone exerted no PKA stimulating activity in the absence of cyclic AMP (data not shown). The membrane associated PKC-β was increased in PMA-stimulated neutrophils and this response was unaffected by acetylshikonin (Figure 5). Analysis of the cyclic AMP levels in neutrophils showed that a significant increase was observed in cells treated with forskolin, an adenylate cyclase activator (Seamon & Daly, 1986). In addition, the cyclic AMP level of fMLP-stimulated neutrophils was increased in the presence of a nonselective phosphodiesterase inhibitor IBMX. In contrast, acetylshikonin concentration-dependently decreased the cellular cyclic AMP levels in neutrophils (Table 1).

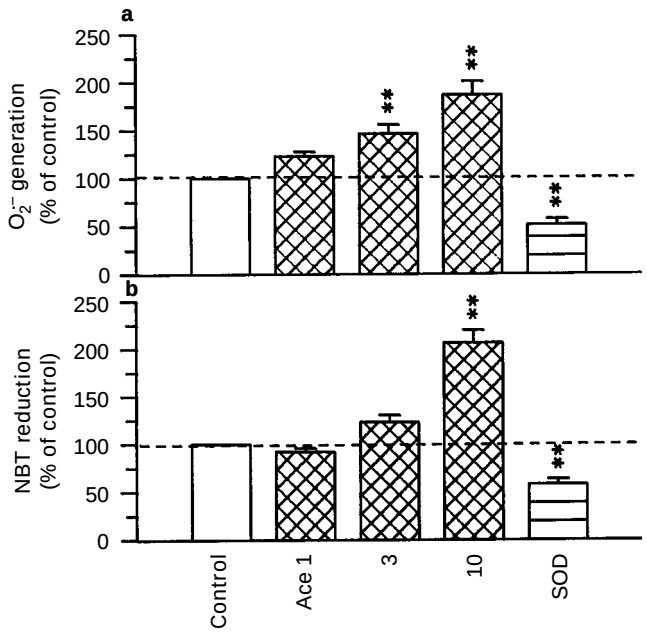

Figure 2 Effect of acetylshikonin (Ace) on $O_2^{\cdot-}$ generation in cell-free systems. Reaction mixtures were preincubated with DMSO (as control), 1–10 μM acetylshikonin or 0.3 μg ml^{-1} superoxide dismutase (SOD) at 37°C for 3 min before addition of (a) xanthine (0.15 mM) to xanthine oxidase (2.5 mu ml^{-1})-contained reaction mixtures, or (b) 0.891 mM dihydroxyfumaric acid (DHF) to start the reaction. Results are expressed as mean ± s.e.mean of 6 separate experiments. $^{**}P<0.01$ compared to the corresponding control values.

Effect of acetylshikonin on protein tyrosine phosphorylation and PLD activity

Protein tyorsine phosphorylation was assessed by Western blotting with a specific monoclonal anti-phosphotyrosine antibody. Figure 6 depicts the pattern of tyrosine phosphorylation in whole-cell lysates prepared from neutrophils. It was noted that several proteins were labelled to a variable extent in the resting cells. fMLP/CB enhanced the tyrosine phosphorylation state of a number of proteins and a prominent band migrating at about 62 kDa (cf lanes 1–2, arrow). Genistein, a tyrosine kinase inhibitor (Akiyama *et al.*, 1987), attenuated the 62 kDa protein tyrosine phosphorylation. Exposure to acetylshikonin caused a concentration-dependent inhibition of the fMLP/CB-induced accumulation of phosphotyrosine on protein 62 kDa (~90% inhibition with 1 μM acetylshikonin). A significant increase in the formation of phosphatidic acid (PA)

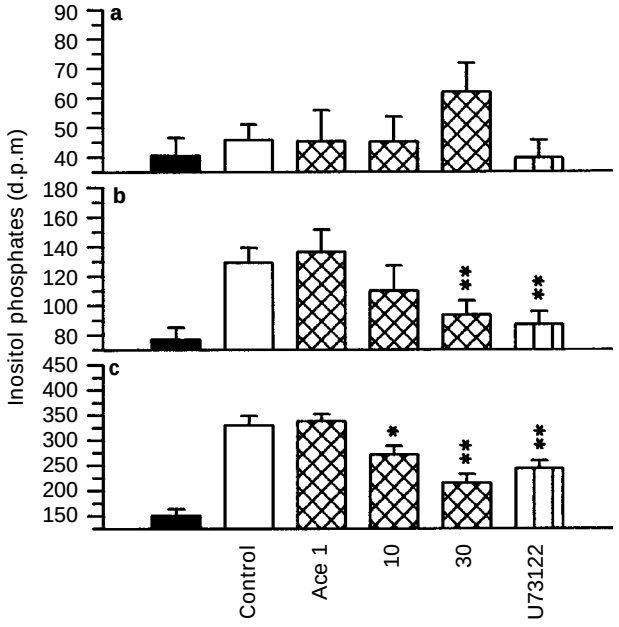

Figure 3 Effect of acetylshikonin (Ace) on formylmethionyl-leucyl-phenylalanine (fMLP)-induced inositol phosphate formation in rat neutrophils. DMSO (as control), 1–30 μM acetylshikonin or 3 μM U73122 was added to the *myo*-[^{3}H]-inositol-loaded cell suspension in the presence of 10 mM LiCl at 37°C for 3 min before addition of 0.3 μM fMLP to start the reaction. Values of (a) IP, (b) IP_2 and (c) IP_3 are expressed as mean ± s.e.mean of 4–5 separate experiments. The resting level of inositol phosphate was measured from the cells exposed to DMSO without fMLP challenge (solid columns). $^{*}P<0.05$, $^{**}P<0.01$ compared to the corresponding control values.

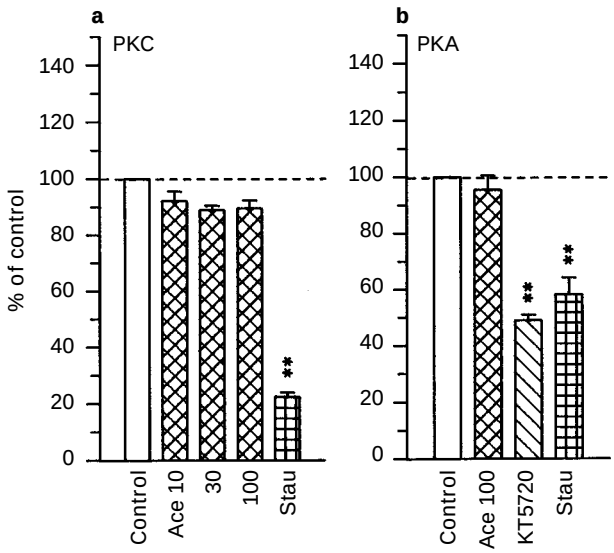

Figure 4 Effect of acetylshikonin (Ace) on protein kinase C (PKC) and PKA activities. (a) Neutrophil cytosolic PKC was preincubated with DMSO (as control), 10–100 μM acetylshikonin or 1 nM staurosporine (Stau) at 25°C for 3 min before the addition of 1 mM $CaCl_2$, 6 mM PS, 2 μg ml^{-1} PMA, 50 μM ATP (0.2 μCi [γ-^{32}P]-ATP per tube) and 75 μM PKC substrate. Reactions were terminated after 15 min by the addition of stop reagent. (b) Porcine heart PKA was preincubated with DMSO (as control), 100 μM acetylshikonin, 30 μM KT5720 or 30 nM staurosporine at 30°C for 3 min before the addition of 100 μM ATP (0.3 μCi [γ-^{32}P]-ATP per tube), 50 μM kemptide and 10 μM cyclic AMP. Values are expressed as mean ± s.e.mean of 5 separate experiments. $^{**}P<0.01$ compared to the corresponding control values.

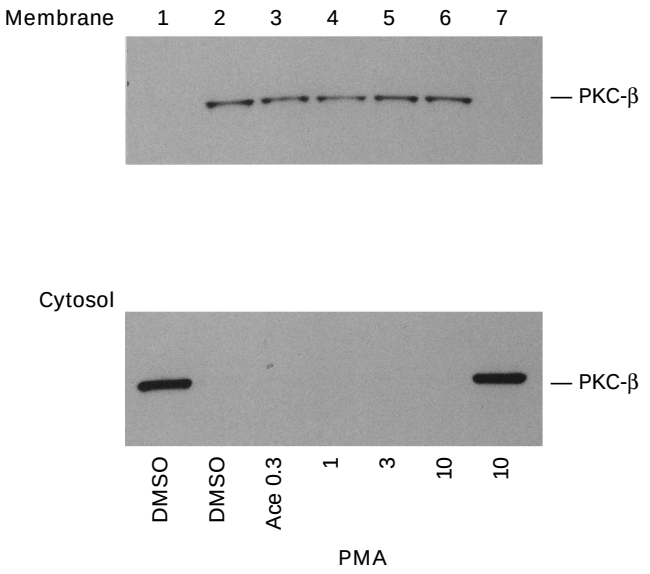

Figure 5 Effect of acetylshikonin (Ace) on phorbol 12-myristate 13-acetate (PMA)-induced PKC-β membrane translocation. Neutrophils were preincubated with DMSO (lane 2) or 0.3–10 μM acetylshikonin (lanes 3–6) for 5 min at 37°C and then stimulated with 0.2 μM PMA for another 5 min. Effect of DMSO (lane 1) or 10 μM acetylshikonin (lane 7) alone on the cells was studied for total 10 min reaction time. After termination of reaction, cells were disrupted, and then the membrane and cytosol proteins were subjected to SDS-PAGE. Each lane contains a 50 μg sample of protein. Analysis was performed by immunoblotting with a monoclonal antibody to PKC-β. The results shown are representative of 3 separate experiments.

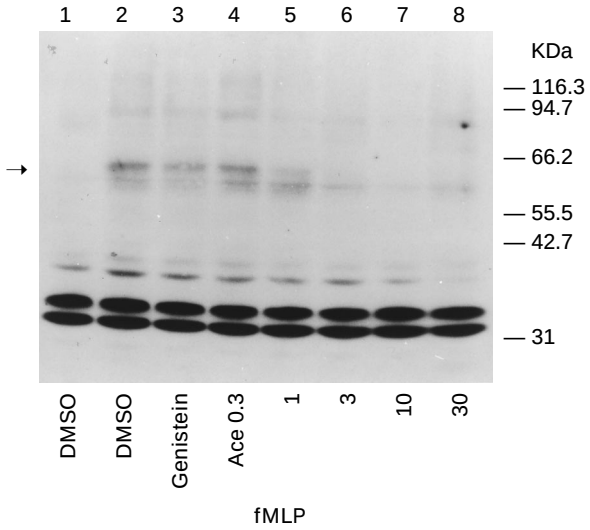

Figure 6 Effect of acetylshikonin (Ace) on formylmethionyl-leucyl-phenylalanine (fMLP)-induced protein tyrosine phosphorylation in rat neutrophils. Neutrophils were preincubated with DMSO (lanes 1–2), 10 μg ml^{-1} genistein (lane 3) or 0.3–30 μM acetylshikonin (lanes 4–8) for 5 min at 37°C before addition of DMSO (lane 1) or 0.1 μM fMLP plus 5 μg ml^{-1} dihydrocytochalasin (CB) (lanes 2–8). One minute later, cells were rapidly sedimented, boiled in Laemmli sample buffer, and subjected to SDS-PAGE. Analysis was performed by immunoblotting with a monoclonal antibody to phosphotyrosine. Mobility of the molecular-mass standard is indicated in kDa. The arrow points to the proteins of 62 kDa. The results shown are representative of three separate experiments.

Table 1 Effect of acetylshikonin on the cyclic AMP level of neutrophils

		Cyclic AMP (pmol per 2×10^6 cells)
Control		0.35±0.04
fMLP	1 μM	0.53±0.04
+acetylshikonin	1 μM	0.18±0.03*
+acetylshikonin	3 μM	0.12±0.01**
+acetylshikonin	10 μM	0.11±0.02**
+IBMX	300 μM	0.95±0.15*
Forskolin	10 μM	1.76±0.21†

Values are expressed as mean±s.e.mean of 5 separate experiments. *$P<0.05$, **$P<0.01$ compared to the fMLP alone-treated group. †$P<0.01$ compared to the control value.

and, in the presence of 0.5% ethanol, the corresponding phosphatidylethanol (PEt) was clearly observed in [^{3}H]-alkyl-lysophosphatidylcholine-loaded neutrophil suspension on exposure to fMLP/CB. The cellular levels of PA and PEt in response to fMLP/CB were reduced in parallel by genistein. In addition, acetylshikonin concentration-dependently inhibited the fMLP/CB-induced PA as well as PEt formation (60.1±7.3 and 63.2±10.5% inhibition, respectively, with 10 μM acetylshikonin) (Figure 7).

Effect of acetylshikonin on NADPH oxidase

In a PMA-activated neutrophil particulate NADPH oxidase preparation, addition of NADPH to initiate $O_2^{\cdot-}$ generation resulted in the reduction of ferricytochrome *c* (1.21±0.02 nmol $O_2^{\cdot-}$ min^{-1} mg^{-1} protein). Trifluoperazine (TFP), an inhibitor of NADPH oxidase (Bellavite *et al*., 1983), induced a concentration-dependent inhibition of NADPH oxidase activity when added to the cells. In contrast, a significantly enhanced oxidase activity was observed with concentrations of acetylshikonin $\geqslant$1 μM (Figure 8). In PMA-activated neutrophils, the membrane associated p47^{phox} was increased, as assessed by Western blotting with a polyclonal anti-p47^{phox} antibody (Figure 9, cf lanes 1–2, arrow). Staurosporine greatly reduced the membrane associated p47^{phox}. Treatment of neutrophils with acetylshikonin also led to a concentration-dependent inhibition of the p47^{phox} membrane translocation in response to PMA (approximately 60 and 80% inhibition with acetylshikonin 1 and 3 μM, respectively).

Discussion

In the present study, we demonstrated that acetylshikonin inhibited the neutrophil $O_2^{\cdot-}$ generation and O_2 consumption in response to fMLP/CB and PMA. Since acetylshikonin did not reduce, but enhanced, the $O_2^{\cdot-}$ generation in cell-free oxygen radical generation systems, this precludes the possibility that acetylshikonin acted as a $O_2^{\cdot-}$ scavenger and suggests that it acted as an inhibitor of certain signal transduction steps that follow after activation of neutrophils.

It is well established that fMLP and PMA elicit the respiratory burst by activating the same NADPH oxidase in neutrophils, but they utilize a different transduction mechanism and are regulated differently (Segal & Abo, 1993). fMLP-activates neutrophils by binding to a G protein-linked receptor on the membrane, which, in turn, activates PLC that catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate IP_3, which increases the $[Ca^{2+}]_i$, and diacylglycerol, which stimulates PKC (Berridge, 1987). There is evidence that fMLP/CB-induced $O_2^{\cdot-}$ generation is a Ca^{2+}-dependent process (Lehmeyer *et al*., 1979). In this study, we found that acetylshikonin, like a PLC inhibitor U73122 (Smith *et al*., 1990), reduced the formation of IP_2 and IP_3 in neutrophils in response to fMLP. Moreover, in the presence of EDTA to remove the extracellular Ca^{2+} and to diminish the Ca^{2+}-influx during cells activation, acetylshikonin inhibited the fMLP-in-

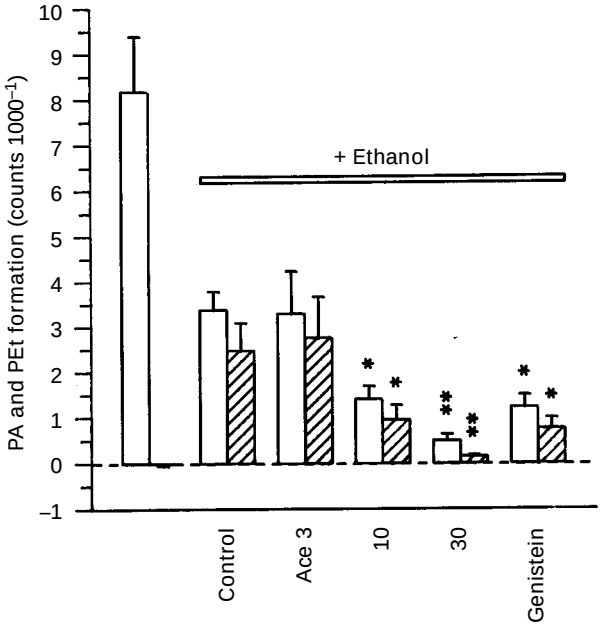

Figure 7 Effect of acetylshikonin (Ace) on PLD activity of rat neutrophils. 1-*O*-[^{3}H]-octadecyl-*sn*-glycero-3-phosphocholine-loaded cells were pretreated with DMSO (as control), 3–30 μM acethylshikonin or 30 μg ml^{-1} genistein for 3 min at 37°C in the presence of 0.5% ethanol before addition of 1 μM fMLP plus 5 μg ml^{-1} dihydrocytochalasin B (CB). The lipids were extracted from the reaction mixture, separated on silica gel 60 plates, and the phosphatidic acid (PA) (open columns) and phosphatidylethanol (PEt) (hatched columns) were quantified by phosphor screen autoradiography. The levels of PA and PEt in fMLP/CB-stimulated cells in the absence of 0.5% ethanol are also shown (the 1st pair of columns). Values are expressed as mean ± s.e.mean of 4 separate experiments. $^*P<0.05$, $^{**}P<0.01$ compared to the corresponding control values.

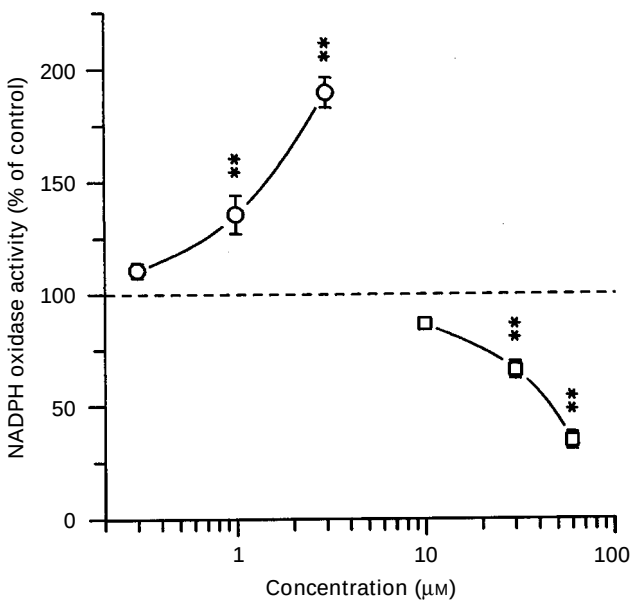

Figure 8 Effect of acetylshikonin on phorbol 12-myristate 13-acetate (PMA)-activated neutrophil particulate NADPH oxidase. NADPH oxidase was preincubated with DMSO (as control), various concentrations of acetylshikonin (○) or trifluoperazine (□) at 28°C for 3 min before addition of 50 μM NADPH to start the reaction. Values are expressed as mean and vertical lines show s.e.mean of 4–5 separate experiments. $^{**}P<0.01$ compared to the control value.

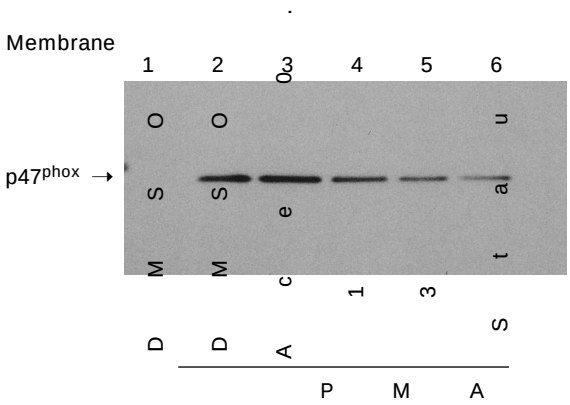

Figure 9 Effect of acetylshikonin (Ace) on phorbol 12-myristate 13-acetate (PMA)-induced p47^{phox} membrane translocation. Neutrophils were preincubated with DMSO (lane 2), 0.3–3 μM acetylshikonin (lanes 3–5) or 0.2 μM staurosporine (Stau) (lane 6) for 5 min at 37°C and then stimulated with 0.2 μM PMA for another 5 min. Cells may also react with DMSO (lane 1) alone for total 10 min reaction time. After termination of reaction, cells were disrupted, and the membrane proteins were subjected to SDS-PAGE. Each lane contained a 50 μg sample of protein. Analysis was performed by immunoblotting with a polyclonal anti-p47^{phox} antibody. The results shown are representative of 3 separate experiments.

duced $[Ca^{2+}]_i$ elevation with an IC_{50} value of about 5.3 μM (data not shown). Since higher concentrations of acetylshikonin had to be used in the PLC/Ca^{2+} assay than in the $O_2^{\cdot-}$ generation assay, these data suggest that the blockade of the PLC/Ca^{2+} pathway may not play a prominent role.

As mentioned previously, the endogenous PKC activator diacylglycerol is generated following activation of the fMLP-receptor. In contrast, PMA bypasses the membrane receptor and activates PKC directly (Castagna *et al*., 1982). Several isoforms of PKC including conventional (α, β_1 and β_2), novel (δ and η) and atypical (ζ) are present in human neutrophils (Lopez *et al*., 1995). PKC activity is primarily cytosolic in unstimulated neutrophils, but becomes firmly associated with the membrane fraction after PMA treatment. PKC-β is the major Ca^{2+}-dependent PKC isoform and is translocated from cytosol to membrane in response to treatment with phorbol ester (Majumdar *et al*., 1991). PKC-β may play a role in the phosphorylation of membrane-associated p47^{phox} and in the assembly or maintenance of an active NADPH oxidase (Majumdar *et al*., 1993). The inability of acetylshikonin to suppress neutrophil cytosolic PKC activity as well as the PMA-induced PKC-β redistribution indicates that the inhibition of respiratory burst by acetylshikonin cannot be attributed to a direct effect on PKC function.

Pharmacologically, increasing intracellular cyclic AMP is one recognized means to down-regulate $O_2^{\cdot-}$ generation in neutrophils (Fantone & Kinnes, 1983). Rap1A appears to be the major PKA substrate (Bokoch *et al*., 1991) and phosphorylation of Rap1A results in the reduction of Rap1A binding to flavocytochrome b_{558} probably contributing to the inhibition of NADPH oxidase by cyclic AMP-elevating agents. In the present study, acetylshikonin did not increase, but reduced, the cellular cyclic AMP level. The finding that porcine heart PKA activity was unaffected by acetylshikonin whether cyclic AMP was present or not indicates that acetylshikonin has no ability to activate PKA directly. However, cellular PKA activity is assumed to be decreased rather than increased by acetylshikonin because of the marked reduction of the cellular cyclic AMP level by this agent. Thus, there is no indication that inhibition of respiratory burst by acetylshikonin is due to an increase of cyclic AMP level or PKA activity.

It has been shown that tyrosine phosphorylation plays a regulatory role in the signal transduction pathway leading to the respiratory burst induced by fMLP (Torres *et al*., 1993). Tyrosine kinases p53/56^{lyn} and p59^{fgr} are involved in the receptor-mediated generation of reactive oxygen intermediates (Hamada *et al*., 1993; Stephens *et al*., 1993). fMLP-induced

tyrosine phosphorylation of 40 and 42 kDa proteins in human neutrophils has also been demonstrated (Torres *et al*., 1993). In the present study, the most prominent phosphotyrosine-containing protein was 62 kDa after stimulation with fMLP/CB. In a similar manner to the tyrosine kinase inhibitor genistein (Akiyama *et al*., 1987), acetylshikonin attenuated the fMLP/CB-induced tyrosine phosphorylation of 62 kDa. Since the inhibition of tyrosine phosphorylation and $O_2^{\cdot-}$ generation by acetylshikonin occurred over the same range of concentrations, the data suggest that the attenuation of protein tyrosine phosphorylation may play an important role in the inhibition of respiratory burst by acetylshikonin.

Since PLD is a downstream effector of fMLP-induced tyrosine kinase activation that leads to activation of NADPH oxidase (Yasui *et al*., 1994), the effect of acetylshikonin on the PLD activity was also determined. PLD catalyzes the hydrolysis primarily of phosphatidylcholine to produce PA. In the presence of ethanol, PA via a transphosphatidylation reaction yields PEt. PA could act on the respiratory burst through activation of PKC or NADPH oxidase (Bellavite *et al*., 1988). Acetylshikonin as well as genistein suppressed both PA and PEt formation in neutrophils in response to fMLP. Since acetylshikonin inhibited the $O_2^{\cdot-}$ generation at a much lower concentration range than was active in the PLD assay, the data suggest that the blockade of PLD pathway is probably not important in its inhibitory effect on the respiratory burst.

The $O_2^{\cdot-}$ generating NADPH oxidase complex in neutrophils consists of a heterodimeric flavocytochrome b_{558} and cytosolic factors, mainly $p47^{phox}$ and $p67^{phox}$ (Segal & Abo, 1993). Upon activation, $p47^{phox}$ is phosphorylated and the polyproline motif of $p47^{phox}$ becomes accessible to the C-terminal SH3 domain of $p67^{phox}$ (Finan *et al*., 1994). This new interaction changes the overall structure of the complex and makes it able to recognize the membrane flavocytochrome b_{558} through the polyproline motif of $p22^{phox}$ and one of the SH3 domains of $p47^{phox}$ (Sumimoto *et al*., 1994), favourable to electron transport, and thereby proceeding to the univalent reduction of O_2 (Segal & Abo, 1993). Acetylshikonin, like staurosporine, decreased the membrane associated $p47^{phox}$ in neutrophils in response to PMA. However, acetylshikonin enhanced the oxidase activity in the presence of NADPH in the PMA-activated neutrophil particulate NADPH oxidase preparation. These results indicate that acetylshikonin influenced the assembly of a functional NADPH oxidase complex, but did not suppress the activity of assembled oxidase. Since acetylshikonin did not attenuate PKC activity, the inhibition by acetylshikonin of the assembly of the oxidase probably occurs through other mechanisms.

A diaphorase activity associated with NADPH oxidase has recently been described (Cross *et al*., 1994). Diaphorase-catalyzed two-electron transfer from NADPH to 1,4-naphthoquinone yields a hydroquinone product and, hence, the $O_2^{\cdot-}$ generation during autoxidation of the latter (Öllinger *et al*., 1989). The autoxidation products can be re-reduced by diaphorase and thereby generate more $O_2^{\cdot-}$ (Buffinton *et al*., 1989). These could account for the observation in the present study that acetylshikonin, a 2-alkyl 5,8-dihydroxy 1,4-naphthoquinone, enhanced $O_2^{\cdot-}$ generation in PMA-activated particulate NADPH oxidase. However, the exact mechanism underlying the enhancement of the $O_2^{\cdot-}$ generation in cell-free oxygen radical generating systems by acetylshikonin is not yet understood. The possibility that acetylshikonin reduces the oxidized reaction products which, in turn can be reused by the systems to generate $O_2^{\cdot-}$ needs further investigation.

In summary, we have shown that acetylshikonin is capable of inhibiting $O_2^{\cdot-}$ generation and O_2 consumption in neutrophils in response to fMLP as well as to PMA. The underlying mechanism of action was also assessed. Acetylshikonin did not act as a $O_2^{\cdot-}$ scavenger. Moreover, acetylshikonin is neither a PKC inhibitor nor a cyclic AMP-elevating agent. Inhibition of respiratory burst in neutrophils by acetylshikonin can probably be attributed mainly to the suppression of tyrosine phosphorylation and $p47^{phox}$ membrane association, since these effects were observed at the same range of concentrations. However, we do not exclude the possibility that the weak blockade of PLC/Ca^{2+} and PLD pathways may also play a certain role to enhance and contribute to the inhibitory effect of acetylshikonin on the respiratory burst.

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