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The signaling mechanisms mediating the inhibitory effect of TCH-1116 on formyl peptide-stimulated superoxide anion generation in neutrophils

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ABSTRACT

In fMLP (formyl-Met-Leu-Phe)-stimulated rat neutrophils, a mixture of regioisomers benzo[*a*]furo[2,3-*c*]phenazine-10-carboxylic acid and benzo[*a*]furo[2,3-*c*]phenazine-11-carboxylic acid (TCH-1116) inhibited $O_2^{\bullet-}$ (superoxide anion) generation, which was not mediated by scavenging the generated $O_2^{\bullet-}$ or by a cytotoxic effect on neutrophils. TCH-1116 had no effect on the arachidonic acid-induced NADPH oxidase activation in a cell-free system, whereas it effectively attenuated the phosphorylation of Ser residues in $p47^{phox}$ and the association between $p47^{phox}$ and $p22^{phox}$ in fMLP-stimulated neutrophils. The interaction of $p47^{phox}$ with PKC (protein kinase C) isoforms (α, βI, βII, δ and ζ) was attenuated by TCH-1116, whereas TCH-1116 did not affect the PKC isoforms membrane translocation, phosphorylation (Ser660) and kinase activity. TCH-1116 effectively attenuated the association between PKB/Akt (protein kinase B) and $p47^{phox}$, Akt phosphorylation (Thr308/Ser473) and kinase activities of Akt and human recombinant PDK (3-phosphoinositide-dependent kinase) 1, whereas it had no effect on recruitment of Akt, phospho-PDK1 (Ser241) and p110γ to membrane. Moreover, the interaction of p21-activated kinase (PAK) 1 with $p47^{phox}$ and the phosphorylation of PAK1 (Thr423 but not Ser144) were inhibited by TCH-1116, but without affecting the membrane recruitment of PAK1. The cellular cyclic AMP level was not changed by TCH-1116. Taken together, these results suggest that TCH-1116 inhibits fMLP-stimulated $O_2^{\bullet-}$ generation in rat neutrophils through the blockade of PKC, Akt and PAK signaling pathways.

1. Introduction

Neutrophils are professional phagocytes that play a central role in a host's defense system. For optimal killing of invading microbial pathogens, neutrophils require the production of $O_2^{\bullet-}$ (superoxide anion) free radical and its toxic metabolites. A defect in $O_2^{\bullet-}$ production is illustrated in chronic granulomatous disease (Holmes et al., 1967), characterized by severe and recurrent infections. However, the extensive production of $O_2^{\bullet-}$ results in undesirable tissue damage, and is probably involved in the pathogenesis of many diseases (Halliwell and Gutteridge, 1990). The enzyme responsible for $O_2^{\bullet-}$ generation is NADPH oxidase, which is an enzymatic complex. The oxidase activity is dependent on the assembly of cytosolic regulatory factors ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac2 GTPase) on the membrane-bound flavocytochrome b_{558} ($p22^{phox}$ and $gp91^{phox}$ heterodimer), which contains FAD and heme redox centers, for univalent reduction of extracellular O_2 by using NADPH as the electron donor. Thus, activated neutrophils evoke a respiratory burst in which the oxygen consumption is increased and large amounts of $O_2^{\bullet-}$ are generated.

The signaling mechanisms responsible for oxidase activation in neutrophils are complex and not clearly defined. It is generally believed that the phosphorylation of $p47^{phox}$, mainly on Ser residues, and subsequent translocation to interact with flavocytochrome b_{558} are essential steps for the activation of NADPH oxidase upon neutrophil activation (Ago et al., 2003). A number of Ser/Thr kinases have been proposed to participate in $p47^{phox}$ phosphorylation events, including PKC (protein kinase C), MAPK (mitogen-activated protein kinase) (El Benna et al., 1996; Regier et al., 1999), PAK (p21-activated kinase) (Martyn et al., 2005) and PKB/Akt (protein kinase B) (Chen et al., 2003). Pharmacological interference with these signaling pathways is expected to modulate $O_2^{\bullet-}$ generation in neutrophils, and this presents a reasonable therapeutic strategy to control the potentially harmful proinflammatory activity of these cells in $O_2^{\bullet-}$-mediated diseases.

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In relation with the above considerations, we designed and efficiently synthesized new benzofurophenazine compounds and evaluated their anti-inflammatory activity (Tseng et al., 2009; Tseng et al., in press). In further screening studies with the goal of identifying potential benzofurophenazine compounds, TCH-1116, a mixture of regioisomers benzo[*a*]furo[2,3-*c*]phenazine-10-carboxylic acid and benzo[*a*]furo[2,3-*c*]phenazine-11-carboxylic acid (Fig. 1), was found to have a potent inhibitory effect on $O_2^{\bullet-}$ generation in rat neutrophils in response to fMLP (formyl-Met-Leu-Phe), the most intensively studied formyl-tripeptide derived from bacterial proteins, through the activation of the G_i-protein-coupled fMLP receptor (Klinker et al., 1996). The present study examined the underlying mechanisms of this inhibitory effect by TCH-1116. The data provide evidence that the inhibition of $O_2^{\bullet-}$ generation by TCH-1116 in rat neutrophils is mediated mainly through the blockade of PKC, PAK and Akt signaling pathways, thereby interfering with the downstream NADPH oxidase assembly and subsequent activation.

2. Materials and methods

2.1. Materials

HBSS was purchased from Invitrogen (Carlsbad, CA, USA). Antibodies against p110γ, phospho-Akt (Ser473), Akt, $p47^{phox}$, $p22^{phox}$, PKCα, PKCβI, PKCβII, PKCδ, PKCζ, and G_β were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-PAK1 (Thr423)/PAK2 (Thr402), PAK1, Akt, phospho-Akt (Thr308), phospho-PDK1 (Ser241), and phospho-PKC (pan) (βII Ser660) were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal phosphoserine antibody, 6BIO ((2′Z,3′E)-6-bromoindirubin-3′-oxime), an Akt kinase activity kit, and a PKC kinase activity kit were purchased from Enzo Life Sciences (Farmingdale, NY, USA). Polyvinylidene difluoride membranes, Immobilon Western chemiluminescent HRP substrate, and antibodies against $p47^{phox}$ and β-actin were purchased from Millipore (Bedford, MA, USA). A cyclic AMP EIA kit was purchased from Cayman Chemical (Ann Arbor, MI, USA). PDK1 assay/inhibitor screening kit was purchased from MBL International (Woburn, MA, USA). Dextran 500, Ficoll-Paque, and protein A sepharose were purchased from GE Healthcare (Piscataway, NJ, USA). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY 294002) was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). 2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (GF 109203X) was obtained from Merck (Taipei, Taiwan). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The final volume of dimethyl sulfoxide (DMSO) in all reaction mixture was <0.5%.

2.2. Synthesis of TCH-1116

To a suspension of naphtho[1,2-*b*]furan-4,5-dione (**1**, 0.20 g, 1.0 mmol) in ethoxyethanol (30 ml) 3,4-diaminobenzoic acid (**2**, 0.30 g, 2.0 mmol) was added. The reaction mixture was heated with stirring under microwave irradiation (100 W) for 30 min (TLC monitoring). The solvent was removed in vacuo and the residue suspended in H_2O (20 ml). The resulting precipitate was recrystallized from EtOH to give benzo[*a*]furo[2,3-*c*]phenazine-10-carboxylic acid (**3**) and benzo[*a*]furo[2,3-*c*]phenazine-11-carboxylic acid (**4**) in regioisomeric mixture (Fig. 1), which was obtained as a yellow solid (0.26 g, 82%, ratio **3**/**4**: 50/50). Mp: 311–313 °C. ^{1}H NMR (400 MHz, DMSO-d_6): 7.61 (m, 1H), 7.84 (m, 1H), 7.94 (m, 1H), 8.24–8.37 (m, 4H), 8.75–8.80 (m, 1H), 9.21 (d, 1H, J=8.0 Hz). Anal. calcd for $C_{19}H_{10}N_2O_3 \cdot 0.2H_2O$: C 71.78, H 3.30, N 8.81; found: C 71.63, H 3.32, N 9.18. With our current methodology, we were unable to successfully purify **3** and **4** from regioisomeric mixture.

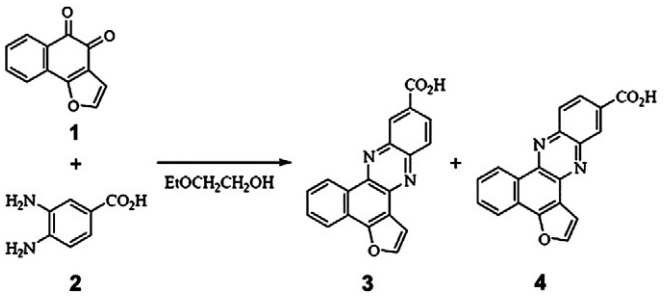

Fig. 1. Synthesis of TCH-1116 regioisomeric mixture.

2.3. Isolation of neutrophils

Rat (Sprague–Dawley) blood was collected from the abdominal aorta and the neutrophils were purified by dextran sedimentation, centrifugation through Ficoll-Paque, and hypotonic lysis of erythrocytes (Wang et al., 2002). Purified neutrophils (>95% viable cells) were resuspended in Hanks' balanced salt solution (HBSS) containing 10 mM HEPES, pH 7.4, and 4 mM $NaHCO_3$, and kept in an ice-bath before use. All experiments in the present study were performed under the guidelines of the Institutional Experimental Laboratory Animal Committee and were in strict accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health.

2.4. Measurement of $O_2^{\bullet-}$

The generation of $O_2^{\bullet-}$ from neutrophils was assessed in superoxide dismutase (SOD)-inhibitable ferricytochrome *c* reduction assay (Wang et al., 2002). Briefly, the reaction mixture contained neutrophils (2×10^6 cells) and 40 μM ferricytochrome *c* in a final volume of 1.5 ml at 37 °C. Cells were pretreated with 5 μM dihydrocytochalasin B for 3 min before stimulation with fMLP. For the determination of $O_2^{\bullet-}$ scavenging effect, $O_2^{\bullet-}$ generation in a cell-free system was assessed by measuring the reduction of nitroblue tetrazolium (0.274 mM) during dihydroxyfumaric acid (0.891 mM) autoxidation. SOD (17.5 U/ml) was added to the reference cuvette at the beginning of the incubation. Absorbance changes were monitored continuously with a double-beam spectrophotometer at 550 nm and 560 nm to determine reductions of ferricytochrome *c* and nitroblue tetrazolium, respectively.

2.5. Measurement of NADPH oxidase activity in a cell-free system

Neutrophils were pretreated with 5 μM dihydrocytochalasin B for 15 min at 37 °C. After being washed, cells were suspended in Tris buffer (0.34 M sucrose, 10 mM Tris–HCl, pH 7.0, 2 mM phenylmethylsulfonyl fluoride and 1 mM benzamidine) (Wang et al., 2002) and disrupted by sonication. The unbroken cells and nuclei were removed by centrifugation (800×*g* for 10 min at 4 °C) and then the supernatants were further centrifuged (123,000×*g* for 30 min at 4 °C) to collect cytosolic and membrane fractions. The reaction mixture contained the membrane fraction (20 μg), the cytosolic fraction (100 μg), 10 μM FAD, 3 μM GTPγS, 200 μM NADPH and 0.5 μg/ml of ferricytochrome *c* in phosphate buffer. The reaction was initiated by the addition of 150 μM arachidonic acid and stopped by 5 U/ml of SOD. Absorbance changes were monitored at 550 nm.

2.6. Immunoblot analysis

Reactions were terminated by the addition of Laemmli sample buffer and then the solution was boiled. Proteins (60 μg per lane) were resolved by 10% SDS-PAGE, and transferred to polyvinylidene

difluoride membranes. The membranes were blocked with 5% (w/v) nonfat dried milk in TBST buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20) and probed with anti-phospho-PAK, anti-phospho-Akt, anti-phospho-PDK1, or anti-phospho-PKC antibody. The blots were then stripped and reprobed with anti-PAK, anti-Akt, anti-PDK1 or anti-PKCα antibody to standardize protein loading in each lane. Detection was performed with the Western chemiluminescent horseradish peroxidase (HRP) substrate. The band intensity was detected by a Luminescent Image Analyzer (Fujifilm LAS-3000) using MultiGauge software.

For the membrane translocation experiments, the reaction was stopped by the addition of ice-cold HBSS, and then resuspended in ice-cold isolation buffer (0.34 M sucrose, 50 mM Tris–HCl, pH 7.5, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 50 mM NaF, 1 mM Na_3VO_4 and 5 μg/ml each of aprotinin, leupeptin and pepstatin A). After sonication, the lysates were centrifuged (800×*g* for 10 min at 4 °C) to remove the unbroken cells and nuclei, and then further centrifuged (130,000×*g* for 30 min at 4 °C) to collect pellets as membrane fractions. Proteins were resolved by SDS-PAGE and subjected to immunoblot analysis.

2.7. Immunoprecipitation

Cells were washed with ice-cold HBSS and lysed in MLB buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 1 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and 5 μg/ml each of leupeptin and aprotinin) on ice for 10 min. After centrifugation (10,000×*g* for 10 min at 4 °C), the supernatant was incubated overnight at 4 °C with anti-$p47^{phox}$ or anti-Akt antibody. The resulting immunocomplexes were precipitated with protein A sepharose beads for 2 h at 4 °C. The beads were washed and then boiled in Laemmli sample buffer. Proteins were resolved by SDS-PAGE and subjected to immunoblot analysis.

2.8. Akt, PDK1 and PKC kinase assays

Cells were washed with ice-cold HBSS and then suspended in lysis buffer (20 mM MOPS, pH 7.2, 1% NP-40, 5 mM EGTA, 2 mM EDTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 50 mM NaF, 1 mM Na_3VO_4, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml each of leupeptin and aprotinin) on ice for 10 min. Lysates were centrifuged (10,000×*g* for 10 min at 4 °C), and supernatants were collected for Akt or PKC kinase assay by using the Akt or PKC kinase ELISA kit according to instructions of the manufacturer. Briefly, supernatant was added to the kinase assay dilution buffer-rinsed Akt substrate microtiter plate well (for Akt assay) or PKC substrate microtiter plate well (for PKC assay) in the presence of ATP and incubated for 90 min at 30 °C with shaking. After the reaction was stopped by emptying contents of each well, phospho-specific substrate antibody was added to each well at room temperature for another 60 min, and then anti-rabbit IgG-HRP conjugate was added with subsequent addition of TMB substrate. Human recombinant PDK1 with PDK1 assay/inhibitor screening kit was used for PDK1 kinase assay. Briefly, purified PDK1 (5 mU) in kinase buffer was pipetted into the substrate-peptide pre-coated microtiter plate well in the presence of ATP and incubated for 30 min at 30 °C with shaking. The amount of phosphorylated substrate is measured by binding it with anti-phospho-Akt (Thr308) antibody for another 30 min at room temperature, and then subsequent addition of anti-rabbit IgG-HRP conjugate and TMB substrate. The color development was stopped with acid stop solution and the intensity of the color was measured in a microplate reader at 450 nm.

2.9. Statistical analysis

Statistical analyses were performed using the Bonferroni's *t*-test method after one way repeated measures ANOVA for multiple comparisons vs. control. $P<0.05$ was considered significant for all tests. After subtraction of the basal level (treatment with DMSO only) from the test values, analysis of the regression line test was used to calculate IC_{50} values.

3. Results

3.1. Effects of TCH-1116 on $O_2^{\bullet-}$ generation in neutrophils and in cell-free systems

As expected, pretreatment of neutrophils with the NADPH oxidase inhibitor diphenylene iodonium (3 μM) greatly attenuated the fMLP-induced $O_2^{\bullet-}$ generation. TCH-1116 treatment inhibited $O_2^{\bullet-}$ generation in a concentration-dependent inhibition with an IC_{50} value of 3.7 ± 1.0 μM. Significant inhibitory effects began at 0.3 μM (Fig. 2A), whereas TCH-1116 alone (in the absence of fMLP) did not affect the basal level. Pretreatment of cells with TCH-1116 for various time-intervals before stimulation with fMLP did not show a prominent time response profile: An initial 65% inhibition was followed by an increase to 73% inhibition around 5 min and then a plateau over the next 10 min preincubation time (Fig. 2A inset). A 5-min preincubation time was used in the following experiments. This inhibitory effect was fully reversed by washing with HBSS before stimulation with fMLP (Fig. 2B). The viability was >95% when cells were treated with 20 μM TCH-1116 for 20 min at 37 °C as assessed by fluorescein diacetate and propidium iodide staining to evaluate live and dead cells, respectively. The $O_2^{\bullet-}$ generation during dihydroxyfumaric acid autoxidation was not affected by TCH-1116 up to 10 μM, whereas SOD exerted a scavenging ability to eliminate the generated $O_2^{\bullet-}$ (Fig. 2C). To determine whether TCH-1116 directly inhibited NADPH oxidase activity, experiment with NADPH oxidase in a cell-free system, in which the complicated cellular signaling mechanisms to activate oxidase can be bypassed, was performed. Addition of arachidonic acid resulted in the assembly of the cytosolic and membrane components of NADPH oxidase and then generation of $O_2^{\bullet-}$ in the presence of NADPH. The amphiphile property of arachidonic acid has been proposed to provide a neutralizing negative charge which allows $p47^{phox}$ to undergo conformational changes in cell-free system that are similar to those occur with phosphorylation in intact cells (Swain et al., 1997). Under these experimental conditions, addition of diphenylene iodonium greatly attenuated the $O_2^{\bullet-}$ generation (Fig. 2D), whereas TCH-1116 up to 10 μM had no inhibitory effect. Therefore, inhibition of $O_2^{\bullet-}$ generation by TCH-1116 in fMLP-stimulated neutrophils is likely occurring through the blockade of intracellular signaling mechanism.

3.2. Effect of TCH-1116 on $p47^{phox}$ activation

It is generally regarded that an AIR (autoinhibitory region) in $p47^{phox}$ combines with the bis-SH3 domain, preventing binding to $p22^{phox}$. Phosphorylation of Ser residues in AIR results in the release of its binding to the bis-SH3 domain, allowing the interaction of $p47^{phox}$ with membrane-bound $p22^{phox}$ (Ago et al., 2003), which mediates the recruitment of the $p40^{phox}$/$p47^{phox}$/$p67^{phox}$ heterotrimeric complex to form the active NADPH oxidase. Assessment of $p47^{phox}$ phosphorylation and its association with $p22^{phox}$ were performed by Western blot analysis of the 47^{phox} immunoprecipitates. As shown in Fig. 3, fMLP stimulation increased the band immunointensities of both $p22^{phox}$ and phosphoserine with expected size for $p47^{phox}$. Pretreatment of cells with TCH-1116 attenuated both $p47^{phox}$ phosphorylation and interaction with $p22^{phox}$ in a parallel concentration-dependent manner with IC_{50} values of 4.4 ± 3.0 μM

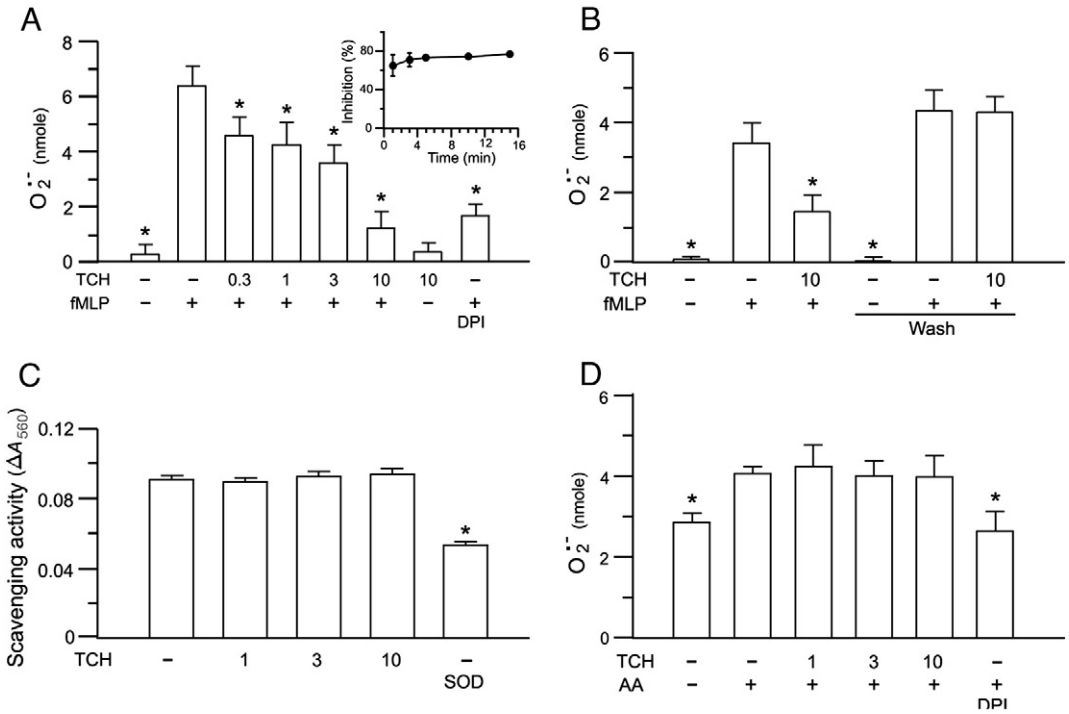

Fig. 2. Effect of TCH-1116 (TCH) on $O_2^{\cdot-}$ generation. (A) Neutrophils were pretreated with DMSO, 3 μM DPI (diphenylene iodonium) or the indicated concentrations (μM) of TCH for 5 min before stimulation or no stimulation with 1 μM fMLP for 5 min. In some experiments, cells were pretreated with 5 μM TCH for the indicated time periods before stimulation with fMLP (inset). The $O_2^{\cdot-}$ generation was determined. *P<0.05, compared with the control value (2nd column). (B) Cells were washed twice with HBSS after treatment with either DMSO or 10 μM TCH, and then stimulated with fMLP. *P<0.05, compared with the corresponding control values (2nd column of each group). (C) DMSO, TCH or 5 U/ml of SOD was added to the dihydroxyfumaric acid autoxidation reaction mixture for 15 min. The absorbance change at 560 nm was determined. *P<0.05, compared with the control value (1st column). (D) DMSO, TCH or DPI was added to the reaction mixture of cytosolic and membrane fractions at 37 °C for 5 min before the addition or no addition of arachidonic acid for another 10 min. *P<0.05, compared with control value (2nd column). Values are expressed as means ± S.D. of 3–4 independent experiments.

and 4.5 ± 4.0 μM, respectively. Therefore, the signaling pathway responsible for p47^{phox} phosphorylation, which was followed by association with p22^{phox}, is likely blocked by TCH-1116.

3.3. Effect of TCH-1116 on PKC activation

In neutrophils and neutrophil-like HL-60 cells, α, β, δ, and ζ appear to be the main PKC isoforms involved in NADPH oxidase activation (Brechard and Tschirhart, 2008). For commencement of the process of PKC-mediated phosphorylation, p47^{phox} requires interaction with PKC. Co-immunoprecipitation experiments revealed that the interaction between PKCα, PKCβI, PKCβII, PKCδ, PKCζ and p47^{phox} takes place in fMLP-stimulated cells. Pretreatment with TCH-1116 attenuated these responses in a concentration-dependent manner, significant inhibitory effects began at 1–3 μM TCH-1116 (Fig. 4A), whereas TCH-1116 alone (in the absence of fMLP) did not affect the basal levels.

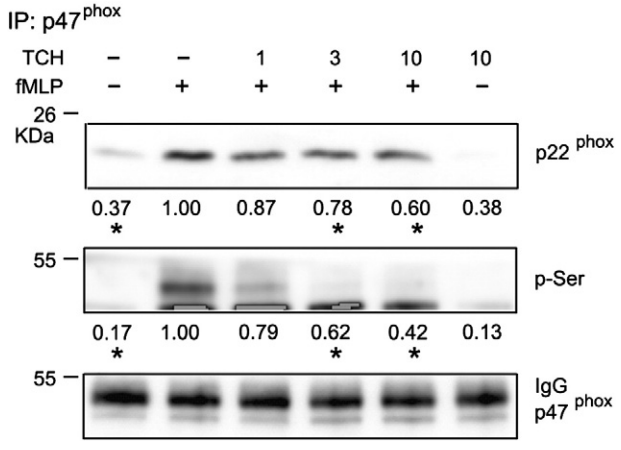

Fig. 3. Effect of TCH-1116 (TCH) on p47^{phox} activation. Neutrophils were pretreated with DMSO or the indicated concentrations (μM) of TCH for 5 min before stimulation or no stimulation with 1 μM fMLP for 1 min. The cell lysates were precipitated with anti-p47^{phox} antibody, and then immunoblotted with the specific antibody against p47^{phox} (as loading control), phospho-Ser or p22^{phox}. The ratio of immunointensity between phospho-Ser, p22^{phox} and the loading control was calculated. *P<0.05, compared with the corresponding control values (2nd lane). The numbers below blots are the mean fold of control values from 3 to 4 independent experiments.

In general, PKC activation is characterized by association of cytosolic PKC to the membrane fraction in intact cells. In the present study, fMLP stimulation resulted in the membrane recruitment of PKCα, PKCβI, PKCβII, PKCδ and PKCζ, as assessed by Western blot analysis. TCH-1116 did not cause prominent attenuation of the immunointensities of these PKC isoforms at any of the concentrations tested, except for the inhibition of PKCα recruitment at 10 μM (P<0.05), whereas TCH-1116 alone (in the absence of fMLP) did not affect the basal levels (Fig. 4B), suggesting the inability to block PKC activation.

The optimum catalysis of PKCs requires the autophosphorylation of C-terminal Ser/Thr (βII Ser660/Thr641) to lock the kinase domains in their active conformation (Parker and Murray-Rust, 2004). Stimulation of neutrophils with fMLP increased the PKC phosphorylation as assessed by immunoblotting with the specific antibody against phospho-PKC (pan) (βII Ser660), which detected PKCα, PKCβI, PKCβII, PKCδ, PKCε, PKCηand PKCθ isoforms when phosphorylated at a C-terminal residue homologous to Ser660 of PKCβII, whereas no significant inhibition was observed when cells were pretreated with TCH-1116 (Fig. 4C). Moreover, by using PKC substrate microtiter plate to assess PKC kinase activity, pretreatment of cells with PKC inhibitor GF 109203X greatly attenuated the fMLP-stimulated PKC kinase activity, whereas TCH-1116 failed to exert a significant change (Fig. 4D). These results are in line with the observation that TCH-1116 did not affect the PKC activation.

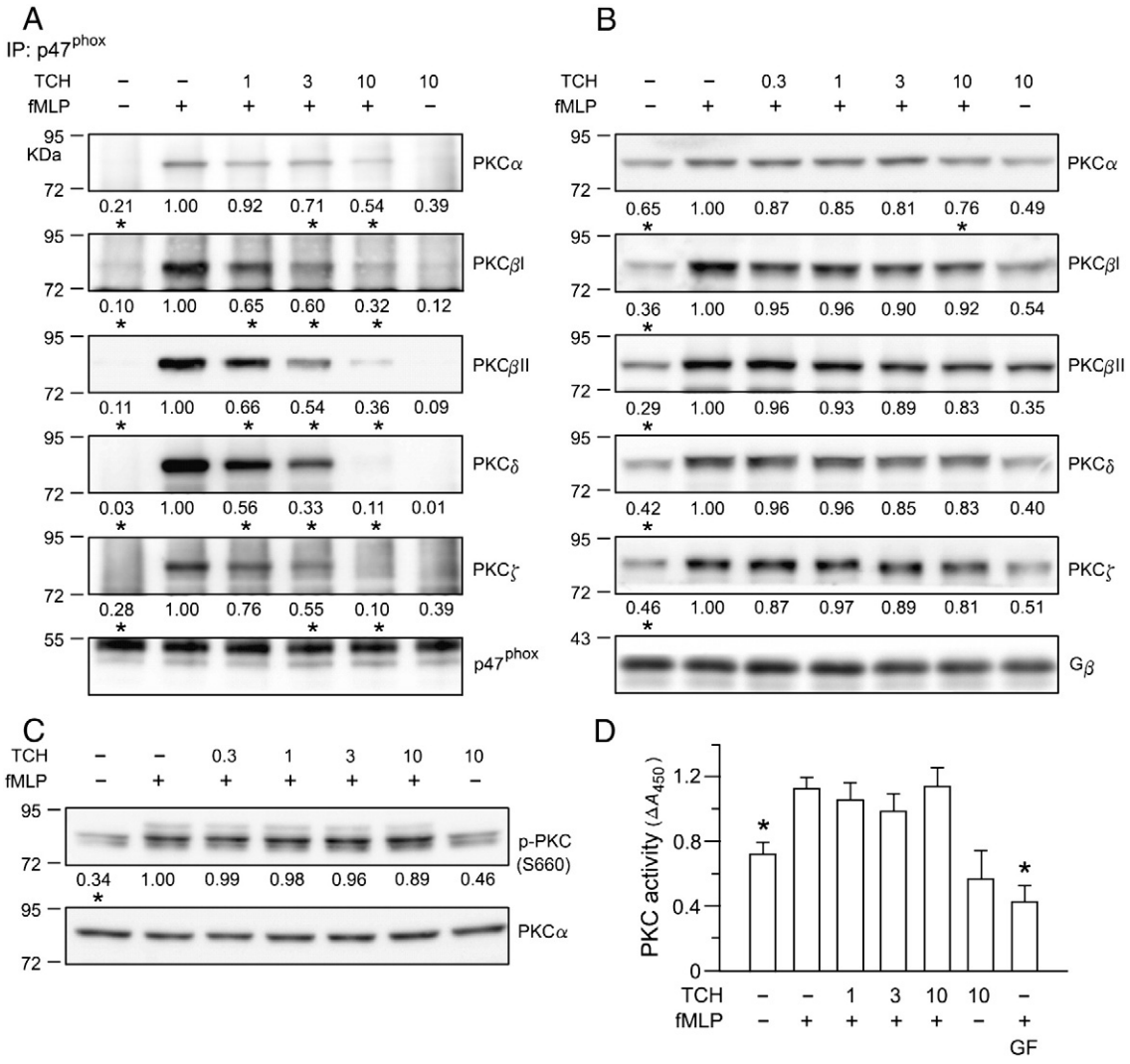

Fig. 4. Effect of TCH-1116 (TCH) on PKC activation. Neutrophils were pretreated with DMSO or the indicated concentrations (μM) of TCH for 5 min before stimulation or no stimulation with 1 μM fMLP for 1 min. (A) The cell lysates were precipitated with anti-p47^{phox} antibody, and then immunoblotted with the specific antibody against p47^{phox} (as loading control) or PKC isoforms. (B) The PKC isoforms and G_{β} (as loading control) in the membrane fractions were determined by Western blot analysis. (C) The phosphorylation of PKC in cell lysates was determined by Western blot analysis, and then the blots were stripped and reprobed with anti-PKCα antibody (as loading control). The ratio of immunointensity between the PKC isoforms, phospho-PKC and the loading control was calculated. *$P<0.05$, compared with the corresponding control values (2nd lane). The numbers below blots are the mean fold of control values from 3 to 4 independent experiments. (D) Cells were pretreated with DMSO or TCH for 5 min or with 1 μM GF109203X (GF) for 10 min before stimulation or no stimulation with fMLP. Cell lysates were prepared for PKC kinase assay. *$P<0.05$ compared with the control value (2nd column). Values expressed as means ± S.D. from 3 independent experiments.

3.4. Effect of TCH-1116 on Akt activation

It is conceivable that 3-phosphorylated phosphoinositides, the product of PI3K (phosphoinositide 3-kinase), recruit PDK1 and inactive Akt to plasma membrane, and in turn facilitates the phosphorylation of Thr308 in the catalytic site and Ser473 in the hydrophobic motif of Akt by PDK1 and PDK2, respectively, for full Akt activation in many cell types, including the fMLP-stimulated neutrophils (Sasaki et al., 2000). The direct phosphorylation of p47^{phox} by Akt in cell-free system has been proposed to mediate a positive stimulatory effect on NADPH oxidase activation (Chen et al., 2003). Therefore, we undertook experiments to assess whether TCH-1116 would have any effect on Akt activation and interaction with p47^{phox}. Stimulation of cells with fMLP for 30 s significantly induced the association between Akt and p47^{phox} as assessed by immunoprecipitation, and TCH-1116 attenuated this interaction in a concentration-dependent manner with an IC_{50} value of 7.3 ± 1.7 μM (Fig. 5A). Moreover, fMLP stimulation induced the phosphorylation of Akt on both Thr308 and Ser473 residues in rat neutrophils. As expected, pretreatment with PI3K inhibitor LY 294002 greatly attenuated the phosphorylation on both residues. Significant inhibitory effect of TCH-1116 on Akt phosphorylation began at 3–10 μM concentration range (about 43% and 55% inhibition for phosphorylation of Thr308 and Ser473, respectively, at 10 μM TCH1116) (Fig. 5B). TCH-1116 alone (in the absence of fMLP) did not affect the basal levels.

PDK1 is a constitutively active enzyme and constitutive autophosphorylation on Ser241 is critical for kinase activity (Casamayor et al., 1999). The result that stimulation of neutrophils with fMLP for a variety of time-intervals did not alter the cellular levels of phospho-PDK1 (Fig. 5C) is in agreement with the finding of this previous report. Fig. 5D shows that fMLP-induced membrane recruitment of phospho-PDK1, Akt and p110γ (catalytic subunit of PI3Kγ) occurred in a time-dependent manner. The parallelism of the membrane translocation of both phospho-PDK1 and Akt was detectable within 5 s after stimulation, reached a maximal level at 15 s then gradually declined, whereas the recruitment of p110γ to membrane reached a peak at around 5 s and declined thereafter. As expected, LY 294002 attenuated the phospho-PDK1 and Akt membrane recruitment, whereas TCH-1116

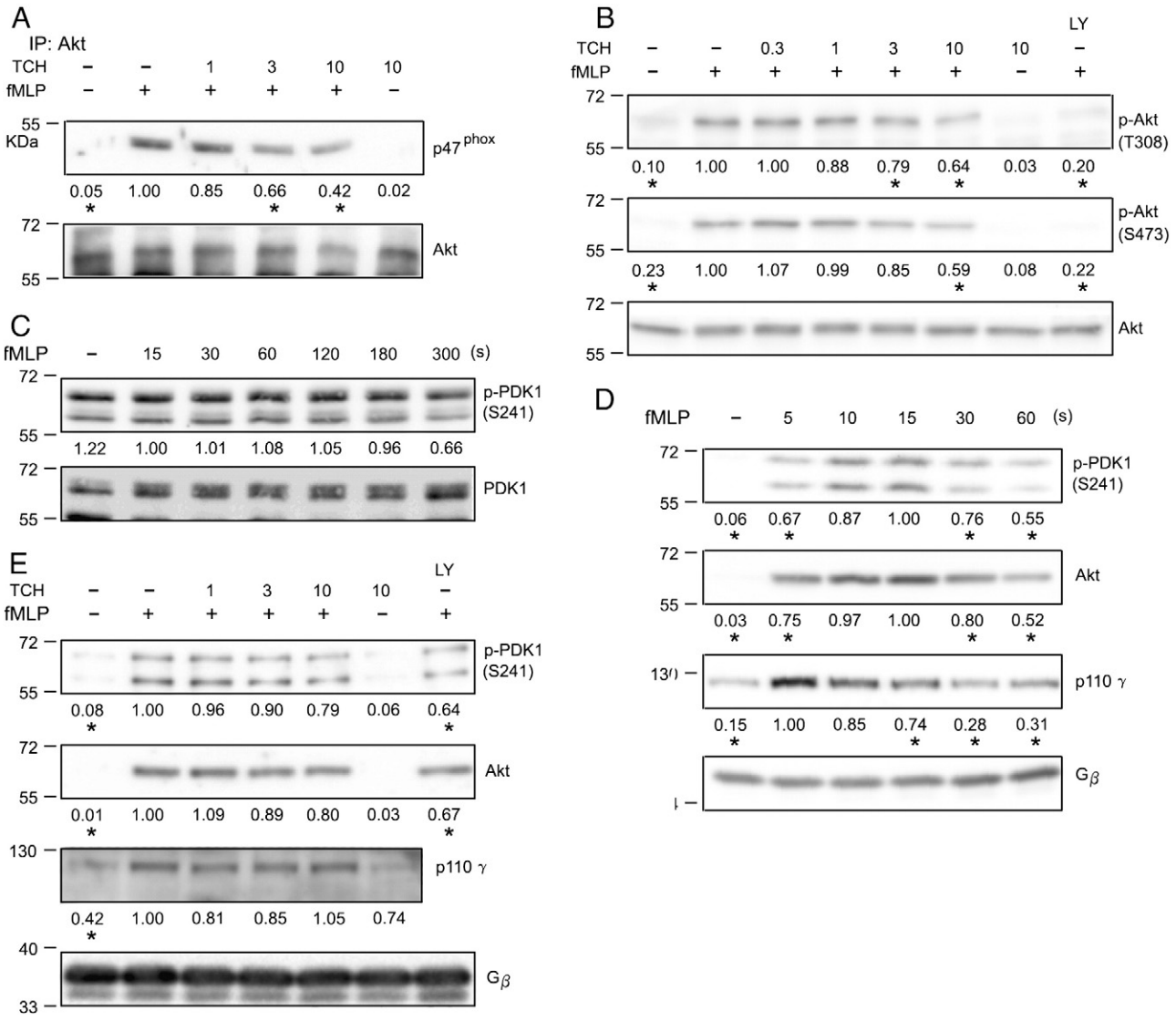

Fig. 5. Effect of TCH-1116 (TCH) on Akt activation. Neutrophils were pretreated with DMSO or the indicated concentrations (μM) of TCH for 5 min or with 10 μM LY 294002 (LY) for 10 min before stimulation or no stimulation with 1 μM fMLP. (A) The cell lysates were precipitated with anti-Akt antibody, and then immunoblotted with the specific antibody against $p47^{phox}$ or Akt (as loading control). (B) The phosphorylation of Akt in cell lysates was determined by Western blot analysis, and then the blots were stripped and reprobed with anti-Akt antibody (as loading control). (E) The phospho-PDK1, Akt, p110γ and G_{β} (as loading control) in the membrane fractions were determined by Western blot analysis. The ratio of immunointensity between $p47^{phox}$, phospho-Akt, phospho-PDK1, Akt, p110γ and the loading control was calculated. $^{*}P<0.05$, compared with the corresponding control values (2nd lane). In some experiments, neutrophils were stimulated with fMLP for the indicated time periods. (C) The phospho-PDK1 and PDK1 (as loading control) in cell lysates were determined by immunoblotting. The ratio of immunointensity between phospho-PDK1 and the loading control was calculated. $^{*}P<0.05$, compared with the control value (2nd lane). (D) The phospho-PDK1, Akt, p110γ and G_{β} (as loading control) in the membrane fractions were determined. The ratio of immunointensity between phospho-PDK1, Akt, p110γ and the loading control was calculated. $^{*}P<0.05$, compared with the corresponding control values (4th lane for phospho-PDK1 and Akt, and 2nd lane for p110γ). The numbers below blots are the mean fold of control values from 3 to 4 independent experiments.

was found to be devoid of inhibitory activity on the membrane translocation of all three proteins in response to optimal fMLP activation (Fig.5E). These data imply that the inability of fMLP to stimulate Akt phosphorylation after TCH-1116 treatment is likely not attributable to a defect in 3-phosphorylated phosphoinositides production.

We next examined the effect of TCH-1116 on Akt kinase activity by using an Akt/PKB substrate microtiter plate. Pretreatment of cells with LY 294002 significantly decreased the fMLP-stimulated Akt kinase activity. TCH-1116 inhibited Akt kinase activity in a concentration-dependent manner with an IC_{50} value of 7.9 ± 3.2 μM (Fig. 6A), whereas incubation of TCH-1116 with the cell lysates prepared from fMLP-stimulated cells had no inhibitory effect (Fig. 6B), excluding the direct inhibition of Akt. Moreover, TCH-1116 treatment resulted in a concentration-dependent inhibition of human recombinant PDK1 kinase activity by using a PDK1 assay/inhibitor screening kit with an IC_{50} value of 7.2 ± 2.0 μM (Fig. 6C). As expected, PDK1 kinase activity was inhibited by a PDK1 inhibitor 6BIO. However, a very weak PDK1 kinase activity was detected in cell lysate (data not shown), making it difficult to evaluate the inhibitory effect of TCH-1116 in neutrophils by using the same assay kit. This problem awaits further investigation.

3.5. Effect of TCH-1116 on PAK activation

The regulatory action of Rac on $p47^{phox}$ occurs through the binding of Rac-GTP to the PAK-PBD domain (Burbelo et al., 1995), which interferes with the inhibitory domain functions and enables PAK to make the transition to an open conformation allowing transphosphorylation of activation loop, including Thr423 residue, and autophosphorylation of N-terminus, including Ser144 residue, of PAK1 (or equivalent residues for other isoforms) for full activation (Lei et al., 2000; Zenke et al., 1999) to phosphorylate $p47^{phox}$ (Martyn et al., 2005). In the present study,

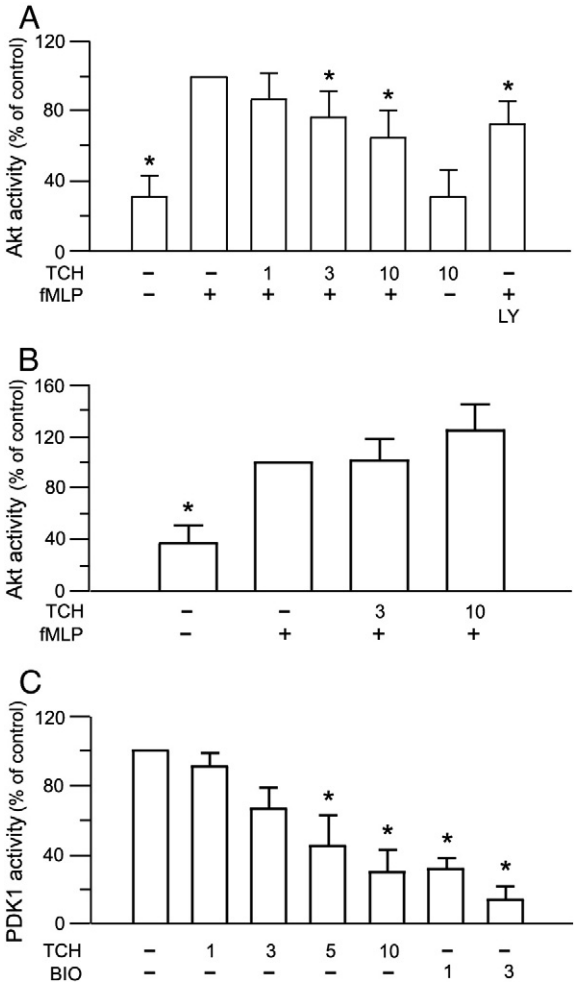

Fig. 6. Effect of TCH-1116 (TCH) on Akt activity. (A) Neutrophils were pretreated with DMSO or the indicated concentrations (μM) of TCH for 5 min or with 10 μM LY 294002 (LY) for 10 min before stimulation or no stimulation with 1 μM fMLP. Cell lysates were prepared for Akt kinase assay. In some experiments, (B) cell lysates of fMLP-stimulated control cells were then pretreated with DMSO or TCH for 5 min followed by Akt kinase assay. (C) Human recombinant PDK1 protein was treated with DMSO, TCH or 6BIO (BIO) for 5 min followed by PDK1 kinase assay. *$P<0.05$, compared with the corresponding control values (2nd column, $A_{450\ nm}$ 0.175 ± 0.086 and 0.595 ± 0.127 for A and B, respectively; 1st column, $A_{450\ nm}$ 1.01 ± 0.13 for C). Values are expressed as means ± S.D. from 3 independent experiments.

p47^{phox} was co-immunoprecipitated with PAK1 in neutrophils stimulated with fMLP for 30 s. Pretreatment with TCH-1116 attenuated the association between p47^{phox} and PAK1 in a concentration-dependent manner with an IC_{50} value of 3.7 ± 1.8 μM (Fig. 7A). In addition, stimulation with fMLP for 15 s resulted in the phosphorylation of PAK as assessed by immunologic detection of cell lysates with anti-phospho-PAK1 (Thr423)/PAK2 (Thr402) antibody. PAK1 phosphorylation was attenuated by TCH-1116 in a concentration-dependent manner with an IC_{50} value of 3.8 ± 1.8 μM (Fig. 7B) and also by 10 μM 6BIO.

During cell activation, PAK, predominantly cytoplasmic protein, is recruited to the membrane and then activated (Zhao et al., 2000). Immunoblotting analysis of the membrane fractions revealed that the membrane-associated PAK was increased in fMLP-stimulated neutrophils, however TCH-1116 did not influence this response (Fig. 7C). We next examined the autophosphorylation of PAK by immunological detection using specific antibody against phospho-PAK1 (Ser144). fMLP stimulation resulted in a time-dependent phosphorylation of PAK1, which reached a maximal level within 15 s after stimulation then gradually declines, and was undetectable at times ≥120 s (Fig. 7D), whereas TCH-1116 treatment did not show any significant inhibitory effect on the optimal response (Fig. 7E).

3.6. Effects of TCH-1116 on cyclic AMP formation

Addition of a β-adrenoceptor agonist isoproterenol (3 μM) greatly increased the cellular concentrations of cyclic AMP (0.5 ± 0.2 as control vs 24.2 ± 2.0 pmol/2×10^6 cells, $P<0.05$) in response to fMLP stimulation as assessed by using a cyclic AMP EIA kit, whereas TCH-1116 up to 10 μM did not affect the cyclic AMP level (0.2 ± 0.1 pmol/2×10^6 cells, $P>0.05$).

4. Discussion

In the present study, we demonstrated that the inhibition of fMLP-stimulated $O_2^{\bullet-}$ generation by TCH-1116 in rat neutrophils is not mediated by cytotoxic effect (viability >95%) on neutrophils, $O_2^{\bullet-}$ scavenging effect (assessed in dihydroxyfumaric acid autoxidation assay), and by inhibition of NADPH oxidase activity (assessed in a cell-free system of arachidonic acid-induced assembly of oxidase). The results that TCH-1116 attenuated both fMLP-induced p47^{phox} phosphorylation and interaction with p22^{phox} over a similar concentration range to inhibit $O_2^{\bullet-}$ generation in neutrophils imply the interference with certain cellular signaling pathways by TCH-1116. A number of kinases have been proposed to participate in p47^{phox} phosphorylation events, including PAK, Akt, PKC, p38 MAPK, and ERK (Chen et al., 2003; El Benna et al., 1996; Martyn et al., 2005). Subsequent studies indicated that TCH-1116 inhibition of $O_2^{\bullet-}$ generation is mediated through the modulation of PKC, Akt and PAK signaling pathways. The results that TCH-1116 had no effect on fMLP-induced PKC membrane translocation and p38 MAPK phosphorylation (data not shown) exclude the possibility of blocking fMLP receptor and/or G_i-protein.

PKC, a key Ser/Thr kinase in signal transduction, is involved in the regulation of diverse cellular processes, including the activation of NADPH oxidase in phagocytes. More than ten isoforms of mammalian PKC have been identified, and phosphorylation of p47^{phox} in cell-free system is mediated by all families of PKC isoforms (Regier et al., 1999). PKC-dependent phosphorylation of p47^{phox} occurred at Ser303, Ser304, Ser315, Ser320, Ser328, Ser359, Ser370, and Ser379 (Fontayne et al., 2002). PKCα, PKCβ, PKCδ, and PKCζ have been implicated in the regulation of NADPH oxidase activity in PKC isoform depleted neutrophil-like HL60 cells or in neutrophils from PKC isoform knockout mice (Brechard and Tschirhart, 2008). PKC isoforms may have discrete specificities for differential phosphorylation of p47^{phox} (Fontayne et al., 2002) or may trigger distinct signaling pathway(s) upstream from p47^{phox}. In rat neutrophils, eight PKC isoforms (PKCα, PKCβI, PKCβII, PKCδ, PKCε, PKCθ, PKCι and PKCζ) have been identified (Tsao and Wang, 1997), however the specific isoform(s) of PKC which is responsible for regulation of NADPH oxidase remains ill defined. Two main obstacles in this respect have been 1) the lack of selective small molecule inhibitors of the specific PKC isoforms, which limits the pharmacological approaches and 2) the short life span of neutrophils, which makes the current molecular biological approaches impracticable. In this study, we provide evidence that TCH-1116 had no effect on fMLP-induced PKC activation based on the following observations of: 1) no prominent effect on PKC membrane recruitment; 2) lack of effect on PKC autophosphorylation; and 3) lack of influence on PKC kinase activity. Nevertheless, the results that both $O_2^{\bullet-}$ generation and the interaction of p47^{phox} with PKC isoforms induced by fMLP were attenuated by TCH-1116 in a parallel effective concentration range imply the involvement of PKC signaling in the inhibition of p47^{phox} phosphorylation by TCH-1116. The precise mechanism underlying the inhibitory effect of TCH-1116 on interaction between p47^{phox} and PKC isoforms remains to be determined.

PI3K, a family of lipid kinases that play a role in phosphorylation of the 3′-OH position of *myo*-inositol, regulates several neutrophil functions, including $O_2^{\bullet-}$ production (Hannigan et al., 2004). Three classes of PI3K have been identified (Vanhaesebroeck et al., 2001), of which

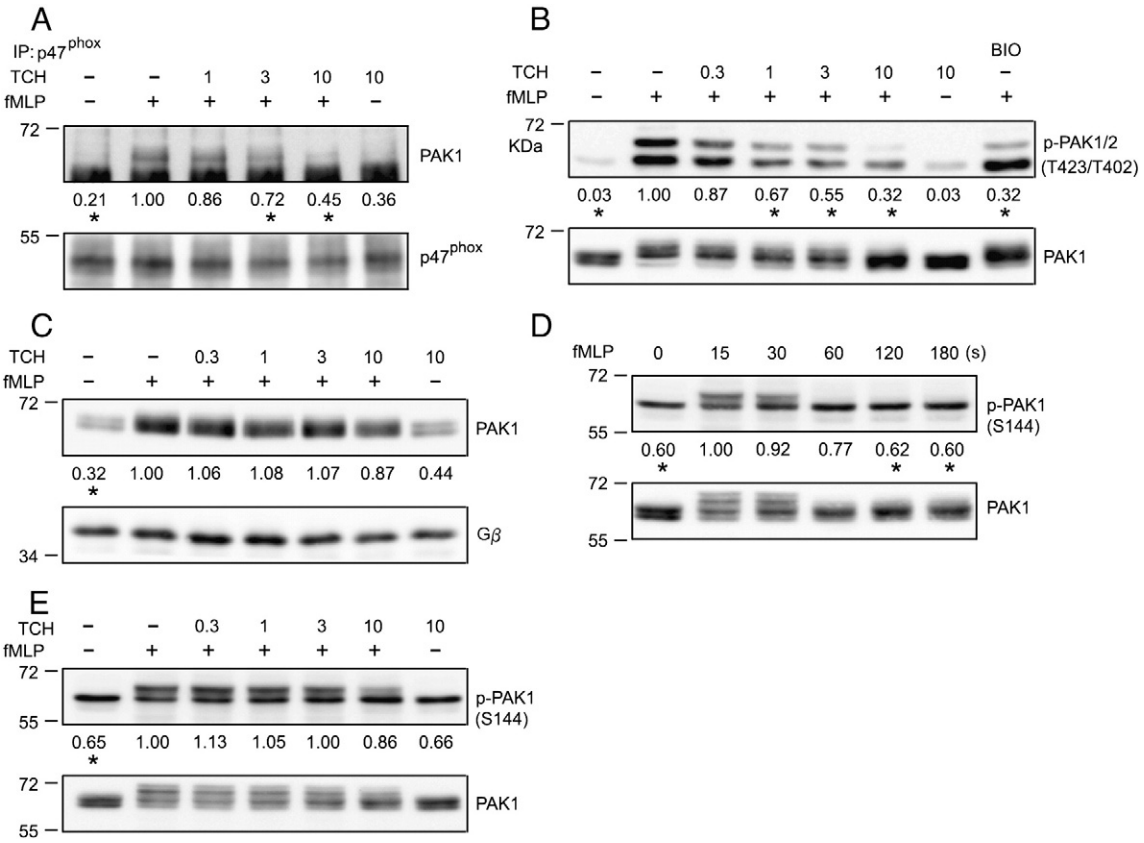

Fig. 7. Effect of TCH-1116 (TCH) on PAK activation. Neutrophils were pretreated with DMSO, 10 μM 6BIO or the indicated concentrations (μM) of TCH for 5 min before stimulation or no stimulation with 1 μM fMLP. (A) The cell lysates were precipitated with anti-$p47^{phox}$ antibody, and then immunoblotted with the specific antibody against PAK1 or $p47^{phox}$ (as loading control). The cell lysates were also analyzed by immunoblotting with (B) anti-phospho-PAK1 (Thr423)/PAK2 (Thr402) or (E) anti-phospho-PAK1 (Ser144) antibody. The blots mentioned above were then stripped and reprobed with anti-PAK1 antibody (as loading control). (C) PAK1 and G_{β} (as loading control) in the membrane fractions were determined by Western blot analysis. In some experiments, (D) cells were stimulated with fMLP for the indicated time periods. Phospho-PAK1 (Ser144) and PAK1 (as loading control) in the cell lysates were determined (as described in E). The ratio of immunointensity between PAK1, phospho-PAK1 and the loading control was calculated. *$P<0.05$, compared with the corresponding control values (2nd lane). The numbers below blots are the mean fold of control values from 3 to 4 independent experiments.

the major isoform of PI3K transmitting the signal from G-protein-coupled receptors is the single class IB PI3Kγ. Activated PI3K participates in the regulation of a number of cell processes through the PDK/Akt-mediated pathway. It has been reported that Akt directly phosphorylates $p47^{phox}$ on Ser304 and Ser328 in a cell-free system (Chen et al., 2003). In the present study, the results that both Akt kinase activity and its interaction with $p47^{phox}$ were attenuated by TCH-1116 over a similar concentration range leading to the inhibition of $O_2^{\bullet-}$ production suggest the involvement of Akt signaling. Inhibition of Akt kinase activity by TCH-1116 is likely to occur through the blockade of cellular signaling pathway. The evidence for the indirect blockade of Akt kinase activity by TCH-1116 comes from the observations that: 1) inhibition of both Akt phosphorylation and kinase activity occurs at a similar effective concentration range of TCH-1116; and 2) TCH-1116 had no inhibitory effect on Akt kinase activity in the cell lysates prepared from fMLP-stimulated cells. Although we did not determine the cellular level of 3-phosphorylated phosphoinositides after TCH-1116 treatment, it is unlikely they decreased and therefore interfered with the subsequent phosphorylation of Akt, because the membrane translocations of PDK1, Akt and PI3Kγ were normal. Inhibition of Akt phosphorylation probably occurs through the blockade of PDK1 kinase activity. Our results are compatible with those of a previous report indicating that PDK1 activity is critical for phosphorylation of Akt on both Ser473 and Thr308 residues in PC-3 cells and PDK1 inhibitor caused a reduction in the levels of phospho-Ser241 with much lower potency than that for reduction of phospho-Thr308 (Feldman et al., 2005). Although the advances have been made in understanding the PI3K/Akt signaling pathway, little information is available regarding the interaction between Akt and downstream substrates in intact cells. Thus, the mechanism mediating the inhibiting effect of TCH-1116 on interaction between $p47^{phox}$ and Akt awaits further investigation. Since PDK1/Akt signaling pathway also plays a key role in cancer cell growth, survival, and tumor angiogenesis and represents a promising target for anticancer drugs (Harris, 2003), it will be undoubtedly of great interest to evaluate the anticancer activity of TCH-1116 in the future study.

It is generally agreed that the fMLP-induced oxidative burst is substantially diminished by cyclic AMP (Anderson et al., 1998). A recent report demonstrated that cyclic AMP-elevating agents suppressed fMLP-induced PI3Kγ activation and translocation of both PDK1 and Akt (Burelout et al., 2007). Therefore, a cyclic AMP-elevating effect cannot account for the inhibition of fMLP-induced $O_2^{\bullet-}$ production by TCH-1116 as evidenced from the following: 1) TCH-1116 did not increase cellular cyclic AMP levels; and 2) the translocation of p110γ induced by fMLP was not altered by TCH-1116.

Previous report indicated that inhibition of PAK kinase activity, by using HIV-1 Tat-mediated protein transduction of PAK inhibitory domain, in human neutrophils reduced fMLP-stimulated $O_2^{\bullet-}$ generation, and the $p47^{phox}$ residues phosphorylated by PAK1, the best-characterized member of the PAK family, in cell-free system were identified to be Ser303, Ser304, Ser320, and Ser328 (Martyn et al., 2005). The results that attenuation of both PAK1 phosphorylation at Thr423, indicating the blockade of PAK1 kinase activity, and interaction with $p47^{phox}$ by TCH-1116 with similar IC_{50} values to those for the inhibition of $O_2^{\bullet-}$ generation imply the involvement of PAK signaling pathway. However, the lack inhibition of both PAK1 autophosphorylation at Ser144 and recruitment to membrane

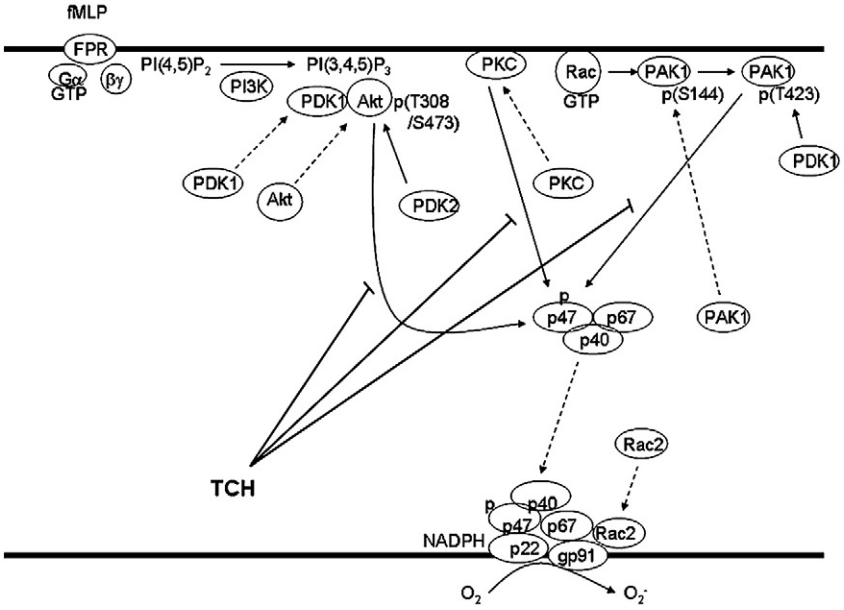

Fig. 8. Schematic diagram showing the mechanisms underlying the inhibition by TCH-1116 (TCH). The signaling molecules generally employed in mediating activation of NADPH oxidase are indicated. Some additional signaling molecules in these pathways have been omitted for the sake of clarity. Broken lines indicated the membrane translocation. Blunt lines indicated the possible sites of action of TCH.

by TCH-1116 suggests an effect on the signaling process at PAK transphosphorylation. Since phosphorylation of Thr423 occurs by an intermolecular mechanism, PAK1 dimer should be relatively efficient in this phosphorylation process (Chong et al., 2001). Alternatively, the biological significance of PDK1 phosphorylation of PAK1 is supported by the observation that these two proteins interact in HeLa cells and PDK1 phosphorylates PAK1 at Thr423 in cell-free system (King et al., 2000). The results that 6BIO inhibited the phosphorylation of PAK1 at Thr423 is compatible with the findings of these previous reports. Therefore, inhibition of PDK1 kinase activity by TCH-1116 may, at least partly, account for the inhibitory effect on Thr423 phosphorylation.

Three major subfamilies of MAPK proteins have been defined: ERK (extracellular signal regulated kinase), the JNK (c-Jun amino-terminal kinase), and the p38 MAPK, of which both p38 MAPK and ERK, but not JNK, phosphorylate $p47^{phox}$ at Ser345 residue (Dang et al., 2006; El Benna et al., 1996). Pharmacological evidence indicated that fMLP-induced neutrophil $O_2^{\bullet-}$ generation was suppressed markedly by the p38 MAPK inhibitor SB 203580, but not by the MEK1/2 (an upstream kinase of ERK) inhibitors PD 98059 and U0126 (Kuan et al., 2005; Zu et al., 1998). However, a p38 MAPK activator anisomycin did not induce but enhanced $O_2^{\bullet-}$ production in fMLP-stimulated human neutrophils (Sakamoto et al., 2006). Moreover, the MAPK recognition sequence in $p47^{phox}$ is not conserved in murine, bovine, bottle-nosed dolphin, and rabbit homologues (Bunger et al., 2000; Inoue et al., 2001; Jackson et al., 1994), suggesting that phosphorylation of this Ser residue by MAPK is of little physiological significance.

In summary, we have demonstrated that inhibition of fMLP-stimulated $O_2^{\bullet-}$ generation by TCH-1116 in rat neutrophils is attributable to the attenuation of $p47^{phox}$ phosphorylation mediated by PKC, Akt and PAK signaling pathways (Fig. 8), thereby implying interference with the subsequent membrane recruitment of $p47^{phox}$ for NADPH oxidase activation. Since neutrophils are relatively insensitive to the conventional anti-inflammatory agents, one may speculate that the inhibition by TCH-1116 of $O_2^{\bullet-}$ generation may have beneficial anti-inflammatory effect.

Acknowledgments

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