European Journal of Pharmacology 682 (2012) 171–180

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and Inflammation

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

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ARTICLE INFO

Article history:
Received 9 December 2011
Received in revised form 31 January 2012
Accepted 9 February 2012
Available online 21 February 2012

Keywords:
Neutrophil
Superoxide anion
p21-Activated kinase
Akt
Protein kinase C
$p47^{phox}$

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.

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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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doi:10.1016/j.ejphar.2012.02.016

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. ^1H 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.

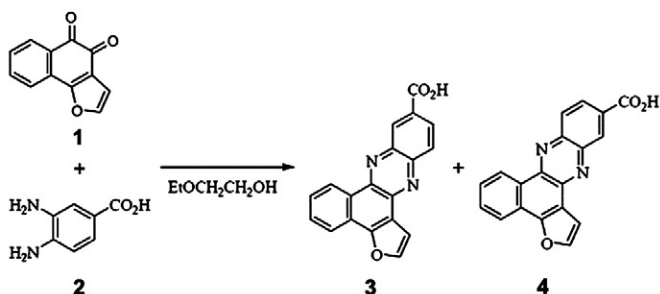

Fig. 1. Synthesis of TCH-1116 regioisomeric mixture.

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

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