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European Journal of Pharmacology 586 (2008) 332–339
www.elsevier.com/locate/ejphar

Inhibitory effects of 16-hydroxycleroda-3,13(14)*E*-dien-15-oic acid on superoxide anion and elastase release in human neutrophils through multiple mechanisms

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Received 3 October 2007; received in revised form 29 January 2008; accepted 14 February 2008
Available online 4 March 2008

Abstract

Reactive oxygen species and granule proteases produced by neutrophils contribute to the pathogenesis of inflammatory diseases. In this study, a cellular model in isolated human neutrophils was established to elucidate the anti-inflammatory functions of 16-hydroxycleroda-3,13(14)*E*-dien-15-oic acid (PL3S), a clerodane diterpenoid from Formosan *Polyalthia longifolia* var. *pendula*. PL3S significantly inhibited the generation of superoxide anion and the release of elastase in formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated human neutrophils in a concentration-dependent fashion with IC_{50} values of 3.06±0.20 and 3.30±0.48 μM, respectively. PL3S did not affect cAMP-dependent pathway, and the inhibitory effect of PL3S was not reversed by protein kinase A inhibitor. PL3S did not display antioxidant or superoxide anion-scavenging ability, and it failed to alter the subcellular NADPH oxidase activity. PL3S concentration-dependently inhibited calcium mobilization caused by FMLP but not thapsigargin. Furthermore, PL3S attenuated the FMLP-induced protein kinase B (AKT) and p38 mitogen-activated protein kinase phosphorylation. However, PL3S had no effect on FMLP-induced phosphorylation of extracellular regulated kinase and *c*-Jun N-terminal kinase. In summary, these results indicate that the suppressive effects of PL3S on human neutrophil respiratory burst and degranulation are at least partly mediated by inhibition of calcium, AKT, and p38 signaling pathways.
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Keywords: Clerodane diterpenoid; Elastase; Neutrophil; *Polyalthia longifolia*; Superoxide anion

1. Introduction

Polyalthia longifolia var. *pendula* (Annonaceae) is native to the drier regions of Sri Lanka and is locally known as 'Ulta Ashok' and is commonly cultivated in Pakistan and India. This plant is used as an antipyretic agent in indigenous systems of medicine (Saleem et al., 2005). Today, *P. longifolia* var. *pendula* is in large-scale cultivation in southern Taiwan as a landscape plant. Pharmacological studies on the bark and leaves of this plant display effective antimicrobial activity (Faizi et al., 2003a,b), cytotoxic function (Chang et al., 2006; Chen et al., 2000), and hypotensive effect (Saleem et al., 2005). In spite of this, the anti-inflammatory effect of *P. longifolia* var. *pendula* remains to be established.

Neutrophils are active phagocytes that act as a crucial component of innate immunity. Although antimicrobial functions of neutrophils are essential to host defense, their extensive or inappropriate activation often causes unwanted tissue damage, such as rheumatoid arthritis, ischemia-reperfusion

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doi:10.1016/j.ejphar.2008.02.041

injury, chronic obstructive pulmonary disease, and asthma (Louis et al., 2000; Mohr et al., 1984; Nathan, 2006; Noguera et al., 2001; Vinten-Johansen, 2004). In response to diverse stimuli, activated neutrophils secrete a series of cytotoxins, such as superoxide anion ($O_2^{\cdot-}$), a precursor of other reactive oxygen species, and granule proteases (Borregaard, 1988; Klebanoff, 2005). Therefore, it is crucial to restrain respiratory burst and degranulation in physiological conditions while potentiating these functions in infected tissues and organs. However, there are only a few currently available agents that directly modulate neutrophil proinflammatory responses in clinical practice. In a search for new anti-inflammatory agents, two clerodane diterpenoids, 16-hydroxycleroda-3,13(14)*E*-dien-15-oic acid methyl ester and 16-hydroxycleroda-3,13(14)*E*-dien-15-oic acid (PL3S) (Fig. 1), from Formosan *P. longifolia* var. *pendula* were found to inhibit the generation of $O_2^{\cdot-}$ in formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated human neutrophils (Chang et al., 2006). The clerodane diterpenoids constitute a large class of natural products. The number of natural clerodane diterpenoids has grown rapidly in recent years, and many of them exhibit a diverse range of biological activities (Ahmad et al., 2005; Cavin et al., 2006; Huang et al., 2004; Lee et al., 2005; Shen et al., 2005). However, there is little research elucidating the anti-inflammatory function of the clerodane diterpenoids. Since ester structure may undergo hydrolysis, the aims of this study were to investigate the effect of PL3S on $O_2^{\cdot-}$ generation and elastase release in human neutrophils and to elucidate the signaling pathways responsible for the PL3S-caused inhibition of the neutrophil responses. In the present study, our data suggest that the suppressive effects of PL3S on $O_2^{\cdot-}$ generation and elastase release in FMLP-induced human neutrophils are at least partly mediated by the regulation of calcium mobilization and inhibition of p38 mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) activation.

2. Materials and methods

2.1. Materials

PL3S was isolated as pure compound from Formosan *P. longifolia* var. *pendula* as described previously (Chen et al., 2000), and was dissolved in dimethyl sulfoxide (DMSO) to make stock solutions. Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), and rolipram were obtained from Calbiochem (La Jolla, CA, USA). Fluo-3 AM was purchased from Molecular Probes (Eugene, OR, USA). 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were obtained from Sigma (St. Louis, MO, USA). When drugs were dissolved in DMSO, the final concentration of DMSO in cell experiments did not exceed 0.4% and did not affect the parameters measured.

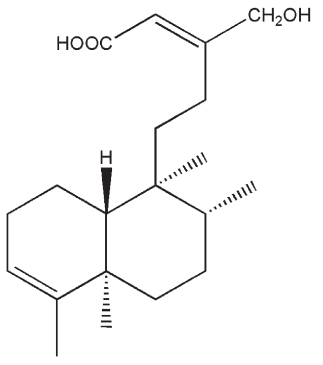

Fig. 1. Chemical structure of 16-hydroxycleroda-3,13(14)*E*-dien-15-oic acid (PL3S).

2.2. Preparation of human neutrophils

Blood was taken from healthy human donors (20–32 years old) by venipuncture, using a protocol approved by the institutional review board at Chang Gung Memorial Hospital. Neutrophils were isolated with a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes (Boyum et al., 1991). Purified neutrophils that contained > 98% viable cells, as determined by the trypan blue exclusion method, were resuspended in a calcium (Ca^{2+})-free HBSS buffer at pH 7.4, and were maintained at 4 °C before use.

2.3. Neutrophil fractionation

Neutrophils were pretreated with 1 mM PMSF for 30 min at 4 °C, disrupted in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, and 10 mM PIPES; pH 7.3) by sonication. Unbroken cells were removed by centrifugation at 300 *g* for 5 min, and the supernatant was then centrifuged at 100,000 *g* for 20 min at 4 °C to produce cytosolic and plasma membrane fractions.

2.4. Measurement of $O_2^{\cdot-}$ generation

The assay of the generation of $O_2^{\cdot-}$ was based on the SOD-inhibitable reduction of ferricytochrome *c* (Babior et al., 1973). In brief, after supplementation with 0.5 mg/ml ferricytochrome *c* and 1 mM Ca^{2+}, neutrophils (1×10^6 cells/ml) were equilibrated at 37 °C for 2 min and incubated with drugs for 5 min. Cells were activated with 100 nM FMLP or 5 nM phorbol myristate acetate (PMA). When FMLP was used as a stimulant, 1 μg/ml cytochalasin B (FMLP/cytochalasin B) was incubated for 3 min before peptide activation. $O_2^{\cdot-}$ generation by isolated neutrophil fractionation was measured after the addition of 160 μM NADPH to 800 μl of relaxation buffer containing 4×10^6 cell equivalents of membrane extract, 1.2×10^7 cell equivalents of cytosol, 2 μM GTP-γ-S, 0.5 mg/ml ferricytochrome *c*, and 100 μM sodium dodecyl sulfate. To facilitate the assembly of NADPH oxidase components, all constituents (excluding NADPH) were incubated at 37 °C for 3 min before the addition of NADPH. Drugs were incubated for 2 min before NADPH oxidase assembly. Changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010,

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