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6-Hydroxy-5,7-dimethoxy-flavone suppresses the neutrophil respiratory burst *via* selective PDE4 inhibition to ameliorate acute lung injury

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ABSTRACT

Over-activated neutrophils produce enormous oxidative stress and play a key role in the development of acute and chronic inflammatory diseases. 6-Hydroxy-5,7-dimethoxy-flavone (UFM24), a flavone isolated from the Annonaceae *Uvaria flexuosa*, showed inhibitory effects on human neutrophil activation and salutary effects on lipopolysaccharide (LPS)-induced acute lung injury (ALI) in mice. UFM24 potently inhibited superoxide anion ($O_2^{\bullet-}$) generation, reactive oxidants, and CD11b expression, but not elastase release, in *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLF)-activated human neutrophils. However, UFM24 failed to scavenge $O_2^{\bullet-}$ and inhibit the activity of subcellular NADPH oxidase. fMLF-induced phosphorylation of protein kinase B (Akt) was inhibited by UFM24. Noticeably, UFM24 increased cyclic adenosine monophosphate (cAMP) concentration and protein kinase (PK) A activity in activated human neutrophils. PKA inhibitors significantly reversed the inhibitory effects of UFM24, suggesting that the effects of UFM24 were through cAMP/PKA-dependent inhibition of Akt activation. Additionally, activity of cAMP-related phosphodiesterase (PDE)4, but not PDE3 or PDE7, was significantly reduced by UFM24. Furthermore, UFM24 attenuated neutrophil infiltration, myeloperoxidase activity, and pulmonary edema in LPS-induced ALI in mice. In conclusion, our data demonstrated that UFM24 inhibits oxidative burst in human neutrophils through inhibition of PDE4 activity. UFM24 also exhibited significant protection against endotoxin-induced ALI in mice. UFM24 has potential as an anti-inflammatory agent for treating neutrophilic lung damage.

1. Introduction

Bacterial endotoxemia in patients with sepsis may contribute to the development of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). ADRS features high morbidity and mortality [1,2]. ALI and ARDS are manifested by massive neutrophil activation and infiltration into lungs to disrupt the alveolar capillary barrier and to impair gas exchange in the respiratory tract [3]. Superoxide anion ($O_2^{\bullet-}$) and reactive oxidants generated from activated neutrophils have been proposed to cause acute and chronic lung injury [4].

Abbreviations: AC, adenylate cyclase; ALI, acute lung injury; Akt, protein kinase B; ARDS, acute respiratory distress syndrome; cAMP, cyclic adenosine monophosphate; CB, cytochalasin B; COPD, chronic obstructive pulmonary disease; fMLF, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; FPR, formyl peptide receptor; GPCR, G protein-coupled receptor; MPO, myeloperoxidase; NADPH, nicotinamide adenosine dinucleotide phosphate; $O_2^{\bullet-}$, superoxide anion; PDE, phosphodiesterase; PGE, prostaglandin E; PKA, protein kinase A; LDH, lactate dehydrogenase; PMA, phorbol myristate acetate; UFM24, 6-Hydroxy-5,7-dimethoxy-flavone

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Cyclic adenosine monophosphate (cAMP) is the essential secondary messenger of neutrophils and other inflammatory cells, and it down-regulates several biological functions of immune or inflammatory cells [5]. An increase in intracellular cAMP concentration reduced the lung damage caused by over-activated neutrophils in animal studies [6,7]. Cyclic nucleotide phosphodiesterase (PDE) is responsible for catalyzing the hydrolysis of cAMP to regulate its intracellular level. In the immune or inflammatory cells, cAMP-specific PDE4 is a major subtype of PDEs. Theophylline, a nonselective PDE inhibitor, has been used to treat pulmonary diseases for more than 70 years. However, its application is usually limited because of concerns about its safety, narrow therapeutic margin, and complex interactions with other drugs [8]. Selective PDE4 inhibitors have drawn a great deal of attention to their anti-inflammatory effects in neutrophils and airway smooth muscle cells as promising therapeutic target for treating lung diseases [9]. Gamble et al. [10] proposed that cilomilast, an oral PDE4 selective inhibitor, reduced cell counts of neutrophils by 37% in subepithelial tissues of the bronchus in patients with chronic obstructive pulmonary disease (COPD). The salutary effects of PDE4 inhibitors have been validated in various models of acute and chronic disease, such as asthma, COPD [11], septic shock [12], rheumatoid arthritis [13], type II diabetes [14], autoimmune diseases [15], psoriasis, and atopic dermatitis [16]. The clinical use of PDE4 inhibitor in patients is restricted because of safety concerns, the discovery of new PDE4 inhibitors has been in the spotlight of pharmaceutical research.

6-Hydroxy-5,7-dimethoxy-flavone ($C_{17}H_{14}O_5$, UFM24) was extracted and purified (Fig. 1A) from the leaves of an endemic traditional medicine, *Uvaria flexuosa* [17]. *U. flexuosa* is found in Vietnam and Cambodia. Available literature regarding *U. flexuosa* has primarily centered on the description of its phylogeny and morphological features. UFM24 was found in many plants and had anti-inflammatory, anti-oxidant, anti-cancer activities *in vitro* [18,19]. However, the effects of UFM24 in neutrophilic inflammation and its molecular mechanism of action remain elusive. Our study sought to investigate the salutary effects of UFM24 on human neutrophil activation *in vitro* and on lipopolysaccharide (LPS)-induced ALI in male C57BL/6 mice, as well as to determine the anti-inflammatory mechanism of UFM24. Together, the present results suggest that UFM24 inhibits $O_2^{\bullet -}$ generation, reactive oxidant production, and CD11b expression in activated human neutrophils by selectively inhibiting PDE4 activity. Inhibition of PDE4 by UFM24 led to significant increases in cAMP accumulation and PKA activation in activated human neutrophils. To our knowledge, this is the first time that UFM24 has been proven as a PDE4 inhibitor. Notably, administration of UFM24 protected against endotoxin-induced ALI in a mouse model, and has emerging potential for clinical application in therapies for pulmonary inflammation.

2. Materials and methods

2.1. Reagents

UFM24 (6-hydroxy-5,7-dimethoxy-flavone, $C_{17}H_{14}O_5$, molecular weight 298) was purified from *U. flexuosa*. The purity of UFM24 exceeded 98%, and it was dissolved in dimethylsulfoxide (DMSO). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) was purchased from Dojindo (Kurnamoto, Japan). Methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide, rolipram, and Ro 318220 (3-(1-(3-(amidinothio)propyl-1H-indol-3-yl)) − 3-(1-methyl-1H-indol-3-yl) maleimide) were purchased from Calbiochem (La Jolla, CA, USA). CytoTox 96 Non-Radioactive Cytotoxicity Assay was purchased from Promega (Maddison, WI, USA). cAMP enzyme immunoassay kits, glycerol and Ficoll-Paque were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Dihydrorhodamine 123 (DHR123), Fluo-3 acetoxymethylester (Fluo-3/AM), and *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys (FNLFNYK) were purchased from Molecular Probes (Eugene, OR, USA). FITC-labeled anti-CD11b was purchased from eBioscience (San Diego, CA, USA). PKA activity kits were purchased from Arbor Assays (Ann Arbor, MI, USA). PDE enzyme subtypes including 3B, 4A1A, 4B2, 4C1, 4D2, and 7A, were purchased from BPS Bioscience (Cornerstone Court West, CA, USA). PDE cAMP enzyme assays were purchased from Cisbio (Bedford, MA, USA). Antibodies to phospho-protein kinase B (Akt) (pan) and phospho-Akt (Ser 473) were purchased from Cell Signaling (Beverly, MA, USA). The other agents were all purchased from Sigma-Aldrich (St. Louis, MO, USA). In cell studies, the final concentration of DMSO was not allowed to exceed 0.4% to prevent bias of results.

2.2. Extraction and isolation

UFM24 was extracted from the fresh leaves of *U. flexuosa*. The structure of UFM24 was elucidated and confirmed by comparing the obtained spectroscopic data to those reported in the literature (Fig. 1A) [20]. UFM24 was a yellow powder and its molecular formula, $C_{17}H_{14}O_5$, was determined by the ESI-MS peak at *m/z* 299 $[M+H]^{+\cdot}$. ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.96 (3H, s, 7-OCH_3), 4.18 (3H, s, 5-OCH_3), 6.99 (1H, s, 8-H), 7.12 (1H, s, 3-H), 7.45–7.48 (3H, m, H-3′, 4′, 5′), 7.91–7.96 (2H, dd, J=8.0, 2.0 Hz, H-2′, 6′); ^{13}C NMR (50 MHz, $CDCl_3$): δ 56.3 (q, 7-OCH_3), 61.7 (q, 5-OCH_3), 96.9 (d, C-8), 108.4 (d, C-3), 113.4 (s, C-10), 126.2 (2 × d, C-2′, 6′), 129.2 (2 × d, C-3′, 5′), 132.2 (d, C-4′), 131.2 (s, C-1′), 139.5 (s, C-6), 145.5 (s, C-9),151.7 (s, C-5), 154.3 (s, C-7), 160.7 (s, C-2), 176.8 (s, C-4). The purity of UFM24 at 99% was assessed using a three-point peak purity method and determined by Shimadzu “Class VP” software.

2.3. Protocol of human neutrophil isolation

The study was designed following the principles of the contemporary revision of the Declaration of Helsinki 1975 and approved by the ethics committee of our hospital (Registration number: IRB 99-3848B). Blood samples were donated by healthy volunteers aged from 20 to 30 years after their written informed consent. Human neutrophils were isolated following standardized procedures as sedimentation in dextran solution, gradient centrifugation in Ficoll-Paque, and hypotonic lysis of erythrocytes [7,21]. The separated neutrophils contained more than 98% viable cells, and then were resuspended in no Ca^{2+} HBSS buffer at 4 °C before experiments.

2.4. Analysis of $O_2^{\bullet -}$ generation

The analysis of $O_2^{\bullet -}$ generation from stimulated neutrophils was measured indirectly by the reduction of ferricytochrome *c*. After 6×10^5 cells/ml neutrophils were added with ferricytochrome *c* (0.5 mg/ml), they were incubated for 2 min at 37 °C. Cells were then treated with DMSO or UFM24 for another 5 min, and were activated with fMLF (0.1 μM) for 10 min or stimulated with phorbol myristate acetate (PMA, 5 nM) for 15 min. Cytochalasin B (CB, 1 μg/ml) was added 3 min before fMLF induction. The change in absorbance was monitored continuously at 550 nm with a spectrophotometer (U-3010; Hitachi, Tokyo, Japan) [22].

2.5. Evaluation of intracellular reactive oxidant production

Human neutrophils (2.5×10^6 cells/ml) were incubated at 37 °C for 10 min with DHR123 (2 μM). These cells were treated with UFM24 for 5 min, and then stimulated with 0.1 μM fMLF for another 15 min. HBSS (at 4 °C) was poured into the sample solution to stop the reaction. Then, a flow cytometry was used to detect the intracellular reactive oxidant contents represented by the conversion of nonfluorescent DHR123 to fluorescent rhodamine 123.