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Development of a new colorimetric assay for lipoxygenase activity

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ABSTRACT

Lipoxygenases (LOXs) are a family of non-heme iron-containing dioxygenases that catalyze the hydroperoxidation of lipids, containing a *cis,cis*-1,4-pentadiene structure. A rapid and reliable colorimetric assay for determination of the activity of three human functional lipoxygenase isoforms (5-lipoxygenase, platelet 12-lipoxygenase, and 15-lipoxygenase-1) is developed in this article. In the new assay, LOX-derived lipid hydroperoxides oxidize the ferrous ion (Fe^{2+}) to the ferric ion (Fe^{3+}), the latter of which binds with thiocyanate (SCN^-) to generate a red ferrithiocyanate (FTC) complex. The absorbance of the FTC complex can be easily measured at 480 nm. Because 5-LOX can be stimulated by many cofactors, the effects of its cofactors (Ca^{2+}, ATP, dithiothreitol, glutathione, L-α-phosphatidylcholine, and ethylenediaminetetraacetic acid) on the color development of the FTC complex are also determined. The assay is adaptive for purified LOXs and cell lysates containing active LOXs. We use the new colorimetric assay in a 96-well format to evaluate several well-known LOX inhibitors, the IC_{50} values of which are in good agreement with previously reported data. The reliability and reproducibility of the assay make it useful for in vitro screening for inhibitors of LOXs and, therefore, should accelerate drug discovery for clinical application.

Lipoxygenases (LOXs)[1] comprise a class of non-heme iron-containing dioxygenases that stereospecifically insert molecular oxygen into *cis-cis*-1,4-pentadiene-containing polyunsaturated fatty acids [1]. Among the six identified functional LOX gene isoforms in humans, 5-lipoxygenase (5-LOX), platelet 12-lipoxygenase (p-12-LOX), and 15-lipoxygenase-1 (15-LOX-1) were originally discovered in leukocytes, platelets, and reticulocytes, respectively, and widely studied in immune and inflammatory diseases [2,3]. Arachidonic acid, the substrate of LOXs, is an essential constituent of cellular membranes that is released by tightly regulated phospholipase cleavage [4]. 5-LOX catalyzes the first two steps in biosynthesis of leukotrienes (LTs): the conversion of arachidonic acid to 5(S)-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid [5(S)-HpETE] and further to leukotriene A4 (LTA4) [5,6]. LTA4 is then catalyzed by LTA4 hydrolase to dihydroxy acid LTB4, one of the most potent chemotactic agents known to date, and by LTC4 synthases to glutathione conjugate LTC4 [7]. The 5-LOX pathway is supposed to promote cardiovascular diseases through increased leukocyte chemotaxis, vascular inflammation, and enhanced permeability [8]. Zileuton (Zyflo), the only marketed drug as a 5-LOX inhibitor within the past 25 years, is used clinically in long-term maintenance of asthma control. However, there is much debate about its clinical efficacy [9]. p-12-LOX can catalyze the conversion of arachidonic acid into 12(S)-HpETE [10]. The expression of p-12-LOX is observed in several tumor tissues, including gastric cancer, prostate cancer, and melanoma [11–13]. p-12-LOX's metabolite (12-HETE) has been implicated in cardiovascular and renal diseases, many types of cancer, and inflammatory responses [14–16]. p-12-LOX may also be involved in maintenance of the epidermal water barrier by catalyzing arachidonic acid to 12-HETE and incorporation into acyl ceramides [2]. The main reaction product of 15-LOX-1 is 15(S)-HpETE. It is reported that the increased expression and activity of 15-LOX-1 in lung epithelial cells is a proinflammatory event in the pathogenesis of asthma and other inflammatory lung disorders [17]. A number of studies have demonstrated that 15-LOX-1 plays a critical role in colonic tumorigenesis [18–20].

During the process of searching for LOX inhibitors as therapeutic agents, various methods have been developed for determining the enzyme activity of LOXs, including (i) spectrophotometric assay, (ii) high-performance liquid chromatography (HPLC), and (iii) chemiluminescence [21,22]. But these methods are too laborious and time-consuming to quickly and effectively identify potential inhibitors of LOXs. A high-throughput fluorescence-based assay of LOXs using the fluorescent probe H_2DCFDA has been

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[1] *Abbreviations used:* LOX, lipoxygenase; 5-LOX, 5-lipoxygenase; p-12-LOX, platelet 12-lipoxygenase; 15-LOX-1, LT, leukotriene; 5(S)-HpETE, 5(S)-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid; LTA4, leukotriene A4; HPLC, high-performance liquid chromatography; FTC, ferric thiocyanate; NDGA, nordihydroguaiaretic acid; Ni-NTA, nickel–nitrilotriacetic acid; PCR, polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; GSH, glutathione; DMSO, dimethyl sulfoxide.

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developed [23]. However, it is not adaptive for purified enzymes because H_2DCFDA contains acetate groups that require esterase to cleave prior to oxidation.

In previous studies, the ferric thiocyanate (FTC) method has been used to detect the lipid hydroperoxides in biologic samples, and the assay system is widely employed to determine the antioxidant activity of natural products and synthetic compounds [24–26]. To our knowledge, there is no study that has used this assay system for the detection of enzyme-catalyzed lipid hydroperoxides. Here, we describe a new, rapid, and reliable 96-well format colorimetric assay for three human LOXs (5-LOX, p-12-LOX, and 15-LOX-1) based on the red FTC complex production and the subsequent determination of inhibition activities of several well-known LOX inhibitors using this assay, including zileuton, nordihydroguaiaretic acid (NDGA), fisetin, and quercetin. IC_{50} values of these compounds determined by the colorimetric assay are in good agreement with previously reported data obtained from other methods [27–30]. The new assay is adaptable to the 96-well microtiter plate format and may be employed as a preliminary screening for the discovery of novel and potent LOX inhibitors. To our knowledge, this is the first time the FTC complex has been used to detect LOX-catalyzed lipid hydroperoxides. Our work may expand the application field of the FTC complex in lipid research.

Materials and methods

Arachidonic acid, 5-HpETE, 12-HpETE, 15-HpETE, zileuton, and NDGA were bought from Cayman. ATP, L-α-phosphatidylcholine, quercetin, fisetin, NH_4SCN, and $FeSO_4$ were purchased from Sigma–Aldrich.

Stable 5-LOX expression and purification

The stable 5-LOX in vector pET14b was kindly provided by Marcia E. Newcomer (Department of Biological Sciences, Louisiana State University). The expression and purification were performed as described previously with minor modifications [31]. Briefly, Rosetta cells containing the stable 5-LOX plasmid were cultured in Terrific Broth containing 34 μg ml^{-1} chloramphenicol and 100 μg ml^{-1} ampicillin at 37 °C for 4 h and then shifted to 22 °C for an additional 24 h. Cells were harvested by centrifugation at 5000*g* for 30 min and stored at −80 °C. The cell pellets were resuspended in lysis buffer (50 mM Tris [pH 8.0], 500 mM NaCl, 20 mM imidazole, and 100 μM phenylmethanesulfonyl fluoride [PMSF]). The suspension was lysed by sonication (300 W; sonication cycle: 10 s on, 10 s off; total time: 30 min) and centrifuged at 11,000*g* for 30 min at 4 °C. The supernatant were loaded on an Ni-NTA (nickel–nitrilotriacetic acid) column preequilibrated with 50 mM Tris (pH 8.0), 500 mM NaCl, and 20 mM imidazole; washed with 50 mM Tris (pH 8.0), 500 mM NaCl, and 50 mM imidazole; and eluted with 50 mM Tris (pH 8.0), 500 mM NaCl, and 200 mM imidazole. The elution was concentrated using Millipore Amicon Ultra immediately for enzymatic assay. The purified enzyme is stable for at least 4 h on ice.

p-12-LOX and 15-LOX-1 expression and purification

The mammalian expression plasmids of pCDNA3–p-12-LOX and pCDNA3–15-LOX-1 were kindly provided by Colin D. Funk (Department of Biochemistry, Queen's University, Canada). The complementary DNA (cDNA) of p-12-LOX and 15-LOX-1 was amplified by polymerase chain reaction (PCR) using the following primers: 5′-CACTCACATATGATGGGCCGCTACCGC-3′ and 5′-GCCTGTCGACTCAGATGGTGACACTGTTC-3′; 5′-CTCTCGCATATGATGCCCTCCTACACGGT-3′ and 5′-GAATGTCGACTCAGATGGCCACACTGTTC-3′. The PCR products were digested with two restriction enzymes, *Nde*I and *Sal*I (New England Biolabs), and then inserted into pET28a. The generated plasmids pET28a–p-12-LOX and pET28a–15-LOX-1 were verified by sequence and then transformed into BL21(DE3). Cells were cultured in Luria–Bertani (LB) medium supplemented with 100 μg/ml kanamycin at 37 °C for approximately 4 h. Protein expression was induced at 20 °C when the cells had grown to an OD_{600} value of 0.6 by the addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 200 μM. After 20 h, Cells were harvested by centrifugation at 4000*g* for 30 min and stored at −80 °C.

The purification procedures of 12-LOX and 15-LOX were similar to that of stable 5-LOX and performed as described previously [32].

Cell culture and transfection

Human embryonic kidney cell line (HEK293T) was purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 U/ml) in a 37 °C incubator with 5% CO_2. The cells were plated and transfected with the mammalian expression vector (pCDNA3–5-LOX, pCDNA3–p-12-LOX, or pCDNA3–15-LOX-1) or control vector (pCDNA3) using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. After 24 h, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and collected by centrifugation (2500*g* for 5 min at 4 °C). The cell pellets were sonicated in 1 ml of ice-cold buffer (100 mM Tris–HCl [pH 7.5] containing protease inhibitors) and centrifuged at 11,000*g* for 10 min at 4 °C. The supernatant was collected for LOX enzymatic assay immediately. The concentrations of individual LOXs in cell lysates were not known. We assume that all LOXs have the same level of expression, and the concentrations used in FTC assay are total protein concentrations of cell lysates.

Spectrophotometric assay

Enzyme activity assays of recombinant LOXs were carried out at room temperature as described previously with minor modifications [33]. The conjugated diene (HpETEs and HETEs) formation catalyzed by LOX can be measured by absorbance at 234 nm using a Hitachi U2910 ultraviolet–visible (UV–Vis) spectrophotometer (assay buffer: 50 mM Tris–HCl [pH 7.5], 0.3 mM $CaCl_2$, 0.1 mM ethylenediaminetetraacetic acid [EDTA], 0.1 mM ATP, and 40 μM arachidonic acid for 5-LOX; 50 mM Tris–HCl [pH 7.5] and 40 μM arachidonic acid for p-12-LOX and 15-LOX-1). All reactions were performed in 1-cm pathlength quartz cuvettes with a total assay buffer volume of 500 μl, and absorbance at 234 nm was recorded continuously for 300 s.

FTC-based LOX activity assay

FTC methods were carried out as reported previously with some modifications [25]. LOX FTC colorimetric assays were performed in 96-well flat-bottom plates in a total assay volume of 200 μl. Recombinant human LOXs or HEK293T cell lysates in 95 μl of assay buffer (50 mM Tris–HCl, pH 7.5) were added to assay wells. The enzymatic reaction was initiated by adding 5 μl of arachidonic acid solution (2 mM stock solution) to a final concentration of 100 μM and was terminated after 6 min at room temperature by adding 100 μl of FTC reagents. FTC reagents were the equivalent volume mixture of reagent I and reagent II mixture (reagent I: 4.5 mM $FeSO_4$ in 0.2 M HCl; reagent II: 3% NH_4SCN methanolic solution). Thestock solution should be protected from oxygen and light and should be stored at 4 °C. The absorbance at 480 nm was monitored