Vol. 96, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1980 Pages 817-822

ROLE OF FERRIC IRON IN PLATELET LIPOXYGENASE ACTIVITY

J.E. Greenwald, M.S. Alexander, R.H. Fertel, C.A. Beach,

L.K. Wong‡ and J.R. Bianchine

Department of Pharmacology, The Ohio State University

College of Medicine, Columbus, Ohio 43210

Received August 14,1980

SUMMARY: Four iron chelating agents, EDTA, EGTA, ferron and orthophenanthrolene, were found to inhibit human platelet lipoxygenase activity in a dose-dependent manner. The inhibition produced by these chelators could be selectively reversed by the addition of ferric ion but not ferrous ion. The ID_{50} for lipoxygenase activity directly correlated with the avidity of these compounds for ferric ion. Thus, human platelet lipoxygenase requires ferric ion for activity.

INTRODUCTION

Human platelet lipoxygenase is an enzyme which catalyzes the conversion of arachidonic acid to 12-hydroperoxyeicosatetraenoic acid (12-HPETE). It previously has been reported that plant lipoxygenases have specific activity requirements for iron (1-4). It also has been reported that ferrous iron catalyzes the non-enzymatic oxidation of arachidonic acid (5). Our laboratory (6) as well as Aharony et al (7) have presented preliminary data suggesting that human platelet lipoxygenase is an iron-dependent enzyme. In order to more fully delineate the biochemical requirements for the mammalian lipoxygenase enzyme isolated from platelets, we have undertaken a study to examine the role of iron in this enzyme's activity.

METHODS

Enzyme Preparation and Lipoxygenase Activity Assay: Outdated platelet concentrates were obtained from The Ohio State University Hospital blood

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‡Department of Pharmaceutics, School of Pharmacy, University of Pittsburgh, Pittsburgh, Pennsylvania

0006-291X/80/180817-06$01.00/0

bank. Platelet concentrates were centrifuged at 1500 x g for 30 min at 4°C and the resulting platelet pellet was suspended in 50 mM phosphate buffer pH 7.0. The platelets were frozen and thawed three times to produce lysis, then centrifuged at 5,000 x g for 15 min at 4°C to settle the membrane debris. The 5,000 x g supernatant fraction was then centrifuged for 60 min at 100,000 x g. The resulting 100,000 x g supernatant fraction was subjected further to $(NH_4)_2SO_4$ fractionation; the resulting precipitate was reconstituted in phosphate buffer to 4 mg/ml protein and stored at -75°C until used. Protein determinations were done by the method of Lowry (8). To assay for lipoxygenase activity, the enzyme was diluted to 100 µg/ml of protein with 50 mM phosphate buffer at pH 7.0 and incubated with 3.81 µM (^{14}C)-arachidonic acid at 25°C for ten minutes. The reaction was stopped by the addition of 1.0N formic acid to achieve a final pH of 2.5-3.0. The amount of arachidonic acid remaining after 10 min was identified by radiochromatographic techniques on thin layer chromatography plates and quantitated by liquid scintillation counting as described previously (9). To control for non-enzymatic oxidation of arachidonic acid, the enzyme was inactivated by boiling for 15 min prior to the incubation. To examine the effects of iron chelators on lipoxygenase activity, EDTA, EGTA, 7-iodo-8-hydroxyquinoline-5-sulfonic acid (ferron)[1] (Sigma Chem. Co., St. Louis, MO.) and orthophenanthrolene (G.F. Smith Chem. Co., Columbus, OH.) were preincubated with the enzyme for 60 min at 4°C. Ferric and ferrous chloride were obtained from Mallinckrodt (St. Louis, MO.).

Determination of Chelator Stability Constants: Apparent stability constants (K) for the ferric ion chelates of EDTA, EGTA, ferron and orthophenanthrolene were determined spectrophotometrically by competition with thioglycolic acid (Matheson, Coleman and Bell Chem. Co., Norwood, OH.), a selective ferric ion chelating indicator. Thioglycolic acid forms a complex with ferric ion which absorbs light at 540 nm in proportion to the concentration of ferric ion present in the complex. A standard curve for absorbance versus ferric ion concentration was constructed, and absorbance was measured after addition of a known amount of EDTA, EGTA, ferron or orthophenanthrolene to an assay medium of 50 mM phosphate buffer containing 20 mM NH_4Cl and 20 mM thioglycolic acid at pH 7.0. The affinity of the chelators for ferric ion was then calculated from the absorbance changes (10).

RESULTS

Human platelet lipoxygenase is a soluble enzyme which has been shown by Nugteren (11) to precipitate mainly between 0 and 35% $(NH_4)_2SO_4$ saturation. Although a series of fractions were tested, the 35% $(NH_4)_2SO_4$ fraction which contains most of the activity, was used in all experiments. The specific activity of enzyme in this fraction was determined to be 2.67×10^{-9} moles arachidonic acid consumed.mg protein^{-1}.min^{-1}. When this fraction was assayed in the presence of 10^{-5}M indomethacin, an inhibitor of cyclooxygenase, no inhibition of activity was observed, indicating absence of a cyclooxygenase enzyme. However, incubation in the presence of 10^{-5}M eicosatetraynoic

1. Abbreviation: Ferron, 7-iodo-8-hydroxyquinoline-5-sulfonic acid

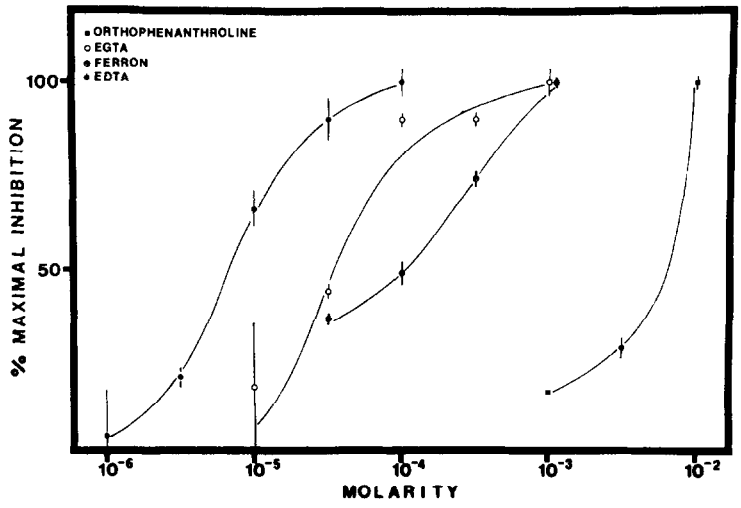

Figure 1. Inhibition of Platelet Lipoxygenase Activity by Four Iron Chelating Agents.
n = 6 for all values.

acid (ETYA), an inhibitor of lipoxygenase, produced an 85% inhibition of enzyme activity, indicating the presence of lipoxygenase activity in our preparation.

Figure 1 represents the ability of four iron chelating agents to inhibit lipoxygenase activity in a dose-dependent manner. The ID_{50} values for EDTA, EGTA, ferron and orthophenanthrolene are 7.61×10^{-6}M, 4.17×10^{-5}M, 1.13×10^{-4}M and 5.26×10^{-3}M respectively.

EDTA and EGTA do not possess a selective iron chelating ability but can chelate a number of di and trivalent cations. Therefore, the next experiment was designed to investigate whether iron was the chelated species responsible for loss of enzymatic activity. When 10^{-4}M EDTA was incubated with the enzyme, maximal inhibition was noted. When challenged with $FeCl_3$, a dose-dependent reversal of inhibition was observed (figure 2). In order to establish reversal of enzymatic inhibition with the other chelates, the experiment in figure 2 was repeated with EGTA and ferron. When 3.3×10^{-3}M $FeCl_3$ was added to the enzyme previously preincubated with the ID_{50} concentrations of EGTA and ferron, a 68% and 34% stimulation of lipoxygenase activity was detected. When similar concentrations of $FeCl_2$ were added to the enzyme, no reversal

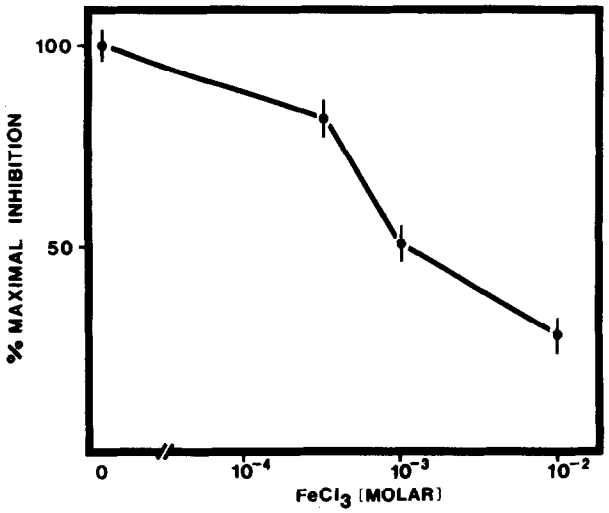

Figure 2. Reversal of Lipoxygenase Inhibition by $FeCl_3$. In all experiments, enzyme was first preincubated with 10^{-4}M EDTA for 60 min at 4°C. n = 6 for all values.

of inhibibition was detected with any of the chelating agents. We conclude from these experiments that the lipoxygenase enzyme is dependent on the ferric ion for activity.

A third set of experiments were designed to establish a correlation between the ability of these compounds to chelate ferric ion and their respective ID_{50} values. The avidity of a chelate for its ligand is dependent on the pH and the ionic composition of the medium. Therefore, we designed a spectrophotometric assay to measure the avidity of a chelate for ferric ion in the same medium and pH that enzyme assays were performed. Under these conditions, the stability constant (K) for EDTA, EGTA, ferron and orthophenanthrolene were 5.5×10^5M, 1.2×10^4M, 6.17×10^3M and 1.7×10^2M respectively. When the log of the apparent K values were plotted versus the log ID_{50} values for all four chelating agents a straight line with a correlation coefficient of 0.97 was generated (figure 3).

DISCUSSION

EDTA, EGTA, ferron and orthophenanthrolene inhibit lipoxygenase activity in a dose-dependent manner; this inhibition can be reversed by ferric but

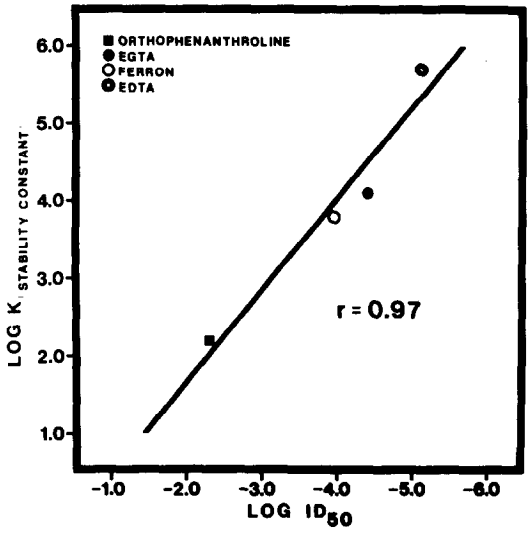

Figure 3. Correlation between Chelator Stability Constants and ID_{50} Potencies of Lipoxygenase Activity.

not ferrous ion. In addition, the ID_{50} for lipoxygenase activity directly correlates with the avidity of these compounds for ferric ion. Rao et al (5) have reported that ferrous ion is a necessary cofactor for arachidonic acid oxidation. However, these experiments were performed in an enzyme free system. In our experiments, when the lipoxygenase enzyme was inactivated by boiling, no autooxidation of arachidonic acid is noted in the presence of ferric or ferrous ion. In addition, since both iron and arachidonic acid are protein bound and their effective free concentration may not be significant, autooxidation is probably not a major factor in a protein system. From our data, we conclude that human platelet lipoxygenase enzyme requires ferric ion, not ferrous ion for activity. Currently, many investigators prevent assay interference from endogenously produced arachidonic acid by inhibiting the activity of phospholipase by chelating calcium with EDTA. Also, it is standard procedure to use EDTA while collecting whole blood by venipuncture to keep the platelets from clotting. It is clear from our data that a preparation of the lipoxygenase enzyme containing EDTA may be compromised and a true reflection of its activity will not be attainable. We

suggest that if a calcium chelator be used, that EGTA rather than EDTA be the chelator of choice. EDTA has a greater avidity for ferric ion than calcium, while EGTA has a greater avidity for calcium ions (12). Since we have found lipoxygenase in mammalian vasculature (9), and others have reported that 12-hydroperoxyeicosatetraenoic acid is an inhibitor of PGI_2 synthesis (13), it is possible that therapeutic agents which affect iron metabolism also may affect PGI_2 synthesis. Finally, 1-phenyl-3-pyrazolidone (14) and a derivative of this compound BW755C (15) have been reported to inhibit platelet lipoxygenase enzyme activity. In our assay system, 1-phenyl-3-pyrazolidone chelates ferric ion (unpublished observations). Therefore, both of the above-mentioned compounds may inhibit platelet lipoxygenase by chelating ferric ion.

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