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Lipoxygenase activity in heart cells

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Abstract Arachidonic acid (AA) metabolism via the lipoxygenase (LOX) pathway in rat hearts and in cultured rat cardiomyocytes was investigated using 1-[^{14}C]AA. LOX activity was detected in the microsomal fraction, in the high speed supernatant prepared from rat hearts and in rat cardiomyocyte supernatant. LOX products from all fractions comigrated in thin layer chromatography as 12-hydroxyeicosatetraenoic acid (12-HETE) and 15-HETE. Enzyme linked immunosorbent assay for 12-HETE showed its formation by the microsomal fraction, the ammonium sulfate (AS) pellet, and by rat cardiomyocyte supernatant, while radioimmunoassay for 15-HETE showed its formation only by the AS pellet. The properties of LOX in each fraction are reported here.

Key words: Lipoxygenase; 12-HETE; 15-HETE; Cardiac muscle; Cardiomyocyte

1. Introduction

Lipoxygenase (LOX) catalyzes the first committed step in biosynthesis of leukotrienes, lipoxins, and many other physiologically active oxygenated fatty acids [1]. Lipoxygenases are dioxygenases which recognize the 1,4-pentadiene structure of polyunsaturated fatty acids and incorporate single molecules of oxygen at specific carbon atoms of substrate fatty acids to produce hydroperoxy acids containing conjugated dienes [2].

Three main mammalian lipoxygenases have been classified according to their oxygenation position in arachidonic acid (AA). 5-LOX was identified in porcine leukocytes, alimentary tract, lung, thymus, cultured rabbit aortic smooth muscle cells and other cells [3]. The products of 5-LOX have a strong biological activity, among which are chemotaxis and chemokinesis of leukocytes [4], the synthesis of leukotrienes, and the contraction of smooth muscle. Human and bovine platelets contain 12-LOX, which oxygenate carbon 12 (C-12) of AA. Products of 12-LOX were found in lungs, spleen, small intestine, mast cells, and other tissues [2]. A 15-LOX was discovered and purified during the study on endogenous respiratory inhibitors in rabbit reticulocytes, and was suggested as being involved in the degradation of reticulocyte mitochondria during red cell maturation [5]. 15-LOX was isolated from other tissues, such as porcine and human leukocytes and macrophages of mice.

Although there are reports in the literature about the physiology and role of 5-LOX and its products, there are only a few indications about the role of the 12-LOX and 15-LOX in mammalian tissues. Recently, Freire-Moar et al. reported the presence of 12-LOX mRNA in murine peritoneal macrophages, lung, spleen, heart, and liver [6]. Bailey et al. reported the activation of 15-LOX in the heart tissue of rabbits by hypercholesterolemia [7].

We describe here the presence of 12-LOX activity in rat heart muscle and in cultured rat cardiomyocytes.

2. Materials and methods

2.1. Preparation of enzyme from rat heart

The microsomal fraction and the cytosolic fraction were prepared as described by Shahin et al. [8]. Fresh hearts taken from Charles River female rats (200–300 g) were homogenized in 0.25 M sucrose and 0.01 M phosphate buffer (pH 7.2–7.4) containing 2.5 mM EGTA (buffer A), at 4°C (3 ml/g tissue), using a Virtis homogenizer. The microsomal and high speed supernatant (S-100) were prepared by differential centrifugation, as described by Shahin et al. [8]. The S-100 was fractionated by 0–55% saturation of ammonium sulfate (AS) at 4°C, and the pellet was collected by centrifugation at $12000 \times g$ for 10 min (AS pellet) [9]. The microsomal fraction was washed with buffer A, containing 0.05 mg/ml fatty acid free bovine serum albumin [10].

2.2. Preparation of cell cultures and preparation of enzyme from cultured rat cardiomyocytes

Cardiomyocytes of rats were prepared, as described by Brik and Shainberg [11]. Cultures were incubated in humidified 5% CO_2, 95% air, at 37°C. The cultures taken for this work were 1–8 days old.

The fraction used for the determination of enzyme activity was prepared as described by Shainberg et al. [12]. The supernatant obtained after centrifugation at $1000 \times g$ for 10 min was used for determination of LOX activity.

2.3. Enzyme assay

LOX activity was determined by the radiochemical method, using labeled AA substrate solution. The analysis of AA products was done by thin-layer chromatography (TLC) and known standards. The reaction mixture (final volume 1 ml) contained 0.1 μCi 1-[^{14}C]AA (50 mCi/mmol, Amersham, UK), enzymatic preparation (0.4–0.6 mg protein) and PBS. After incubation at 37°C for 30 min, the extraction and separation of the products were performed, as described by Grossman et al. [13]. The radioactivity was counted by a Kontron liquid scintillation spectrometer (Basel, Switzerland). The Rf values for AA, 12-HETE and 15-HETE were 1.0, 0.65 and 0.68, respectively.

2.4. Enzyme linked immunosorbent assay (ELISA) for determination of 12-HETE

In this ELISA kit (Advanced Magnetics, Cambridge, MA, USA), the activity and quantity of the metabolite 12-HETE were detected. The products were prepared as described above, using unlabeled AA and extraction with ethyl acetate. The ELISA was performed according to the manufacturer's instructions.

2.5. Radioimmunoassay (RIA) for determination of 15-HETE

In this radioactive RIA kit (Advanced Magnetics), the activity and quantity of the metabolite 15-HETE were detected. The RIA was performed according to the manufacturer's instructions.

3. Results

3.1. LOX activity in rat heart

The activity of LOX was found in the microsomal fraction and in the supernatant after precipitation with ammonium

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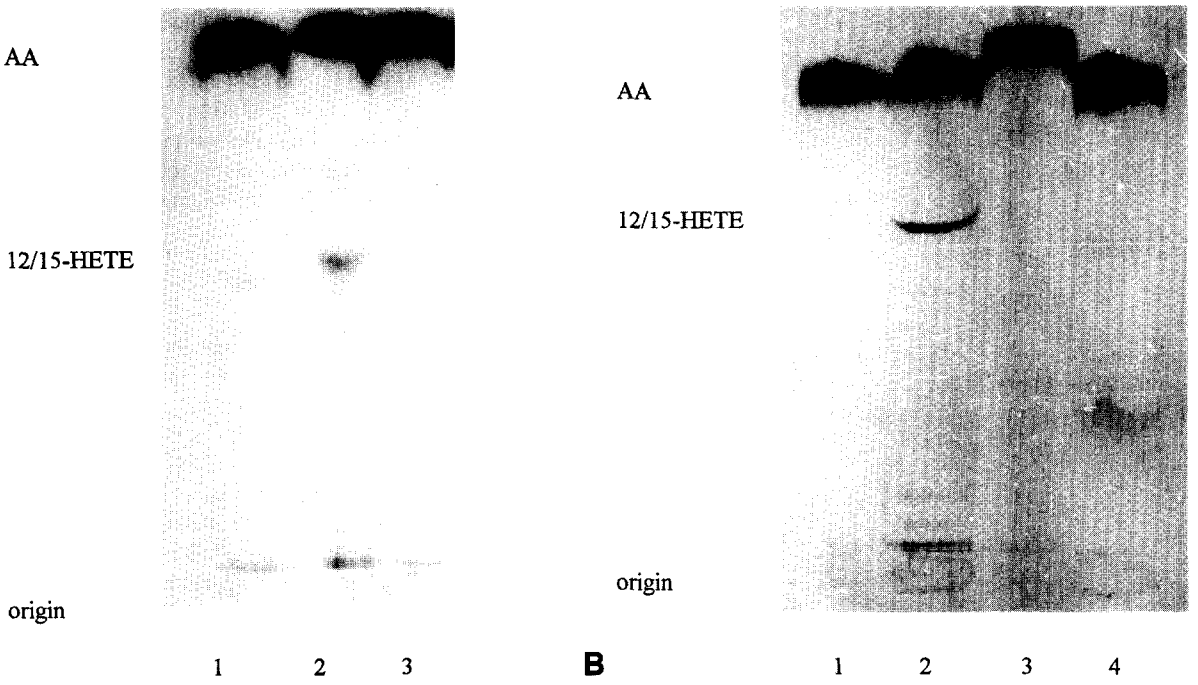

Fig. 1. Autoradiographs of LOX products in subcellular fractions of rat heart. A: Rat heart microsomes. Lane 1: blank, the enzyme was boiled for 30 min before adding AA; 2: LOX activity; 3: LOX activity in the presence of 100 μM NDGA. B: Rat heart S-100. Lanes 1–3 after ammonium sulfate precipitation. Lane 1: blank, the enzyme was boiled for 30 min before adding AA; 2: LOX activity; 3: LOX activity in the presence of 100 μM NDGA; 4: S-100 without ammonium sulfate precipitation.

sulfate (AS pellet). No activity was detected in the lysosomal-mitochondrial fraction. The autoradiograph of the LOX products after TLC revealed a major band at Rf 0.67, corresponding to 12-HETE or 15-HETE, which are usually characterized by an Rf of 0.65 or 0.68, respectively (Fig. 1A,B). LOX activity in the microsomal fraction and the AS pellet was inhibited over 90% by the addition of NDGA, a known LOX inhibitor. Only 10% of the activity was detected in assays containing heat denatured enzyme.

It should be pointed out that the LOX activity in the supernatant could be found only after ammonium sulfate precipitation. It is possible that before the precipitation, the HETE is decomposed by the hemoglobin or myoglobin present in this supernatant [14]. Another possible explanation is that the enzyme is inhibited by an inhibitor present in the supernatant.

The activity of LOX was determined in samples of up to 8 day old cultured rat cardiomyocytes, but was detected only in the 8 day old sample. The autoradiograph of the LOX products after TLC revealed a major band at an Rf of 0.67 (Fig. 2), which was identified as 12-HETE or 15-HETE, as mentioned above. The enzyme activity was 80% inhibited by NDGA.

3.2. *Quantitation of LOX activity by ELISA and RIA*

The data presented in the autoradiographs indicate the presence of 12-HETE or 15-HETE as products from LOX activity of the supernatant or microsomal fractions. To verify these findings, the formation of 12-HETE, and 15-HETE was determined by ELISA and RIA, respectively. As shown in Table 1, the main product in both fractions is 12-HETE, which represents close to 100% of the microsomes and AS pellet activities. The microsomal fraction produces only 12-HETE and this activity is 4 times less than in the AS pellet of S-100. The cultured rat cardiomyocytes produce only 12-HETE. The activities from all three sources were highly inhibited by the LOX inhibitor NDGA.

Table 1
Determination of 12-HETE and 15-HETE by ELISA and RIA after incubation of rat heart microsomes, rat heart AS pellets of S-100 and cultured rat cardiomyocytes with AA

Fraction	12-HETE		15-HETE	
	ng/mg protein	I (%)	ng/mg protein	I (%)
Microsomes	44.8 ± 9.1		nd	
Microsomes with 100 μM NDGA	3.9 ± 0.6	91.3	nd	
AS pellet of S-100	175.4 ± 5.5		0.610 ± 0.090	
AS pellet of S-100 with 100 μM NDGA	10.0 ± 2.3	94.3	0.020 ± 0.004	96.7
Cultured rat cardiomyocytes	30.4 ± 4.8		nd	
Cultured rat cardiomyocytes with 100 μM NDGA	9.3 ± 3.6	69.4	nd	

The quantity of products was determined as described in Section 2. I (%) represents % inhibition by NDGA.
nd, not detected.

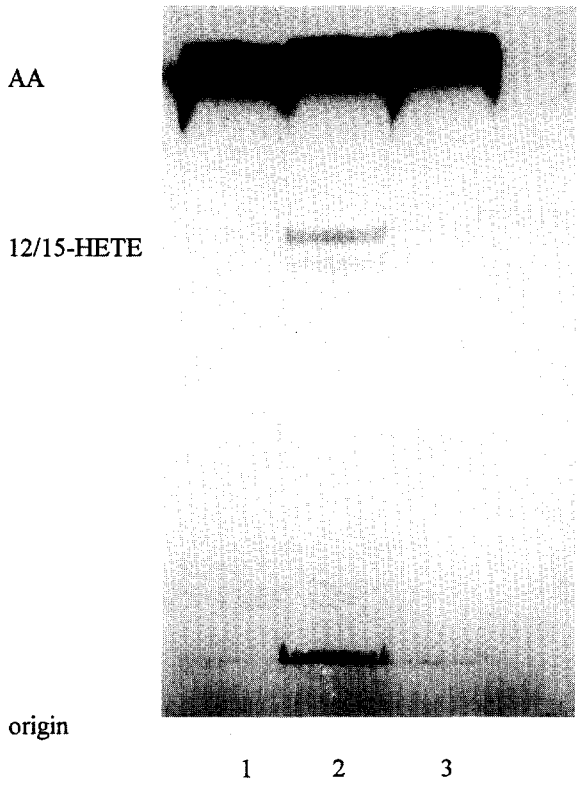

Fig. 2. Autoradiograph of LOX activity in cultured rat cardiomyocytes. Lane 1: blank; 2: LOX activity; 3: LOX activity in the presence of 100 μM NDGA.

3.3. Characterization of LOX activity in rat heart and in cultured rat cardiomyocytes

The dependence of LOX activity on AA concentration was measured at a range of 2–100 μM AA. The K_m values were calculated from the Lineweaver-Burk curves (Fig. 3) and were found to be 97 μM, 12.5 μM and 10.6 μM for the microsomes, and for AS pellets or cultured rat cardiomyocytes, respectively.

LOX activity was characterized at various pH values at a range of 6.0–8.0. An optimal pH of 6.8 was found for the microsomal activity, 7.0–7.2 for the AS pellet, and 7.3 for LOX from cultured rat cardiomyocytes.

In order to further characterize LOX activity, several known inhibitors of eicosanoid metabolism were investigated. The results are summarized in Table 2.

NDGA, a known specific inhibitor of LOX, shows above 90% inhibition in all preparations used. Indomethacin, an inhibitor of COX, shows low inhibition in AS pellets and in cultured rat heart cardiomyocytes (10% and 4% inhibition, respectively), with relatively high inhibition (55%) in the microsomes. Since LOX from various mammalian sources contains ferric ion (0.45 mol atom/mol enzyme) [15], we determined the effect of bipyridin, a chelator of ferric ion, on LOX activity. The data revealed relatively low inhibition by bipyridin in all fractions tested. Caffeic acid, an inhibitor of 5-LOX, also shows low inhibition. Esculetin, an inhibitor of 12-LOX [16], shows high inhibition in AS pellets and in cultured rat cardiomyocytes (85% and 90% inhibition, respectively) and relatively low inhibition (35%) in the microsomal fraction (Table 2).

4. Discussion

The activity of LOX was characterized for the first time in various fractions prepared from rat hearts and from cardiomyocyte tissue cultures. Analysis of the LOX products by TLC revealed one band with an Rf of 0.67, which is the average value of the Rfs for 12-HETE and 15-HETE (0.65 and 0.68, respectively). Identification of these two products was made by using specific immunoassays (see Table 1). These products were identified in the microsomal fraction and in the cytosol (AS pellet) of rat hearts and in cardiomyocytes in culture. No activity could be found in the lysosomal-mitochondrial fraction. In monocytes and neutrophils, the activity of 12-LOX is localized in the cytosol, and no activity was found in the microsomal fraction [17]. The microsomal fraction contains intracellular and plasma membranes. It is possible that the localization of 12-LOX and 15-LOX in the microsomal fraction of heart muscle cells is important for the function of the sarcoplasmic reticulum (SR) or plasma membrane. Since part of the LOX activity was found in the cytosol, it is possible that the enzyme can be translocated to the SR or to the plasma membrane under certain conditions, as was shown in polymorphonuclear cells [18]. It was shown that hepoxilin A_3, a metabolite of 12(S)-HPETE, can also stimulate the release of Ca^{2+} from ER by activating neutrophil phospholipase C to produce inositol triphosphate [19].

In the heart fractions, a very low activity of 15-LOX was found only in the AS pellet (Table 1). This may be due to the ability of 12-LOX to produce 15-HETE, as reported in the literature [16,20].

A comparison of LOX activity in all fractions checked showed a pH optimum of 6.8–7.3, which is similar to the optimum of 6.8–7.2 obtained for the enzyme in human leukocytes [21]. In human platelets, the pH optimum was in the range of 7.5–8.0 [22], and at the range of 7.0–8.0 in porcine epidermis [23].

The K_m value for AA found for LOX from human platelets was 10 μM [23], showing a similarity to the K_m values found here for the enzyme from AS pellet and cultured rat cardiomyocytes (12.5 μM and 10.6 μM, respectively), while the microsomal LOX showed a higher value of 97 μM. The similar K_m values found in AS pellet and in cultured rat cardiomyocytes raise the possibility of identity of LOX from the two sources. The high K_m value (low affinity) found in the micro-

Table 2
Effects of several inhibitors on rat heart microsomes, AS pellets, and cultured rat heart cardiomyocytes

Fraction	Indomethacin (50 μM)	NDGA (100 μM)	Bipyridine (500 μM)	Caffeic acid (100 μM)	Esculetin (100 μM)
Microsomes	55 ± 0.5	92 ± 0.7	50 ± 1.5	20 ± 0.8	35 ± 1.4
AS pellet	10 ± 0.5	90 ± 5.5	22 ± 1.7	55 ± 1.8	85 ± 1.5
Cultured rat cardiomyocytes	4 ± 0.2	95 ± 1.6	37 ± 1.1	45 ± 1.0	90 ± 2.3

LOX activity was determined by the radiochemical assay. The results are expressed as % inhibition (n=4).

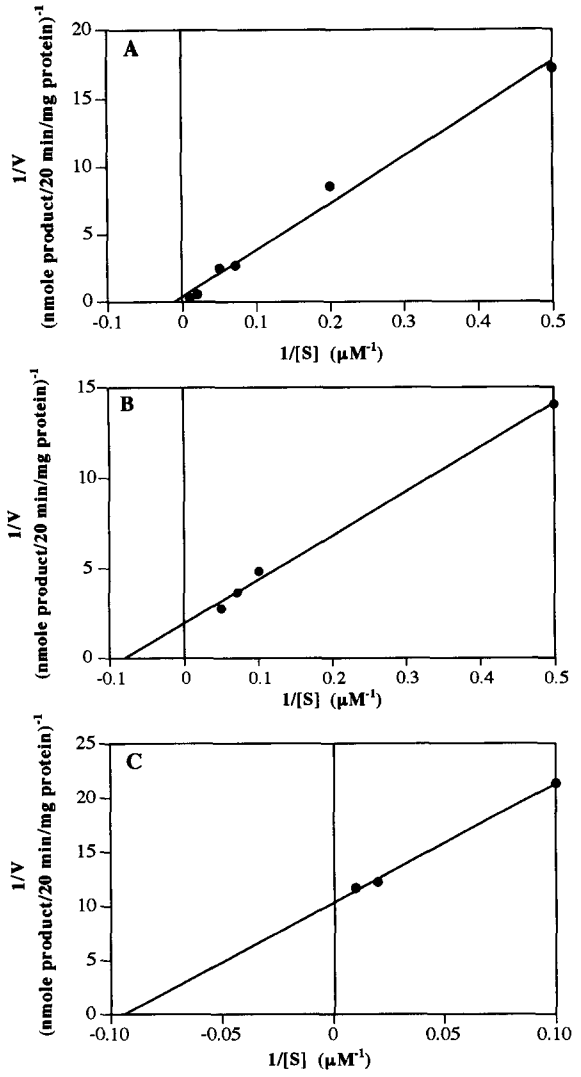

Fig. 3. Lineweaver-Burk curves of subcellular fractions of rat heart and cultured rat cardiomyocytes. LOX activity of rat heart microsomes and AS pellet and cultured rat cardiomyocytes was determined at various AA concentrations by the radiochemical assay. Lineweaver-Burk curves were plotted from the results ($n=4$). K_m values for microsomes, AS pellet, and cultured rat cardiomyocytes were found to be 97.0 μM, 12.5 μM, and 10.6 μM, respectively. A: Microsomes. B: AS pellet. C: Cultured rat cardiomyocytes.

somal fraction may be due to the fact that this fraction is not purified and contains non-specific binding sites for the AA substrate. It is also possible that the microsomal fraction contains COX activity and other enzymes which participate in the biosynthesis of phospholipids and triglycerides and use AA as a substrate.

The effect of different inhibitors on the LOX activity in all three fractions was studied (Table 2). Over 90% inhibition in all fractions was obtained with NDGA, which is the most accepted and specific inhibitor for LOX. This supports our conclusion concerning the existence of LOX activity in the heart. Additional support is derived from the findings that indomethacin, a specific inhibitor of COX, showed low inhibition in AS pellets and in cultured rat cardiomyocytes (10% and 4%, respectively), indicating the specificity of our results. The relatively high inhibition (55%) in the microsomal fraction is not clear, but there is a possibility that the high concentration (50 μM) inhibits microsomal LOX activity, as suggested elsewhere [24]. The inhibition of LOX activity by the ferric chelator biptridin suggests the involvement of ferric ion in LOX activity. These data support the conclusions concerning the presence of LOX activity in the tested preparations. Caffeic acid, which is a more specific inhibitor for 5-LOX, shows relatively low inhibition. As expected, esculetin, a 12-LOX inhibitor [16], inhibited the 12-LOX activity in AS pellets and in cultured rat heart cardiomyocytes (85% and 90%, respectively). The relatively low inhibition by esculetin of the activity in the microsomal fraction (35%, Table 2) is probably due to the fact that the enzyme is masked.

In conclusion, the data presented in this study indicate the presence of 12-LOX activity in rat heart microsomes and cytosol (AS pellet) and in cultured rat cardiomyocytes. There are several similarities among the 12-LOX activities found in the rat heart cytosol and in cultured rat cardiomyocytes. The enzymes from these two sources show similar Km values for AA, similar inhibition by NDGA, esculetin, caffeic acid, bipyridin, and indomethacin, and similar optimal pH. These data imply an identity between 12-LOX from rat heart cytosol and from cultured rat cardiomyocytes.

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