The Rat Leukocyte-type 12-Lipoxygenase Exhibits an Intrinsic Hepoxilin A₃ Synthase Activity*

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Hepoxilins are biologically relevant eicosanoids formed via the 12-lipoxygenase pathway of the arachidonic acid cascade. Although these eicosanoids exhibit a myriad of biological activities, their biosynthetic mechanism has not been investigated in detail. We examined the arachidonic acid metabolism of RINm5F rat islet cells and found that they constitutively express a leukocyte-type 12S-lipoxygenase. Moreover, we observed that RINm5F cells exhibit an active hepoxilin A₃ synthase that converts exogenous 12S-HpETE (12S-5Z,8Z,10E,14Z-12-hydro(pero)xy-eicosa-5,8,10,14-tetraenoic acid) or arachidonic acid predominantly to hepoxilin A₃, 12S-lipoxygenase and hepoxilin A₃ synthase activities were co-localized in the cytosol; immunoprecipitation with an anti-12S-lipoxygenase antibody co-precipitated the two catalytic activities. These data suggested that hepoxilin A₃ synthase activity may be considered an intrinsic catalytic property of the leukocyte-type 12S-lipoxygenase. To test this hypothesis we cloned the leukocyte-type 12S-LOX from RINm5F cells, expressed it in Pichia pastoris, and found that the recombinant enzyme exhibited both 12S-lipoxygenase and hepoxilin A₃ synthase activities. The recombinant human platelet-type 12S-lipoxygenase and the porcine leukocyte-type 12S-lipoxygenase also exhibited hepoxilin A₃ synthase activity. In contrast, the native rabbit reticulocyte-type 15S-lipoxygenase did not convert 12S-HpETE to hepoxilin isomers. These data suggest that the positional specificity of lipoxygenases may be crucial for this catalytic function. This hypothesis was confirmed by site-directed mutagenesis studies that altered the positional specificity of the rat leukocyte-type 12S- and the rabbit reticulocyte-type 15S-lipoxygenase. In summary, it may be concluded that naturally occurring 12S-lipoxygenases exhibit an intrinsic hepoxilin A₃ synthase activity that is minimal in lipoxygenase isoforms with different positional specificity.

Hepoxilins form a family of eicosanoids that are biosynthesized via the 12S-lipoxygenase (12S-LOX)¹ pathway of the arachidonic acid (AA) cascade. Chemically they constitute epoxy-hydroxy eicosanoids that can be classified as HxA₃ (8-hydroxy-11,12-epoxyicosatetraenoic acid) and HxB₃ (10-hydroxy-11,12-epoxyicosatrienoic acid) (1). Hepoxilins exhibit a myriad of biological activities. They stimulate glucose-induced secretion of insulin and increase the intracellular calcium levels in rat pancreatic islets cells (2). In human neutrophils a dose-dependent increase in intracellular calcium concentration (3, 4) and an augmented cellular diacylglycerol content (5) were observed. The latter effect was blocked by pertussis toxin, suggesting a receptor-mediated mechanism involving GTP-binding proteins. In neurons, HxA₃ induces hyperpolarization of the membrane potential and increases the amplitude and duration of the inhibitory postsynaptic potential (6, 7). In platelets and aplysia neurons, HxA₃ antagonized the expansion of cell volume and the opening of potassium channels (8, 9).

Although hepoxilin biosynthesis has been studied for a long time, some mechanistic details remain unclear. The first step of hepoxilin formation is conversion of AA to 12S-HpETE by a 12S-LOX (10). In mammalian cells this primary metabolite may be further metabolized via two alternative routes: (i) reduction to 12S-HETE (reductive pathway), and (ii) isomerization to hepoxilins (hepoxilin synthase pathway). When the reductive pathway, which involves glutathione peroxidase isoforms, is up-regulated hepoxilin biosynthesis is minimal because of substrate exhaustion (11). In contrast, cells with diminished reductive capacity are capable of biosynthesizing large amounts of hepoxilins provided that they express a hepoxilin synthase (11). A critical point in the mechanism of cellular hepoxilin formation has been the question of whether or not intracellular conversion of 12S-HpETE to hepoxilin isomers is an enzymatic process. Recent investigations on the stereospecificity of hepoxilin formation in rat pineal glands suggested an enzymatic pathway. This conclusion was based on the stereoselective conversion of 12S-HpETE. Under strictly comparable conditions, 12R-HPTE remained unmetabolized (10). Moreover, it became evident during the past several years that an active hepoxilin biosynthesis was always associated with a high expression level of the 12S-LOXs. This observation was usually explained by the fact that a 12S-LOX was required to convert AA to 12S-HpETE, the immediate substrate of hepoxilin biosynthesis (12). On the other hand, these data

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1 The abbreviations and trivial terms used are: LOX, lipoxygenase; GC-MS, gas chromatography-mass spectrometry; HxA₃, hepoxilin A₃, 8(S)-R-hydroxy-11,12-epoxy-icosatetraenoic acid; HxB₃, hepoxilin B₃, (10S-hydroxy-11,12-epoxy-icosatetraenoic acid); 12S-HpETE, 12S-5Z,8Z,10E,14Z-12-hydro(pero)xy-eicosa-5,8,10,14-tetraenoic acid; OPP, 4-(2-oxapentadeca-4-yne)phenylpropanoic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; TrxA₃, trioxilin A₃; PBS, phosphate-buffered saline; AA, arachidonic acid.

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prompted the possibility that 12S-LOX itself might be involved in the isomerization of 12S-HpETE to hepoxilins (11).

LOXs are multifunctional enzymes that exhibit a hydroperoxide oxidase (13–15), a leukotriene synthase (16, 17), and a lipoxin synthase activity (18–20) in addition to their oxygenase function. We recently observed that RINm5F rat insulinoma cells convert exogenous arachidonic acid or 12S-HpETE to hepoxilins, but not HxA3, as the major metabolite (11). More detailed mechanistic investigations revealed that these cells are devoid of glutathione peroxidase activities that reduce hydroperoxy fatty acids to the corresponding hydroxy compounds but express a leukocyte-type 12S-LOX at relatively high levels (11).

In the present study, we cloned a HxA3 synthase from RINm5F rat insulinoma cells and characterized the enzyme as leukocyte-type 12S-LOX. Alterations of the positional specificity of this enzyme by site-directed mutagenesis were paralleled by changes in the intrinsic hepoxilin synthase activity. An inverse mutagenesis strategy converting the rabbit 15S-LOX to a 12-lipoxygenating enzyme species induced significant HxA3 synthase activity. These data as well as HxA3 synthase activity assays of other LOX isoforms indicated that mammalian 12S-LOXs exhibit an intrinsic HxA3 synthase activity.

** MATERIALS AND METHODS **

**Cell Culture**—The permanent rat insulinoma cell line RINm5F (a kind gift from Dr. Tiedge, Hannover, Germany) was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The cells were grown at 37 °C in a humidified atmosphere containing 5% CO2.

Activity Assays—HxA3 synthase activity was assayed by incubating intact cells or enriched enzyme preparations with exogenous AA (100 μM) or 12S-HpETE (20 μM) for 30 min at 37 °C. The formation of hepoxilin isomers was quantified by gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC). The reaction was stopped by acidification to pH 3.0, the mixtures were allowed to incubate at room temperature under acidic conditions for 15 min (epoxide hydrolysis), and the lipids were twice extracted with three volumes of ethyl acetate. The solvent was evaporated, the lipids were reconstituted in 0.1 ml of methanol, and aliquots were directly injected in HPLC or, after making suitable derivatives, in GC-MS.

For measurements of the cellular HxA3 synthase activity, intact RINm5F cells (about 106 cells) were resuspended in 0.1 ml of PBS. 0.1 mM AA was then added, and the reaction was allowed to proceed at 37 °C for 30 min. The lipids were extracted by adding a mixture of diethyl ether/methanol/0.1 M citrate (155:15:1, v/v/v), and after phase separation the upper organic layer was recovered. The solvents were evaporated, the residues were reconstituted in 0.1 ml of ethyl acetate, and aliquots were converted to methylsilyl derivatives for GC-MS. For convenient detection in HPLC a fluorophore was introduced at the carboxylic group by the reaction with 100 μM of 9-anthryldiazomethane for one hour at room temperature (21). HPLC analysis was carried out on a NovaPak-C18 column (250 × 46 mm; 5-μm particle size) using acetonitrile/methanol/water (90:6:4; by vol) as a mobile phase. The absorbance was monitored at 235 nm. A calibration curve (6-point measurement) for conjugated dienes was established using 13-HODE as standard.

**Immunoblotting**—For electrophoresis the cells were reconstituted in lysis buffer (PBS, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and pepstatin and leupeptin, 1 μg/ml each), and the debris was removed by centrifugation at 12,000 rpm at 4 °C. Protein concentrations were determined using Lowry’s assay (Bio-Rad). Lysates were electrophoresed on 10% SDS-PAGE, and the protein bands were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) using a semidyblotting procedure. After blocking with 5% skimmed milk in PBS containing 0.05% Tween 20, the blots were probed with an antiporcine leukocyte-type 12-LOX rabbit antibody (kind gift from Prof. S. Yamamoto, Kyoto, Japan) for 1 h and stained using the ECL detection system (Amersham Biosciences). The antibody used cross-reacts with both the rabbit reticulocyte-type 15-LOX and the porcine leukocyte-type 12-LOX but does not recognize platelet-type 12-LOXs.

**Immunoprecipitation**—RINm5F cell lysates were subjected to immunoprecipitation using the antiporcine leukocyte-type 12-LOX antibody (20 μg/ml) and protein A-Sepharose (1 h of incubation). The total lysate, the resuspended immunoprecipitate, and the depleted precipitation supernatant were investigated with 20 μl 12S-HpETE. The identity of trioxilin A3 (TrxA3), the major hydrolysis product of HxA3, was determined by GC-MS. Similarly, LOX activity was determined by incubating the different immunoprecipitate fractions with 100 μM AA and analyzing the LOX products by HPLC as described above. 13S-Hydoxy-octadecenoic acid was used as an internal standard for quantification of both TrxA3 and 12S-HpETE.

**Chiral Phase HPLC Analysis**—Chiral phase HPLC of the fluorescence derivative (9-anthryldiazomethane ester) of HxA3 was performed on a Supelco LC(R)-DNB-PC chiral column (4.6 mm × 250 mm; Sigma) using the solvent system n-hexane/isopropanol (96:4, v/v) after preparative purification of the 9-anthryldiazomethane derivatives by RP-HPLC. Authentic standards of 8(13S,13R)-13-HpETE and 8(13S,13R)-HpETE (kind gifts from Dr. Cecil R. Pace-Asciak, Toronto, Canada) were used as reference compounds.

**Gas Chromatography-Mass Spectrometry**—Prior to GC-MS the free fatty acid derivatives were converted to their methyl esters by the addition of 300 μl of ethereal diazomethane. After 5 min of incubation the solvent was evaporated, 30 μl of N-methyl-N-(trimethylsilyl) trifluoroacetamide were added, and the hydroxy groups were silylated for 30 min at 60 °C in dry pyridine. GC-MS was performed on a Varian Saturn 4D GC-MS-MS system (Varian, Darmstadt, Germany) equipped with a Supelco DB5-MS column (30 μm × 0.25 mm; 0.25 μm). The temperature program was started at 150 °C and increased to a rate of 5 °C/min to reach a temperature of 250 °C for 20 min. The injector and transfer line temperatures were adjusted at 230 °C, respectively.

**Cloning of Hepoxilin A3 Synthase from RINm5F Cells**—According to our working hypothesis, the leukocyte-type 12S-LOX of RINm5F cells might exhibit HxA3 synthase activity. To test this hypothesis, we cloned the leukocyte-type 12S-LOX from these cells. For this purpose we first isolated total RNA from RINm5F cells using an RNAeasy kit from Qiagen (Hilden, Germany). Then 3 μg of the RNA preparation were reverse-transcribed at 42 °C for 60 min with 150 pmol of oligo(dT) primer and 15 units of AMV reverse transcriptase (Roche Applied Science). For amplification of the leukocyte-type 12S-LOX cDNA, 1 μl of the cDNA or transcription reaction was used and the following primers were added to the amplification mixture: 5′-cat atg tgg tga cag cgg c-3′ (forward primer), 5′-ttg ctc gac tga ggc cag cct gtt-3′ (reverse primer). After initial denaturation for 4 min at 94 °C, 30 cycles of PCR were performed. Each cycle consisted of a denaturing period (40 s at 94 °C), an annealing phase of 30 s at 60 °C, and a synthesis phase (90 s at 72 °C). After the last cycle, all samples were kept for an additional 10 min at 72 °C. PCR products were separated by 1% agarose gel electrophoresis, and the DNA bands were stained with ethidium bromide. The purified 12S-LOX PCR fragment was cloned into the NdeI and XhoI sites in PET 15b vector (Novagen, Bad Soden, Germany), and LOX-positive colonies were isolated. Their sequence insert was confirmed by automated fluorescent DNA sequencing.

**Expression of the Cloned LOX in P. pastoris GS115**—The 12S-LOX cDNA was cloned into the pPICZ, and the native stop codon prevented formation of a C-terminal His tag fusion protein. The plasmid was linearized and electroeptorated into competent P. pastoris GS115 cells.
LOX-positive colonies were selected using zoein as selection marker. The mutant phenotypes of the transformants were tested by incubating them on minimal medium containing histidine and methanol or dextrose. All positive clones turned out to be mutants. Some of them were randomly selected and then cultured in minimal medium containing glycerol, biotin, and histidine for 3 h at 37 °C with constant stirring to an optical A_{600} of 2. Cells were pelleted, resuspended in 1/10 of the initial culture volume (minimal medium containing histidine and 0.5% methanol), and further grown at 30 °C. After 24 h the cells were harvested and lysed using glass beads. The lysates were centrifuged, and the stroma-free lysate supernatant was used as enzyme source.

Expression of Rabbit 15S-LOX Mutants in Escherichia coli—The recombinant wild-type and mutant rabbit 15S-LOX and the various mutants were expressed in E. coli as His-tagged fusion proteins. For this purpose the 15S-LOX cDNA was cloned into the pQE-9 expression plasmid (Qiagen) between the SalI and Hind III restriction sites. Bac-

Site-directed Mutagenesis—Site-directed mutagenesis of leukocyte-
type 12S-LOX was carried out using the QuikChange mutagenesis kit (Stratagene, Heidelberg, Germany). The following amino acid changes were performed: L147Q, I1418A, N592S, V593I, and H366L. The mutated plasmids were transformed into E. coli, and five colonies were selected for each mutant for activity assays. The sequence of these colonies was confirmed by automated DNA sequencing.

RESULTS

Rat Insulinoma RINm5F Cells Express a Leukocyte-type 12S-LOX and Convert 12S-HpETE to HxA_3—When RINm5F cells were incubated with high concentrations of exogenous AA (100 μM), 12S-HETE was detected as a major oxygenation product (Fig. 1A). Western blot analysis of cytosolic extracts using a polyclonal antiporcine leukocyte-type 12S-LOX antibody indicated an immunoreactive band migrating with an apparent molecular mass of 75 kDa (Fig. 1B). Moreover, RT-PCR using a set of rat leukocyte-type 12S-LOX-specific primers revealed a fragment with the expected molecular mass (not shown). Preincubation of the cells with 10 μM of the leukocyte-type 12S-LOX inhibitor 4-[(2-oxapentadeca-4-ene)phenylpropanoic acid (OPP) (22, 23) abrogated formation of both 12S-Z,9Z,10E,14Z-12-hydro(peroxy)xy-eicosa-5,8,10,14-tetraenoic acid and HxA_3 (data not shown). To obtain more detailed information on the identity of the leukocyte-type 12S-LOX of RINm5F cells, we cloned its cDNA following a RT-PCR-based strategy. Sequencing the isolated cDNA clone, we observed 100% identity with the published sequence of the rat leukocyte-type 12S-LOX (GenBankTM accession number NM_031010). These data indicate that RINm5F cells express the rat leukocyte-type 12S-LOX.

Earlier we reported that RINm5F cells exhibit HxA_3 synthase activity (11); these results were confirmed in the present study. To characterize the enzymes involved in HxA_3 biosynthesis, intact RINm5F cells as well as cell lysates were incubated with exogenous 12S-HpETE and the lipid extracts were analyzed by HPLC for the presence of hepoxilin isomers. From Fig. 2 it can be seen that substantial amounts of HxA_3 were formed with intact cells (trace A) or cellular lysate (trace B). In contrast, we did not find any HxA_3 formation when a boiled cell lysate was used as enzyme source (trace C). Preincubation of the cells with the 12S-LOX inhibitor OPP strongly impaired HxA_3 formation; similar results were obtained with arachidonic acid as substrate (data not shown). From these results one may conclude that RINm5F cells are capable of oxygenating AA to 12S-HpETE and further convert this primary LOX product to hepoxilin A_3 via a heat-sensitive metabolic pathway that can be inhibited by a 12S-LOX inhibitor. Thus, 12S-LOX appears to be involved in the conversion of 12S-HpETE to HxA_3.

To obtain additional evidence for the enzymatic character of the hepoxilin A_3 synthase reaction, the enantioselectivity of HxA_3 biosynthesis was tested. For this purpose, lysed RINm5F cells were incubated with 12S-HpETE, the lipids were extracted, and HxA_3-9-anthryldiazomethane derivatives were prepared and purified by RP-HPLC (Fig. 2). Chiral phase HPLC for the separation of hepoxilin A_3 enantiomers was then...
Lipoxygenases are multifunctional enzymes that, in addition to their fatty acid oxygenase activity, exhibit a hydroperoxidase (14, 15), a leukotriene synthase (16, 17), and a lipoxin synthase activity (18, 19). To test whether the leukocyte-type 12S-LOX of RINm5F cells may be involved in HxA3 synthesis, we immunoprecipitated the enzyme from the cell lysis supernatant using an anti-12S-LOX antibody. Subsequently, the two catalytic activities (12S-LOX and HxA3 synthase) were assayed in the lysate supernatant, in the precipitate, and in the 12S-LOX-depleted precipitation supernatant. From Fig. 4 it can be seen that immunoprecipitation removed the majority of both 12S-LOX and HxA3 synthase activities from the lysate supernatant. Control precipitations carried out with a non-immune antiserum did not precipitate any catalytic activity (data not shown). These data suggest that the leukocyte-type 12S-LOX in RINm5F cells is responsible for their HxA3 synthase activity.

**Recombinant Rat Leukocyte-type 12S-LOX Exhibits HxA3 Synthase Activity**—Although the immunoprecipitation experiments suggest that HxA3 synthase activity may be an intrinsic catalytic property of the rat leukocyte-type 12S-LOX, our data...
do not prove this working hypothesis. To obtain more convincing evidence we expressed the rat leukocyte 12S-LOX in P. pastoris GS115 and tested the two catalytic activities for the recombinant enzyme. Lysates of yeast cells transformed with the 12S-LOX-containing plasmid converted arachidonic acid to 12S-HpETE (5.3 ± 1.5 µg of 12-HETE/ml of lysis supernatant) as indicated by RP-HPLC analysis (Table II). In contrast, wild-type yeast cells did not exhibit measurable LOX activity. Next, we checked the hepoxilin A₃ synthase activity of the cell lysate using 12S-HpETE as substrate. From Fig. 5 it can be seen that large amounts of HxA₃ (measured as the corresponding trioxin derivative) are formed when a crude enzyme preparation was incubated with 12S-HpETE (trace I). For the rabbit reticulocyte-type 15S-LOX and HxA₃ synthase activities were determined in the total cell lysate, in the immunoprecipitate, and in the precipitate-depleted supernatant as described under “Materials and Methods” (n = 3). 13S-Hydroxy-octadecenoic acid was used as an internal standard for quantification of both TrxA₃ (measured for HxA₃) and 12S-HETE. Values for 12S-HETE and TrxA₃, formation are indicated as mean ± S.D. Activity of the cell lysate was set at 100%. Significances between the different samples are indicated (*, p < 0.05, **, p < 0.01).

To obtain additional support for the hypothesis that hepoxilin A₃ synthase is an intrinsic catalytic property of mammalian 12S-LOXs, we performed site-directed mutagenesis studies to alter the positional specificity of the rat leukocyte-type 12S-LOX and the rabbit reticulocyte 15S-LOX. For the rabbit and human 15-LOX1, sequence determinants for the positional specificity have previously been identified, and site-directed mutagenesis of these residues shifted the positional specificity of the enzymes toward arachidonic acid 12-lipooxygenation (24–27). However, when a similar mutagenesis strategy was carried out with the rat leukocyte-type 12S-LOX, an A418I exchange did not alter positional specificity (28). Construction of chimeric LOX species and additional mutagenesis experiments suggested that other amino acids residues, in particular Leu-353, appear to be more important for the positional specificity of rat and mouse leukocyte-type 12S-LOXs (26). Based on these findings, we first tested the HxA₃ synthase activity of a 12-lipooxygenating mutant (I418A) of the rabbit reticulocyte-type 15S-LOX. In contrast to the wild-type enzyme, we detected significant HxA₃ formation with this mutant (Table II). To identify target amino acids for mutagenesis in the rat leukocyte-type 12S-LOX, we performed an amino acid alignment with the rabbit reticulocyte-type 15S-LOX (Fig. 6). From this alignment it can be seen that the sequence determinants identified for the rabbit enzyme (Phe-353, Gln-417, Ile-418, Ile-593) align with Leu-353, Lys-417, Ala-418, and Val-593 of the rat 12S-LOX. To stress the positional specificity of the rat leukocyte-type 12S-LOX, we constructed several enzyme mutants by interchanging small amino acids with residues carrying more space-filling side chains. The results of these experiments, shown in Table III, can be summarized as follows: (i) all mutants created were enzymatically active, and their specific activities were comparable; (ii) 12-lipooxygenating enzyme mutants exhibit a major HxA₃ synthase activity; and (iii) in contrast, the 15-lipooxygenating mutant (L353F) exhibited a reduced HxA₃ synthase activity. The residual HxA₃ synthase activity of this mutant may be because of its residual 12S-LOX activity. In summary, one may conclude that 12-lipooxygenating enzyme species exhibit a strong HxA₃ synthase activity that is impaired when the positional specificity of arachidonic acid oxygenation is altered in favor of 15-lipooxygenation. In this respect, there appears to be a correlation between AA 12-oxygenation and HxA₃ formation.

Kinetic Investigation on 12S-HpETE Conversion—Next we determined basic kinetic parameters for 12S-HpETE conversion by a HxA₃-synthesizing enzyme (I418A mutant of the rabbit 15S-LOX) and its wild-type counterpart, which is not capable of catalyzing HxA₃ formation. We found that the reaction kinetics followed Michaelis–Menten equation for both LOX isoforms (data not shown), suggesting an enzymatic character of the reaction. The kinetic constants obtained (Table IV) allow the following conclusions: (i) binding affinity of 12S-HpETE at the active site of both enzyme species was lower than that for arachidonic acid. These data are consistent with previous observations indicating that hydroxylated fatty acids exhibit an impaired binding affinity at the active site of LOXs (29); (ii) the reaction rate of arachidonic acid oxygenation was higher for both enzyme species when compared with 12S-HpETE conversion. Thus, the catalytic efficiency (Vₘₐₓ/Kₘₐₓ ratio) of LOX-catalyzed conversion of 12S-HpETE is lower than that of arachidonic acid oxygenation; and (iii) the wild-type rabbit 15S-LOX, which is not capable of synthesizing HxA₃ (Table III), exhibits a higher affinity for 12S-HpETE than the HxA₃-synthesizing I418A mutant. In contrast, Vₘₐₓ of 12S-

\[ \text{Max} \times \text{Km} \]
Hepoxilin Synthesis

Wild-type and mutant LOX isoforms were expressed in E. coli as described under “Materials and Methods.” Crude lysate supernatants were used as enzyme source for activity measurements. The LOX and HxA3 synthase activities are expressed as μg of HETE or HxA3 (determined as TrXA3) formation/ml of fermentation culture during a 15- or a 30-min incubation period, respectively. HETE formation was quantified by RP-HPLC after borohydride reduction and TrXA3 formation by GC-MS after making suitable methylsilyl derivatives. The activity data represent the mean ± error range of duplicate experiments. * Single activity determination.

**Table II.** LOX and HxA3 synthase activities of rabbit 15S- and rat 12S-LOX mutants

| Enzyme                  | 12-HETE/15-HETE ratio | LOX activity | HxA3 synthase activity |
|-------------------------|------------------------|--------------|------------------------|
| Rabbit 15S-LOX (wild-type) | 3.97                   | 5.9 ± 0.7    | <0.01                  |
| Rabbit 15S-LOX (I418A)   | 9.19                   | 5.8 ± 1.0    | 0.4 ± 0.1              |
| Rat 12S-LOX (wild-type)  | 88.12                  | 5.3 ± 1.8    | 0.88 ± 0.15            |
| Rat 12S-LOX (A418I)      | 87.13                  | 5.8 ± 0.8    | 0.58 ± 0.1             |
| Rat 12S-LOX (K417Q)      | 94.6                   | 6.2 ± 1.4    | 0.80 ± 0.2             |
| Rat 12-LOX (K417Q + A418I)* | 84.16                | 6.9          | 0.8                    |
| Rat 12S-LOX (L335F)      | 25.75                  | 5.1 ± 1.7    | 0.20 ± 0.05            |

Hepoxilin bioactivity, in particular the question of whether or not the secondary isomerization of 12S-HpETE to hepoxilins is catalyzed by enzymes or by non-enzymic catalysts, has been a matter of discussion for many years. The apparent stereospecificity of the reaction with the preferential use of 12S-HpETE (12R-HpETE is not a suitable substrate for HxA3 formation) as well as the heat sensitivity of HxA3 formation suggested an enzymatic process (10–12). However, the enzymes involved in hepoxilin biosynthesis in vivo have not been characterized so far. We recently presented experimental evidence suggesting that HxA3 formation in RINm5F cells from AA requires an oxidative environment and raised the question of whether second- ary conversion of 12S-HpETE to hepoxilins may proceed enzyme-controlled (11). However, the proof of this hypothesis was lacking. Here we have presented four lines of experimental evidence implicating the rat leukocyte-type 12S-LOX, which is expressed in RINm5F cells at relatively high level, in cellular HxA3 synthase activity starting from 12S-HpETE: (i) 12S-LOX and HxA3 synthase are localized in the same subcellular compartment (Table I); (ii) immunoprecipitation with an anti-12S-LOX antibody co-precipitated both the 12S-LOX and the HxA3 synthase activity (Fig. 4); (iii) the recombinant rat leukocyte-type 12S-LOX-cloned RINm5F cells exhibit an intrinsic HxA3 synthase activity that is abolished by a specific 12S-LOX inhibitor, OPP (Fig. 5); (iv) conversion of AA or 12S-HpETE by RINm5F cell lysates led to regio- (Fig. 5) and enantioselective synthesis of the 8(S)-HxA3 isomer (Fig. 3). These data also indicate for the first time that pure 12S-LOXs exhibit an intrinsic hepoxilin A3 synthase activity. Thus, in vivo hepoxilin formation may involve two consecutive LOX-catalyzed steps (primary step 12S-lipoxygenation of arachidonic acid to 12S-HpETE, followed by secondary step isomerization 12S-HpETE). The chemical mechanism of the secondary step has been discussed for quite a while, but here we provide rigorous experimental evidence to prove its enzymatic nature: (i) pure 12S-LOXs (native and recombinant species) are capable of converting 12S-HpETE to HxA3; (ii) heat denaturation abolished both their LOX and HxA3 synthase activities; (iii) the reaction is highly stereoselective with respect to substrate (12R-HETE is no substrate) and product (8R-epimer is not formed); (iv) the basic kinetic parameters (Michaelis-Menten kinetics) are consistent with an enzymatic reaction; (v) 12S-LOX inhibitors, such as OPP, abolish hepoxilin A3 formation from 12S-HpETE. As reported earlier, 5S-LOXs catalyze two consecutive steps during leukotriene biosynthesis (5-lipoxygenation of arachidonic acid to 5S-HpETE and secondary 5S-HpETE dehydration) (16), and multiple LOX reactions are involved in lipoxin formation (19). Here we report that 12S-LOXs can catalyze two consecutive reactions during hepoxilin biosynthesis. Abstracting these findings, one may conclude that LOXs are involved in different (primary and secondary) steps of eicosanoid biosynthesis. This conclu-
Wild-type LOX isoforms were expressed in *E. coli* as described, and crude lysis supernatants were used as enzyme source for activity measurements. LOX and HxA3 synthase activities are expressed in μg of HETE or μg of HxA3 (determined as TrxA3-isomers) formation/ml of fermentation culture during a 15-min incubation period. HETE formation was quantified by RP-HPLC after borohydride reduction and TrxA3 formation by GC-MS after making suitable methylsilyl derivatives. The activity data represent the mean of triplicate experiments. *, to adjust comparable arachidonic acid oxygenase activities, the bacterial lysis supernatant was diluted with PBS 1:10. n.a., not applicable.

![TABLE III](image)

**TABLE III**  
Kinetic constants of arachidonic acid oxygenation and 12S-HpETE conversion by the 12-lipoxygenating 1418A mutant of rabbit 15S-LOX

| Enzyme species | Substrate | Kinetic constants of arachidonic acid oxygenation | 12S-HpETE conversion by 12-lipoxygenating 1418A mutant of rabbit 15S-LOX |
|----------------|-----------|--------------------------------------------------|-------------------------------------------------------------------------|
| Human 5S-LOX   | Arachidonic acid | Km = 20.5, Vmax = 22.2 | 12-HETE/15-HETE ratio = 260 |
| Human platelet-type 12S-LOX | Arachidonic acid | Km = 99.1, Vmax = 2.2 | 12-HETE/15-HETE ratio = 1080 |
| Rabbit reticulocyte-type 15S-LOX | Arachidonic acid | Km = 3.97, Vmax = 0.4 | 12-HETE/15-HETE ratio = 4.6 |
| Rat leukocyte-type 12S-LOX | Arachidonic acid | Km = 98.2, Vmax = 0.4 | 12-HETE/15-HETE ratio = 103 |

**FIG. 6.** Amino acid alignment between the rabbit reticulocyte-type 15S- and the rat leukocyte-type 12S-LOX. The amino acids that align with the sequence determinants identified for the rabbit 15S-LOX are boxed. These residues were targeted by site-directed mutagenesis.

**TABLE IV**  
Kinetic constants of arachidonic acid oxygenation and 12S-HpETE conversion by the 12-lipoxygenating 1418A mutant of rabbit 15S-LOX

| Enzyme species | Substrate | Km (μM) | Vmax (s⁻¹) | Vmax/Km (μM s⁻¹) |
|----------------|-----------|---------|------------|------------------|
| Wild-type      | Arachidonic acid | 20.5    | 22.2       | 1080             |
| 12S-HpETE      | Arachidonic acid | 91.5    | 1.34       | 15               |
| 1418A          | Arachidonic acid | 26.0    | 6.67       | 260              |
| 12S-HpETE      | Arachidonic acid | 481.5   | 1.16       | 2                |

Clavage of the peroxy group forming radical intermediates (14, 30), and (ii) radical stabilization via the formation of various secondary products (epoxy-hydroxy isomers, ketodienes, short chain aldehydes, alkane, etc.). During LOX-catalyzed hydroperoxidase reactions, the first step appears to be enzyme-controlled (30), whereas the second step may proceed non-enzymatically. This second non-enzymatic share might be responsible for the complexity of secondary reaction products. During LOX-catalyzed HxA3 synthesis, both reaction steps may proceed enzyme-controlled; thus, a much simpler product pattern is expected. Indeed, we observed that exogenous 12S-HpETE is converted by the recombinant rat leukocyte-type 12S-LOX mainly to HxA3, suggesting tight enzymatic control. The mechanistic details for this remarkable specificity remain unclear, but obviously the enzyme prefers to direct the hydroxy group away from the epoxide ring to it at C8 of the hydrocarbon backbone in the S position. Taken together, one may conclude that the HxA3 synthase activity of certain LOX isoforms may be considered as intrinsic catalytical property of mammalian 12S-LOXs.

LOXs are multifunctional enzymes that in addition to their oxygenase activity may also exhibit hydroperoxidase (14, 15), leukotriene synthase (16, 17), and lipoxin synthase activities (18–20). Our findings that various LOX isoforms are capable of converting 12S-HpETE to HxA3 increase the functional multiplicity of this enzyme class. Hepoxilin A3 synthase from 12S-HpETE resembles the hydroperoxidase reaction (12, 14), which, unlike the oxygenase activity, does not require insertion of atmospheric oxygen. In principle, the hydroperoxidase reaction can be divided into two major steps: (i) homolytic cleavage of the peroxy group forming radical intermediates (14, 30), and (ii) radical stabilization via the formation of various secondary products (epoxy-hydroxy isomers, ketodienes, short chain aldehydes, alkane, etc.). During LOX-catalyzed hydroperoxidase reactions, the first step appears to be enzyme-controlled (30), whereas the second step may proceed non-enzymatically. This second non-enzymatic share might be responsible for the complexity of secondary reaction products. During LOX-catalyzed HxA3 synthesis, both reaction steps may proceed enzyme-controlled; thus, a much simpler product pattern is expected. Indeed, we observed that exogenous 12S-HpETE is converted by the recombinant rat leukocyte-type 12S-LOX mainly to HxA3, suggesting tight enzymatic control. The mechanistic details for this remarkable specificity remain unclear, but obviously the enzyme prefers to direct the hydroxy group away from the epoxide ring to it at C8 of the hydrocarbon backbone in the S position. Taken together, one may conclude that the HxA3 synthase activity of certain LOX isoforms may be considered as intrinsic catalytical property of mammalian 12S-LOXs.

HxA3 has not been detected in all cells and tissues that express either leukocyte-type or platelet-type 12S-LOXs. The mechanistic reason for this observation may be because different cells exhibit different peroxide-reducing capacities. In most mammalian cells peroxide-reducing enzymes, such as selenium-dependent and -independent glutathione peroxidases, are expressed at high levels. These enzymes reduce the cellular peroxides, including 12S-HpETE, and thus remove the substrates for hepoxilin biosynthesis (11). However, when the activity of the cellular glutathione peroxidases is down-regulated by lowering the intracellular glutathione concentration, e.g. by incubation of the cells with diethyl maleate, HxA3 formation may be enhanced (11).

Thus, our experimental data indicate that mammalian 12S-LOXs exhibit a hepoxilin A3 synthase activity in reconstituted *in vitro* systems as well as in intact mammalian cells. However,
the results do not rule out the possibility that other proteins exhibit a similar catalytic activity. In fact, it appears as if different peroxide-metabolizing enzymes or transition metal-containing proteins catalyze a similar reaction. Hence, cellular hepoxilin biosynthesis may be considered as the result of several enzymatic and/or pseudoenzymatic processes, and a variety of potential catalysts including 12S-LOXs might be involved. These considerations leave sufficient room for LOX-independent hepoxilin formation. More research is obviously needed to work out the mechanisms involved in hepoxilin biosynthesis in other cells and organs.

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Hepoxilin Synthesis
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