PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **188**, 370–374 (1988)

# Lipoxygenases in Rat Embryo Tissue (42749)

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*Abstract.* It has previously been reported that rat embryonic tissue produces various prostanoids. This report demonstrates that rat embryo homogenates synthesized various lipoxygenase metabolites, including 12-hydroxyeicosatetraenoic acid (12-HETE) as the major metabolite, 5-HETE, and 15-HETE. The cyclooxygenase product 11-HETE was also formed. Product identification was based on radioimmunoassay and comparison of reverse-phase– and straight-phase–high-pressure liquid chromatography retention times with authentic standards. Additional evidence was the observation that the lipoxygenase inhibitor nordihydroguaiaretic acid inhibited HETE formation. It appears that, under the same (though not necessarily optimal) experimental conditions, lipoxygenase metabolites predominate quantitatively over cyclooxygenase pathway products and that 11-day embryonic tissue produces more HETEs than either 12-day or 13-day embryo homogenates. 

Arachidonic acid is the precursor of a variety of biologically active compounds. Oxygenation of this polyunsaturated fatty acid can occur via two pathways. Prostaglandins and thromboxanes are formed via the cyclooxygenase enzyme system whereas hydroxyeicosatetraenoic acids (HETEs), leukotrienes, and lipoxins are produced through the action of various lipoxygenases (1). Lipoxygenase enzymes constitute a family of closely related enzymes that are widely distributed and catalyze the insertion of oxygen at different carbon atoms of arachidonic acid. The role of arachidonic metabolites in both normal and abnormal development is an unexplored area of biology. Both direct and indirect evidence suggest that arachidonic acid metabolites play a role in embryogenesis. Klein and co-workers (2) previously demonstrated that embryonic tissue produces prostaglandins. Since there is very little or no information in the literature as to the capacity of embryonic tissue to synthesize arachidonic acid metabolites via lipoxygenase pathways, the objective of this study was to determine whether lipoxygenase enzymes are present in rat embryonic tissue and can metabolize arachidonic acid to different HETEs.

**Materials and Methods.** Young adult female rats of a Wistar-derived strain (Royalhart) were caged overnight with males of the same strain. The next morning the females were checked for the presence of sperm by vaginal lavage. If sperm were found, 9:00 AM was considered time 0 of gestation. Embryos were removed at 3:00 PM on Day 11, 12, or 13, dissected free from their investing membranes in 0.9% saline, immediately frozen in liquid nitrogen, and stored at −80°C until assayed. The 11-, 12-, and 13-day postimplantation embryos were used primarily because this is the major period of organogenesis and, in addition, we have previously reported on certain teratological aspects with these gestational age embryos (2).

[$^{14}$C]arachidonic acid and reagents for RIA of 6-ketoPGF$_{1\alpha}$ were purchased from DuPont NEN Research Products (Boston, MA) and RIA kits for 5-HETE, 12-HETE, and 15-HETE were obtained from Advanced Magnetics, Inc. (Cambridge, MA). Standard curves for each eicosanoid were generated following the manufacturer's instructions and all experimental eicosanoid quantitations were extrapolated from the appropriate standard curves using sample concentrations that were within the range of the standard curve. Data were normalized and expressed as ng eicosanoid/mg protein. Calcium ionophore A23187 was obtained from Calbiochem-Behring Diagnostics (San Diego, CA) and nordihydroguaiaretic acid was obtained from Sigma Chemical Co. (St. Louis, MO).



0037-9727/88 $1.50

Organic HPLC solvents and HPLC grade water were purchased from Fisher Scientific Co.

Frozen rat embryos were placed in a graduated tube and embryo pellets equivalent to a volume of 5 ml were allowed to thaw to 0°C and homogenized in a Dounce-Ball tissue homogenizer (six to eight strokes). The homogenate was kept at 0°C and the protein concentration was measured and adjusted to 15 mg/ml with Dulbecco's phosphate-buffered saline (pH 7.0). Rat homogenate (1 ml) was incubated at 37°C with 1 $\mu$l of ethanol (the vehicle for the drugs tested below). After 2 min, [$^{14}$C]arachidonic acid (55 $\mu$Ci/$\mu$mole, 33 $\mu M$ final concentration) was added. After 15 min, the incubation was stopped by the addition of 3 ml of methanol. Tissue was removed by centrifugation and the supernatant was extracted with chloroform (6 ml). The chloroform extract was evaporated under $N_2$ and analyzed by HPLC, whereas the unlabeled extract was analyzed by RIA. In some experiments, the embryonic homogenate was pretreated with certain drugs or the calcium ionophore A23187 for 2 min at 37°C. Extraction efficiencies of eicosanoid metabolites were determined in separate samples without radioactive substrate but using radioactive tracers. It was determined that the extraction efficiency of [$^{3}$H]$PGE_2$, a typical cycloxygenase product, was 59 ± 7% ($n$ = 5) and of [$^{14}$C]15-HETE, a typical lipoxygenase product, was 56 ± 6% ($n$ = 4).

Reverse-phase–high-pressure liquid chromatography (RP–HPLC) analyses were carried out on a Perkin-Elmer Series 4 liquid chromatograph equipped with a LC-85B variable wavelength detector using a Zorbax ODS column (4.6 × 250 mm, DuPont). The mobile phase (flow rate, 1 ml/min) for the first 23 min consisted of 36% acetonitrile, pH 3.7, while the effluent was monitored for prostanoids at 194 nm. Following a 5-min gradient, the solvent composition was changed to 50% acetonitrile and the effluent was monitored for diHETEs at 280 nm. After 25 min, another 5-min gradient changed the mobile phase to acetonitrile/methanol/water (50:15:35), pH 3.7, with uv detection for HETEs monitored at 235 nm. This solvent composition was maintained for 25 min. Finally, less polar metabolites as well as unreacted arachidonic acid were eluted with pure methanol. Fractions (0.3 ml) were collected and aliquots were assayed for radioactivity by liquid scintillation counting. Each sample to be analyzed contained authentic HETE standards to correlate HPLC retention times of the standards with those of the radioactive products formed in the incubation mixtures. Straight-phase–high-pressure liquid chromatography (SP–HPLC) analyses were performed on a Hewlett-Packard 1084A instrument equipped with a constant wavelength detector (254 nm) and fitted with a Lichrosorb-100 column (1.0 × 25 cm, Alltech Associates, Inc.). The solvent system used was 90% hexane/acetic acid (999:1, Solvent A) and 10% hexane/isopropyl alcohol/acetic acid (899:100:1, Solvent B) at a flow rate of 4 ml/min. After 20 min, a linear solvent gradient was established to reach the composition of 10% Solvent A and 90% Solvent B by 35 min after injection.

**Results.** It is now generally recognized that high-pressure liquid chromatography is a very suitable method to analyze a variety of arachidonic acid metabolites formed by different lipoxygenase enzymes (3). A RP–HPLC chromatogram of lipids extracted from Day 11 gestational age rat embryonic tissue homogenate incubated with exogenous [$^{14}$C]arachidonic acid is shown in Fig. 1A. The major peaks chromatographed with the same retention times as authentic 5-HETE, 12-HETE, and 15-HETE. A minor peak of 11-HETE was also observed. Additional confirmation for the structural identities of these lipoxygenase metabolites was obtained by analyzing the extract on SP–HPLC. Figure 1B indicates a similar product profile as indicated by cochromatography of the radioactive peaks with authentic standards. Usually about 0.05–0.1% of exogenous [$^{14}$C]arachidonic acid was converted to lipoxygenase products. Supporting evidence that these metabolites were indeed lipoxygenase-catalyzed products was obtained using the lipoxygenase inhibitor NDGA (Table I). When 13-day embryonic homogenate was preincubated with 100 $\mu M$ NDGA followed by the addition of [$^{14}$C]arachidonic acid, the conversion to [$^{14}$C]12-HETE and [$^{14}$C]15-HETE was inhibited ($P < 0.05$) by 68 and 49%, respectively. The calcium iono-

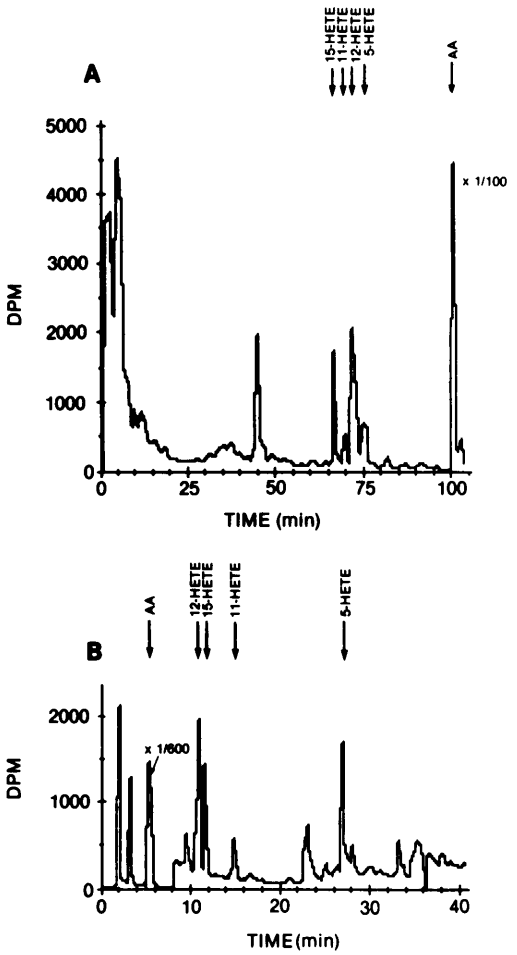


FIG. 1. HPLC chromatograms of radiolabeled arachidonic acid metabolites formed after incubation of [$^{14}$C]arachidonic acid with rat embryonic homogenate. Rat embryonic homogenate (11-day, 15 mg/ml protein) was incubated with [$^{14}$C]arachidonic acid (33 $\mu M$) for 15 min at 37°C. After extraction of products, half the extract was analyzed by RP-HPLC (A) and the other half by SP-HPLC (B) as described under Materials and Methods. Fractions (20 sec) were collected and the radioactivity was measured. The retention times of authentic compounds are designated by arrows.

phore A23187 also had a statistically significant inhibitory effect on [$^{14}$C]12-HETE formation.

To determine whether or not gestational age influences either the qualitative or quantitative product profile, embryonic tissue taken on Days 11, 12, and 13 was incubated with [$^{14}$C]arachidonic acid and the lipoxygenase products formed were analyzed using HPLC. The results in Table II show that qualitatively, the product profile was very similar. The quantities of [$^{14}$C]5-HETE, [$^{14}$C]11-HETE, and [$^{14}$C]15-HETE produced from Day 11 embryonic tissue were statistically different from the quantities of these metabolites formed from Day 12 and Day 13 tissues. Furthermore, under the experimental conditions used, the 11-day embryonic homogenate produced about 40–70% more metabolites than either the 12- ($P < 0.05$) or 13-day tissue.

We have also investigated whether embryonic homogenates synthesize and release HETEs from endogenous arachidonic acid. The data shown in Table III indicate that embryonic homogenates produce several arachidonic acid metabolites as measured by RIA. The predominant products were formed via the lipoxygenase pathways with the cyclooxygenase product 6-ketoPGF$_{1\alpha}$, a minor product. The major lipoxygenase metabolite was 12-HETE (as was observed with exogenous arachidonic acid, see Tables I and II) whereas 5-HETE and 15-HETE were produced in smaller amounts. Calcium ionophore did not significantly affect arachidonic acid metabolism from endogenous stores.

**Discussion.** Incubation of Day 11 rat embryonic tissue with exogenously added [$^{14}$C]arachidonic acid revealed that several lipoxygenase products were formed. The major products were 12-HETE, 15-HETE, and 5-HETE with 11-HETE a minor metabolite. A similar pattern was observed with Day 12 homogenates whereas 5-HETE was the minor metabolite in Day 13 embryonic tissue. These metabolites were identified on the basis of identical retention times with authentic standards on both SP- and RP-HPLC. Production of these metabolites was partially inhibited by the lipoxygenase inhibitor NDGA (100 $\mu M$) which lends additional support for these assignments. Since aspirin (2.5 m$M$) inhibited 15-HETE formation by 23% and 11-HETE production by 60% (results not shown), it appears that the major source of 15-HETE was the lipoxygenase pathway whereas the cyclooxygenase pathway contributed most to the formation of 11-HETE (4). Although the calcium iono-

TABLE I. INFLUENCE OF THE CALCIUM IONOPHORE $A_{23187}$ AND THE LIPOXYGENASE INHIBITOR NDGA ON THE [$^{14}$C]ARACHIDONIC ACID METABOLITE DISTRIBUTION PRODUCED BY 13-DAY RAT EMBRYONIC HOMOGENATES[a]

| Agent added | [$^{14}$C]5-HETE | [$^{14}$C]11-HETE | [$^{14}$C]12-HETE | [$^{14}$C]15-HETE |
|---|---|---|---|---|
| | | dpm/10 mg protein | | |
| — | 280 ± 57 | 417 ± 61 | 807 ± 99 | 735 ± 71 |
| $A_{23187}$ (4.5 $\mu M$) | 159 ± 33 | 266 ± 79 | 478 ± 38* | 586 ± 116 |
| NDGA (100 $\mu M$) | 177 ± 28 | 128 ± 42* | 262 ± 47* | 377 ± 65* |

[a] Rat embryonic homogenate was incubated at 37°C with ethanol (the vehicle), A23187, or NDGA. After 2 min, [$^{14}$C]arachidonic acid (33 $\mu M$) was added. After 15 min, the reaction was stopped by the addition of methanol and the products were extracted and analyzed by HPLC as described under Materials and Methods. The results are expressed as means ± SEM ($n$ = 5). Inactivated homogenate did not metabolize any arachidonic acid.

* $P < 0.05$, compared to controls.

phore A23187 has been reported to stimulate arachidonic acid metabolism via the cyclooxygenase and 12-lipoxygenase pathways in rat mast cells and the 5-lipoxygenase in human neutrophils (5, 6), it is somewhat surprising that A23187 had no appreciable effect on the formation of endogenous eicosanoid formation. One possible explanation is that homogenization of rat embryos may have maximally stimulated the embryonic lipoxygenases. On the other hand, in the presence of exogenous [$^{14}$C]arachidonic acid, A23187 was found to significantly inhibit the rat embryonic 12-lipoxygenase. It is possible that A23187 induced large concentrations of calcium in the vicinity of this lipoxygenase which inhibited this enzyme. In support of this role for calcium is the report that certain plant lipoxygenase isoenzymes were inhibited by calcium (7).

RIA measurements indicate that rat embryonic homogenates produce appreciable amounts of lipoxygenase products from endogenous arachidonic acid stores. Apparently, homogenization caused deacylation of cellular lipids to release arachidonic acid followed by *de novo* metabolism to a variety of products. It is interesting to note that endogenous lipoxygenase metabolite production was about 20- to 70-fold greater than product formation from exogenously added [$^{14}$C]arachidonic acid. Since the cyclooxygenase and lipoxygenase products are readily formed as a result of mechanical trauma, including homogenization (8), and the cyclooxygenase and lipoxygenase enzymes become inactivated during their catalytic action (9), it appears likely that when [$^{14}$C]arachidonic acid is added to the embryonic homogenate, these enzymes are near the end of their catalytic effectiveness and have lost most of their capacity to metabolize arachidonic acid.

Considering that 5-HETE, 12-HETE, and 15-HETE have been reported to affect cellular migration, secretion of cellular constituents, and fatty acid oxygenases and phospholipases (10), and given the major modulatory role various eicosanoids play in adult

TABLE II. DISTRIBUTION OF HYDROXYEICOSATETRAENOIC ACIDS (HETEs) FORMED FROM [$^{14}$C]ARACHIDONIC ACID AND RAT EMBRYONIC HOMOGENATES[a]

| Day of gestation | [$^{14}$C]5-HETE | [$^{14}$C]11-HETE | [$^{14}$C]12-HETE | [$^{14}$C]15-HETE |
|---|---|---|---|---|
| | | dpm/10 mg protein | | |
| 11 | 927 ± 31 | 238 ± 52 | 1059 ± 461 | 1046 ± 173 |
| 12 | 376 ± 137* | 139 ± 40* | 796 ± 247 | 508 ± 51* |
| 13 | 280 ± 57* | 417 ± 61* | 807 ± 99 | 735 ± 71* |

[a] The protocol followed was identical to that described in the legend of Table I. Results are expressed as means ± SEM ($n$ = 4–5).

* $P < 0.05$, compared to the formation of the particular HETE in the Day 11 sample.

TABLE III. ENDOGENOUS ARACHIDONIC ACID METABOLISM BY RAT EMBRYONIC HOMOGENATES[a]

| Agent | i-5-HETE | i-12-HETE | i-15-HETE | i-6-ketoPGF$_{1\alpha}$ |
|---|---|---|---|---|
| | | ng/mg protein | | |
| — | 5.4 ± 1.5 | 8.6 ± 2.1 | 4.0 ± 1.6 | 0.69 ± 0.30* |
| A23187 (3.6 $\mu M$) | 5.4 ± 1.4 | 9.7 ± 1.6 | 4.9 ± 2.6 | N.D. |

[a] Rat embryos (13 day) were homogenized and the homogenate (15 mg/ml) was incubated at 37°C for 15 min. The products were extracted and analyzed by RIA as described under Materials and Methods. Values of eicosanoid formation are expressed as the means ± SEM ($n$ = 4–6).

* The amount of the major cyclooxygenase metabolite of 6-ketoPGF$_{1\alpha}$ formed was significantly ($P < 0.05$) less than the total quantity of lipoxygenase metabolites produced.

** There were no statistically significant differences observed between the control and A23187-treated samples.

mammalian physiology, it is conceivable that these metabolites may be involved with one or more of these functions during embryogenesis. Another interesting aspect of this study is the observation that 11-day embryonic tissue appears to have a larger capacity to metabolize [$^{14}$C]arachidonic acid than tissues from 12- or 13-day embryos. This suggests that arachidonic acid metabolites may play a role during different stages of development. Furthermore this suggests that arachidonic acid metabolism may be critical during organogenesis and that interference with eicosanoid metabolism via various drugs may be teratogenic, as is the case with aspirin (2).

In summary, this study is the first to report that embryonic tissue metabolizes arachidonic acid to lipoxygenase products. Second, it was found that the extent of lipoxygenase metabolism is different at different stages of embryogenesis. It would be interesting to determine if these arachidonic acid metabolites play a regulatory role during organogenesis and teratogenesis.

We thank Ms. Mary T. Karmin, Ms. Lysle Wilhelmi, and Mrs. Elisabeth Resnick for excellent technical assistance. This work was supported by Grant HD19651 from the National Institutes of Health.

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Received October 19, 1987. P.S.E.B.M. 1988, Vol. 188.
Accepted April 5, 1988.