A Novel Cyclooxygenase Metabolite of Arachidonic Acid*

(AReceived for publication, March 19, 1984)

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The 8000 × g pellet of rabbit placenta transformed arachidonic acid into a number of lipoxygenase and cyclooxygenase products of known structure. A metabolite was also produced which was inhibited by indomethacin and required calcium for its formation. This compound had a UV absorption maximum at 227 nm under acidic or neutral conditions and gave a bathochromic shift to 281 nm under alkaline conditions. Reduction of this metabolite with sodium borohydride increased the molecular weight by four mass units, in-dicating the presence of two double bonds. Based on the mass spectrum of the derivatized metabolite, the structure proved to be PGE2, PGF2α, or PGD2. The compound is suppressed by NAD or NADP. PGH2 and PGG2 are not converted to similar products generated from the rabbit placenta. We performed a subcellular fractionation of placental homogenates, sequen-tially centrifuging at 1,000 g, 8,000 × g, and 100,000 × g. The 100,000 × g pellet (presumably microsomal enriched) was anticipated to contain the majority of the cyclooxygenase activity which would be inhibited by indomethacin and not affected by calcium. Similarly, we expected to find much of the lipoxygenase activity in the 100,000 × g supernatant, anticipating that some lipoxygenases (e.g. in platelets) are independent of the presence of calcium and others (e.g. 5-lipoxygenase) are calcium-dependent (8). The application of these criteria led us to the surprising finding that the 8,000 × g pellet possessed considerable arachidionate metabolic activity that was enhanced by calcium addition and blocked by indomethacin. The preparation, isolation, and identification of an unusual metabolite produced by the 8,090 × g pellet is the subject of this investigation.

EXPERIMENTAL PROCEDURES

Methods—22-31-day pregnant New Zealand White rabbits were anesthetized with pentobarbital (30 mg/kg). The uterus was excised and placed in cold (4°C) 50 mM Tris, pH 7.2, buffer containing 2 mM EGTA. The uterine horns were opened and the placenta was dissected away from the uterus and fetal membranes (amnion and chorion). Isolated placentas were then weighed and placed in 3-4 volumes of fresh buffer (w/v) and homogenized with a tissueizer (Tekmar Co., Cincinnati, OH). The homogenate was centrifuged at 1000 × g for 10 min, and the supernatant was then centrifuged for 20 min at 8000 × g. The 8000 × g pellet was resuspended and recentrifuged at 8000 × g for 20 min. The pellet was suspended in an equal volume (w/v) of buffer equivalent to the original wet weight of placenta (protein concentration 7-12 mg/ml). Incubations were performed at 37°C for 10 min and contained enzyme suspension, cofactors, and [1-14C] arachidonic acid (17 μM) in a total volume of 150 μl. The reaction was terminated with 4 volumes of MeOH. Following centrifugation, the pellet was extracted with 2 volumes of MeOH. The MeOH fraction was then evaporated to dryness under N2 and subjected to silica gel thin-layer chromatography. In large incubations, 1 ml of 8000 × g pellet suspension was used in multiple incubations. At the end of incubations, the reactions were stopped with methanol pooled and...
acidified to pH 3.5 with 1 N HCl, followed by the addition of 4 volumes of diethyl ether and 2 volumes of distilled H₂O. The aqueous layer was discarded, and the ether phase was washed with 1 volume of H₂O.

The ether extract was evaporated to dryness after addition of absolute ethanol. Autoradiograms were obtained with Kodak X-Omat film.

The etheral extract was evaporated to dryness after addition of PGH₂ and separated by silicic acid chromatography. Incubations of PGX and PGH₂ were carried out with and without addition of Ca²⁺.

Chromatography—Thin-layer chromatography was carried out on silica plates (20 x 20 cm) obtained from E. Merck. The mobile phase was benzene-diethyl ether:ethanol:acetic acid (50:40:2:1). The plates were then dried in air, and autoradiograms were obtained with Kodak X-Omat film.

HPLC was carried out using a Waters system consisting of two 6000 A pumps, controller, UV variable wavelength detector, and U6K injector. A flow-through HPLC radioactive detector (Radiomatic Instruments, Tampa, FL) with splitter was coupled in series to the UV detector and a fraction (5-10%) counted for radioactivity, and the remainder was diverted to a fraction collector.

UV Spectrometry and Derivatization—UV absorption spectrometry was carried out in methanol on a Beckman DU-8 scanning spectrophotometer. Methyl esters were obtained from excess ethereal diazomethane. Methoximes were prepared by incubation in 3% methoxamine hydrochloride in pyridine overnight at room temperature. Trimethylsilyl derivatives were prepared from N₂O-bis(triethylsilyl)trifluoroacetamide or N₂O-bis(trimethylsilyl)acetamide in pyridine by heating at 40 °C for 15 min. Reduction was carried out in 150 µl of ethanol using palladium on charcoal as a catalyst (100 µg) and bubbled with H₂ for 1 min. To this reaction, 500 µl of H₂O were added and extracted twice with ethyl acetate.

Gas Chromatography—Mass Spectrometry—C values were obtained on a column 3 feet long with an inner diameter of 2 mm packed with 5% OV-1 in a Hewlett-Packard 5380 gas chromatograph run isothermally at 220 °C. Mass spectrometry was carried out on a Hewlett-Packard 5895B equipped with a Grob type injector at 275 °C. The column used was a wide bore capillary column of cross-linked OV-1 programmed from 85 to 240 °C at 30 °C/min. Mass spectra were obtained under electron-impact conditions at 70 eV. Carrier gas was helium.

Materials—[1-¹⁴C]Arachidonic acid (55 mCi/mmol) was purchased from New England Nuclear; NADPH, NADP, NAD, sodium borohydrate, indomethacin, triphenylphosphine, and EGTA were from Sigma. Diazald was from Aldrich; methoxamine hydrochloride and N₂O-bis(trimethylsilyl)trifluoroacetamide were from Pierce Chemical Co.; and N₂O-bis(trimethylsilyl)acetamide (99.2 atom % deuterium) was from Merck (Canada).

RESULTS

[¹⁴C]Arachidonic acid incubated with rabbit placental 8,000 x g pellet suspension resulted in the production of classical prostaglandins, and nonpolar products with RF values on thin-layer chromatography of 0.75 and 0.68 (Fig. 1). Addition of Ca²⁺ (5 mM) produced a novel peak with an RF value of 0.26. This peak was inhibited by indomethacin. Thus, the mitochondrial fraction produced a compound "X" which required Ca²⁺ for its synthesis and was indomethacin-inhibitable. The concentration of indomethacin used was 5 µg/ml (14 µM) which was 100% effective in inhibiting cyclooxygenase. However, the IC₅₀ for inhibition of compound X was 0.43 µM, which was similar to the IC₅₀ for cyclooxygenase inhibition in addition, 300 µM acetyl salicylic acid which inhibition compound X formation by 70% also decreased PGE₂ production by 70%. The 100,000 x g supernatant fraction did not produce compound X but did possess a Ca²⁺-dependent, lipoxygenase pathway (Fig. 1, right).

Large-scale incubations were carried out to generate adequate material for separation by normal-phase HPLC (Fig. 2). The radioactive peak obtained at 76 min was compound X. Subsequent purification was facilitated by conversion to the methyl ester in excess ethereal diazomethane and rechromatography with 1 ml of hexane and rechromatography (Fig. 1).

FIG. 1. Autoradiogram of thin-layer chromatogram of placental arachidonate metabolites. The solvent system employed was benzene:ethanol:acetic acid (50:40:2:1). Incubations of the 8,000 x g pellet and 100,000 x g supernatant were carried out with and without 5 mM Ca²⁺ and with 5 mM Ca²⁺ plus 5 µg/ml indomethacin (indO). AA, arachidonic acid.

FIG. 2. HPLC tracing of arachidonate metabolites generated by placental 8000 x g pellet. The separation was carried out on a semipreparative Porasil column at 3 ml/min. The mobile phase was initially hexane:ethanol:acetic acid (984:6:1) for 25 min, followed by a linear gradient to 80% solvent, hexane:ethanol:acetic acid (90:10:1) over 60 min.
matography on a Resolve® straight-phase 5-μm particulate silica column (150 × 3.4 mm; Waters, Milford, MA) with an isocratic mobile phase of hexane:isopropyl alcohol:acetic acid (92:7:1). The ultraviolet spectrum of the methyl ester of compound X showed $\lambda_{\text{max}}^\text{MeOH} = 227$ nm (ε ≈ 9000 M$^{-1}$ cm$^{-1}$) (Fig. 3). The spectrum of the methyl ester under basic conditions (pH 9.0 for 1 h) showed a bathochromic shift to $\lambda_{\text{max}}^\text{MeOH} = 281$ nm.

Compound X was converted to the methoxime with methoxyamine HCl and $d_5$-methoxyamine, HCl and both compounds were converted to trimethylsilyl ether derivatives with N,O-bis(trimethylsilyl)trifluoroacetamide for gas chromatography-mass spectrometric analysis. Fig. 4, upper, shows the mass spectrum of compound X, methyl ester, methoxime trimethylsilyl ether, and Fig. 4, lower, shows the mass spectrum of compound X methyl ester deuterated methoxime trimethylsilyl ether. The methyl ester methoxime trimethylsilyl ester of compound X gave two isomers on gas chromatography: a minor isomer with a C value 24.2 and the major isomer with a C value of 24.6. The mass spectrum of the major isomer showed ions at $m/z$ 494 (M$^+$), 479 (M − 15), 463 (M − 31, loss of OMeH), 404 (M − 90, loss of trimethylsilanol), 373 (M − (90 + 31)), 321 (M − 173, loss of carbons 9, 10, and 11 with methoxime and OSiMe$_3$), and 180 (321-141), 321 minus C-8 side chain as base peak (Fig. 4, upper). The mass spectrum of the methyl ester deuterated methoxime trimethylsilyl ether showed ions at $m/z$ 500 (M$^+$), 485 (M − 15), 469 (M − 31), 466 (M − 34, loss of OCD$_3$), 410 (M − 90), 376 (M − (90 + 34)), 324 (M − (176 loss of carbons 9, 10, and 11, methoxime, and OSiMe$_3$)), 290 (324 − 34), and 183 (324-141, 183).
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loss of C-8 side chain as base peak (Fig. 4, lower)). The spectrum of methyl ester methoxime of compound X also showed ions at m/z 503 (M⁺), 495 (M – 18), 472 (M – 31), 404 (M – 99, loss of HOSi(CD₃)₃), 321 (loss of carbons 9, 10, and 11, methoxime, and OSi(CD₃)₃), 290 (321-31), and 180 (321-141, loss of C-8 side chain).

The increase in molecular weight by 6 mass units on making the deuterated methoxime confirmed the presence of two carbonyl functions in the molecule and suggested the structure of the absence of any hydroperoxy functions. On the other hand, reduction with NaBH₄ gave a mass spectrum identical to that of derivatized PGF₂α. The mass spectrum of the borohydride-reduced compound as the methyl ester Me₃Si ether is shown in Fig. 5. The major ions are m/z 584 (M⁺), 513 (M – 71), 494 (M – 90), and 404 (M – 90 × 2) and are consistent with the published spectrum of PGF₂α methyl ester Me₃Si ether (10).

The mass spectra were obtained of the methyl ester trimethylsilyl ether of compound X before and after hydrogenation with palladium on charcoal. The methyl ester Me₃Si ether of compound X showed ions at m/z 436 (M⁺), 421 (M – 15), 405 (M – 31), 365 (M – 71, loss of (CH₃)₂CH₂), 346 (M – 90, loss of trimethylsilyl), 315 (M – 90 × 31), and base peak at 206 (M – 199 + 31), loss of CH₂=CH·OSi(CH₃)₃CH₂CH₂CH₂CH₂OCH₃). The hydrogenated methyl ester Me₃Si ether of compound X showed ions at m/z 440 (M⁺), 425 (M – 15), 369 (M – 71), 350 (M – 90), 319 (M – 90 × 31), 311 (M – 129, loss of (CH₂)₃COCH₃), 208 (M – 201 + 31), loss of (CH₂)₂CH·OSi(CH₃)₃CH₂CH₂CH₂OCH₃). The hydrogenation increased the molecular ion by 4 mass units, which confirmed the presence of two double bonds in the molecule.

Thus, the structure of the unusual metabolite produced by the placenta is 9,15-dixo-11-hydroxyprosta-5,13-dienoic acid. Incubations of [H]PGF₂α and [H]PGF₂β with the 8000 × g pellet in the presence and absence of Ca²⁺ did not produce any metabolites with the same R₂ value as compound X on TLC (data not shown). Thus, we have no evidence that compound X in our in vitro system is a metabolite of PGF₂α or PGF₂β. Incubation of PGH₂ and PGG₂ with the 8000 × g pellet also did not produce compound X.

DISCUSSION

The novel metabolite of arachidonate acid produced by the placental 8000 × g pellet has some unique features. The synthesis of this compound is Ca²⁺-dependent and is indomethacin-inhibitable. The structure of this novel compound is established by: (a) the UV absorption at 227 nm under acidic conditions with a bathochromic shift to 281 nm under basic conditions, suggesting the dehydration of the cyclopentane ring to give a dieneone system; (b) the increase by 6 mass units when the methoxime was prepared with d₅-methoxyamine HCl versus undeuterated methoxyamine HCl, confirming the presence of two carbonyl functions in the molecule; (c) the reduction by sodium borohydride to PGF₂α, suggesting the presence of a cyclopentane ring; and (d) the mass spectrum, which indicates that the compound is 9,15-dixo-11-hydroxyprosta-5,13-dienoic acid.

The formation of 9,15-dixo-15-hydroxyprosta-5,13-dienoic acid is usually considered to be formed via a 15-PG dehydrogenase present in the soluble fractions of cells (11–14). Furthermore, GSH reportedly potentiates the activity of the 15-hydroxyprostaglandin dehydrogenase (15), whereas in our system, GSH reduced markedly the formation of compound X. Furthermore, in the 8,000 × g pellet, Ca²⁺ markedly increased the formation of this compound, while it has been previously reported (16, 17) that Ca²⁺ inhibits the 15-hydroxyprostaglandin dehydrogenase. Finally, in addition to PGF₂α, this novel compound is the major metabolite of arachidonic acid, but unlike PGF₂α, its synthesis by the 8,000 × g pellet particulate fraction of placenta requires the presence of Ca²⁺.

Similar incubations of placenta 100,000 × g pellet produce small amounts of the 9,15-dixo-11-hydroxyprosta-5,13-dienoic acid, but this is not stimulated by Ca²⁺ (data not shown). In the 8,000 × g pellet, the ratio of PGF₂α to compound X is 2 or 3 to 1. Since PGH₂ is not converted to this compound under the conditions from which it is formed by the 8,000 × g pellet, it is suggested that PGH₂ may be the source from which it is formed. The formation could thus be nonenzymatic by rearrangement of the hydroperoxy function at C-15 or through a specific enzyme which converts the hydroperoxy function to a carbonyl. Incubations of placenta 8,000 × g pellet and PGH₂ with and without Ca²⁺ did not produce compound X. This therefore suggests a novel enzymatic synthesis of this compound.

Initial attention should be directed at the role of the material in placental function. The placenta mediates physiologic exchange between the mother and the developing embryo-fetus; it transports gases and metabolites to and from the fetus and synthesizes a variety of steroid and peptide hormones (18, 19). In addition, placental metabolites may participate in the regulation of fetal growth, the protection of the fetus from the maternal immune system, and the events that occur at delivery (20, 21). This work provides a first example of a calcium-dependent product formed from cyclooxygenase (i.e. indomethacin-suppressible), thereby suggesting the existence of a novel enzyme apparently uniquely distributed in the mitochondrial fraction of the placenta.

REFERENCES

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Natl. Acad. Sci. U. S. A. 72, 2994-2998