Characterization of 11-HETE and 15-HETE, together with prostacyclin, as major products of the cyclooxygenase pathway in cultured rat aorta smooth muscle cells

J. Martyn Bailey, R. W. Bryant, J. Whiting, and Kalman Salata

Department of Biochemistry, The George Washington University School of Medicine, Washington, DC 20037

Abstract Arachidonic acid is the precursor of several potent derivatives that regulate physiological functions in the cardiovascular system. Thromboxane (TXA_2) and prostacyclin (PGI_2) are synthesized by the cyclooxygenase enzyme. The proaggregatory and vasoconstrictive TXA_2 produced by platelets is opposed in vivo by the antiaggregatory and vasodilating activity of PGI_2 synthesized by blood vessels. Arachidonic acid is also converted via a 5-lipoxygenase to leukotrienes, the vasoconstrictive components of SRSA. We have shown that this latter pathway is regulated by 15-HETE, a product of the 15-lipoxygenase present in lymphocytes. Confluent cultures of rat aorta smooth muscle cells (RSM) were superfused briefly with [^{14}C]arachidonic acid. The products were isolated and analyzed by thin-layer chromatography–radioautography, high performance liquid chromatography, and gas–liquid chromatography–mass spectrometry. Prostacyclin (PGI_2) was identified as the major product both by its biological properties in a platelet aggregation assay and by the mass spectrum of its tetra-trimethylsilylether-methyl ester derivative. Minor quantities of PGE_2, PGD_2, and $PGF_{2\alpha}$ were also synthesized. Three other compounds with chromatographic properties of mono-hydroxy eicosanoic acids were also formed in major amounts. These were shown to be cyclooxygenase products since their synthesis, together with that of prostacyclin, was blocked by the cyclooxygenase inhibitors aspirin (0.2 mM) and indomethacin (10 μM). Quantities of the hydroxy-eicosanoids were isolated from large scale incubations by silicic acid chromatography. Following methylation and reduction with platinum oxide/H_2, the compounds were converted to their trimethylsilylether derivatives and analyzed by gas–liquid chromatography–mass spectrometry. The compounds were identified as 11-hydroxy-5,8,12,14-eicosatetraenoic acid (11-HETE), 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE), and hydroxy-5,8,10-heptadeca-trienoic acid (HHT) by simultaneous ion monitoring of characteristic ions at M/e ratios of 287, 258, 229 for 11-HETE and 343, 314, 173 for 15-HETE, and by comparison with the mass spectra of authentic samples. Rat smooth muscle cells, prelabeled by 24-hour incubation with [^{14}C]arachidonic acid, released large amounts of prostacyclin together with enhanced amounts of 11- and 15-HETE in response to physiological levels of thrombin (0.5–5 units/ml). These experiments demonstrate that, in addition to the thromboxane antagonist prostacyclin, vascular smooth muscle cells produce significant quantities of the leukotriene inhibitor 15-HETE via the cyclooxygenase pathway in response to physiological stimuli such as thrombin. The release of both prostacyclin and 15-HETE by vascular smooth muscle cells may thus play an important role in vascular homeostasis.—**Bailey, J. M., R. W. Bryant, J. Whiting, and K. Salata.** Characterization of 11-HETE and 15-HETE, together with prostacyclin, as major products of the cyclooxygenase pathway in cultured rat aorta smooth muscle cells. *J. Lipid Res.* 1983. **24:** 1419–1428.

Supplementary key words leukotrienes • arachidonic acid • lipoxygenase

Arachidonic acid, released from cellular phospholipids by various stimuli, is the precursor of many physiologically active compounds including the prostaglandins PGE_2, PGD_2, $PGF_{2\alpha}$, prostacyclin PGI_2, and thromboxane TXA_2 (1–3). These are all formed, via the common endoperoxide precursor PGG_2, by a cyclooxygenase enzyme that is inhibited by common anti-inflammatory drugs such as aspirin and indomethacin (4). Thromboxane A_2, formed in platelets during aggregation and also in sensitized lungs and other tissues in response to antigenic stimuli, has been shown to be identical to the labile RCS, or rabbit aorta contracting substance released during anaphylaxis (5). Prostacyclin (PGI_2) formed in blood vessels (6) is a primary physiological antagonist of TXA_2 both as an inhibitor of platelet aggregation and as a potent vasodilator and vascular smooth muscle relaxant.

Abbreviations: HETE, hydroxy eicosatetraenoic acid; HHT, hydroxy-5,8,10-heptadeca-trienoic acid; PG, prostaglandin; TX, thromboxane; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; MS, mass spectrometry; RCS, rabbit aorta contracting substance; THETE, trihydroxy eicosatetraenoic acid; HEPA, hydroxy-epoxy eicosatetraenoic acid; PMN, polymorphonuclear cells; ASA, acetyl salicylic acid; RSM cells, rat smooth muscle cells; BFT, boron trifluoride reagent; TMS, trimethylsilyl; PRP, platelet-rich plasma.

In addition to these cyclooxygenase derivatives, arachidonic acid is also converted by several lipoxygenase enzymes to other compounds of physiological importance. A 5-lipoxygenase found in polymorphonuclear cells (PMNs) and macrophages converts arachidonic acid to a 5,6-epoxide intermediate, leukotriene A_4. This is converted via a specific hydrolase to the highly chemotactic agent leukotriene B_4, or by glutathionyl-S transferases to the sulfido peptide derivatives, leukotrienes C_4, D_4, and E_4 (7). These are the principal components of slow-reacting substance of anaphylaxis (SRSA), and are potent contractile agents for smooth muscle both in vivo and in vitro (8, 9). The 12-lipoxygenase pathway, which is particularly active in platelets, produces 12-hydroxyeicosatetraenoic acid (12-HETE) as the major product together with trihydroxyeicosatriaenoic acids (THETE's) and monohydroxy-epoxy eicosatriaenoic acids (α-HEPA's) (10) for which no significant physiological function has yet been defined. A 15-lipoxygenase found in PMNs, certain T-lymphocytes, and reticulocytes (11–13) synthesizes primarily 15-hydroxyeicosatetraenoic acid (15-HETE). 15-HETE has been shown to be a potent and selective (vis a vis the cyclooxygenase) inhibitor of other lipoxygenases including the 12-lipoxygenase pathway in platelets and the 5-lipoxygenase-leukotriene pathway in rat PMNs (11). In the PT-18 line of mast-basophil cells, 15-HETE at low levels activates a cryptic 5-lipoxygenase present in these cells (14). 15-HETE produced by circulating lymphocytes may therefore function as an important regulator for synthesis of vasoconstrictive leukotrienes.

We report here that a line of cultured rat aorta smooth muscle cells synthesizes both prostacyclin and significant quantities of 11- and 15-HETE via the cyclooxygenase pathway in response either to arachidonic acid or to physiological stimuli such as thrombin.

MATERIALS AND METHODS

Cell culture procedures

Confluent cultures of rat aorta smooth muscle cells were obtained from Dr. E. A. Brown, NIH, Bethesda, MD. These were isolated from the aortas of Wistar rats by sequential elution of the cannulated vessel with collagenase and trypsin according to the general procedure described by Gimbrone, Cotran, and Folkman (15). Cells were maintained in 25-sq-cm or 75-sq-cm flasks (Costar); for assay of prostacyclin synthesis following aspirin treatment, they were plated out in replicate cultures in 10-sq-cm or 25-sq-cm culture plates (Costar). Cells were grown in NCTC-135 (Flow Laboratories) medium buffered with HEPES (Fisher) (hereinafter referred to as HNCTC-135) and supplemented with 10% fetal bovine serum (M.A. Bioproducts). Antibiotics (Gibco) gentamycin (50 μg/ml), penicillin (50 units/ml), and streptomycin (50 μg/ml) were added to all cultures. Cells were harvested for subculturing using 0.25% trypsin (Gibco) in calcium-free (CMF) Hanks medium plus EDTA (0.54 mM) and incubated at 37°C for 2–4 min to release the cells. Cell populations were determined using a Coulter Counter and cell protein content was determined by a modification of the procedure of Lowry et al. (16). Serum-containing growth medium was then added and the resulting suspension was aliquoted into culture flasks or wells at a subculturing ratio of 1:4. Cultures became confluent within 3–4 days at which time they were used for assay of prostacyclin formation as described below.

Incubation of cells with [^{14}C]arachidonic acid and product extraction

Medium was removed from confluent cultures and the cells were washed twice with HNCTC-135 (pH 7.4) at 37°C using 2 × 1-ml portions for 25-sq-cm flasks and 2 × 0.5-ml portions for well cultures. [1-^{14}C]Arachidonic acid (0.75 μCi, 4 μg per ml) was added as follows: 1 ml per 25-sq-cm flask, 0.5 ml per 10-sq-cm well, and 0.25 ml per 2-sq-cm well. All cultures were incubated at 37° for 5 min. The medium was collected and added to tubes containing 5 μg each of 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_2, PGD_2, and 15-HETE as carriers. Samples were acidified to pH 3 with 1 N HCl and extracted three times with two volumes of ethyl acetate or once with six volumes of chloroform–methanol 2:1. Ethyl acetate layers were collected and backwashed with one volume of distilled water. When extracting media-containing serum, samples were extracted with six volumes of chloroform–methanol, and methanol-aqueous layer was extracted twice more with chloroform, and the organic layers were separated and stored at −20° until analyzed.

Thin-layer chromatography–radioautography procedures

Silica gel G TLC plates (Analtech) were used to separate prostaglandins and hydroxy fatty acids, using solvent system Iw (17), which consisted of the organic layer of a mixture of ethyl acetate–isooctane–water–acetic acid in the proportions 11:5:10:2. After sample application, plates were equilibrated in water vapor for 30 min before development. A separate lane of TLC standards was incubated with each plate and the compounds in this lane only were visualized by spraying with 10% phosphomolybdic acid in methanol followed by heating at 110°C for 5 min. Radioactive compounds were analyzed by three different procedures. The plates were

first scanned using a Vanguard radioactivity scanner (Model 930 Autoscanner, scanner gas 1.3% butane in helium) with an efficiency for ^{14}C of approximately 20%. The plates were then wrapped in plastic wrap (Union Carbide) and placed on 8 in × 10 in sheets of XAR X-OMat X-ray film (Kodak) in the dark for 5–7 days. The exposed film was developed for 5 min in Kodak X-ray developer at 25°C and fixed in Kodak Rapid Fix for 3 min. After separation by TLC and visualization by radioautography, the radioactive bands were tentatively identified by comparison with the authentic standards and scraped from the plates into 4 ml of Aquasol (New England Nuclear) for quantitative determination by liquid scintillation counting. All data were converted to dpm using a quench curve.

Platelet aggregation studies

Confluent cultures of rat smooth muscle cells (25 sq cm) were washed twice with HNCTC-135 medium and superfused with arachidonic acid (50 μg/ml) in 1 ml of HNCTC-135 for 5 min at 37°C. The medium from these experiments was treated in several ways before testing its effect on platelet aggregation. *1*) It was allowed to incubate at room temperature for 0, 1, 6, 11, 16, and 21 min; *2*) it was heated at 100°C for 30 sec; and *3*) the pH was briefly lowered to 3.0 and returned to pH 7.0. In other studies the cell monolayers were incubated for 30 min at 37°C with 1 ml of HNCTC-135 containing 0.2 mM aspirin and washed before the incubation with arachidonic acid was begun.

Rabbit platelet-rich plasma (PRP) was prepared from blood obtained from the central ear artery of 3-kg New Zealand White rabbits and anticoagulated with 3.8% sodium citrate (1:9) as previously described (18). Aliquots (0.4 ml) of PRP were incubated with 0.1-ml portions of the cell superfusates for 1 min. Aggregation was then initiated by the addition of arachidonic acid (50 μg in 2 μl of ethanol) in siliconized glass cuvettes with continuous stirring at 37°C. The progress of the aggregation process was followed using a Chronolog Aggregometer coupled to a chart recorder.

Thrombin-induced release of prostacyclin and hydroxyeicosanoids

Confluent cultures of RSM cells were prelabeled by incubation for 24 hr with 1 μCi (4 μg) of arachidonic acid per ml of medium in 10-sq-cm Costar well cultures. The medium was removed and the cells were washed twice with HNCTC-135. The cultures were then superfused with 1 ml of HNCTC-135 containing from 0.5 to 5 units of Thrombin for 15 min at 37°C. The radioactive products were extracted from the medium and analyzed as previously described.

Derivatization procedures and GLC–mass spectrometry

Ethyl acetate extracts of the radioactive arachidonate metabolites were dried under N_2 and the residue was incubated with ethereal diazomethane (10 μl) for 5 min to convert free carboxylic acid groups to the methyl esters. For analysis of 6-keto $PGF_{1\alpha}$ (the stable hydrolysis product of PGI_2), methylated samples were treated with hydroxylamine in pyridine (20 μl, 25 mg/ml) for 60 min at 70°C to convert keto groups to the oximes. The pyridine was evaporated under N_2 and trimethylsilylimidazole reagent (Pierce, 10 μl diluted 1:10 in benzene) was added to convert hydroxyl groups to the corresponding trimethylsilylethers. The resulting tetratrimethylsilyl-oxime-methyl ester derivative was immediately injected onto the column for GLC–MS analysis. For hydroxy fatty acids, the cell-derived materials were first purified on a column of 0.1 g of Biosil (Supelco). The sample dissolved in 0.2 ml of ethyl acetate containing 0.1 g of Biosil was diluted to 2 ml with hexane and applied to the column. The column was sequentially eluted with hexane–ethyl acetate 95:5, hexane–ethyl acetate 80:20, and finally ethyl acetate–methanol 90:10. The hexane–ethyl acetate 80:20 fraction containing the mono-HETE's was evaporated under N_2 and methylated with diazomethane. The methyl esters were then hydrogenated by dissolving them in methanol containing 2 mg of platinum oxide (PtO) and bubbling a gentle stream of H_2 for 5 min. The resulting reaction mixture was poured over a Biosil column (100 mg) to remove the catalyst. The samples were then treated with Sylon-BFT reagent (Pierce, 10 μl) to convert them to their monotrimethylsilylether derivatives.

GLC–MS studies were performed on a Hewlett-Packard model 5992A GLC–MS system using electron ionization with the voltage of the MS source set at 70 e.v. The injection port temperature was 240°C for all studies and the carrier gas (helium) flow was 20 ml/min. Prostaglandins were analyzed using a 3 ft × 2 mm silanized glass column packed with 3% SP-2250 (Supelco) on 100/120 mesh Supelcoport. The column was maintained isothermally at 210°C. Hydroxy fatty acids, including HHT, 11- and 15-HETE were analyzed on a 3 ft × 2 mm silanized glass column packed with 1% SE-30 on 100/120 mesh Gaschrom Q (Supelco). The temperature was maintained isothermally for 1 min at 190°C, then increased at 10°/min to 230°C and maintained for 4 min at this temperature.

RESULTS

Confluent monolayers of rat smooth muscle cells were superfused with arachidonic acid as described in

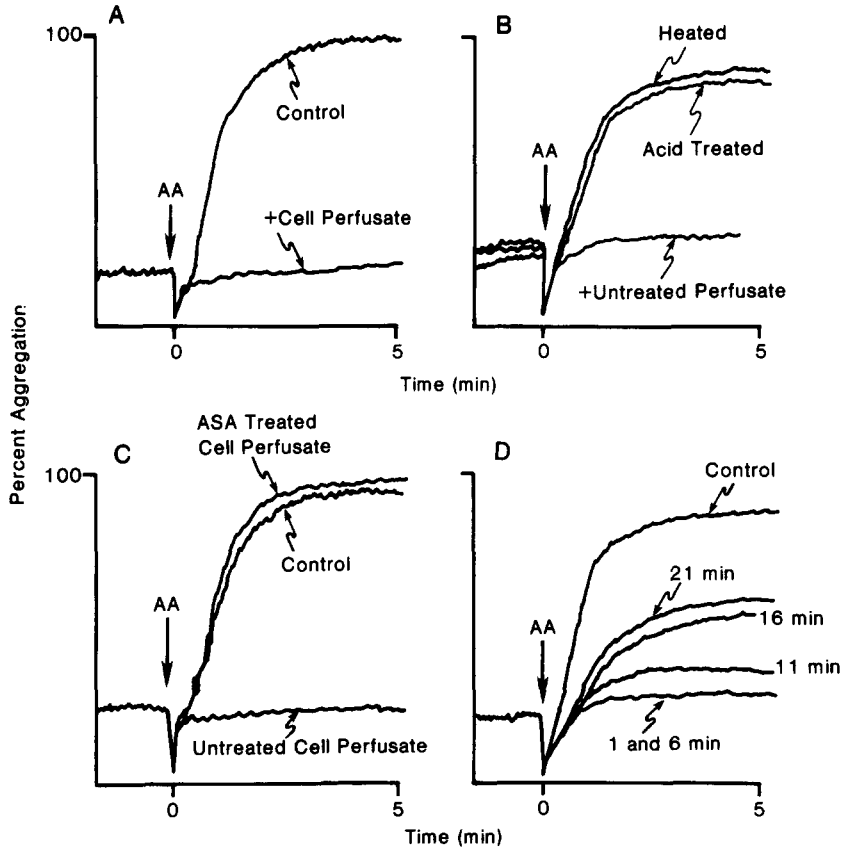

Fig. 1. Properties of platelet inhibitory substance produced by rat aortic smooth muscle cell monolayers following superfusion with arachidonic acid. Confluent 25-sq-cm cultures of rat aorta smooth muscle cells were incubated at 37°C for 5 min with 1 ml of HNCTC-135 medium containing 50 μg of arachidonic acid. Aliquots (0.1 ml) of the cell superfusates were incubated for 1 min with 0.4 ml of rabbit platelet-rich plasma (PRP) in the cuvettes of a Chronolog Aggregometer with stirring. Aggregation was initiated with arachidonic acid (50 μg in 2 μl of ethanol) and recorded for 5 min. Panel A: Upper curve, control platelets; lower curve, preincubated with cell superfusate. Note the complete inhibition of aggregation. Panel B: Upper two curves, medium heated on boiling water bath for 30 sec and medium pH lowered to 3.0 and readjusted to pH 7.4; lower curve, control. Panel C. Upper curve, cells preincubated with 200 μM acetyl salicylic acid (ASA) for 30 min were washed before addition of arachidonic acid and a further 5-min incubation. Note that ASA completely inhibits synthesis of the anti-platelet activity. Panel D. Superfusate from cells incubated for various time periods at 25°C before addition to platelets. Note gradual decay of anti-platelet activity with increasing time of incubation.

Methods and the superfusates were tested for their ability to inhibit platelet aggregation in a standard aggregometer test system using rabbit platelet-rich plasma. Control platelets were titrated with arachidonic acid to determine the dose that gave optimum aggregation (**Fig. 1,** panel A). Using this optimum dose (50 μg of arachidonic acid in 2 μl of ethanol), 100% aggregation was obtained within 2 min. Preincubation of platelets for 1 min with cell superfusates completely inhibited aggregation (panel A). The anti-platelet activity was destroyed by heating at 100°C for 30 sec and also by briefly dropping the pH of the solution to 3.0 before readjusting to pH 7.4 (panel B). In addition, the ability of the cell monolayers to inhibit platelet aggregation was destroyed by preincubation for 30 min with aspirin (0.2 mM, Fig. 1, panel C). Aliquots of the cell superfusate were held at 25°C for various intervals before testing for antiaggregatory activity (Fig. 1, panel D). The biological activity decreased progressively with increasing time of incubation at 25°C. When the percentage of the activity remaining was plotted as a logarithmic function of the time, the decay was found to be of first order with a half-life of 9 min. This half-life is similar to that reported as the biological half-life of authentic prostacyclin under similar conditions of incubation (19).

Release of prostacyclin progressively increased with increasing concentrations of arachidonic acid added over the range from 3 μM to 25 μM. The release of prostacyclin was extremely rapid, occurring in a pulse lasting only 1 to 2 min following addition of arachidonic acid. The reaction is self-limiting and does not terminate

due to exhaustion of substrate since an excess of arachidonic acid remained in the incubations at the end of the reaction.

Analysis of the products formed following superfusion with [^{14}C]arachidonic acid gave the pattern of metabolites illustrated in **Fig. 2.** In a typical incubation approximately 25% of the arachidonic acid was converted to products which were released into the superfusing medium. The products fell into two main groups. One major and three minor bands were observed in the prostaglandin region and two or three major overlapping bands were present in the hydroxy fatty acid region that traveled immediately behind the [^{14}C]arachidonic acid band. The major product in the prostaglandin region traveled with an R_f value similar to that of an authentic sample of 6-keto-$PGF_{1\alpha}$. The minor products in this region were similarly tentatively identified as PGE_2, PGD_2, and $PGF_{2\alpha}$. Synthesis of all compounds in the prostaglandin region was inhibited by pretreatment of the cell monolayers for 30 min with either 0.2 mM aspirin or with 10 μM indomethacin. Surprisingly, however, synthesis of the three compounds in the mono-hydroxy-fatty acid region of the chromatogram, usually considered as probable lipoxygenase metabolites, was also inhibited by aspirin and indomethacin.

The products of a large scale incubation of cells with nonradioactive arachidonic acid (50 μg/ml) were derivatized as described for prostacyclin in Methods and analyzed by GLC–MS. Three major peaks eluted, one of which showed a significant abundance profile for the 687.4 molecular ion characteristic of the tetra-trimethylsilyl derivative of 6-keto-$PGF_{1\alpha}$. The mass spectrum of this peak, shown in the lower panel of **Fig. 3,** was essentially identical in its major ion proportions to that of a sample of authentic 6-keto-$PGF_{1\alpha}$ subjected to the same derivatization procedure. The remaining com-

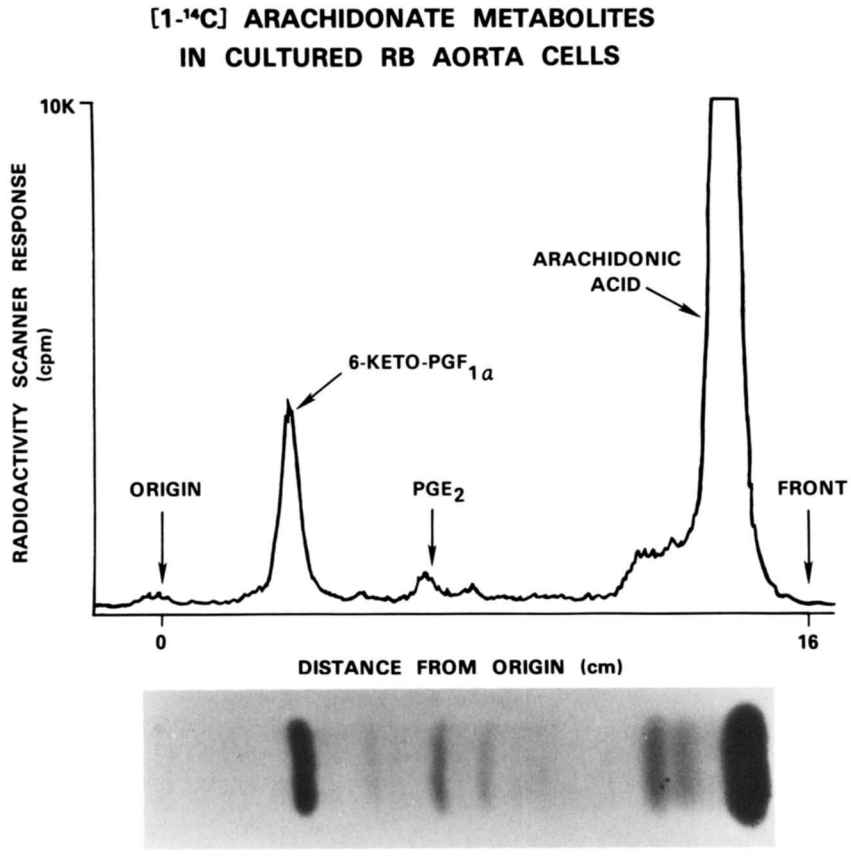

Fig. 2. Analysis of products formed during incubation of rat smooth muscle cells with [1-^{14}C]arachidonic acid. Confluent cultures were incubated with [1-^{14}C]arachidonic acid (0.75 μCi, 55 Ci/mol) in 1 ml of HNCTC-135 for 5 min at 37°C. The products were extracted as described in Methods and separated in silica gel G plates in solvent Iw. The resulting chromatographic plate was scanned for radioactive compounds using a Vanguard Model 930 Autoscanner (upper panel). The TLC plate was then analyzed by radioautography by exposure to Kodak X-OMat X-ray film for 7 days (lower panel). Note the major band corresponding to 6-keto-$PGF_{1\alpha}$, the stable breakdown product of prostacyclin (PGI_2), and also the considerable quantities of compounds corresponding to mono-hydroxy eicosatetraenoic acids that chromatograph in the region immediately behind the [^{14}C]arachidonic acid substrate.

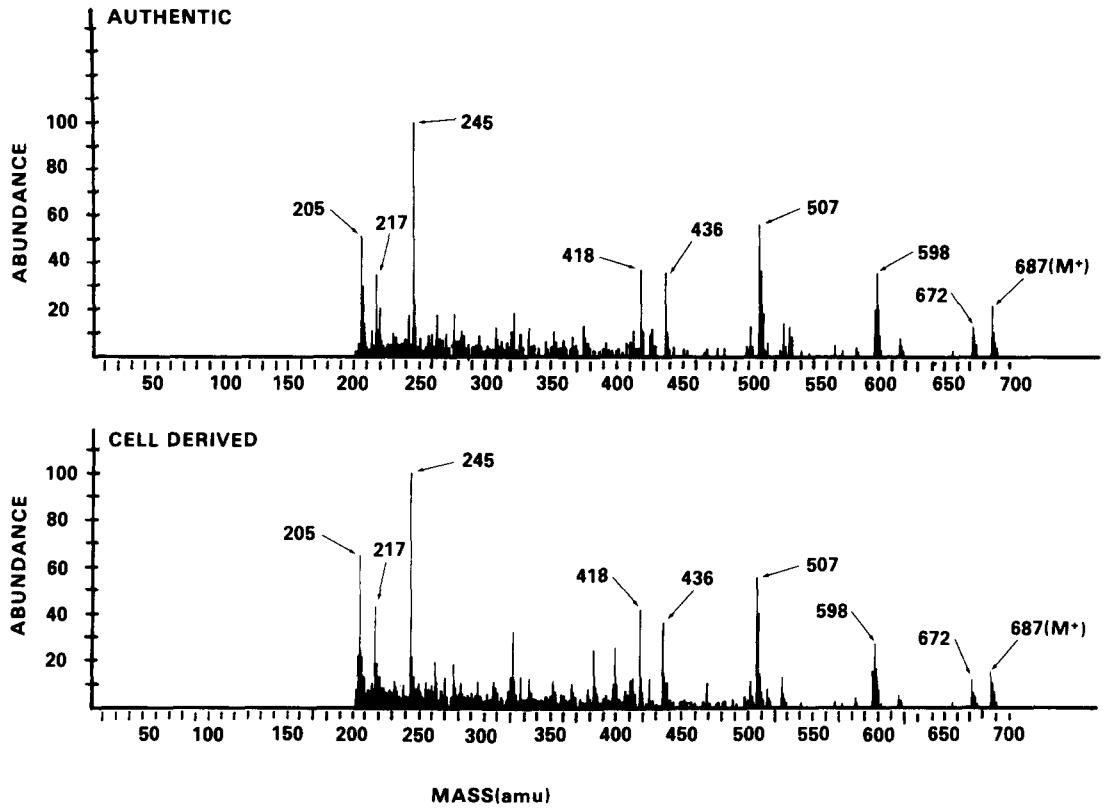

Fig. 3. Mass spectra of derivatized 6-keto-$PGF_{1\alpha}$: authentic and cell derived material. Confluent cultures of rat smooth muscle cells (25 sq cm) were superfused with HNCTC-135 medium containing 50 μg of arachidonic acid for 5 min at 37°C. The products were extracted and derivatized by sequential methylation, oxime formation, and conversion to the TMS derivatives, and analyzed by GLC–MS as described in Methods. The total ion profile was monitored. The mass spectra of the tetra TMS derivative of authentic 6-keto-$PGF_{1\alpha}$ and of the cell-derived material eluting with the same retention time are shown.

pounds in the prostaglandin region were not further identified by GLC–MS because of their relatively small amounts. However, the second most abundant peak in this region was confirmed as PGE_2 by treatment with 1 N KOH for 30 min at 37°C followed by rechromatography. The chromatographic profiles before and after base treatment demonstrated a quantitative conversion of PGE_2 to a compound migrating with the characteristics of PGB_2. This quantitative conversion also indicates that very little, if any, TXB_2 (which co-chromatographs with PGE_2 in this system) was formed by the smooth muscle cells.

The compounds traveling in the hydroxy fatty acid region of the chromatograms were isolated from large-scale incubations (50 μg of arachidonic acid/ml) using twenty 25-sq-cm flasks, and subjected to preliminary fractionation on Biosil columns as described in Methods. Since it is difficult to differentiate 11- and 15-HETEs from the mass spectrum of the polyunsaturated mono-HETEs, the samples were converted to their corresponding saturated mono-hydroxy-eicosanoic-acid methyl esters followed by TMS derivatization. The TMS derivatives of 11-HETE and 15-HETE had very similar retention times on the SE-30 column used (**Fig. 4**). The mass spectra of these two compounds, however, were quite distinct and it proved possible to analyze them in samples which eluted with partially overlapping retention times, by simultaneously monitoring several ions that were unique to the mass spectrum of one or the other across the elution profile as indicated in **Fig. 5.** The hydroxy fatty acid derivatives from the Biosil columns gave a broad peak (the third peak in Fig. 4) for the total ion chromatogram which eluted with a retention time slightly faster than that of authentic 15-HETE derivatized in the same manner and which contained an ion profile for the 343 ion characteristic of 15-HETE. The GLC–MS data were then analyzed for particular ions and the elution profile of these ions was reconstructed. Ions 229, 287, and 258 were used for the 11-HETE derivative and 173, 314, and 343 were used for the 15-HETE derivative. The simultaneous monitoring of these ions across the profile of the elution is shown in Fig. 5. The three ions for 11-HETE eluted slightly faster than the three ions for 15-HETE, thus confirming the presence of the two compounds. The mass spectra were then plotted for retention times of

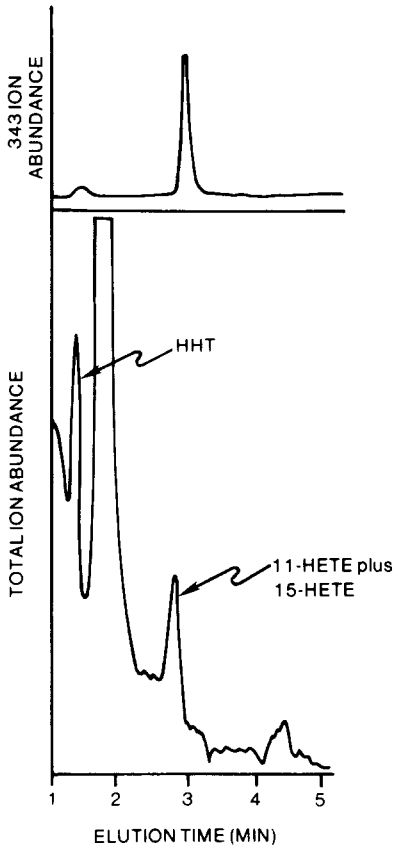

Fig. 4. The ion GLC–MS chromatographic profile of TMS derivatives of monohydroxy-eicosanoids produced by rat smooth muscle cells. Thirty confluent cultures (10-sq-cm) were incubated with 0.5 ml of HNCTC-135 containing arachidonic acid (50 μg/ml) for 5 min at 37°C. The products were extracted, methylated with diazo methane, reduced using PtO/H_2, and converted to the mono TMS derivatives as described in Methods. The derivatives were analyzed on a 3-ft column packed with 3% SE-30 on 100/120 mesh Gaschrom Q. Oven temperature was 190°C for 1 min and increased at 10°/min to a final temperature of 230°C. The abundance of the 343 ion, characteristic of authentic 15-HETE derivatized in the same manner, was monitored simultaneously (upper curve). The first major peak was identified as HHT and the third as a mixture of 11-HETE and 15-HETE (see Figs. 5 and 6). The central large peak was not an arachidonate derivative, and was not further studied.

2.68 min and 2.94 min where the peaks for the 11- and 15-hydroxy-eicosanoate-TMS-methyl ester derivatives overlapped the least. These spectra are shown in **Fig. 6.** In the upper panel (2.68 min), the ions characteristic of 11-HETE (229, 258, and 287) clearly predominate and in the bottom panel (2.94 min), the ions characteristic of authentic 15-HETE (173, 314, and 343) are prominent. The major central peak in Fig. 4 is probably not an arachidonate metabolite and was not further identified. The mass spectrum of the compound eluting at 1.3 min contained major ions at M/e ratios of 173, 272, 301, 341, and 357 which are characteristic of the hydrogenated methyl ester TMS derivative of authentic HHT. The relative proportions of 11-HETE and 15-HETE in the mixture determined from the single ion chromatographic profile were approximately 1.5:1.

Cells prelabeled for 24 hr with [^{14}C]arachidonic acid were exposed to bovine thrombin (Parke-Davis Co., 5 units/ml) in NCTC-135 medium for 5 min, as shown in **Fig. 7** (hydroxy eicosanoids and prostacyclin were the predominant compounds formed during thrombin stimulation accounting for over 75% of the total products formed). A similar pattern of products was also formed with lower doses of thrombin (0.5 units/ml). Appropriate controls were performed which showed that no prostacyclin or hydroxy fatty acid release occurred in cells that were treated with NCTC-135 medium alone.

DISCUSSION

Synthesis of prostacyclin by intact aorta strips was first demonstrated by Moncada et al. (6). By sectioning

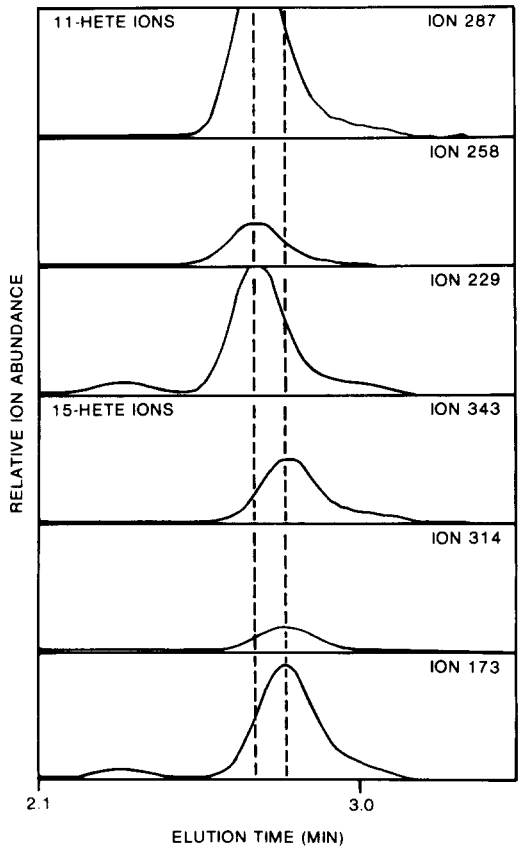

Fig. 5. Selected ion monitoring of 11-HETE and 15-HETE production by cultured rat smooth muscle cells. Products isolated from cultures of RSM incubated with arachidonic acid were isolated, derivatized, and analyzed by GLC–MS as described in Fig. 4. During analysis, two trios of ions characteristic of the derivatives of 15-HETE (173, 314, 343) and 11-HETE (229, 258, 287) were monitored. Note that the ions for 15-HETE coeluted from the column with a slightly longer retention time than those for 11-HETE.

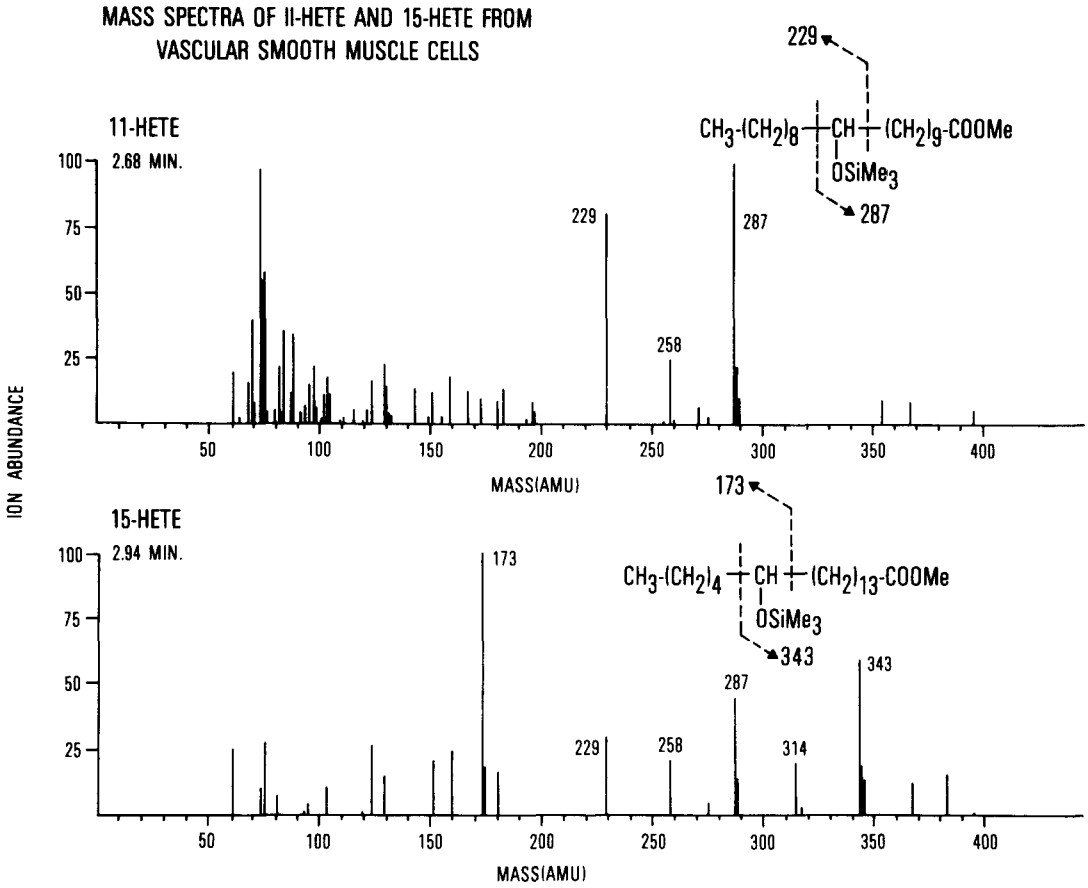

Fig. 6. Mass spectra of TMS-methyl ester derivatives of hydrogenated 11-HETE and 15-HETE. During the analysis of the mono-hydroxy-eicosanoid fraction shown in Fig. 5, mass spectra were taken at retention times where the two compounds overlapped the least. In the upper spectrum (2.68 min) the presence of the ions characteristic of 11-HETE (229, 258, and 287) are clearly seen while in the lower spectrum those for 15-HETE (173, 314, and 343) predominate.

the tissue into layers they showed that the inner or endothelial cell layer produced the largest amounts of biologically active material but that significant amounts were also produced by the outer layers of the tissue. It has been shown that pure cultures of vascular endothelial cells synthesize prostacyclin (20, 21). The present studies demonstrate that pure cultures of smooth muscle cells also synthesize considerable amounts of PGI_2 averaging 90 pmol per mg of cells per min. The endothelial cell component of vascular tissues is in general only one cell layer in thickness. The smooth muscle cell component is present in multiple layers throughout the elastic lamina. The smooth muscle cells are thus the major potential contributors to the total prostacyclin-synthesizing capacity of the vessel wall. The extent to which this potential contribution by smooth muscle cells buried deep in the elastic lamina is utilized, in comparison to that of the endothelial cells exposed directly to circulating and in situ generated agonists such as thrombin, is unclear. It is possible that prostacyclin synthesized in different locations in the vessel wall may have different functions. That synthesized by the endothelial cells may contribute primarily to the anti-platelet activity of the tissue, whereas that synthesized by the smooth muscle cells may function via its properties as a smooth muscle cell relaxant and vasodilator. It is of interest, however, that the smooth muscle cells respond readily to thrombin by releasing prostacyclin. It is believed that release of prostacyclin by endothelial cells in response to thrombin represents a form of feedback control of the thrombotic properties of this compound (22, 23). The thrombin sensitivity of smooth muscle cells in the deeper layers of the intima and media may relate to a requirement for anti-thrombotic activity at these locations in response to injurious conditions. This could include degenerative conditions such as atherosclerosis, where the integrity of the endothelial and intimal layers is disturbed.

The synthesis of considerable quantities of the two monohydroxy fatty acids identified as 15-HETE and 11-HETE is an unusual feature in these cells. The unexpected inhibition of synthesis of these compounds by

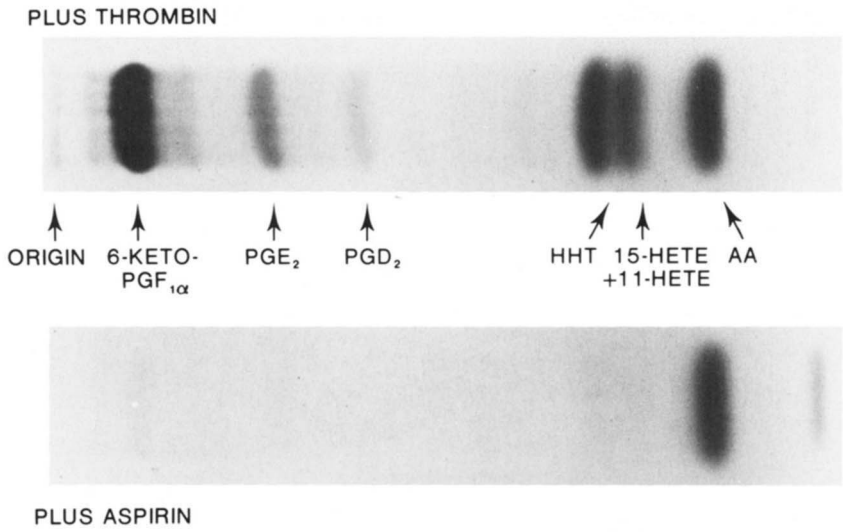

Fig. 7. TLC-radioautograph of products released from rat smooth muscle cells by thrombin before and after aspirin treatment. Confluent cultures (25-sq-cm) of RSM cells were prelabeled by incubation for 24 hr with [1-^{14}C]arachidonic acid. The washed, prelabeled cultures were superfused with a maximally stimulated dose of bovine thrombin (Parke-Davis, Co., 5 units/ml) for 5 min and the products were extracted and analyzed by TLC in solvent system Iw. The plates were radioautographed for 5 days. Upper panel: thrombin alone. Lower panel: thrombin plus aspirin, prelabeled cells were incubated for 30 min with acetyl salicylic acid (200 μM) for 30 min and washed before addition of thrombin. Note significant release of PGI_2 and hydroxy eicosanoids under physiological conditions of stimulation. Note also complete inactivation of prostaglandin and hydroxy eicosanoid synthesis, but not arachidonic acid release, by aspirin, indicating that the hydroxy eicosanoids are cyclooxygenase products.

aspirin and indomethacin demonstrates that they are products of the cyclooxygenase enzyme. The data indicate that, in contrast to the usual situation in which HHT synthesis is accompanied by thromboxane production (24), smooth muscle cells do not make significant amounts of thromboxane. Base treatment of the chromatographic region corresponding to PGE_2, in which TXB_2 co-chromatographs in this solvent system, gave quantitative conversion of the radioactivity to PGB_2. The absence of significant radioactive bands in the hydroxy eicosanoid region in the presence of cyclooxygenase inhibitors indicates that smooth muscle cells contain very little lipoxygenase activity per se. In most cells which make 15-HETE, such as lymphocytes and reticulocytes, the synthesis is catalyzed by a 15-lipoxygenase enzyme (13).

The normal reaction sequence of prostaglandin synthesis by cyclooxygenase involves introduction of an oxygen function at both the 11- and 15-positions. 11- and 15-HETE have previously been identified as minor side-products of the cyclooxygenase reaction (25). The reason for enhancement of this secondary function of the cyclooxygenase in smooth muscle cells, leading to the production of large quantities of mono-oxygenated products, is not known. It is unlikely to be an abnormal response caused by direct addition of free [^{14}C]arachidonic acid substrate since it was observed (Fig. 7) that in prelabeled cells the proportions of 11- and 15-HETE released in response to the physiological mediator thrombin were even larger. This suggests that release of monohydroxy fatty acids by vascular smooth muscle cells may have some physiological function.

15-HETE has been shown recently to be an important potential regulator of other lipoxygenase pathways. It is a potent and selective inhibitor of the platelet 12-lipoxygenase (26), completely inhibiting synthesis of 12-HETE without affecting thromboxane production. In this regard, it is of particular interest that 12-HETE (10^{-10}–10^{-8} M) has been shown to stimulate aortic smooth muscle cell migration (27). In rabbit glycogen-elicited peritoneal macrophages, we have shown that 15-HETE is a specific inhibitor of the 5-lipoxygenase pathway and of leukotriene synthesis. In the PT-18 mast basophil cell line, however, we have demonstrated that 15-HETE can activate a cryptic 5-lipoxygenase-leukotriene pathway in these cells (14). In other experimental systems, 15-HETE has been shown to inhibit certain components of inflammatory tissue reactions since it blocks both synthesis of the chemotactic agent leukotriene B_4 and also the proliferative responses of T-lymphocytes to mitogenic agents (28, 29). It is possible that these widespread anti-lipoxygenase-mediated activities of 15-HETE may be functionally important in vascular tissues. Thus, the release of both prostacyclin and 15-HETE by vascular smooth muscle cells may play

an important role in the maintenance of vascular homeostasis.

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