Vol. 112, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1983 Pages 242-249

FORMATION OF MONOHYDROXYEICOSATETRAENOIC ACIDS FROM ARACHIDONIC ACID BY CULTURED RABBIT AORTIC SMOOTH MUSCLE CELLS

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Received February 28, 1983

In addition to the well established cyclooxygenase pathway, cultured aortic smooth muscle cells convert arachidonic acid to several polar metabolites identified by high performance liquid chromatography and gaz chromatography - mass spectrometry. 15-Hydroxyeicosatetraenoic acid, 12-Hydroxyeicosatetraenoic acid and 5-Hydroxyeicosatetraenoic acid are the major products formed. These observations indicate that the rabbit aortic smooth muscle cells are a potential source of lipoxygenase products and raise the possibility that this pathway of arachidonic acid metabolism can influence the biological functions of arterial myocytes under normal and pathological conditions.

Vascular smooth muscle cells are significantly involved in the prostacyclin (PGI_2) generation by arteries (1, 2). This property is maintained under culture conditions (3). We recently demonstrated that both cyclooxygenase and PGI_2-synthetase activities were reduced in cultured cells originated from atherosclerotic aortas (4). These findings were consistent with an intrinsic modulation of prostaglandin formation in vascular smooth muscle cells (5). The hydroperoxy precursors of monohydroxyeicosatetraenoic acids, have been shown to be potent inhibitors of PGI_2 formation (6) and may represent candidates for a self-regulation of the oxydative pathway of arachidonic acid as previously hypothesized by Greenwald et al. (7) in rabbit aorta.

Abbreviations used :

AA : Arachidonic Acid ; PGI_2 : Prostacyclin ; 6 $KF_{1\alpha}$: 6 Keto Prostaglandin $F_{1\alpha}$; $PGF_{2\alpha}$, PGE_2 : Prostaglandins $F_{2\alpha}$ and E_2 ; HETE : Hydroxyeicosatetraenoic Acid ; HPETE : Hydroperoxyeicosatetraenoic acid ; SMC : Smooth Muscle Cell ; HPLC : High Pressure Liquid Chromatography ; GC-MS : Gas Chromatography Mass Spectrometry ; NDGA : Nordehydroguaiaretic acid ; ETYA : Eicosatetraynoic acid.

0006-291X/83 $1.50

The present study was performed to investigate the lipoxygenase activity in cultured aortic smooth muscle cells and report on the nature of the monohydroxylated products formed.

MATERIAL AND METHODS :

Arachidonic acid purchased from SIGMA chemical Co, and [1.^{14}C] 20:4 from the radiochemical Center AMERSHAM were always purified on silicic acid column immediatly prior to use in order to remove small amounts of contaminating material which had a retention time on high pressure liquid chromatography and thin layer chromatography similar to that of 15-hydroxy arachidonic acid. Hydroxyeicosatetraenoic acid standards, namely the 15, 12 and 5 HETEs were a gift of Dr J. Maclouf. 15-HPETE was prepared from arachidonic acid by soybean lipoxygenase type IV catalysed oxygenation and purified by HPLC (8). HAM F10 medium and fetal calf serum were purchased from SEROMED. Organic solvants of HPLC grade were obtained from MERCK.

Cells : Aortic smooth muscle cells were obtained from explants of thoracic aorta from adult male rabbits weighing 2 - 2.5 kg essentially as described by Ross (9). Cells were grown at 37°C in 25 cm^2 plastic flasks in an atmosphere of 5 % CO_2 in air using HAM F10 growth medium supplemented by 20 % fetal calf serum during the first weeks. When confluency was achieved, cells were tripsinized and subcultivated in a 1:3 split ratio in 10 % fetal calf serum supplemented HAM F10 medium. All experiments were done using cells below passage 5.

Metabolism of [1.^{14}C] AA by cell homogenates : Cells were scraped with a rubber policeman, suspended in 2 ml of 0.05 M Tris-HCl, 0.15 M NaCl (pH : 7.4) buffer and homogenized. Sonication was avoided in order to minimize free radicals formation leading to a disturbance of oxygenation reaction. Aliquots (0.2 ml) of resultant suspension were used for protein determination according to the method of Bradford (10). The reaction mixture contained the following components at the indicated amounts : cell homogenate 1 mg/proteins, [1.^{14}C] AA (50 Ci/M) 10 nmoles in 10 µl absolute ethanol vehicle. The final volume reaction was 1 ml. In some experiments, the cell homogenate was boiled 10 min. prior to incubation and was used as a control for nonenzymatic conversion of AA. When inhibitors were used (Indomethacin, HPETE), they were added to the incubation mixture 10 min. prior the addition of AA. Incubations were carried out at 37° C for 20 min. with shaking and stopped by addition of 1.5 volumes of methanol.

Then the mixture was centrifuged, the supernatant collected and the pellet washed with 0.5 ml of methanol. The pooled supernatants were acidified (citric acid) to pH : 3 and extracted with diethyl ether. Ether extracts were washed several times and then evaporated to dryness. The dry residue was dissolved in 0.5 ml of hexane - diethyl ether (90, 10 v/v) and applied on silicic acid column in order to elute unreacted arachidonic acid with hexane ether (90, 10 v/v). The more polar metabolites were eluted with ether-methanol (95, 5 v/v). The ether methanol fraction was evaporated, dissolved in methanol and treated 10 min. at 4° C in darkness with diazomethane in ether to form the methyl esters. After evaporation, dry residues were dissolved in absolute methanol and applied on a µ BONDAPACK C_{18} analytical column (WATERS Associates). The mobile phase was composed of 75 % methanol, 25 % water, 0.02 acetic acid (pH adjusted to 5.7 with NH_4OH). Isocratic elution was performed at a flow at 1.5 ml/min. Column effluent was continuously maintained for UV absorbance at 232 nm, and 1.5 ml fractions were collected. Portions of

each 1.5 ml fraction were assessed for radioactivity by scintillation counting. In some experiments, column effluent was directly monitored for counting in a Flow One (KONTRON) radiometer using Lumaflow II as liquid scintillator.

Either the entire extracted material or the fractions isolated by HPLC were analysed by GC-MS . Prior to analysis, the extracts methylated as described above and the HPLC isolated fractions were submitted to trimethylsilylation with bis - trimethylsilyl - trifluoroacetamide containing 1 % trimethyl chlorosilane for 30 min. One aliquot was analysed by GC-MS computer system (LKB 2091-061) equipped with a home-made high efficiency glass capillary column (20 m x 0.28 mm, stationary phase OV_1, 0.15 µm thick). Mass fragmentometry analysis were performed using the following m/e for characteristic fragments of methyl (trimethyl silyl) derivatives - 12 HETE : 295, 15 HETE : 225, 5 HETE : 255, 11 HETE : 225, 8 HETE : 265, 9 HETE : 255. After catalytic hydrogenation these products yield the corresponding hydroxylated arachidonic acid.

Metabolism of endogenous AA by SMC : Confluent SMC were washed with serum free medium, then preincubated 16 h. with [^{14}C]AA (0.1 µCi/ml). After labelling, the medium was removed and the cell layer washed twice. 3 ml of TRIS-HCl buffer were added in the flasks, then the cells were activated by scraping and homogeneization. The homogenates were centrifuged, incubation media were collected and treated as described above.

RESULTS :

- Monohydroxyeicosatetraenoic acid (HETE) derivatives formed by incubation of [1. ^{14}C] AA with SMC homogenates :

Incubation with crude SMC homogenates revealed enzyme activity which produced several metabolites of AA. Despite the fact that different cultures exhibited different rates of conversion of AA to its metabolites, non polar metabolites represented around 18 % of the total transformed AA, the major part of oxydative derivatives being under the form of polar metabolites -namely prostacyclin -(tab. I). HPLC analyse revealed 3 major peaks of radioactivity each coinciding with a peak detected by UV absorbtion at 232 mn with retention time similar to 15 HETE, 12 HETE and 5 HETE authentic standards. When either of these three

Table I

Eicosanoids formed by incubation of [1-^{14}C]AA with SMC homogenates.

	Eicosanoids			
	6 $KF_{1\alpha}$	$PGF_{2\alpha}$	PGE_2	HETEs
Control	3.80 ± 0.30	0.90 ± 0.27	0.80 ± 0.40	1.20 ± 0.38
Boiled Cells	0.21 ± 0.08	0.10 ± 0.07	0.10 ± 0.08	0.12 ± 0.04

Results are expressed as percent of the total radioactivity recovered from the incubation mixture and are the mean of 3 experiments with the same cell strain.

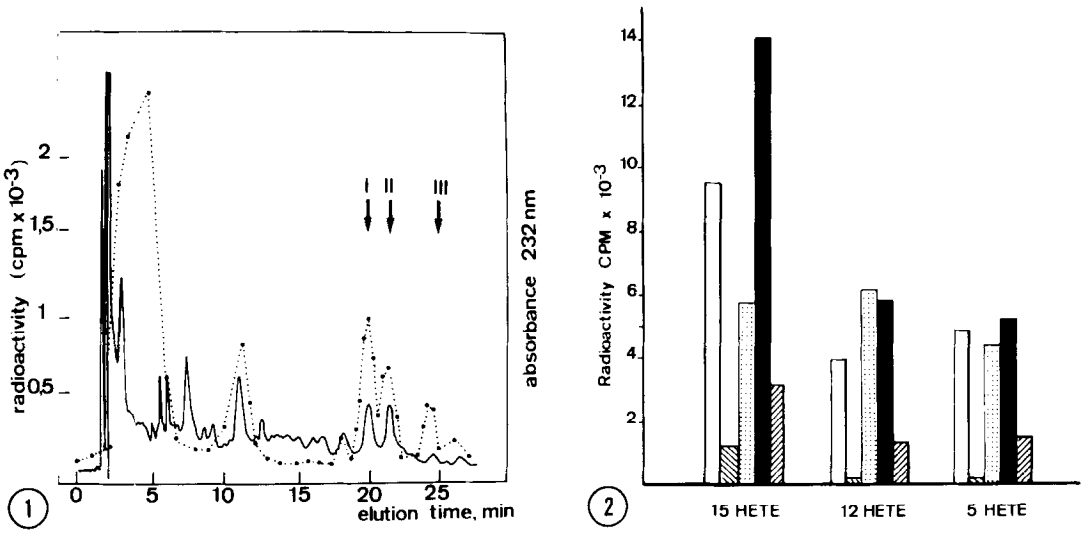

Fig. 1 Reverse HPLC chromatography of incubation media of [1-^{14}C] AA with homogenate of SMC. —— Absorbance 232 nm ····Radioactivity (cpm). ↑ Indicate the position of authentic standards. I : 15 HETE, II : 12 HETE, III : 5 HETE.

Fig. 2 HPLC data obtained from homogeneized cultured SMC incubated with 10 nM [1-^{14}C] AA under the presence of inhibitors. □ Control, ▨ Boiled cells, ⁘ Indomethacin 10^{-4}M, ■ HPETE 10^{-7} M, ▨ HPETE 3.10^{-6} M. For incubation details, see methods.

peaks were collected and reinjected, the retention time did not change. Additionaly, two unidentified peaks represented around 10 % each of the radioactivity recovered from the non polar metabolites (fig. 1) were detected . The formation of these 5 compounds was practically abolished after boiling the cells (6 % of the control activity) (tab. I).

Factors influencing HETE formation

As demonstrated in fig. 2 under the presence of a cyclooxygenase inhibitor (Indomethacin 10^{-4} M) the total formation of monohydroxylated products did not significantly change. Despite this, the relative concentration of 15 HETE was reduced when compared to 12 HETE. Exogenously added 15 HPETE enhanced the formation of 15 and 12 HETE respectively at low concentrations (10^{-7} M). Higher concentrations ($3. 10^{-6}$ M) significantly inhibited all the three HETE without any change in their relative distribution (tab. II). The relatively selective inhibitor of lipoxygenase, NDGA (10 µg/ml) yielded about the same results as the boiled control.

Table II

Mass Fragmentometry of Monohydroxylated derivatives

HETE Types	Percent of total HETEs	
	HPLC	GC-MS
- " X "	13.4 ± 3.1	N.D.
- 15 HETE	30.7 ± 9.9	58.0 ± 7.1
- 12 HETE	23.0 ± 4.1	16.9 ± 7.3
- 5 HETE	20.1 ± 2.8	17.8 ± 3.9
- " Y "	12.7 ± 11.1	N.D.
- 11 HETE	N.D.	16.2 ± 3.6
- 8 HETE	N.D.	6.0 ± 0.6
- 9 HETE	N.D.	3.4 ± 0.4

Analysis of HETE derivatives formed during 20 min. incubation of 10 nM AA with SMC homogenates. For mass fragmentrometry, the relative concentrations were calculated using authentic standards according to ref. 22. Analysis were obtained from 5 experiments using different cell strains. Results are expressed as the mean ± S.D. N.D. Not detected peak.

GC-MS Analyses

Monohydroxylated derivatives were further characterized by gaz chromatography - mass spectrometry using the methylsilyl ethers derivatives before and after catalytic hydrogenation. Mass fragmentometry analyses of non reduced

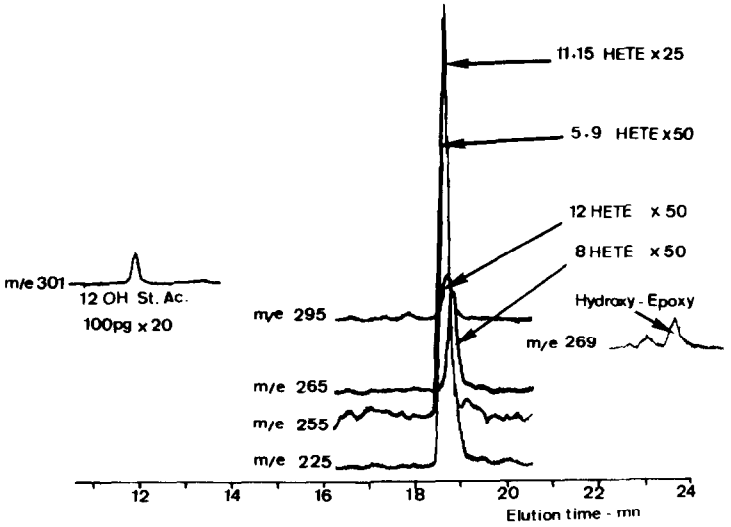

Fig. 3 Mass fragmentometry recording of the methyl ester trimethyl silyl ether derivatives of HETEs (12 OH stearic acid was used as internal standard).

extracts revealed the presence of 4 different isomers 15, 12, 5 and 8 HETE respectively (fig. 3). Upon treatment of the extracts with etheral triphenylphosphine, two additional derivatives were measured with characteristic intense ions consistant with the presence of 11 HETE and 9 HETE.

Monohydroxyeicosanoids formed from endogenous AA

In order to define further the enzyme activity leading to the formation of mono HETE, we tested the metabolism of endogenous AA after activation of $[1\text{-}^{14}C]$ AA prelabelled cells by scraping.

Incubation medium contained around 11 % of the total radioactivity recovered under eicosanoids forms. Among these derivatives, 2.1 % were monohydroxylated compounds. 12 HETE represented more than 63 % of these monoHETE derivatives.

DISCUSSION

The metabolism of arachidonic acid in mammalian cells generates several oxydative derivatives, the amount of them being characteristic of the cellular source. Prostaglandin generation in aortic smooth muscle cells has been extensively studied (11). In rabbit arterial smooth muscle cells under cultures conditions, PGI_2, revealed as 6 Keto $PGF_{1\alpha}$ is the major component, following by PGE_2 and $PGF_{2\alpha}$ (4). In addition, we demonstrate the production of several HETEs, the relative amount of them depending on the experimental conditions. In our study, homogenized smooth muscle cells were first incubated with $[1.^{14}C]$ AA. The major monohydroxylated compounds formed were identified by HPLC as 15 HETE, 12 HETE and 5 HETE. Additional identification was obtained by GC-MS which unequivocally demonstrated the presence of 15 HETE, 12 HETE, 5 HETE and also 11, 8 and 9 HETE in SMC incubates. The formation of these products was practically abolished after boiling the cells. When SMC homogenates were incubated under the presence of the cyclooxygenase inhibitor indomethacin (10^{-4} M), we did not observed a true shift of the metabolism of arachidonic acid from the cyclooxygenase to the lipoxygenase pathway. In fact, the total level of monohydroxylated compounds remained identical. Despite this, the relative level of 12 HETE was enhanced to the depends of 15 HETE. Such an activation of the 12 HETE formation has been recently reported in fetal calf aorta under indomethacin treatment (12). The lipoxygenase inhibitor 15 HPETE (13) (3.10^{-6} M) produced a large decrease of both 15 and 12 HETE synthesis,

without any significant reduction of the cyclooxygenase activity (3.2 %vs 3.9 % of the total AA transformation in the control).

We have also investigated the lipoxygenase activity on the endogenous substrate after activation by scraping. Under these conditions, the three main metabolites 15, 12 and 5 HETEs were produced from rabbit SMC. Despite some variations between different cell strains, the major metabolites was 12 HETE. This metabolite has previously been reported to be synthesized from exogenously added AA by rabbit aortic rings (7). Its formation was enhanced by indomethacin and inhibited by lipoxygenase inhibitor such as ETYA. Although adult vascular tissue has not previously been shown to convert AA to others mono HETE, several of these compounds have been identified in a number of mammalian tissues (14, 19) including fetal calf aorta(12). It is not certain that all of these products were originated from lipoxygenase activities and that their production is a general property of arterial SMC, but it is of importance, however, that the biological activities of their hydroperoxy intermediates (namely 15 HPETE) include inhibition of vascular prostacyclin formation (6) and that some of them exhibit chemotactic properties for several cell types including SMC or monocytes (20, 21). So, despite the fact that the physiological and pathological implications of these derivatives remain to define further under in vivo situation, they may exhibit a particular interest regarding the progression of the atherosclerotic process.

ACKNOWLEDGEMENTS

This work was supported by the Institut National de la Santé et de la Recherche Medicale (CRL 81.5.037) and by the University of Bordeaux II.

The authors wish to thank Mrs N. Grillon for typing the manuscript.

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