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Synthesis of monohydroxylated fatty acids from linoleic acid by rat aortic smooth muscle cells and tissues: influence on prostacyclin production

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Abstract We have investigated whether cellular metabolism of linoleic acid (18:2) can influence prostacyclin (PGI_2) production by cultured rat aortic smooth muscle cells (SMC) and tissues. Incubation of rat SMC homogenates with [1-^{14}C]18:2 results in the enzymatic synthesis of [^{14}C]13-HODE (hydroxyoctadecadienoic acid) and to a lesser extent [^{14}C]9-HODE as defined by gas-liquid chromatography-mass spectrometry (GLC-MS). The observed changes, in percent enzymatically synthesized 13-HODE in the presence of indomethacin, aspirin, metyrapone, 15-HPETE (hydroperoxyeicosatetraenoic acid), and NDGA, suggest that it is formed from the PGH (prostaglandin endoperoxide) synthase pathway. Incubation of intact adherent SMC with [^{14}C]linoleic acid demonstrates that the monohydroxylated compounds are predominantly esterified within the membrane phospholipids and not released into the incubation medium. The simultaneous incubation or a short-term preincubation of 18:2 and arachidonic acid (20:4) do not modify the enzymatic profile of 20:4 transformation. By contrast, long-term preincubation of cells with 18:2 or 13-HODE stimulates the transformation of exogenously added [^{14}C]20:4 to [^{14}C]6-keto $PGF_{1\alpha}$. However, exogenous 13-HODE does not enhance [^{14}C]6-keto $PGF_{1\alpha}$ recovery from [^{14}C]20:4 prelabeled SMCs. Our results demonstrate that 18:2 is a substrate for PGH-synthase in rat aortic SMC and tissues. The 13-HODE formed is essentially esterified in cell phospholipids and remains without any significant effects on the release of [^{14}C]6-keto $PGF_{1\alpha}$ from [^{14}C]20:4 prelabeled SMC.—**Daret, D., P. Blin, B. Dorian, M. Rigaud, and J. Larrue.** Synthesis of monohydroxylated fatty acids from linoleic acid by rat aortic smooth muscle cells and tissues: influence on prostacyclin production. *J. Lipid Res.* 1993. **34:** 1473–1482.

Supplementary key words PGH-synthase • lipoxygenase • PGI_2 production

Monohydroxylated derivatives, namely hydroxy octadecadienoic acids (HODE), have been shown to esterify cholesterol in large proportions in advanced human atherosclerotic lesions (1, 2). These compounds are derived from linoleic acid (18:2), one of the major polyunsaturated fatty acids in humans, which may become predominant depending on the diet composition (3), and competes with arachidonic acid (20:4) for incorporation into phospholipids (4–6).

Linoleic acid may be transformed to 13-HODE and 9-HODE by arterial vascular endothelial (7, 8) and smooth muscle cells (SMCs) (9, 10). The enzymatic course remains essentially questionable as either lipoxygenase or PGH-synthase activities have been implicated (7–12).

The effects of linoleic acid or its monohydroxylated derivatives on vascular wall biology and PGI_2 production remain a matter of debate. Linoleic acid has been shown to produce considerable reduction of prostacyclin (PGI_2) release during incubation of pieces of rat aortic tissue (13) or human umbilical vein endothelial cells (HUVEC) (14). In contrast, it stimulated PGI_2 formation by perfused rat aorta (4). In addition, its hydroxylated derivative 13-HODE has been reported either as a potent stimulator of PGI_2 formation by cultured fetal bovine aortic endothelial cells (10) and inhibitor of platelet adhesion to vascular endothelial cells under static conditions (9) or, together with 9-HODE, as an inhibitor of PGI_2 synthesis (11) in human umbilical vein endothelial cells.

In this study, we demonstrate that rat aortic SMCs and tissues metabolize linoleic acid essentially to 13-HODE through the PGH-synthase pathway. This compound is predominantly esterified within membrane phospholipids and not secreted. A long-term preincubation of SMCs with linoleic acid may produce a weak stimulation of the transformation of exogenous arachidonic acid to PGI_2, but as 13-HODE, linoleic acid remains without any significant effect on the capacity of SMCs to produce [^{14}C]PGI_2 from [^{14}C]20:4-prelabeled cells.

Abbreviations: PGI_2, prostacyclin; SMC, smooth muscle cells; HODE, hydroxyoctadecadienoic acid; PGH-synthase, prostaglandin endoperoxide synthase; HETE, hydroxyeicosatetraenoic acid; TLC, thin-layer chromatography; RP-HPLC, reverse phase-high performance liquid chromatography; GLC-MS, gas-liquid chromatography-mass spectrometry; HPETE, hydroperoxyeicosatetraenoic acid; HUVEC, human umbilical vein endothelial cells; HHT, 12-hydroxyheptadecatrienoic acid; NDGA, nordihydroguaiaretic acid.

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MATERIAL AND METHODS

Material

Reference monohydroxylated products and standards were obtained from Calbiochem or Paesel. The [1-^{14}C]linoleic acid (18:2) and [1-^{14}C]arachidonic acid (20:4) (50-60 mCi/mmol) were purchased from Amersham.

Smooth muscle cell cultures

Aortic SMC were obtained from explants of thoracic aorta of adult male Wistar rats weighing 300-350 g, essentially as described by Ross (15). 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 trypsinized and subcultivated in a 1:3 split ratio in 10% fetal calf serum supplemented with HAM F10 medium. All experiments were done using cells below passage nb 8.

Preparation of rat aortic tissues

Normal adult rat thoracic aortas were excised, cleaned, rinsed, and immediately frozen in liquid N_2, then maintained at −80°C until used. The tissues (100 mg) were cryoground, suspended in 1 ml of 50 mM Tris-HCl, 0.15 M NaCl (pH 7.4), and incubated with [^{14}C]18:2 at a final concentration of 3 μM for 20 min. When inhibitors were used, the tissues were preincubated 10 min with 10^{-5} M indomethacin, before transformation of linoleic acid. The incubation extracts were analyzed by RP-HPLC.

Metabolism of ^{14}C-labeled fatty acids by cell homogenates

Rat aortic SMC were suspended in 50 mM Tris-HCl, 0.15 M NaCl (pH 7.4) buffer and homogenized; 1 ml (10^7 cells) of cell suspension was incubated with either [^{14}C]18:2 or 20:4 (200,000 dpm) at the final desired concentration for 2-20 min. In incubations carried out in the presence of inhibitors, the cell suspension was preincubated for 10 min at 37°C in the incubation medium containing either metyrapone (10^{-6}-10^{-4} M), NDGA (10^{-5}-10^{-3} M), 15-HPETE (10^{-6}-10^{-5} M), aspirin (10^{-4}-10^{-3} M), or indomethacin (5.10^{-7}-10^{-4} M) and the enzymatic reactions were started by addition of fatty acids.

Extraction and thin-layer chromatography

At the end of the incubation period, the mixture was acidified to pH 3 (1 N HCl) and then extracted twice with 3 ml of ethyl acetate. The organic extracts were evaporated under a stream of nitrogen, resuspended in a minimal volume of ethyl acetate, and analyzed by thin-layer chromatography on 0.25-mm silica gel G plates (Whatman). Chromatograms were developed in an ascending solvent of the organic phase of ethyl acetate-iso-octane-acetic acid-water 110:50:20:100 (v/v). Products were located by autoradiography (X-OMAT Kodak) and the appropriate regions were scraped and counted (β scintillation) or measured by scanning (radioscan Berthold).

High performance liquid chromatography

Alternatively, ethyl acetate extracts were evaporated and the dry residues were dissolved in absolute ethanol and then analyzed by RP-HPLC. Analyses were performed on 10-μm particles of a μBondapak analytical column (Waters Associates) and eluted with a mobile phase composed of 70% methanol, 30% water, and 0.02% acetic acid. Isocratic elution was performed at a flow rate of 1 ml/min. Column effluent was continuously monitored for UV absorbance at 234 nm and radioactivity (Berthold). The retention times (R_t) of the monohydroxylated products were HHT, 19.1-19.5 min; 13-HODE, 35.7-36.1 min; 9-HODE, 41.7-42.1 min; and 12-HETE, 42.3-42.7 min; untransformed fatty acids were eluted after 60 min in 100% methanol.

Chiral stationary phase HPLC separation

The separation of optical isomers of 13-HODE was performed as follows (16). The 13-HODE peak isolated from RP-HPLC as described was methylated with diazomethane and analyzed using two 25-cm Bakerbond Chiral phase LC columns in series (DNBPG 5μ Baker Research products). The mobile phase mixture consisted of hexane-2-propanol 995:5, with a flow rate of 0.8 ml/min.

Gas combined liquid chromatography-mass spectrometry

The 35.7- and 41.7-min peaks isolated by RP-HPLC were analyzed by GLC-MS. Prior to analysis, the fractions were methylated by a 10-min treatment at 4°C in darkness with diazomethane in ether to form the methyl esters and then submitted to trimethylsilylation with bis-trimethylsilyl-trifluoro-acetamide containing 1% trimethyl chlorosilane for 30 min. Electron ionization mass spectra of the derivatives were acquired via the use of an LKB 2091-061 GC-MS computer system equipped with a home-made high efficiency glass capillary column (25 m × 0.32 mm, stationary phase OVI, 0.20-μm thick). The analysis was performed in a programmed thermal gradient from 170°C to 320°C (2°C/min) with helium (35 ml/min) as carrier gas.

Metabolism of ^{14}C-labeled fatty acids by intact adherent SMC

Confluent adherent SMC were incubated with 2 μM ^{14}C-labeled fatty acid for either 1 h or 20 h as previously described (17). The incubation medium was recovered, the cell layer was washed twice, and wash and incubation media were pooled. Alternatively, 5 ml Tris-HCl buffer containing 2 mM $CaCl_2$ was added to the flask and the

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cells were incubated in the absence or presence of the Ca^{2+} ionophore A23187 (10 μM) for 30 min at 37°C.

Cell and incubation media were separately extracted with 3 vol chloroform-methanol 2:1 (v/v) containing 0.05% of butylated hydroxytoluene (BHT) in order to prevent autooxidation during the preparation of samples for analysis, especially in the fractions subjected to hydrolysis. The dry residues of the extracts were dissolved either in a minimal volume of chloroform for TLC analysis or in ethanol for RP-HPLC analysis. When hydrolysis was required, extracts were dissolved in 0.2 N sodium hydroxyde in 80% methanol and allowed to stand 20 h at 45°C. The TLC solvent was chloroform-methanol-acetic acid-water 195:129:3:9 (v/v). Under these conditions, the yield of monohydroxylated compounds varied between 35% after alkaline hydrolysis of esterified material to 70% in direct extraction of incubation medium. Corrected values were expressed as pmoles of labeled compounds.

Effects of linoleic acid and 13-HODE on arachidonic acid transformation by SMC

Confluent adherent SMCs were incubated with 2 μM of 18:2, 13-HODE, or 20:4 in a serum-free HAM F10 medium for either 1 h or 20 h. Thereafter, the cell layer was washed twice, scraped, homogenized, and incubated with 3 μM of [1-^{14}C]20:4 or 18:2, respectively. The products formed were extracted twice from incubation medium by 3 volumes of ethyl acetate and analyzed by TLC or RP-HPLC as described.

RESULTS

Transformation of [^{14}C]linoleic acid by rat aortic smooth muscle cell homogenates

Incubation of rat aortic SMC homogenates with [1-^{14}C]18:2 resulted in the enzymatic synthesis of metabolites that were extracted together with 18:2 from the whole homogenate (cells + incubation media) by ethyl acetate, pH 3. These compounds eluted from an RP-HPLC column with retention times that corresponded to 13-HODE and 9-HODE standards and represent 5.9 ± 1.9% and 1.6 ± 0.4%, respectively, of the total radioactivity applied to the column (**Fig. 1A**). This transformation was practically abolished after boiling the cells, as the quantities of 13-HODE and 9-HODE recovered did not exceed 0.6% and 0.4% of the original substrate concentration (Fig. 1B).

The electron-ionization mass spectrum of the Me_3Si derivatives of the methyl ester of the compound contained in the 35.7-min peak of the RP-HPLC analysis is shown in **Fig. 2A.** Ions of the *m/z* 382(M^+), 367(M^+–15), 351(M^+–31), 311(M^+–71), 292(M^+–90), 225(M^+–157) indicated that the compound was a monohydroxylated C18 fatty acid containing two double bonds. The position of

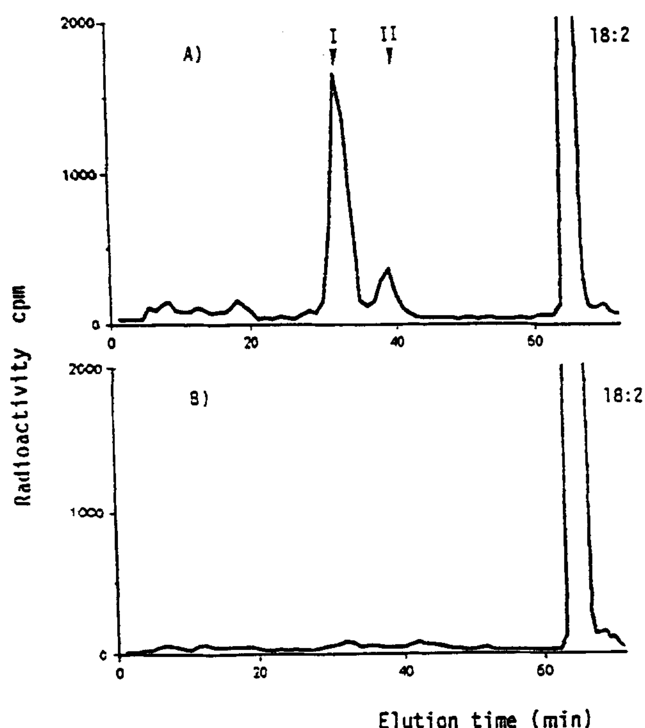

Fig. 1. RP-HPLC of the products formed during 20 min incubation of 3 μM [^{14}C]18:2 with homogenate of aortic SMCs (A) or with boiled cells (B). The whole cell suspension was extracted with ethyl acetate and the organic extract was analyzed as described in Material and Methods. I, 13-HODE; II, 9-HODE. Radioactive products eluted between 0 and 20 min were not characterized.

the hydroxyl moiety was determined from the mass spectra of the Me_3Si derivative of the methyl ester of the catalytically hydrogenated compound (Fig. 2B). The major fragment ions of the hydrogenated compound contained in the 35.7-min peak at *m/z* 315(M^+–71) and 173 (M^+–213) indicate the presence of an hydroxyl moiety at C13 and cleavage of carbon bonds either between C13 and C14 (*m/z* 315) or between C12 and C13 (*m/z* 173). The same experiment with the compound contained in the 41.7-min peak, after catalytic hydrogenation, demonstrates the presence of major fragment ions at *m/z* 259 (M^+–127) and 229 (M^+–157) which indicate the presence of the Me_3Si derivative of an hydroxyl moiety at C9 and cleavage of carbon bond between C9 and C10 and C8 and C9, respectively. Taken together, these results demonstrate that the compounds formed are 13-HODE and 9-HODE.

Time course experiments are reported in **Fig. 3.** The formation of 13-HODE and 9-HODE reached a plateau at 10 min and 5 min, respectively. At these times, around 6% and 1.2% of the substrate [^{14}C]18:2 were transformed to [^{14}C]13-HODE and [^{14}C]9-HODE, respectively.

When the concentration of 18:2 increased, the increase of 13-HODE formation was linear until a substrate concentration of 30 μM; thereafter, the percentage transformation decreased and did not represent more than 2.2% at 90 μM. By contrast, 9-HODE formation was linear

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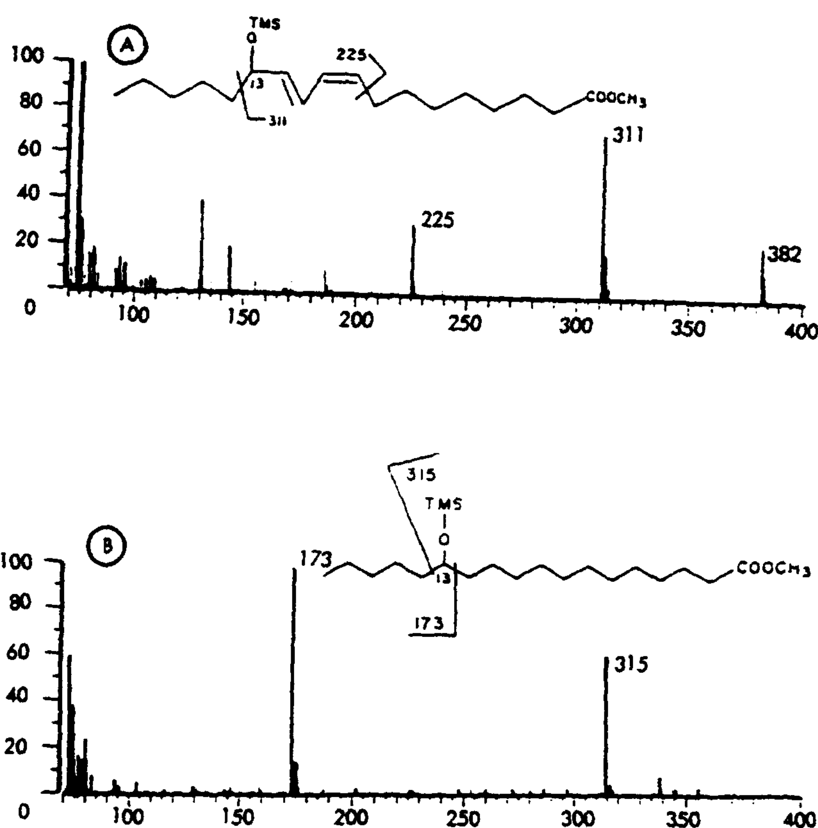

Fig. 2. Mass spectra of 13-HODE. The presence of the major ions at *m/z* 311 and 225 in the electron ionization mass spectrum of the compound contained in the 35.7-min peak of the RP-HPLC analysis (A) and at *m/z* 315 and 173 after catalytic hydrogenation (B) indicate that the product is a C13 monohydroxylated C18 fatty acid with two double bonds.

until 90 μM concentration of 18:2 (1.4%) (**Fig. 4**). The total concentrations of HODE achieved were 2- to 3-times higher than those of monohydroxylated compounds formed from 20:4 under the same experimental conditions.

Transformation of [^{14}C]18:2 by rat aortic tissues

Aortic tissues metabolize linoleic acid to monohydroxylated compounds, identified by RP-HPLC. The main product, 13-HODE, represents 8.3 ± 1.2% of [^{14}C]18:2 applied (**Fig. 5A**). This synthesis was inhibited by 90% with indomethacin 10^{-5} M (Fig. 5B).

Enzymatic pathways of 18:2 metabolism

Chiral phase HPLC analysis was carried out on the 13-HODE peak obtained from RP-HPLC. The results showed that the S-enantiomer of 13-HODE predominated (66–74%) over the R form (33–26%), suggesting that a significant part of 13-HODE has an enzymatic origin. The starting [^{14}C]18:2 material was examined for the presence of autooxidation products and showed 0.40% and 0.28% of 13-HODE and 9-HODE, respectively. Ex-

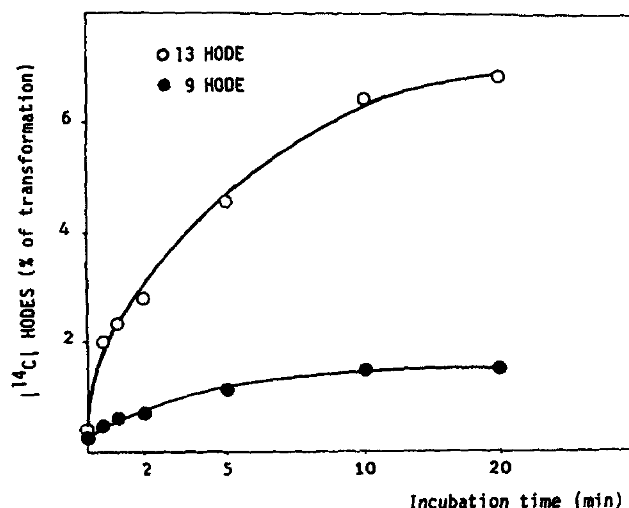

Fig. 3. Time course of 13-HODE and 9-HODE formation in homogenates of rat aortic SMC. The hydroxylated derivatives were extracted and analyzed by RP-HPLC from incubated cell suspensions as described in Material and Methods. Data are expressed as the mean percent of transformation from triplicates of a representative experiment.

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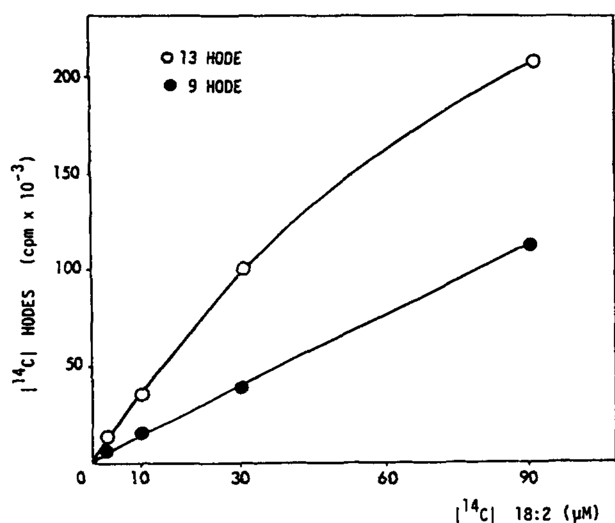

Fig. 4. [14C]18:2 concentration dependence in the formation of [14C]13-HODE and [14C]9-HODE in homogenates of rat aortic SMC. Cells were incubated 20 min with increasing concentrations of [14C]18:2 (2–90 μM). Extraction was performed on the whole incubation mixture with ethyl acetate and products were analyzed by RP-HPLC as described. Results are expressed as the cpm of products formed from duplicate experiments.

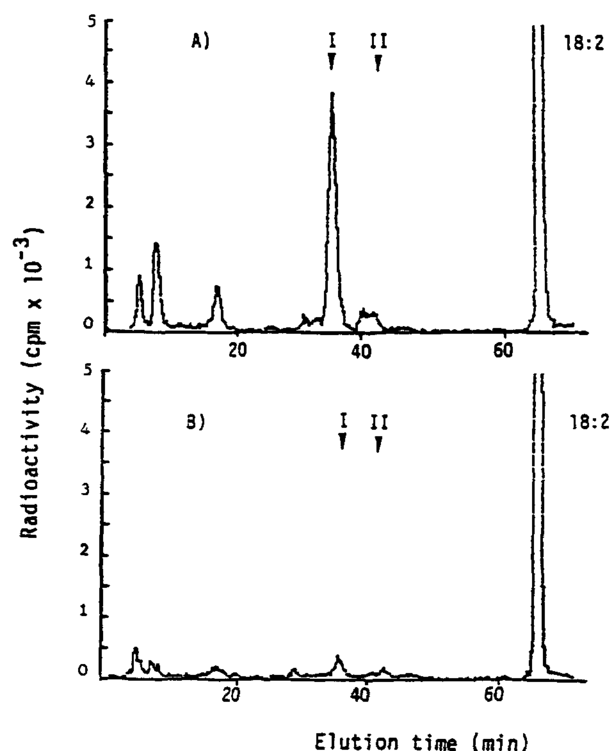

Fig. 5. RP-HPLC of the products formed during 20 min incubation of 3 μM [14C]18:2 with 100 mg rat aortic tissue without (A) or with indomethacin preincubation (B). The whole tissue suspension was extracted with ethyl acetate and the organic extract was analyzed as described in Material and Methods. I, 13-HODE; II, 9-HODE. Radioactive products eluted between 0 and 20 min were not characterized.

periments with boiled cells demonstrated roughly the same proportion of autooxidation products; after 20 min incubation, 13-HODE and 9-HODE represented 0.44% and 0.29%, respectively, of the radioactivity recovered in the ethyl acetate extract.

In addition, preincubation of SMCs with NDGA inhibited 13-HODE and 9-HODE recovery (40 and 95% inhibition for 10^{-4} and 10^{-3} M, respectively) but was without any significant inhibitory action at 10^{-5} M. Exogenously added 15-HPETE (10^{-6} to 10^{-5} M) enhanced the formation of 13-HODE and 9-HODE, and metyrapone (10^{-6} to 10^{-4} M), a cytochrome P450 inhibitor, did not modify the recovery of the monohydroxylated compounds. In contrast, pretreatment of cells with aspirin (10^{-4} to 10^{-3} M) inhibited the formation of 13-HODE (20–90%) and exerted a weaker effect on 9-HODE formation (20%). Finally, indomethacin (5.10^{-7} to 10^{-5} M) dependently inhibited 13-HODE synthesis, (64–90%) without any effect on 9-HODE recovery. Indomethacin inhibition of 13-HODE formation was at a similar extent in rat aortic tissue and in cultured rat aortic SMC.

Metabolism of [14C]linoleic acid by adherent rat aortic SMC

Short-term (1 h) incubation of 5×10^6 intact adherent rat aortic SMC with 12 nmol [14C]18:2 in the presence of albumin resulted in lower incorporation of radioactivity (19.2 ± 2.4% of the total applied) than that observed with [14C]20:4 (51.6 ± 6.2%). Incorporation preferentially occurred in neutral lipids (7.5%) and phosphatidylcholine (9.5%) (**Table 1**). Long-term (20 h) incubation of cells with [14C]18:2 resulted in the incorporation of 69.4 ± 4.8% of the radioactivity applied with a distribution essentially different from that of [14C]20:4

TABLE 1. Comparative incorporation of [14C]18:2 and [14C]20:4 in lipids of rat aortic smooth muscle cells

Incubation	Free Fatty Acid	Neutral Lipid	PC	PS	PI	PE	Total
	pmol/5 × 10⁶ cells						
1 Hour							
[14C]18:2	38	784	988	56	46	76	2100
[14C]20:4	57	673	3574	331	644	376	5700
20 Hours							
[14C]18:2	397	583	4046	402	215	1288	6940
[14C]20:4	344	632	2781	558	1414	3376	9300

Adherent smooth muscle cells (5×10^6) were incubated for 1 h or 20 h in the presence of 12 nmol of either [14C]18:2 or [14C]20:4 in a final volume of 5 ml. The cell layers were extensively washed with Tris-HCl medium plus serum albumin, and then extracted with chloroform–methanol with 0.05% BHT as described in Material and Methods. Results are the mean of two separate experiments with cells at the same passage. PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

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(93 ± 5%). The [^{14}C]18:2 was preferentially incorporated into the phosphatidylcholine fraction (55.8%) at that time compared to 27.7% for [^{14}C]20:4. [^{14}C]20:4 was recovered mainly in the phosphatidylethanolamine fraction (42.4%). The RP-HPLC analysis of radioactivity recovered from the incubation medium demonstrated that 98.3% was recovered as [^{14}C]18:2, and that [^{14}C]13-HODE and [^{14}C]9-HODE did not represent more than 0.6 and 0.4%, respectively (**Fig. 6A**).

Practically all (99%) of the material recovered as free acid in extracts of the cell layer was intact 18:2 (5.7% of the total radioactivity applied); monohydroxylated derivatives were undetectable (data not shown).

By contrast, RP-HPLC analysis of cell extracts after alkaline hydrolysis demonstrated the presence of significant amounts of [^{14}C]13-HODE (1.3%) and, to a lesser extent, 9-HODE (0.4%) (Fig. 6B). In addition, there was a slight accumulation of [^{14}C]13-HODE after 20 h incubation of intact cells in the presence of [^{14}C]18:2 in contrast to [^{14}C]9-HODE, which remained constant (**Fig. 7**).

The results summarized in **Table 2** clearly indicate that the major part (80.8%) of 13-HODE synthesized by intact, adherent SMC from 18:2 was not released into the incubation medium but remained within the cells in esterified form.

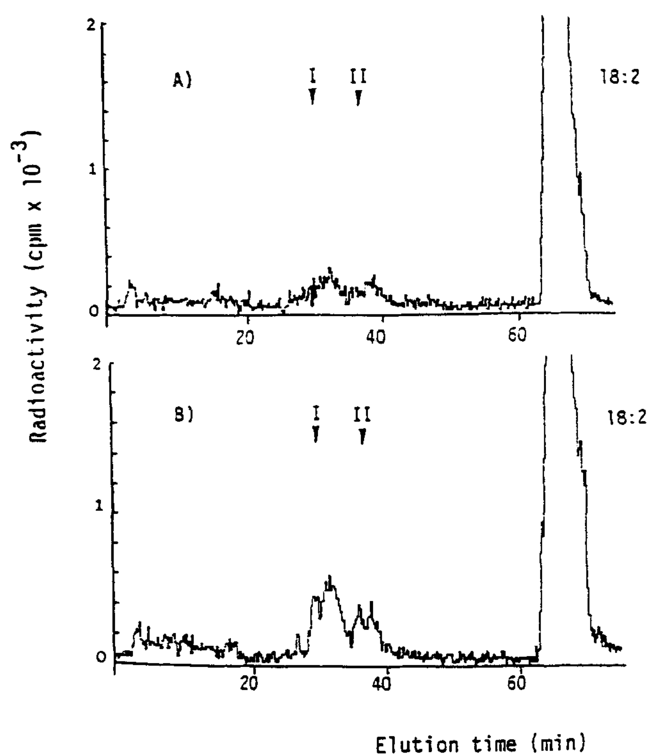

Fig. 6. RP-HPLC analysis of fatty acid compounds formed in incubation medium (A) or cell layer (B) during 20 h incubation of 5 × 10^6 rat adherent aortic SMC with 12 nmol [^{14}C]18:2. Cell layers and incubation media were separately extracted with chloroform-methanol and the cell layer extracts were submitted to hydrolysis as described. The products were analyzed by RP-HPLC; I, 13-HODE; II, 9-HODE.

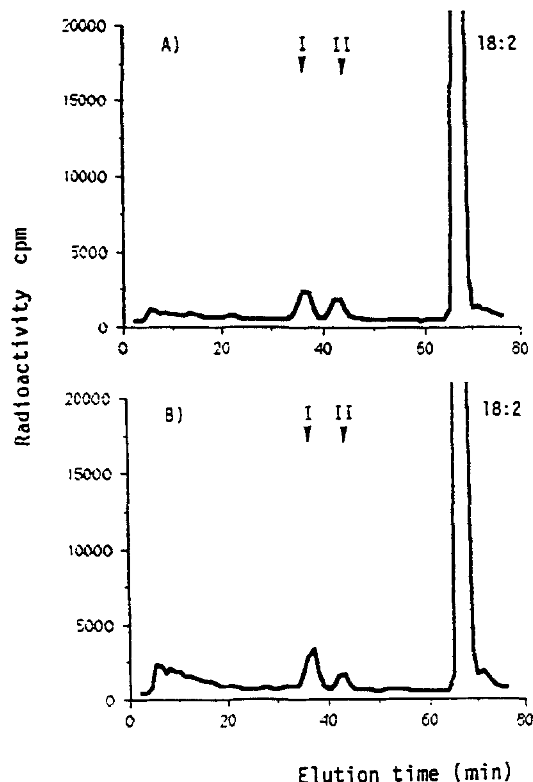

Fig. 7. Preferential accumulation of esterified [^{14}C]13-HODE in rat adherent aortic SMC with time. Rat adherent aortic SMC (5 × 10^6) were labeled during 1 h (A) or 20 h (B) with 12 nmol [^{14}C]18:2. The cell layers were extracted with chloroform-methanol and submitted to hydrolysis as described. The products were analyzed by RP-HPLC; I, 13-HODE; II, 9-HODE.

The treatment of [^{14}C]18:2-prelabeled cells with 10 μM calcium ionophore A23187 for 20 h stimulated the release of radioactivity from phosphatidylcholine and phosphatidylethanolamine, but was unable to produce any measurable secretion of [^{14}C]13-HODE and did not significantly modify the relative concentrations of the esterified [^{14}C]13-HODE and 9-HODE (data not shown).

Effects of linoleic acid on arachidonic acid incorporation and metabolism by SMC

The simultaneous incubation, or the short-term preincubation (1 h), of rat aortic SMC with 2 μM 18:2 neither modified the incorporation of exogenously added [^{14}C]20:4 nor its transformation to [^{14}C]6-keto $PGF_{1\alpha}$. Long-term (20 h) preincubation of cells with 18:2, despite any significant quantitative changes in the incorporation of exogenous [^{14}C]20:4 (1100 vs. 1140 pmol/10^6 cells), stimulated its transformation leading to an increase of [^{14}C]6-keto $PGF_{1\alpha}$ recovery in the incubation medium (**Table 3**).

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TABLE 2. Metabolism of [^{14}C]18:2 by adherent rat aortic smooth muscle cells

Sample	Total Metabolites	Free			Esterified		
		18:2	13-HODE	9-HODE	18:2	13-HODE	9-HODE
	pmol/5 × 10^6 cells						
Incubation medium	3600	3450	21.6	14.4	ND	ND	ND
Cell layer	6940	397	ND	ND	6425	90.2	27.8

Adherent smooth muscle cells (5 × 10^6) were incubated 20 h with 12 nmol [^{14}C]18:2. Cell layers and incubation media were separately extracted with chloroform–methanol as described, and subjected to alkaline hydrolysis when required. The products were analyzed by RP-HPLC. Results are the mean of two separate experiments. ND, not detected.

Effects of 13-HODE on arachidonic acid metabolism by SMC

Short-term (1 h) incubation of SMCs with increasing concentrations of 13-HODE (2–10 μM) did not produce measurable incorporation of the monohydroxylated derivative or alteration of [^{14}C]6-keto $PGF_{1\alpha}$ formation from exogenously added [^{14}C]20:4.

By contrast, significant incorporation of 13-HODE occurred after 20 h incubation of 2–10 μM 13-HODE. The esterification of 13-HODE in cell phospholipids (roughly 30 pmol/10^6 cells) was correlated with a slight stimulation of exogenous [^{14}C]20:4 transformation and [^{14}C]6-keto $PGF_{1\alpha}$ formation ($P < 0.04$) as shown in **Table 4.**

Finally, when 3–30 μM 13-HODE was added to [^{14}C]20:4-prelabeled cells, a stimulation of the release of radioactivity was observed only for the maximal concentration of 13-HODE (30 μM) ($P < 0.05$) without any changes in the recovery of [^{14}C]6-keto $PGF_{1\alpha}$ in the incubation medium (**Table 5**).

In contrast with the situation in cell culture, 30 μM 13-HODE enhanced [^{14}C]6-keto $PGF_{1\alpha}$ formation by [^{14}C]20:4-prelabeled rat aortic tissue (35.11 vs. 23.06 pmol/100 mg tissue).

DISCUSSION

The results indicate that rat aortic SMCs incorporate and metabolize [^{14}C]18:2 mainly to 13-HODE and, to a lesser extent, to 9-HODE. Chiral phase HPLC and the use of inhibitors clearly indicate that 13-HODE is essentially of enzymatic origin and that the PGH-synthase is involved. The predominant part of monohydroxylated compounds formed is incorporated into membrane phospholipids and not secreted; by this, they seem to be able to promote PGI_2 synthesis from exogenous sources. By contrast, exogenously added 13-HODE (3–30 μM) remains without any significant action on PGI_2 formation from endogenous sources despite a slight effect on arachidonic acid release.

Recent investigations have demonstrated that 18:2 is metabolized by vascular cells or tissue to monohydroxylated derivatives 13-HODE and 9-HODE by different enzymatic pathways (7–12). These compounds have been reported to exert metabolic and physiological effects. Intracellular levels of 13-HODE influence interactions of several circulating cells and tumor cells with the vascular endothelium (18), and exogenous 13-HODE differentially modulates prostacyclin production, when added to endothelial cells, as a function of cell origin (8, 11). The 9- and 13-HODE and their hydroperoxy precursors are weak vasoconstrictors of isolated rabbit blood vessels and inhibitors of arachidonic acid-induced platelet aggregation and release (19). Finally, 13-HODE is formed by rabbit atherosclerotic aortas (20) and together with 9-HODE accumulate in human atherosclerotic plaques (1, 2).

Under our experimental conditions, the incorporation of [^{14}C]18:2 in intact adherent SMC occurs to an extent similar to that of [^{14}C]20:4 after long-term incubation (20 h) (Table 1), but the distribution within phospholipids differs dramatically. [^{14}C]18:2 is incorporated preferentially into phosphatidylcholine in contrast to [^{14}C]20:4 which is present mainly in phosphatidylethanolamine and

TABLE 3. Effects of 18:2 on the [^{14}C]20:4 transformation and 6-keto $PGF_{1\alpha}$ formation

Experiment	Untransformed 20:4	6-Keto $PGF_{1\alpha}$
	pmol	
Control: 3 μM [^{14}C]20:4 in HAM	1983 ± 213	762 ± 105
3 μM [^{14}C]20:4 + 3 μM 18:2	1965 ± 126	714 ± 150
3 μM [^{14}C]20:4 after 20 h preincubation with 2 μM 18:2	1677 ± 135	1047 ± 90*

Smooth muscle cells (5 × 10^6) were incubated or preincubated with 18:2, then treated 20 min at 37°C with 3 μM [^{14}C]20:4. The whole incubation mixture was extracted with ethyl acetate and analyzed by TLC as described. Results are expressed as mean pmol formed ± SD from triplicate experiments; *, $P < 0.05$ (paired Student's t test).

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TABLE 4. Effects of 13-HODE preincubation on [^{14}C]20:4 transformation

Experiment	Total Transformed	Cyclooxygenase-Derived Products	6-Keto $PGF_{1\alpha}$
		pmol	
Control	1269 ± 147	1095 ± 132	822 ± 63
13-HODE, 2 μM, 20 h	1494 ± 78	1326 ± 63	1002 ± 168*

Smooth muscle cells (5 × 10^6) were preincubated with 13-HODE, then treated 20 min at 37°C with 3 μM [^{14}C]20:4. The incubation mixture was extracted with ethyl acetate and analyzed by TLC as described. Results are expressed as mean pmoles formed ± SD from four experiments; *, $P \leq 0.04$ (paired Student's t test).

phosphatidylinositol at that time. According to Spector et al. (6) the preincubation of cells with a low concentration (2 μM) of 18:2 does not modify the subsequent incorporation of [^{14}C]20:4 after either short (1 h) or long (20 h) term experiments.

Under these experimental conditions, we observed significant synthesis of 13-HODE of which the major part (80%) remained esterified within phospholipids.

On the other hand, exogenous 13-HODE was poorly incorporated into cultured SMC. Maximal incorporation (1.3%) was in the phospholipid fraction after 20 h incubation of 10 μM 13-HODE. This is in accordance with a report that [^{14}C]HODE incorporation into aortic tissue does not reach more than 11% of the amount of [^{14}C]18:2 incorporation (10). In addition, 13-HODE neither binds nor incorporates HUVEC under experimental conditions similar to those used in this study (18). There are several possible mechanisms for the formation of the esterified oxygenated forms of 18:2. As free 13-HODE was not well incorporated into intact adherent vascular smooth muscle cells, it seems likely that esterified 18:2 was oxygenated. Oxygenation of polyenoic fatty acids in various types of biological membranes has been previously reported for reticulocyte lipoxygenase (21).

The metabolic study of [^{14}C]18:2 by homogenates of aortic smooth muscle cells demonstrates that two major compounds are formed, recognized as 13-HODE and 9-HODE on the basis of their mass spectrometry characteristics. Stereochemical analysis and the use of inhibitors indicate that the major part of [^{14}C]18:2 transformation to 13-HODE occurs through PGH-synthase pathway. Additional experiments using cryoground rat aortic tissue confirm that PGH-synthase is also operating in the whole aorta as the formation (8.3% of the total [^{14}C]18:2 applied) of [^{14}C]13-HODE is inhibited by 90% in the presence of 10^{-5} M indomethacin (Fig. 5B). Several mammalian tissue homogenates (22–24), including particulate fractions of aortic tissue (9), convert 18:2 to 13-HODE through the PGH-synthase pathway. On the other hand, a lipoxygenase origin has been proposed for 13-HODE formation in vascular endothelial cells (7) and tissues (10, 20), and in porcine leukocytes (25). The discrepancies observed may be related to experimental conditions (homogenates vs. intact cells or tissue preparations), as we have previously reported a different enzymatic origin for 13-HODE formation by intact platelets and 100,000 g-supernatants (26). It is possible that similar mechanisms may be involved in SMC homogenates and intact adherent SMC. In addition, at least in rat aortic SMC homogenates, we (19) and others (20) have demonstrated that the formation of 11- and 15-HETE may occur through the PGH-synthase pathway (17, 27). Thus, even if we cannot exclude totally the participation of a lipoxygenase pathway in the formation of 13-HODE in homogenates of rat vascular SMC, the major part of this metabolite appears to be formed through the PGH-synthase activity.

TABLE 5. Effects of 13-HODE on endogenous 20:4 metabolism

Experiment	Total Eicosanoids	Prostaglandins
	cpm released	
Control	4710 ± 100	2430 ± 51
13-HODE		
3 μM	3327 ± 647	1800 ± 183
10 μM	4152 ± 260	2070 ± 160
30 μM	6500 ± 248*	2600 ± 95

[^{14}C]20:4-prelabeled cells (5 × 10^6) were treated for 1 h at 37°C with 13-HODE. The incubation medium was extracted with ethyl acetate and analyzed by TLC. Results are expressed as mean radioactivity (cpm) released ± SD from triplicate experiments; *, $P < 0.05$.

Considerable alterations of the fatty acid composition of HUVEC and fibroblasts in culture have been reported as a function of fatty acid composition of the serum used (5), and 18:2 (150 μM) has been shown to reduce arachidonate incorporation into HUVEC phospholipids (6). Under our experimental conditions, 2 μM 18:2 does not produce such alterations. Accordingly, coincubation of cells with 2 μM [^{3}H]20:4 and [^{14}C]18:2 results in the formation of [^{3}H]6-keto $PGF_{1\alpha}$ and [^{14}C]13-HODE at the same rate as in experiments with one fatty acid alone (not shown). Long-term incubation of cells with 2 μM 18:2 changes the metabolic transformation of exogenously added arachidonic acid and enhances the recovery of

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6-keto $PGF_{1\alpha}$ in the incubation medium. The mechanism of this activation remains puzzling. As we show that long-term incubation of SMCs with 18:2 results in the esterification of measurable amounts of monohydroxylated derivatives in membrane phospholipids, we can speculate that activation may depend on the presence of increasing amounts of esterified 13-HODE. In our experiments, a long-term (20 h) incubation of 13-HODE with aortic SMC results in a limited incorporation of the monohydroxylated compounds, and a slight but significant activation of the transformation of exogenously added [^{14}C]20:4 is observed.

However, in contrast to other studies (8, 11), we do not observe any significant alteration of PGI_2 production during short-term incubation of SMC with increasing amounts (1–30 μM) of 13-HODE. The fact that exogenously added 13-HODE is not actually incorporated into SMC in these experimental conditions (10, 18) may explain the lack of effect.

Taken together, our results demonstrate that rat aortic SMCs incorporate and metabolize 18:2 into monohydroxylated compounds and that PGH-synthase is involved. The derivatives formed are poorly secreted but are esterified in the cell phospholipids, namely phosphatidylcholine. The presence of 13-HODE inside the cell membrane seems to be able to facilitate the transformation of exogenous 20:4 to prostacylin. In contrast, exogenous 13-HODE is poorly incorporated and remains without any significant action in short-term incubation, in contrast with previous results using endothelial cells (8, 11).

13-HODE and 9-HODE accumulate in atherosclerotic plaques in humans (1, 2), and 13-HODE is actively synthesized from linoleic acid (20) by atherosclerotic rabbit aortas (22) in which it represents the main monohydroxylated-derived fatty acid together with the arachidonic acid-derived 15-HETE (28, 29). Whether or not this accumulation of monohydroxylated compounds in membrane phospholipids may represent part of any specific protective response of the vasculature to the atherosclerotic process remains to be established, in accordance with the weak capacity of such compounds to significantly modulate PGI_2 secretion. ■

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