Platelet–Neutrophil–Smooth Muscle Cell Interactions: Lipoxygenase-Derived Mono- and Dihydroxy Acids Activate Cholesteryl Ester Hydrolysis by the Cyclic AMP Dependent Protein Kinase Cascade†

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ABSTRACT: Fluid-phase interactions between hematologic cells and those of the vessel wall were studied in order to define a role for lipoxygenase products as cell signals in the control of vascular cholesterol metabolism. A functional parameter for hydroxy acids in this system has not been previously demonstrated. We report herein for the first time a biochemical effect of lipoxygenase-derived eicosanoids in the modulation of cholesterol metabolism in smooth muscle cells. Products of platelet–neutrophil interactions served as cell signals in vitro to modulate cholesterol metabolism. We demonstrate that 12-HETE, 12,20-DiHETE, and 12-HETE-1,20-dioic acid activate both lysosomal and cytoplasmic cholesteryl ester (CE) hydrolytic activities, although no effect was observed on CE synthetic (ACAT) activity. The platelet lipoxygenase product, 12-HETE, was the most effective stimulator of CE hydrolysis in the smooth muscle cell, and its conversion to 12,20-DiHETE and the dioic acid derivative by the neutrophils was not necessary for the activation of CE hydrolase. A 2-fold enhancement on CE hydrolysis occurred and was independent of any "cross-activation" by hydroxy acids on production of cyclooxygenase or other lipoxygenase products. The activation of cytoplasmic CE hydrolysis had a lesser cofactor dependence on bile salts in the presence of 12-HETE. This suggested a reduced requirement for surface-active agents in an enzyme–substrate interaction where enzymes are hydrolyzing insoluble lipid substrates. Moreover, 12-HETE induced an additive effect with several lipolytic hormones in the activation of CE catabolism. Dose-dependent, increased enhancement of lysosomal and cytoplasmic CE hydrolase activities by these hydroxy and dihydroxy acids was also cyclic AMP dependent since (1) there was no stimulatory effect on CE hydrolysis in the presence of an inhibitor of adenylate cyclase, which maintained cyclic AMP at basal levels, and (2) these acids enhanced cyclic AMP levels almost 2-fold in the cell, which paralleled the increased level of hydrolytic activities. Mechanistic data further revealed that the cytoplasmic CE hydrolase was activated by the cyclic AMP dependent protein kinase in the presence of these eicosanoid agonists. In other experiments, we found that incubation of 12-HETE with endothelial or smooth muscle cells did not result in production of 12,20-DiHETE or 12-HETE-1,20-dioic acid. Therefore, the cytochrome P-450 12-HETE ω-hydroxylase characteristically present in human neutrophils is absent from these arterial cells. Thus, under conditions of vascular damage, when smooth muscle cells gain access to cell components in the circulation, lipoxygenase products derived from platelet–neutrophil interactions could serve in an "antiatherosclerotic" capacity by enhancement of CE catabolism. Collectively, these novel findings, especially with platelets, represent heretofore unrecognized properties of eicosanoid metabolites formed through cell–cell interactions in the circulatory microenvironment.

Arterial injury results in exposure of subendothelium to circulating platelets which immediately adhere to the damaged surface (Ross, 1986). Proteins in the extracellular matrix of basement membrane, such as collagen, promote platelet adhesion, activation, and recruitment (Marcus, 1988a,b). Platelet activation, possibly induced by viruses, circulating immune complexes, or endothelial injury itself, culminates in formation of thrombin, fibrinogen polymerization, and release of platelet constituents which predispose to proliferation of smooth muscle cells (Ross, 1981). Moreover, collagen and thrombin are agonists which promote release and activation of eicosanoid precursors, intermediates, and metabolic end products in both platelets and endothelial cells (Marcus et al., 1980; Marcus, 1988a,b). The autacoids produced are capable of regulating intracellular metabolism (Hajjar et al., 1982, 1987; Hajjar & Weksler, 1983; Etingin et al., 1986).

Inflammation, thrombosis, and atherosclerosis may represent multicellular processes involving the aforementioned cells as well as smooth muscle. Close apposition of cells in the microenvironment provides the opportunity for transcellular exchange between eicosanoid precursors and intermediates including products which neither cell can produce individually

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(Marcus, 1986). We recently demonstrated that 12-hydroxyeicosatetraenoic acid (12-HETE),[1] the lipoxygenase product produced by activated platelets, is metabolized to 12,20-dihydroxyeicosatetraenoic acid (12,20-DiHETE) by unstimulated neutrophils via an ω-hydroxylation process involving the cytochrome P-450 system (Marcus et al., 1984, 1987). We also found that 12,20-DiHETE can be further metabolized via an NAD-dependent neutrophil dehydrogenase to 12-HETE-1,20-dioic acid (Marcus et al., 1988). Several biological functions have been attributed to 12-HETE, such as increased epidermal cell proliferation (Kupfer, 1980), chemotactic activity for human neutrophils in vitro (Goetzl et al., 1980), promotion of mononuclear procoagulant activity (Lorenzet et al., 1986), involvement in glomerular immune injury (Lianos et al., 1985), and aldosterone secretion (Nadler et al., 1987). Functional implications of 12,20-DiHETE and 12-HETE-1,20-dioic acid formation indicate that these autacoids, synthesized during the hemostatic process, and the inflammatory response accompanying it may modulate host defense mechanisms (Marcus, 1988a,b). Neutrophils are among the first cells recruited to areas of vessel injury which eventually lead to atherosclerotic lesions (Marcus, 1988a,b). Therefore, their participation in cell–cell interactions at these sites is of potential pathophysiological importance.

We previously found that endothelial cells were capable of regulating intracellular cholesterol metabolism in underlying smooth muscle (Hajjar et al., 1987), and that eicosanoids can regulate these metabolic events (Hajjar, 1985, 1986). Prostacyclin (PGI_2), a cyclooxygenase metabolite, was shown to stimulate the lysosomal (acid) cholesteryl ester (CE) hydrolase and the cytoplasmic (neutral) CE hydrolase (Hajjar et al., 1982). This neutral CE hydrolase is bound to cholesteryl ester enriched lipid droplets in atherosclerotic foam cells (Hajjar et al., 1983). We now describe a role and a mechanism for lipoxygenase-derived, aspirin-insensitive eicosanoids in the regulation of cholesterol metabolism. These autacoids, which are derived from stimulated platelet–unstimulated neutrophil interactions, appear to serve as "metabolic signals" in vitro for cells in the vasculature.

Materials and Methods

Materials. Cholesteryl [1-^{14}C]oleate (sp act. 55.6 mCi/mmol), [1-^{14}C]oleic acid (sp act. 59.0 mCi/mmol), and [1-^{14}C]oleoyl-CoA (sp act. 57.4 mCi/mmol) were purchased from Du Pont-New England Nuclear, Boston, MA. Unlabeled oleic acid, cholesterol, and cholesteryl oleate were obtained from Applied Science Laboratory, Inc., State college, PA. Phospholipids and bile salts were purchased from Supelco, Inc., Bellefonte, PA. The following were obtained from Sigma, St. Louis, MO: acetylsalicyclic acid (ASA), bovine serum albumin (fraction V), 2′,5′-dideoxyadenosine, epinephrine, glucagon, isoproterenol, protein kinase (bovine heart), protein kinase inhibitor (type II, bovine heart), and 2-mercaptoethanol. All other chemicals were reagent grade. Tissue culture media, antibiotics, and amino acids were purchased from Flow Laboratories, Inc., McLean, VA. Screened fetal calf sera (heat inactivated) were obtained from M. A. Bioproducts, Walkersville, MD, and tissue culture supplies were purchased from Becton-Dickinson Labware, Oxnard, CA. Silica gel G thin-layer chromatography (TLC) plates were obtained from Analtech, Inc., Newark, DE.

12-HETE was purchased from Cayman Chemicals, Ann Arbor, MI. 12,20-DiHETE and 12-HETE-1,20-dioic acid were prepared by chemical synthesis as described elsewhere (Marcus et al., 1988). 5,8,11,14-Eicosatetraynoic acid (ETYA) was purchased from Hoffmann-La Roche, Nutley, NJ.

Cell Culture. Culture medium used to propagate bovine arterial smooth muscle cells consisted of Eagle's minimum essential medium (MEM) supplemented with 20% fetal calf serum, 1% glutamine, 1% penicillin–streptomycin, and 1% Fungizone. Cells were cultured in Falconware in a 37 °C incubator containing 5% CO_2–95% air.

Smooth muscle cells were obtained from bovine arteries as described previously (Hajjar et al., 1985). Maintenance, propagation, and harvesting of these cells have been previously described (Hajjar et al., 1985). Smooth muscle cells were subpassaged on an average of three to four times prior to use.

Experimental Design and Biochemical Methods. To assess CE metabolic activity in response to various concentrations of hydroxy acids, 2×10^5 smooth muscle cells were placed per well in six-well, 35 mm diameter cluster plates in MEM with 10% fetal calf serum. Cells were allowed to adhere for 24–48 h. Cell monolayers were then washed twice with PBS. One milliliter of MEM was then added to each well. Aspirin was added to designated wells at a final concentration of 0.1 mM for 20 min prior to eicosanoid addition in order to inhibit the production of cyclooxygenase metabolites (Hajjar et al., 1987). Cells were washed free of aspirin prior to the experiment. Various concentrations of eicosanoids were prepared in ethanol. Control wells contained buffer plus a similar volume of ethanol. Except for the protein kinase experiments where cell homogenates were prepared first prior to addition of effectors, eicosanoids (100 ng/mL) were added to cell monolayers for 30 min (the optimal concentration and time period for activation, as determined in preliminary studies) at 37 °C prior to harvesting and assay of enzyme activities. When required, 2′,5′-dideoxyadenosine (DDA), an adenylate cyclase inhibitor, was added at a final concentration of 0.1 mM for 30 min prior to addition of eicosanoids. This was done to maintain cyclic AMP at basal levels following addition of the eicosanoids (Hajjar et al., 1982). The cells were exposed to DDA during the entire course of the experiment.

Isobutylmethylxanthine (MIX), a cyclic AMP phosphodiesterase inhibitor (Hajjar et al., 1982; Hajjar & Weksler, 1983), was also added to all wells at a final concentration of 1.0 mM for 20 min prior to eicosanoid addition in order to prevent cyclic AMP breakdown and thus maximize intracellular cyclic AMP levels (Hajjar et al., 1982). This inhibitor was prepared in HEPES–Hanks buffer (pH 7.0) by sonication over ice for 15 min.

All experiments were terminated after 30 min. Supernatants were recovered for PGI_2 analysis. Cells were washed twice with PBS over ice, after which 1 mL of ice-cold isotonic sucrose buffer (250 mM sucrose, 5 μM EDTA, and 50 mM Tris-HCl, pH 7.0) was added to each well. Cells were harvested with a rubber policeman, and aliquots were taken for assays of CE metabolic activities, cyclic AMP levels, and protein determinations (Hajjar et al., 1982, 1987). Since there were no effects of the hydroxy acids on protein synthesis or smooth

[1] Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; ACEH, acid (lysosomal) cholesteryl ester hydrolase; ASA, aspirin; CE, cholesteryl esters; DDA, dideoxyadenosine; ETYA, 5,8,11,14-eicosatetraynoic acid; 12-HETE, 12(*S*)-hydroxy-5,8-*cis*,10-*trans*,14-*cis*-eicosatetraenoic acid; 12,20-DiHETE, 12(*S*),20-dihydroxy-5,8,10,14-eicosatetraenoic acid; 12-HETE-1,20-dioic acid, 12(*S*)-hydroxy-5,8,10,14-eicosatetraen-1,20-dioic acid; HPLC, high-performance liquid chromatography; MEM, minimum essential medium; NaTC, sodium taurocholate; NCEH, neutral (cytoplasmic) cholesteryl ester hydrolase; PGI_2, prostacyclin; PK, protein kinase; PKI, protein kinase inhibitor.

muscle cell proliferation in vitro during the experimental time period, we expressed the data on a cell protein basis. Protein determinations were done with BSA as standard (Lowry et al., 1951).

Preparation of Cell Homogenates. For assay of enzyme activities following addition of hydroxy acids, protein kinase, and protein kinase inhibitor, cell monolayers were washed twice with 2.0 mL of ice-cold isotonic sucrose buffer. Cells were then harvested by gentle scraping with a rubber policeman in 2.0 mL of ice-cold sucrose buffer (Hajjar et al., 1982, 1987) and homogenized over ice for 1 min. Cell homogenates were prepared prior to addition of protein kinase, protein kinase inhibitor, and 0.1 mM MgATP since protein kinase and protein kinase inhibitor do not effectively traverse intact cell membranes (Hajjar, 1986). Aliquots were taken for assay of lysosomal and cytoplasmic CE hydrolase activities and protein content.

Lysosomal (Acid) CE Hydrolase Activity. Lysosomal CE hydrolase activity was assayed in smooth muscle cells at pH 3.9 (Haley et al., 1980). Cholesteryl [1-^{14}C]oleate was used as substrate, prepared as an egg lecithin–digitonin dispersion (Haley et al., 1980). Substrate was incubated with cell homogenates (ca. 150 μg of protein per assay tube) for 60 min at 37 °C. The final reaction mixture contained enzyme, 12.7 μM cholesteryl [1-^{14}C]oleate in 1.3 mM egg lecithin, 50 mM sodium acetate buffer (pH 3.9), 2.0 mM sodium taurocholate, and 0.005% digitonin. Substrate blanks (which contained sucrose buffer in place of enzyme) were run in parallel. Released oleic acid was separated from unhydrolyzed cholesteryl oleate by adding 16.5 mL of methanol–chloroform–heptane (1.4/1.25/1, v/v/v) and 3.5 mL of 50 mM K_2CO_3–H_3BO_3 buffer (pH 10) (Hajjar et al., 1983). The preparation was shaken and then centrifuged to separate the organic layer and aqueous phases (Hajjar et al., 1983). Aliquots (1 mL) of released radiolabeled oleic acid in the upper aqueous phase were counted. Quenching was corrected by automatic external standardization. Efficiency of extraction of radiolabeled oleic acid averaged 80%.

Cytoplasmic (Neutral) CE Hydrolase Activity. Cholesteryl [1-^{14}C]oleate was prepared in a mixed micelle of egg lecithin/sodium taurocholate/cholesteryl oleate as substrate to assay cytoplasmic CE hydrolase activity in arterial homogenates (Hajjar et al., 1983).

For the enzyme assay, 50 μL of micellar cholesteryl [1-^{14}C]oleate was added to 800 μL of 100 mM potassium phosphate buffer (pH 7.0) containing 0.05% BSA and 150 μL of cell homogenate (ca. 200 μg of protein) for 60 min. Final reaction mixture contained enzyme, 6.0 μM cholesterol [1-^{14}C]oleate, 23.7 μM egg lecithin, 12.5 μM sodium taurocholate (ca. 10 μg/mL), 0.04% serum albumin, and 85 mM potassium phosphate buffer, pH 7.0 (Hajjar et al., 1983). Released oleic acid was separated and counted as described in the previous section.

Activation of Cholesteryl Esterase by Agonists. Activation of cytoplasmic CE hydrolase by several agonists (protein kinase, glucagon, epinephrine, isoproterenol) was carried out by a preincubation step as previously described (Hajjar et al., 1983). Effectors were added in a 2-fold higher concentration for the preincubation step and were then diluted 1/1 (v/v) with substrate prior to the assay of the CE hydrolase activities (Hajjar et al., 1983).

Acyl-CoA:Cholesterol O-Acyltransferase Activity (ACAT). Activity of microsomal ACAT was assayed by measuring the synthesis of cholesteryl oleate from radioactive oleoyl-CoA and exogenous free cholesterol (Hajjar et al., 1981). Oleoyl-CoA and cholesterol were prepared as unilamellar liposomes as described previously (Hajjar et al., 1981).

Assay for Prostacyclin (PGI_2) Production. Following exposure of confluent cell monolayers to MEM (no serum) and ASA, DDA, or the hydroxy acids for 30 min, supernatants were recovered for measurement of PGI_2 release with the use of a radioimmunoassay for 6-keto-$PGF_{1\alpha}$ (Hajjar et al., 1982; Hajjar & Weksler, 1983). Prior to initiation of the experiment, cells were washed twice with PBS to remove any residual serum.

Assay of Intracellular Cyclic AMP. Intracellular levels of cyclic AMP in arterial smooth muscle cells were measured following addition of hydroxy and dihydroxy acids by radioimmunoassay as described previously (Hajjar et al., 1982; Hajjar & Weksler, 1983). Cells were confluent at the start of the 30-min experiment. Only MEM (no serum) was present.

Assay for 12,20-DiHETE Production by Endothelial or Smooth Muscle Cells. Human umbilical cord endothelial cells (fifth passage) or bovine arterial smooth muscle cells (second passage) from ten T 75-cm^2 flasks were treated with 1 mM aspirin for 30 min. Endothelial or smooth muscle cells were removed from the flasks with collagenase–EDTA, and then resuspended in 2.8 mL of buffer (5 mM HEPES, 140 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, pH 7.5) (Marcus et al., 1982). For each assay, 500 μL of cell suspensions containing 3×10^6 cells was used. Total volume in each Falcon tube was adjusted to 1 mL with buffer. Cells were preincubated for 1 min at 37 °C (with stirring), at which time 12-HETE (sodium salt) was added to a final concentration of 4.4 μM (Marcus et al., 1987). Reactions were stopped at 15-, 30-, 60-, and 120-min intervals by addition of 1.5 mL of acetone and placement on ice. Samples were extracted, and reversed-phase HPLC was carried out on a μBondapak C18 column. The eluting solvent was methanol–water–acetic acid (70/30/0.10, v/v/v), pH 6.1 (Marcus et al., 1988).

Statistical Analyses. Comparisons of cyclic AMP levels or enzyme activities following treatment of the cells with the hydroxy acids, enzyme inhibitors, or protein kinase were analyzed with the use of single-factor analyses of variance. If analyses revealed significant interaction between additive factors, then simple-effects analysis was performed.

Results and Discussion

12-HETE is the major lipoxygenase-derived hydroxy acid produced by activated platelets (Marcus et al., 1984, 1987, 1988), arterial endothelial cells (Hajjar et al., 1987), and smooth muscle cells (Hajjar et al., 1987). Of these cell types, platelets are a more abundant source of 12-HETE than smooth muscle or endothelial cells (Marcus et al., 1984; Hajjar et al., 1987). The specific interaction of activated platelets with unstimulated neutrophils results in production of two metabolites of 12-HETE, viz., 12,20-DiHETE and 12-HETE-1,20-dioic acid (Marcus et al., 1987, 1988). As shown in Figure 1 and Tables I and II, all of these hydroxy acids possess biological activity in that they activate CE hydrolysis in a dose-dependent manner by raising cyclic AMP levels in the cell. Interestingly, they do not exert a significant effect on CE synthesis.

As a corollary to these results, it was important to determine whether unstimulated endothelial or smooth muscle cells had the cytochrome P-450 ω-hydroxylase activity necessary to convert platelet-derived 12-HETE to 12,20-DiHETE, as in the case of neutrophils (Marcus et al., 1987). When we incubated aspirin-treated endothelial or smooth muscle cells with 12-HETE for periods of up to 2 h, 12,20-DiHETE formation

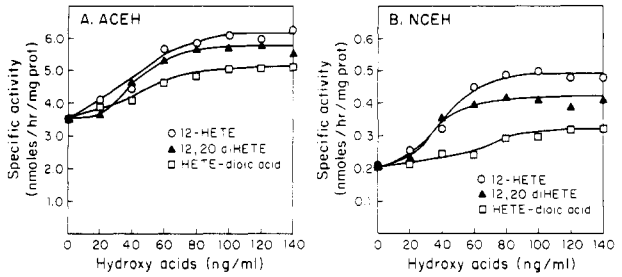

FIGURE 1: Concentration-dependent effects of 12-HETE, 12,20-DiHETE, and 12-HETE-1,20-dioic acid on lysosomal ACEH (panel A) and cytoplasmic NCEH (panel B) activities in arterial smooth muscle cells. Variable doses of the hydroxy acids were added to washed cells for 30 min at 37 °C, which was the optimal time period for enzyme activation. Cells had been previously treated with 0.1 mM aspirin for 20 min to inhibit cyclooxygenase activity; the cells were washed prior to hydroxy acid treatment. After the 30-min activation time period with the hydroxy acids in MEM (no serum), cells were harvested on ice and enzymic activities assessed (Materials and Methods).

Table I: Cyclic AMP Dependent Activation of CE Hydrolase by Hydroxy Acids[a]

	nmol h^{-1} (mg of protein)$^{-1}$ [b]		
	ACEH	NCEH	ACAT
control	3.5 ± 0.2[a,b,c]	0.22 ± 0.01[i,j,k]	5.0 ± 0.9
12-HETE	6.3 ± 0.3[a,d]	0.42 ± 0.04[i,l]	5.4 ± 0.6
12,20-DiHETE	5.8 ± 0.2[b,e]	0.38 ± 0.05[j,m]	6.3 ± 0.3
12-HETE-1,20-dioic acid	4.9 ± 0.4[c,f]	0.35 ± 0.01[k,n]	7.2 ± 1.4
DDA	3.8 ± 0.3	0.26 ± 0.02	
12-HETE + DDA	3.7 ± 0.2[d]	0.27 ± 0.03[l]	
12,20-DiHETE + DDA	3.7 ± 0.3[e]	0.29 ± 0.02[m]	
12-HETE-1,20-dioic acid + DDA	3.8 ± 0.2[f]	0.28 ± 0.01[n]	
ASA	3.1 ± 0.4[g]	0.21 ± 0.01[o]	5.6 ± 0.3
ETYA	3.6 ± 0.1[h]	0.27 ± 0.01[p]	5.2 ± 0.3
12,20-DiHETE + ASA	4.6 ± 0.7[g]	0.43 ± 0.06[o]	4.7 ± 1.5
12,20-DiHETE + ETYA	4.4 ± 0.3[h]	0.37 ± 0.02[p]	5.6 ± 0.4

[a] Experimental protocols are described in detail under Materials and Methods. Final concentrations: 100 ng/mL hydroxy acids, 0.1 mM ASA, 0.1 mM DDA, and 0.1 mM ETYA. These were the optimal concentrations, as previously determined from preliminary experiments. Values indicated by the same superscript Roman letters are significantly different ($p < 0.05$). [b] Mean ± SD. Three separate experiments were done, each in quadruplicate.

Table II: Effects of Hydroxy Acids on Cyclic AMP and PGI_2 Production

	intracellular cyclic AMP (pmol/mg of protein)[b]	PGI_2 release (ng/mg of protein)[b]
untreated	0.9 ± 0.1[a,b,c]	5.0 ± 0.5[d]
ASA	ND	1.3 ± 0.3[d]
DDA	0.2 ± 0.1[a,b,c]	4.9 ± 0.9
12-HETE	1.9 ± 0.2[a]	6.8 ± 1.3
12,20-DiHETE	1.6 ± 0.1[b]	7.8 ± 1.7
12-HETE-1,20-dioic acid	1.3 ± 0.1[c]	9.5 ± 3.4

[a] Experimental protocols are described in detail under Materials and Methods. Final, optimal concentrations: 100 ng/mL hydroxy acids, 0.1 mM ASA, and 0.1 mM DDA. Values indicated by the same superscript Roman letters are significantly different ($p < 0.05$). [b] Mean ± SD. Two separate experiments were done, each in triplicate. ND = not done.

was not detectable by reversed-phase HPLC (data not shown). There are two implications of these results: First, the cytochrome P-450 ω-hydroxylase activity is specific for the neutrophil as compared to the endothelial or smooth muscle cell. Second, at sites of vascular damage where smooth muscle cells are exposed, 12,20-DiHETE produced by "interacting" platelets and neutrophils can directly exert biological effects on smooth muscle as described herein.

Table III: Cyclic AMP Dependent Protein Kinase Activation of Neutral CE Hydrolase by Hydroxy Acids in Smooth Muscle Cell Homogenates[a]

	nmol h^{-1} (mg of protein)$^{-1}$ [b]	
	ACEH	NCEH
untreated homogenates	2.2 ± 0.4[a,b,c]	0.13 ± 0.05[m,n,o]
protein kinase inhibitor (PKI)	1.8 ± 0.6[d,e,f]	0.17 ± 0.08
protein kinase (PK)	1.4 ± 0.8[g,h,i]	0.24 ± 0.08[s,t,u]
12-HETE	3.4 ± 0.3[a]	0.28 ± 0.02[m]
12,20-DiHETE	3.1 ± 0.1[b]	0.25 ± 0.03[n]
12-HETE-1,20-dioic acid	3.2 ± 0.4[c]	0.27 ± 0.01[o]
12-HETE + PKI	3.2 ± 0.1[d]	0.17 ± 0.04[p]
12,20-DiHETE + PKI	2.8 ± 0.1[e]	0.21 ± 0.03[q]
12-HETE-1,20-dioic acid + PKI	3.4 ± 0.6[f]	0.18 ± 0.01[r]
12-HETE + PKI + PK	3.2 ± 0.3	0.46 ± 0.05[p]
12,20-DiHETE + PKI + PK	3.3 ± 0.2	0.40 ± 0.01[q]
12-HETE-1,20-dioic acid + PKI + PK	3.0 ± 0.1	0.42 ± 0.03[r]
12-HETE + PK	3.3 ± 0.3[g,j]	0.41 ± 0.02[s,v]
12,20-DiHETE + PK	3.7 ± 0.4[h,k]	0.39 ± 0.08[t,w]
12-HETE-1,20-dioic acid + PK	3.1 ± 0.6[i,l]	0.37 ± 0.01[u,x]
12-HETE + DDA + PK	2.1 ± 0.3[j]	0.20 ± 0.01[v]
12,20-DiHETE + DDA + PK	2.0 ± 0.4[k]	0.21 ± 0.03[w]
12-HETE-1,20-dioic acid + DDA + PK	1.9 ± 0.4[l]	0.16 ± 0.05[x]

[a] Experimental protocols are described in detail under Materials and Methods. Final, optimal concentrations: 100 ng/mL hydroxy acids, 10 μg/mL PKI, 50 μg/mL PK, and 0.1 mM DDA. Values indicated by the same superscript Roman letters are significantly different ($p < 0.05$).

In the metabolic cascade from 12-HETE to the hydroxylated 12,20-DiHETE to the ω-carboxy 12-HETE-1,20-dioic acid, there is a progressive decrease in the pronounced stimulatory effect on cyclic AMP (Table II). As shown in Table I or Figure 1, this results in a parallel decrease in stimulation of lysosomal (ACEH) and cytoplasmic (NCEH) CE hydrolase activities in the smooth muscle cell (ACEH, 80% → 66% → 40%; NCEH, 91% → 75% → 59%). In the case of NCEH activity, where we observed for the first time that the hydroxy acids stimulate NCEH through the cyclic AMP dependent protein kinase (Table III), there was also a reduced effect on lipolysis in parallel with the stepwise metabolic hydroxylation to 12,20-DiHETE and then its ω-carboxylation to dioic acid.

It is especially noteworthy that the hydroxy acid effects observed on CE metabolism occurred in the absence of parallel stimulation of a cyclooxygenase or another lipoxygenase metabolite. Evidence for this conclusion was that 12,20-DiHETE induced identical effects whether or not cyclooxygenase (ASA) or lipoxygenase inhibitors (ETYA) were present (Table I). Importantly, the hydroxy acids had no effect whatsoever on PGI_2 release in this cell system (Table II).

The 40–80% increase in ACEH activity and the 59–91% stimulation of NCEH activity by the hydroxy acids are great enough to substantially reduce CE accumulation in arterial smooth muscle cells (Hajjar et al., 1981, 1983). Our results show that the hydroxy acids studied herein activated ACEH and NCEH of the CE cycle by also elevating cellular cyclic AMP; this stimulatory effect of the hydroxy acids was not observed in cells pretreated with the adenylate cyclase inhibitor, DDA (Table I). Cyclic AMP activation has been shown previously to enhance cholesterol efflux from arterial smooth muscle cells (Numano et al., 1976; Tertov et al., 1987). Our results, coupled with our observations that all three hydroxy acids activate the *cytoplasmic* CE hydrolase via the cyclic AMP dependent protein kinase (Table III), suggest that the hydroxy acids act as "metabolic signals" to induce secondary

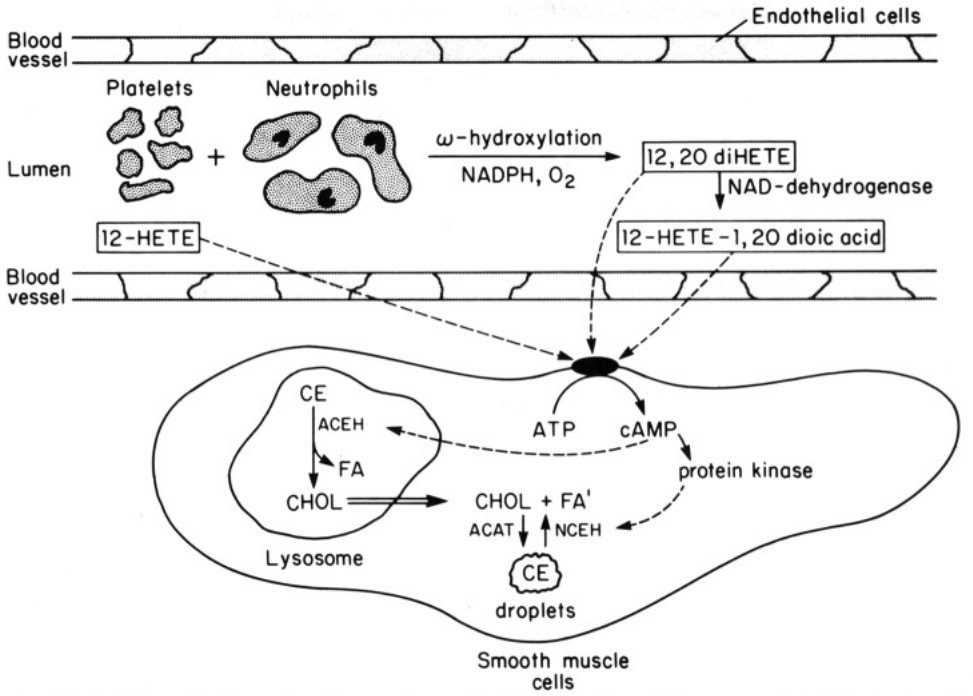

FIGURE 2: Hypothetical model depicting platelet–neutrophil–smooth muscle cell interactions which can regulate cholesterol metabolism. Stimulated platelets synthesize 12-HETE, which is then metabolized by a cytochrome P-450 ω-hydroxylase in unstimulated neutrophils to 12,20-DiHETE. Neither endothelial nor smooth muscle cells metabolize 12-HETE to 12,20-DiHETE. This hydroxy acid is further processed to 12-HETE-1,20-dioic acid by an NAD-dependent neutrophil dehydrogenase. 12-HETE, 12,20-DiHETE, and 12-HETE-1,20-dioic acid, which can traverse the endothelial cell barrier, stimulate (dotted lines) cyclic AMP production in vascular smooth muscle cells. Cyclic AMP can, in turn, increase lysosomal (acid) CE hydrolase (ACEH) activity or, through the cyclic AMP dependent protein kinase, can activate cytoplasmic (neutral) CE hydrolase activity (NCEH). We speculate that these hydroxy acids, through their induction of lipolytic activity, may promote breakdown of CE droplets in smooth muscle cells following release of hydroxy acids during platelet–neutrophil interactions.

messengers to activate intracellular CE hydrolysis. Interestingly, only the activation of NCEH by 12-HETE, 12,20-DiHETE, and 12-HETE-1,20-dioic acid occurs through the MgATP–protein kinase which is dependent on cyclic AMP (Table III). This new finding was demonstrated by experiments in which preincubation of the cells with DDA, followed by addition of protein kinase and the hydroxy acids, blocked activation of NCEH (Table III). We conclude from these experiments that the sequence of metabolic events is as follows: 12-HETE > 12,20-DiHETE > 12-HETE-1,20-dioic acid → stimulate cyclic AMP → stimulate protein kinase activity → NCEH activation → increase CE hydrolysis. Additional evidence to support this metabolic sequence is based on our observations that preincubation of cell homogenates with a protein kinase inhibitor blocked NCEH activation by 12-HETE. The activation could subsequently be restored by protein kinase (Table III). These results provide additional evidence that the activity of NCEH in smooth muscle cells is regulated by covalent phosphorylation as in hormone-sensitive tissues. Definitive proof will require the demonstration that phosphate is incorporated into the active site of the enzyme.

We also observed that the activation of cytoplasmic CE hydrolase (NCEH), but not lysosomal CE hydrolase (ACEH), had a lesser cofactor requirement for sodium taurocholate in the presence of 12-HETE (Table IV). These findings suggest a reduced need for surface-active agents such as bile salts during enzyme–substrate interactions where lipid-insoluble substrates are interacting with the enzyme's active site. Since 12-HETE can penetrate cells, it may serve to substitute for bile salts in the assay of cytoplasmic CE hydrolase activity, an enzyme which is solubilized in the cytoplasm (Hajjar et al., 1983). In the case of the lysosomal enzyme (ACEH), 12-HETE cannot substitute for sodium taurocholate since this bile salt is necessary to decrease lysosomal latency, thereby releasing membrane-bound ACEH activity for assay (Haley et al., 1980). 12-HETE also induced an additive effect with two lipolytic hormones (glucagon, epinephrine) and isoproterenol, a β-adrenergic receptor agonist (Table V).

Table IV: Effect of Enzyme Cofactor Concentration on 12-HETE-Induced Activation of CE Hydrolysis[a]

	nmol h^{-1} (mg of protein)$^{-1}$ [b]	
	ACEH	NCEH
NaTC (μg/mL)		
0	0.9 ± 0.1	0.05 ± 0.02
1	2.8 ± 0.2	0.11 ± 0.01[b]
5	3.7 ± 0.2	0.18 ± 0.02[c]
10	3.6 ± 0.4[a]	0.21 ± 0.02[d]
NaTC (μg/mL) + 100 ng/mL 12-HETE		
0	1.3 ± 0.1	0.10 ± 0.01
2	1.9 ± 0.1	0.44 ± 0.02[b]
5	3.6 ± 0.2	0.39 ± 0.02[c]
10	6.9 ± 0.2[a]	0.46 ± 0.03[d]

[a] Experimental protocol is described in detail under Materials and Methods. Variable concentrations of sodium taurocholate (NaTC) were used in the preparation of the liposomal substrate (ACEH) and micellar substrate (NCEH) to assay the enzyme activities. Smooth muscle cells were activated with a fixed amount of 12-HETE (100 ng/mL) (see text) prior to the assay of the enzyme activities. Values indicated by the same superscript Roman letter are significantly different ($p < 0.05$). [b] Mean ± SD. Two separate experiments were done, each in triplicate.

In previous studies, these agents have been demonstrated to enhance cytoplasmic CE catabolism by elevating intracellular levels of cyclic AMP (Hajjar et al., 1983; Hajjar, 1986). PGI_2, another eicosanoid which can enhance CE catabolism, did not exert a similar effect because MIX could not be used in these particular assays; hence, the lack of stability of PGI_2 at physiological pH was a contributory factor

Table V: Additive Effect of 12-HETE on the Activation of Cytoplasmic CE Hydrolysis by Lipolytic Hormones and a β-Adrenergic Receptor Agonist[a]

	% activation of NCEH activity[b]	
	-12-HETE	12-HETE
no additions	0	106
5 μM epinephrine	85	125
10 μM glucagon	105	215
5 μM isoproterenol	223	328

[a] Experimental protocol for assaying NCEH activity is described in the text. Base-line NCEH activity (no additions, minus 12-HETE) was 0.25 ± 0.03 nmol h^{-1} (mg of protein)$^{-1}$. Concentration of 12-HETE used in this experiment was 100 ng/mL. Confluent cells (3×10^5 cells/well) were washed free of serum, harvested, and then homogenized over ice. Effectors were added in the presence or absence of 12-HETE for 30 min at 37 °C in a Dubnoff shaking water bath; after which, NCEH activity was assayed (see text). Unlike the experiments described in Tables I–IV, the experiments described herein were not carried out in the presence of MIX, because this phosphodiesterase inhibitor interfered with the solubility of the agonists used. [b] Average of two separate analyses, each done in triplicate.

to the negative effect observed. All three of these compounds have been shown to enhance CE catabolism (Hajjar et al., 1983; Hajjar, 1986), and from this study, 12-HETE appears to increase the lipolytic effect of these agonists.

It has been suggested that lipoxygenase products of the eicosanoid pathway play a pivotal role in "stimulus–secretion coupling events" in various cells, particularly those under hormonal control (Dix et al., 1984; Sullivan & Cooke, 1985). Cyclic AMP and the cyclic AMP dependent protein kinase, in particular, have been shown to be involved in hormone-stimulated steroidogenesis, in CE hydrolysis in macrophages (Khoo et al., 1981), and in aortae of hypercholesterolemic animals or in lipid-laden smooth muscle cells (Hajjar et al., 1983). The hypothesis has also been proposed that lipoxygenase metabolites elevate cyclic AMP and, thus, redirect cholesterol toward steroidogenesis or toward transport of CE for further metabolism to pregnenolone in testicular Leydig cells (Dix et al., 1984). Mathur et al. have shown that cholesterol-rich resident mouse peritoneal macrophages increase their production of lipoxygenase products (principally 12-HETE) by over 2-fold as compared to macrophages which are not lipid-laden (Mathur et al., 1985). This increase in 12-HETE may result from "injury" to these cells as a consequence of lipid overload. Such events may explain the initial rise in total CE hydrolysis during the lipid loading process (enzyme activation) that has been reported in a variety of cell types (Brecher et al., 1977; Hajjar et al., 1981). This phenomenon diminishes as the cells become metabolically overloaded with lipid and thence they degenerate into the atherosclerotic foam cell (Hajjar et al., 1981). Alternatively, in studies utilizing hormone-sensitive tissues, such as aorta (Eldor et al., 1982), there is a decrease in eicosanoid synthesis in the lipid-laden monocyte (Saito et al., 1986) and smooth muscle cell (Larrue et al., 1982; Pomerantz & Hajjar, 1989). This has been correlated with altered phospholipase A_2 activity (Pomerantz & Hajjar, 1989) and cyclic AMP levels (Numano et al., 1976).

Our demonstration that lipoxygenase-catalyzed, aspirin-insensitive eicosanoids possess biological activity is the first report which links lipoxygenase metabolites, i.e., 12-HETE, with an antiatherosclerotic process, such as increased CE catabolism. These results have been summarized in a hypothetical model depicted in Figure 2. We propose that hydroxy acids can serve as "intracellular signals" in the regulation of cholesterol metabolism by activating the cyclic AMP dependent protein kinase cascade. In addition, acid and neutral lipases can hydrolyze esterified lipid found in lysosomal compartments and in cytoplasmic lipid droplets of the cell (Hajjar et al., 1983). For this reason, we propose a similar role for hydroxy acids in the regulation of triacylglycerol hydrolysis. Owing to the hormone sensitivity of adipose and adrenal tissue, both of which store esterified lipid, additional studies are warranted which attempt to define the role of eicosanoid metabolites derived from specific interactions of blood cells with tissues that primarily store lipid. Since activated platelets and neutrophils in close apposition interact during the thrombotic and inflammatory processes (Marcus et al., 1987, 1988), metabolic interactions of biologically active eicosanoids produced as a result of these interactions could contribute to regulation of lipid hydrolysis in vivo.

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Three Novel Oligosaccharides with the Sialyl-Le^a Structure in Human Milk: Isolation by Immunoaffinity Chromatography†

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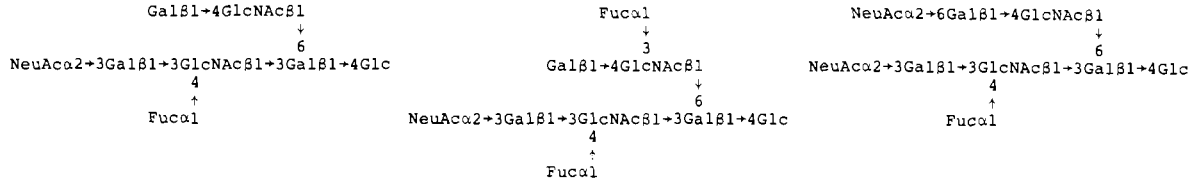
ABSTRACT: We have determined the structures of three novel oligosaccharides isolated from human milk using the monoclonal antibody MSW 113. These oligosaccharides were purified by affinity chromatography on a column of the immobilized monoclonal antibody and by high-performance liquid chromatography. From the results of 500-MHz ^{1}H NMR spectroscopy and fast atom bombardment–mass spectrometry, their structures were deduced to be



These oligosaccharides bound to MSW 113 to nearly the same extent as sialyl-Le^a hexasaccharide but bound to another sialyl-Le^a structure-directed monoclonal antibody, NS 19-9, only weakly.

Many of the sialyloligosaccharides that occur in human milk have been found to correspond to the carbohydrate moieties of glycolipids and glycoproteins on cell surfaces. Therefore, they have been used as models for studies on the acceptor specificities of glycosyl transferases, the substrate specificities of glycosidases, and the structures of antigenic determinants (Kobata et al., 1968, 1969, 1972).

We have recently established several monoclonal antibodies directed toward mucin carbohydrates (Fukui et al., 1988; Kurosaka et al., 1987, 1988; Kitagawa et al., 1988a). During studies to determine the epitopic structure for one of these antibodies, MSW 113, we found that acidic oligosaccharides from human milk significantly inhibited the antigen–antibody reaction. By means of immunoaffinity chromatography with MSW 113, we were able to isolate the inhibitory oligosaccharides. One of the immunoreactive oligosaccharides was

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