Biological activity and metabolism of 20-hydroxyeicosatetraenoic acid in the human platelet

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1 The cytochrome P-450 metabolite of arachidonic acid, 20-hydroxyeicosatetraenoic acid (20-HETE), was found to be a potent, dose-dependent inhibitor of platelet aggregation and inhibitor of thromboxane biosynthesis induced by arachidonic acid (IC_{50} $5.2 \pm 1.5 \mu M$), A23187 (IC_{50} $16.2 \pm 5.4 \mu M$), and U46619 (IC_{50} $7.8 \pm 2.4 \mu M$). 20-HETE did not inhibit thrombin-induced aggregation.

2 The human platelet metabolized 20-HETE to a series of novel metabolites formed by cyclo-oxygenase as well as lipoxygenase pathways. The structures of the metabolites were identified by mass spectrometry as 20-hydroxy-thromboxane B₂, 12,17-dihydroxyheptadecatrienoic acid, 12,20-dihydroxy-eicosatetraenoic acid, and 11,20-dihydroxyeicosatetraenoic acid.

3 The identification of the 11-hydroxy metabolite of 20-HETE suggests that 20-HETE is less efficiently cyclized to an endoperoxide intermediate by cyclo-oxygenase than is arachidonate.

4 Although some biological activity of 20-HETE may be related to competition with endogenous arachidonate for cyclo-oxygenase metabolism, the predominant mechanism of action of 20-HETE appears to be through antagonism of the prostaglandin H₂/thromboxane A₂ receptor.

Keywords: 20-Hydroxyeicosatetraenoic acid (20-HETE); cytochrome P450; mass spectrometry, platelets; cyclo-oxygenase metabolism; lipoxygenase metabolism; platelet aggregation

Introduction

It is now widely appreciated that arachidonic acid can be oxidatively converted into biologically active eicosanoids by three distinct metabolic pathways. The cyclo-oxygenase pathway yields prostaglandins and thromboxanes (Smith & Marnett, 1991). Various lipoxygenase pathways yield 5-, 12-, and 15-hydroxyeicosatetraenoic acids (HETEs) with the 5-lipoxygenase also catalyzing the formation of leukotriene A₄ which is subsequently converted into the biologically active leukotrienes (Samuelsson *et al.*, 1987). The cytochrome P-450 mono-oxygenase pathway yields regioisomeric *cis*-epoxides as well as lipoxygenase-like products such as 12(R)-HETE and also ω -oxidized metabolites of arachidonic acid (Fitzpatrick & Murphy, 1989). Hepatocytes (Capdevila *et al.*, 1990), renal cortical, and medulla preparations (Morrison & Pascoe, 1981; Oliw *et al.*, 1981; Schwartzman *et al.*, 1986; Takahashi *et al.*, 1990) contain an active cytochrome P-450 which readily catalyzes the formation of 20-hydroxyeicosatetraenoic acid (20-HETE) as well as 19-hydroxyeicosatetraenoic acid (19-HETE). In addition, 20-HETE is a major metabolite of arachidonic acid formed within the resting polymorphonuclear leukocyte (PMN) following the addition of exogenous arachidonic acid (Hatzelmann & Ullrich, 1988). Recently, 20-HETE has been found to possess diverse biological properties, including the ability to inhibit rabbit renal medulla microsomal Na⁺/K⁺-ATPase (Carroll *et al.*, 1991), stimulate erythropoietin-dependent stem cell growth in human bone marrow (Abraham *et al.*, 1991), and constrict rat aortic rings (Escalante *et al.*, 1989). Interestingly, the vasoconstrictor properties of 20-HETE were found to be dependent upon further metabolic action of endothelial cyclo-oxygenase to yield 20-hydroxy-prostaglandin H₂ (PGH₂) (Schwartzman *et al.*, 1989). Furthermore, the endoperoxides/thromboxane receptor antagonist, SQ29548, was found to block the vascular reactivity of 20-HETE.

These results suggest that the thromboxane receptor as well as metabolism of 20-HETE may play an important role in biological activities of this molecule. However, little is known about the metabolism and biological effects of 20-HETE on cells which are found in the blood, in particular the platelet which is known to have a thromboxane receptor as well as specific enzymes which can metabolize arachidonic acid including cyclo-oxygenase, thromboxane synthase, and 12-lipoxygenase (Marcus, 1978). We show here that 20-HETE can interact with platelets in three ways: (i) it can inhibit platelet aggregation induced by several, but not all agonists; (ii) platelets can metabolically transform 20-HETE via 12-lipoxygenase- as well as cyclo-oxygenase-dependent mechanisms into platelet inactive products; and (iii) it inhibits formation of thromboxane A₂ (TxA₂) during platelet activation.

Methods

Preparation of human platelets

Venous blood was obtained from adult human volunteers who had not received medication for a period of at least one week before collection. The blood was anticoagulated with acid citrate-dextrose anticoagulant (ACD; National Institutes of Health [NIH] formula A) and centrifuged for 15 min at 120 g at 25°C. The platelet-rich plasma (PRP) was separated and platelets isolated and washed in citrate buffer containing 100 nM PGE₁ and resuspended in Hank's balanced salt solution (HBSS) at $2-4 \times 10^8$ platelets ml⁻¹ as previously described (MacLough & Murphy, 1988).

Platelet aggregation measurements

Platelet suspensions (0.6 ml) were incubated for 2 min at 37°C with 20-HETE prior to application of stimuli which included arachidonic acid (1 μM), ionophore A23187 (0.1 μM), thrombin (0.1 u ml⁻¹) or U46619 (11 α , 9 α -epoxymethano-

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PGH₂, 0.5 μ M), a prostaglandin H₂/thromboxane A₂ (PGH₂/TxA₂)-mimetic. For certain experiments PRP was incubated with aspirin (1 mM, 30 min) before the platelets were washed. For all experiments organic solvents were less than 0.1% final concentration in the platelet suspensions. Aggregation was monitored photometrically and was quantitated by measurement of light transmittance 3 min after the addition of the stimulus. In some experiments the platelet suspensions were centrifuged (1000 *g*) after aggregation for 3 min and supernatants frozen (-20°C) for subsequent analysis of TxB₂ by enzyme immunoassay (Pradelles *et al.*, 1985).

To determine whether 20-HETE inhibition of platelet aggregation was dependent on lipoxygenase metabolism, aspirin-treated platelets were incubated with nordihydroguaiaretic acid (NDGA, 10 μ M, 2 min) followed by 20-HETE (0.1 μ Ci [³H]-20-HETE diluted to 30 μ M with cold 20-HETE, 2 min) followed by U46619 (0.5 μ M). Aggregation was monitored for 3 min after U46619 addition and then platelet incubations were stopped with 1 vol methanol and the metabolites analyzed by reverse phase high performance liquid chromatography (r.p.h.p.l.c.) as described below.

Adenosine 3':5'-cyclic monophosphate

(Cyclic AMP) measurements Washed platelets (2×10^9 /ml) were incubated with 0.2 mM isobutylmethylxanthine (a phosphodiesterase inhibitor) and either PGE₁ (30 μ M) or 20-HETE (30 μ M) at 37°C for 30 s. Platelets were centrifuged at 250 *g* for 5 min and the cell pellet extracted twice with 1 ml ethanol. The ethanol was evaporated and residue was assayed for cyclic AMP by immunoassay (Gilman, 1970).

Identification of 20-HETE metabolites in platelets

Platelets (20 ml) were incubated with 0.02 μ Ci [³H]-20-HETE diluted with cold 20-HETE (1 μ M) for 2 min at 37°C . The incubation was stopped with 2 vol of ice cold methanol and the cells centrifuged at 2000 *g* for 10 min. The organic solvent was removed from the supernatant by rotary evaporation and the aqueous residue diluted with 2 vol of sodium acetate buffer (pH 4.0) and applied to a prewashed C₁₈ solid phase extraction column (Analytichem International, CA, U.S.A.). The column was washed with distilled water and eicosanoids eluted with pure methanol. The methanol solvent was removed under reduced pressure and the eicosanoids redissolved in 1 ml 20% methanol/water for analysis by r.p.h.p.l.c.

R.p.h.p.l.c. was performed on an Ultramex 5 μ m C₁₈ column (250×4.6 mm) (Phenomenex, Torrance, CA, U.S.A.) by use of methanol/water/acetic acid (55:45:0.05 pH 5.7 adjusted with NH₄OH) at 1.0 ml min⁻¹. Gradient elution was carried out from 55–70% methanol (20 min), 70–80% methanol (15 min), and 80–100% methanol (5 min). Fractions were collected at 0.5 min intervals. In some experiments, the h.p.l.c. effluent was monitored for u.v. absorbance and ³H with an online radioactive detector (Radiomatic, Tampa, FL, U.S.A.), with a Flow-Scint II (Packard Radiomatic, Tampa, FL) and a scintillation mixture in the ratio of 3:1 h.p.l.c. effluent to scintillation cocktail.

An aliquot of the radioactive fractions was derivatized to the pentafluorobenzyl ester, trimethylsilyl ether and analyzed by electron capture gas chromatograph-mass spectrometry (g.c.-m.s.) using methane as moderating gas (Strife & Murphy, 1984). Another aliquot of the radioactive fraction was derivatized with ethereal diazomethane, then *bis*(trimethylsilyl)trifluoroacetamide (BSTFA) and analyzed by electron ionization g.c.-m.s. The remainder of the radioactive fraction was reduced by use of 5% rhodium on alumina at room temperature for 3 min (Murphy & Sala, 1990) prior to conversion into the pentafluorobenzyl esters or methyl esters, and trimethylsilyl ethers for analysis by g.c.-m.s. The methyl esters of a radioactive dihydroxy metabolite fraction were

further purified by normal phase (n.p.)-h.p.l.c. with a 5 μ m silica column (250×4.6 mm, Ultramex, Phenomenex, CA, U.S.A.) with 2% isopropanol in hexane, 2 ml min⁻¹ before analysis by electron ionization g.c.-m.s. G.c.-m.s. was performed on a Finnigan-MAT SSQ70 mass spectrometer with a 10 m DB-1 capillary column and injector held at 280°C . The column temperature was programmed from 100°C – 300°C at $15^{\circ}\text{C min}^{-1}$ using helium as carrier gas with a linear velocity of 10 m s⁻¹.

Materials

20-HETE (5,6,8,9,11,12,14,15-[³H]20-HETE) (240 Ci mmol⁻¹) was obtained from Dupont-New England Nuclear. Synthetic 20-HETE and 19(S)-HETE were gifts from Dr J.R. Falck, University of Texas, Southwest Medical Center, Dallas, TX, U.S.A. All solvents employed were h.p.l.c. grade (Fisher) and other reagents were the highest grade commercially available. *Bis*(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Supelco. Rhodium (5%) on alumina powder was obtained from Fluka AG, Switzerland and pentafluorobenzyl bromide was obtained from Aldrich Chemical Co.

Results

Platelet-antiaggregatory properties of 20-HETE

At concentrations ≤ 30 μ M, 20-HETE had no detectable agonist activity on human platelets. However, it inhibited arachidonic acid-induced aggregation of platelets in a dose-dependent manner with an IC₅₀ of 5.2 ± 1.5 μ M (Figure 1a). 20-HETE also inhibited TxB₂ formation in a dose-dependent manner, reducing it from a control level of 308 ± 16 ng

Figure 1 Effect of 20-HETE on aggregation and thromboxane B₂ (TxB₂) fraction by platelets stimulated by (a) 1 μ M arachidonic acid; (b) 0.1 μ M A23187; (c) 0.1 μ M thrombin; (d) 0.5 μ M U46619 (aspirin-treated platelets). Aggregation trace are from a single representative experiment. TxB₂ concentrations are a mean of three experiments; s.e. shown by vertical bars.

TxB₂/10⁸ platelets to 41 ± 10 ng TxB₂/10⁸ with 30 μ M 20-HETE. The positional isomer, 19(S)-HETE was less potent, inhibiting platelet aggregation by only 23% at this concentration.

The A23187-induced aggregation was also inhibited by 20-HETE with an IC₅₀ of 16.2 ± 5.4 μ M with a parallel decline in TxB₂ production from 147 ± 27 to 28 ± 5 ng/10⁸ cells (Figure 1b). Aggregation by the PGH₂/TxA₂ agonist, U46619, was inhibited by 20-HETE with an IC₅₀ concentration of 7.8 ± 2.4 μ M in aspirin-treated platelets (Figure 1d). In contrast to other agonists, thrombin-induced aggregation was only inhibited by 14% in the presence of 30 μ M 20-HETE despite a 4 fold decrease in TxB₂ formation (Figure 1c).

There was no significant difference between the levels of cyclic AMP observed in resting and 20-HETE (30 μ M)-treated platelets (Table 1) suggesting that elevation of platelet cyclic AMP was not the primary mechanism for its effects.

When aspirin-treated platelets were incubated with NDGA (10 μ M) for 2 min before 20-HETE incubation (30 μ M), U46619-induced platelet aggregation was completely inhibited (Figure 2). However, NDGA decreased production of the 20-HETE metabolite 12,20-diHETE by 80% (data not shown). This suggested that a 12-hydroperoxy intermediate was not responsible for 20-HETE inhibition of platelet aggregation. In contrast to platelets, 20-HETE had no effects on the neutrophil activation. At 30 μ M it did not induce shape change, cause Ca²⁺ mobilization, or inhibit leukotriene B₄ (LTB₄)-induced calcium mobilization (Scanlon *et al.*, 1987).

Identification of platelet 20-HETE metabolites

When [³H]-20-HETE (1 μ M) was incubated with platelets for 2 min at 37°C, several metabolites were formed (Figure 3). Their relative concentration and extent of 20-HETE metabolism was dependent upon the initial concentration of 20-HETE, the number of platelets, and the time of incubation. In all incubations less than 2% of the added 20-HETE remained associated with the pellet after methanol extraction suggesting that it was not readily incorporated into platelet phospholipids.

Metabolite I (Figure 3) displayed no u.v. absorption above 210 nm. Electron capture g.c.-m.s. of the pentafluorobenzyl (PFB) ester trimethylsilyl (TMS) ether derivative of metabolite I (equivalent carbon value of 25.6) produced an abundant ion at m/z 673 (M-181) with additional ions at m/z 583 (M-181-90), m/z 493 (M-181-2(90)), and m/z 403 (M-181-3(90)). These results were consistent with a molecule having at least three hydroxyl groups and molecular weight of 386 daltons. The electron ionization of the methyl ester, trimethylsilyl ether derivative of metabolite I yielded a mass spectrum similar to that previously described for 20-hydroxy-TxB₂ (Powell, 1979), but with an equivalent carbon value of 26.6 This was lower than that previously measured (equivalent carbon value of 27.5) (Powell, 1979). Prominent ions were observed at m/z 598 (M-90), 508 (M-2(90)), 439 (loss of •(CH₂)₅OSiMe₃ and SiMe₃OH), 389 (Me₃SiO⁺ = CH-

Figure 2 (a) Nordihydroguaiaretic acid (NDGA, 10 μ M, 2 min) by itself has no effect on platelet aggregation induced by 0.5 μ M U46619 as seen by the increase transmittance in light in the aggregometer during the 3 min incubation. (b) 20-HETE blocks U46619-induced aggregation. (c) NDGA does not block the antiaggregatory effect of 20-HETE although extraction of this incubation revealed inhibition of 12-lipoxygenase.

CH = CH-CH (OSiMe₃)-(CH₂)₄-CH₂OSiMe₃) 323, 313, 256 (Me₃SiO⁺ = CH = CH-CH₂-CH = CH-(CH₂)₃-CO₂Me), 217, 129 and a base peak at m/z 73. It was concluded that metabolite I was 20-hydroxy-TxB₂ formed by the action of platelet cyclo-oxygenase on 20-HETE and subsequent action of thromboxane synthase on the intermediate 20-hydroxy-PGH₂.

Metabolite II displayed an ultraviolet spectrum with a $\lambda_{\max}$ 232 nm typical of a conjugated diene. Electron capture g.c.-m.s. of the PFB ester, TMS ether of metabolite II produced ions at m/z 439 (M-181), 349 (M-181-90) and 259 (M-181-2(90)) which were consistent with two hydroxy groups in the metabolite and a molecular weight of 296 daltons. Reduction of the metabolite increased the equivalent carbon value from 21.6 to 21.8 for the PFB ester TMS ether and resulted in an electron capture mass spectrum with prominent ions at m/z 445 (M-181), 355 (M-181-90), and 265 (M-181-2(90)) consistent with the reduction of three double bonds and the presence of two hydroxy groups within a C17 carboxylic acid. Electron ionization of the PFB ester TMS ether of

Table 1 Effect of 20-HETE on cyclic AMP levels in platelets¹

Treatment	Cyclic AMP (pmol/10 ⁹ platelets)
Control	4.8 ± 0.7
20-HETE (30 μ M)	3.8 ± 0.1
PGE ₁ (30 μ M)	55.2 ± 7.7

¹Platelets were incubated with 0.2 μ M isobutylmethylxanthine and either prostaglandin E₁ (PGE₁) or 20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE) for 30 s at 37°C. Platelets were centrifuged and the cell pellet extracted with ethanol. Values are mean $\pm$ s.e. of 4 experiments.

Figure 3 Reverse phase high performance liquid chromatography analysis of metabolites formed from the incubation of 20-HETE with human platelets. Platelets (20 ml) were incubated with 0.02 μCi [^3H]-20-HETE (1 μM) for 2 min at 37°C.

metabolite II produced ions at m/z 461 (which were observed at m/z 467 following reduction) consistent with α -cleavage at C-12 and loss of $\bullet(\text{CH}_2)_4\text{CH}_2\text{OSiMe}_3$, consistent with a C_{17} carboxylic acid. Electron ionization mass spectrometry of the trimethylsilyl ether methyl ester of metabolite II (equivalent carbon value 22.3) produced ions at m/z 454 (M^+), 295 (α -cleavage at C-12, loss of $\bullet(\text{CH}_2)_4\text{CH}_2\text{OSiMe}_3$) and m/z 313 (Figure 4a). Trimethylsilylation with [^3H]-BSTFA shifted m/z 313 to m/z 331 indicating that this ion contained two intact trimethylsilyl groups. Thus m/z 313 could correspond to a rearrangement ion suggested to be a stabilized 6-membered ring structure illustrated in Figure 5.

The electron ionization of the reduced molecule did not yield a corresponding ion (Figure 4b). However, abundant ions were observed for the methyl ester TMS derivative of reduced metabolite II (equivalent carbon value 22.6) at m/z 445 ($\text{M}-15$), at 429 ($\text{M}-31$), 413 ($\text{M}-15-32$), 355 ($\text{M}-15-90$), 301 (α -cleavage at C-12, loss of $\bullet(\text{CH}_2)_4\text{CH}_2\text{OSiMe}_3$), 261 (α -cleavage at C-12, loss of $\bullet(\text{CH}_2)_{10}\text{COOCH}_3$), and m/z 272 (rearrangement of the trimethylsilyl group to the carboxyl group (VanRollins & Murphy, 1984)). These data were consistent with the structure of metabolite II as 12,17-dihydroxy-5,8,10-heptadecatrienoic acid formed by the action of platelet cyclo-oxygenase on 20-HETE, followed by decomposition and loss of malonyldialdehyde from the cyclic endoperoxide intermediate compatible with the established mechanism for arachidonic acid (Hamberg & Samuelsson, 1974).

Electron capture g.c.-m.s. of PFB ester, TMS ether of metabolite III (equivalent carbon value of 23.7) resulted in abundant ions at m/z 479 ($\text{M}-181$) and 389 ($\text{M}-181-90$). The electron capture mass spectrum of the reduced molecule (equivalent carbon value of 24.9) produced ions at m/z 487 ($\text{M}-181$), 397 ($\text{M}-181-90$), and 307 ($\text{M}-181-2(90)$). These

results are consistent with a dihydroxy metabolite having a molecular weight of 336 daltons. The methyl ester of metabolite III was further purified on n.p.-h.p.l.c. and resulted in the separation of two components with retention times of 30.9 (IIIA) and 32.4 min (IIIB) and u.v. absorption maxima at 234 and 232 nm, respectively. The retention times and u.v. spectra of these metabolites were identical to that

Figure 4 (a) Electron ionization (70 eV) mass spectrum of the methyl ester trimethylsilyl (TMS) ether of metabolite II. (b) Electron ionization (70 eV) mass spectrum of metabolite II following catalytic hydrogenation and derivatization as the methyl ester TMS ether.

Figure 5 Mechanism of formation of ion m/z 313 in the electron ionization mass spectrum of methyl 12,17-trimethylsiloxyheptadecatrienoate (metabolite II).

for 12,20-diHETE and 11,20-diHETE respectively (authentic standards were synthesized from the incubation of 12-HETE and 11-HETE with human neutrophils).

Electron ionization g.c.-m.s. of the methyl ester TMS ether of IIIA (equivalent carbon value of 24.6) revealed ions at m/z 479 (M-15), 404 (M-90), 353 (M-141, loss of $\bullet C_6H_{10}COOCH_3$) and an abundant ions at m/z 295 (α -cleavage at C-12, loss of $\bullet CH_2CH_2OSiMe_3$, with a base peak at m/z 73. Reduction of IIIA (equivalent carbon value 25.4) resulted in major ions at m/z 303 (α -cleavage at C-12, loss of $\bullet (CH_2)_{10}COOCH_3$), 301 (α -cleavage at C-12, loss of $\bullet (CH_2)_7CH_2OSiMe_3$), and 272 (trimethylsilyl rearrangement to the carboxyl moiety). Both the electron ionization mass spectra of the native and reduced metabolite IIIA were identical to those described previously for 12,20-dihydroxy-eicosatetraenoic acid by Marcus *et al.* (1984), and had the same gas chromatographic retention parameter and u.v. spectra as 12,20-diHETE prepared by the incubation of 12-HETE with neutrophils in this laboratory.

Electron ionization of the methyl ester, trimethylsilyl ether of metabolite IIIB revealed ions at m/z 479 (M-15), 313 (α -cleavage at C-11, loss of $\bullet C_9H_{14}COOCH_3$) 295 and 223 (313-90) (Figure 6a). The mass spectrum and the gas chromatographic retention value (equivalent carbon value 24.6) was exactly the same as that for the 11,20-diHETE standards prepared by the incubation of 11-HETE with neutrophils with the exception of the ion at m/z 295. This was most likely to be a residual contaminant of 12,20-diHETE identified as metabolite IIIA which was incompletely separated on the n.p.-h.p.l.c. system.

Reduction of IIIB (equivalent carbon value 25.4) followed by mass spectrometric analysis of the methyl ester trimethylsilyl ether derivative (Figure 6b) revealed ions at m/z 487 (M-15), 365 (M-90-47), 317 (α -cleavage at C-11, loss of $\bullet C_9H_{16}COOCH_3$) and a prominent ion at m/z 387 (α -cleavage at C-11, loss of $\bullet (CH_2)_7CH_2OSiMe_3$). An ion at m/z 258 would correspond to the rearrangement of trimethylsilyl group to carboxyl moiety (VanRollins & Murphy, 1984). With the exception of the contaminant ions at m/z 301 and

303 from the reduced 12,20-diHETE, this mass spectrum was identical to that of derivatized 11,20-diHETE. From h.p.l.c. and g.c. retention data, u.v. data, and mass spectral data, metabolite IIIB corresponds to 11,20-dihydroxy-5,8,12,14-eicosatetraenoic acid. Selected ion monitoring of the α -cleavage ions produced by the electron ionization of the methyl ester trimethylsilyl ether of the native diHETEs (m/z 295, 12,20-diHETE, m/z 11,20-diHETE) showed that both diHETEs were produced in similar concentrations in platelets. However, only 12,20-diHETE was produced in aspirin-treated platelets (data not shown) which confirmed 11,20-diHETE was a product of cyclo-oxygenase metabolism.

Insufficient material was available for further structural characterization of metabolite IV. None of the four metabolites had stimulatory or inhibitory effects on platelet aggregation.

Discussion

Interest in the cytochrome P-450 pathway of arachidonic acid metabolism and specifically the formation of the ω -hydroxylation product, 20-HETE, stems from several reports of biological activities, especially in the renal system (McGiff, 1991). Reported renal activities for 20-HETE include inhibition of $(Na^+/K^+)ATPase$ (Carroll *et al.*, 1991), effect on ion transport in the loop of Henle (Escalante *et al.*, 1991), and effects on natriuresis without affecting renal hemodynamics or glomerular filtration rate (Takahashi *et al.*, 1990). This metabolite also has potent vascular effects causing contractions of the aorta that are dependent upon metabolism of 20-HETE by cyclo-oxygenase (Escalante *et al.*, 1989). Interestingly 20-HETE has been found to be a stimulator of erythropoietin-dependent stem cell clonal growth in human bone marrow even at 10^{-11} M (Abraham *et al.*, 1991).

Biological effects of 20-HETE on the human platelet have not been previously reported. Human platelet aggregation induced by arachidonic acid, ionophore (A23187), and U46619 could be inhibited by 20-HETE in a dose-dependent manner. Importantly, thrombin-induced platelet aggregation was not inhibited. With each agonist, TxB_2 formation significantly declined in a dose-dependent manner to the amount of added 20-HETE, suggesting that one action of 20-HETE was to inhibit cyclo-oxygenase. However, cyclo-oxygenase inhibition by 20-HETE cannot completely account for platelet antiaggregatory activity since A23187-induced aggregation is independent of cyclo-oxygenase-catalyzed metabolism and phospholipase C (PLC)-catalyzed phosphoinositide hydrolysis (Levy-Toledano *et al.*, 1982). Inhibition of platelet aggregation by 20-HETE could not be blocked by aspirin even though cyclo-oxygenase was inhibited, further suggesting an independent mechanism. Inhibition of A23187-induced aggregation suggests that 20-HETE directly inhibits $[Ca^{2+}]$ increase in the platelet. The lack of inhibition of thrombin-induced aggregation suggests that phospholipase C is not inhibited because platelet activation by thrombin is associated with activation of phospholipase C and subsequent activation of phospholipase A_2 . 20-HETE also inhibits U46619-induced aggregation in platelets which were treated with aspirin to block cyclo-oxygenase. Thus, antagonism of the PGH_2/TxA_2 receptor by 2-HETE could account for the inhibition of arachidonic acid and U46619-induced aggregation. Agonists that stimulate adenylate cyclase, such as PGE_1 , inhibit aggregation induced by thrombin or ionophore. In this study, thrombin-induced aggregation was not inhibited by 20-HETE and 20-HETE did not increase cyclic AMP which argues against stimulation of adenylate cyclase as a mechanism for antiaggregatory action of this eicosanoid.

This platelet effect of 20-HETE resembles that of another group of cytochrome P-450 metabolites, the epoxyeicosatetraenoic acids (EETs) (Bernstrom *et al.*, 1991). However, inhibition of platelet aggregation by 20-HETE appears to be specific for this positional HETE isomer, in that no

Figure 6 (a) Electron ionization (70 eV) mass spectrum of the methyl ester trimethylsilyl (TMS) ether of metabolite IIIB. (b) Electron ionization (70 eV) mass spectra of metabolite IIIB following catalytic hydrogenation and derivatization as the methyl ester TMS ether.

significant antiaggregatory effect has been reported for other HETEs including 19(S)-HETE described here. In addition, 12,20-diHETE did not inhibit arachidonic acid-induced aggregation of platelets. It is important to note that 12-HPETE has been previously reported to inhibit platelet aggregation (Aharony *et al.*, 1982; Siegel *et al.*, 1979; Brune & Ullrich, 1991) and this effect has been found to involve an elevation of cyclic GMP (Grune & Ullrich, 1991). Our failure to block 20-HETE inhibition of platelet aggregation while inhibiting 12-lipoxygenase in the platelet with NDGA suggests that 20-HETE is not exerting its effect through the formation of a 12-hydroperoxide intermediate. However, such considerations did lead to a study of the metabolism of 20-HETE in the platelet described here.

Platelet metabolism of 20-HETE was rapid and extensive as outlined in Figure 7. Within 2 min over 50% of 20-HETE (1 μ M) could be metabolized to cyclo-oxygenase (and thromboxane synthase) products such as 20-hydroxy-TxB₂, 12,17-dihydroxyheptadecatrienoic acid (12,17-diHHTrE) and 11,20-diHETE as well as the 12-lipoxygenase product 12,20-diHETE. The rapid metabolism of 20-HETE by cyclo-oxygenase and 12-lipoxygenase suggests that 20-HETE may effectively compete for metabolism with released arachidonic acid as substrates. This would explain, in part, the significant decrease in TxB₂ formation with increasing concentrations of 20-HETE when platelets were stimulated with arachidonic acid, ionophore, and thrombin. It is not known whether the

unstable metabolites, 20-hydroxy-TxA₂ or 20-hydroxy-PGH₂, inhibit the pharmacological activity of TxA₂ and PGH₂ either at the TxA₂/PGH₂ receptor or by receptor desensitization associated with the mobilization of the intracellular stores of Ca²⁺.

The 20-HETE metabolite, 11,20-diHETE, is likely to be formed as an initial intermediate in the cyclo-oxygenase catalyzed addition of oxygen to 20-HETE before the formation of a cyclic endoperoxide (Figure 7). Hamberg & Samuelsson (1967) showed that the initial step in the formation of prostaglandins from arachidonic acid was the stereospecific removal of a hydrogen at C-13 followed by the introduction of oxygen at C-11 in a radical centred process. The 11-hydroperoxy radical of 8,12,14-eicosatetraenoic acid can then be cyclized into an endoperoxide by attack of the C-11 hydroperoxy radical and cyclization to form the 9,11-dioxo bridge (Samuelsson, 1972). This leaves a carbon-centred radical at C-8 which can attack C-12 to generate a free radical at C-15 to which a second oxygen molecule attaches to give a hydroperoxy radical at C-15. There have been data to support an intermediate 11-hydroperoxy radical as shown by a peroxidase reduction product, 11-HETE, which has been found as a minor metabolite in some studies (Morrison & Crowley, 1984; Hemler *et al.*, 1978; Bailey *et al.*, 1983). However, it would appear that the rate of cyclization of the 11-hydroperoxy radical intermediate by cyclo-oxygenase may be compromised when 20-HETE is substrate, leading to

Figure 7 Metabolism of 20-HETE by platelet cyclo-oxygenase and 12-lipoxygenase pathways.

accumulation of a 11-hydroperoxy radical intermediate. This intermediate would be reduced by peroxidase to 11,20-diHETE.

In our laboratory, studies have shown that 20-HETE has no effect on neutrophil function as measured by shape change of neutrophil or alteration in intracellular free calcium concentrations. However, it is interesting to note that in the ionophore-stimulated neutrophil, 20-HETE serves as a substrate for 5-lipoxygenase and is rapidly metabolized to 5,20-dihydroxyeicosatetraenoic acid and to a lesser extent, 20-hydroxy-LTB₄ (data not shown).

Recent investigations have revealed 20-HETE to be a significant metabolite of arachidonic acid produced by various cell types. Isolated cells from the thick ascending loop of Henle in the kidney release 20-HETE following stimulation with arginine vasopressin (10^{-8} to 10^{-7} M) and calcitonin (10^{-9} to 10^{-8} M) (Schwartzman *et al.*, 1985). Hepatocytes as well as unstimulated neutrophils have also been found to transform exogenous free arachidonic acid into this metabolite (Hatzelmann & Ullrich, 1988; Capdevila *et al.*, 1990). Recently, 20-HETE biosynthesis was found to be elevated in the kidneys of young spontaneous hypertensive

rats (Sacerdoti *et al.*, 1988). Collectively, these experiments suggest that this cytochrome P450 metabolite of arachidonic acid is produced by various cells and may escape into the circulation to exert a biological effect. The results of the experiments described here show that 20-HETE can be rapidly metabolized by the platelet but, if the platelet has been stimulated by certain agonists, 20-HETE may exert anti-aggregatory properties if present in sufficient quantities.

In summary, 20-HETE but not 19-HETE inhibits platelet aggregation, most probably by antagonism of the PGH₂/TxA₂ receptor. 20-HETE competes with endogenous arachidonic acid for cyclo-oxygenase and 12-lipoxygenase metabolism within the platelet. It is rapidly converted into 20-hydroxy-TxA₂ and the 12,17-dihydroxyheptadecatrienoic acid via 20-hydroxyendoperoxide intermediates which previously had been shown to be potent vasoconstrictors (Schwartzman *et al.*, 1989). Further investigations are under way to determine the amount of endogenous 20-HETE and metabolites produced during inflammatory reactions.

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