15-Hydroxy-5,8,11,13-eicosatetraenoic Acid Inhibits Human Vascular Cyclooxygenase

Potential Role in Diabetic Vascular Disease

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Abstract

Human umbilical arteries converted arachidonic acid to three hydroxyeicosatetraenoic acids (HETEs) as well as prostaglandins. The mono-HETEs have been identified by reverse-phase high pressure liquid chromatography and gas chromatography-mass spectroscopy as 15-HETE and 11-HETE. 15-HETE in arterial segments appears to be derived mainly via the 15-lipoxygenase pathway, whereas 11-HETE, and the presumed di-HETE(s) were products of cyclooxygenase. Nordihydroguaiaretic acid, a lipoxygenase inhibitor, stimulated prostanoid production with a concomitant inhibition of 15-HETE formation. These results suggested that 15-HETE may function as an endogenous regulator of prostacyclin. In human umbilical arterial microsomes, 15-HETE was found to inhibit 6-keto-prostaglandin $F_{1\alpha}$ and total prostanoid production in a concentration-dependent manner (median inhibition constant [IC_{50}] of 52±3 and 63±4 μM, respectively). The relative distribution of prostaglandins, however, remained unaffected, indicating that the site of action was cyclooxygenase. Kinetic analysis revealed that 15-HETE was a competitive inhibitor of the enzyme. Although no changes in maximum velocity occurred, the apparent K_m was significantly different (9.3±6.9 μM [1 SD] for control vs. 37.6±17.7 μM for the 15-HETE-treated enzyme). Furthermore, the inhibitory effect of 15-HETE on prostacyclin production was confirmed using cultured bovine endothelial cells. In this cell system, not only did 15-HETE inhibit endogenous prostacyclin production, but also the conversion of exogenous [1-^{14}C]arachidonic acid to prostacyclin (IC_{50} of 40±17 μM). No effect on arachidonic acid release was noted.

To investigate whether our in vitro finding that 15-HETE inhibited prostacyclin production could be relevant to the in vivo situation, our final studies were performed on vasculature obtained from the diabetic milieu. We found that the production of 15-HETE was significantly increased in vasculature obtained from the infant of the diabetic mother (1.14±0.26 pmol/mg) when compared to control neonates (0.77±0.22; $P < 0.01$). A concomitant decrease in prostacyclin production was seen (51.6±12.6 pmol/mg in infants of diabetic mothers vs. 71±22.3 in controls). Moreover, an inverse correlation between these two eicosanoids was also noted. Our results suggest a potential in vivo regulatory role for 15-HETE on prostacyclin production.

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Introduction

15-Hydroperoxy-5,8,11,13-eicosatetraenoic acid has been found to enhance the release of anaphylactic mediators from guinea pig lung (1), and to block prostacyclin synthetase (2). These biologic activities however have not been described for the corresponding hydroxyacid, 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE).[1] We have recently reported that 15-HETE (a major lipoxygenase metabolite of arachidonic acid in human endothelial cells [3], leukocytes [4], and lung [5]) enhances endothelial cell migration (6), an initial and obligatory step in angiogenesis and neovascular proliferation (7, 8). In this report we provide evidence that 15-HETE is a competitive inhibitor of vascular cyclooxygenase, is the major lipoxygenase metabolite in human vasculature, and is significantly increased in vessels obtained from the diabetic milieu. Our results suggest a potential role for this hydroxyacid as an endogenous regulator of vascular prostacyclin biosynthesis.

Methods

Materials

15-HETE was prepared by incubating arachidonic acid with soybean lipoxygenase (9). 12-HETE was prepared by incubating arachidonic acid with indomethacin-treated platelets (10). The resulting hydroperoxy derivatives were reduced to the respective hydroxy acids using triphenyl phosphine and then sodium borohydride (11). Both 15-HETE and 12-HETE were purified by silicic acid column chromatography followed by reverse-phase high pressure liquid chromatography (HPLC) and then stored at −70°C in methanol. The purity of these preparations was >99%, as tested by analytical reverse-phase HPLC and ultraviolet absorption spectroscopy. The structures were confirmed by gas chromatography-mass spectroscopy (GC-MS). The mass spectrum of the trimethyl silyl ether methyl ester of 15-HETE showed prominent ions (M^+, molecular ion; the relative abundance and probable mode of origin are given in parentheses) at the mass-to-charge ratio (m/e) 335 (4%, M^+-71); 316 (7%, M^+-90); 225 (50%, M^+-181); 173 (15%, M^+-233) and 73 (100%). The mass spectrum of the trimethyl silyl ether methyl ester of 12-HETE showed prominent ions at m/e 391 (0.6%, M^+-15); 375 (0.8%, M^+-31); 316 (0.9%, M^+-90); 295 (65%, M^+-111); 229 (15%); 205 (10%); and 73 (100%). These spectra are in agreement with the published spectra of 15-HETE and 12-HETE, respectively (12, 13).

Bradykinin, reduced glutathione (GSH), hydroquinone, hemin, tri-

1. *Abbreviations used in this paper:* CMFH, calcium- and magnesium-free Hanks' balanced salt solution; di-HETEs, dihydroxyeicosatetraenoic acids; ETYA, eicosatetraynoic acid; GC-MS, gas chromatography-mass spectroscopy; HETE, hydroxyeicosatetraenoic acid; HPLC, high pressure liquid chromatography; IC_{50}, median inhibition constant; IDM, infant of a diabetic mother; 6k$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$; M^+, molecular ion; m/e, mass-to-charge ratio; MEM, minimal essential medium; NDGA, nordihydroguaiaretic acid; PG, prostaglandin; R_f, retention factor; RIA, radioimmunoassay; TLC, thin-layer chromatography; TX, thromboxane; V_{max}, maximum velocity.

olein, ricinoleic acid, and soybean lipoxygenase were obtained from Sigma Chemical Co. (St. Louis, MO). Arachidonic acid (purity >99%) was purchased from Nu-Chek Prep., Inc. (Elysian, MN). Prostaglandin standards and 1,2-diolein were obtained from P-L Biochemicals, Inc. (Milwaukee, WI) or The Upjohn Co. (Kalamazoo, MI). The calcium ionophore A23187 was obtained from Calbiochem-Behring Corp. (La Jolla, CA). [1-^{14}C]Arachidonic acid (50–60 mCi/mmol) and tritium-labeled prostaglandins and HETEs were obtained from Amersham Corp. (Arlington Heights, IL) or New England Nuclear (Boston, MA). Minimal essential medium (MEM), calcium- and magnesium-free Hanks' balanced salt solution (CMFH), trypsin-EDTA, and fetal calf serum were purchased from Gibco Laboratories (Grand Island, NY). 8,15-, 5,12-, and 14,15-dihydroxyeicostatraenoic acids (di-HETEs), as well as 11-HETE and 5-HETE, were obtained from Biomol Research Laboratories (Philadelphia, PA). 11-HETE used in the experiments to be described was repurified by reverse-phase HPLC.

Umbilical cords were obtained from control infants (n = 9) at the time of delivery, and from infants of diabetic mothers (IDM; n = 11) of comparable gestational age. Classification of maternal diabetes mellitus was established according to standard criteria and included class A (n = 2), class B (n = 6), class C (n = 1), class D (n = 1), and class R (n = 1). No control or diabetic mother had ingested aspirin within 2 wk of delivery, and the mean gestational ages in both groups was similar (38.8±0.9 for controls vs. 38.2±1.2 for diabetics). This study was approved by the Human Institutional Experimentation Committee.

Metabolism of arachidonic acid by intact human umbilical arterial segments to prostaglandins and HETEs and their identification

The umbilical cords were dissected at ice temperature and the arteries were isolated, cut into 5-mm segments, and opened longitudinally before use. Histologic sections of the vessels were concomitantly evaluated revealing an intact endothelial layer in all arteries studied. The arterial segments were suspended in Tris-buffered CMFH, pH 7.4, with calcium chloride (5 mM) and magnesium chloride (1 mM) (Tris-Hanks) and preincubated for 10 min in the presence or absence of 30 μM indomethacin, 15 or 30 μM nordihydroguaiaretic acid (NDGA), or 60 μM 5,8,11,14-eicosatetraynoic acid (ETYA). [1-^{14}C]Arachidonic acid (10 μM) was then added and the incubation was continued for a further 30 min. The reaction was arrested by adding 2 vol of methanol-chloroform (2:1, by vol) and the metabolites were extracted as described by Bligh and Dyer (14). Extracts were then dried over anhydrous magnesium sulfate, evaporated under a stream of nitrogen, and dissolved in 100 μl of chloroform for chromatographic analysis. Thin-layer chromatography (TLC) was performed on silica gel G plates (Anal Tech, Inc., Newark, DE) using two solvent systems either to resolve prostanoids (15) (ethyl acetate–acetic acid, 99:1 by vol) or to resolve HETEs (16) (hexane-ether–acetic acid, 50:50:1 by vol). Appropriate standards were run on the same plate and were visualized by spraying with iodine in ethanol. After radioautography, the prostaglandin bands were identified by comparing their retention factor (R_f) values with those of authentic standards. The R_f values for reference prostaglandins and HETEs in the ethyl acetate–acetic acid system were as follows: 6-keto-prostaglandin $F_{1\alpha}$ (6kPGF$_{1\alpha}$), 0.08±0.02 (1 SD); prostaglandin (PG) $F_{2\alpha}$, 0.12±0.02; PGE_2, 0.23±0.04; thromboxane (TX) B_2, 0.3±0.02; PGI_2, 0.41±0.02; PGD_2, 0.46±0.03; PGB_2, 0.54±0.02; 15-HETE, 0.63±0.03; 12-HETE, 0.63±0.03; arachidonic acid, 0.68±0.02; diolein, 0.79±0.02; triolein, 0.85±0.02. The R_f values for PGB_2, 5-HETE, 12-HETE, 15-HETE, and arachidonic acid in the hexane-ether-acetic acid system were 0.07±0.01, 0.21±0.02, 0.3±0.03, 0.32±0.03, and 0.5±0.02, respectively. Radioactive bands were scraped into scintillation vials, extracted with 500 μl of methanol and counted in an LKB Mini-Beta Counter (LKB Instruments, Inc., Gaithersburg, MD) using ACS scintillant (Amersham Corp.). The nonenzymatic formation of HETEs was estimated by incubating [1-^{14}C]arachidonic acid in buffer alone (blank) and all reported values were corrected by subtraction of blank values. Recovery of individual eicosanoids was determined using [^{3}H]-labeled metabolites and was found to be 95%, 96%, 98%, 98%, 98%, and 97%, for 6kPGF$_{1\alpha}$, PGF$_{2\alpha}$, PGE_2, TXB_2, 15-HETE, and arachidonic acid, respectively. Prostaglandin and HETE production was calculated from the specific radioactivity of the added substrate and necessary corrections were made for the recovery.

For structural identification, HETEs were prepared by incubating the intact arterial segments with either labeled or unlabeled arachidonic acid as described above. The products were purified by silicic acid column chromatography and analyzed by reverse-phase HPLC in methanol–0.1% acetic acid (72:28, by vol) using a 4.6 × 250-mm Waters C-18 column (Waters Associates, Milford, MA) and a Beckman liquid chromatograph (Beckman Instruments, Inc., Palo Alto, CA). The flow rate was 1 ml per min. The column effluent was monitored continuously at 237 nm (for the elution of mono-HETEs) and 270 nm (for di-HETE elution) with a Beckman model 165 variable wavelength detector. For radiolabeled products, 0.5-ml fractions were collected, a 0.25-ml aliquot from each fraction was counted, and the remaining peak fractions were pooled, individually rechromatographed in the same system, and analyzed on silica gel G plates in the hexane-ether–acetic acid solvent system. For GC-MS analysis, the unlabeled metabolites were purified by silicic acid column chromatography followed by reverse-phase HPLC. The trimethyl silyl ether methyl ester derivatives of HPLC-purified HETEs were analyzed on a Hewlett-Packard 5985 GC-MS (Hewlett-Packard Co., Palo Alto, CA) with a column of 1% SE-30. The mass spectra were recorded at 70 eV.

Metabolism of [1-^{14}C]arachidonic acid by human umbilical arterial microsomes after exposure to HETEs

The arterial segments were homogenized using a Sorvall Omni Mixer (Sorvall-DuPont, Newtown, CT) in 50 mM Tris buffer, pH 8.0, containing 2 mM reduced glutathione (Tris-GSH) and the microsomal fractions were prepared by differential centrifugation (17). Microsomal pellets were suspended in Tris-GSH and the protein contents were determined (18). The hydroxy fatty acids (provided as ammonium salts) were dissolved in Tris-GSH with sonication to a final concentration of 200 μM. Control buffer was used to make all dilutions. Umbilical arterial microsomes were preincubated with the indicated concentrations of 15-HETE, 11-HETE, 12-HETE, or ricinoleic acid for 5 min at room temperature in Tris-GSH buffer. Hydroquinone (250 μM) and hemin (1 μM) were then added, and the reaction was initiated by the addition of [1-^{14}C]arachidonic acid (10 μM). The reaction was terminated at 10 min by the addition of cold ethyl acetate, products were extracted (19), and analyzed by TLC in the ethyl acetate-acetic acid system. In five further experiments, kinetic analyses of the effects of 15-HETE (100 μM) on the metabolism of [1-^{14}C]arachidonic acid by umbilical arterial microsomes were performed by varying substrate concentration between 5 and 40 μM, and terminating the reaction at 5 min. Apparent K_m and maximum velocity (V_{max}) for cyclooxygenase were calculated using the sum of all prostanoids produced.

Endogenous production of 6kPGF$_{1\alpha}$ and 15-HETE by human umbilical arteries

Vascular segments were incubated in Tris-Hanks at 37°C. Aliquots of incubation medium were removed at 1, 3, 5, 15, 30, and 60 min for assay of 6kPGF$_{1\alpha}$ and 15-HETE production by radioimmunoassay (RIA). For the evaluation of 6kPGF$_{1\alpha}$ and 15-HETE production by normal or diabetic blood vessels, arterial segments were incubated for 30 min at 37°C in Tris-Hanks', and aliquots of incubation medium were obtained for RIA.

Studies of the effect of 15-HETE on bovine endothelial cell arachidonic acid metabolism

Culture of endothelial cells. Endothelial cells derived from fetal bovine aorta were kindly provided by Drs. Bert Glaser and Janet Graeber (Johns Hopkins Medical Center, Baltimore, MD) and cultured as previously described (20). Cells (passages 10–17) from confluent cultures were detached with trypsin (0.05%)-EDTA (0.02%) and 100,000 cells/well were seeded into 9-cm^2 wells of six-well plates. The cells reached confluency within 5–6 d of seeding, and were used within 1–3 d after confluency was reached. At confluency each well contained 6–8 × 10^5 cells, with an average variance of ±5% among the wells of an individual plate. Before use, the culture medium was removed and the monolayers were

gently rinsed with 2.5 ml of CMFH. The cells were then incubated with prewarmed control or test medium. All experiments were done in serum-free medium at 37°C in an atmosphere of 5% CO_2 in air.

Basal or bradykinin-stimulted release of 6kPGF$_{1\alpha}$. 15-HETE provided as ammonium salt was dissolved in MEM with sonication to a final concentration of 100 μM. Control medium was used to make all 15-HETE dilutions. The endothelial cell monolayers were incubated with 1 ml of control medium, or test medium containing the indicated concentrations of 15-HETE. After incubation, cell culture supernatant aliquots were removed at the indicated times for assay of 6kPGF$_{1\alpha}$ production. A second set of monolayers (control and experimental) were stimulated with bradykinin (10 μg/well). The production of 6kPGF$_{1\alpha}$ was measured by RIA.

Metabolism of endogenous [1-^{14}C]arachidonic acid. Endothelial cells were prelabeled by incubation for 20 h at 37° in 1 ml of MEM plus 10% fetal calf serum containing 5 μM [1-^{14}C]arachidonic acid. The radiolabeled medium was removed, and the monolayers were rinsed twice with 2.5 ml of unlabeled medium, followed by 2.5 ml of CMFH. Monolayers were then preincubated in the presence or absence of 15-HETE (50 or 100 μM) for 15 min at 37°C, and stimulated with 10 μM A23187 for 60 min. Pooled cells and media were extracted, and extracts were dried over anhydrous magnesium sulfate, evaporated under a stream of nitrogen, and dissolved in 50 μl of chloroform for analysis by TLC.

Metabolism of exogenously provided [1-^{14}C]arachidonic acid. Monolayers were preincubated in the presence of varying concentrations of 15-HETE, 12-HETE, 11-HETE, or ricinoleic acid for 15 min at 37°C. [1-^{14}C]Arachidonic acid (5 μM) was then added to each well, and the incubation was continued for a further 60 min. The medium and cells were processed as described above.

Kinetics of [1-^{14}C]arachidonic acid metabolism by bovine endothelial cell microsomal cyclooxygenase. Endothelial cells from 40 confluent 75-cm^2 flasks were trypsinized, and the combined cell suspension was pelleted, washed once with CMFH, and suspended in 5 ml of Tris-GSH. The cells were disrupted by sonication, the microsomal fraction prepared (21), and the pellet was suspended in Tris-GSH. Kinetic experiments were performed as described previously.

Radioimmunoassay of 6kPGF$_{1\alpha}$ and 15-HETE

RIAs for 6kPGF$_{1\alpha}$ and 15-HETE in the incubation medium were performed essentially as described by Granström and Kindahl (22). The antisera for 6kPGF$_{1\alpha}$ and 15-HETE were purchased from Seragen Inc. (Boston, MA) and used according to the manufacturer's directions. These RIAs routinely provided linear results for 6kPGF$_{1\alpha}$ or 15-HETE vs. sample volume, and coefficient of variability of the assay was <10%. In addition to the cross-reactivities reported by the manufacturer, the 6kPGF$_{1\alpha}$ antiserum was found to be <0.04% cross-reactive to 15-HETE, whereas the 15-HETE antiserum did not cross-react with 6kPGF$_{1\alpha}$. No cross-reactive material was detected when incubation medium alone or medium containing 10 μg of bradykinin per milliliter was assayed. Results were expressed as picomoles of product released per milligram of tissue dry weight, or per 10^6 endothelial cells.

Statistical analysis

Statistical evaluation was performed by the paired Student's *t* test when only one treatment group was involved. The significance of differences in a treatment series was determined by randomized complete block analysis of variance (F ratio), and individual treatments were compared with the control by the Dunnett's test (23). The unpaired Student's *t* test was used to compare 6kPGF$_{1\alpha}$ and the HETEs generated by normal and diabetic umbilical arteries.

Results

Identification of arachidonic acid metabolites generated by human umbilical arteries

Incubation of intact human umbilical arterial segments with [1-^{14}C]arachidonic acid led to the formation of radiolabeled 6kPGF$_{1\alpha}$, PGI_2, PGE_2, and TXB_2 as well as HETEs. Vascular prostaglandins were identified by comparing their R_f values to those of authentic prostanoid standards. When the arachidonic acid metabolites were analyzed by TLC in a hexane-ether–acetic acid system, three major HETE products were routinely observed designated as HETE-1, HETE-2, and HETE-3 in increasing order of mobility (Fig. 1). The R_f values for these metabolites were 0.26±0.01 (1 SD), 0.30±0.02, and 0.33±0.02, respectively. The silicic acid column-purified vascular HETEs were resolved into three radioactive peaks by reverse-phase HPLC (Fig. 2). The rechromatographed HPLC peaks having retention times of 26 min (peak II) and 28 min (peak III) were found to be single components by TLC in the hexane-ether–acetic acid system with R_f values identical to HETE-3 and -2, respectively. HPLC-purified radioactive peak-II coeluted with reference 15-HETE and peak-III coeluted with reference 11-HETE. Peak I (*a* and *b*), which eluted in the di-HETE region, was found to be a mixture of components that proved refractory to separation by either reverse-phase HPLC or HPLC on a Partisil silica column (Whatman, Inc., Clifton, NJ). This fraction was therefore not subjected to further analysis. Trimethyl silyl ether methyl ester derivatives of unlabeled peak II and peak III had similar retention times (6.2 and 6.1 min, respectively), when analyzed by GC-MS using a 1% SE-30 column. Authentic 15-HETE, derivatized in the same manner and analyzed concomitantly under identical conditions, had a retention time of 6.2 min, suggesting that C values for peak II and peak III were similar to that of 15-HETE. The mass spectrum of the trimethyl silyl ether methyl ester derivative of peak II showed prominent ions at m/e 335 (M^+-71), 316 (M^+-90), 225 (M^+-181), 173 (M^+-233), and 73 and was identical to that reported for 15-HETE (12, 13) and to standard 15-HETE analyzed concomitantly. The mass spectrum of the trimethyl silyl ether methyl ester derivative of peak III showed prominent ions at m/e 316 (M^+-90), 239, 225 (M^+-181), and 73, identical to that reported for 11-HETE (12, 13). Although the spectra for peaks II and III had prominent ions at m/e 225, their spectra were clearly different in that the m/e 335 ion (loss of terminal pentyl group, C_{16}-C_{20} fragment), characteristic of 15-HETE fragmentation, was prominent in the spectrum of peak II and not detected in that of peak III. Based on these analyses, peaks II and III were identified as 15-HETE and 11-HETE, respectively.

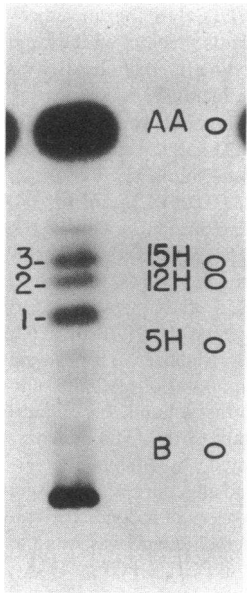

Figure 1. Thin-layer radiochromatogram of human umbilical arterial [1-^{14}C]arachidonic acid metabolites. Intact arterial segments (30 mg, dry weight) were incubated with [1-^{14}C]arachidonic acid (10 μM) for 30 min in 2 ml of Tris-Hanks'; the metabolites were extracted with chloroform-methanol and analyzed in the hexane-ether–acetic acid system. The positions of reference compounds are indicated. *B*, PGB_2; *5H*, 5-HETE; 12H, 12-HETE; 15H, 15-HETE; AA, arachidonic acid.

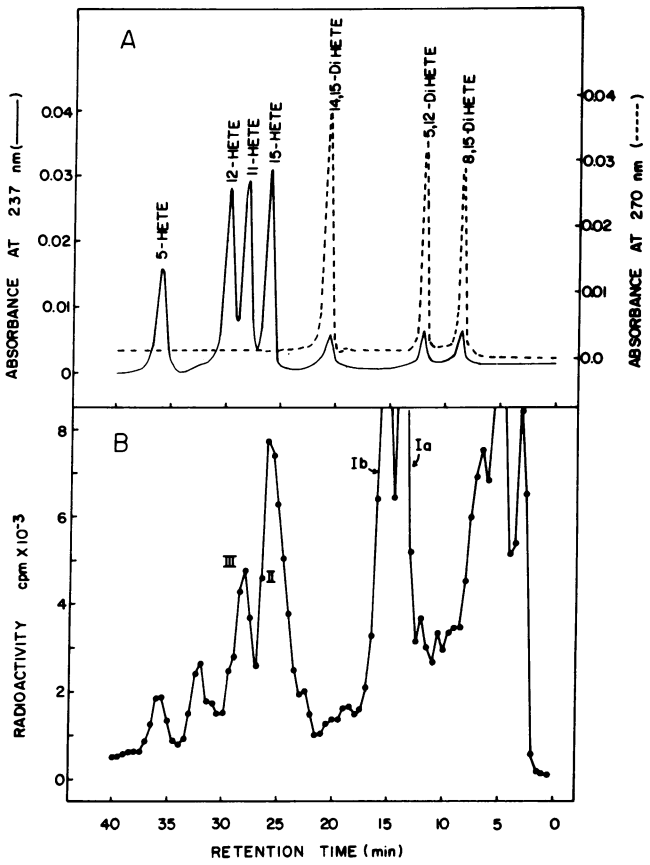

Figure 2. Reverse-phase HPLC of silicic acid column-purified human umbilical arterial HETEs. Intact human umbilical arterial segments (200 mg, dry weight) were incubated with [1-^{14}C]arachidonic acid (10 μM) for 30 min in 10 ml of Tris-Hanks'. Incubation medium was extracted as described. After silicic acid column chromatography, [1-^{14}C]HETEs were analyzed by reverse-phase HPLC as described in Methods. Fractions of 0.5 ml were collected, and radioactivity in 0.25-ml aliquots from each fraction was determined by liquid scintillation counting (*B*). Reference 5-, 11-, 12,- and 15-HETE (monitored at 237 nm) as well as 5,12-, 8,15-, and 14,15-diHETE at 270 nm) were chromatographed under identical HPLC conditions (*A*).

As depicted in Fig. 3, ETYA (60 μM), an inhibitor of both the cyclooxygenase and lipoxygenase pathways (24), inhibited the production of prostaglandins (71±9% [1 SE]; $P < 0.01$) as well as the vascular HETEs including 11-HETE (98±2%; $P < 0.05$), 15-HETE (71±15%; $P < 0.01$) and the di-HETE (61±16%; $P < 0.05$). NDGA, a lipoxygenase inhibitor (25) showed differential inhibitory effects on vascular HETE production. At a concentration of 30 μM, NDGA inhibited 15-HETE production by 66±6% ($P < 0.01$) whereas it stimulated the production of prostacyclin, total prostanoids, 11-HETE, and the di-HETE by 86±25%, 52±25%, 160±60%, and 49±10% over control values, respectively ($P < 0.05$ to $P < 0.01$). Similar effects of NDGA were also noted at a concentration of 15 μM (data not shown). Indomethacin (30 μM) significantly inhibited prostacyclin, total prostanoids, 11-HETE, and di-HETE production by 83±7%, 78±8% 98±2%, and 81±9%, respectively ($P < 0.01$). 15-HETE production was only minimally inhibited (20±3%; $P = 0.05$). These results indicate that, whereas 11-HETE and the di-HETEs are enzymatic products of cyclooxygenase in human umbilical arteries, 15-HETE is mainly a lipoxygenase product with minimal production of this metabolite occurring via cyclooxygenase. Table I summarizes the conversion of radiolabeled arachidonic acid to various metabolites by intact human umbilical arteries. Prostacyclin was the major metabolite of arachidonic acid in these tissue preparations. Significant amounts of $PGF_{2\alpha}$, PGE_2, di-HETEs, and 15-HETE were also generated. The ratio of prostacyclin to 15-HETE was ~6:1.

Inhibition of prostaglandin production by 15-HETE

Stimulation of prostacyclin production by a lipoxygenase inhibitor with a concomitant inhibition of 15-HETE production suggested to us that lipoxygenase metabolites of arachidonic acid could function as endogenous regulators of prostacyclin production in human vascular tissue. We therefore studied the metabolism of arachidonic acid by human umbilical arterial microsomal cyclooxygenase in the presence or absence of 15-HETE. Whereas the production of $6kPGF_{1\alpha}$ was inhibited by 15-HETE (Fig. 4 *A*), the relative distribution among the prostaglandins was essentially unaltered. The ratios of $6kPGF_{1\alpha}$ to $PGF_{2\alpha}$ to PGE_2 produced was 3:1:1 in both control and 15-HETE-treated microsomes. These results suggested that 15-HETE inhibited cyclooxygenase. The inhibition was concentration-dependent with an IC_{50} of 52±3 (mean±1 SE) and 63±4 μM for $6kPGF_{1\alpha}$ and total prostanoid formation, respectively (Fig. 4 *A*). At concentrations of 25, 50, and 100 μM 15-HETE, inhibition of vascular $6kPGF_{1\alpha}$ production was 22%, 52%, and 76%, respectively ($P < 0.01$). Inhibition of total prostanoid production by 15-HETE was found to parallel that observed for $6kPGF_{1\alpha}$ (12%, 42%, and 69% respectively). We also tested the effects of 11-HETE, another mono-HETE made by vascular segments; 12-HETE the platelet lipoxygenase product; and ricinoleic acid, a C_{18} hydroxy fatty acid on vascular cyclooxygenase activity (Fig. 4 *B*). Both 11-HETE and 12-HETE were found to inhibit total prostanoid production (with a parallel decrease in $6kPGF_{1\alpha}$) with IC_{50} of 74 and 78 μM, respectively. Ricinoleic acid had no effect on microsomal prostanoid production.

Conditions for the kinetic analyses of human umbilical arterial cyclooxygenase were previously established (19). Kinetic analyses of 15-HETE inhibition of arterial microsomal cyclooxygenase revealed that the V_{max} of the conversion of arachidonic acid to prostaglandins remained essentially unaltered. The apparent affinity of the enzyme for substrate (apparent K_m) was, however, decreased in the presence of 15-HETE. Such changes were seen in each of the five different experiments, a representative example of which is depicted in Fig. 5. The results of all experiments are seen in Table II. The mean V_{max} remained unaltered whereas the apparent K_m was significantly different ($P < 0.05$) in the paired 15-HETE-treated microsomes (37.6±17.7 μM, I SD) when compared to the control microsomes (9.3±6.9 μM). These results indicate that 15-HETE is a competitive inhibitor of cyclooxygenase.

Evaluation of endogenous 15-HETE and $6kPGF_{1\alpha}$ production by normal and diabetic human vascular tissues

Production of $6kPGF_{1\alpha}$ and 15-HETE by human umbilical arterial segments increased linearly for the first 5 and 15 min, respectively. By 30 min, maximum $6kPGF_{1}\alpha$ and 81±2% of 15-HETE production was established in both control and IDM vessels. When the production of $6kPGF_{1\alpha}$ and 15-HETE by vascular segments from controls and IDM were compared, significant differences were observed. Production of 15-HETE in the infants born to diabetic mothers was significantly greater ($P < 0.01$) than in control neonates [1.14±0.26 pmol/mg (mean±1 SD) vs. 0.77±0.22 pmol/mg, respectively]. A concomitant decrease in

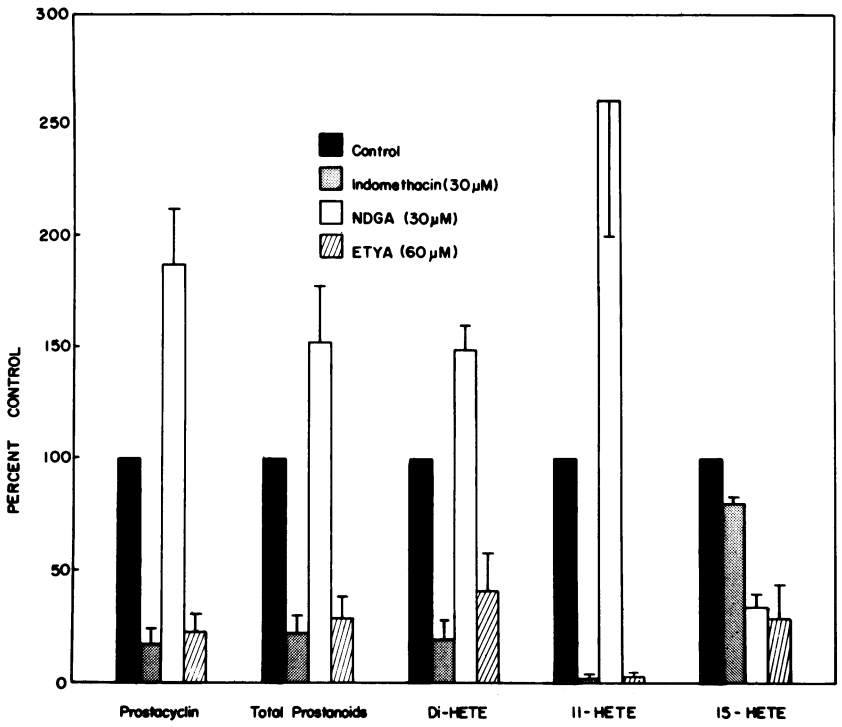

Figure 3. Effect of indomethacin, NDGA, and ETYA on [1-^{14}C]arachidonic acid metabolism by human umbilical arteries. Intact arterial segments (20–30 mg, dry weight) were preincubated at 37°C for 10 min in 2 ml of Tris-Hanks' alone or containing indomethacin (30 μM), NDGA (30 μM), or ETYA (60 μM). [1-^{14}C]Arachidonic acid (10 μM) was then added, and the incubation was continued for a further 30 min. The products were analyzed as described in Methods. Values represent the mean±1 SE of five experiments. Total prostanoids are the sum of 6kPGF$_{1\alpha}$, PGF$_{2\alpha}$, PGE$_2$, TXB$_2$, and PGI$_2$ production. Total prostacyclin is the sum of 6kPGF$_{1\alpha}$ and PGI$_2$ production.

6kPGF$_{1\alpha}$ production (51. 6±12.6 pmol/mg in the IDM vs. 71±22.3 pmol in controls; $P < 0.05$) was noted. An inverse correlation was also observed between endogenous production of 6kPGF$_{1\alpha}$ and 15-HETE ($r = -0.60$; $P < 0.02$; Fig. 6).

Effect of 15-HETE on endothelial cell arachidonic acid metabolism

Effect of 15-HETE on the release of immunoreactive 6kPGF$_{1\alpha}$. Incubation of bovine endothelial cells with MEM led to the appearance in the medium of prostacyclin measured immunologically as its stable hydrolysis product 6kPGF$_{1\alpha}$. The appearance of 6kPGF$_{1\alpha}$ in the medium increased markedly in the first 10 min after the addition of fresh serum-free medium and rose more slowly up to 60 min remaining essentially unchanged thereafter (Fig. 7 *A*). When the endothelial cells were stimulated with bradykinin, the release of immunoreactive 6kPGF$_{1\alpha}$ increased two- to three-fold (Fig. 7 *B*). Basal release of 6kPGF$_{1\alpha}$ was inhibited by 65% and 85% in the presence of 50 and 100 μM 15-HETE, respectively, at 60 min. Bradhykinin-stimulated release was also inhibited (83%) by 15-HETE at 60 min.

Inhibition of endothelial cell cyclooxygenase by 15-HETE. We studied the metabolism of exogenous [1-^{14}C]arachidonic acid by intact endothelial cells in the presence or absence of 15-HETE. The production of prostacyclin as well as total prostanoids was inhibited by 15-HETE in a parallel concentration-dependent manner (Fig. 8). The IC$_{50}$ for prostacyclin and total prostanoid formation were 39.6±16.9 μM (mean±1 SD) and 36.9±13.7 μM, respectively. When similar concentrations of 12-HETE and ricinoleic acid (1–100 μM) were tested for their effects on endothelial cell arachidonic acid metabolism, 12-HETE was found to inhibit total prostanoid formation with an IC$_{50}$ of 80 μM, whereas ricinoleic acid had no effect on prostaglandin formation (data not shown).

15-HETE also inhibited endothelial cell cyclooxygenase activity in isolated microsomes. Kinetic analyses of 15-HETE inhibition of microsomal cyclooxygenase revealed that the V_{max}

Table I. Metabolism of [1-^{14}C]Arachidonic Acid by Intact Human Umbilical Arteries

Product	Conversion
	pmol/mg dry tissue wt
Total prostacyclin	9.52±2.39
PGF$_{2\alpha}$	1.63±1.09
PGE$_2$	1.35±0.95
TxB$_2$	0.68±0.63
Total prostanoids	13.19±4.67
Di-HETEs	1.82±0.27
11-HETE	0.28±0.14
15-HETE	1.74±0.96

Intact arterial segments (20–30 mg, dry weight) were incubated with [1-^{14}C]arachidonic acid (10 μM) for 30 min in 2 ml of Tris-Hanks', and the metabolites were extracted and analyzed as described in Methods. Values represent the mean±1 SD, of five experiments, each performed in duplicate. Total prostacyclin is sum of 6kPGF$_{1\alpha}$ and PGI$_2$.

Table II. Effects of 15-HETE (100 μM) on Kinetic Parameters of Human Umbilical Arterial Microsomal Cyclooxygenase

Treatment of arterial microsomes	V_{max}	Apparent K_m
	pmol/min·mg	*μM*
Control	281.6±193.9	9.3±6.9
15-HETE treated	253.6±178.9	37.6±17.7
	(NS; $P > 0.2$)	($P < 0.05$)

Kinetic parameters of arterial microsomal cyclooxygenase were calculated from the Lineweaver–Burk plots (Fig. 5). Values represent the mean±1 SD of five paired experiments.

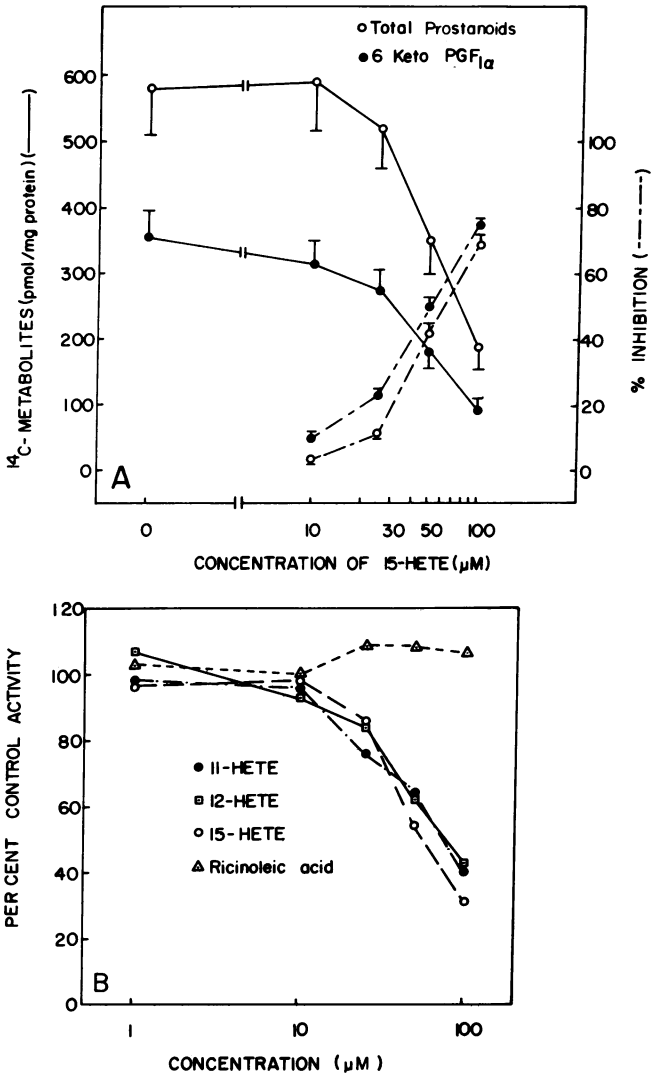

Figure 4. Concentration-dependent inhibition of human umbilical arterial microsomal cyclooxygenase by HETEs. Human umbilical arterial microsomes (500 μg in 500 μl of Tris-GSH) were preincubated with the indicated concentrations of HETEs (1–100 μM) for 5 min at room temperature. The cyclooxygenase reaction was initiated by addition of [1-^{14}C]arachidonic acid (10 μM) and terminated after 10 min. The products were analyzed by TLC radioautography–liquid scintillation countings. Total prostanoids are sum of 6kPGF$_{1\alpha}$, PGF$_{2\alpha}$, and PGE$_2$ production (no PGD$_2$, TXB$_2$, or PGI$_2$ was detected in these experiments). (*A*) Effect of 15-HETE (10–100 μM) on total prostanoids and 6kPGF$_{1\alpha}$ production. Values represent the mean±1 SE of four different experiments each performed in duplicate. (*B*) Effect of 11-HETE, 12-HETE, 15-HETE, and ricinoleic acid (1–100 μM) on total prostanoids and 6kPGF$_{1\alpha}$ production. Results depicted are from a representative experiment.

of prostaglandin formation remained essentially unaltered (1,100 pmol/mg·min control vs. 1,130 pmol/mg·min for 15-HETE-treated enzyme). The apparent affinity of the enzyme for substrate (apparent K_m) was decreased in the presence of 15-HETE (5.7 μM for control vs. 17.0 μM for 15-HETE-treated enzyme) (Fig. 9). The inhibition constant (K_i) for 15-HETE against bovine endothelial cell microsomal cyclooxygenase was estimated from a Dixon plot (26) to be 43 μM (Fig. 9, *inset*), a value quite similar to the IC$_{50}$ determined in the intact cells.

Effect of 15-HETE on ionophore A23187-stimulated release

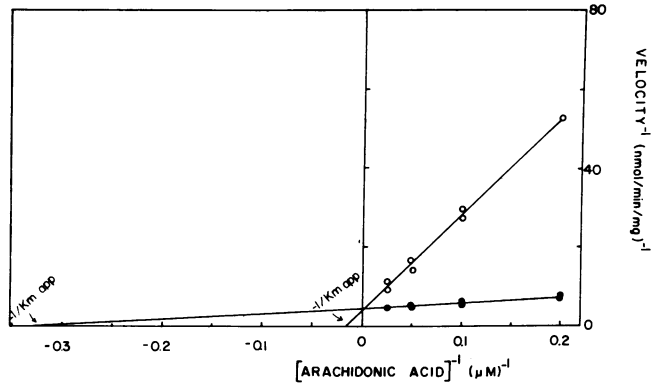

Figure 5. Kinetic analyses of inhibition by 15-HETE of the enzymatic conversion of [1-^{14}C]arachidonic acid to prostaglandins by cyclooxygenase from human umbilical arterial microsomes. Arterial microsomal protein (400 μg in 500 μl of Tris-GSH) was preincubated in the presence (o) or absence of 15-HETE (100 μM) (•) for 5 min at room temperature. Cyclooxygenase activity was assayed with varying concentrations of [1-^{14}C]arachidonic acid (5–40 μM) at 37°C for 5 min. Cyclooxygenase activity is expressed as the sum of 6kPGF$_{1\alpha}$, PGE$_2$, and PGF$_{2\alpha}$ production (no PGI$_2$, TXB$_2$, or PGD$_2$ was detected in these experiments). Lineweaver–Burk plots were generated using linear regression analyses.

and metabolism of [1-^{14}C]arachidonic acid. To determine whether 15-HETE affected other enzymes of the arachidonic acid cascade, bovine aortic endothelial cells were prelabeled with [1-^{14}C]arachidonic acid and stimulated by A23187 in the presence or absence of 15-HETE. Formation of radiolabeled prostacyclin was inhibited in the presence of 15-HETE (56% and 72% inhibition at 50 and 100 μM, respectively) (Table III). 15-HETE also inhibited the formation of other prostaglandins to a similar extent yielding an inhibition of total cyclooxygenase product formation by 54% and 68% at 50 and 100 μM, respectively. In addition to its effects on cyclooxygenase, the net appearance of free arachidonic acid and oxygenated metabolites was inhibited by 41% and 45% at 50 and 100 μM 15-HETE, respectively. An increase in radioactivity in the neutral lipid fraction, equal to the decrease in free arachidonic acid and me-

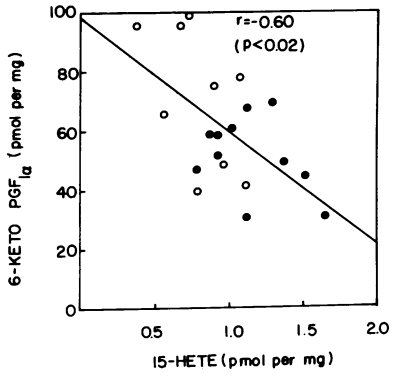

Figure 6. Evaluation of 15-HETE and 6kPGF$_{1\alpha}$ release by normal and diabetic human umbilical arteries. Umbilical arterial segments were incubated in Tris-Hanks' at 37°C for 30 min, and the medium was assayed for 15-HETE and 6kPGF$_{1\alpha}$ content by RIA. Values represent product released in picomoles per milligram of tissue dry weight. (•) Diabetic tissues; (o) normal tissues. The correlation coefficient was determined using linear regression analysis.

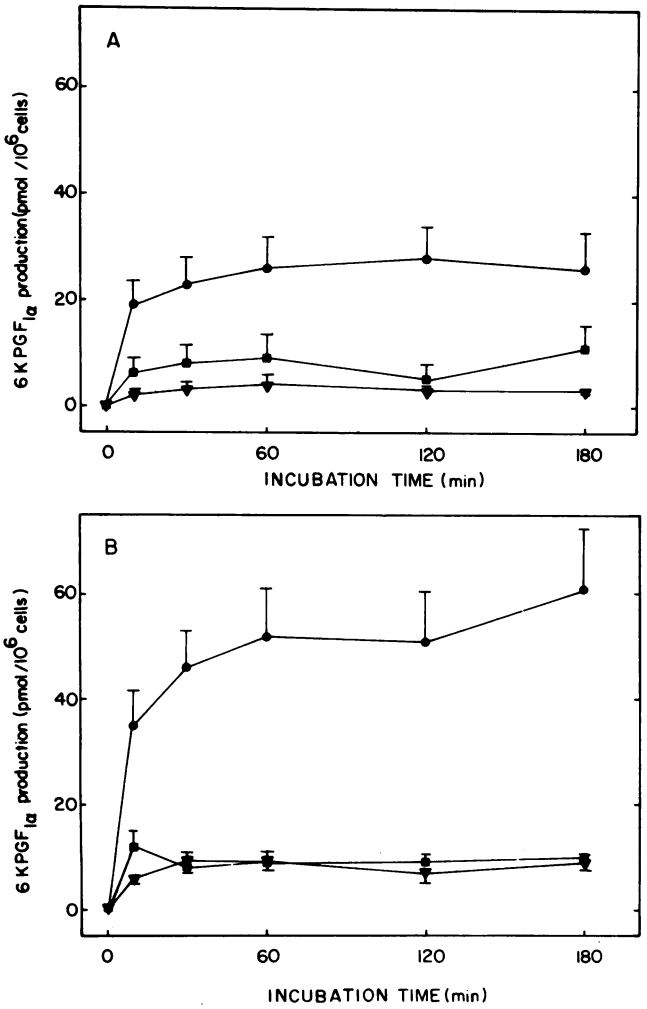

Figure 7. Effect of 15-HETE on basal and bradykinin-stimulated release of immunoreactive 6kPGF$_{1\alpha}$. Fetal bovine aortic endothelial cells were incubated in the presence or absence of varying concentrations of 15-HETE in 1.0 ml of MEM. Aliquots of cell culture media were removed at indicated times and assayed for 6kPGF$_{1\alpha}$ content by RIA. At the end of the experiment, cells were trypsinized and counted. Values are the mean±1 SE of four different experiments corrected for cross-reactivity of 15-HETE at zero time. (*A*) Basal release of 6kPGF$_{1\alpha}$; (*B*) bradykinin (10 μg/ml)-stimulated release of 6kPGF$_{1\alpha}$. (●) Control; (■) 50 μM 15-HETE; (▼) 100 μM 15-HETE.

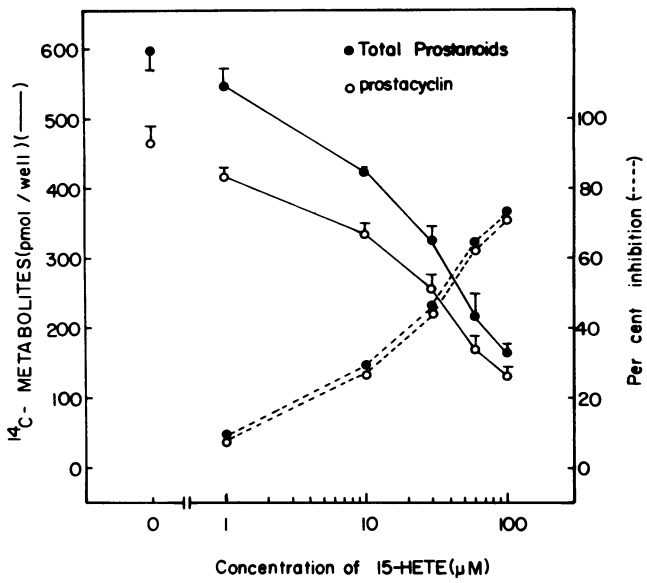

Figure 8. Concentration-dependent inhibition by 15-HETE of the conversion of exogenous [1-^{14}C]arachidonic acid to prostaglandins. Fetal bovine aortic endothelial cell monolayers were preincubated for 15 min with the indicated concentrations of 15-HETE (1–100 μM), in 750 μl of MEM. [1-^{14}C]arachidonic (5 μM) was then added and the incubation was continued for a further 60 min. The products were extracted in chloroform-methanol as previously described and analyzed by TLC radioautography–liquid scintillation counting. Values represent the mean±1 SD of four different experiments. Total prostacyclin is sum of 6kPGF$_{1\alpha}$ and PGI$_2$ production. Total prostanoids are sum of 6kPGF$_{1\alpha}$, PGF$_{2\alpha}$, PGE$_{2\alpha}$, PGI$_2$, and TXB$_2$ production. No PGD$_2$ was detected in these experiments.

tabolites was observed. Thus, although 15-HETE did not inhibit arachidonic acid mobilization from phospholipids, it did affect the metabolic fate of the mobilized arachidonic acid.

Discussion

Intact human umbilical arteries generated prostaglandins as well as mono-, and di-HETEs from arachidonic acid. The major product of arachidonic metabolism was prostacyclin. The arterial mono-HETEs were identified by reverse-phase HPLC and GC-MS as 11-HETE and 15-HETE and these were produced in the ratio of 1:6. Our results also suggest that both mono- and di-HETEs are enzymatically generated in arterial segments, with 15-HETE being the major lipoxygenase metabolite. A minor portion (~20%) of total 15-HETE production together with 11-HETE and the presumed di-HETEs are derived via the cyclooxygenase pathway in umbilical vascular segments. The production of 15-HETE via the 15-lipoxygenase pathway has also been reported by others in cultured human umbilical endothelial cells (3). Our initial exeriments with inhibitors also suggested that endogenous products of the lipoxygenase reaction could modulate the production of prostacyclin in vascular tissue. This suggestion was supported by our observation that NDGA stimulated prostacylcin with a concomitant inhibition in the production of 15-HETE (Fig. 3). To test this hypothesis, we next evaluated the effects of 15-HETE on arachidonic acid metabolism via the cyclooxygenase pathway using two different systems including human umbilical arterial microsomal cyclooxygenase and cultured bovine endothelial cells. Our results indicate that 15-HETE causes a parallel inhibition of both prostacyclin synthesis and the formation of the other cyclooxygenase metabolites both in isolated microsomes from human umbilical arteries, as well as in endothelial cells. Thus, this hydroxy acid appears to inhibit prostaglandin formation at the level of cyclooxygenase without affecting prostacyclin synthetase. 15-HETE was also found to be a reversible substrate competitor (competitive inhibitor) rather than a direct inhibitor of cyclooxygenase both in microsomes and in intact cells, in that the inhibition could be reversed by increasing substrate concentration. The inhibitory effects of 15-HETE on cyclooxygenase were not only documented in two different systems (umbilical vascular microsomes and isolated endothelial cells), but also using two different assay techniques, i.e., radioactive precursor labeling and radioimmunoassay studies.

In addition to its direct effect on cyclooxygenase, 15-HETE also affected the net appearance of free arachidonic acid and its oxygenated metabolites in stimulated cells. Although the net

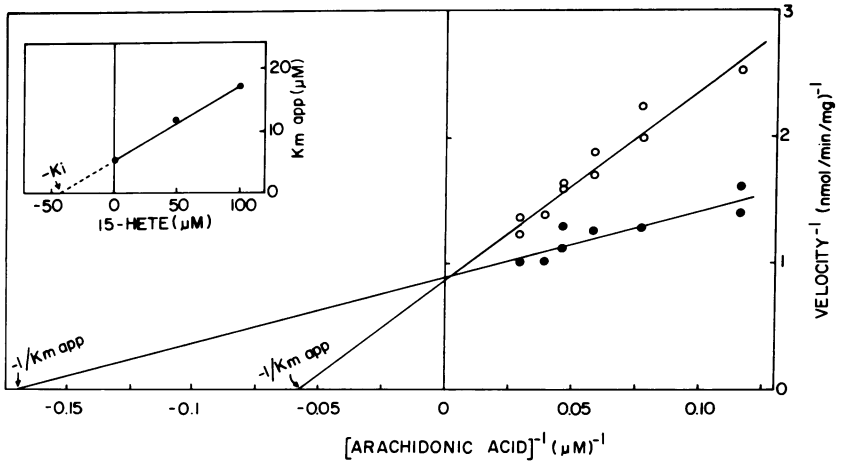

Figure 9. Kinetic analyses of inhibition by 15-HETE of the enzymatic conversion of [1-^{14}C]arachidonic acid to prostaglandins by cyclooxygenase from endothelial cell microsomes. Endothelial cell microsomal protein (250 μg in 500 μl of Tris-GSH) was preincubated in the presence (○) or absence (●) of 15-HETE (100 μM) for 5 min at room temperature. Cyclooxygenase activity was assayed with varying concentrations of [1-^{14}C]arachidonic acid (8.6–34.5 μM) at 37°C for 5 min. Cyclooxygenase activity is expressed as the sum of 6kPGF$_{1\alpha}$, PGE$_2$, TXB$_2$, and PGF$_{2\alpha}$ production (no PGD$_2$ or PGI$_2$ was detected in these experiments). Lineweaver–Burk plots were generated using linear regression analyses. (*Inset*) K_m apparent from experiments at 0, 50, and 100 μM 15-HETE were calculated and K_i determined from linear regression analysis of a Dixon plot.

release of arachidonic acid from cellular phospholipids in response to the calcium ionophore A23187 stimulation remained largely unchanged, substantial amounts of radiolabel accumulated in neutral lipids. This could be due to the increased availability of free arachidonic acid for the synthesis of triglycerides, which may play an important role in the turnover of arachidonic acid in endothelial cells (27). Other mono-HETEs including 11- and 12-HETE elicit similar inhibitory effects on vascular cyclooxygenase, although 15-HETE is the principal monoHETE, and appears to be the only metabolite produced by the lipoxygenase pathway in human vasculature tissue. That our in vitro findings on the effects of 15-HETE on vascular cyclooxygenase activity may be relevant to the in vivo situation was further studied in a human pathalogic state known to be associated with abnormal vascular prostacyclin production. For these final studies we chose pathalogic vascular tissue obtained from the diabetic milieu, i.e., the infant of the diabetic mother (IDM). We found that vascular tissue obtained from the IDM produced significantly more 15-HETE and less 6kPGF$_{1\alpha}$ when compared to control tissues. Moreover, an inverse correlation between 15-HETE and 6kPGF$_{1\alpha}$ production was also observed.

Based on our findings, we suggest that the hydroxy acid 15-HETE could potentially play a role in vascular pathologic states associated with both macrovascular and microvascular changes, as occurs in diabetes mellitus. Vascular disease is the major cause of morbidity and mortality in diabetes, and includes both large vessel disease (atherosclerotic or macrovascular), and abnormalities affecting the capillaries, i.e., diabetic "microangiopathy" (28). Both in humans with diabetes mellitus and in the animal model, abnormalities in platelet-vascular arachidonic acid metabolism have been observed that may contribute to the development of atherosclerotic or macrovascular disease. These include enhanced platelet production of proaggregatory TXA$_2$, together with a decrease in vascular prostacyclin production (29–32). The term microangiopathy hides a fundamental lack of knowledge regarding the basic biochemical and cellular level

Table III. Effects of 15-HETE on the A23187-stimulated Release and Metabolism of [1-^{14}C]Arachidonic Acid from Prelabeled Endothelial Cells

	Unstimulated	Stimulated		
Product		Control	15-HETE (50 μM)	15-HETE (100 μM)
	cpm × 10³ per well	*cpm × 10³ per well*	*cpm × 10³ per well*	*cpm × 10³ per well*
Prostacyclin	3.5±2.4	7.5±4.5	‡3.3±1.9	‡2.1±1.0
Cyclooxygenase products	5.4±3.6	10.4±5.6	‡4.8±2.5	‡3.3±1.0
HETEs	1.0±0.7	2.2±0.7	1.7±0.3	2.0±0
Free arachidonic acid	1.7±1.1	3.8±2.6	3.3±0.5	3.8±1.0
Free and metabolized arachidonic acid	8.1±5.2	16.4±7.8	*9.7±2.5	*9.1±1.0
Neutral lipids	4.6±2.7	7.7±4.9	11.9±7.5	‡14.8±9.0
Neutral lipids + free and metabolized arachidonic acid	12.7±7.7	24.1±12.0	21.5±9.2	23.8±10.0
Phospholipids	280.3±178.5	264.7±169.5	267.5±175.1	259.4±169.7

Confluent endothelial cells were prelabeled with 5 μM [1-^{14}C]arachidonic acid. 96±1% (n = 24) of radiolabel was taken up by the cells. Cells were preincubated in the presence or absence of 15-HETE (50 or 100 μM) in 750 μl of MEM for 15 min. The calcium ionophore A23187 (10 μM) in dimethyl sulfoxide (0.1% final concentration) was then added, and cells were incubated for a further 60 min. The metabolites were extracted with chloroform-methanol, and analyzed by TLC in the ethyl acetate–acetic acid system. Values represent the mean±1 SD of six different experiments (cpm × 10^3 per well). Total prostacyclin is the sum of 6kPGF$_{1\alpha}$ and PGI$_2$ production; cyclooxygenase products are the sum of 6kPGF$_{1\alpha}$, PGI$_2$, PGF$_{2\alpha}$, PGE$_2$, and TXB$_2$; free and metabolized arachidonic acid is the sum of cyclooxygenase products, HETEs, and free arachidonic acid. Values significantly different from control *$P < 0.05$; ‡$P < 0.01$.

changes that affect the retinal and glomerular capillaries in this disease. Diabetic retinopathy begins with the formation of microaneurysms and ultimately leads to neovascularization and proliferative retinopathy, a cause of vitreous hemorrhage, retinal detachment, and blindness (33). We have previously shown that 15-HETE enhances endothelial cell migration in vitro, and elicits neovascularization in an in vivo angiogenic system, i.e., the rabbit corneal pocket assay (6). An increase in 15-HETE production in diabetes mellitus could therefore not only modulate endogenous vascular prostacyclin production, but could also play a role in the abnormal neovascularization observed in the microcirculation of the patient with diabetes.

The umbilical vessels offer an unique opportunity to evaluate vascular tissue obtained from normal and various pathologic environments. Moreover, because the IDM has been observed to exhibit both an increased incidence of thromboses and thromboembolic phenomena (34, 35), together with abnormalities of the microcirculation (36), changes observed in IDM vasculature may closely simulate those observed in the adult with diabetes mellitus. In a previous study of platelets from IDM, we had demonstrated that the metabolism of arachidonic acid by the neonatal platelet at birth accurately reflected diabetic maternal platelet arachidonate metabolism (37). The presence of increased amounts of 15-HETE in vasculature obtained from the diabetic milieu with a concomitant decrease in $6kPGF_{1\alpha}$ formation may therefore be of potential significance, and point the way to further studies on the diabetic patient himself.

The studies of Vanderhoek (38) have recently begun to elucidate a possible regulatory role for 15-HETE in the formation of lipoxygenase arachidonate metabolites in platelets, polymorphonuclear leukocytes, and T lymphocytes. This author has demonstrated that 15-HETE is a selective inhibitor of platelet lipoxygenase. This hydroxy acid was also found to inhibit the formation of the 5-lipoxygenase arachidonic acid metabolites in rabbit leukocytes, and in human T lymphocytes. Our observation that 15-HETE is a competitive inhibitor of vascular cyclooxygenase, and that this compound is increased in vasculature obtained from the diabetic milieu (a disease state associated with vascular pathology and decreased prostacyclin production), should lay the groundwork for future investigations into the role of 15-HETE as an endogenous regulator of prostacyclin and neovascularization in various disorders associated with vascular pathology.

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