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Formation of Hydroxyeicosatetraenoic Acids (HETE) in Blood From Adults *Versus* Neonates: Reduced Production of 12-HETE in Cord Blood

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ABSTRACT. **Hydoxyeicosatetraenoic acids (HETE) are major arachidonic acid metabolites of a number of cells found in blood and blood vessels. These products have been implicated in physiologic responses as diverse as platelet aggregation, cell migration, and cell proliferation. Using a sensitive and specific assay, GC/selected ion monitoring after high-performance liquid chromatography separation, we have measured the levels of three HETE isomers of biologic significance 12-HETE, 15-HETE, and 5-HETE in plasma, serum and stimulated serum (formed in the presence of arachidonic acid and calcium ionophore), obtained from normal adults and cord blood from normal neonates. Whereas there were no significant differences between the two groups for 5- or 15-HETE in any of the samples, stimulated serum from adults produced 12 times as much 12-HETE when compared to cord blood. When platelets were isolated from adult and cord blood, 12-HETE production by neonatal platelets, stimulated with 10 μM arachidonic acid, was less than one-fourth that of adults. Although no role for 12-HETE in normal platelet responses has yet been established, it has been reported that those individuals with myeloproliferative syndromes who demonstrate a concomitant decrease in platelet 12-HETE synthetic ability have an increased bleeding tendency. It needs to be further evaluated if this already depressed level of 12-lipoxygenase in neonatal platelets may contribute to pathologic bleeding in those infants subjected to additional stress (such as prematurity or birth asphyxia). (*Pediatr Res* 24: 563–567, 1988)**

Abbreviations

HETE, hydroxyeicosatetraenoic acid
HPLC, high-performance liquid chromatography
PPP, platelet-poor plasma

Hydroxyeicosanoids are synthesized by many of the formed elements of blood. In fact, the major arachidonic acid metabolite produced by platelets is 12-HETE (1). Neutrophils and macrophages produce 5-HETE as a side-product in the biosynthesis of leukotrienes (2) and can synthesize 15-HETE (3). In addition, vascular endothelial cells have been shown to produce 15-HETE (4, 5). Although the discovery of these compounds predates the discovery of such important eicosanoids as thromboxane A_2, prostacyclin, and the leukotrienes, there is no clear-cut biologic function ascribed to them. Work in this laboratory, and in others, has suggested a role for such compounds in modulating migration (6–9) and proliferation (10) of endothelial cells that line the vascular wall. Additionally, there are suggestions that some HETE modulate the production of agents such as thromboxane and prostacyclin, which directly participate in platelet-vessel interactions (11).

In ^{14}C-arachidonic acid prelabeled platelets, using strong agonists, we have previously demonstrated differences in arachidonic acid release and metabolism between neonatal and adult platelets (12). No data are available on leukocyte or monocyte arachidonate metabolism in the neonate. We therefore have investigated the levels of the various HETE of biologic significance found in adult and neonatal plasmas and the levels of these compounds in serum formed either spontaneously, or when stimulated with levels of arachidonic acid and calcium ionophore found to maximize HETE production in adult platelets.

MATERIALS AND METHODS

Donors and collection of blood. Blood was drawn by venipuncture from 12 normal adults (six females, six males) between the ages 23 and 47. After informed consent of the mother, umbilical venous blood was drawn from 16 normal, full-term neonates (described in Table 1). Immediately after delivery (either vaginally or by cesarean section) clamps were placed on the umbilical cord, a 19-gauge needle was introduced into the umbilical vein near the placenta, and blood was withdrawn by a two-syringe technique. None of the adult donors or mothers had ingested any medications known to effect cyclooxygenase or lipoxygenase activity. White blood cell and platelet counts were determined to be in the normal range.

Aliquots were collected into tubes containing no anticoagulant to form serum, or tubes containing 2 mM arachidonic acid and 25 μM A23187 to produce stimulated serum. Preliminary experiments with adult samples had demonstrated that the presence of arachidonic acid during serum formation increased 12-HETE production and that the stimulation was maximal between 1 and 2 mM arachidonate. Further stimulation was produced by the calcium ionophore A23187, and this stimulation was maximal at 25 μM. These tubes were immediately brought to 37° C and maintained at that temperature for 1 h. Sera were then prepared by centrifugation at 1200 × *g* for 5 min. Additional aliquots were collected in tubes anticoagulated with 0.0894 M bisodium

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Table 1. *Vital statistics of cord blood donors*

Parameter	Mean	SEM	Maximum	Minimum
Gestational age (wk)	39.4	0.3	42	36
Birth wt (g)	3648	131	4820	2837
Apgar 1	8.1	0.2	9	6
Apgar 2	8.9	0.2	10	7
Platelets ($10^3/\mu l$)	294	19		
White cells ($10^3/\mu l$)	12.7	1.4		
Delivery				
Vaginal	3			
Cesarean	13			
Sex				
Male	8			
Female	8			

citrate, 0.0156 M citric acid, 0.016 M citric acid and 0.01418 M dextrose. PPP was then prepared by centrifugation at 1200 × *g* for 10 min in a Beckman J6B centrifuge (Beckman Instruments, Fullerton, CA). A plasma sample was also incubated with arachidonate and A23187 to control for noncell-mediated production of HETE under these conditions.

Extraction and isolation of HETE from blood samples. Each of the samples was treated with an equal volume of ice cold (−4° C) ethanol (to precipitate protein), acidified with one-tenth volume of 1.0 N HCl and extracted with five volumes of ethyl acetate. The ethyl acetate phase was removed and replaced with an equal volume of ethyl acetate and the extraction repeated. The combined ethyl acetate phases were dried over anhydrous $MgSO_4$, and evaporated under a nitrogen stream. The extracts were dissolved in a minimal volume of $CHCl_4$ and applied to a dry-packed silicic acid column (1 g/each 5 ml or smaller portion of serum). The columns were washed with 10 column volumes of hexane/ether (9:1), to elute di- and triglycerides, nonhdyroxylated fatty acids, and other neutral lipids. The column was then eluted with 10 column volumes of hexane/ether (50:50). This fraction contained the monohydroxy-eicosanoids. Solvent was removed without heating under reduced pressure in a Savant Speed Vac sample concentrator.

Before HPLC analysis, the sample was dissolved in methanol, diluted with water to a final concentration of 75% methanol, then injected onto a Waters C_{18}-reverse phase column (Waters Associates, Milford, MA). The column was eluted with 72% methanol/28% acetic acid (0.1%) at a flow of 1.0 ml/min, a modification of the method of Borgeat *et al.* (13). Column effluent was monitored at A_{237}nm. Fractions corresponding to the retention regions of authentic standards of 5-HETE, 12-HETE, and 15-HETE were collected for GC/MS analysis. An appropriate amount of ricinoleic acid (usually 1.0 nmol) was added as an internal standard to each peak before derivatization.

Derivatization of samples and standards for GC/SIM. Standard curves were generated from ricinoleic acid (1.0 nmol) (Applied Sciences, Belefonte, PA) mixed with known amounts (from 0.01 to 3.0 nmol) of either 5-, 12-, or 15-HETE. Standards were purchased from either Biomol (Philadelphia, PA) or Cayman (Ann Arbor, MI). Standard purity was assessed by reverse phase HPLC and concentration was determined by UV absorbance at 237 nM using a molar extinction coefficient of 30,500 (10). Standard mixtures or samples were treated with 100 μl of ethereal diazomethane [prepared from N,N,dimethyl-nitrosoguanidine in a millimolar diazomethane generator (Pierce, Rockford, IL)] containing 10% anhydrous methanol, for 3 h at room temperature in a Teflon capped vial. Solvent was evaporated under a nitrogen stream, the methyl esters were converted to trimethylsilyl ethers by reaction with 50 μl BSTFA (Pierce) overnight at room temperature. Samples were either analyzed immediately or stored at −20° C in that reagent until use. Just before GC analysis, the BSTFA was evaporated under a nitrogen stream and the sample was dissolved in approximately 5 μl of hexane.

Approximately 0.5 μl of the solution was injected onto the capillary column using a J & W (Folsom, CA) cool on-column injector coupled to a 1 m length of inactivated, but uncoated, capillary column. This was butted to a 6 m 0.25 mm id, cross-linked methyl silicone column (Hewlett-Packard Co., Palo Alto, CA) maintained at 185° C in a Hewlett-Packard 5880 gas chromatograph. The column was connected through a capillary-direct interface maintained at 280° C to a Hewlett-Packard 5970 mass selective detector operated in the scan mode. Head pressure on the column was maintained at 5 psi of helium resulting in a flow rate of 0.5 ml/min. Source, lens, and detector settings were established by the manufacturer's autotune software. Ion chromatograms were integrated, post-run, using software provided by the manufacturer. Standard curves were generated by linear regression analysis for (area of HETE ion peak/area of RA ion peak) *versus* (mass of HETE/mass of RA). Using this procedure we have demonstrated a linear response from 0.3 to 3 nmol of each HETE. Recovery of authentic standards added to plasma or serum averaged 89.5 ± 2.6% (14).

Metabolism of radiolabeled arachidonic acid by washed platelets. Platelets isolated from 3.0 ml platelet-rich plasma by centrifugation at 1500 rpm for 10 min was brought to 10 μM in EDTA. Platelets were washed with calcium-magnesium-free Hanks' balanced salt solution, 25 mM Tris, pH 7.4, and resuspended in 3.0 ml of that buffer. These washed platelets were incubated at 37° C and were brought to a final concentration of 1.0 mM in calcium chloride just before use. Platelets were incubated with ^{14}C-arachidonic acid (60 μCi/μmol) at concentrations of 10, 30, and 100 μM at 37° C in a final volume of 0.25 ml. After 2.5 min the reactions were stopped by the addition of 1.0 ml chloroform-methanol (1:2 vol/vol). Radiolabeled products and substrate were extracted essentially as described by Bligh and Dyer (15). 12-HETE was resolved from other products by thin-layer chromatography on silica gel G developed in hexane-diethyl ether-acetic acid (50:50:1 vol/vol/vol) and radioactivity of spots comigrating with authentic standard was determined by liquid scintillation spectroscopy in Liquiscint. Mass of HETE produced was calculated from specific activity of the radiolabeled arachidonic acid, corrected for total recovery of radioactivity through the procedure.

RESULTS

Platelet-poor plasmas from both adults and cord blood contained little of any of the HETE (Table 1). The means for each of the HETE in both populations were 0.11 μM or less. Although plasma from cord blood contained twice as much 5- or 15-HETE as adult plasma, these differences were not statistically significant. The average level of each of the HETE was significantly higher in serum than in plasma. 12-HETE was 10-fold more concentrated in both adult and neonatal sera than plasmas (Table 2). The differences between adult and cord blood serum 12-HETE were not significant ($p > 0.10$), due to the large variance in each population, even though the adult average was 60% more than the neonatal average. There were virtually no differences between adult and neonatal sera with regard to either 5-HETE or 15-HETE levels.

A striking difference was observed between the ability of adult and cord blood cells to produce 12-HETE when provided with arachidonic acid and A23187 (Fig. 1; Table 2). Serum from adults, generated in the presence of these compounds, contained an average of 21.3 μM 12-HETE, whereas cord serum reached only 1.75 μM ($p < 0.001$). The lowest level of 12-HETE production in adult blood was greater than the highest level seen in cord blood (Fig. 2). When ratios between stimulated and basal serum levels were determined for individuals, the average adult serum formed in the presence of arachidonate and A23187 contained 33 times as much 12-HETE as unstimulated serum, whereas the average ratio for the neonate was only four.

These differences in 12-HETE levels apparently did not result

Table 2. *HETE content of plasma and serum from adults and neonates (mean ± SE)**

Source	Treatment	HETE isomer (nmol/ml) 5-HETE	12-HETE	15-HETE
Adult	Plasma	0.051 ± 0.021	0.065 ± 0.038	0.018 ± 0.012
	Serum	0.22 ± 0.08	0.82 ± 0.22	0.069 ± 0.025
	Stimulated serum	3.64 ± 0.48	21.3 ± 2.78	1.01 ± 0.36
Neonate	Plasma	0.11 ± 0.055	0.047 ± 0.016	0.041 ± 0.012
	Serum	0.21 ± 0.072	0.55 ± 0.11	0.080 ± 0.025
	Stimulated serum	2.61 ± 0.496	1.75 ± 0.46	1.11 ± 0.37

* The indicate HETE isomer concentration was determined for each of the blood fractions for adult venous blood and neonatal cord blood.

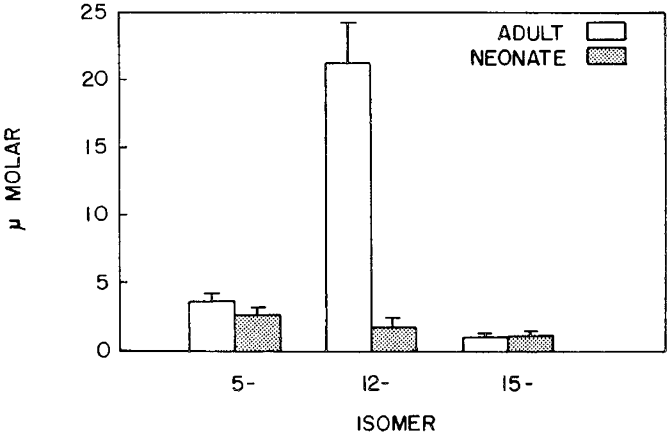

Fig. 1. HETE concentrations in stimulated sera from adults and neonates. Mean concentration (in μM) of the indicated HETE positional isomer in stimulated serum obtained from adult blood (*open bars*) or cord blood (*hatched bars*) plus 1 SD is depicted.

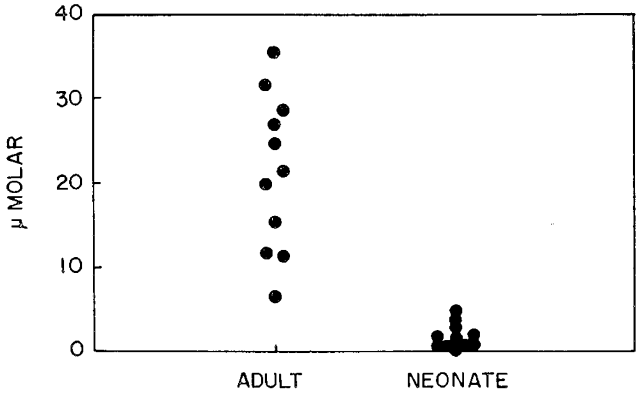

Fig. 2. 12-HETE concentrations in stimulated sera from individual adults and neonates.

from differences in the numbers of platelets in the blood from adults *versus* umbilical cords. Platelet counts in adult and neonate were nearly identical (263 ± 14 *versus* 294 ± 19 × $10^3/\mu l$, respectively, *cf.* Table 1). Thus, neither the slight reduction in 12-HETE in cord blood plasma and serum nor the enormous reduction in stimulated cord serum (compared to adults) can be explained by a corresponding quantitative platelet abnormality. Cord blood contained an average of twice as many white blood cells as did adult blood (12.7 ± 1.4 × $10^3/\mu l$ *versus* 6.3 ± 0.6, *cf.* Table 1). This difference might be responsible for the higher level of 5-HETE in plasma from neonates. However, despite the increased number of white cells, serum from cord blood, either spontaneously generated or formed during stimulation with arachidonate and ionophore, produced less or similar amounts of 5- and 15-HETE as did adult sera.

In exploring possible correlations between production of different HETE, by the same or different fractions within individuals, only the following correlations were found. In adults, there was a positive correlation between production of 12 HETE in PPP and serum. That is, those individuals with the higher plasma levels of 12-HETE also tended to produce the higher levels of 12-HETE in their serum ($r = 0.77$). In addition, there was a correlation between 12- and 15-HETE levels in adult serum ($r = 0.74$) and also in plasma ($r = 0.79$). In neonates, the only observed correlation was between the 5-HETE and 12-HETE content of plasma ($r = 0.76$).

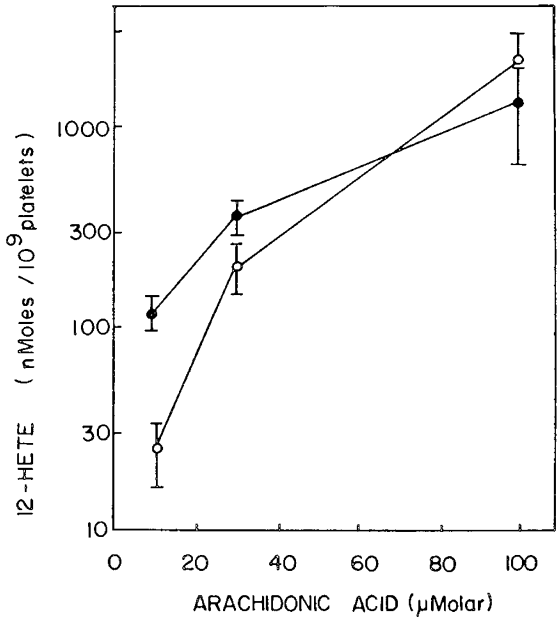

Fig. 3. Production of 12-HETE by washed platelets from three adults (*closed circles*) and four neonates (*open circles*) (mean ± SD). Platelets were incubated for 150 s with the indicated concentration of 1-^{14}C-arachidonic acid (60 μCi/μmol). Note logarithmic ordinate.

We also investigated if different groups of neonates differed from the mean or from each other with regard to any of the HETE measured in the various blood preparations. Cord blood from female neonates produced nearly twice as much 12-HETE in stimulated serum as did blood from males (2.1 ± 1.7 μM *versus* 1.1 ± 1.1) but the difference was not statistically significant ($p > 0.2$). No other differences were obvious between male and female neonates. When method of delivery was investigated, it was found that vaginally delivered infants had cord serum levels of 12-HETE twice those delivered by cesarean section (1.08 ± 0.52 μM *versus* 0.45 ± 0.29, $p < 0.05$). No significant differences in the other HETE were observed in serum, and neither stimulated serum nor plasma HETE values differed among these two groups.

To determine if the altered production of 12-HETE in stimulated serum resulted from differences between adult and cord blood platelets themselves, rather than differences in other blood

components, we examined the production of 12-HETE from arachidonic acid by washed platelets. The results depicted in Figure 3 demonstrate that cord blood platelets do differ from adult platelets in their ability to produce 12-HETE when provided with arachidonic acid. At 10 μM arachidonic acid, well below the apparent K_m of lipoxygenase for that substrate (16), adult platelets produced nearly five times more 12-HETE than did neonatal platelets (118 ± 24 nmol/10^9 adult platelets *versus* 25 ± 9 for neonates. $p < 0.01$). At 30 μM arachidonate the disparity was not as great (353 ± 74 nmol/10^9 platelets for adults *versus* 199 ± 55 for neonates) and at 100 μM, neonatal platelets produced more 12-HETE than did adult platelets (1300 ± 68 nmol/10^9 platelets for adults *versus* 2160 ± 790 for cord blood platelets) although these latter differences were not statistically significant ($p > 0.10$). It is worth noting that the levels of 12-HETE produced by washed platelets from adults stimulated with 10 μM arachidonic acid are similar to the amounts produced in stimulated serum. Assuming 0.25×10^9 platelets/ml the washed platelets should produce 12-HETE concentrations of 29 μM for adults (*versus* 21.3 μM actually measured). However, the calculated value of 6 μM for neonatal platelets was greater than the observed value of 1.75 μM (Table 1).

DISCUSSION

Hydroxyeicosanoids have been found to be involved in a number of biologic responses. Perhaps the first of these to be characterized was the chemoattraction of neutrophils toward 12-HETE (16). With the discovery of leukotrienes, important products of lipoxygenase-initiated metabolism in neutrophils, interest in the functions of HETE was reawakened. 5-HETE was found to be a stimulus of neutrophil secretory reactions (18). 15-HETE was found to modulate other reactions of arachidonic acid, inhibiting platelet 12-lipoxygeanse (19), but activating the 5-lipoxygenase (20) in other cells. Our laboratory has discovered effects of HETE on cell motility and cell division. 15-HETE has been found to promote neovascularization, a process that is fundamental to many physiologic and pathologic states (6–8). 15-HETE (0.1 mM) stimulates the migration of capillary endothelial cells in an *in vitro* Boyden chamber assay and also enhances cell proliferation. Further studies have also revealed an *in vivo* effect of 15-HETE on neovascularization in the rabbit corneal pocket assay (16, 17). Studies using lipoxygenase inhibitors have implicated HETE in vascular endothelial cell proliferation (10), and stimulus activation of macrophages (21) and neutrophils (22).

When the levels of HETE in plasma from normal adults or neonates was determined, the mean level of the HETE was not sufficient to evoke any of the known biologic responses. However, when blood was allowed to clot, the resulting levels of 12-HETE were sufficient to serve as chemoattractants for neutrophils (1 μM), and levels of 15-HETE were similar to those demonstrated to cause endothelial cell migration and proliferation (9). Blood cells have the capacity to produce far more HETE than formed during clotting. When presented with excess substrate and the calcium ionophore A23187, production of 12-HETE by adult serum was stimulated to a mean of 22 μM in 1 h. This level surpasses the concentration at which 12-HETE inhibits cyclooxygenase (11), or stimulates smooth muscle cell migration (9) and approaches the concentration shown to inhibit endothelial cell migration (6). 5-HETE also accumulated to a level at which stimulation of neutrophil secretion has been observed (18). Under these conditions, 15-HETE reached a concentration of over 1 μmol, similar to that required for activation of leukotriene synthesis (20).

Although plasma and serum samples from cord bloods contained similar levels of all the HETE measured (Table 2), in response to the additional stimuli arachidonic acid and A23187, neonatal blood produced only one-twelfth the amount of 12-HETE as did adult blood. This discrepancy was unique to 12-HETE, inasmuch as adult and neonatal stimulated sera contained similar levels of 5- and 15-HETE. This discrepancy could result from any of at least three causes. First, lipoxygenase activity in neonatal platelets might be reduced or altered in kinetic properties. Second, neonatal blood might contain increased levels of proteins or other components that bind or otherwise remove arachidonic acid from access to the platelet. Third, neonatal blood might contain increased levels of components that bind, sequester, or metabolize the 12-HETE that was produced.

When we measured 12-HETE production by washed platelets, we observed that at 10 μM arachidonic acid, neonatal platelets appeared to have only one-quarter the activity of their adult counterparts. When the concentration of substrate was raised to 100 μM, the difference disappeared. Whereas it is difficult to draw kinetic conclusions from whole cell experiments, these results are consistent with an increased K_m (decreased affinity) of neonatal platelet lipoxygenase for arachidonic acid, but a similar maximal level of enzyme activity when saturating substrate is available. If this explanation is the reason for the reduced 12-HETE level in neonatal stimulated sera, it also suggests that even at 2 μM arachidonic acid and 25 μM A23187, the concentration of substrate actually experienced by the platelet enzyme is quite low. It is possible that some component of neonatal serum further reduces the concentration of arachidonic acid available to the enzyme. However, it is unlikely that this arachidonic acid sink is plasma albumin or another plasma protein because we have previously shown that plasma from neonates has less "binding capacity" for arachidonic acid than that found in adult plasma (23).

The addition of arachidonic acid at much higher levels than is found in normal blood along with the calcium ionophore is certainly an artificial system. We have used it to determine the maximal level of HETE that might be produced by blood cells. Yet the levels of HETE produced in response to these extraordinary stimuli are not that different than might be produced in more physiologic settings. Collagen stimulation of platelet-rich plasma has been seen to produce 5 to 10 μM 12-HETE (24).

Although 12-HETE has been implicated in some reports in the second phase or disaggregation phase of responses to stimuli (25), there is as yet no generally agreed on, clearly defined role for this compound in platelet function or hemostasis. The only reported pathologic entity in which 12-HETE production is reduced is the myeloproliferative syndrome (26). In this condition, an increased bleeding tendency has been correlated with the platelet 12-lipoxygenase deficiency. Whereas the normal neonate does not manifest a bleeding tendency and has a normal bleeding time (27), severe bleeding can occur in preterm infants (28, 29). Should 12-HETE play an heretofore undiscovered essential role in hemostasis, it is possible that pathologic bleeding in the neonate might result from further reduction in the already impaired platelet 12-HETE synthetic ability.

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