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14,15-EPOXYEICOSATRIENOIC ACID PROMOTES ENDOTHELIAL CELL DEPENDENT ADHESION OF HUMAN MONOCYTIC TUMOR U937 CELLS

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SUMMARY: Arachidonic acid (AA) can be metabolized in endothelial cells (EC) to a series of epoxides via cytochrome P-450 epoxygenase with 14,15 epoxyeicosatrienoic acid (14,15-EET) as the major product. In this communication we report that 14,15-EET significantly enhances U937 cell attachment to EC with maximal cell attachment at 2.5 to 5 x 10^{-7}M 14,15-EET. Thus, 14,15-EET may play a substantial role in inflammation and/or atherogenesis by inducing monocyte attachment to EC.

Monocyte attachment to the endothelium represents an important step in both inflammation and atherogensis [1,2]. When atherogenic low density lipoprotein (LDL) concentrations are incubated with endothelial cells, there is increased synthesis and release of epoxyeicosatrienoic acid (EET) products [3] as well as increased monocyte attachment [4]. The mechanism of EET production appears, in large part, related to P-450 enzyme system induction [3]. Physiologic effects of EET products include proinflammatory events such as increased vascular permeability [5], polymorphonuclear leukocyte chemotaxis [5,6], and Ca^{2+} mobilization [7]. For these reasons, we explored the hypothesis that 14,15-EET is a potent stimulator of monocyte attachment to EC.

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Abbreviations: 14,15 epoxyeicosatrienoic acid (14,15-EET); cytochrome P-450 (P-450); human umbilical vein endothelial cells (EC); arachidonic acid (AA); low density lipoprotein (LDL); endothelial cell growth factor (ECGF); fetal bovine serum (FBS); American Type Tissue Culture (ATTC); phorbol myristate acetate (PMA); 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM); dimethylsulfoxide (DMSO); and lactate dehydrogenase (LDH); hydroxyheptadecanoic acid (HHT); hydroxyeicosatetraenoic acid (HT); polymorphonuclear leukocytes (PMN).

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Using as a model U937 cells, a human monocytic tumor cell-line, we pretreated EC with 14,15-EET and then exposed the treated EC to U937 cells. Results indicate that EC exposure to 14,15-EET substantially enhances U937 cell attachment to EC.

Materials and Methods

Cell Cultures

Human umbilical vein endothelial cells (EC) were obtained as described [3,8]. EC were cultured in M-199 media with Earl's salts, 25 mM $NaHCO_3$ (Gibco, Grand Island, NY), supplemented with 20% human serum, 250 ug ECGF/mL [9], and 90 ug heparin/mL (Gibco, Grand Island, NY), pH 7.45. Eicosanoid analysis was performed on second passage EC in Primaria T75cm^2 flasks Falcon from Becton-Dickinson & Co. (Lincoln Park, NJ). Cell attachment assays were performed on second passage EC in 24 or 48 well plates; Primaria and Costar (Cambridge, MA) respectively. All wells were coated with cryoprecipitate of human plasma. EC were maintained at confluence for two to three days prior to experiments.

The human monocytic tumor cell line, U937, was purchased from ATTC and maintained at 0.56 to 1.0 million cells/mL in RPMI media supplemented with 10% heat inactivated fetal bovine serum (FBS) and antibiotics/antimicotics. Cells were split with fresh media 1:5 every two to three days. Cell counts were performed routinely to maintain low population density.

EC Eicosanoid Analysis

Confluent EC in T75cm^2 flasks were thrombin stimulated in the presence of ^{14}C-arachidonic acid as described [3] with the following modifications. The buffer used to wash EC monolayers and to stimulate with thrombin was M199 containing Hanks salts from Sigma Chemical Co. (St. Louis, MO) and 10 mM HEPES from Gibco Inc. (Grand Island, NY) instead of the HEPES buffer previously used [3]. Solid phase extraction of ^{14}C-eicosanoids was performed on the media and EC was described [3]. HPLC separation, identification and quantification was performed as previously described [3].

Cell Attachment Assay

A) EC Pretreatment: Confluent EC monolayers were incubated with the following agents for four hours at 37^{o}C: arachidonic acid from Nu Chek Prep (Elsian, MN), 12-HETE, 15-HETE and 14,15-EET from Biomol (Plymouth Meeting, PA), and phorbol myristate acetate (PMA) from Sigma. These agents were removed and EC monolayers washed with M-199 media before the U937 cell attachment assay. The attachment assays were performed by either morphometric analysis or fluorescent dye incorporation.

B) Morphometric Analysis: U937 cells were centrifuged, washed with M-199 media, and resuspended in EC media (defined above) to approximately one million cells per milliliter. Cell suspensions (0.5 ml for 48 well plates and 1.0 ml for 24 well plates) were added to each test well of washed EC and

incubated at 37°C, 95% air, 5% CO_2 and 100% humidity for 30 minutes. The U937 cell suspension was withdrawn and the well was washed with M-199 media to remove unattached U937 cells by the method of Charo et al. [10]. The wells were then fixed with 10% formaldehyde and morphometric analysis was performed with phase contrast microscopy. EC and bound U937 cells were counted using a TMS Nikon phase contrast microscope at 20X magnification. Five fields per well were counted, the ratio, U937 cells per 100 EC, was calculated, results averaged per well and then finally results from each well averaged for each test condition.

C) Fluorescent Dye Incorporation: U937 cells were loaded with fluorescent dye to determine cell attachment to EC. Fifty micrograms of the fluorescent dye BCECF-AM from Molecular Probes (Eugene, OR) was dissolved in fifty microliters of DMSO from Fisher Scientific (Springfield, NJ) which was then mixed with five milliliters of RPMI media (Gibco, Inc.) containing 2% FBS. Thirty million U937 cells were pelleted and resuspended in the dye mixture for 30 minutes at 37°C, Dye loading was halted by adding RPMI media to a final volume of fifty milliliters and then centrifuging at 200 x g for 20 minutes at 20°C. BCECF-loaded cells were resuspended in EC media to a final concentration of one million cells per milliliter. A volume of this cell suspension (as above) was added to each test well of washed EC and incubated at 37°C, 95% air, 5% CO2, 100% humidity for 30 minutes. The U937 cell suspension was withdrawn, and well washed as described above [10]. The BCECF dye was released from the attached U937 cells with 0.1% Triton X-100 (Sigma) in 100 mM Tris (Sigma), pH 8.0, and fluorescence was measured in a Shimazdu spectrofluorimeter (EX 485, EM 535). The number of attached cells per well was determined by comparing the fluorescence per well to a standard curve of fluorescence per cell.

Results

Thrombin stimulated EC generate several hydroxy-eicosanoids and four arachidonic acid epoxides (EET) (Figure 1). The epoxide formed from exogenous

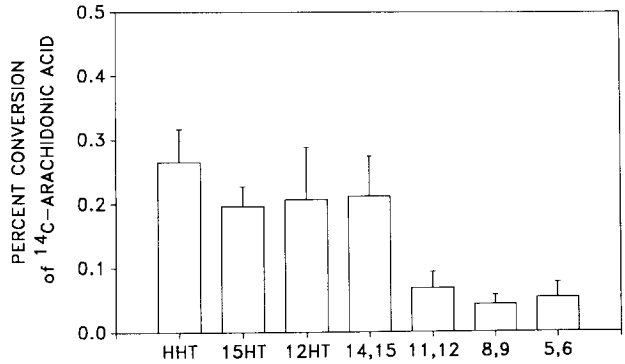

Fig. 1. This figure shows the percent conversion of [^{14}C]-arachidonic acid to [^{14}C]-eicosanoids by thrombin (1U/mL) stimulated EC. Data represent mean +/- SEM (n=8).

[^{14}C]-arachidonic acid in the greatest amount is 14,15-EET with minor amounts of the other EETs (11,12-; 8,9-; and 5,6-EET). Thus 14,15-EET is the major arachidonic acid epoxide formed by normal confluent EC during thrombin stimulation.

Enhanced U937 cell attachment to EC was established by exposing EC to PMA. PMA was used as a positive control and as an index of maximum U937 cell attachment. PMA is a potent inducer of EC adhesion protein expression [11]. Maximal attachment (PMA-MAX) was seen at a PMA concentration of 1×10^{-8} M. Cell binding to EC after PMA treatment was 0.066×10^{6} cells per well $\pm$ 0.01 SEM (n=15). U937 cell attachment measured with all other compounds was compared to PMA max and expressed at %PMA-MAX.

EC exposure to 14,15-EET produced enhancement of U937 attachment to EC in a dose dependent manner (Figure 2). EC exposure to 14,15-EET concentrations greater than 7.5×10^{-7} M produced EC retraction and detachment. Neither this nor overt cytotoxicity, as measured by LDH release (Table 1), was observed at concentrations of 0.0625 to 5.0 $\times 10^{-7}$M of 14,15-EET. 14,15-EET promotes the attachment of human PMNs but to a lesser degree than U937 cells and has no effect on human platelets (Table 2).

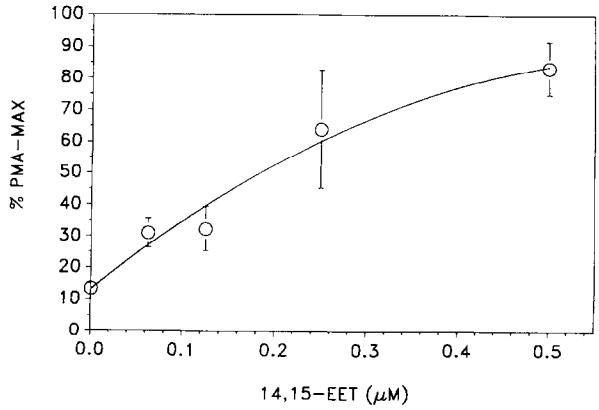

Fig. 2. This figure shows the effects of 14,15-EET on EC function with respect to binding of U937 cells. After a 4 hour EET incubation, EC bind U937 cells nearly as much as 10 nM PMA (PMA-MAX).

Table 1

14,15-EET (uM)	0.0	0.0625	0.125	0.250	0.500
LDH (U/L)	181.8	182.0	181.7	183.9	181.6
+/- SEM	0.56	0.34	0.68	1.0	0.98
(n=4)					

This table shows the effects of 14,15-EET on EC viability. Lactate dehydrogenase activity was measured in EC media after 4 hours exposure to the above concentrations. 14,15-EET had no effect on the release of LDH from EC as evidenced by no change in enzyme activities.

Discussion

EETs represent a recently described group of eicosanoids with a wide variety of effects as previously mentioned [4-6]. EC can produce EETs when stimulated with thrombin [3]. Furthermore, Pritchard and colleagues have recently observed that EC, when exposed to atherogenic levels of LDL for four days, produce increased 14,15-EET via cytochrome P-450 epoxygenase dependent pathway [3] and also exhibit increased attachment of human monocytes [4]. An important mechanism linking enhanced monocyte attachment to EC may be attributed to the production of 14,15-EET. Such a mechanism may involve EET induction of EC adhesion proteins, which in turn may facilitate monocyte attachment in atherosclerotic and inflammatory processes. This report demonstrates that 14,15-EET induces increased EC dependent monocyte attachment. 14,15-EET inhibits platelet aggregation [12] and adhesion to EC

Table 2

	Platelet	PMN	Monocyte
14,15-EET	-	++	+++
Control Media	-	-	-

This table shows EC effects by 14,15-EET (0.25 uM) with respect to platelet, PMN and monocyte binding. (+ = promotes binding; - = lack of binding)

monolayers. In contrast, 14,15-EET strongly promotes attachment of U937 cells, a monocyte-like cell, and PMN cells. Furthermore, the activation of the P-450 pathway in EC by LDL may lead to excessive generation of arachidonic acid epoxides thus promoting monocyte attachment to the endothelium and further augmenting cell to cell interactions in the atherosclerotic process.

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