Increases in plasma trans-EETs and blood pressure reduction in spontaneously hypertensive rats

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1Department of Pharmacology, New York Medical College, Valhalla, New York; 2Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas; and 3Entomology and Cancer Center, University of California, Davis, California

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Jiang H, Quilley J, Doumad AB, Zhu AG, Falck JR, Hammock BD, Stier CT, Jr, Carroll MA. Increases in plasma trans-EETs and blood pressure reduction in spontaneous hypertensive rats. Am J Physiol Heart Circ Physiol 300: H1990–H1996, 2011. First published March 11, 2011; doi:10.1152/ajpheart.01267.2010.—Epoxyeicosatrienoic acids (EETs) are vasodilator, natriuretic, and antiinflammatory lipid mediators. Both cis- and trans-EETs are stored in phospholipids and in red blood cells (RBCs) in the circulation; the maximal velocity (V_{max}) of trans-EET hydrolysis by soluble epoxide hydrolase (sEH) is threefold that of cis-EETs. Because RBCs of the spontaneously hypertensive rat (SHR) exhibit increased sEH activity, a deficiency of all EET isomers, and the rate of EET hydrolysis decreased two- to threefold sequentially from 14,15-, 11,12-, and 8,9- to 5,6-EETs (29). Inhibition of sEH will favor the preservation of EETs. Increased sEH activity has been associated with hypertension (1, 18, 24, 55). Inhibition of EET hydrolysis by sEH inhibitors reduced BP by 20 to 30 mmHg in rats with angiotensin II (ANG II)-/salt-induced hypertension (21, 24, 37), as well as in the SHR (55) and the stroke-prone SHR (34). Deletion of the sEH gene reduced BP by 16 mmHg compared with wild-type DOCA-salt hypertensive mice (39). Inhibition of sEH is a potential therapeutic approach for control of BP, vascular inflammation, and for providing cardiac and renal protection by raising levels of EETs in vivo (45, 49).

RBCs represent a major source of sEH in the circulation for the hydration of cis- and trans-EETs, producing threeo- and erythro-DHETs, respectively (29). The maximal velocity (V_{max}) for the hydration of trans-EETs is approximately three times greater than for their respective cis-isomers (29). The 14,15-trans-EET is hydrolyzed the fastest by rat RBCs among all EET isomers, and the rate of EET hydrolysis decreased two- to threefold sequentially from 14,15-, 11,12-, and 8,9- to 5,6-EETs (29). Inhibition of sEH will favor the preservation of trans-EETs over cis-EETs, since trans-EETs are hydrolyzed three times more rapidly than cis-EETs by sEH, and the Michaelis constant for trans- vs. cis-EETs is on average 1.6 vs. 1.0 μM, respectively (29). There is greater sEH expression and activity in SHR than in the normotensive Wistar-Kyoto rat (WKY) control strain (18, 48, 55).

RBCs are also reservoirs and carriers of EETs in the circulation (22, 27). RBCs, in addition to O2 delivery in the microvasculature, participate in the regulation of microvascular tone by releasing vasoactive factors, including EETs (25, 28). The role of RBC sEH in the regulation of circulating EETs may be particularly significant when considering that there are
effects of EETs on the rheological and hemodynamic determinants of the circulation. Inhibition of RBC sEH presumably contributes to elevating effects of EETs on regional blood flows to a greater degree than inhibition of sEH localized “in the smooth muscle layers of the arterial wall” (54). The present studies focused on effects of the potent sEH inhibitor, cis-4-[4-(3-adamantan-1-ylreido)cyclohexyloxy]benzoic acid (AUCB) (23, 29), on BP and EET levels in the circulation in relation to the activity of RBC sEH in the SHR compared with the WKY, as well as the direct vasorelaxant activity of cis- vs. trans-EETs in renal microvessels.

MATERIALS AND METHODS

Animals. The experimental procedures in animal use were approved by the Institutional Animal Care and Use Committee of New York Medical College and conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. Ten-week-old male SHR and WKY rats were purchased from Taconic Farms. Seven-week-old male Sprague-Dawley (SD) rats were from Charles River Laboratories. Rats were maintained at 22°C with alternating cycles of light and darkness and fed ad libitum with standard rat chow (Purina) and water. AUCB (0.01 mg/ml to provide a dose of 2 mg·kg⁻¹·day⁻¹) was dissolved in drinking water containing 0.2% ethanol and administered to 11-wk-old SHR and WKY rats for 7 days while the vehicle control rats were given tap water containing 0.2% ethanol.

BP measurements. Noninvasive BP measurements were made using a volume-pressure tail-cuff system (Kent Scientific) (15). Each rat was subjected to five acclimation cycles followed by five measurement cycles for BP readings. In some cases, BP was measured by radiotelemetry (35) to confirm the changes measured by tail cuff.

Reagents. Standard racemic trans-EETs were synthesized as described, and the purity as analyzed by HPLC was over 99% (14, 26). Standard cis-EETs were from Cayman Chemical (Ann Arbor, MI). AUCB was provided by Dr. Hammock and has been characterized as a potent sEH inhibitor (23, 29). BSA and HPLC-grade organic solvents were obtained from Fisher Scientific (Pittsburgh, PA), and EET-d8 standards were from Enzo Life Sciences (Plymouth Meeting, PA). Other reagents were purchased from Sigma (St. Louis, MO).

Preparation and incubation of rat RBCs was performed as previously described (29). Briefly, up to 10 ml blood were drawn from the inferior vena cava of pentobarbital-anesthetized rats. RBCs were isolated by centrifugation at 400–800 g and then washed with cold physiological salt solution after the buffy layer was discarded. RBC incubations to compare the hydrolysis of EETs by rat RBCs were carried out using 16 ng of cis- or trans-EETs in 50 µl prewarmed 5 × 10⁴ RBCs in PBS at 37°C for 10 min with shaking at 600 rpm in a VWR 3-mm orbital shaker.

Plasma and urine EET extraction. Rat plasma was obtained after centrifuging blood at 2,000 g for 10 min and mixed with polymer-bound triphenylphosphine (TPP, 1 mg/ml) to quench free radical-induced lipid peroxidation. Phospholipid was extracted from 0.4 ml plasma using the Bligh-Dyer (3) method and hydrolyzed with 1 M NaOH for 90 min at room temperature. The hydrolysis mixture was then neutralized with 1 M HCl and extracted two times with 2 ml ethyl acetate. The ethyl acetate extract was dried under a gentle stream of nitrogen and dissolved in acetonitrile (20 µl) for immediate LC/MS/MS analysis (27). Rat urines (24 h) were collected in tubes containing 5 mg polymer-bound TPP. Urine samples (2 ml) with added d11-labeled 8,9- and 14,15-DHET and d8-labeled 8,9-, 11,12-, and 14,15-EET (1 ng each) were vigorously mixed two times with 3 ml hexane-ethyl acetate (1:1) to extract EETs and DHETs. The combined organic phase was backwashed with 4 ml of water, dried under a gentle stream of N₂, and dissolved in 80 µl acetonitrile for HPLC separation and GC/MS analysis as described (26, 41).

Mass spectrometry analyses. ESI LC/MS/MS analyses of EETs and DHETs were carried out as described (27, 29). Briefly, a Finnigan LCQ Advantage quadrupole ion-trap mass spectrometer equipped with ESI source run by XCALIBUR software was used. MS/MS breakdown for mass-to-charge ratio (m/z) 319 was at an energy level of 35% set by the instrument, and a seven-point Gaussian smoothing was applied in the mass data processing. Quantification of individual EETs was based on standard curves (r = 0.99) between their respective characteristic fragmentation ions with reference to an internal standard of 2 ng of d8–11,12-EET.

For quantification using electron-capture negative-chemical ionization GC/MS, purified DHET samples were derivatized to trimethylsilyl ether pentafluorobenzyl (PFB) esters, and EETs were derivatized to PFB esters as described (26, 28). The ions m/z of 481 and 492 were monitored for endogenous and d11-labeled DHETs; the ions m/z of 319 and 327 were monitored for endogenous and d8-labeled EETs.

Western blot analysis of sEH. RBC cytosol was obtained by centrifugation of lysed RBCs at 10,000 g for 1 h and then diluted 1:2 with 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1% SDS on ice. Total protein concentration was quantified with the Pierce BSA assay using Fraction V BSA as the calibrating standard. For each sample, 50 µg of protein were loaded on a 12% SDS-PAGE, and Western blot analysis was carried out as described (29). Expression of GAPDH was detected using a monoclonal mouse antibody and a goat anti-mouse IgG labeled with horseradish peroxidase. Bands were visualized using the ECL kit from Amersham and results calculated as a ratio relative to GAPDH expression.

Rat renal arcuate artery studies. Activities of cis- and trans-EET regioisomers were evaluated in microdissected SD rat arcuate arteries (~100 µm internal diameter) that were cannulated and pressurized to 80 mmHg as described (12). Arcuate arteries were chosen because of their exquisite sensitivity to cis-EETs (12). We determined that neither indomethacin (10 µM), a cyclooxygenase inhibitor, nor l-NAME (200 µM), a nitric oxide synthase inhibitor, affected the vasoactivity of cis- and trans-EETs (data not shown). The vessels were preconstricted with phenylephrine (10⁻⁷ mol/L), and cumulative concentration responses to individual EET regioisomers were recorded before and after application of 14,15-EEZE (10⁻⁶ mol/L), a selective EET antagonist (20). We also examined the activity of the 14,15-EET isomers on arcuate arteries obtained from 12-wk-old SHR and WKY rats. In these experiments, arteries were pressurized to 80 and 120 mmHg to address any effects of different baseline pressures that exist in SHR and WKY rats.

Data analysis. Results are presented as means ± SE and analyzed using GraphPad Prism 5 (San Diego, CA) software. Comparisons among multiple groups were made by ANOVA followed by Tukey’s test for differences between groups. Paired and unpaired t-tests were used for comparison between two groups where appropriate. A P value <0.05 was considered as statistically significant.

RESULTS

Increased RBC sEH activity and expression in SHR compared with WKY. We examined RBC sEH activity and expression in SHR and WKY rats, since elevated sEH expression has been reported to occur in both the kidney and the brain of SHR (18, 48, 55). Incubation of cis- and trans-isomers of 14,15-, 11,12-, and 8,9-EETs (1 µM each) with RBCs from the 11-wk-old SHR and WKY revealed significant differences in the hydrolysis of EETs. Specific rates of EET hydrolysis by RBCs of the SHR are on average more than double that of the WKY (Fig. 1A). Greater hydrolysis of trans- than cis-EETs by RBCs from both the WKY and the SHR was also found (Fig. 1A), which is consistent with a Vₘₐₓ for the hydrolysis of trans-EETs that is three times greater than that of cis-EETs (29).
with sEH inhibition (vehicle vs. AUCB-treated rats: 2.4 EETs. A decrease in plasma DHETs was not found as expected both strains when treated with the sEH inhibitor (Table 1). sEH (10, 29), other trans-

plasma the WKY and the SHR (Table 1). However, AUCB increased EETs in control WKY rats (18.3 vs. 7.8 ng/ml). AUCB

seen in the SHR (Table 1). An increase in plasma total EETs (Table 1). Plasma total EETs was twofold greater than trans-EETs in WKY and SHR treated with vehicle or AUCB (2 mg · kg⁻¹ · day⁻¹ in the drinking water) for 7 days. *P < 0.05 compared with plasma concentrations of vehicle rats of the same strain.

Values were determined by LC/MS/MS and expressed as means ± SE (ng/ml); n = 6–8 rats in each group. EET, epoxyeicosatrienoic acid; AUCB, cis-4-[4-(3-adamantan-1-ylureido)cyclohexyloxy]benzoic acid. Eleven-week-old Wistar-Kyoto (WKY) and spontaneously hypertensive (SHR) rats were treated with vehicle or AUCB (2 mg · kg⁻¹ · day⁻¹ in the drinking water) for 7 days. *P < 0.05 compared with plasma concentrations of vehicle rats of the same strain.

Table 1. Plasma concentrations of individual cis- and trans-EETs in WKY and SHR treated with vehicle or AUCB for 1 wk

<table>
<thead>
<tr>
<th>EETs</th>
<th>WKY</th>
<th>AUCB</th>
<th>SEH</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>14,15-cis-EET</td>
<td>5.8 ± 0.6</td>
<td>5.2 ± 0.7</td>
<td>4.5 ± 0.6</td>
<td>4.4 ± 0.7</td>
</tr>
<tr>
<td>14,15-trans-EET</td>
<td>1.6 ± 0.2</td>
<td>3.4 ± 0.6*</td>
<td>1.1 ± 0.1</td>
<td>2.3 ± 0.7*</td>
</tr>
<tr>
<td>11,12-cis-EET</td>
<td>2.1 ± 0.3</td>
<td>2.7 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>11,12-trans-EET</td>
<td>2.0 ± 0.2</td>
<td>3.6 ± 0.8*</td>
<td>0.9 ± 0.1</td>
<td>2.0 ± 0.5*</td>
</tr>
<tr>
<td>8,9-cis-EET</td>
<td>4.9 ± 0.4</td>
<td>3.3 ± 0.4</td>
<td>3.2 ± 0.4</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>8,9-trans-EET</td>
<td>1.8 ± 0.3</td>
<td>2.7 ± 0.7*</td>
<td>0.9 ± 0.1</td>
<td>1.9 ± 0.3*</td>
</tr>
<tr>
<td>5,6-cis-EET</td>
<td>5.5 ± 0.4</td>
<td>4.8 ± 0.3</td>
<td>3.2 ± 0.4</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>5,6-trans-EET</td>
<td>2.4 ± 0.1</td>
<td>2.8 ± 0.5</td>
<td>1.2 ± 0.1</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>Total</td>
<td>18.3 ± 1.4</td>
<td>16.0 ± 1.5</td>
<td>12.3 ± 1.6</td>
<td>12.1 ± 1.3</td>
</tr>
<tr>
<td>trans-EET</td>
<td>7.8 ± 0.5</td>
<td>12.5 ± 1.9*</td>
<td>4.1 ± 0.2</td>
<td>7.9 ± 1.5*</td>
</tr>
<tr>
<td>Overall EETs</td>
<td>26.1 ± 1.8</td>
<td>28.5 ± 3.2</td>
<td>16.4 ± 1.6</td>
<td>20.0 ± 2.4*</td>
</tr>
</tbody>
</table>

AJP-Heart Circ Physiol • VOL 300 • JUNE 2011 • www.ajpheart.org
Table 2. Conversion of 14,15-cis-EET to 14,15-threo-DHET and 14,15-trans-EET to 14,15-erythro-DHET by RBCs of vehicle- and AUCB-treated WKY and SHR

<table>
<thead>
<tr>
<th></th>
<th>Rat RBCs</th>
<th>14,15-threo-DHET Produced, pmol·min⁻¹·10⁻⁹ RBCs</th>
<th>14,15-erythro-DHET Produced, pmol·min⁻¹·10⁻⁹ RBCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY vehicle</td>
<td></td>
<td>2.82 ± 0.34</td>
<td>4.37 ± 0.52#</td>
</tr>
<tr>
<td>WKY AUCB</td>
<td></td>
<td>1.67 ± 0.20*</td>
<td>1.84 ± 0.22*</td>
</tr>
<tr>
<td>SHR vehicle</td>
<td></td>
<td>4.40 ± 0.53</td>
<td>7.02 ± 0.70#</td>
</tr>
<tr>
<td>SHR AUCB</td>
<td></td>
<td>2.59 ± 0.31*</td>
<td>2.81 ± 0.34*</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 6–8 rats in each group. DHET, dihydroxyeicosatrienoic acid; RBC, red blood cell. 14,15-cis- and 14,15-trans-EET (1 μM each) were added to RBCs (5 × 10⁶ RBCs in 50 μl PBS) of the vehicle control and the AUCB-treated SHR and WKY for 10 min at 37°C. *P < 0.05 compared with vehicle control rat RBCs for hydrolysis of the same 14,15-EET isomer. #P < 0.05 compared with vehicle control rat RBCs for hydrolysis of the same 14,15-EET isomer by the same RBCs.

by vehicle-treated WKY RBCs compared with that of the vehicle-treated SHR once again reflects differences in RBC sEH activity in the two strains. As was the case for SHR, sEH inhibition in WKY resulted in equalization of erythro- and threo-DHET production, viz., 1.84 vs. 1.67 pmol·min⁻¹·10⁻⁹ RBCs, respectively (Table 2).

BP reduction in the SHR is associated with increases in plasma trans-EETs. Mean arterial BP fell from 176 ± 8 to 153 ± 5 mmHg in the SHR, whereas it was unaffected in the WKY after 1 wk of treatment with AUCB (Fig. 2A). The reduction in BP with AUCB treatment in SHR reached a maximum in a week although BP was significantly reduced by day 3. Continued AUCB treatment did not result in a further decrease in BP. We anticipate that the effect of AUCB was maximal based on other studies of the potency of AUCB in inhibiting sEH (23, 29). The heart rate was not changed with AUCB treatment in either WKY rats or SHR (Fig. 2B).

The BP reduction is consistent with significant increases in plasma trans-EET concentrations in the SHR (Table 1). Although inhibition of sEH also increased trans-EETs in the plasma of normotensive WKY rats, the relative increase was less than that for the SHR and was not associated with a reduction in BP, which is not surprising when considering that most antihypertensive agents exert little or no effect on BP in normotensive individuals. The results suggest that the biological activities of trans-EETs may have contributed to the fall in the elevated BP in the SHR. There is also evidence of enhanced sensitivity of the renal vasculature to the vasodilator action of EETs in the SHR (43).

Comparison of cis- vs. trans-EETs on relaxation of preconstricted rat arcuate arteries. Because only trans-EETs were increased by treatment with AUCB, we compared the activity of cis- and trans-EETs on a preconstricted rat arcuate artery preparation (Fig. 3). We found that the 14,15-trans-EET was more potent (ED₅₀ 5 × 10⁻¹¹ M; 59 ± 15 μM dilation at 10⁻⁷M) than 14,15-cis-EET. The 11,12-EET cis- and trans-isomers were equipotent dilators (ED₅₀ 10⁻¹⁰ M) as were the 8,9-EET isomers (ED₅₀ 5 × 10⁻¹⁰ M). The vasodilator activity of EETs, except for 8,9-EETs, was significantly reduced in the presence of 14,15-EEZE (10⁻⁶ mol/l), an EET antagonist (20). The responses to 8,9-cis-EET were unaffected by preincubation with 14,15-EEZE, whereas the potency of 8,9-trans-EET was significantly enhanced in the presence of 14,15-EEZE.

Because the 14,15-EET isomers exhibited the major difference in dilator activity, we compared the activity of 14,15-trans-EET and 14,15-cis-EET in arteries obtained from SHR and WKY rats pressurized to 80 and 120 mmHg. We observed...
no difference in basal diameters, sensitivity to phenylephrine, or responsiveness to EETs between SHR and WKY arteries. The 14,15-trans-EET was more potent than 14,15-cis-EET in both SHR and WKY arteries. No differences in the activities of 14,15-trans-EET and 14,15-cis-EET were apparent at 80 mmHg (Fig. 4) or 120 mmHg (data not shown).

DISCUSSION

The major new findings from this study are: 1) that plasma levels of EETs, especially trans-EETs, are reduced in the SHR; 2) the reduction in BP in SHR in response to sEH inhibition is associated with increased levels of plasma trans-EETs; and 3) the renal microvascular vasodilator activity of 14,15-trans-EET is greater than 14,15-cis-EET. In addition, the finding of increased expression and activity of sEH in RBCs of SHR compared with WKY is consistent with earlier studies showing increased expression and activity of sEH in RBCs of SHR. 14,15-cis-EET on SHR arcuate arteries, and 14,15-trans-EET on WKY arcuate arteries. 14,15-cis-EET and 14,15-trans-EET were apparent at 80 mmHg (Fig. 4) or 120 mmHg (data not shown).

EETs have direct actions to relax vascular smooth muscle, presumably via activation of potassium channels (5, 31), and EETs may also potentiate the dilator effects of other hormones (5, 30). Consequently, an increase in trans-EETs in response to inhibition of sEH might account for the hypotensive effect of AUCB in SHR through direct and indirect mechanisms. Of the EET isomers, 14,15-trans-EET and 11,12-cis-EET were equipotent vasodilators. The 14,15-trans isomers exhibited the greatest differences in potency with the 14,15-trans-EET being a more potent renal vasodilator than 14,15-cis-EET. Falck et al. (14) have reported less potency for 14,15-trans- vs. 14,15-cis-EET in bovine coronary arteries (14), and this discrepancy should be of no surprise considering the different potencies of EETs in various vascular beds and species (5, 9, 12, 44). However, in isolated arcuate arteries obtained from SHR and WKY rats, the greater vasodilator responses to 14,15-trans-compared with 14,15-cis-EET were consistent with those obtained from normotensive SD rats, and no difference in responsiveness was observed between arteries from SHR and WKY rats. This is in contrast to the results obtained in the isolated perfused kidney of SHRs where vasodilator responses to 5,6-EET and its precursor, arachidonic acid, were markedly enhanced compared with those of control rats (42, 43). The vasodilator activity of the cis- and trans-EETs, except for 8,9-EETs, was attenuated in the presence of the putative EET antagonist 14,15-EEZE, suggesting a common mechanism for vasorelaxation. In contrast, the responses to 8,9-cis-EET were not attenuated by 14,15-EEZE, and those of 8,9-trans-EET were enhanced, suggesting an EET receptor-independent mechanism for 8,9-trans-EET that is amplified with 14,15-EEZE.

A role for cis-EETs in BP reduction in the SHR with sEH inhibition was not directly supported by this study of circulating EETs; however, an elevation in both cis- and trans-EETs in tissues such as the kidney that are involved in BP regulation cannot be discounted. Imig et al. (24) reported decreased urinary EET excretion in rats with ANG II-induced hypertension and elevated excretion of 14,15-DHET, consistent with increased activity of sEH. Inhibition of sEH increased urinary EETs, decreased 14,15-DHET, and resulted in a diuresis that paralleled the fall in BP (24). In contrast to those results, we were unable to discern any effect of AUCB treatment of SHR and WKY rats for 7 days on the urinary excretion of EETs or
DHETs, possibly signifying a lack of effect of AUCB to inhibit sEH in our study. This possibility was highly unlikely, since RBCs obtained from rats treated with AUCB showed a marked impairment in the transformation of 14,15-cis- and 14,15-trans-EET to 14,15-threo- and 14,15-erythro-DHET, respectively. Similar to the results of Imig et al. for the urinary excretion of EETs in the ANG II hypertensive rat, we found that excretion of urinary EETs in the SHR was ~50% less than that in the WKY; however, we saw no increase in DHET excretion. It should be noted that the urinary EETs and DHETs analyzed in this study represented total amounts measured by GC/MS, which cannot distinguish between individual isomers.

The results of the present study confirm the beneficial effects of sEH inhibitors that have been reported for a variety of pathological conditions, including hypertension (45, 49), but extend these findings to invoke a novel mechanism for the hypotensive effect of AUCB that we postulate is mediated by an increase in plasma trans-EETs. The consideration of a role for RBCs in vascular regulation derives from their ability to release and hydrolyze EETs (25, 27, 28). Because RBCs are a major source of sEH in the circulation, inhibition of sEH elevated plasma EETs. This reflects a substantial increase in trans-EETs with little or no change in cis-EETs and is consistent with the preferential hydrolysis of trans- over cis-EETs by the sEH (29). Increased plasma levels of trans-EETs and greater vasodilator activity of trans- than cis-EETs may account for the reduction in BP by sEH inhibition in the SHR. Because activity of many CYP isoforms can be driven by lipid hydroperoxides (4, 40), formation of cis- and trans-EETs should be increased with enhanced endothelial expression of CYP epoxygenases, resulting in BP reduction (32). Thus, increasing CYP metabolism that consumes lipid hydroperoxides and forms trans-EETs may be a significant antioxidant and antihypertensive remedy (4, 7, 52).

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DISCLOSURES

B. D. Hammock is a George and Judy Marcus Senior Fellow of the American Asthma Foundation.

REFERENCES


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