Vitamin C activation of the biosynthesis of epoxyeicosatrienoic acids

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ABSTRACT

The cardiovascular effects of vitamin C (VitC) could be mediated by epoxyeicosatrienoic acids (EETs). We aimed to study the mechanism of VitC-dependent microsomal formation of cis- and trans-EETs and the regulation of EET levels in rat isolated perfused kidneys and in vivo. VitC biphasically stimulated rat kidney microsomal cis- and trans-EET formation in a ratio of 1:2, involving the participation of lipid hydroperoxides (LOOHs), Fe^{2+}, and cytochrome P450 (CYP). Levels of LOOHs correlated with microsomal EET production. LOOH stimulation of CYP isoforms resulted in preferred trans-over cis-EET formation from arachidonic acid and was associated with the cleavage of LOOHs, which indicated a CYP peroxygenase activity. EETs contributed to VitC-induced vasodilator responses in rat isolated perfused kidneys. VitC (1 mg/ml) given in the drinking water for 9 days doubled rat urinary EET excretion, increased plasma levels of EETs, mostly trans-EETs, by 40%, and reduced plasma levels of 20-hydroxyeicosatetraenoic acid. Depletion of VitC in brain cortex and kidney tissues by more than 20- and 50-fold, respectively, in gulonolactone oxidase-knockout mice was associated with mild increases in tissue EETs. These data suggest that LOOHs are a determinant factor for EET formation in vivo in which VitC exerts a key regulatory effect. VitC-activated CYP peroxygenase activity may represent a CYP interaction with lipooxygenases and cyclooxygenases to mediate the cardiovascular effects of VitC via formation of EETs.

Keywords: Vitamin C; Epoxyeicosatrienoic Acids; Cytochrome P450; Lipid Hydroperoxides; Peroxygenase

1. INTRODUCTION

Vitamin C (VitC, ascorbic acid) is an antioxidant with implicated antihypertensive, antiinflammatory, antitherogenic, antiarrhythmic, antiapoptotic and natriuretic functions [1-6]. However, the mechanism of VitC actions is not clear. Even the antioxidant mechanism of VitC is debated, since millimolar concentrations of VitC are required to scavenge superoxide [7], and VitC mobilizes the release of Fe^{2+} from ferritin which can potentially cause lipid peroxidation.

Epoxyeicosatrienoic acids (EETs), cytochrome P450 (CYP) products from arachidonic acid (AA), have potent functions similar to the cardiovascular effects of VitC. EETs are vasodilatory, antiinflammatory and natriuretic lipid mediators [8,9]. They activate K^+ channels and are proposed to function as endothelium-derived hyperpolarizing factors (EDHF). EETs inhibit high salt- and angiotensin II-induced elevation of blood pressure [10]. EETs exert potent antiinflammatory effects [11]. EETs also possess fibrinolytic, angiogenic, analgesic, antiapoptotic and antiatherosclerotic functions. EETs protect the heart from ischemic damage and postschismic electrocardiogram abnormalities [12].

VitC is a potent dilator of human radial arteries [3]. VitC-dependent endothelium NO formation [13] is consistent with EETs as activators of NO synthase. In addition, the antiinflammatory effect of VitC may occur through inhibition of nuclear factor κB via EETs. VitC is beneficial in ameliorating the earliest stages of atherosclerosis. VitC modulates electronic signaling via vascular gap junctions at physiological concentrations [14], which is also consistent with EETs modulating endothelium-dependent gap junctions [15,16]. The antiarrhythmic effect of VitC [17] is consistent with a role of EETs in protecting against cardiac electrophysiologic dysfunction [12]. VitC therapy could be effective for the preven-
tion and treatment of diseases if the right protocols were used [18].

When examining the production of trans-EETs by rat kidney microsomes, we found that formation of cis- and particularly trans-EETs was activated by the addition of VitC to microsomes. We have been puzzled by the source of trans-EETs because NADPH-dependent CYP metabolism of AA produces only cis-EETs and 20-hydroxyeicosatetraenoic acid (20-HETE), whereas there are four cis- and four trans-EETs, totaling sixteen EET stereoisomers in vivo [10,19]. EETs are present at concentrations more than 10 ng/ml in rat and human plasma, which is much greater than that of isoprostanes in plasma produced by lipid peroxidation. Mechanisms of CYP-mediated metabolism in the absence of NADPH-CYP reductase has been the subject of intense study. CYP enzymes have demonstrated peroxygenase [20,21] and isomerase [22] activities in addition to NADPH-dependent monooxygenase activity. The peroxygenase or peroxidase activity breaks down lipid hydroperoxides (LOOHs) through homolytic or heterolytic cleavages while resulting in co-oxidation of other substrates. Hydroperoxides are activators of CYP metabolism activity and attempts have been made to explain the CYP peroxygenase activity using a monooxygenase mechanism [23,24].

While most of the biological activities of EETs have been described based on cis-EETs, cis- and trans-EETs have been found to possess equipotent platelet antiaggregatory properties; 14,15-trans- and 5,6-trans-EETs are more potent than their corresponding cis-isomers in relaxing rat preconstricted renal arteries [10,19]. EETs are hydrolyzed by soluble epoxide hydrolase (sEH) forming dihydroxyeicosatrienoic acids (DHETs), which have less biological activities. The hydrolysis of trans-EETs by sEH is, on average, three-fold faster than that of cis-EETs [25].

In humans, VitC acts as a cofactor for eight different enzymes that are essential for the synthesis of collagen, carnitine and tyrosine [18]. Interactions of VitC with CYP expression and metabolism in vivo have been reported [26]. Although humans have lost the ability to synthesize VitC from glucose, many human tissues avidly accumulate VitC against a concentration gradient. The cardiovascular effects of VitC have been attributed to the antioxidant effect in most studies; however, this is potentially problematic considering that VitC at levels under 150 μM will not protect NO from the interaction with superoxide [7]. Identifying the biochemical mechanism of action of VitC may lead to new and effective therapeutic approaches in the treatment of hypertension and other cardiovascular diseases. Thus, we carried out studies to investigate the mechanism of VitC-dependent microsomal cis- and trans-EET formation and the regulation of EET formation in rat isolated kidneys and in vivo by VitC. Formation of EETs activated by VitC may provide a mechanism for vasodilator, antiarrhythmic, antiatherogenic, antiapoptotic and other cardiovascular effects of VitC.

2. MATERIALS AND METHODS

2.1. Materials

Human recombinant CYP Supersomes (0.5 or 1 nmol in 0.5 ml), CYP1A1, CYP2B1, CYP2E1 and CYP3A4, were purchased from BD Biosciences (San Jose, CA). Human CYP2C8 and CYP2J2 (4 - 6 μM) were co-expressed with NADPH-CYP reductase in SF9 insect cells using the baculovirus system [27]. Acetonitrile, methanol and chloroform (all HPLC grade) were obtained from Fisher Scientific. CYP inhibitors, methylsulphonylpropargyloxyphenyl hexanamide (MSPPOH) and 17-octadecenoic acid (ODYA), lipid hydroperoxides, hydroperoxyeicosatetraenoic acids (HPETEs), d6-20-HETE, d11-DHETs, (+)-13-hydroperoxy-9Z, 11E-octadecadienoic acid (13-HPODE) and 13S-hydroperoxy-9Z, 11E, 15Z-octadecatrienoic acid (13-HPOTrE), as well as other eicosanoid standards were from Cayman, while deuterated (d8) EETs were from Enzo Life Sciences. AA, diethylenetriamine pentaacetate (DTPA), and all other reagents were from Sigma-Aldrich. AA was used within a week after further HPLC purification and dissolving in argon-saturated 0.1 M Tris buffer (pH = 8).

2.2. Animals

Six-week-old male Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). Dulonolactone oxidase (gulo) is required for VitC biosynthesis. Heterozygous gulo-knockout mice were originally obtained from Mutant Mouse Regional Resource Centers (http://www.mmrrc.org, #000015-UCD) and bred together to create a colony of gulo+/- mice that were maintained on a C57BL/6J background (stock #000664; Jackson laboratories, Bar Harbor, ME). Animals were housed in groups of 3 to 5 in tub cages in a temperature- and humidity-controlled vivarium. Animals were kept on a 12:12-hour light:dark cycle with lights on at 6 am. All animals had free access to food and water for the duration of the experiment. All procedures were approved by New York Medical College or the Vanderbilt University Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.3. Rat Kidney Microsome Preparation

Male Sprague-Dawley rats were anesthetized with sodium pentobarbital (65 mg/kg i.p.). Kidneys were re-
moved after laparotomy, and the cortex was dissected and homogenized in a buffer containing 0.1 M Tris-HCl and 0.15 M KCl, pH 7.4. Homogenates were centrifuged at 10,000 g for 30 min. Microsomes were obtained by centrifugation of the supernatant at 100,000 g for 90 min and resuspended in 0.1 M Tris buffer containing 0.25 M sucrose and stored at ~80°C. Protein concentrations in microsomes were measured using the Bradford assay.

2.4. Microsome and CYP Incubations

Microsomes were diluted with incubation buffer containing 0.05 M Tris, 10 mM MgCl2, and 0.15 M KCl, pH 7.4 to 0.5 mg/ml and warmed up to 37°C before incubations. Reagents were prepared in stock solutions over 100× targeted concentrations. AA and other reagents were preincubated with microsomes for 5 min before transferring 1 ml of the mixture to test tubes with NADPH and/or VitC to initiate the reaction. Abundant AA (0.1 mM) was used in incubations to delineate the magnitude of EET formation in dose-/time-dependent and kinetic studies; while AA of 10 μM was used in incubations to effectively determine factors inhibiting and stimulating microsomal EET formation.

To determine the $K_m$ and $V_{max}$ of LOOH-dependent EET formation by CYP isoforms, 50 μM and six progressive 1:3 dilutions of LOOH were incubated in 0.1 ml reaction mixtures containing 0.1 mM AA and 100 mM CYP isoforms at 37°C. All reactions were terminated after 10 min by the addition of 2 ml of ice-cold ethyl acetate. Control experiments were carried out and the background EET formation from AA oxidized by LOOHs was subtracted in calculating LOOH-dependent CYP activity. Formation of EETs was analyzed by LC/MS/MS. $K_m$ and $V_{max}$ were calculated using the Michaelis-Menten kinetics.

2.5. Rat Isolated Kidney Perfusion

Sprague-Dawley rats, weigh 300 g, were anesthetized with pentobarbital, 65 mg/kg i.p., and the right kidney was isolated for perfusion as described [28]. Briefly, following a midline laparotomy, the right kidney was cannulated via the mesenteric artery to prevent interruption of blood flow and perfused with warmed (37°C), oxygenated Krebs-Henseleit buffer at constant flow to obtain a baseline renal perfusion pressure (RPP) of approximately 50 to 75 mmHg. The vena cava was ligated above and below the right renal vein and cut to allow exit of the perfusate, and the ureter was transected. The kidney was then removed and suspended in a water-jacketed organ bath. Once a stable RPP was obtained, pressure was elevated with phenylephrine (0.5 × 10⁻⁶ M) to approximately 150 to 220 mmHg to amplify vasodilator responses, and the response to sodium nitroprusside (SNP, 0.2 μg) was determined. Indomethacin (5.6 μM) and $N_{\text{ω}}$-nitro L-arginine methyl ester (100 μM) were tested without effect and were included in the buffer in all experiments to minimize contributions of prostaglandins and NO, respectively, to vasodilator responses.

Since the experiment was designed to address the contribution of EET formation to potential vasodilator effect of VitC, a potent sEH inhibitor, $trans$-4-[4-(3-adamantanyl-1-ylureido) cyclohexyloxy]benzoic acid (tAUCCB, 1 μM), was added to the buffer to amplify the effect of EETs by inhibiting EET hydrolysis in the kidney. In some experiments, the effluent was collected for 1 min before the VitC challenge and at the major response after VitC challenge for measurements of EETs/DHETs and 20-HETE.

2.6. Rat Metabolism Cage Study

Seven-week-old Sprague-Dawley rats were housed in metabolism cages and VitC (1 mg/ml, 130 mg/kg/day) in the drinking water was administered to half of the rats for 9 days to test the effects of VitC on in vivo EET formation and on urinary EET excretion. Urine samples (24 h) were collected and analyzed on days 0, 1, 3 and 9. Food and water consumption, body weight and urine volumes were recorded. Urinary Na⁺ was analyzed with an IL 943 flame photometer (Instrumentation Laboratory, MA) after centrifugation at 2000 g for 10 min and a 5-fold dilution in distilled water.

2.7. Gulo⁻/⁻ Mouse Treatments

At weaning (21 days) all mice were given deionized water that was supplemented with 0.33 g/L VitC with 20 μL/0.5 mM EDTA to increase stability of VitC in solution. This is the standard supplement level that provides adult gulo⁻/⁻ mice with approximately wild-type levels of VitC in tissues when administered long-term [29]. One week later gulo⁻/⁻ mice were divided into treatment groups and were provided with high (3.33 g/L), standard (0.33 g/L) or low VitC (0.033 g/L). A final group (gulo⁻/⁻/low) received standard VitC supplements for one additional week, then VitC supplements were removed entirely for 3 weeks (0 g/L). Water bottles were refilled with fresh VitC solutions twice each week. Gulo⁻/⁻ wild-type mice were given tap water with no VitC added. Mice remained on these treatments for 4 weeks until sacrificed at 8 weeks of age.

2.8. VitC Detection

VitC levels were measured in the brain cortex and the kidney in 5 to 7 mice per group. Tissues were kept at
–80°C until assayed. Tissue samples were homogenized with 25% aqueous metaphosphoric acid and 100 mM sodium phosphate buffer containing 5 mM EDTA, premixed in a ratio of 2:7. A total of 10 µl of buffer solution was used for each mg of tissue. The samples were then centrifuged at 13,600 g for 4 min at 4°C, and aliquots of the supernatant were taken for assay of VitC following appropriate dilution with HPLC mobile phase. Concentrations were measured by ion pair HPLC and electrochemical detection as previously described with tetrapentyl ammonium bromide used as the ion pair reagent [29].

2.9. Eicosanoid Extractions

Eicosanoids produced in microsomal incubations were extracted twice with 2 ml ethyl acetate containing 0.1 mM butylated hydroxytoluene (BHT) after addition of deuterated (d11-) 8,9- and 14,15-DHETs, (d8-) 8,9-, 11,12- and 14,15-EETs and (d6-) 20-HETE (2 ng each) as internal standards (the same internal standards were used in all sample analyses). For measurement of residual LOOHs in some CYP isofrom incubations, no antioxidant was applied during extraction, but corresponding control experiments were carried out. All LC/MS/MS experiments for microsomal incubations were finished within hours of the extraction and EET formation from lipid peroxidation during extraction at neutral pH was found to be insignificant in test experiments.

Mouse brain cortex and kidney tissues (20 mg) were added 2 ml of chloroform/methanol (2:1) containing 0.1 mM BHT. The tissues were minced to small pieces and homogenized for less than 1 min on ice. Total phospholipids were extracted with the Folch method after addition of d11-DHETs, d8-EETs and d6-20-HETE (2 ng each) as internal standards. EET formation from AA oxidation during the process of tissue analysis was insignificant as verified by adding freshly purified d8-AA (5 µg/mg wet tissue) to samples. Rat plasma was obtained after centrifuging blood at 2000 g for 10 min and mixed with polymer bound triphenylphosphine (1 mg/ml) to quench free radical-induced lipid peroxidation. Phospholipids were extracted from 0.4 ml plasma using the Bligh-Dyer method after adding the same deuterated internal standards. Extracted phospholipids were subsequently hydrolyzed with 1 M NaOH for 90 min at room temperature. The hydrolysis mixture was then carefully neutralized with 1 M HCl and extracted twice with 2 ml ethyl acetate. The ethyl acetate extract was dried under a gentle stream of nitrogen and dissolved in acetonitrile (20 µl) for LC/MS/MS analysis [25].

Half of the collected rat kidney perfusate (6 ml) was directly extracted twice with 3 ml ethyl acetate containing 0.1 mM BHT after adding the deuterated internal standards. Rat urine samples (24 h) were collected into tubes containing 10 mg polymer-bound triphenylphosphine. Rat urine samples (2 ml each), after centrifugation at 2000 g for 10 min, were added the internal standards and extracted twice with 2 ml hexane/ethyl acetate (1:1). The organic phase was combined, backwashed with 3 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 [19].

2.10. Mass Spectrometry Analysis

ESI LC/MS/MS analyses were carried out for in vitro incubations and plasma and tissue EETs as described [25]. HPLC was run through a Kinetex 2.6 µ C18 150 × 2.1 mm column (Phenomenex, Torrance, CA) maintained at 48°C with an isocratic gradient of acetonitrile/water/methanol/ acetic acid (53:37:10:0.05) at a flow of 0.38 ml/min. A Thermo Finnigan LCQ Advantage quadrupole ion-trap mass spectrometer equipped with ESI source run by XCALIBUR software was used for mass spectrometry analysis. MS/MS breakdown of the precursor ions of EETs and DHETs, m/z 319 and m/z 337, respectively, was at an energy level of 35% set by the instrument and a 7-point Gaussian smoothing was applied in data processing. Quantification of individual EETs and DHETs was based on standard curves (r² > 0.99) between selected characteristic fragmentation ions with reference to that of an internal standard of d8-11,12-EET and d11-14, 15-DHET, respectively. The accuracy and sensitivity of the LC/MS/MS analysis were examined with standard eicosanoid and internal standard mixtures before sample analysis. Levels of HPETEs and 5/12/15-HETEs were calculated by integrating peaks of selected ions, m/z 335 and m/z 319, respectively, with reference to the internal standard d8-11,12-EET.

Analyses of total levels of DHETs, EETs and 20-HETE in rat plasma, kidney perfusate and urine samples were carried out by electron capture negative chemical ionization GC/MS due to the superior quantification limit for eicosanoids at 1 pg far exceeding that of the LC/MS system (50 to 100 pg) used in this study. Purified DHETs samples were derivatized to trimethylsilyl ether pentfluorobenzyl esters, and EETs were derivatized to pentafluorobenzyl esters as described [19]. The ions m/z of 481 and 492 were monitored for endogenous and d11-DHETs, the ions m/z of 319 and 327 were monitored for endogenous and d8-EETs, and the ions m/z of 391 and 397 were monitored for endogenous and d6-20-HETE. Total plasma levels of EETs from GC/MS analysis confirmed the results obtained from LC/MS/MS analysis.

2.11. Statistical Analyses

Results were presented as mean ± standard error 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 less than 0.05 was considered as statistically significant. VitC levels were analyzed using SPSS version 19 for Mac with univariate ANOVA with Fisher’s LSD post hoc tests to establish differences among the groups.

3. RESULTS

3.1. VitC Activation of Kidney Microsomal EET Formation

Microsomal CYP metabolism of AA was activated by NADPH producing cis-EETs and 20-HETE (Figure 1(a)). In an effort to investigate microsomal trans-EET formation, we tested and found no effects caused by reagents including 0.1 mM tert-butylic hydroperoxide; however, addition of 0.1 mM VitC to microsomes potently activated more trans- than cis-EET formation without formation of 20-HETE (Figure 1(b)). EET formation activated by 0.1 mM NADPH was about 4-fold more than by 0.1 mM VitC. Combination of VitC with NADPH (0.1 mM each) did not result in more total EET formation than NADPH alone; however, the resultant EET profile represented a mixture of their individual products (Figure 1(c)), suggesting overlapping microsomal CYP activation by VitC and NADPH. Identification of these peaks was accomplished by matching retention times and tandem mass spectra with those of authentic standards [19,25]. In microsomal incubations, some of the cis- and trans-EETs were further converted to threeo- and erythro-DHETs, respectively, which was greatly inhibited by the sEH inhibitor, tAUCB, at 1 μM (data not shown) [25].

Control microsomal incubations with AA (0.1 mM) also produced 5/12/15-HETEs and basal levels of DHETs (Figure 1(d)). Levels of 5/12/15-HETEs produced in AA incubations with microsomes were about 3-fold higher than in control AA incubations in buffer only, suggesting formation of HPETEs by the microsomal lipoxygenase activity. HPETEs (m/z 335) produced in microsomal incubations were not apparent because of isomerization to a series of peaks at similar abundances in addition to conversion to HETEs.

In the selected ion chromatograms (Figure 1), the first peak of DHETs, 14,15-erythro-DHET derived from 14, 15-trans-EET, and the last peak of EETs, 5,6-trans-EET, were unique indicators of the level of trans-EETs. The preferred trans- over cis-EET formation from microsomes stimulated by VitC could be further demonstrated in chromatograms of the selected reaction monitoring analysis (Figure 2). NADPH-dependent microsomal metabolism of AA produced cis-EETs, which was evident in the study although trace amounts of trans-EETs were found in microsomal incubations with NADPH (Figure 1(a)). Analysis of VitC-stimulated EET formation by LC/MS/MS revealed 2-fold more trans- than cis-EETs with comparable composition of individual regioisomers (Table 1). EETs were major products activated by VitC, however, VitC-dependent EET formation was associated with increased formation of 5-, 12- and 15-HETEs (Figures 1(b) and (c) compared to (d)). VitC did not activate the formation of 20-HETE as did NADPH (Figure 1(b)). Instead, VitC inhibited NADPH-dependent 20-HETE formation by 18% ± 3% (VitC, NADPH and AA 0.1 mM each, microsomal protein 0.5 mg/ml; p < 0.05). Since NADPH-dependent CYP metabolism for EET formation has been well studied, we only focused on results of VitC-activated EET formation in this study. The maximal effect of VitC on EET formation from kidney microsomes was at 0.1 mM VitC (Figure 3(a)). VitC at 1 mM produced 30% less EET formation compared to VitC at 0.1 mM. Microsomal VitC-dependent EET formation was linearly time-dependent from 2 to 30 min (Figure 3(b)).

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inhibition by a superoxide scavenger, tempol (0.1 mM), and by iron chelators, DTPA and deferoxamine (0.1 mM each), suggested involvement of peroxidation and free iron ions in microsomal VitC-dependent EET formation. Addition of Fe^{2+} (10 μM) or ferritin (1 mg/ml) to the microsomes doubled VitC-dependent EET formation while Fe^{3+} (10 μM) had no effect. Mobilization and stabilization of ferritin-iron by VitC have been documented [30,31].

While microsomal preparations may contain residual catalase activity, addition of bovine liver catalase (3500 units) to microsomes stimulated VitC-dependent EET formation (Figure 4), but was without effect on NADPH-dependent EET formation (data not shown).

A group of investigators have reported effects of vasoconstriction and blockade of EDHF-mediated vasodilation by VitC [32]. However, the enhancement of the vasoconstriction by Cu^{2+} and abolishment by catalase [32] is consistent with potential EET formation associated with VitC based on this study (Figure 4). The inhibition of VitC-stimulated EET formation by vitamin E was likely a result of the tendency of the α-tocopheroxyl radical to accept electrons from VitC.

Figure 2. Analysis of DHETs and EETs produced from VitC-stimulated rat kidney microsomal metabolism of AA by selected reaction monitoring LC/MS/MS. VitC and AA (0.1 mM each) were incubated with rat kidney microsomes (0.5 mg protein) for 10 min at 37°C. Precursor ions of DHETs (m/z 337) and EETs (m/z 319) were presented as the top chromatogram. Breakdown of the ion m/z 337 for product ions m/z 207, 197, 185, and 145 produced peaks for 14,15-, 11,12-, 8,9- and 5,6-erythro- and threo-DHETs, respectively, as labeled. Similarly, breakdown of the ion m/z 319 for product ions m/z 219, 195, 155, and 191 produced peaks for 14,15-, 11,12-, 8,9- and 5,6-cis- and trans-EETs, respectively, as labeled. Notably, more trans- than cis-EETs are converted to DHETs in microsomal incubations.

3.2. Factors Affecting VitC-Dependent EET Formation

Microsomal VitC-dependent EET formation relied on CYP enzymatic activity because it was greatly reduced in boiled microsomes and by general and specific CYP inhibitors of AA metabolism (Figure 4). Miconazole (0.1 and 1 mM), CO (1 mM), MSPPOH (24 μM), ODYA (30 μM) and Cu^{2+} (10 μM) all inhibited VitC- as well as NADPH-dependent microsomal EET formation (data not shown for NADPH-dependent metabolism). Specific

Table 1. VitC-dependent EET formation by rat renal microsomes (ng/min/mg protein).

<table>
<thead>
<tr>
<th></th>
<th>14,15-</th>
<th>11,12-</th>
<th>8,9-</th>
<th>5,6-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>trans-EET</td>
<td>0.46 ± 0.05*</td>
<td>0.39 ± 0.05*</td>
<td>0.29 ± 0.03*</td>
<td>0.37 ± 0.05*</td>
<td>1.52 ± 0.17*</td>
</tr>
<tr>
<td>cis-EET</td>
<td>0.18 ± 0.02</td>
<td>0.17 ± 0.03</td>
<td>0.13 ± 0.02</td>
<td>0.18 ± 0.03</td>
<td>0.66 ± 0.08</td>
</tr>
<tr>
<td>trans/cis</td>
<td>2.50</td>
<td>2.30</td>
<td>2.25</td>
<td>2.06</td>
<td>2.28 ± 0.09</td>
</tr>
</tbody>
</table>

Results represented LC/MS/MS analysis of EETs, combined with corresponding DHETs, respectively, from incubations with rat kidney microsomes (1 ml, 0.5 mg protein) and AA (0.1 mM) at 37°C for 10 min. n = 5 - 9, *p < 0.01 vs cis-EET.
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Figure 3. VitC-dependent rat kidney microsomal EET formation. (a) Dose-dependent VitC-activated EET formation at 37°C in 10 min incubations; (b) Time-dependent EET formation. Rat kidney microsomes (1 ml, 0.4 mg protein) and AA (0.1 mM) were incubated at 37°C, n = 4 - 6. Production of EETs including DHETs was analyzed by LC/MS/MS analysis after extraction.

Figure 4. Inhibition and stimulation of VitC-dependent microsomal EET formation. VitC (0.1 mM) was applied to all incubations except for the control with rat kidney microsomes (1 ml, 0.5 mg protein) and AA (10 μM) for 30 min at 37°C and EET formation including DHETs was analyzed with LC/MS/MS. *p < 0.05 vs VitC 0.1 mM, n = 3 - 6.

Figure 5. Kinetic analysis of CYP peroxygenase activity using 13-HPOTrE as a LOOH for the two typical CYP epoxygenases, CYP2C8 and CYP2J2, revealed a Km of 2.4 and 4.8 μM, respectively (Figure 5), suggesting a high affinity of CYP isoforms to LOOHs [20]. The Vmax for 13-HPOTrE-activated EET formation by CYP2C8 and CYP2J2 was 0.31 ± 0.02 and 0.40 ± 0.03 nmol EETs/nmol P450/min, respectively, indicating an activity about 20% that of NADPH-dependent CYP epoxygenases [33,34]. The profile of EETs generated by CYP2C8 and CYP2J2 stimulated by 50 μM of 13-HPOTrE (Figure 5(c)) was no different from VitC-dependent microsomal EET formation.

3.4. EETs Contributed to VitC-Induced Vasodilator Responses in Rat Isolated Perfused Kidneys

Hemodialysis patients exhibit reduced systemic concentrations of VitC and VitC diminishes reperfusion injury in patients undergoing renal transplantation [36,37]. Based on microsomal VitC-dependent EET formation,
Figure 5. Kinetics and CYP peroxygenase EET formation from AA stimulated by 13-HPOTrE for CYP2C8 and CYP2J2. (a) CYP2C8; (b) CYP2J2; (c) Selected reaction monitoring analysis of EETs produced by CYP2J2 stimulated by 13-HPOTrE (50 μM) in which EETs from nonzymatic peroxidation constituted about 30% based on control experiments. The EET precursor ion (m/z 319) was isolated and fragmented to different product ions in the selected reaction monitoring analysis of EET isomers as labeled. 13-HPOTrE (50 μM) and six progressive 1:3 dilutions were incubated with AA (0.1 mM) and 100 nM CYP isoform in 0.1 ml Tris buffer (pH 7.4) at 37°C for 10 min (n = 3 - 4). EET formation from 13-HPOTrE-dependent AA oxidation in control experiments was subtracted in calculating the kinetics for the CYP peroxygenase activity.

Table 2. LOOH-dependent EET formation by CYP isoforms (nmol EETs/nmol P450/min).

<table>
<thead>
<tr>
<th>CYP Isoform</th>
<th>15-HPETE</th>
<th>12-HPETE</th>
<th>5-HPETE</th>
<th>13-HPODE</th>
<th>13-HPOTrE</th>
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</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>0.40</td>
<td>0.38</td>
<td>0.42</td>
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<tr>
<td>CYP2B1</td>
<td>0.33</td>
<td>0.28</td>
<td>0.27</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>0.43</td>
<td>0.28</td>
<td>0.30</td>
<td>0.26</td>
<td>0.30</td>
</tr>
<tr>
<td>CYP3A4</td>
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<td>0.31</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>0.48</td>
<td>0.20</td>
<td>0.43</td>
<td>0.29</td>
<td>0.31</td>
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<tr>
<td>CYP2J2</td>
<td>0.26</td>
<td>0.20</td>
<td>0.15</td>
<td>0.28</td>
<td>0.36</td>
</tr>
</tbody>
</table>

No apparent regioselectivity in EET formation was observed. The ratio of trans-cis-EETs produced was about 2:1. Nonenzymatic LOOH-oxidized EET formation in control experiments, equal cis- and trans-EETs with a total at most 30% of EETs produced by CYP isoforms, was deducted. Experiments were carried out in duplicate with 50 μM of LOOHs, 0.1 mM AA and 10 pmol CYP isoforms in 0.1 ml buffer at 37°C for 10 min. Variations between duplicate experiments were within 10%.
and the vasodilator properties of EETs in the kidney, we investigated the role of EETs in rat isolated perfused kidneys challenged with bolus doses of VitC (Figure 6).

VitC challenges at 0.1, 1 and 5 mg induced decreases in RPP. The major decrease in RPP and duration of the effect of VitC at 1 and 5 mg were significantly greater with the addition of \( \tau \text{AUCB of } 1 \mu \text{M} \) in the perfusion buffer (Figures 6(b) and (c)), suggesting a role of EETs. The major decrease in RPP corresponded to increased DHET and decreased 20-HETE release in the renal effluent after challenge with 5 mg of VitC (Figure 6(d)). The addition of \( \tau \text{AUCB} (1 \mu \text{M}) \) in the perfusion buffer more than doubled EET concentration in the effluent after challenge with 5 mg VitC. Notably, the release of EETs from rat isolated kidneys was barely detectable before challenges with VitC without the application of \( \tau \text{AUCB} \), and the levels of EETs in the effluent was less than that of DHETs even with the application of \( \tau \text{AUCB} \) (Figure 6(d)), suggesting that EETs were more likely retained than DHETs by the kidney. The increased EET/ DHET associated with reduced 20-HETE release in isolated kidneys supported VitC-activated CYP activity, and disputed possible effects of increased phospholipase A2 or increased NADPH-dependent P450 activity. The initial drop in RPP responding to bolus doses of VitC was likely a transient effect of pH.

Figure 6. EETs contributed to VitC-induced vasodilator responses in rat isolated perfused kidneys. (a) A representative trace of vascular responses to VitC (0.1, 1 and 5 mg) before and after the application of \( \tau \text{AUCB} (1 \mu \text{M}) \) in Sprague-Dawley rat isolated perfused kidney constricted with phenylephrine (PE, \( 5 \times 10^{-7} \text{ mol/L} \)) at RPP about 200 mm Hg; (b) Potentiation of the decrease in RPP with \( \tau \text{AUCB} \). *\( p < 0.05 \) compared to without \( \tau \text{AUCB}, n = 6 - 9 \); (c) Increased time for the pressure to recover after VitC challenges in the presence of \( \tau \text{AUCB} \). *\( p < 0.01 \) compared to without \( \tau \text{AUCB}, n = 6 - 9 \) and (d) Increased excretion of DHETs and EETs and decreased excretion of 20-HETE by the challenge of 5 mg VitC. *\( p < 0.05 \) vs before challenges with 5 mg VitC. *\( p < 0.05 \) compared to without \( \tau \text{AUCB}, n = 6 - 9 \). EETs, DHETs and 20-HETE in renal effluent were quantitated by GC/MS analysis. L-NAME, N\( \omega \)-nitro L-arginine methyl ester; RPP, renal perfusion pressure; SNP, sodium nitroprusside.

Figure 7. Effects of VitC administration on Sprague-Dawley rat urinary excretion and plasma eicosanoids. (a) Urinary excretion of EETs and DHETs; (b) Plasma levels of EETs and DHETs (day 9) and (c) Plasma levels of 20-HETE (day 9) *\( p < 0.05 \), vs control rats, \( n = 9 \). VitC (1 mg/ml) was given in the drinking water to rats for 9 days. Plasma and urinary eicosanoids were quantitated by GC/MS analysis.
3.5. Rat VitC Administration Increased EET Formation and Improved Daily Sodium Balance

VitC (1 mg/ml) in the drinking water was administered to seven-week-old rats for 9 days to test the effects of VitC on in vivo EET formation. Selection of rats for the experiments is challenging because rats can synthesize VitC from glucose. Nevertheless, rat urinary excretion of EETs and DHETs doubled on day 1; plasma EETs and DHETs increased by 40% on day 9, while plasma levels of 20-HETE were reduced by 30% (Figure 7). DHETs were major metabolites excreted in rat urine and levels of EETs accounted for no more than 10% that of DHETs [10]. No 20-HETE in rat urine could be detected by GC/MS analysis.

LC/MS/MS analysis of individual EET regioisomers in rat plasma revealed major increases in trans-EETs after VitC treatment (Table 3). The ratio of trans-over cis-EETs in rat plasma was greatly increased in VitC-treated rats (0.87 ± 0.07 vs 0.53 ± 0.07, p < 0.05). Also, levels of 15-, 12- and 5-HETE in the plasma of VitC-treated rats tended to be higher than that of control rats, particularly for 12-HETE (15, 12- and 5-HETE: 8.0 ± 1.7, 6.7 ± 1.8 and 8.0 ± 1.8 ng/ml in control rats; 10.2 ± 2.0, 10.2 ± 1.9 and 10.6 ± 1.5 ng/ml in VitC-treated rats, respectively), indicating that platelet 12-lipoxygenase might contribute to plasma EET formation.

While rat urinary sodium excretion did not increase significantly with VitC treatment (31.3 ± 2.4 vs 33.6 ± 2.8 μmol/h/100 g b.w., control vs VitC on day 9), the daily sodium balance measured as a ratio of daily urinary sodium excretion over food sodium intake showed a significant improvement for the VitC-treated rats on day 9 (day 0: 5.7% ± 3.2% vs 15.6% ± 3.6%, *p < 0.05). Food consumption did not differ between the control and the VitC-treated group (21.2 ± 0.8 vs 20.4 ± 0.7 g/rat/day, control vs VitC on day 9), nor did water consumption (35.8 ± 2.4 vs 42.6 ± 7.5 ml/day/rat, control vs VitC on day 9) and urine volume (9.1 ± 1.3 vs 12.0 ± 3.5 ml/day/rat, control vs VitC on day 9).

3.6. Effect of Decreased VitC in vivo on EET Levels in Gulo−/− Mouse Brain and Kidney Tissues

VitC variation in the drinking water for gulo−/− mice led to significant differences among groups in tissue VitC levels in both the brain cortex and the kidney (p < 0.001; Table 4). In the brain, VitC levels did not differ between wild-type and gulo−/− high VitC treated mice, but significantly decreased in other VitC treated gulo−/− groups (p < 0.05). In the kidney, VitC levels were similar among wild-type, gulo−/− high VitC and gulo−/− standard VitC groups but significantly decreased (p < 0.05) in gulo−/− low VitC and water treated groups, although these latter two groups did not differ from each other. Unlike VitC in the mouse kidney, VitC in the brain cortex was not totally depleted after 4-week water treatment of the gulo−/− mice (Table 4).

An analysis of EETs after the four-week treatment revealed significant inverse correlations between VitC levels in brain cortex and kidney tissues and EET levels (Figure 8). The result was the opposite as initially expected. However, EETs were only increased about 30% in contrast to more than 20- and 50-fold decreases in VitC concentrations in brain cortex and kidney tissues, respectively. The result was in fact consistent with LOOHs as the determinant factor in VitC-dependent EET formation in microsomal and CYP studies. Levels of trans-EETs were comparable to those of cis-EETs in both the mouse brain and kidney tissues and no dominance of any one EET regioisomer was evident, which was similar to what has been published in a LC/MS chromatogram analysis of brain tissues by Yue H, et al. although these peaks of

Table 3. Plasma concentrations of individual cis- and trans-EETs in control and VitC-treated Sprague-Dawley rats.

<table>
<thead>
<tr>
<th>Group (VitC in tap water)</th>
<th>Brain cortex</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gulo−/− wild-type (0 g/L)</td>
<td>4.77 ± 0.16a</td>
<td>0.20 ± 0.03a</td>
</tr>
<tr>
<td>Gulo−/− high (3.33 g/L)</td>
<td>4.38 ± 0.09a</td>
<td>0.34 ± 0.08a</td>
</tr>
<tr>
<td>Gulo−/− standard (0.33 g/L)</td>
<td>3.41 ± 0.13b</td>
<td>0.22 ± 0.08b</td>
</tr>
<tr>
<td>Gulo−/− low (0.033 g/L)</td>
<td>0.72 ± 0.23c</td>
<td>0.004 ± 0.002c</td>
</tr>
<tr>
<td>Gulo−/− water (0 g/L)</td>
<td>0.19 ± 0.05d</td>
<td>0f</td>
</tr>
</tbody>
</table>

Data are group ±s.e.m. expressed as μmol per g of wet tissue. Values within each tissue type that do not share a letter are significantly different (n = 5 - 7, p < 0.05).
trans-EETs were not pointed out [38].

4. DISCUSSION

This study pointed out LOOHs as a determinant factor and VitC as having a key regulatory effect on cis- and trans-EET formation by the CYP peroxygenase activity. VitC-dependent microsomal cis- and trans-EET formation involved CYP enzymes and LOOHs without the requirement of NADPH and CYP reductase. LOOHs stimulated preferred trans- over cis-EET formation from AA by CYP isoforms. EETs contributed to VitC-induced vasodilator responses in rat isolated perfused kidneys. VitC (1 mg/ml) in the drinking water doubled rat urinary excretion of DHETs, increased plasma trans-EETs, but decreased plasma levels of 20-HETE. Increased trans-EET formation in rats by VitC administration was indicative of the VitC-activated CYP peroxygenase activity in vivo, which was supported by reduced 20-HETE formation (Figures 7 and 8). In fact, it was unfair to compare VitC- and NADPH-dependent microsomal EET formation as studied (Figures 1 and 5), since abundant NADPH was provided while the level of LOOHs in microsomal incubations was limited. Notably, HPETEs at 5 μM increased VitC-dependent microsomal EET formation by 4.8 fold. VitC-dependent cis- and trans-EET formation by the CYP peroxygenase activity consumes LOOHs, indicating that even the pro-oxidant effect of VitC is essentially antioxidant.

A mechanism for VitC-activated CYP peroxygenase activity is proposed in Figure 9. Fe^{2+} is essential for the homolytic O-O bond reductive cleavage [39], which explains the role of Fe^{2+} in microsomal VitC-dependent EET formation. The EET formation was not mediated by Fenton reaction (H_{2}O_{2} + Fe^{2+} → Fe^{3+} + OH^{-} + HO^\bullet) because catalase stimulated VitC-dependent EET formation (Figure 4). H_{2}O_{2} has been found to decrease Fe^{2+}-initiated LOOHs cleavages in cells enriched with docosahexaenoic acid [40]. Fe^{2+} alone does not initiate lipid peroxidation, which was confirmed by insignificant EET formation in control experiments. The scheme of VitC-dependent EET formation is supported by studies on homolytic O-O bond cleavage and CYP epoxygenation involving an oxenoid iron in a high-spin state [41,42]. Cleavage of HPETEs results in the formation of 5-, 12- and 15-HETEs, and is characteristic of the CYP peroxygenase activity [20]. The participation of free Fe^{2+} in EET formation likely reflected a synergy of diverse reaction mechanisms and a pseudo-enzymatic nature of the CYP peroxygenase activity.

While cis- and trans-EETs represent monoxygenation products of AA, the lack of regioselectivity in their formation by CYP isoforms stimulated by LOOHs is uncharacteristic of CYP monoxygenase activity. For example, NADPH-dependent CYP2C8 metabolism of AA produced 14,15- and 11,12-cis-EETs only [34], however, LOOH-dependent CYP2C8 produced four cis-EETs and more abundantly four trans-EETs without any apparent regioselectivity. The preferred trans-EET formation that requires free rotation of C-C bond likely overrides the provision for regioselectivity, since VitC activated preferred nonselective trans- over cis-EET formation from AA (10 μM) that was expected to be embedded in restricted active sites of CYP enzymes in microsomal incubations. While CYP inhibitors inhibited microsomal VitC-dependent EET formation, chiral analysis of cis- and trans-EETs produced by microsomes and CYP isoforms stimulated by LOOHs should confirm the CYP peroxygenase activity; however, the diverse and multiple mechanisms of CYP reactions may not yield results conforming to model enzymatic reactions.

Contributions of CYP peroxygenase, isomerase and monoxygenase activities for AA metabolism in vivo will depend on concentrations of LOOHs produced by
Lipooxygenases and prostaglandin H synthases. The low $K_m$ values for CYP2C8 and CYP2J2 binding of 13-HPOTrE (Figure 5) was consistent with the great affinity of CYP isoforms to LOOHs in epoxygenation by the peroxygenase activity [20]. In rats, levels of plasma $trans$-EETs from the CYP peroxygenase activity (Table 3) were 10-fold higher than that of $20$-HETE (Figure 7), the product characteristic of NADPH-dependent CYP monooxygenase activity, which suggested a prominent contribution of CYP peroxygenase metabolism of AA under normal physiological conditions. In microsomal incubations in the absence of exogenous LOOHs, HPETEs derived from the microsomal lipooxygenase activity and lipid peroxidation fulfilled the role, which explained the inhibition of microsomal EET formation by tempol (Figure 4). Epoxidation by VitC/Fe$^{2+}$ represents an unspecific peroxidation mechanism, which will not likely prevail in vivo because of the presence of multiple antioxidant systems and the limited mobilization and release of Fe$^{2+}$ from ferritin [31]. Sub-micromolar concentrations of HPETEs could not stimulate significant microsomal EET formation without VitC as determined by the weak LOOH-dependent CYP peroxygenase activity. However, under oxidative stress, such as conditions of VitC deficiency in vivo, increased LOOH concentrations may stimulate the CYP peroxygenase activity for EET formation.

The confluence of two opposing factors of VitC and LOOH, one antioxidant and one oxidant, in activating EET formation resulted in a biphasic effect of VitC-dependent microsomal EET formation and a seemingly paradoxical EET regulation by VitC in vivo. The biphasic effect of VitC on microsomal EET formation was outlined based on very low levels of LOOHs; it would be of interest to assess the effect of VitC on microsomal EET formation at increased concentrations of LOOHs. VitC administration increased plasma EETs and urinary EET excretion in rats, confirming a role of VitC in stimulating EET formation in a rat model without deficiency of VitC. Depletion of VitC in tissues correlated with moderately increased EET levels in both the brain cortex and kidney tissues in gulo$^{+/}$ mice, which indicated increased levels of LOOHs associated with VitC deficiency. Though it is difficult to compare EET levels in rat plasma and urine with EETs in mouse tissues, these values should all reflect EET levels in vivo. More than 20- and 50-fold decreases in brain and kidney VitC concentrations, respectively, were only associated with a 30% increase in tissue EET levels, which was consistent with the weak potency of LOOH-dependent CYP peroxygenase activity for EET formation in the absence of VitC.

The minor increases in vasoprotective EETs associated with VitC depletion are apparently not enough to alleviate increased oxidative injuries and cardiovascular risks [29,43]. The result suggested the importance of maintaining sufficient VitC for the benefit of cardiovascular protection by EETs. Considering factors of absorption and distribution, large doses and extended periods of VitC supplementation have been recommended for therapeutic effect against critical illness, major trauma, and chronic inflammation [18]. VitC of 0.1% in the drinking water for 9 days in this study improved rat daily sodium balance defined as urinary sodium excretion over food sodium intake, which may reflect the natriuretic effect of VitC [6] and EETs as well as a potential role of EETs in reducing appetite and increasing metabolism [44].

The potential role of EETs in mediating the cardiovascular effects of VitC was demonstrated in experiments with rat isolated perfused kidneys. Increased vasodilator responses to VitC were associated with increased EET excretion from the kidney and the inhibition of EET hydrolysis (Figure 6). Although individual EET isomers could not be quantitated by GC/MS analysis, increases in 14,15-$trans$-EET associated with the inhibition of sEH should play a major role based on the preferred hydrolysis of 14,15-$trans$-EET by sEH and its potent vasodilator activity in rat renal arcuate arteries [10]. Vasodilator and natriuretic actions of EETs likely contributed to the effects of VitC in the kidney and could provide a direct explanation for the renal protective effects of VitC in reperfusion injuries in renal transplantation [36,37]. Inhibition of EET hydrolysis in the spontaneously hypertensive rat has increased plasma $trans$-EETs and reduced blood pressure [10]. Reduced levels of plasma $trans$-EETs in hypertension are consistent with reduced levels
of VitC in hypertensive rats [45] as well as in humans with hypertension [2,4]. However, VitC-dependent EET formation is also associated with the formation of 5/12/15-HETEs that are potentially involved in prohypertensive processes. Vitamin E, also known as an inhibitor of CYP metabolism, inhibited VitC-activated microsomal EET formation (Figure 4), which may explain why large clinical studies using a combination of VitC and vitamin E failed to register significant cardiovascular protective effects.

The study demonstrated potential interactions between CYP metabolism and pathways of lipoxigenases and cyclooxygenases. VitC as a cofactor for activating EET formation by the CYP peroxygenase activity broadens the approach for EET regulation. VitC may be particularly efficient for EET formation in diseases associated with inflammation and increased lipoxigenase activities. The cardiovascular functions of VitC are potentially effected by activation of EET formation under specific pathophysiological conditions.

5. ACKNOWLEDGEMENTS

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REFERENCES


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