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Original Research Article

A novel mechanism of ascorbate direct modulation of soluble epoxide hydrolase

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ABSTRACT

To test the hypothesis that VitC downregulates soluble epoxide hydrolase (sEH, responsible for converting EETs to DHETs) to stabilize tissue EETs, the heart, lung, liver, kidney, and mesenteric arteries isolated from normal rats were incubated with VitC (1000 μM) for 72 h, and tissue sEH expression, along with EET and DHET profiles were assessed. VitC caused significant reductions in sEH mRNA and protein content in the liver, heart and vessels, but had no effect on renal and pulmonary sEH expression, revealing a tissue-specific regulatory mechanism. The functional consequence of reduced sEH expression was validated by LC/MS/MS-based analysis, indicating that in VitC-treated tissues that displayed downregulation of sEH mRNA and protein expression, total DHETs were significantly lower, accompanied with a greater ratio of EETs/DHETs than those in VitC-untreated groups. Thus, VitC elicits a transcriptional downregulation of sEH in normal liver, heart, and vessels to reduce EET degradation and increase EET bioavailability.

1. Introduction

The cardioprotective activity of vitamin C (VitC, ascorbic acid) is mainly attributed to its antioxidant properties via its role as a key co-factor in various hydroxylation and amidation processes [24]. VitC, through its water-soluble characteristics acts in the cytosol and extracellular fluid to interact directly with free radicals, thus preventing oxidative insults such as damage to cellular componentes (DNA, proteins and lipids), endothelial dysfunction, activating cellular apoptosis and promoting protein degradation, etc. Alternatively, VitC does not merely act as an anti-oxidant agent, it also possesses a potential to become pro-oxidant by means of the fenton reaction in the presence of metal ions [30]. In this context, currently reported clinical trials have approved that infusion of high doses of VitC as a potential stragedy improves outcomes of standard cancer treatment, through the auto-oxidative property of VitC to generate steady-state levels of hydrogen peroxide (H_2O_2), which then, is capable of selectively sensitizing cancer, but not normal cells by disrupting intracellular iron metabolism [46]. Moreover, activation of cytochrome P450 (CYP)-2C isozyme(s) is associated with the production of superoxide [9,49], a free radical species that is indispensible for CYP2C-dependent synthesis of epoxyeicosatrienoic acids (EETs) when transferred to CYP 2C epoxygenase [2]. In this regard, the scavenging of superoxide by VitC might interrupt EET synthesis.

EETs are arachidonic acid metabolites produced by CYP epoxygenases, enzymes such as CYP2C, 2J, 2E and 2B isozymes, which have been reported to catalyze the formation of EETs in various tissues across many species [43] to evoke anti-oxidative, anti-inflammatory and thrombolytic activities, as well as systemic vasodilation [6,43]. Additionally, EETs promote angiogenesis in response to ischemia [28,53] and prevent vascular remodeling via inhibition of smooth muscle cell proliferation [50]. In this context, the development of cardiovascular diseases has been correlated to the impaired CYP/EET activity in the cardiovascular system [14]. Alternatively, conversion of EET epoxides to their corresponding diols (DHETs) by soluble epoxide hydrolase (sEH) minimizes bioavailability of EETs and compromises their cardioprotective actions. To this end, a mechanistically-based rationale for the use of sEH inhibitors (sEHI) to stabilize EETs, followed by potentiating EET bioavailability was proposed [14]. Indeed, genetic deletion or downregulation of sEH gene, or pharmacologic inhibition of sEH activity has been demonstrated to be beneficial in a variety of tissues including cardiovascular, renal, hepatic, and cerebral systems [7,10,12,14,15,23,38,39,43,48]. As such, in addition to EET synthase, sEH also warrants consideration as a therapeutic target, since the

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elevation of EET bioavailability can be triggered by either an increase in EET synthesis or a decrease in its metabolism.

To date, studies involving VitC and EETs/CYP have predominantly focused on the drug metabolism-based relationship between these two players [45]. For instance, a deficiency in VitC is concomitantly associated with a reduction in hepatic CYP content [23], whereas, a high-dose of VitC propels CYP activity [51] and prevents sepsis-induced CYP dysfunction [22] in the liver. In the renal system, Jiang, et al. have proposed a particular mechanism by which VitC-induced microsomal formation and regulation of EET synthesis are dependent upon the concert-like actions of lipid hydroperoxides (LOOHs), Fe^{2+} and CYP enzyme without requirement of NADPH and CYP reductase [16]. Of note however, most of the aforementioned studies were focused on VitC-induced changes in hepatic EET *synthesis*. Fewer studies have explored roles of VitC in the *degradation* of EETs, in terms of modulation of sEH in extra-hepatic systems. To this end, we tested the hypothesis that VitC specifically downregulates sEH to stabilize tissue EETs, by chronic exposure of rat tissues to VitC. The specific signaling responsible for the VitC-dependent regulation of the sEH gene, sEH protein, and its product profile of EETs and DHETs were assessed by molecular and LC/MS/MS analyses.

2. Material and methods

2.1. Animals and tissue incubation

Male Sprague-Dawley rats (350–400 g) were purchased from Charles River laboratories (Wilmington, MA). Rats were sacrificed by inhalation of carbon dioxide (CO_2) and the heart, lung, liver, kidney, and mesenteric arteries were isolated and rinsed gently with MOPS-buffered physiological salt solution three times. Each isolated tissue was cut into small pieces and distributed to ~150 mg/sample/well, except for mesenteric arteries, where one well consisted of ~10 second-order arteries. Based on established tissue culture protocols, samples were transferred into a 24-well dish filled with enzyme solution that contains collagenase (1 mg/ml), soybean trypsin inhibitor (1 mg/ml) and antibiotics (1%) mixed with HBSS, and then incubated at 37 °C in 5% CO_2 for ~2 h. After that, samples were exposed to DMEM containing 1% antibiotics (Antimycin solution 100x) with different concentrations (zero as a time-course control, 50, 100, 500 and 1000 μM respectively) of Vitamin C (L-ascorbic acid, Sigma #A5960, St. Louis, MO), and Vitamin E (1000 μM; (±)-α-Tocopherol, Sigma T3251, St. Louis, MO) as a control for 72 h. The entire procedure was performed under sterile conditions. DMEM containing VitC was prepared fresh and replaced on a daily-basis. During the procedure, a few cells were indeed attached to the well at the end of the incubation period. Instead of a single type of cells, the majority of the cell population is from the tissue mass of the corresponding organ. To this end, the study was defined as tissue culture-based experiments. At the conclusion of the incubation period, tissue samples were collected and pulverized in liquid N_2 for molecular and biochemistry analyses.

All protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conformed to the guidelines of the National Institutes of Health and the American Physiological Society for the use and care of laboratory animals.

2.2. Quantitative real-time RT-PCR

Extracted tissue RNA from samples was subjected to real-time quantitative PCR (LightCycler, Roche Diagnostics, Indianapolis, IN). Oligonucleotide primers for the Ephx2 gene encoding for the sEH protein were designed in house and synthesized by Fisher Scientific customer services (Morris Plains, NJ). Forward primer: GAAGATCTTGGTCCCTGCCC and Reverse primer: AATCTGGTTCA CCTCTGCCG with annealing temperature at 55 °C and a product length of 166 bp. The sEH expression was normalized to GAPDH. A relative quantitation method (ΔΔCt) was used to evaluate the expression of the gene in different groups of samples. All primer products were verified on a 1.5% Agarose gel.

2.3. Western blot analysis

Equal amounts of total protein (25 μg) extracted from tissue samples were loaded on and separated by a 10% SDS-PAGE gel and transferred to a PVDF membrane. The PVDF membrane was probed with specific primary antibodies for sEH, CYP2J (Santa Cruz Biotechnology, INC), or CYP2C (Biodesign Inc.) and appropriate secondary antibodies conjugated with horseradish peroxidase. Specific bands were visualized with a chemiluminescence kit and normalized to GAPDH. The X-ray film was scanned into a computer and band densitometry was digitalized with UN-SCAN-IT software.

2.4. LC/MS/MS-based measurements for tissue EETs and DHETs

The method for measurement of EETs and DHETs in the heart, lung, and vessels has been described in detail previously [10,19,38,39,48]. Briefly, cultured tissue samples were pulverized in liquid nitrogen. EETs and DHETs were extracted following alkali hydrolysis to release esterified EETs or DHETs and quantified with a Q-trap 3200 linear ion trap quadrupole LC/MS/MS equipped with a Turbo V ion source operated in negative electrospray mode (Applied Biosystems, Foster City, CA). NaOH (1 mol/l, 1 ml) was added to samples after extraction for their dissolution, and then, protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA) and used to normalize the detected lipids. Data are presented as total EETs and DHETs that include the sum of all free and esterified four regioisomeric EETs and DHETs, and expressed as ng/mg protein.

2.5. Tissue superoxide production

Cytosolic and mitochondrial superoxides were measured using an established HPLC/fluorescence detector-based analysis [37]. Specifically, VitC-treated and −untreated tissues were incubated with either 5 μM dihydroethidium (DHE, Invitrogen, Eugene, Oregon, USA), or MitoSox (mitochondrial superoxide indicator, Invitrogen, Eugene, Oregon, USA) for one hour in the dark. After incubation, samples were washed with PBS and homogenized in acetonitrile/water (1:1), and then centrifuged for 10 min. The supernatant fraction was collected for HPLC/fluorescence detection of 2-hydroxyethidium (EOH) or 2-hydroxy-mito-ethidium (mito-EOH), products of the reaction of superoxide with DHE or MitoSox, with excitation and emission wave lengths of 480/580 nm for EOH and 510/590 nm for mito-EOH, respectively. The centrifuge precipitates were used for protein measurement using Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Final concentration of superoxide in each sample was normalized to its protein content, and expressed as picomoles of superoxide (EOH or mito-EOH) per milligram of protein.

2.6. Statistical analysis

Data are expressed as means ± SEM. N refers to the number of rats or Western blots. Statistical analysis was performed using repeated-measures of ANOVA followed by the Tukey-Kramer post hoc test and Student's t-Test. Statistical significance was accepted at a level of $p < 0.05$.

3. Results

3.1. Tissue protein content of sEH

The liver is a major organ responsible for EET production and has the highest epoxide hydrolase activity for converting EETs to DHETs by