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SELENIUM-DEPENDENT DOWN REGULATION OF SOLUBLE EPOXIDE HYDROLASE IN INFLAMED MURINE BONE-DERIVED MACROPHAGES

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Selenium-dependent down regulation of soluble epoxide hydrolase in inflamed murine bone marrow-derived macrophages

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Abstract

The metabolism of anti-inflammatory epoxyeicosatrienoic acids (EETs) to inflammatory dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH) is a critical, but elusive pathway, which contributes to the inflammatory immune response that underlies many diseases. Selenium is an essential micronutrient that functions through its co-translational incorporation into proteins as the 21st amino acid, selenocysteine (Sec) via a highly regulated process involving a specific tRNA$^{[Sec]}$. Expression of selenoproteins in murine bone marrow-derived macrophages (BMDMs) is critical to mitigate signaling pathways that culminate in the expression of inflammatory mediators upon stimulation with bacterial endotoxin lipopolysaccharide (LPS). However, the role of selenium in the regulation of sEH has not been investigated before. Therefore, we hypothesized that selenium supplementation (as selenite; 250 nM) may inhibit sEH to contribute to the alleviation of inflammation through down regulation of EET metabolism. Our results indicate that even though sEH mRNA increased in the selenium supplemented group similar to those in the selenium-adequate group (50 nM selenite; baseline selenium), the levels of sEH protein decreased only in the former group; while such a regulation was absent in the latter group. This suggests that sEH is regulated post-transcriptionally or post-translationally. Furthermore, BMDMs from *Trsp*$^{fl/f}$Cre^{LysM} mice that are unable to express selenoproteins confirmed the importance of selenoproteins in the decreased production of sEH. Our studies suggest that selenium supplementation has the ability to inhibit LPS-dependent increase in sEH and skew the metabolism of arachidonic acid metabolites towards anti-inflammatory mediators. In summary, our studies have discovered a new link to implicate the anti-inflammatory activities of selenoproteins via inhibition of sEH.

1. Introduction

Inflammation is a common underlying mechanism of many diseases including cancers, hypertension, autoimmune disorders, diabetes and neurodegenerative diseases [1-5]. As part of a complex biological response, inflammation provides the first line of immune defense against harmful stimuli, infections, and pathogenic invasions; however, in the absence of a temporal control of these inflammatory insults, a wide range of detrimental effects that result from the collateral damage could exacerbate leading to uncontrolled inflammation. Factors that influence inflammation include toxins, highly saturated fat diet, stress, genetics, and the environment [6-9]. In response to these stimuli, specific mediators are released from tissues and cells to activate inflammatory pathways [10]. Mediators such as cytokines and bioactive lipids initiate controlled inflammation, which can progress into chronic inflammation if the balance between pro- and anti-inflammatory mechanisms is disrupted [11].

An important group of bioactive lipid mediators are the epoxyeicosatrienoic acids (EETs). EETs comprise a group of fatty acid metabolites that are derived from the omega-6 polyunsaturated fatty acid, arachidonic acid (C20:4), through the cytochrome P450 (CYP) pathway [12]. Arachidonic acid is also converted to other eicosanoid mediators by cyclooxygenase and lipoxygenase pathways [12]. Previous research has demonstrated a pivotal role for EETs in anti-inflammatory mechanisms, where EET-activated vascular relaxation and downregulation of NF-kB transcription [13]. EETs also act as potent inhibitors of cellular adhesion molecule (CAM) expression, which leads to reduced adhesion of inflammatory cells [13]. Their broad spectrum of anti-inflammatory activity mitigates the damaging effects of cardiovascular and renal disease [14,15].

Soluble epoxide hydrolases (sEH) are cytosolic enzymes that convert fatty acid epoxides into less active diols, resulting in the loss of their cell signaling actions and consequently protective functions [16]. In cells, EETs can be further hydrolyzed by sEH into corresponding dihydroxyeicosatrienoic acids (DHETs) [12]. Increased levels of sEH can be associated with increased cellular inflammation due to sEH converting anti-inflammatory EETs into inflammatory DHETs. Therefore, sEH is considered a potential therapeutic target for diseases characterized by inflammation. Previous research has shown that pharmacological inhibitors of sEH, AUDA-nBE

and t-AUCB, modulate pro-inflammatory pathways, leading to mitigated colitis and acute systemic inflammation [17,18].

Although literature is replete with the causal association of EET metabolites in inflammation, the mechanisms underlying the role of CYP pathways and the effects of EET metabolism on inflammation are still not well understood. Therapeutic inhibitors of sEH are currently a novel area of research. Epidemiological data suggests a positive association between selenium deficiency and the prevalence of infections where chronic inflammation forms the underlying basis of the disease [19].

Selenium is an environmentally derived nutrient that is important for various aspects of human health [20]. Dietary selenium, mainly through its incorporation into selenoproteins, plays an important role in inflammation and immunity by modulating certain inflammatory pathways. Selenoproteins, such as GPX, are created by the incorporation of selenocysteine amino acid residues into proteins. Previous studies in our laboratory have demonstrated the anti-inflammatory role of selenoproteins in pathologies involving inflammation by acting on arachidonic acid metabolites [21, 22, 23]. Arachidonic acid contributes to inflammation through its metabolism by the cyclooxygenase (COX) pathway, the lipoxygenase (LOX) pathway, and the cytochrome P-450 (CYP) pathway. Selonoproteins skew the COX pathway towards production of the anti-inflammatory cyclopentenone PGJ_2 metabolites rather than PGE_2 and TXA_2 [21]. Along with that, studies from our lab have shown that selenium supplementation increases the polarization of macrophages form a M1 to M2 phenotype, thereby decreasing inflammation [23]. In all, selenoproteins modulate metabolic pathways through redox mechanisms, which aids in alleviating inflammation.

The main goal of this study was to explore the role of selenium in the metabolism of anti-inflammatory EETs into pro-inflammatory DHETs by sEH in immune cells, specifically macrophages. It is currently unknown if dietary selenium modulates this pathway through an anti-inflammatory mechanism, as it has shown to do in the COX and LOX pathways of arachidonic acid metabolism. This study investigates the ability of selenium supplementation to inhibit EET

metabolism by downregulating the expression of sEH. The results will contribute to the search for better treatment plans for many diseases where sEH plays a critical role in inflammation.

2. Materials and Methods

2.1. Mice

Four month old C57BL/6 mice were utilized to harvest bone marrow stem cells that would be induced to differentiate into macrophages that would grow in varying concentrations of selenium. As a control, a transgenic C57BL/6 line carrying a lysozyme M Cre (Cre^{LysM}) transgene was crossed to a C57Bl/6 mouse with a floxed *Trsp* ($Trsp^{fl/fl}$) allele, as previously described [23]. The extent of Trsp deletion was determined by PCR analysis of the floxed region of the gene, as previously described [24]. Studies were preapproved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee at Penn State University.

2.2. Isolation and culturing of BMDM's

Macrophages were isolated from the bone marrow of murine ($Trsp^{fl/fl}$ WT) femurs and were brought to a single cell suspension. The bone-marrow-derived macrophages (BMDMs) were cultured in BMDM media containing Dulbecco's modified Eagle's medium (DMEM) containing 5% defined fetal bovine serum, 200 mM L-glutamine, penicillin (10,000 units/ml), and streptomycin (10,000 ug/ml), and 10% (v/v) L929 fibroblast media as the source of macrophage colony stimulating factor (M-CSF) for 48 h. These cells were incubated with 50 nM and 250 nM selenium (in the form of sodium selenite) for 96 h to simulate adequate and supraphysiological (supplemental) levels of selenium, respectively. The murine macrophages were treated with 100 ng/ml LPS for 4h and 24h. Cells that were not treated with LPS (0hr) were used as a control for a normal, non-inflamed cell environment. Similar procedures and treatments were performed with $Trsp^{fl/fl}$ Cre^{LysM} (KO) mice to understand if selenium-dependent selenoprotein expression was essential in the regulation of sEH expression.

2.3. Harvesting of LPS-treated Macrophages

Inflamed murine macrophages that were intended for protein extraction and protein activity determination were washed twice with sterile and cold PBS, after the BMDM media was removed. The cells were harvested with PBS and centrifuged at 2500g for 10 min. The supernatant was aspirated out and the cell pellets were stored at -80°C until further analysis. Inflamed murine macrophages that were intended for RNA isolation were extracted with TRIZOL at 4°C for 15 min, after the BMDM media was removed. The TRIZOL/cell suspension was collected and stored in -80°C.

2.4. Protein Extraction and Estimation

Protease inhibitor cocktail (containing PMSF, leupeptin, aprotinin, pepstatin, sodium orthovanadate) and Mammalian Protein Extraction Reagent (M-PER™) reagent (Thermo-Pierce) were added to the cell pellets on ice, which were then vortexed every 5 min for 20 min, followed by centrifugation at 12,000g for 15 min. The supernatant was transferred to a new tube for analysis. The concentration of protein in each sample was determined using a bovine serum albumin (BSA) in the BCA protein assay that was measured absorbance at 562 nm (Thermo Pierce).

2.5. RNA Isolation

Chloroform was centrifuged with the cells at 12,000 g for 15 min. The aqueous phase was vortexed with isopropanol and then centrifuged at 12,000 g for 10 min. The supernatant was discarded and the RNA pellet was washed by adding 75% ethanol and centrifuging at 7500 g for 5 min. The supernatant was removed, the RNA pellet was briefly air dried, and was re-dissolved in RNase free water. The absorbance at 260 and 280 nm were recorded and those with a ratio of ≥1.8 were used for qPCR analysis.

2.6. Lipid Extraction

A glass syringe and column was used to extract lipids from the BMDM media. Acidified media was passed through the column dropwise using a glass hypodermic syringe and was collected. The collected media was added into the column again and was pushed though dropwise. PBS was used to wash the column. Hexane was pushed through dropwise and the column was flash aired twice. Methanol was used to collect the elute. The vials of lipid were dried under nitrogen gas and dissolved in ethyl acetate. The samples were stored at -80ºC until further analysis.

2.7. Western Blot Analysis

Western immunoblot analysis was performed with proteins isolated from inflamed murine macrophages using prevalidated polyclonal antibodies for Gpx-1 [Abcam, anti-rabbit], β-actin [Cayman, anti-mouse], and sEH [Abcam, anti-rabbit]. The isolated proteins were separated using a discontinuous SDS/PAGE (% T= 10) and then electroblot transferred onto nitrocellulose membranes. The membranes were incubated with anti-rabbit or anti-mouse IgG and washed. Secondary antibodies were visualized by a SuperSignal West Pico Substrate chemiluminescence detection system and imaged using GeneSys G:Box Chemi-XR5 software. The immunodetectable bands were quantified by densitometry using Image J (NIH).

2.8. qPCR

RNA was isolated from murine-derived macrophages using TRIzol reagent as per the instructions of the supplier (Invitrogen). RNA concentrations were determined by UV-Vis spectroscopy. RNA (1 ug) was reverse-transcribed into cDNA. A TaqMan probe for *sEH* was used to quantitate cDNA. Amplifications were performed using PerfeCTa qPCR SuperMix Master Mix in a 7300 Real time PCR system. Data was analyzed according to the method of Livak and Schmittgen with normalization to 18*S* rRNA [25].

2.9. Specific Activity of sEH

Macrophage cell pellets were suspended in sodium phosphate buffer (20mM, pH 7.4) containing 5mM EDTA, 1mM DTT, 1mM PMSF, and 0.01% of Tween 20. The activity of the mixture was measure with [^{3}H]-tDPPO 50 μM for 90-120 minutes at 37 ^{o}C. Protein concentration was measured with the BCA method using BSA as a standard.

2.10. DHET and EET Analysis // Pending- to be performed by Dr. Hammock's laboratory at UCD//

2.11. Statistics

All data have been represented as mean ± S.E.M. The differences between groups were analyzed using two-way ANOVA on GraphPad® Prism and paired t-test was used to compare the mean between treatment groups. The criterion for statistical significance was $p \leq 0.05$. All experiments were performed in biological triplicate for a range of $n = 3$ to n=5 per sample.

3. Results

3.1. Selenium supplementation increases sEH mRNA levels in macrophages in vitro over time for both adequate and supplemented cells

We tested the effect of selenium supplementation on EET metabolism by investigating the modulation of expression of sEH in a murine macrophage model of LPS-induced inflammation. *Trsp* $^{f/fl}$ WT BMDMs were cultured in media that contained either 50 nM Se or 250 nM Se. Cells at each selenium concentration were stimulated with LPS for either 4 hours or 24 hours to trigger inflammation. The cells that did not receive any LPS served as a negative or untreated controls. Total RNA was isolated from all cells and the mRNA levels of sEH was determined using qPCR (Fig. 1). As shown in Fig. 1A, the mRNA levels did not differ between the 50 nM (adequate concentration) and 250 nM Se (supplemented concentration) cells in unstimulated or stimulated cells. However, there was a significant increase in sEH mRNA levels over time in both Se concentrations. The fold change increased ~200% between hour 4 and 24.

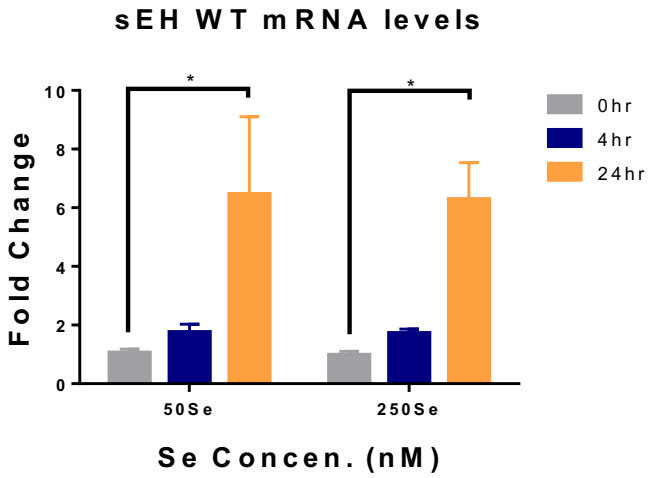

Fig. 1. LPS-dependent increase in sEH mRNA expression in selenium-adequate (50 nM) and selenium-supplemented (250 nM) BMDMs. Cells were treated with 100 ng/ml E.coli LPS for indicated time periods. Total RNA was used in qPCR assays. sEH mRNA levels increased over time for both adequate and supplemented cells. Representative of *n*=5. Mean ± SEM shown.

3.2. Selenium supplementation decreases sEH protein levels in macrophages in vitro

We further examined the effect of selenium supplementation on sEH levels through analysis of protein expression. Total cellular protein were isolated with M-PER reagent and the levels of sEH protein were analyzed through western blotting (Fig. 2). To confirm that the cells expressed selenoproteins upon exogenous supply of selenium (as selenite), Gpx1 was used. Gpx-1 is a selenoprotein that is the first one to be expressed when cells are treated with selenium. Expression of Gpx-1 increased marginally in the 250 nM selenium treatment group compared to the 50 nM group. Interestingly, as shown in Fig. 2A-B, the sEH protein levels decreased by ~50% upon selenium supplementation when compared to cells with adequate concentrations of selenium during 24h post LPS. A decrease in sEH protein levels over time in Se supplemented cells was clearly seen.

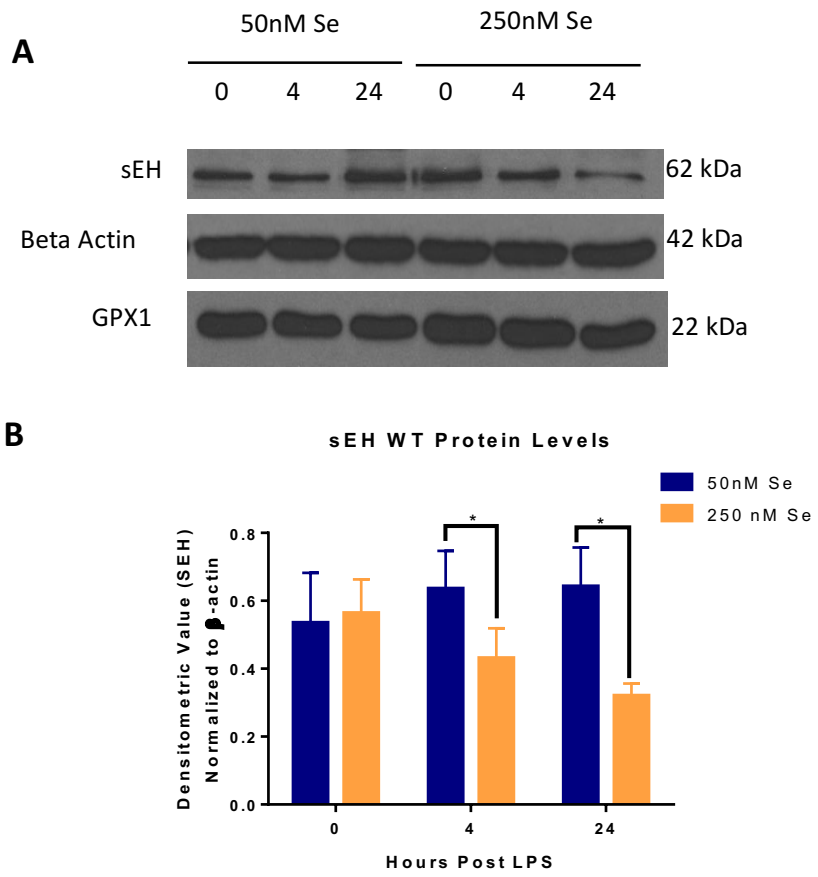

Fig. 2. Selenium supplementation of LPS-stimulated murine macrophages decreases sEH protein levels when compared to adequately supplemented macrophages. (A) Western blot of WT macrophage proteins shows an increase in sEH protein levels in adequate cells and a decrease in supplemented cells. The presence of GPX1 indicates that selenoproteins are expressed. (B) There is a lower level of sEH protein expression in supplemented cells when compared to adequate cells. Representative of *n*=5 shown. Densitometric data are Mean ± SEM.

3.3.Decreasing Trend in sEH Enzymatic Activity Levels in Selenium Supplemented Cells

To test if sEH enzymatic activity coincided with the above data showing decreased protein levels in selenium supplemented cells, enzymatic activity of sEH was analyzed at the Hammock laboratory at University of California, Davis. As shown in Fig 3, the fold change in activity was determined in WT BMDMs. Even in unstimulated cells, there was a decreased activity seen in the selenium supplemented cells. Stimulation with LPS for 4 h increased the activity in both groups, similar to the mRNA and protein. However, there was a slight decrease in activity in selenium supplemented group, which did not reach statistical significance. With further stimulation, the pattern remained the same even though the activity at 24 h was less than that at

4h. Though the data is not statistically significant, activity levels showed a general decrease over time, which is encouraging. These results correlate with the overall decreased levels of sEH protein in supplemented cells that was determined earlier in the experiment. It is clear that the number of replicates will have to be increased in future experiments in order to determine statistical significance.

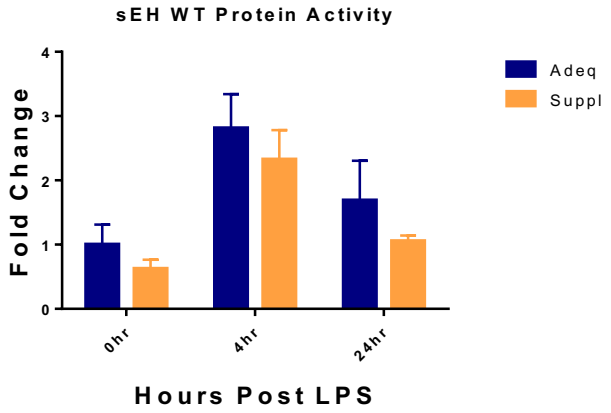

Fig. 3. Selenium supplementation of LPS-stimulated murine macrophages creates a decreasing trend in sEH enzymatic activity. Cells were lysed and total cell protein was used for activity analysis. Specific activity was measured and used to calculate fold change in activity using the corresponding selenium-adequate samples at specific time points. The fold change in enzymatic activity shows a decreasing trend in selenium supplemented cells between 4hr and 24hr. There is an overall trend of lower activity levels in supplemented cells when compared to adequate cells. Representative *n*=3.

3.4. Depletion of selenoprotein levels affect sEH mRNA and protein levels in inflamed Trsp KO BMDMs

To investigate if selenium-dependent effect on sEH expression was mediated through selenoprotein expression, *Trsp*$^{fl/fl}$ LysM^{Cre+} KO murine-derived BMDMs were subjected to LPS stimulation as described in the above experiments. Apart from genotyping, we confirmed the selenoprotein status of these BMDMs, cultured in the presence of 50 and 250 nM selenium (as selenite), by examining the expression of Gpx-1. As shown in Fig. 4A, GPX1 expression was completely absent in the KO mice, cultured with selenium, in the absence or presence of LPS stimulation. Fig. 4B shows that there is no statistical difference of protein levels between adequate and supplemented cell. The same results are true for that of mRNA levels (Fig. 4C).

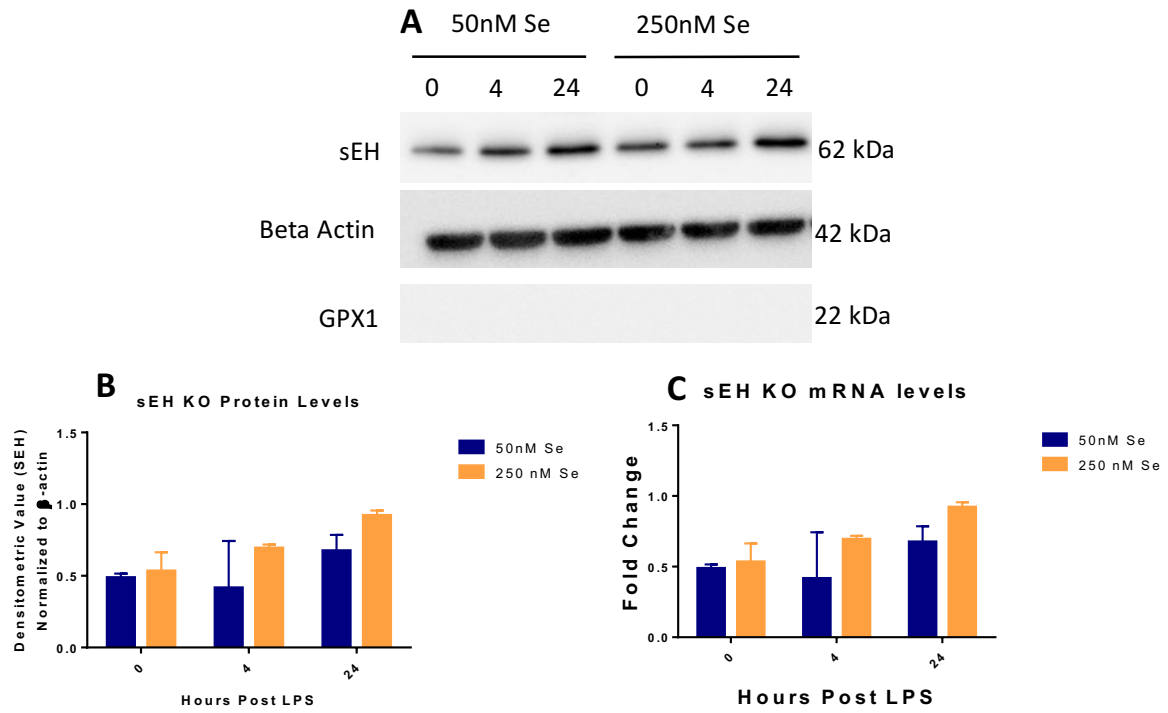

Fig. 4. Selenium supplementation of LPS-stimulated murine macrophages does not significantly change sEH mRNA or protein. (A) Western blot of Trsp KO macrophage proteins shows a lack of GPX1, which indicates that selenoproteins are not present. Representative of *n*=3 shown. (B) Densitometric evaluation of the sEH protein levels between adequate and supplemented cells. *N*= 3; Mean ± SEM. (C) There is no difference in mRNA levels between adequate and supplemented cells. Representative of *n*=3 shown.

3.5. EET RESULTS // Pending- being performed at Dr. Hammock's laboratory at UC Davis//

3.6. DHET RESULTS // Pending- being performed at Dr. Hammock's laboratory at UC Davis//

4. Discussion

Past research has demonstrated the extensive contribution inflammation has in a variety of diseases. The widespread, and sometimes detrimental, influence that the inflammatory response has on so many pathologies makes it an important consideration when treating such illnesses. Dietary selenium plays a critical role in modulating inflammatory processes within the body. Studies have shown that selenium supplementation regulates ROS in phagocytes, inhibits the

activation of NF-kB, suppresses CRP production, and inhibits expression of VCAM-1 [26-28] all of which contribute to reduced inflammation.

Arachidonic acid metabolism is comprised of three separate pathways which all contribute to the development of inflammation; the cyclooxygenase (COX) pathway, the lipoxygenase (LOX) pathway, and the cytochrome P-450 (CYP) pathway. Many studies have characterized the mechanisms of the COX and LOX pathways [29-31] with few emphasizing the role of the CYP pathway in inflammation and cancer [12, 32]. Research has shown that selenium inhibits the production of lipid metabolites through the COX and LOX pathways [29, 33], which contributes to the overall alleviation of inflammation in the disease process. To our knowledge, there is no report on the metabolic regulation of the CYP pathway by selenium. It is known that through the CYP pathway anti-inflammatory EETs are produced, which are hydrolyzed to pro-inflammatory DHETs by soluble epoxide hydrolase (sEH) [12]. Therefore, sEH has been a target in past studies and small molecule inhibitors of sEH have been shown to reduce inflammation in a number of mammalian disease models [34-36]. Due to the side effects of these inhibitors, there has been interest in the discovery of a more therapeutic alternative. Our studies, for the first time, show that dietary selenium supplementation decreases the expression and activity of sEH in primary macrophages, which could indicate reduced inflammation in certain pathologies. However, this result being further confirmed upon analysis of EETs and DHETs that are currently underway.

Our data demonstrates the ability of selenium supplementation decrease protein levels of sEH in an LPS-stimulated murine-derived macrophage model. The sEH activity level results coincide with this observation, as they too show a decreased trend in supplemented cells, when compared to adequate cells. These results suggest that the quantities of cellular DHET and EET may also follow a similar pattern, though it remains to be seen. This is very likely since a decrease in sEH protein corresponds to a subsequent decrease in DHET and increase in EET, as shown in previous studies involving sEH inhibitors [18, 37]. Such a modulation in lipid mediator concentrations creates a shift towards anti-inflammatory properties associated with EETs. Previous studies have described the role EETs play in decreasing inflammation in a variety of diseases including hyperalgesia, cardiovascular disease, and chronic obstructive pulmonary disease [38-40]. EETs display the ability to down regulate COX-2 and its associated metabolites, along with a decreasing 5-LOX metabolites [41]. Research has also shown that the anti-inflammatory properties of EET are mediated by the inhibition NF-kB and VCAM-1 expression [42,43]. Therefore, by way of

increasing EET concentration through inhibition of sEH via selenium supplementation, inflammation can be significantly reduced.

The inverse relationship amongst sEH mRNA and protein levels insinuate possible post-transcriptional or post-translation modifications. Over time cellular mRNA levels increased while protein levels decreased in selenium supplemented macrophages. Therefore, selenium-dependent effects may be mediated via initiating inhibition of mRNA translation or via rapid degradation of the sEH protein. With regards to post-transcriptional processing of sEH gene transcripts, many modification steps such as cap formation, poly A addition, and splicing could be defective and result in degradation of the transcripts. Possible post-translation modifications should be tested using a proteasome or ubiquitin inhibitor, which will determine if the protein is being degraded.

Given that the KO cells were unable to produce selenoproteins and consequently had no change or an increase in sEH protein levels, it appears that selenoprotein activity is necessary for the inhibitory effect of selenium supplementation on EET metabolism. This assumption is corroborated by previous research stating a difference in selenium supplementation activity when a nonbioavailable source of selenium is used [21]. Therefore, these studies suggest that the beneficial effects of selenium on sEH expression are mediated through its incorporation into selenoproteins. It appears to be seen if these results can be replicated in-vivo models of inflammation.

In conclusion, we propose that the micronutrient selenium, through incorporation into selenoproteins, downregulates EET metabolism via inhibition of sEH activity in macrophages. These studies pave the way for research on the dietary micronutrient-mediated inhibition of sEH and its role in mitigating inflammation.

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Education

B.S. in Microbiology, Class of 2018
The Millennium Scholars Program
The Pennsylvania State University, University Park, PA

Thesis Title: Selenium-Dependent Down Regulation of Soluble Epoxide Hydrolase in Inflamed Murine Bone-Derived Macrophages
Thesis Supervisor: Dr. K. Sandeep Prabhu

Research Experience

Penn State University Research Lab, Dr. K. Sandeep Prabhu 2016-Present
- Studying the effects of selenium on sEH metabolism in bone marrow derived macrophages
- Cultured macrophages, Analyzed protein with Western blotting and RNA with qPCR

Computational Biology Summer Program, Rutgers University, Dr. David Salas Summer 2016
- Independently devised and conducted an experiment to optimize the biofuel production process
- Developed new procedure to analyze cellulose degradation by fungal enzymes in a 3-D model

Undergraduate Clinical Research Internship Program, Vanderbilt University, Dr. Agnes Fogo Summer 2015
- Research pertained to fibrotic kidneys and the effects of PAI-1 on the symptoms of chronic kidney disease
- Managed care and treatment of 6 mice; including I.P. injections and assistance during UUO surgery

Research Conferences and Presentations

Undergraduate Poster Exhibition, Penn State University October 2015, 2016
- Deletion of PAI-1 in Fibroblasts and Interstitial Cellular Infiltration
- Enzymatic Degradation of Biomaterials by Neurospora crassa

Annual Biomedical Research Conference for Minority Students; Seattle, Washington November 2015
- Deletion of PAI-1 in Fibroblasts and Interstitial Cellular Infiltration

Invited Oral Presentation at HHMI; UMBC-PSU-UNC Partnership Annual Retreat October 2015
Stonewall Resort, West Virginia
- Deletion of PAI-1 in Fibroblasts and Interstitial Cellular Infiltration

Community Service

Volunteer at Mount Nittany Hospital 2016-Present
- Discharge patients and help nurses with day to day tasks

Science LionPride Member 2015- Present
- Lead tours for prospective science students and their families
- Organize science outreach programs for kids in grades K-12

Penn State University Learning Assistant Spring 2017
- Aid students learning and understanding in a microbiology lab class
- Explain class topics, answer questions, present techniques to be used for class assignments

Awards / Grants

Penn State Millennium Scholars Program 2014- Present
Erickson Discovery Research Grant Summer 2017
Dean's List 2014-2017
Schraer Scholarship for Women in Science 2014-2017
Bunton Waller Merit Award 2014-2017
ABRCMS Student Travel Award Fall 2015