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Reduced macrophage selenoprotein expression alters oxidized lipid metabolite biosynthesis from arachidonic and linoleic acid☆

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Abstract

Uncontrolled inflammation is an underlying etiology for multiple diseases and macrophages orchestrate inflammation largely through the production of oxidized fatty acids known as oxylipids. Previous studies showed that selenium (Se) status altered the expression of oxylipids and magnitude of inflammatory responses. Although selenoproteins are thought to mediate many of the biological effects of Se, the direct effect of selenoproteins on the production of oxylipids is unknown. Therefore, the role of decreased selenoprotein activity in modulating the production of biologically active oxylipids from macrophages was investigated. Thioglycollate-elicited peritoneal macrophages were collected from wild-type and myeloid-cell-specific selenoprotein knockout mice to analyze oxylipid production by liquid chromatography/mass spectrometry as well as oxylipid biosynthetic enzyme and inflammatory marker gene expression by quantitative real-time polymerase chain reaction. Decreased selenoprotein activity resulted in the accumulation of reactive oxygen species, enhanced cyclooxygenase and lipoxygenase expression and decreased oxylipids with known anti-inflammatory properties such as arachidonic acid-derived lipoxin A_4 (LXA_4) and linoleic acid-derived 9-oxo-octadecadienoic acid (9-oxoODE). Treating RAW 264.7 macrophages with LXA_4 or 9-oxoODE diminished oxidant-induced macrophage inflammatory response as indicated by decreased production of TNFα. The results show for the first time that selenoproteins are important for the balanced biosynthesis of pro- and anti-inflammatory oxylipids during inflammation. A better understanding of the Se-dependent control mechanisms governing oxylipid biosynthesis may uncover nutritional intervention strategies to counteract the harmful effects of uncontrolled inflammation due to oxylipids.

Keywords: Selenium; Selenoproteins; Macrophage; Oxylipids; Eicosanoids

1. Introduction

Inflammation is an essential component of innate immune defenses that works to eliminate infectious microbes and other causes of tissue damage. Optimal inflammatory events should be robust enough to destroy pathogens but resolve quickly to restore normal organ function and eliminate the source of injury [1]. Uncontrolled inflammation, however, can contribute significantly to several disease pathologies by causing tissue damage. Uncontrolled inflammation can be defined as either an exacerbated acute inflammation, such as that seen during sepsis [2], or a chronic, low-grade inflammation, such as that observed during atherosclerosis [3]. Therefore, tight regulation and timing of inflammatory events are crucial to effectively eliminate the insult and prevent host tissue damage [1].

Macrophages are pivotal in orchestrating and resolving inflammation. They produce reactive oxygen species (ROS) to phagocytize pathogens and secrete cytokines to control immune cell diapedesis and promote tissue remodeling [4]. In addition, macrophages are a major source of oxidized lipid mediators, such as the linoleic acid (LA)-derived oxidized LA metabolites or the arachidonic acid (AA)-derived eicosanoids, collectively called oxylipids. Oxylipids such as fatty acid hydroperoxides (FAHP) from LA and AA (HPODEs and HPETEs, respectively) can be produced enzymatically by cyclooxygenases (COX) 1 and 2 and lipoxygenases (LOX) enzymes [5]. These hydroperoxides are also formed by non-enzymatic oxidation by free radicals, making them suitable markers of oxidative stress [6]. FAHP from LA and AA can be chemically reduced to hydroxyls (HODEs and HETEs) and these hydroxyls can undergo dehydrogenation to produce ketone derivatives (oxoODEs and oxoETEs) [6]. Oxylipid biosynthesis can be regulated by enzymatic expression and activity, oxidative tone of the cell/tissue and feedback from other oxylipids; all of which must be tightly controlled. Unregulated oxylipid biosynthesis can contribute significantly to inflammatory-based disease pathologies [3]. Furthermore, some hydroxyl metabolites, 15-HETE, 12-HETE, 9-HODE and 13-HODE, were shown to be pro- or anti-inflammatory in atherosclerosis, arthritis and cancer models [7–9]. Therefore, it will be necessary to understand how the synthesis of AA- and LA-derived

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oxylipids and their inflammatory properties are regulated in different disease models.

Selenium (Se) is an essential nutrient in the mammalian diet that has anti-inflammatory properties in cancer, cardiovascular disease, mastitis and other inflammatory disease models [2,10]. Se can affect oxylipid biosynthesis in several ways. In murine macrophages, supplementation with Se compounds decreased prostaglandin E_2 (PGE_2) production by diminishing protein expression of COX2 through regulation of NFκB signaling [11,12]. In bovine endothelial cells, Se deficiency increased the ratio of AA-derived hydroperoxide to hydroxyl, 15-HPETE:15-HETE, and 15-HPETE exhibited pro-inflammatory effects by inhibiting the synthesis of prostacyclin (PGI_2) [13]. Overexpression of 15-LOX in Se-deficient endothelial cells resulted in increased production of 15-HPETE and expression of the intercellular adhesion molecule, ICAM-1 [14]. In rat aortas, Se deficiency significantly decreased production of the LA hydroxyl, 9-HODE and a downstream product of PGI_2, 6-keto $PGF_{2\alpha}$, which can have implications on endothelial cell function [15]. In contrast, increased LA-derived hydroxyl 13-HODE resulted from both Se deficiency and free radical insult in Jurkat T-cells [16]. Whereas much of the previous research characterizes oxylipid biosynthesis as a function of dietary Se, much less is known on how other Se metabolites, such as selenoproteins, affect the oxylipid signaling networks.

Se is incorporated into selenoproteins via the Se-containing amino acid selenocysteine (Sec), that is, biosynthesized on its tRNA, Sec $tRNA^{[Ser]Sec}$, which in turn reads a "UGA" codon ensuring proper Sec insertion into protein [17]. The antioxidant functioning glutathione peroxidases (GPx) and thioredoxin reductases (TrxR) are the most well characterized selenoproteins and are expressed within macrophages [18]. In cancer models, manipulation of GPx2 or GPx4 resulted in altered COX2 expression and PGE_2 production [19,20]. In macrophages specifically, research regarding Se's effect on oxylipid biosynthesis focuses largely on specific AA-derived oxylipids such as prostaglandins [21–23]. However, LA-derived oxylipids are also essential in promoting and diminishing inflammation associated with disease [24]. Furthermore, antioxidant functioning GPx and TrxR can directly regulate both AA- and LA-derived oxylipid production, such as HPETEs and HPODEs, because these FAHP can be synthesized by free radicals during conditions of oxidant stress. Since the oxylipid signaling network is complex, there is a need to characterize the effect of selenoprotein activity on macrophage-derived, biologically active oxylipids that affect inflammation in order to uncover specific mechanisms behind Se's potential anti-inflammatory properties. Therefore, the hypothesis of this study was that decreased macrophage selenoprotein activity reduces the biosynthesis of oxylipids with anti-inflammatory properties.

2. Materials and methods

2.1. Mice and macrophage samples

In vivo selenoprotein status was manipulated in a murine model using a conditional knockout of the selenocysteine tRNA gene (*Trsp*) driven by the *Cre*-recombinase system. C57BL/6 mice carrying a floxed *Trsp* gene were generated as described previously and served as control mice [25]. Briefly, control mice were mated with a transgenic C57BL/6 line carrying the *Lysozyme-M-Cre* transgene from the Jackson Laboratory to generate *Trsp* knockout mice ($\Delta Trsp^M$). This knockout system was driven by the lysozyme M promoter that restricted *Trsp* knockout to myeloid-derived cells including macrophages. All animals were maintained according to protocols approved by Institutional Animal Care and Use Committees and in accordance with the National Institutes of Health institutional guidelines. Peritoneal fluid and peritoneal exudate macrophages (PEM) were isolated and prepared as described previously [25]. Briefly, mice received an intraperitoneal injection of 2 ml of a 4% thioglycolate solution. After 4 days, mice were euthanized then given an intraperitoneal injection of 10 ml ice-cold phosphate-buffered saline (PBS). The peritoneal fluid was extracted from the mouse then centrifuged at 1000 rpm for 10 min to isolate cells. The supernatant fluid was collected as peritoneal fluid, while the cell pellet was washed in PBS and collected for isolation of RNA as described below.

Detection of decreased Sec $tRNA^{[Ser]Sec}$ gene expression was measured *in vivo* using Northern blot to compare levels of Sec tRNA to control Ser tRNA in wild-type and selenoprotein knockout $\Delta Trsp^M$ murine macrophages. Protein expression was quantified by Western blot. Macrophage-derived proteins were labeled with 25 μCi/ml of ^{75}Se for 24 h, electrophoresed on gels, stained with Coomassie Brilliant Blue and exposed to a Phosphor Imager as described [25]. Free radical production was also observed from *ex vivo* PEM by flow cytometry using carboxy-H_2DCFDA (Life Technologies, Grand Island, NY) as a fluorescent indicator of ROS.

2.2. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated from *in vivo* murine PEM using Trizol (Invitrogen, Carlsbad, CA) and 1 μg of total RNA was reverse transcribed using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), according to the manufacturer's instructions. All primers used in the present study were derived from the *Mus musculus* genome (GenBank). Each sample was amplified using TaqMan PreAmp Kit (Applied Biosystems). qPCR was carried out in a 7500 Fast Real-Time Polymerase Chain Reaction (PCR) system (Applied Biosystems) using pre-designed TaqMan minor groove binding probes from Applied Biosystems. The PCR was performed in triplicate using a 20-μl reaction mixture per well, containing 10 μl of TaqMan Gene Expression PCR Master Mix (2×, Applied Biosystems), 1 μl of (20×) TaqMan Gene Expression Assay Mix (Applied Biosystems) and 5 μl of amplified cDNA, and the balance was nuclease-free water. Targeted genes were amplified with the reaction mixture described above. Pre-designed (20×) TaqMan Gene Expression Assays for murine β-glucuronidase, glyceraldehyde 3-phosphate dehydrogenase and β_2 microglobulin from Applied Biosystems were used as reference genes. Each PCR plate included a non-template control to ensure no contamination was present. A non-reverse transcribed control was run to ensure genomic DNA was not being amplified. The following thermal cycling conditions for 2-step PCR were used: stage 1 enzyme activation, 50 °C for 2 min; stage 2, 95 °C for 10 min; stage 3, 95 °C for 15 s; stage 4, 60 °C for 1 min with 40 replications through stages 3 and 4. Quantification was carried out with the relative quantification method [26]. The abundance of target genes, normalized to the average of the 3 reference genes and relative to a calibrator, are calculated by $2^{-\Delta\Delta Ct}$, where C_t is the cycle number at which the fluorescence signal of the product crosses an arbitrary threshold set with exponential phase of the PCR and $\Delta\Delta Ct = (Ct_{\text{target gene unknown sample}} - Ct_{\text{average of 3 endogenous control genes unknown sample}}) - (Ct_{\text{target gene calibrator sample}} - Ct_{\text{average of 3 endogenous control genes calibrator sample}})$. Averaged abundance of target genes in control PEM was used as the calibrator sample for all subsequent samples.

2.3. Solid-phase lipid extractions and liquid chromatography/mass spectrometry (LC/MS)

Macrophage cell pellets, peritoneal fluid and RAW 264.7 macrophage media supernatant samples were collected as follows. Cell pellets were first suspended in 600 μl 1× PBS and sonicated. All samples were collected in formic acid 0.1% solution by volume (1 μl/ml), an antioxidant/reducing agent that reduced hydroperoxides to their corresponding hydroxyls containing ethylenediaminetetraacetic acid, butylhydroxy toluene, triphenylphosphine, indomethacin (4 μl/ml) and a mixture of internal standards. Samples contain 200 μl of the following deuterated oxylipids (0.1 ng/μl, 20 ng total): $LTB_{4\text{-}d4}$, $TxB_{2\text{-}d4}$, $PGF_{2\alpha\text{-}d4}$, $PGE_{2\text{-}d4}$, $PGD_{2\text{-}d4}$, 13(*S*)-HODE$_{\text{-}d4}$, 6-keto $PGF_{1\alpha\text{-}d4}$, 13-oxoODE$_{\text{-}d3}$, 9-oxoODE$_{\text{-}d3}$, 12(*S*)-HETE$_{\text{-}d8}$, 15(*S*)-HETE$_{\text{-}d8}$ and 8-iso-$PGF_{2\alpha\text{-}d4}$ (Cayman Chemical, Ann Arbor, MI). Samples received 60% total (v/v) of methanol (MeOH) and were kept at −80 °C for 30 min to precipitate protein. Samples were then centrifuged at 4000*g* for 30 min at 4 °C for peritoneal fluid and media supernatants or 14,000*g* for 15 min for cell pellets. Lipids were isolated from the samples by solid-phase extraction using a Phenomenex Strata-X 33u Polymeric Reverse-Phase Column (200 mg/6 ml, 8B-S100-FCH; Phenomenex, Torrance, CA) for cell pellets or an Oasis HLB 12 cc (500 mg) LP Extraction Cartridge (186000116; Waters, Milford, MA) for peritoneal fluid and media supernatant. Columns were first conditioned with 6 ml MeOH then 6 ml water. Samples were diluted to 10% (v/v) MeOH with water then run through the column, washed with 40% MeOH, dried and eluted from the columns in MeOH/acetonitrile (50:50; v/v). Samples were then dried in a Sevant SVD121P SpeedVac (Thermo Scientific, Waltham, MA), suspended in acetonitrile/water/formic acid (37:63:0.02; v/v/v) and centrifuged at 14,000*g* for 30 min prior to analyzing by LC/MS.

Oxylipids were analyzed using two distinct LC/MS methods. Both utilized reverse-phase LC on a Waters ACQUITY UPLC BEH C18 1.7-μm column (2.1×100 mm) at a flow rate of 0.6 ml/min at 35 °C and a quadrupole mass spectrometer (Waters ACQUITY SQD H-Class) in electrospray negative ionization mode. The electrospray voltage was −3 kV and the turbo ion spray source temperature was 450 °C. Nitrogen was used as the drying gas. For each method, 10-μl samples were injected in triplicates. An isocratic mobile phase consisting of acetonitrile:water:0.1% formic acid (35:55:10; v/v/v) with an analysis time of 15 min was used to analyze 8-iso $PGF_{2\alpha}$, LTB_4, PGE_2, PGD_2, lipoxin A_4 (LXA_4), $PGF_{2\alpha}$, TxB_2, 6-keto $PGF_{1\alpha}$, resolvin D_1 and resolvin D_2. The second method utilized an isocratic mobile phase of acetonitrile:methanol:water:0.1% formic acid (47.4:15.8:26.8:10; v/v/v/v) and an analysis time of 10 min to analyze 9(*S*)-HODE, 13(*S*)-HODE, 15-oxoETE, 5-oxoETE, 5(*S*)-HETE, 11(*S*)-HETE, 12(*S*)-HETE, 15(*S*)-HETE, 9-oxoODE, 13-oxoODE, 7(*S*)-Maresin1 (MaR1), protectin D_1 (PD_1) and LTD_4. Oxylipids were identified in samples by matching their deprotonated (i.e., [M-H]-) *m/z* values and LC retention times with those of a pure standard.