# SELENOPROTEINS MODIFY OXYLIPIDS FROM LINOLEIC ACID IN MACROPHAGES

By

Sarah Asheley Peek

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ABSTRACT

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Inflammatory diseases are characterized by uncontrolled inflammation and remain the leading cause of death in humans. Selenium (**Se**) is an essential nutrient in the mammalian diet and its bioactivities are critical for optimum immune function. Se exhibits immune-modulatory effects through antioxidant-functioning selenoproteins that can exert control over oxidative tone of cells and the expression of pro-inflammatory mediators. Oxylipids are among the more potent, redox-regulated inflammatory mediators that orchestrate the degree and duration of inflammation. Whereas previous works show Se-deficiency results in enhanced pro-inflammatory, arachidonic acid-derived oxylipid synthesis by macrophages, there is a need to define how antioxidant selenoprotein activity might control the balance between pro- and anti-inflammatory oxylipid biosynthesis. Therefore the objective of this work was to investigate the role of decreased selenoprotein activity in modulating the production of biologically active oxylipids from macrophages. Reduced selenoprotein activity increased free radicals, enhanced inflammatory cytokine expression, and decreased LA-derived oxylipids from both *in vivo* and *in vitro* macrophages. When these oxylipids were added to *in vitro* macrophages subjected to a pro-oxidant challenge, inflammatory TNFα production was abrogated, suggesting an anti-inflammatory action for these LA-derived oxylipids. Future studies should focus on which antioxidant selenoproteins have an impact on oxylipid biosynthesis and the mechanisms behind their effect in order to help prevent pathologies associated with uncontrolled inflammation.

I dedicate this thesis to my husband Joshua, my parents Linda, Thomas and Peggy, Colleen and John, and the countless family and friends for their continuous love and support of my educational and professional ambitions.

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KEY TO ABBREVIATIONS

AA: Arachidonic acid

COX: Cyclooxygenase

DHA: Docosahexaenoic acid

EPA: Eicosapentaenoic acid

FAHP: Fatty acid hydroperoxide

GSH: Glutathione

GSSG: Glutathione disulfide

GPx: Glutathione peroxidase

$H_2O_2$: Hydrogen peroxide

HETE: Hydroxyeicosatetraenoic acid

HPETE: Hydroperoxyeicosatetraenoic acid

HODE: Hydroxyoctadecadienoic acid

HPODE: Hydroperoxyoctadecadienoic acid

IsoP: Isoprostane

LA: Linoleic acid

LOX: Lipoxygenase

LT: Leukotriene

LXA: Lipoxin

MaR: Maresin

oxoETE: oxo-eicosatetraenoic acid

oxoODE: oxo-octadecadienoic acid

PG: Prostaglandin

PD: Protectin

ROS: Reactive oxygen species

RNS: Reactive nitrogen species

Rv: Resolvin

Se: Selenium

Sec: Selenocysteine

Trx: Thioredoxin

TrxR: Thioredoxin Reductase

Tx: Thromboxane

CHAPTER 1

# Regulation of Inflammation by Selenium and Selenoproteins: Impact on Oxylipid Biosynthesis

S.A. Peek[1], B.A. Carlson[2], L.M. Sordillo[1]

[1]College of Veterinary Medicine,
Michigan State University, East Lansing MI, 48824

[2]Section on the Molecular Biology of Selenium, Laboratory of Cancer Prevention,
National Cancer Institute, NIH, Bethesda, MD 20892, USA

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**Abstract**

Uncontrolled inflammation is a contributing factor to many leading causes of human morbidity and mortality including atherosclerosis, cancer, and diabetes. Selenium (**Se**) is an essential nutrient in the mammalian diet that has some anti-inflammatory properties and, at sufficient amounts in the diet, was shown to be protective in various inflammatory-based disease models. More recently, Se was shown to alter the expression of oxylipids that orchestrate the initiation, magnitude, and resolution of inflammation. Many of the health benefits of Se are thought to be due to antioxidant and redox-regulating properties of certain selenoproteins. This review will discuss the existing evidence that supports the concept that optimal Se intake can mitigate dysfunctional inflammatory responses, in part, through the regulation of oxylipid metabolism. The ability of selenoproteins to alter the biosynthesis of oxylipids by reducing oxidative stress and/or by modifying redox regulated signaling pathways also will be discussed. Based on the current literature, however, it is clear that more research is necessary to uncover the specific beneficial mechanisms behind the anti-inflammatory properties of selenoproteins and other Se-metabolites, especially as related to oxylipid biosynthesis. A better understanding of the mechanisms involved in Se-mediated regulation of host inflammatory responses may lead to the development of dietary intervention strategies that take optimal advantage of its biological potency.

Key Words: Selenium, selenoproteins, oxylipid biosynthesis, inflammation

## Introduction

Uncontrolled inflammatory responses can contribute to the pathogenesis of many health disorders. Dysfunctional or uncontrolled inflammation can be characterized as a chronic low-grade inflammation such as that observed in diabetes, obesity, and atherosclerosis (1, 2). Alternatively, uncontrolled inflammation also may manifest as an exacerbated acute inflammation as observed in diseases such as sepsis and mastitis (3). Oxylipids are a class of lipid mediators that constitute one of the several pathways that regulate the inflammatory response and are biosynthesized by many cell-types including endothelial cells and leukocytes. During uncontrolled inflammation, a combination of the over-production of pro-inflammatory oxylipids and a diminished synthesis of anti-inflammatory oxylipids can contribute to an improper and incomplete resolution process. Current non-steroidal anti-inflammatory drug therapies that target specific enzymes involved in oxylipid biosynthesis have limited efficacy in controlling some inflammatory-based diseases and can cause adverse side effects in both humans and veterinary species (4). Therefore, there is a growing interest to identify alternate therapeutic strategies to regulate uncontrolled inflammation through dietary intervention. The potential of optimizing host inflammatory responses by modifying Se dietary intake was explored in several inflammatory-based disease models such as cancer (5), cardiovascular disease (6), mastitis (7), and osteoporosis (8). Although Se nutritional status was often associated with the magnitude and duration of inflammation, the underlying beneficial mechanisms ascribed to this micronutrient are not fully described. The aim of this review is to assess how the antioxidant and redox-regulating properties of certain selenoproteins can contribute to the beneficial properties of Se-nutrition in controlling inflammatory-based diseases. The ability of selenoproteins to regulate oxylipid biosynthetic pathways in both in whole animal models of disease and in individual cell-

types will be critically evaluated as potential anti-inflammatory mechanisms resulting from optimal Se intake. A greater understanding of the factors that can regulate the delicate balance between the initiation and resolution of inflammatory responses is needed in order to help diminish the morbidity and mortality associated with the pathology of inflammatory-based diseases.

## Selenium: An Essential Micronutrient with Anti-inflammatory Properties

***Selenium and Inflammatory Diseases.*** Se was once considered a toxin when livestock and poultry suffered from alkali disease after consuming grass containing 10-20ppm Se. Subsequent studies confirmed the potential for Se poisoning when laboratory rodents were supplemented with 5-15ppm of dietary Se displayed varying degrees of pathology (9). In contrast, others found that Se-deficiency (diets containing less than 0.1ppm Se) caused diseases such as white muscle disease in cattle and lambs (10) and Keshan disease in humans (11). Based on these earlier studies, Se is now understood to be an essential micronutrient in the mammalian diet and our knowledge of its metabolism (Figure 2) and beneficial functions has grown immensely. Current recommendations indicate the upper tolerable intake of Se is between 90-400 μg/day (recommended daily intake between 30-55 μg/day) for humans (12) and 0.4 mg/kg body weight in rodents (13). In a review and meta-analysis of the literature, Huang et al. (14) found that supplementation with Se (between 500-2000 μg/day for various durations) in critically ill patients decreased mortality rates associated with sepsis. Additionally, women with normal pregnancies exhibited significantly higher blood Se concentrations compared to women with preeclampsia, the leading cause of perinatal and maternal mortality globally (15). In a model of inflammatory bowel disease, rats fed a high Se diet (2 μg/g body weight) for 21d, exhibited decreased colonic tissue necrosis (16). It is important to note, however, that not all

clinical trials involving Se supplementation improved health outcomes in a significant way. Recently published results from The Selenium and Vitamin E Cancer Prevention Trial (SELECT) showed that Se supplementation (200 μg/day), alone or with vitamin E for a period between 7-12 years, did not prevent diseases such as prostate, lung, or colon cancers and there were no significant differences in cardiovascular events or diabetes between treatment groups in men (17). Based on these equivocal findings, it is now clear that more research is required to better understand the underlying mechanisms of Se's beneficial health properties in order to design nutritional intervention strategies that yield more consistent and positive results across a range of human health disorders.

***Selenium Functions as an Antioxidant through the Activity of Selenoproteins.*** Although the importance of Se to health is not fully understood, one well-characterized function of Se is its ability to mitigate oxidative stress through antioxidant functioning selenoproteins (Table 1), including the well-studied glutathione peroxidases (**GPx**) and thioredoxin reductases (**TrxR**) families (18, 19). Oxidative stress occurs when the production of free radicals, including reactive oxygen species (**ROS**), reactive nitrogen species (**RNS**), oxidized proteins, and oxidized lipids, outweighs an organism's antioxidant capabilities resulting in cellular/tissue damage (20). The GPx and TrxR selenoproteins contain a selenocysteine in their active site making them suitable for oxidation/reduction reactions (Figure 3). Whereas GPx1 can reduce ROS in the cytoplasm, GPx4 has the ability to reduce fatty acid hydroperoxides (**FAHP**) and phospholipid hydroperoxides within cellular membranes (Figure 3a) (21, 22). A longer, alternative transcript of GPx4 also was localized to mitochondrial membranes (23) and shown to maintain ATP production during oxidative stress which could have implications on cellular activity and function during disease (24). Thioredoxin (**Trx**) reduces a variety of radicals including lipid

hydroperoxides, protein thiols, and ROS/RNS. Oxidized Trx is then restored to its reduced form by TrxR selenoproteins (Figure 3b). Selenoproteins W, K, and P (**Sepw1, Selk, Sepp1**) also were suggested to have antioxidant capabilities, but mechanisms are less understood (25, 26).

Oxidative stress is a contributing factor in inflammatory disease pathologies including atherosclerosis (27), diabetes (28), and mastitis (29) among others. There is ample evidence to indicate that selenoproteins can interrupt disease pathogenesis through antioxidant-dependent mechanisms. Numerous studies in humans, food-animal species, and rodent models demonstrated a negative correlation between measures of selenoprotein activity and disease severity due to oxidative stress (30-32). Direct evidence of the importance of selenoproteins in mitigating oxidative stress was demonstrated in transgenic studies where overexpression of GPx4 significantly reduced lipid peroxidation in atherosclerosis and ischemia/reperfusion mouse models (33, 34). Several in vitro studies also demonstrated that TrxR1 and selenoprotein P could directly reduce the lipid hydroperoxide, 15-HPETE, to its corresponding hydroxyl (**15-HETE**) (35-37), thus having implications in reducing atherosclerotic lesion formation as a consequence of oxidative stress (38). Collectively, these studies support the contention that optimally functioning antioxidant selenoproteins may be crucial for reducing excess free radicals accumulation and preventing oxidative tissue damage during acute or chronic inflammation.

***Role of Selenoproteins in Cellular Redox Signaling.*** Another way in which selenoproteins may protect against immunopathology associated with uncontrolled inflammatory responses is through redox-regulation of inflammatory signaling. The redox state of cells or tissues can be defined as the ratio of oxidized and reduced forms of specific redox couples (39). Some redox couples relevant to inflammation include NADP+: NADPH, glutathione disulfide (**GSSG**): 2 glutathiones (**GSH**), and oxidized thioredoxin (**Trx(SS)**): reduced thioredoxin

(**Trx(SH)2**). Thioredoxin and glutathione redox couples function with the help of TrxR and GPx selenoproteins, respectively. Into et al. found that GSH was capable of modifying nitrosylated forms of the myeloid differentiation factor 88 (**MyD88**) adaptor protein which enhanced signaling through the toll-like receptor (**TLR4**) pathway during acute inflammation and resulted in altered IL-8 and IL-6 expression (40). Mitogen-activated protein kinase (**MAPK**) signaling also can be affected by redox tone. Apoptosis signal-regulating kinase 1 (**ASK-1**) is a MAPK intermediate that activates downstream pro-inflammatory and pro-apoptotic signaling cascades (41, 42). Mammalian Trx is a direct inhibitor of ASK-1 kinase activity and a negative regulator of ASK-1-dependent gene expression (41). The interaction between ASK-1 and Trx was found to be highly dependent on redox status since oxidation of Trx by ROS results in ASK-1 activation. In contrast, the reduced Trx blocked ASK-1 dependent signaling indicating a protective role of selenoproteins in regulation of apoptosis during oxidative stress (43).

Known as the central regulator of inflammatory gene expression, NFκB similarly can be redox regulated at several levels. Vunta et al. reported an association between increased pro-inflammatory NFκB activation, increased TNFα production and decreased GPx1 activity when macrophages were cultured in Se-deficient media that contained only 6 pmoles/ml of Se when compared to cells cultures with 2 nmoles/ml of Se (44, 45). Decreased plasma Se (0.37 ± 0.05 compared to 0.85 ± 0.09 μmol/L) (46) and decreased selenoprotein synthesis (47) in HIV patients was associated with enhanced oxidative stress-induced activation of NFκB which promoted HIV viral transcription. In the cytoplasm, ROS-mediated activation of NFκB can be facilitated through activation of protein kinase A (**PKAc**) which results in release of NFκB from inhibitor of κB (**IKβ**) (48), and overexpression of Trx caused a decrease in ROS-mediated NFκB activity (49). In the nucleus however, Trx can enhance NFκB DNA-binding by reducing

oxidized cysteine resides on NFκB (50). Hirota et al. (51) showed that reduced Trx is primarily found within the cytoplasm of cells; but upon oxidant stimulation, Trx migrates to the nucleus to enhance NFκB-DNA binding. These few examples demonstrate how selenoproteins can both positively and negatively control cell signaling depending on the inflammatory pathway and/or cellular location. Overall, Se nutrition and selenoprotein activity have the potential to improve inflammatory response outcomes in several ways including combating oxidative stress in cells/tissues and through the redox-regulation of inflammatory signaling pathways that lead to cytokine/chemokine production. However, another potentially important but less studied mechanism underlying the health benefits of Se may involve the biosynthesis of bioactive lipid mediators that include the oxylipids (Figure 1).

**Can Se and Selenoproteins Impact Inflammation Through Oxylipid Biosynthesis?**

***Regulation of Inflammation by Oxylipids.*** Oxylipids are a class of lipid mediators that contribute to the orchestration of inflammatory responses. Oxylipids are synthesized from polyunsaturated fatty acid substrates primarily found in the cellular membrane including the omega-6 arachidonic (**AA**) and linoleic (**LA**) or the omega-3 eicosapentaenoic (**EPA**) and docosahexaenoic (**DHA**) acids (52). These fatty acid substrates are oxidized non-enzymatically by free radicals or through different enzymatic pathways including the cyclooxygenases (**COX**), lipoxygenases (**LOX**), and cytochrome P450 pathways to produce both pro-inflammatory and resolving oxylipids (Figure 4). Non-enzymatic oxidation of AA produces the isoprostane series of prostaglandin-like oxylipids. These lipid mediators have been characterized as biomarkers for oxidative stress (53). As such, they have been quantified in models of inflammatory disease, like atherosclerosis, to identify relationships between disease progression and oxidative damage (54). In addition to the isoprostanes, non-enzymatic oxidation of AA or LA can also produce

hydroperoxide metabolites HPETEs or HPODEs respectively that are enhanced during oxidative stress (55). Two isoforms of COX enzymes are involved in the enzymatic oxidation pathways. Whereas COX1 is constitutively expressed in cells, COX2 expression is inducible during inflammation (56, 57). COXs catalyze the oxidation of omega-6 AA to prostaglandin (**PG**) $PGG_2$ and $PGH_2$ (58). From $PGH_2$, downstream PG synthases produce $PGE_2$, $PGD_2$, $PGI_2$, $PGF_{2\alpha}$, among others. Alternatively, thromboxane **(TX)** synthases convert $PGH_2$ to $TXA_2$, and $TXB_2$. Similar to the COX family, there are several isoforms of LOX involved in the enzymatic oxidation of fatty acids. For example, 5LOX catalyzes the oxidation of omega-6 AA to 5-hydroperoxyeicosatetraenoic acid (**5-HPETE**) which can be further metabolized to produce leukotrienes (**LT**). Both 15LOX-1 (12LOX in mice) and 15LOX-2 (8LOX in mice) oxidize AA to 12/15- hydroperoxyeicosatetraenoic acid (**12/15-HPETE**) (59). More recent studies have led to the discovery of anti-inflammatory lipoxins (**LX**) that are produced from the metabolism of 12/15HPETE intermediates by the 5LOX pathway (60). Likewise, 12/15LOX-1 can oxidize the omega-6 LA into 9-hydroperoxy-10E,12Z-octadecadienoic acid (**9-HPODE**) and 13S-hydroperoxy-9Z,11E-octadecadienoic acid (**13-HPODE**) (61). Hydroperoxides can then be reduced to form hydroxyl intermediates (HETEs and HODEs) and further dehydrogenated to form ketone intermediates (oxoETEs and oxoODEs) (62). Omega-3 fatty acids also can be oxidized by COXs and LOXs to produce oxylipids with more anti-inflammatory or resolving properties (52). EPA is metabolized by 5LOX and modified forms of COX2 to produce E-series resolvins (**Rv**) (63), whereas 12/15LOX converts DHA to the D-series Rvs, protectins (**PD1**), and the macrophage-specific maresin (**MaR1**). During uncontrolled inflammation, a combination of exacerbated production of pro-inflammatory oxylipids and diminished production of anti-

inflammatory oxylipids prevents full resolution and restoration of homeostasis (64). Therefore, the balance between production of pro- and anti-inflammatory oxylipids is one factor that determines the inflammatory phenotype of a cell/surrounding microenvironment (65).

Oxylipid abundance and timing of their production are crucial to successfully initiate and resolve inflammation. Oxylipid biosynthesis is regulated at several levels and both Se and selenoproteins have been studied in the context of: 1) altering oxylipid profiles as a function of manipulating dietary Se, 2) feedback-loops from other oxylipids, 3) chemically reducing lipid hydroperoxides, and 4) modifying expression and activity of COX/LOX enzymes (Table 2 and Figure 5). However, research has just begun to uncover the underlying mechanisms of how Se can influence oxylipid biosynthesis at each level of regulation.

***Selenium and Oxylipid Profiles.*** Previous studies have documented how dietary Se impacts the biosynthesis of oxylipids in several different species. Following 2 years of supplementation, increased Se in the diet of humans (100 μg/day) was correlated with a decreased ratio of urinary 11-dehydro $TXB_2$/2,3 dinor 6-keto $PGF_{1\alpha}$. Increased ratios of $TXB_2$/6-keto $PGF_{1\alpha}$ are an indicative biomarker for thrombosis and atherosclerosis (66). Previous research by Meydani (67) then Haberland et al. (68) confirmed that adequate Se intake (300 μg Se/kg and 0.2 ppm, respectively) in rats can decrease the ratio of $TXB_2/PGF_{1\alpha}$ following short term (2mo) and long term (8 generations) of dietary modulation, respectively. In dairy cattle with mastitis, Se-sufficient diets (0.05 mg Se/kg) were associated with decreased pro-inflammatory $TXB_2$, $PGE_2$, and $LTB_4$ oxylipid production and secretion in milk compared to cows with deficient Se intake after 1yr of dietary interventions (69). Taken together, these

results indicate that dietary Se could potentially diminish pro-inflammatory oxylipid biosynthesis during inflammatory diseases.

Se can also alter feedback loops involved with oxylipid biosynthesis. One example was reported on the positive feedback loop involving the ability of 15-deoxy-$^{\Delta 12,14}PGJ_2$ (**15d-$PGJ_2$**) to perpetuate anti-inflammatory oxylipid production by enhancing the expression of its upstream synthesis enzyme in macrophages. Compared to Se-deficiency (6 pmoles/ml of Se from media FBS compared to cells supplemented with 250 nM), culturing murine macrophages with Se to maximize GPx activity enhanced 15d-$PGJ_2$ production; 15d-$PGJ_2$ is a ligand for PPARγ that once activated, enhanced H-PGDS expression. H-PGDS converts $PGH_2$ to $PGD_2$, which is an upstream metabolite of 15d-$PGJ_2$ (70). Thus, depending on the level of regulation, Se could potentially dampen pro-inflammatory oxylipid biosynthesis and enhance more anti-inflammatory oxylipid production; however more research is needed to determine the specific mechanisms involved at different levels of regulation of oxylipid biosynthesis and which selenoproteins could have an effect.

***Antioxidant-dependent Regulation of Oxylipid Biosynthesis.*** There is evidence that certain selenoproteins are at least partially responsible for the ability of Se to modify oxylipid biosynthesis. A direct cause and effect relationship between GPx4 and LT production in cancer cells was previously explored by Imai et al. (71). At the metabolite level, GPx4 overexpression was shown to reduce FAHPs from the 5LOX pathway (5-HPETE to 5-HETE), thus preventing the production of LTB4 and C4 in the leukemia cell line (71). The proposed mechanism was the antioxidant capabilities of GPx4 and the ability to reduce FAHP to hydroxyl-derivatives. Others

found that GPx4 reduced 15-HPETE to 15-HETE and preincubation endothelial cells with GPx4 could prevent peroxide formation (72). Both TrxR and Sepp1 also were shown to have lipid hydryperoxidase activity for 15-HPETE, thus supporting the contention that these selenoproteins can function as antioxidant enzymes against highly reactive hydroperoxy intermediates formed during oxylipid metabolism (37, 73). Collectively, these studies suggest that selenoproteins have an important role in protecting cells against oxidative damage caused by lipid hydroperoxides found in the oxylipid network.

Individual selenoproteins also can modify oxylipid biosynthesis through controlling the activity of COX/LOX enzymes. Walther et al. described how the Se-containing compound ebselen inhibited 15LOX activity by altering the oxidation status of the active-site iron molecule (74). The activation of COX enzymes also requires oxidation of their active site heme iron to form a tyrosyl-radical that is then capable of oxidizing AA and other fatty acid substrates (75). GPx1 can inhibit COX enzyme activity by chemically reducing hydroperoxides that could otherwise activate enzymatic oxidation (76). An abundance of oxylipid metabolites and other radicals, however, can also inhibit the activity of oxylipid enzymes through what is known as "suicide inactivation," as described for COX (77), prostaglandin I synthase (78), and TXAS (79). A decrease in COX activity was described in human endothelial cells due to a buildup of peroxides during diminished GPx1 activity (80). These findings suggest that cellular levels of FAHP are critical in COX enzyme activity; both an excess of FAHP or absence of these radicals can result in COX inhibition. This is interesting because GPx-mediated reduction of FAHP could have different effects on COX or LOX activity depending on the accumulation of FAHP. FAHP generated by the 15LOX pathway were shown to be affected by another selenoprotein *in vitro*. Sepp1, a selenoprotein present in plasma, was shown to chemically reduce 15-HPETE into 15-

HETE (37). Additionally, Sepp1 decreased the production of free radicals following stimulation with 15-HPETE *in vitro* (37). This study highlighted the antioxidant properties of the plasma selenoprotein, Sepp1, which could have significant implications in preventing oxidative stress associated with vascular inflammatory diseases, such as atherosclerosis.

***Redox-Regulation of Oxylipid Biosynthesis.*** Another way that Se can affect oxylipid profiles is through the redox-regulation of oxylipid enzyme expression. Pretreating chondrocytes with physiological levels of Se-Met (0.5 μM) for 24h, for example, decreased IL1β-induced gene expression of COX2 and consequent synthesis of $PGE_2$ (8). Hwang et al. showed in mice, that supplementation with 30μg selenate /g body weight for 2wks decreased tumor size and COX2 expression in a model of colon cancer (81). Addition of various supraphysiological doses of Se (250-500 μM) to cultured HT-29 cells dampened ERK signaling following stimulation with a tumor promoting agent, 12-O-tetradecanoylphorbol-13-acetate (TPA), and increased MAPK signaling; both of which decreased COX2 expression (81). In another model, prostate cancer cells (PC3) pretreated with sodium selenite (0.5 μM–5 μM) for 24 or 48hr had significantly decreased NFκB activity, which is another pathway known to control COX2 expression (82). As described earlier, the redox control of these signaling pathways can occur at several signaling intermediates. Collectively, these studies support the concept that Se can decrease COX2 expression, at least in part, through the regulation of various redox-dependent signaling pathways. More research is needed, however, to characterize cause and effect relationships identifying where specific selenoproteins could be regulating COX2 expression through other redox-regulated signaling pathways.

***Se Can Affect Oxylipid Biosynthesis in Cancer Models.*** Inflammatory pathways can play an important role in cancer development through regulation of cell proliferation and migration (83). For example, oxylipids can play an important role in tumorgenesis by regulating apoptosis and proliferation of cancer cells (84, 85) and Se may exert anti-cancerous properties through the manipulation of oxylipid signaling. For example, Ghosh et al. reported that supplementation with various Se doses (0-3 μM) for 72h induced apoptosis of LNCaP human prostate cancer cells but not of normal PrEC prostate cells (86). Additionally, they noted that stimulation of LNCaP with 5LOX-derived oxylipids, 5-HETE and 5-oxoETE, reversed Se's apoptotic effect and enhanced growth of cancerous cells; thus indicating that 5LOX-derived oxylipids may play a role in promoting cancerous cell growth in prostate cancer (86). Other researchers explored the relationship between specific selenoproteins and oxylipid regulation in models of colon cancer. In GPx2-silenced HT-29 colon cancer cells, an increase in COX2 and mPGES-1 enzyme expression with a concomitant increase in $PGE_2$ production was reported (5). The authors proposed that GPx2 disrupted the positive feedback-loop of $PGE_2$-dependent expression of COX2, representing a unique role specific for GPx2 in the colon cancer model (5). This same feedback-loop also was studied in the context of GPx4 and a fibrosarcoma cancer model. In L29 fibrosarcoma tumor cells, overexpression of GPx4 prevented tumor growth, decreased COX2 expression, $PGE_2$ production, and abrogated $PGE_2$-dependent COX2 expression (87). These studies provide examples in cancer models that the redox-regulating properties of certain selenoproteins could decrease pro-inflammatory oxylipid production and reduce inflammatory-dependent tumor progression.

***Se's Effect on Oxylipid Biosynthesis in Cardiovascular Disease Models.*** Atherosclerosis is another inflammatory-based disease that remains the leading cause of death in the developed world (1). As such, an interest is growing in understanding how Se may be beneficial in cardiovascular disease models. Oxidative stress plays a significant role in the etiology of cardiovascular lesion development by promoting the production of oxidized lipoproteins (**oxLDL**) and lipids such as the non-enzymatically oxidized oxylipids, prostaglandin-like F2 isoprostanes (**F2-IsoP**) (54). These radicals (oxLDL in particular) are recognized and internalized by circulating monocytes which initiates foam cell development and macrophage infiltration into blood vessels (88). The lipid hydroperoxide scavenging GPx4 was overexpressed in a mouse model of atherosclerosis (ApoE-/- mice) which resulted in decreased overall atherosclerotic lesion development (33). The mechanisms behind the protective effect of GPx4 in this study were thought to be enhanced through GPx4's antioxidant capabilities to decrease the accumulation of hydroperoxide radicals and diminish oxidative stress. In support of this theory, both F2-IsoP production and accumulation of intercellular and secreted hydroperoxides were significantly decreased in GPx4 overexpressing mouse aortic endothelial cells compared to atherosclerotic cells (33). When mitochondrial GPx4 was overexpressed in a mouse ischemia/reperfusion model, researchers documented significantly increased cardiac function and decreased lipid peroxidation (34). In another atherosclerosis model, ApoE-/- and GPx1 double knockout mice exhibited significantly increased atherosclerotic lesion development suggesting that GPx1 may also play a role in disease progression (89). Taken together, these data suggest that of GPxs could be a potential therapeutic target during heart disease due to their antioxidant properties and their capability to reduce lipid hydroperoxides and other radicals to less reactive lipid alcohols.

In addition to the antioxidant properties of selenoproteins, other possible mechanisms to explain Se's protective effects in an atherosclerosis disease model were examined. For example, Paniker et al. explored the impact of fatty acid substrate availability and downstream oxylipid enzymatic expression (90). In their study, sodium selenite (8 μg/100 g body weight) supplementation for 30d in isoproterenol-induced myocardial infarction in rats decreased LOX activity, leukotriene A4 hydrolase (**LTA4H**) expression, and $LTB_4$ production in monocytes (90). Se-supplementation also decreased the amount of non-esterified fatty acids (**NEFA**) in the heart which can serve as substrates for LOX enzymatic pathways. The expression of LTA4H was diminished and resulted in decreased $LTB_4$ concentrations. By diminishing the expression of LTA4H, the intermediate lipid metabolite $LTA_4$, is prevented from being metabolized to the more pro-inflammatory oxylipid, $LTB_4$, and preserved for the biosynthesis of resolving oxylipids, such as $LXA_4$. Although the mechanism behind the decrease in LTA4H in Se-treated animals was not explored, evidence suggests that specific enzymatic pathways are potential target for Se-mediated treatment of uncontrolled inflammation. The current findings support the concept that antioxidant selenoproteins could play a role in controlling both non-enzymatic and COX/LOX-mediated oxidation of lipid mediators during cardiovascular disease. Further research is needed, however, to determine which antioxidant selenoproteins are most critical for regulating oxylipid biosynthesis and lipid peroxide-mediated disease progression.

***Se's Impact on Oxylipids in Specific Cell-types: Endothelial Cells.*** Since many different cell-types function in concert during inflammation, studies have focused on characterizing the effects of Se on single cell cultures to determine their role in inflammatory disease. Endothelial

cells are an important component of the immune system. They are the barrier between the blood and tissue, regulate immune cell trafficking, and have been the focus of a number of studies on Se-nutrition and oxylipid biosynthesis. Confirmation that selenoprotein expression within endothelial cells are essential to survival was demonstrated when targeted knock out of selenoproteins in murine endothelial cells resulted in embryonic death due to hemorrhaging and erythrocyte immaturity (91). The ability of Se to reduce lipid radicals accumulation in endothelial cells was explored in early studies by Cao et al. (92). Se-deficient bovine aortic endothelial cells (**BAEC**) cultured in the presence of only 0.01ppm Se were characterized by a significant decrease in GPx1 activity with a concomitant increases in 15-HPETE and $TXB_2$ compared to cells supplemented with 10 ng/ml sodium selenite (92). The same group then explored the association between diminished Se-status of endothelial cells and the ability of 15-HPETE to elicit signs of oxidative stress (36), enhanced adhesion molecule expression (93), higher rates of apoptosis (94), and dampened expression $PGI_2$ (95). Collectively, these studies support the concept that the antioxidant ability of selenoproteins are necessary to mitigate the pro-inflammatory effects of 15-HPETE and reduce endothelial cell death as a consequence of oxidative stress. Evidence also supports a direct effect of TrxR in controlling oxidative stress and inflammation in vascular endothelial cells. Trigona et al. examined the role that TrxR activity may have on the differential regulation of the antioxidant enzyme heme-oxygenase (HO-1) in 15-HPETE challenged endothelial cells (36). Silencing TrxR expression and activity prevented the compensatory increase in HO-1 when endothelial cells were stimulated with 15-HPETE. Additional experiments demonstrated that HO-1 induction was dependent on the TrxR redox activity since restoring intracellular levels of reduced Trx was sufficient to increase HO-1 expression when endothelial cells were cultured in Se-deficient media (less than 0.1ppm Se)

(36). This area requires more attention in future research, especially in the context of 15LOX activity and redox-regulation of signaling that controls 15LOX-derived metabolite formation as there are some conflicting reports of the role of this pathway in disease progression. Whereas some researchers have found that enhancing 15LOX enzyme activity leads to resolving oxylipid production (65), others have found enhanced pro-inflammatory effects (96). It will be necessary to identify how selenoproteins, such as TrxR1, affect the balance of pro- and anti-inflammatory oxylipids as a function of 15LOX activity in endothelial cells to better understand their role in inflammatory responses.

***Impact of Se on Oxylipids in Specific Cell-types: Leukocyte Function.*** Lymphocytes are critical responders to inflammatory stimuli. They play a major role in inflammatory-based diseases including cardiovascular disease by producing chemoattractants such as macrophage chemoattractant protein-1 (**MCP-1**) to enhance macrophage infiltration (97). Lymphocytes are also important sources of oxylipids and were studied in the context of Se-nutrition. One group found significant decreases in oxylipid production from lymphocytes obtained from rats fed a Se deficient diet containing only <0.05 mg Se/kg (98). The underlying mechanism behind the decrease in oxylipid biosynthesis was proposed to be that Se-deficient lymphocytes had significantly diminished phospholipase D activation which is responsible for liberating fatty acid substrates from cellular membranes. Future studies should focus on determining how antioxidant selenoproteins can specifically affect the expression and activity of phospholipases, potentially through redox regulation, and how this may affect the oxylipids produced during inflammation.

Macrophages are especially crucial in pathogen recognition and orchestration of inflammation. Since macrophages synthesize copious amount of ROS to aid in pathogen

destruction, they rely on selenoprotein antioxidants to reduce excess radicals that have the potential to cause self-damage (99). Macrophages were acknowledged as a key cell-type in the early development of atherosclerosis because they are responsible for recognizing and ingesting oxLDL (88). Macrophages were the focus of several reports characterizing oxylipid regulation as a function of Se-status. Prabhu et al. were interested in exploring the relationship between Se-nutrition and the pro-inflammatory signaling pathway, NFκB (100). These investigators described an association between enhanced NFκB activity in macrophages cultured in media containing only 6 pmoles/ml of Se when compared to compared to cells supplemented with 2 nmoles/ml of sodium selenite (100). Additional studies proved that a significant increase in COX2 enzyme expression during Se-deficiency was mediated through increased NFκB activity (101). In contrast, Se-supplementation (20-50 μM) was able to decrease NFκB activation and COX2 expression through the TLR4 pathway (102). In microglial cells (macrophages specific to the central nervous system and brain), pretreating cells with Se-containing compounds (0-10 μM) decreased LPS-induced NFκB activation, COX2 expression, and $PGE_2$ production (103). Collectively, these studies suggest that Se, through the activity of antioxidant selenoproteins, could mediate oxylipid biosynthesis by controlling NFκB-dependent COX2 expression. Other signaling pathways also may be involved in regulating COX2 expression and the subsequent metabolism of lipids through this pathway. For example, LPS-stimulated macrophages cultured in Se supplemented media (0.1 μM sodium selenite) lead to a significant decrease in LPS-induced expression of COX2 and TNFα by inhibition of the MAPK signaling pathway (45). Additional experiments demonstrated that mice maintained on a Se-deficient diet had significant increases in LPS-mediated infiltration of lung macrophages when compared to animals maintained on a Se adequate diet (45). One way that Se status was suggested to alter macrophage

inflammatory properties was through changes in the profile of COX-derived oxylipids. Macrophages cultured in Se supplemented media (0.1 μM sodium selenite) demonstrated a time-dependent increase in the production of 15d-$PGJ_2$ which is an endogenous inhibitor of NFκB activation (44). Recently, reports showed that downstream oxylipid synthase enzymes also are affected by selenoproteins (70). Se-supplementation (0.1 μM) enhanced macrophage expression of H-PGDS and the subsequent increase in Δ12-$PGJ_2$ and 15d-$PGJ_2$ production. These effects where mediated by selenoproteins as confirmed by silencing selenoprotein expression through selenophosphate synthatase 2 in macrophages. On the other hand, microsomal- $PGE_2$ synthase (**m-PGES**) and TXAS were decreased during Se-supplementation (70). Together, these studies have begun to demonstrate the association between antioxidant selenoproteins and different levels of oxylipid regulation in macrophages through several different mechanisms including modification of signaling (i.e. NFκB, MAPK) to affect COX/LOX expression, manipulating downstream oxylipid synthase expression, altering the production of specific oxylipids, and disrupting oxylipid feedback-loops. However, more research is warranted to determine which specific selenoproteins are responsible for these effects in order to gain a better understanding of where in oxylipid cascade that Se nutritional intervention may be possible.

**Conclusions**

Uncontrolled inflammation, governed in part by oxylipids, is recognized to play a prominent role in the major life-threatening diseases of the developed world. Although the beneficial anti-inflammatory properties of Se have been appreciated for many years, the underlying mechanisms of action are not fully understood. There is ample evidence to suggest that optimal Se nutrition can combat uncontrolled inflammation, at least in part, because of the

antioxidant and redox-regulating capabilities of selenoproteins. Considerably less is known, however, about the specific selenoproteins that are responsible for these regulatory mechanisms and dynamic changes in their activity that occur during inflammatory processes. More recently, there is a growing body of evidence that further highlights the importance of selenoprotein-dependent regulation of oxylipid biosynthesis in controlling inflammatory responses. Antioxidant selenoproteins can reduce FAHP and lipid radicals directly, affecting oxylipid stability as well as phospholipase and COX/LOX activity. Certain selenoproteins also can regulate cellular redox tone which has implications on cell signaling through NFκB and MAPK pathways, all of which can control expression of COX/LOX enzymes. A major gap in the existing literature, however, is knowledge of how specific selenoproteins can modify oxylipid networks in such a way as to switch from a pro-inflammatory to resolution state and thereby mitigate uncontrolled inflammatory responses that lead to disease pathogenesis. With the advent of new lipidomic analytical techniques (104), it should now be possible to conduct more detailed investigations of how specific selenoproteins, acting individually or in concert with others, can alter the global expression of oxylipid relevant to specific disease models. Genomic-based approaches also will be necessary to evaluate the differential expression of selenoproteins in various tissues and how selenoprotein activity can affect oxylipid biosynthesis in different cells in involved in the inflammatory response. Some of the equivocal findings from existing clinical studies involving Se nutritional status can be attributed to the lack of information that links dietary intakes of Se-rich foods with tissue levels of selenoproteins that are needed to modify specific inflammatory-regulating biological responses. More precise details of how selenoproteins can modify oxylipid metabolism may not only identify relevant therapeutic targets, but also provide accurate biomarkers for assessing optimal Se intake. A better

understanding of the mechanisms involved in Se-mediated regulation of host inflammatory responses will lead to more efficient and consistent nutritional intervention strategies than what has been achieved to date.

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CHAPTER 2

# Reduced Selenoprotein Activity Alters the Production of Oxidized Lipid Metabolites from Arachidonic and Linoleic Acid in Murine Macrophages

S.A. Peek[1], B.A. Carlson[2], J.C. Gandy[1], and L.M. Sordillo[1]

[1]College of Veterinary Medicine,
Michigan State University, East Lansing MI, 48824

[2]Molecular Biology of Selenium Section, Mouse Cancer Genetics Program,
Center for Cancer Research, National Cancer Institute,
National Institutes of Health, Bethesda, MD 20892

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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 LC/MS as well as oxylipid biosynthetic enzyme and inflammatory marker gene expression by qPCR. Decreased selenoprotein activity resulted in the accumulation of reactive oxygen species, enhanced COX and LOX 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.

Key Words: selenium, selenoproteins, macrophage, oxylipids, eicosanoids

## 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 (65). 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 (19); or a chronic, low-grade inflammation, such as that observed during atherosclerosis (64). Therefore, tight regulation and timing of inflammatory events are crucial to effectively eliminate the insult and prevent host damage (65).

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 (107). 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 (52). These hydroperoxides are also formed by non-enzymatic oxidation by free radicals, making them suitable markers of oxidative stress (108). 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**) (108). 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 (64). 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 (109-111). Therefore, it will be necessary to understand how the synthesis of AA and LA-derived 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, mastitis and other inflammatory disease models (19, 30). 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 (101, 103). In bovine endothelial cells, Se-deficiency increased the ratio of AA-derived hydroperoxide: hydroxyl, 15-HPETE:15-HETE, and 15-HPETE exhibited pro-inflammatory effects by inhibiting the synthesis of prostacyclin (**$PGI_2$**) (95). 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 (93). In rat aortas, Se-deficiency significantly decreased production of the LA hydroxyl, 9-HODE and a downstream product of $PGI_2$, 6-keto $PGF_{1\alpha}$, which can have implications on endothelial cell function (112). In contrast, increased LA-derived hydroxyl 13-HODE resulted from both Se-deficiency and free radical insult in Jurkat T-cells (113). 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 (114). The antioxidant functioning glutathione peroxidases (**GPx**) and thioredoxin reductases (**TrxR**) are the most well-characterized selenoproteins and are expressed within macrophages (115). In cancer models, manipulation of GPx2 or GPx4 resulted in altered COX2 expression and $PGE_2$ production (5, 87). In macrophages specifically, research regarding Se's effect on oxylipid biosynthesis focuses largely on specific AA-derived oxylipids such as prostaglandins (44, 70, 116). However, LA-derived oxylipids are also essential in promoting and diminishing inflammation associated with disease (117). 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.

## Materials & Methods

### *Mice & 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 (99). 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 (***ΔTrsp*$^{M}$**). This knockout system was driven by the lysozyme M promoter which restricted *Trsp* knockout to myeloid-derived cells including macrophages. All animals were maintained according to IACUC-approved protocols 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 (99).

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 *ΔTrsp*$^{M}$ murine macrophages. Protein expression was quantified by Western blot. Macrophage-derived proteins were labeled with 25 μCi/ml of $^{75}$Se for 24h, electrophoresed on gels, stained with Coomassie Brilliant Blue, and exposed to a Phosphor Imager as described (99). Free radical production was also observed from *ex vivo* PEM by flow cytometry using carboxy-$H_2$DCFDA (Life Technologies, Grand Island, NY) as a fluorescent indicator of ROS.

***Quantitative Real-Time PCR (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$^{\mathbf{TM}}$ 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). Quantitative real-time PCR (**qPCR**)

was carried out in a 7500 Fast Real-Time 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 (2x, Applied Biosystems), 1 µL of (20x) TaqMan Gene Expression Assay Mix (Applied Biosystems), 5 µL of amplified cDNA, and the balance was nuclease-free water. Targeted genes were amplified with the reaction mixture described above. Pre-designed (20x) Taqman Gene Expression Assays for murine beta-glucuronidase (**GUSB**), glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**), and $\beta_2$ microglobulin (**B2M**) from Applied Biosystems were used as reference genes. Each PCR plate included a non-template control to ensure no contamination was present. A non-RT control was run to ensure genomic DNA was not being amplified. The 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 (118). 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.

***Solid Phase Lipid Extractions & 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 1x 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 (**EDTA**), butylhydroxy toluene (**BHT)**, triphenylphosphine (**TPP**), indomethacin (4 µL/mL), and a mixture of internal standards. Samples contain 200ul 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)-$HETE_{\text{-}d8}$, 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$^{o}$C for 30 min to precipitate protein. Samples were then centrifuged at 4000 xG for 30 min at 4$^{o}$C for peritoneal fluid and media supernatants or 14,000 xG 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/6mL (8B-S100-FCH, Phenomenex, Torrance, CA) for cell pellets or an Oasis HLB 12cc(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 xG 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 1.7µm column (2.1 X 100mm) at a flow rate of 0.6 ml/min at 35 $^{o}$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 $^{o}$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 $A_4$, $PGF_{2\alpha}$, $TxB_2$, 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.

Quantitative Oxylipid Analysis was performed with Waters Empower 2 software. A linear calibration curve with 5 points ($r^2 > .99$) was generated for each oxylipid with standards and internal standards purchased from Cayman Chemical (Ann Arbor, MI). The curves range from 0.002 ng to 2.38 ng/µl. Empower 2 identifies the sample peak by matching its retention time with the standard. A response is calculated for each matched peak by dividing the sample peak's response by its internal standard's response. This response is multiplied by the concentration of the internal standard for each matched peak. The amount for each sample is

calculated using the response peak, injection volume, and DNA concentration of the sample to yield ng of oxylipid/ng of DNA which is expressed in figures as a fold change to control samples. DNA was quantified using Quant-iT DNA Assay Kit, Broad Range according to manufacturer's instructions (Life Technologies).

***RAW 264.7 Macrophage Culture & Oxylipid Stimulation***

Murine-derived RAW 264.7 macrophages were cultured in Se-sufficient (**+Se**) media or Se-deficient (**-Se**) media to model decreased selenoprotein-activity *in vitro*. Enzyme activity assays (GPx and TrxR) and ROS accumulation were calculated to confirm diminished selenoprotein activity and increased ROS from –Se samples as described previously by our group (36, 92). Macrophages were obtained from the ATCC (TIB-71; ATCC, Manassas, VA). Briefly, cells were grown in a T75 culture flask at 37°C with 5% $CO_2$. Once cells reached 75 to 90% confluence, they were split and transferred into a T225 culture flask either with or without Se. Before all experiments, RAW 264.7 were incubated with 50 μM LA complexed with fatty acid free albumin for 24 h as previously described (119) to mimic the lipid content of mouse PEM. The macrophages were cultured in RPMI 1640 medium (17-105-CV; Cellgro, Manassas, VA) containing 5% fetal bovine serum (FBS), antibiotics and antimycotics (100 U/mL), L-glutamine (300 mg/mL), and sodium selenite (0.1 μM, for +Se media).

To determine the inflammatory effect of certain oxylipids that were significantly correlated with TNFα expression, macrophages were stimulated with the LA-derived ketone, 9-oxoODE, or the AA-derived lipid hydroperoxide, 15-HPETE or lipoxin $A_4$ (**$LXA_4$**). RAW 264.7 macrophages were seeded into 100 mm dishes, incubated overnight, and stimulated with a free radical inducer (200 μM SIN-1, Cayman) for 1 h then $LXA_4$ or 9-oxoODE (100 nM for 2 h,

Cayman Chemical), or with 15-HPETE (20 μM for 2 h, synthesized by our group as previously described (93)). Ethanol served as the vehicle did not exceed 0.1% of culture medium. Pro- and anti-inflammatory cytokine production was quantified using a cytometric bead array as described below to characterize the inflammatory response of the macrophages. Cellular viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay according to manufactures instructions (Promega, Madison, WI).

***Cytometric Bead Array***

Mouse inflammation multi-plex bead assay kits were run according to manufacturer's instructions to quantify the production of inflammatory mediators in RAW 264.7 macrophage samples (552364, BD Biosciences, San Jose, CA). Cytokine production was quantified as pg of cytokine per ng of DNA and expressed in figures as fold change to controls. DNA was quantified using Quant-iT DNA Assay Kit, Broad Range (Life Technologies) according to manufacturer's instructions.

***Statistical Analyses***

All statistical analyses were conducted using SAS, version 9.1.2 for Windows (SAS Institute Inc., Cary, NC). Pearson correlation coefficients were computed to determine relationships between oxylipid production and TNFα expression as a function of selenoprotein activity. The effect of selenoprotein-status or other treatments in macrophages on the relative abundance of mRNA, protein expression, cytokine production and oxylipid synthesis were tested by the MIXED procedure of SAS (using mouse as the random effect) or by student's t-test for RAW 264.7 macrophage data. Tukey's protected least significant difference was used to

compare least squares means and data are reported as least squares means ± standard error of the means. Significant differences were declared at $p \leq 0.05$.

## Results

### *Selenoprotein Expression from In Vivo Macrophages.*

Northern blot analysis was used to compare Sec tRNA to control Ser tRNA in wild-type and selenoprotein condition knockout $\Delta Trsp^{M}$ murine macrophages (99). A substantial decrease of Sec tRNA was found in $\Delta Trsp^{M}$ when compared to control mice. To confirm that no selenoproteins were being synthesized, proteins were labeled with $^{75}Se$ and electrophoresed on an SDS gel (Figure 6) (99). When compared to control macrophages, $\Delta Trsp^{M}$ had considerably decreased selenoprotein expression. ROS accumulation was examined previously as a functional consequence of reducing antioxidant selenoprotein activity. Notably increased production of ROS was observed in $\Delta Trsp^{M}$ macrophages compared to controls (99).

### *Selenoprotein Activity Modifies Inflammatory Gene Expression in PEM.*

Gene expression of oxylipid biosynthetic enzymes and inflammatory cytokines from *in vivo* PEM is outlined in Figures 7 and 8, respectively. A significant increase in gene expression of the COX2, 15-LOX1, 15-LOX2, and 5-LOX oxylipid biosynthetic enzymes (Figure 7) was observed in $\Delta Trsp^{M}$ macrophages compared to control PEM. Macrophages from $\Delta Trsp^{M}$ mice exhibited a significant increase in the mRNA expression of the pro-inflammatory cytokine TNFα, and a cadherin that can mediate macrophage migration during inflammation CHD11

(Figure 8). There was no difference in the expression of monocyte chemotactic protein-1 (MCP-1) or IL-1β (Figure 8).

***Oxylipid Production in $\Delta Trsp^{M}$ Mice & RAW 264.7 Macrophages.***

Oxylipids derived from LA or AA were quantified in peritoneal fluid as outlined in Figure 9 and from media supernatant of RAW 264.7 macrophages in Figure 10. There was very little oxylipid accumulation found within the cell, most oxylipids where present in the peritoneal fluid and media supernatant. In the peritoneal fluid, when compared to controls, $\Delta Trsp^{M}$ mice had significantly diminished production of $LXA_4$ ($p \leq 0.05$), as well as the AA-derived hydroxyl 12-HETE ($p=0.06$) (Figure 9a-b). LA-derived oxylipids including: fatty acid hydroxyl 9-HODE ($p=0.13$), and fatty acid ketone 9-oxoODE ($p \leq 0.05$) were also diminished in $\Delta Trsp^{M}$ peritoneal fluid compared to controls (Figure 9c). In RAW 264.7 macrophage media supernatant, oxylipids that were significantly decreased in –Se compared to +Se control cells are outlined in Figure 10 and included: $TxB_2$, $PGF_{2\alpha}$ (Figure 10a), AA-derived hydroxyls, 15-HETE, 11-HETE and 12-HETE (Figure 10b), LA-derived hydroxyls, 13-HODE and 9-HODE, LA-derived ketones 13-oxoODE and 9-oxoODE (Figure 10c). Oxylipids that did not change as a function of selenoprotein activity included: $LTB_4$, $PGE_2$, $PGD_2$, RvD1, RvD2, 15-OxoETE, 5-OxoETE, 5-HETE, MaR1, $PD_1$, and $LTD_4$ (data not shown).

***Oxylipid Treatment Affects TNFα Production in RAW 264.7 Macrophages.***

Pearson correlations between oxylipid production and TNFα expression as a function of selenoprotein activity are outlined in Table 3. Significant correlations were found for several

oxylipids and TNFα from Se supplemented macrophage samples that expressed selenoproteins activity. Oxylipids derived from LA; 13-HODE, 9-HODE, and 9-oxoDE were all negatively correlated with TNFα. Conversely, the AA-derived oxylipid, 15-HETE, was positively correlated with TNFα expression in the Se supplemented macrophage samples.

In order to get a better understanding of how oxylipids might affect the inflammatory response of macrophages during oxidative stress, RAW 264.7 macrophages were cultured either without Se or stimulated with SIN-1 to elicit ROS production (Figure 11). The RAW 264.7 macrophages cultured under these pro-oxidant conditions were then treated with $LXA_4$ or 9-oxoODE to determine if enhanced TNFα production could be reduced to the amounts observed in the Se supplemented cells. Alternatively, the Se supplemented cells were treated with the pro-oxidant oxylipids, 15-HPETE, to determine the effect on TNFα. Compared to +Se macrophages, $LXA_4$ and 9-oxoODE significantly decreased TNFα, whereas 15-HPETE significantly increased TNFα (Figure 12). Cellular viability did not change as a function of any treatment groups (data not shown).

**Discussion**

This study is the first to directly link loss of selenoproteins activity to changes in macrophage oxylipids biosynthesis that can impact inflammatory phenotype. A macrophage-specific selenoprotein knock out model ($\Delta Trsp^{M}$) was used to investigate the direct effects of altered selenoproteins activity on oxylipids biosynthesis. As previously confirmed by Carlson et al. (99), $\Delta Trsp^{M}$ macrophages exhibit significantly decreased Sec tRNA gene expression, selenoprotein expression, and increased accumulation of ROS. This pro-oxidant phenotype is

consistent with other studies that investigated effects of Se deficiency in bovine endothelial cells, rodent lymphocytes and macrophages, and whole animal murine models (44, 92, 99). Using this $\Delta Trsp^{M}$ model, the impact that ablated selenoproteins activity has on macrophage inflammatory markers relevant to acute and chronic inflammation were characterized. Expression of a cadherin (CHD11) in $\Delta Trsp^{M}$ macrophages was increased in this study and is an indicator of inflammatory response. Cadherins are a family of transmembrane proteins that play an important role in cell adhesion and the maintenance of tissue architecture. CHD11 expression is also known to increase in inflamed synovial fluid and selective down regulation of CHD11 significantly reduced joint inflammation in experimental arthritis (120). Earlier reports using bone marrow-derived macrophages (**BMDM**) obtained from $\Delta Trsp^{M}$ mice also found an increased expression of CHD11 (99). Thus, the increased expression of CHD11 by peritoneal macrophages obtained from $\Delta Trsp^{M}$ mice can function as a marker of inflammation during diminished selenoprotein activity. This study also reported a significant increase in the pro-inflammatory mediator TNFα from $\Delta Trsp^{M}$ macrophages. Increased TNFα as a consequence of decreased Se in murine macrophages was formerly established (45). Additionally, TNFα expression was used as a marker of inflammatory diseases such as atherosclerosis (121) and mastitis (122). However, TNFα was not increased in BMDM when selenoprotein expression was decreased, which suggests that macrophages in different stages of maturity and location will have different inflammatory responses during decreased selenoprotein activity (99). The increased expression of both CHD11 and TNFα in the peritoneal exudate obtained from $\Delta Trsp^{M}$

mice, however, suggests that reduced selenoprotein activity results in enhance pro-inflammatory phenotype.

Since enzymatic oxidation of fatty acids by COX and LOX enzymes is a significant source of oxylipids, the gene expression of these enzymes as a function of selenoprotein activity was assessed. This study showed that $\Delta Trsp^{M}$ macrophages had significantly increased COX2, 15-LOX1, and 15-LOX2 gene expression compared to controls. These findings are of significant interest since this is the first direct evidence that selenoproteins are involved in regulating these oxidizing enzymes. Alterations in overall Se status were previously linked with modifications of these enzymatic pathways. For example, enhanced expression of COX2 was well documented in Se-deficient macrophages in several previous studies (45, 100, 101). A suggested mechanism behind increased COX2 expression during Se-deficiency involved the redox-sensitive transcription factor, NFκB, which was explored in RAW 264.7 macrophages by Zamamiri-Davis et al. (101). By mutating NFκB binding sites in the COX2 promoter and blocking NFκB activation with a chemical inhibitor, they found that NFκB facilitates COX2 expression during Se-deficiency. However, less is known about how Se and selenoproteins specifically may affect 15-LOX expression. Previous studies did show that the enzyme activity of purified 15-LOX was decreased with increasing concentrations of the seleno-organic compound ebselen (74). The proposed mechanism involved is that Se could be interacting with the oxidation status of the active iron in 15-LOX; thus reducing its activity. During decreased selenoprotein expression in this study, increased expression of both 15-LOX-1, and 15-LOX-2 was found. Further research would be necessary to determine how selenoproteins such as GPx and TrxR could specifically affect transcriptional and post-transcriptional regulation of expression and activity of COXs,

LOXs, and cytochrome P450 (**CYP450**) enzymes that are involved in oxylipid production in macrophages.

After showing that COX/LOX expression and free radicals were increased as a function of decreased selenoprotein expression, a targeted lipidomic profile of oxylipids implicated in the regulation of inflammation during disease was characterized. Oxylipids derived from LA and AA were specifically included in the profile since both are found in significant quantities in human Western diets and are major components of cellular membranes (123). No difference in the production of several well-characterized pro-inflammatory oxylipids was observed in peritoneal fluid of $\Delta Trsp^{M}$ mice, including $PGE_2$, $PGD_2$, $LTB_4$, and 5-HETE. These results were surprising since prior studies described a decrease in macrophage-derived $PGE_2$ following Se-supplementation (124). A possible explanation for these disparate findings may be due to the analytical method used for oxylipid detection. Whereas earlier reports quantified oxylipids by enzyme immunoassays (124), the current study is among the first to document changes in oxylipid production as a function of selenoprotein activity in murine macrophages using LC/MS which is capable of yielding a higher degree of specificity. More specific analysis of oxylipid biosynthesis is becoming increasingly important due to the low production and unstable nature of many oxylipids. Another possible explanation for reported differences may be due to the method used to induce oxylipids biosynthesis. For example, previous studies documented increased $PGE_2$ production using a TLR4-mediated inflammatory response induced by lipopolysaccharide (**LPS**) activation in RAW 264.7 macrophages, while the present study characterized peritoneal thioglycollate-elicited macrophages and RAW 264.7 macrophages exposed to pro-oxidant challenge (101, 124). In bovine endothelial cells, however, Se-deficiency significantly altered

productions of $PGE_2$, $TxB_2$, and 5-HETE without LPS induction (92). Taken together, these results suggest that differing oxylipid responses depend on cell-type and the specific inflammatory model studied.

This study did report for the first time, however, that $\Delta Trsp^{M}$ selenoprotein knockout mice have a significant decrease in several LA- and AA-derived oxylipids. Both $LXA_4$ and 9oxoODE production was significantly reduced in peritoneal fluid of $\Delta Trsp^{M}$ mice when compared to controls. The appearance of LX is thought to signal the resolution of inflammation and plays an important role in controlling the pathogenesis of inflammatory-based diseases. For example, $LXA_4$ was shown to diminish macrophage pro-inflammatory cytokine production and prevent the development of atherosclerosis (64). Similarly, the LA-derived ketone 9oxoODE was also associated with anti-inflammatory functions. This ketone is formed through the reduction of 9-HPODE to the hydroxyl 9-HODE which is then oxidized through the actions of a dehydrogenase to form 9-oxoODE. The LA-derived hydroxyls (9-HODE and 13-HODE) and their oxidized ketones (9-oxoODE and 13-oxoODE) are natural ligands for PPARγ signaling that can inhibit inflammation by suppressing NFκB activation (125). On the other hand, LA-derived hydroxyls and ketones were found to increase inflammatory pain in the spinal cord by activating pain receptors (126). Whereas only 9oxoODE was decreased in the peritoneal fluid of $\Delta Trsp^{M}$ mice, macrophages cultured in Se deficient media exhibited significant decreases in all of these LA-derived metabolites suggesting that selenoprotein activity maybe a critical part of their metabolic pathway.

Free radical-mediated oxylipid metabolism could prove to be important in macrophages with altered selenoprotein activity since the primary selenoproteins expressed in macrophages include the antioxidant functioning GPx and TrxR (99). In bovine endothelial cells, increased ROS during Se-deficiency resulted in enhanced production of the hydroperoxide, 15-HPETE, whereas Se-sufficient cells had increased production of the reduced hydroxyl, 15-HETE (93). In rat aortas, Se-deficiency resulted in significantly diminished GPx activity and 9-HODE production (112). Our data is consistent with these previous reports in that we also found that increased ROS during diminished selenoprotein activity coincided with decreased 9-HODE and 15-HETE. As described in the extraction procedure to measure oxylipids, the addition of an antioxidant and reducing agent to inhibit autoxidation prevented us from measuring the highly unstable lipid hydroperoxides derived from AA and LA. Therefore, more research would be needed to characterize AA- and LA-derived FAHP during decreased selenoprotein activity. Additionally, it will be important to determine the role each oxylipid plays during inflammation and disease as a function of selenoprotein activity in both different cell-types and disease models.

To obtain a better insight into how oxylipids that are produced as a consequence of selenoprotein expression might affect inflammation, TNFα expression was correlated with the production of oxylipids. Interestingly, several LA-derived oxylipids that were increased in control macrophages, including the hydroxyls 13-HODE and 9-HODE, and the 9-oxoODE ketone, where all negatively correlated with TNFα expression. These results suggest the increased productions of these LA oxylipids during sufficient selenoprotein activity may exert anti-inflammatory effects. Conversely, 15-HETE production was positively correlated with TNFα expression in macrophages that expressed selenoprotein activity. Previously, our group

explored the production and effect of 15-HPETE and 15-HETE in bovine endothelial cells (93). When +Se endothelial cells were stimulated with 15-HPETE, adhesion molecule expression increased to values statistically the same as –Se cells, while 15-HETE had no effect (93). Since 15-HETE was decreased in macrophages that expressed selenoprotein activity in our model, the present results may suggest that the activity of the upstream hydroperoxide, 15-HPETE, may be pro-inflammatory in macrophages. Furthermore, because the oxylipid extraction method used in this study reduced all hydroperoxides to their corresponding hydroxyl forms, more research is needed to specifically quantify lipid hydroperoxides in macrophages as a function of selenoprotein expression.

To further explore the impact of altered oxylipids biosynthesis on macrophage inflammatory phenotype, RAW 264.7 macrophages cultured under pro-oxidant conditions were stimulated with $LXA_4$, 9-oxoODE, or 15-HPETE. After addition of $LXA_4$ or 9-oxoODE, there was a significant decrease in the production of TNFα. Unlike $LXA_4$, which was previously shown to decrease TNFα in macrophages (64), this is the first study to quantify TNFα following stimulation with 9-oxoODE. Previously, oxidation products of linoleic acid, including 9-oxoODE, were found to be significant components of atherosclerotic plaques, although there was no correlation between these oxylipids and symptomatic vs. asymptomatic patients (127). Interestingly, 9-oxoODE also serves as an agonist of PPARγ, which inhibits NFκB signaling, and can bind with greater affinity than the hydroxyl HODE metabolites of LA (125). The current results suggest an anti-inflammatory role for 9-oxoODE in macrophages. Oppositely, when 15-HPETE was added to macrophages, there was a significant increase in the production of TNFα which was previously shown in endothelial cells (93). These results suggest that the balance of

15-HETE and 15-HPETE production is critical in regulating inflammation compared to the addition of the hydroperoxide metabolite alone. Future studies should aim at identifying the significance in the balance of hydroperoxide to hydroxyl production and how this ratio could affect the inflammatory response. Overall, our study demonstrated that selenoproteins play a role in macrophage-derived oxylipid biosynthesis. Further studies are warranted to determine the mechanisms by which selenoproteins regulate inflammation through oxylipid production such as their capacity to: (1) reduce FAHP, (2) mitigate oxidative tone, and (3) regulate COX/LOX enzymatic expression and activity.

**Conclusion**

Since Se was shown to play a beneficial role in inflammation, and the anti-inflammatory properties of Se are thought to occur though selenoproteins, it was important to characterize how selenoprotein activity affects the oxylipid network in the context of the inflammatory response. Adequate Se-intake to maximize selenoprotein activity is associated with decreased production of pro-inflammatory, AA-derived oxylipids in macrophages (8, 101). In the current study, for the first time, oxylipid biosynthesis was characterized in murine macrophages as a function of selenoprotein activity directly, focusing on oxylipids derived from AA and LA that mediate inflammation. Furthermore, this study examined how the inflammatory response was altered as a consequence of specific oxylipids. Overall, selenoprotein-status had a significant effect on hydroxyl and ketone oxylipids metabolized from AA and LA. Additionally, some of these oxylipids have the potential to mediate the inflammatory response of macrophages during pro-oxidant challenge. It is important to study how oxylipids from AA and LA are affected by selenoproteins, because both AA and LA make up a significant portion of the human diet, are predominate in cellular membranes, and have been implicated in inflammatory diseases.

Moreover, free radical-mediated oxidation of AA and LA could play a significant role in controlling inflammation during oxidative stress, which could potentially be mediated by selenoproteins. Future studies are needed to uncover how these oxylipids are produced (i.e. by enzymes and/or by free radical oxidation), which selenoproteins have an effect, and what affect these oxylipids have on macrophage-derived inflammatory responses as a function of selenoprotein activity.

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# APPENDIX

Table 1. Summary of mammalian selenoproteins with characterized functions.

| Selenoprotein | Proposed Function |
|---|---|
| GPx: 1,2,3,4,6* | Antioxidant/Modify Redox Tone |
| TrxR: 1,2,3 | |
| Sepw1, Selk, Sepp1 | Antioxidant |
| SelR | Reduction of Methyl Sulphoxy Groups |
| Sepp1 | Se Transport in Blood |
| Sephs2 | Selenoprotein Synthesis |
| Sep15, Selm, Seln, Sels | Involved in Misfolded Protein Response in the ER |
| SelH | Redox Sensitive DNA-Binding Protein |
| SelI | Phospholipid Synthesis |
| Sepn1 | Calcium Signaling in the ER |
| DIO1,2,3 | Thyroid Hormone Synthesis |
| Sel O, V | Unknown Function |

Adapted from (31, 105, 106)

*GPx6 contains a Sec in humans and a Cys in rodents.

Table 2. The impact of Se and Selenoproteins on Oxylipid Biosynthesis.

| **Se-metabolite** | **Outcome Resulting from Sufficient Levels of Se-metabolite** | **Level of Oxylipid Regulation** |
|---|---|---|
| Selenium | ↓Phospholipase D Activity | Substrate |
| Selenium | ↑H-PDGS | Enzyme Expression |
| | ↓mPGES-1 | |
| | ↑PGIS | |
| | ↓TXAS | |
| | ↓$LTA_4H$ | |
| | ↓COX2 | |
| | ↓15LOX activity (heme oxidation) | Enzyme Activity |
| GPx4 | ↓Isoprostanes | Oxylipid Production |
| Selenium | ↓$TXB_2$:6keto-$PGF_{1\alpha}$ ratio | |
| | ↓$TXB_2$ | |
| | ↓$LTB_4$ | |
| | ↓$PGE_2$, $PGF_{2\alpha}$ | |
| GPx1,4 | Reduces HPETEs to HETEs | |
| GPx1,4 | Reduces HPODEs to HODEs | |

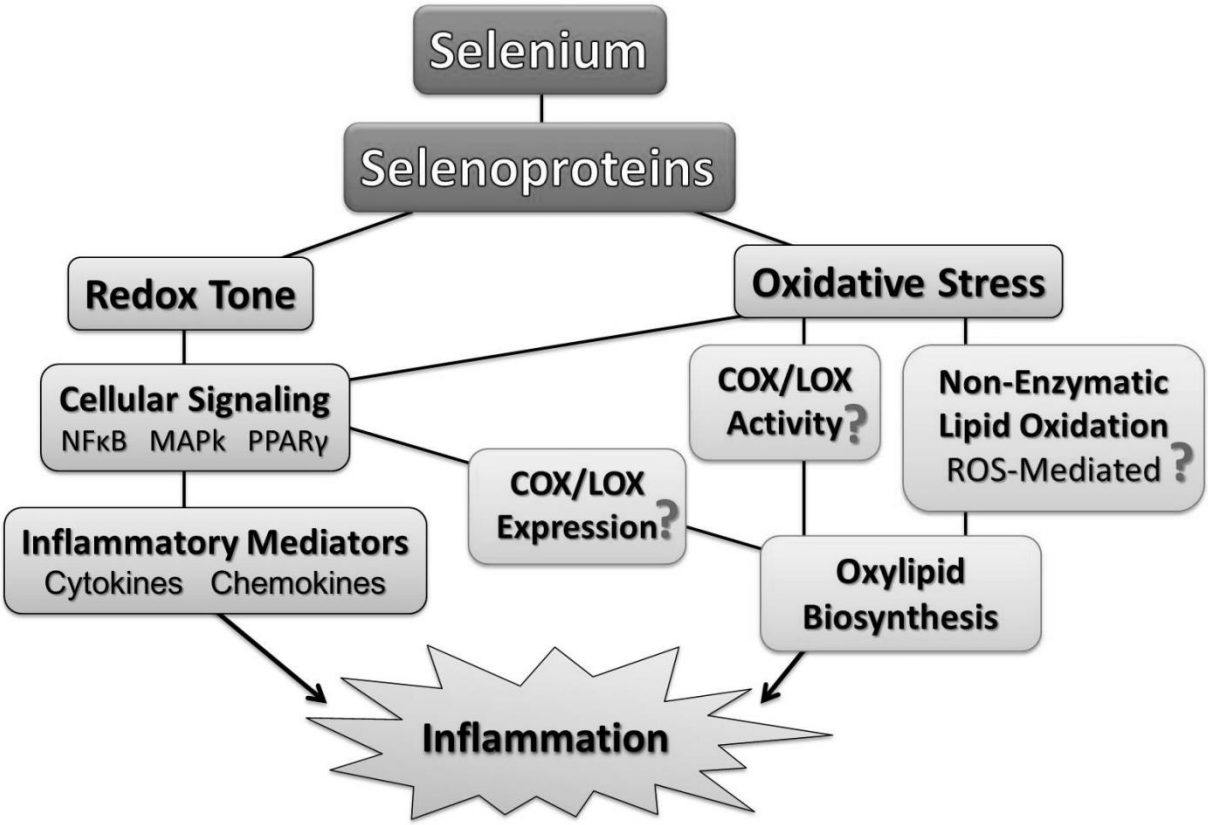



Figure 1. Selenium's Impact on the Regulation of Inflammation.

Some of the several ways in which inflammation is mediated include: 1) signaling through the NFκB, MAP-kinase, and PPARγ pathways, 2) cellular redox tone, 3) the production of inflammatory mediators such as cytokines, and chemokines, 4) oxidative stress, and 5) oxylipid biosynthesis. Selenium was shown to affect each of these regulators and this review will focus specifically on Se's impact on the production of oxylipids.

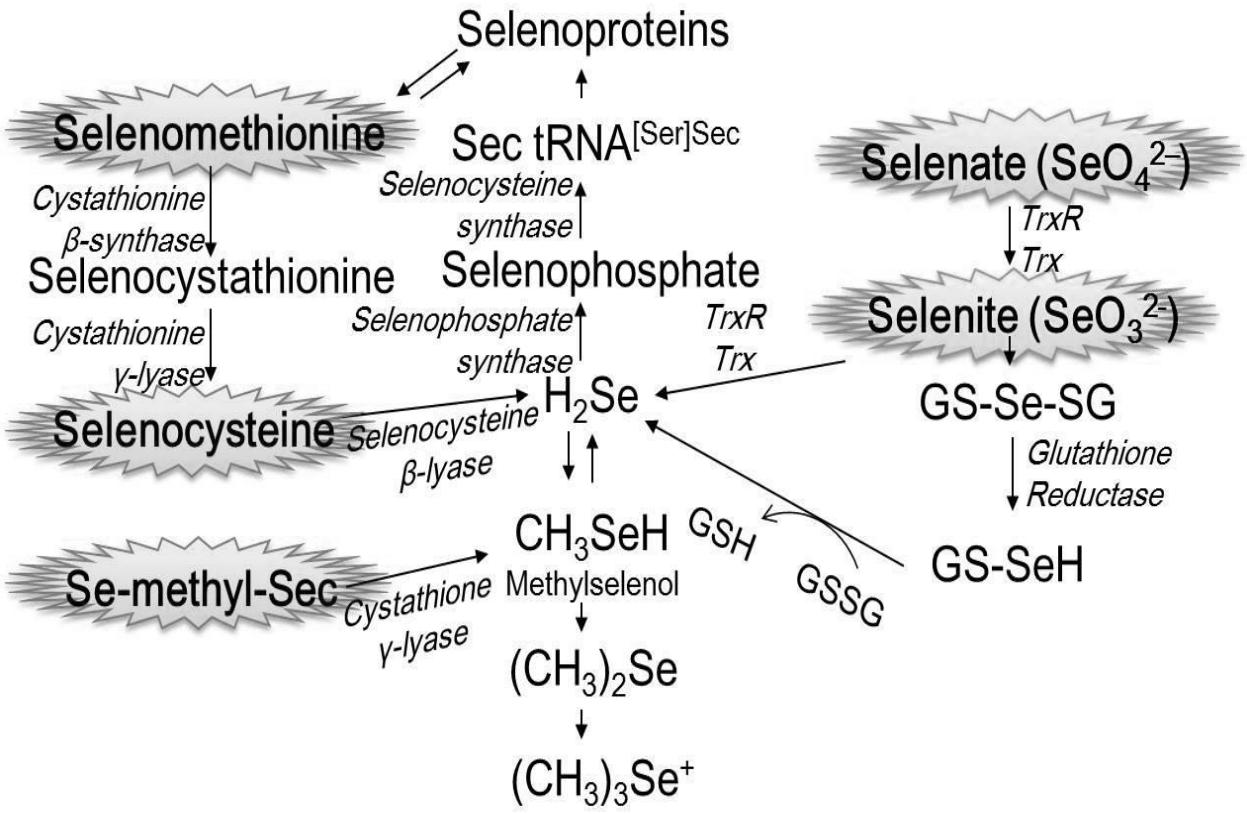
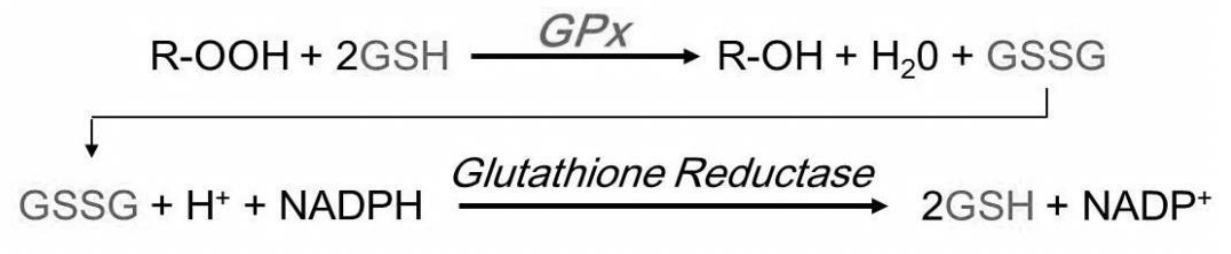



Figure 2. Se metabolism from different dietary sources.

Dietary intake sources of Se include the inorganic selenate and selenite (depicted in the right stars); whereas organic sources (depicted in the left stars) are obtained from animal and plant sources that provide Se in the form of selenocysteine, selenomethionine, and Se-methylselenocysteine (Se-methyl-Sec). Inorganic forms of Se are reduced by TrxR and Trx or converted to selenodiglutathione (GS-Se-SG) by GSSG, reduced by glutathione reductase to glutathioselenol, then converted to hydrogen selenide ($H_2Se$) in a reaction with GSSG.

Selenoproteins are broken down by lyases to form H2Se in intestinal enterocytes. $H_2Se$ can then be converted into selenophosphate by selenophosphate synthase and selenocysteine by selenocysteine synthase for incorporation of Sec into selenoproteins. Hydrogen selenide can also be converted into methylated metabolites by methyltransferases which are primarily excreted through exhalation, urine and feces.

**A)**

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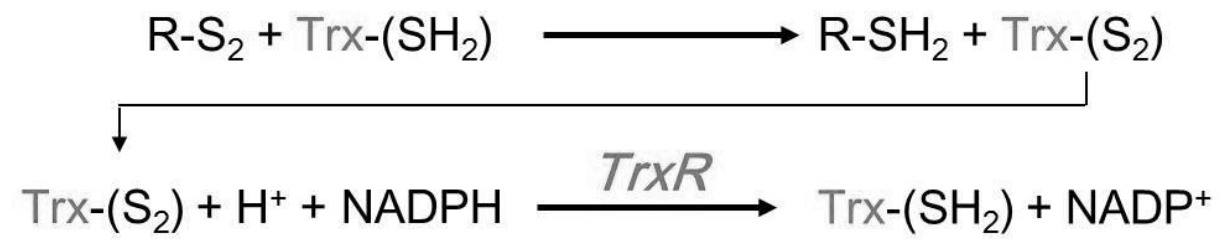



Figure 3. General reaction mechanisms for antioxidant GPxs and TrxRs.

A) GPxs catalyze the chemical reduction of lipid or hydrogen peroxides to respective alcohols and water by glutathione (GSH) which forms glutathione disulfide (GSSG). Glutathione reductase (GSR) catalyzes the reduction of GSSG back to GSH in the presence of NADPH. B) Oxidized protein disulfides and other free radicals are reduced to their corresponding thiols by thioredoxin (Trx). TrxR then catalyzes the reduction of oxidized Trx in the presence of NADPH.

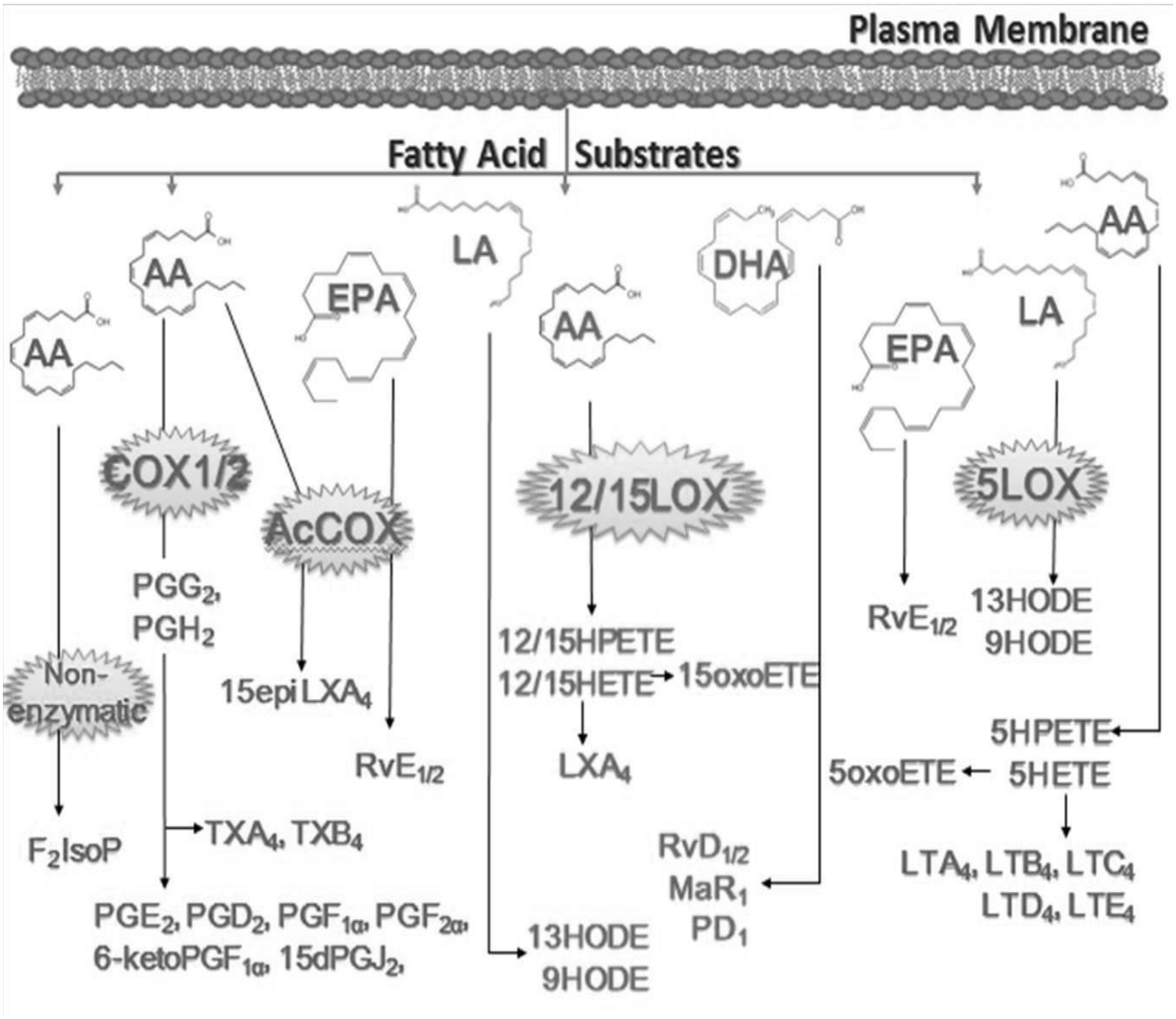
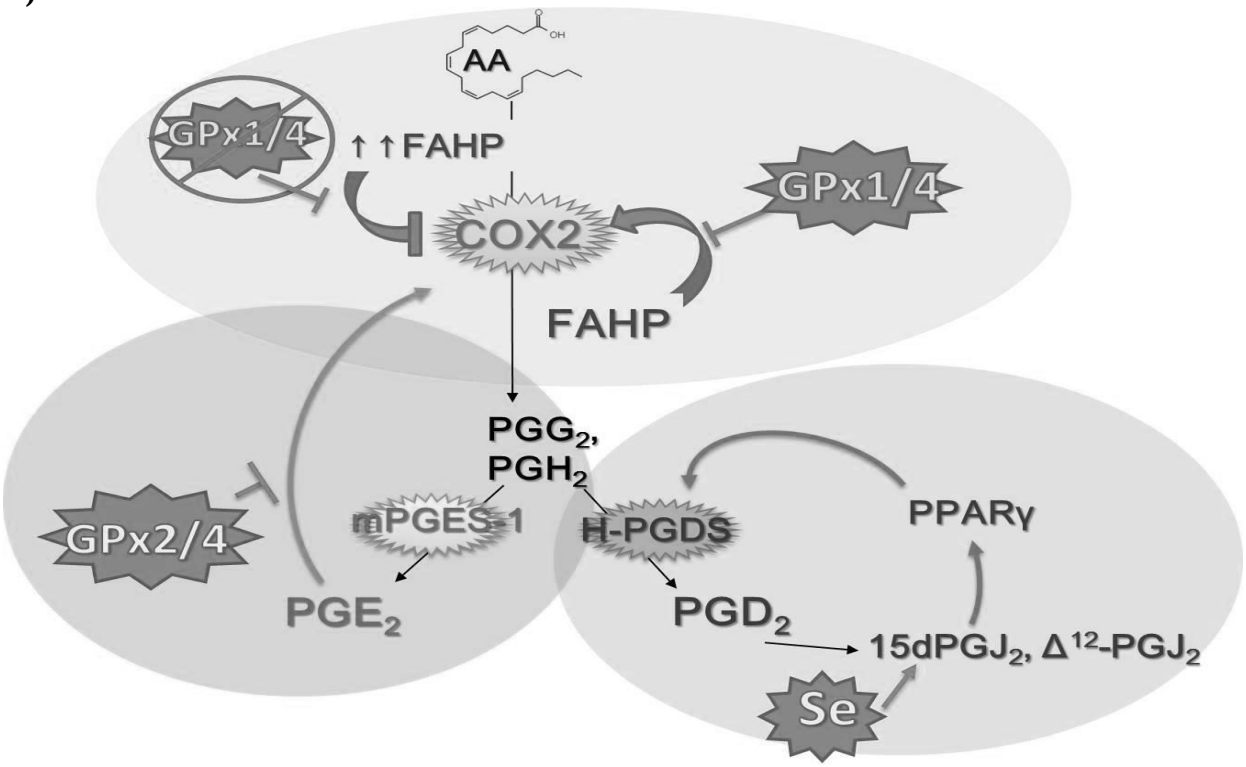



Figure 4. Oxylipid Biosynthesis Pathways.

Omega-3 and omega-6 fatty acids are released from the cellular membrane by phospholipase enzymes. Long-chain, polyunsaturated fatty acids (PUFAs) are oxidized either non-enzymatically by free radicals or by COX1/2, 15LOX, and 5LOX enzymes to produce oxylipid signaling metabolites. Isoprostanes; $F_2$IsoP, prostaglandins; PG, thromboxanes; TX, resolvin E/D series; Rv, lipoxins E/D series; LX, protectin; PD, Maresin; MaR, leukotrienes; LT.

**A)**

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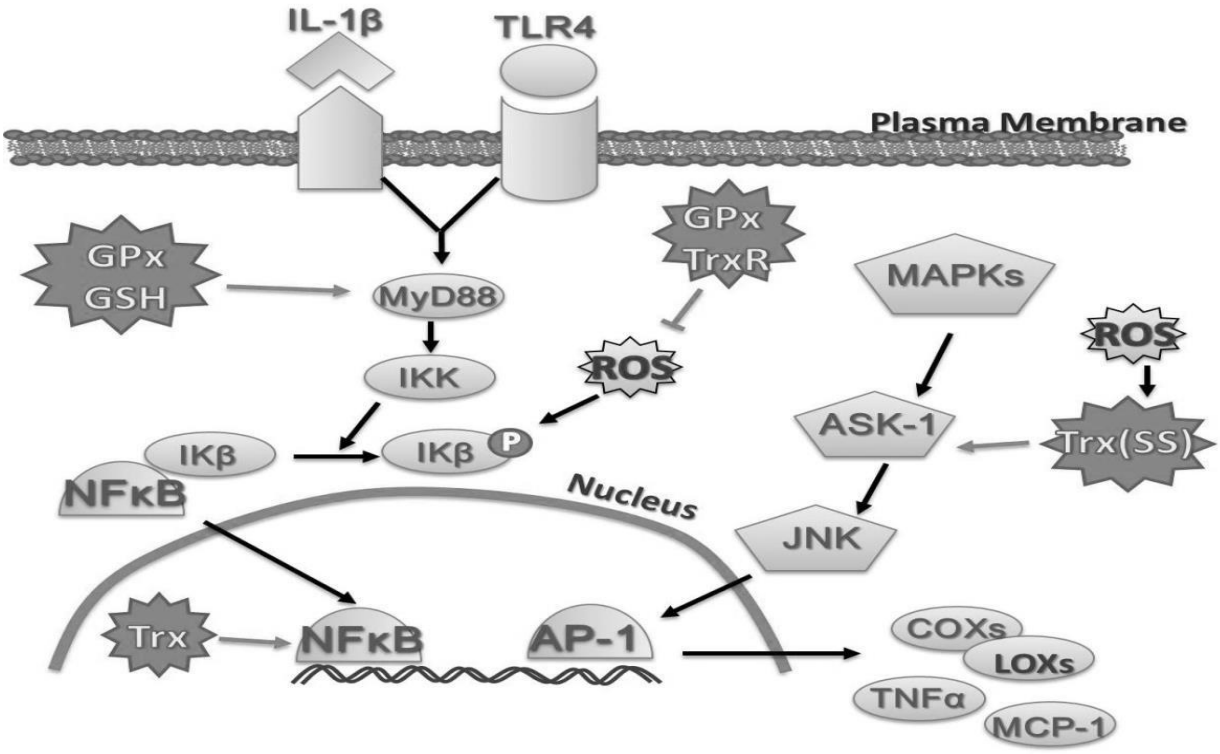


Figure 5. Se's Interaction with Oxylipid Biosynthesis Pathways.

**Figure 5 (cont'd)**
A) Selenium and selenoproteins interfere with oxylipid feedback loops. While GPx1 and 4 can reduce fatty acid hydroperoxides (FAHP) to decrease COX2 activity, a buildup of FAHP, when GPx activity is lacking, can also inhibit COX2. GPx2 and 4 diminish $PGE_2$-dependent expression of COX2. Se enhances 15d-$PGJ_2$ production which is a ligand for PPARγ. PPARγ signaling enhances H-PGDS, which synthesizes PGD2, an upstream metabolite of 15d-$PGJ_2$. B) Antioxidant selenoproteins can affect different signaling pathways leading to activation of NFκB and AP-1 and expression of COX/LOX and other inflammatory mediators such as TNFα and MCP-1. GPxs can alter the redox state of the MyD88 adaptor protein, when MyD88 is denitrosylated by GPx with GSH, signaling is enhanced. ROS-mediated phosphorylation of IKβ can be dampened when antioxidant selenoproteins are present to scavenge ROS. The MAP-kinases can also be affected; ROS-mediated oxidation of Trx causes its dissociation from ASK-1 kinase, enhancing signaling activity. In the nucleus, Trx can reduce oxidized Cys residues on NFκB, enhancing DNA binding and transcription.

Table 3. Pearson Correlations for Oxylipids Produced by Control or $\Delta Trsp^{M}$ Knockout Mice and TNFα, n=5.

| Selenoprotein Activity | Oxylipid Metabolite | | TNFα |
|---|---|---|---|
| Control | $LXA_4$ | *R* | 0.69050 |
| | | *p* | *N.S*[1] |
| | 9-HODE | *R* | -0.9709 |
| | | *p* | ***0.0291*** |
| | 9-oxoODE | *R* | -0.9623 |
| | | *p* | ***0.0377*** |
| | 13-HODE | *R* | -0.988 |
| | | *p* | ***0.012*** |
| | 13-oxoODE | *R* | -0.8361 |
| | | *p* | *N.S*[1] |
| | 15-HETE | *R* | 0.96408 |
| | | *p* | ***0.0359*** |
| $\Delta Trsp^{M}$ | $LXA_4$ | *R* | 0.20771 |
| | | *p* | *N.S*[1] |
| | 9-HODE | *R* | -0.3264 |
| | | *p* | *N.S*[1] |
| | 9-oxoODE | *R* | -0.8985 |
| | | *p* | *N.S*[1] |
| | 13-HODE | *R* | 0.33796 |
| | | *p* | *N.S*[1] |
| | 13-oxoODE | *R* | 0.67470 |
| | | *p* | *N.S*[1] |
| | 15-HETE | *R* | -0.8627 |
| | | *p* | *N.S*[1] |

*N.S.*[1] Not significant, $p>0.05$.

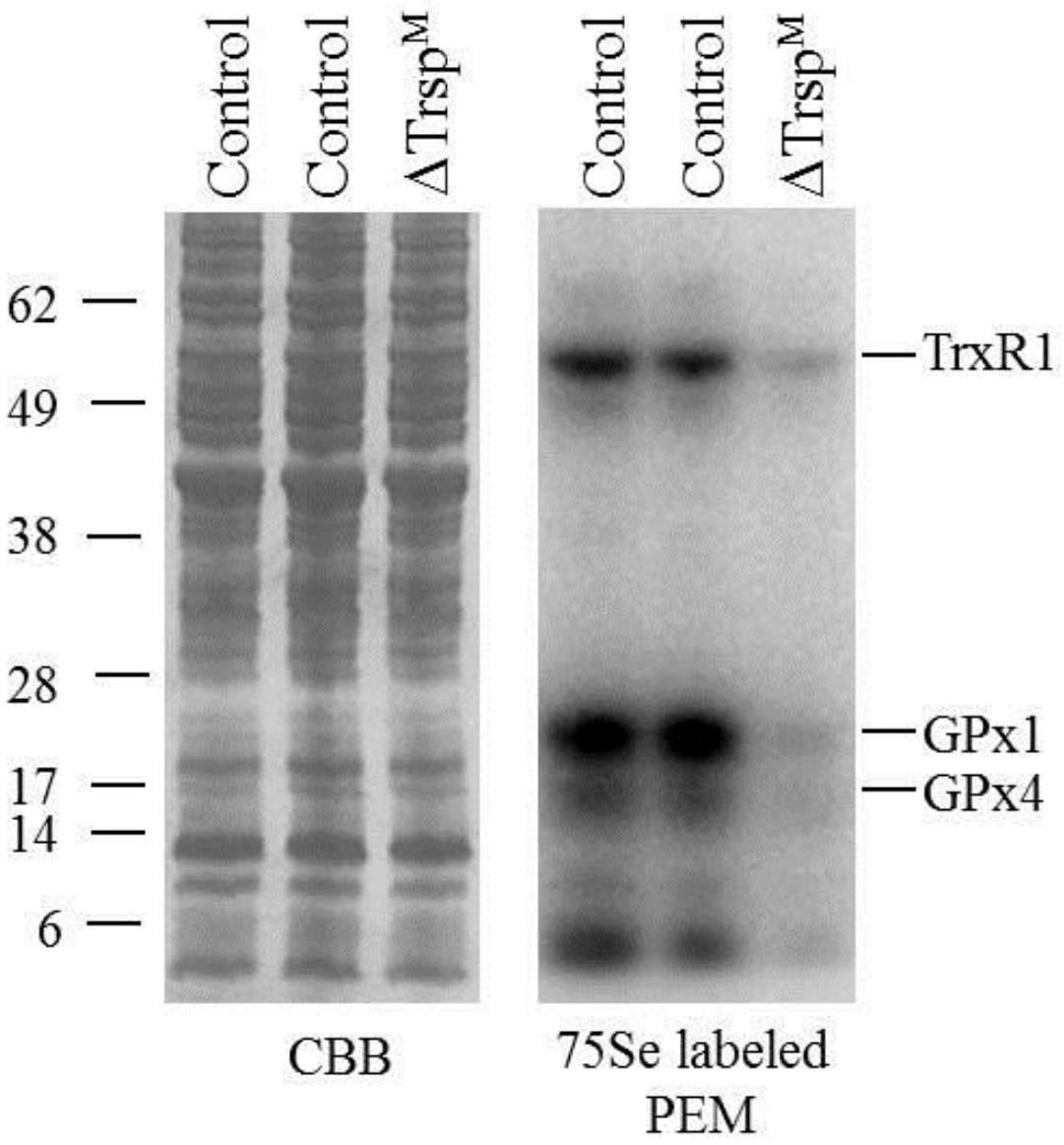


Figure 6. Selenoprotein Knockout in Murine Macrophages.

Peritoneal elicited macrophages (PEM) were labeled with radioactive $^{75}$Se and electrophoresed. The left panel depicts Coomassie Brilliant Blue staining (CBB) which served as the loading control, and the right panel depicts the labeled selenoproteins. Lanes 1 and 2 represent control mice with ample selenoprotein expression while lane 3 represents a *ΔTrsp*$^{M}$ knockout mouse lacking selenoprotein expression in macrophages.

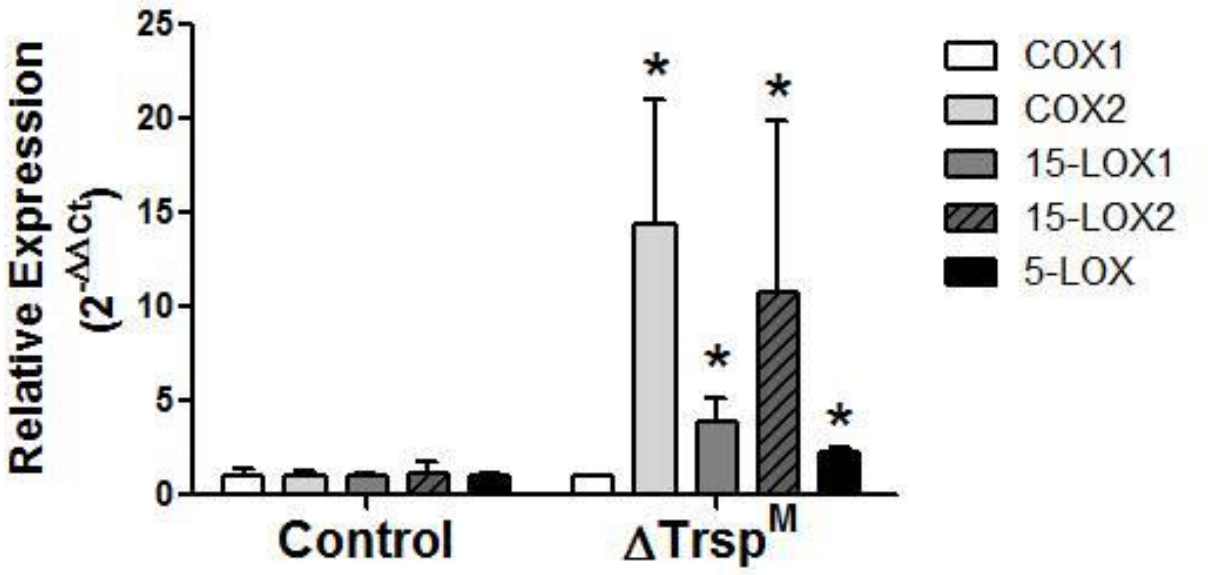


Figure 7. Oxylipid Biosynthetic Enzyme Expression in Macrophages.

*In vivo* PEM were collected from control and $\Delta Trsp^{M}$ knockout mice. Cells were collected for gene expression COX1, COX2, 15-LOX1, 15-LOX2, and 5-LOX. Quantification was carried out with the $2^{-\Delta\Delta Ct}$ relative quantification method [26]. Averaged abundance of target genes for control samples was used as the calibrator sample for all subsequent samples, *Significance $p \leq 0.05$, n=4.

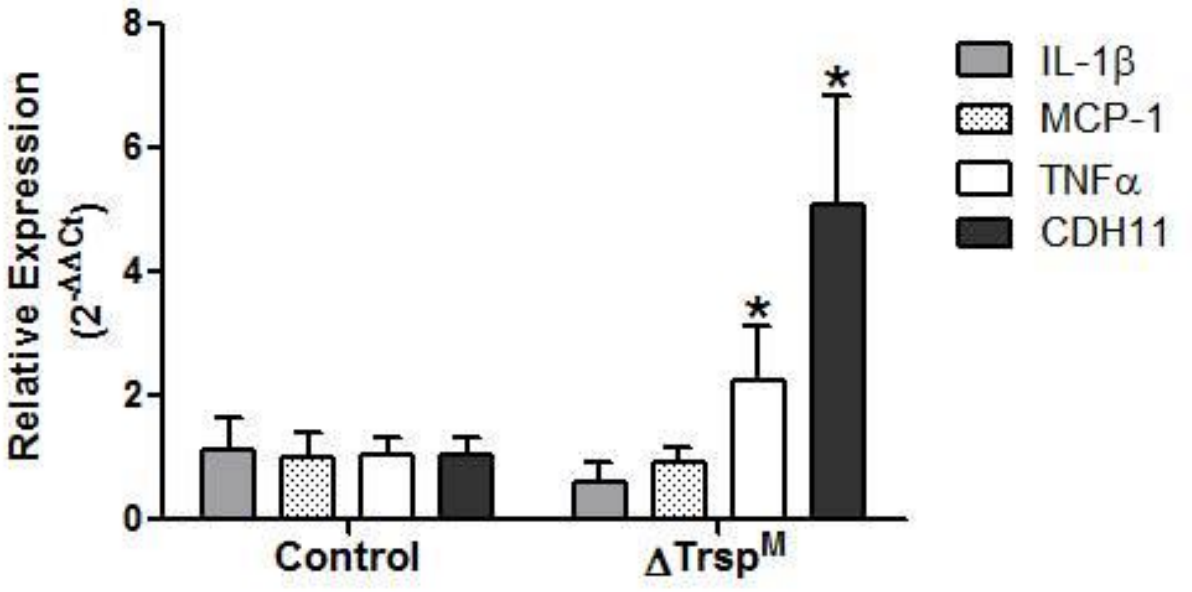


Figure 8. Inflammatory Cytokine Expression by Macrophages.

*In vivo* PEM were collected from control and $\Delta Trsp^{M}$ knockout mice. Cells were collected for gene expression of IL-1β, MCP-1, TNFα, and CDH11. Quantification was carried out with the $2^{-\Delta\Delta Ct}$ relative quantification method [26]. Averaged abundance of target genes for control samples was used as the calibrator sample for all subsequent samples, *Significance $p \leq 0.05$, n=4.

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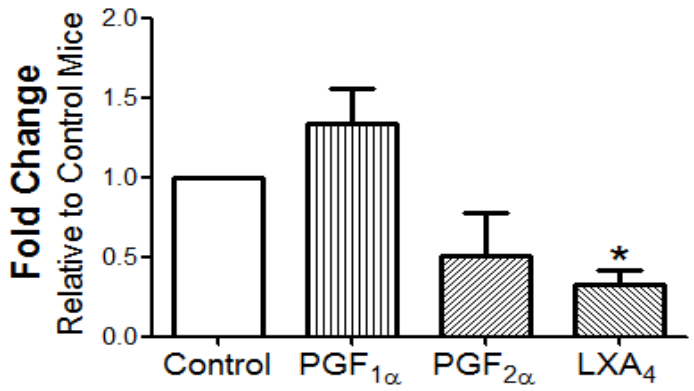


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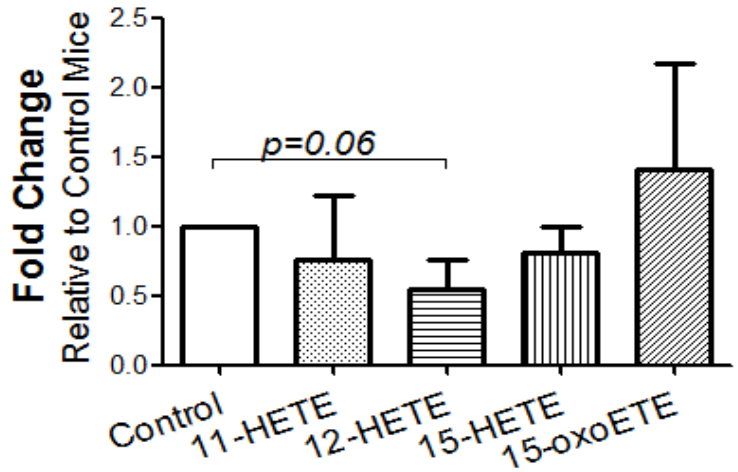


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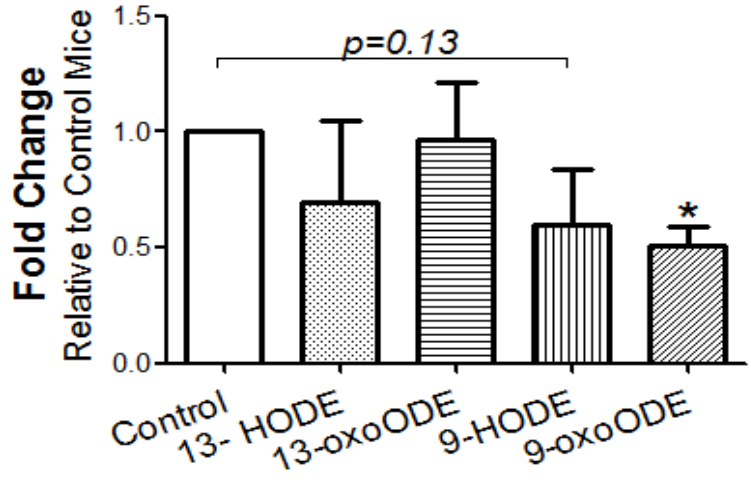
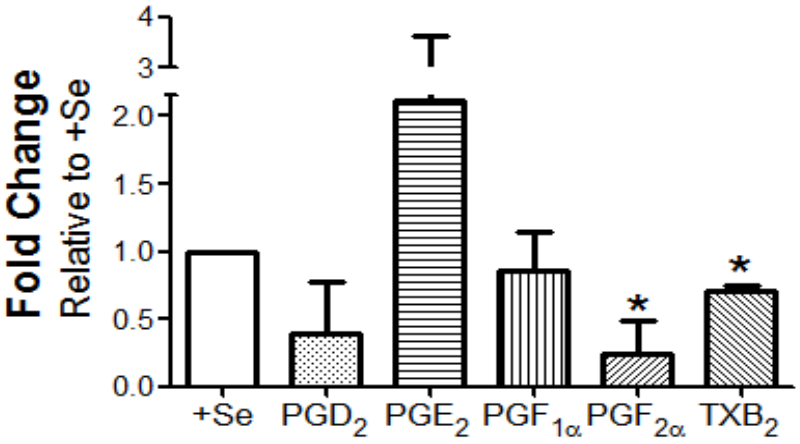



Figure 9. Oxylipid Biosynthesis in the Absence of Selenoproteins.

**Figure 9 (cont'd)**
Oxylipid production is represented from *in vivo* peritoneal fluid from control (white bar) or selenoprotein knockout ($\Delta Trsp^{M}$, filled bars). (A) Production of AA-derived prostaglandins and lipoxin $A_4$. (B) Production of AA-derived 11-HETE, 12-HETE, 15-HETE, and 15-oxoETE. (C) Production of LA-derived 13-HODE, 13-oxoODE, 9-HODE, and 9-oxoODE. Oxylipids are expressed as ng of oxylipid metabolite per mL total peritoneal fluid and depicted as a fold over the control mouse samples. *Significance $p \leq 0.05$ compared to control mice, n=5.

**A)**

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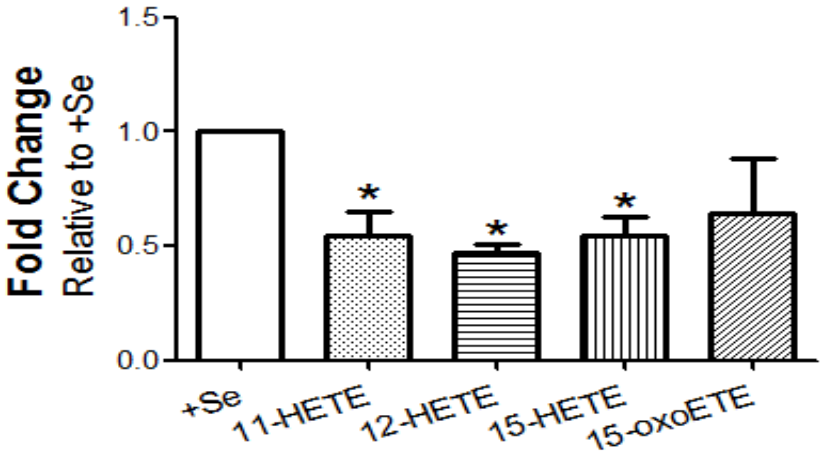


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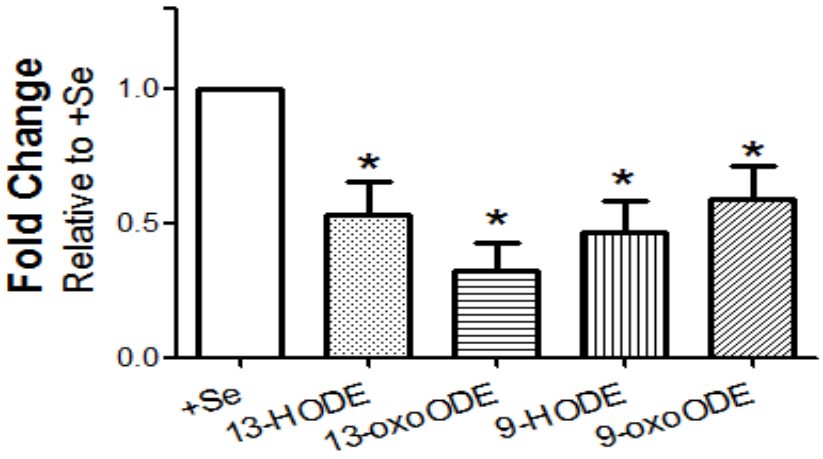



Figure 10. Oxylipid Biosynthesis from RAW 264.7 Macrophages.

**Figure 10 (cont'd)**
Oxylipid production is represented from media supernatants of +Se (white bar) or –Se (filled bars). (A) Production of AA-derived prostaglandins and thromboxane. (B) Production of AA-derived 11-HETE, 12-HETE, 15-HETE, and 15-oxoETE. (C) Production of LA-derived 13-HODE, 13-oxoODE, 9-HODE, and 9-oxoODE. Production is expressed as ng of oxylipid metabolite per ng DNA and depicted as a fold over the +Se samples. *Significance $p \leq 0.05$ compared to +Se controls, n=4.

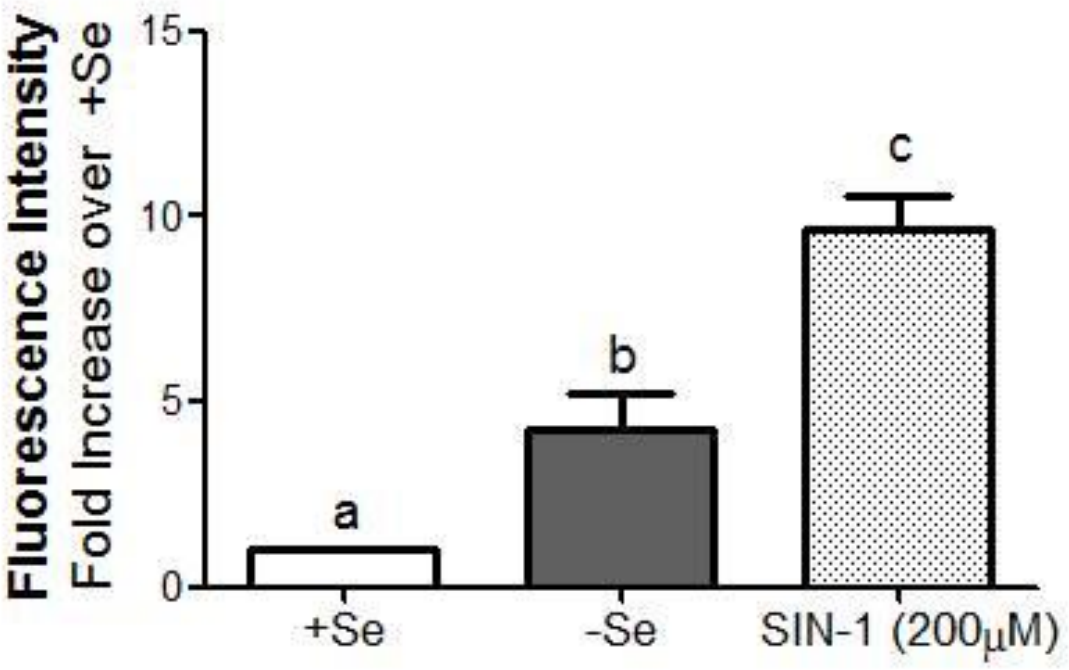



Figure 11. ROS Production Following Pro-oxidant Challenge.

Flow cytometric analysis of ROS production from +Se (white bar), -Se (grey bar), and +Se stimulated with SIN-1 (200 μM, 1 h, spotted bar) RAW 264.7 macrophages quantified using the redox-sensitive fluorescence dye, $H_2$-DCFDA (Life Technologies).

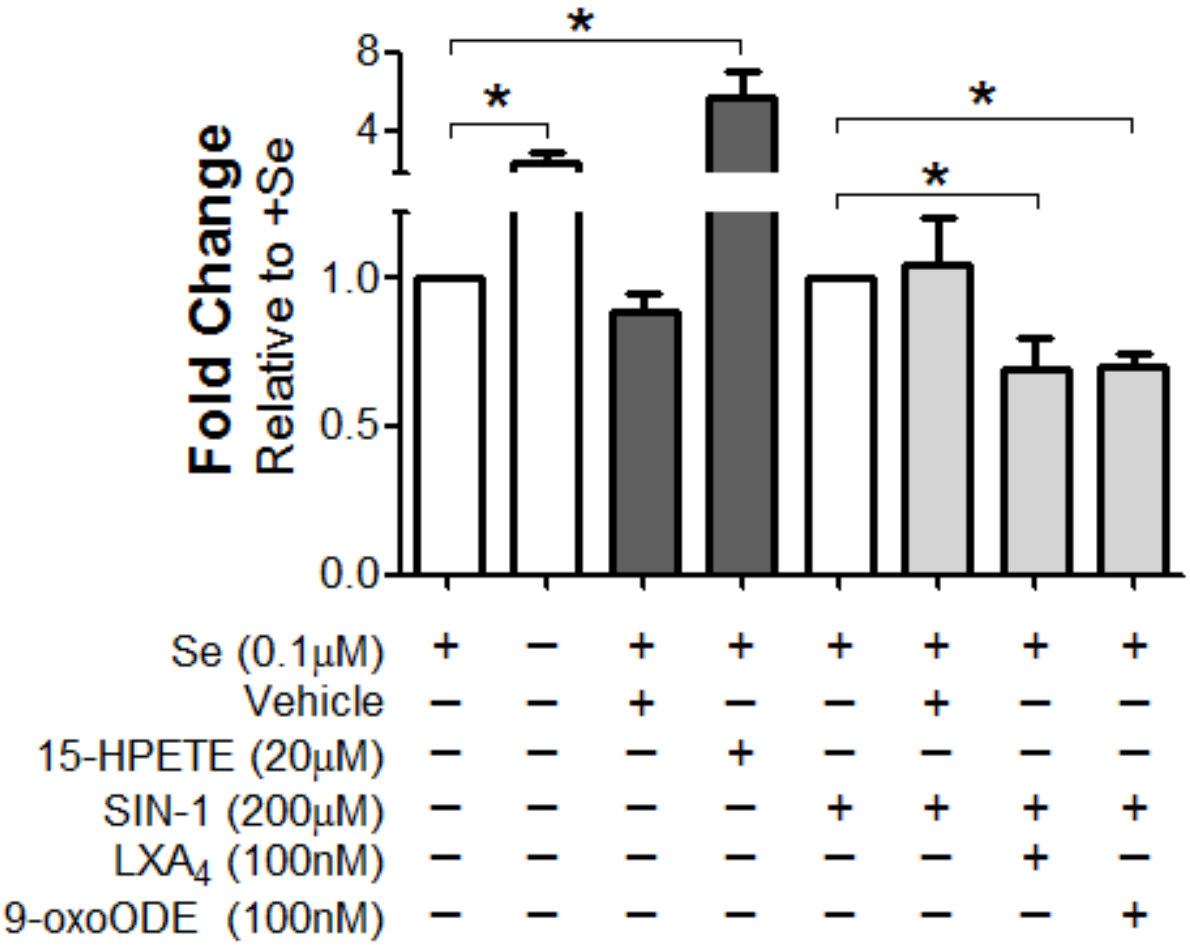



Figure 12. Effect of Oxylipid Stimulation on RAW 264.7

Macrophage TNFα Production. When macrophages were stimulated with 20 μM 15-HPETE for 2 h, production of TNFα was significantly increased compared to controls. When macrophages were stimulated with 100 nM of either $LXA_4$ or 9-oxoODE for 2 h, TNFα was significantly decreased compared to controls. Vehicle represents ethanol without any oxylipid. Production of TNFα was quantified as pg of cytokine per ng DNA and expressed as a fold over control cells. DNA was quantified using Quant-iT DNA Assay Kit, Broad Range. *Significance $p \leq 0.05$ compared to controls, n=4.

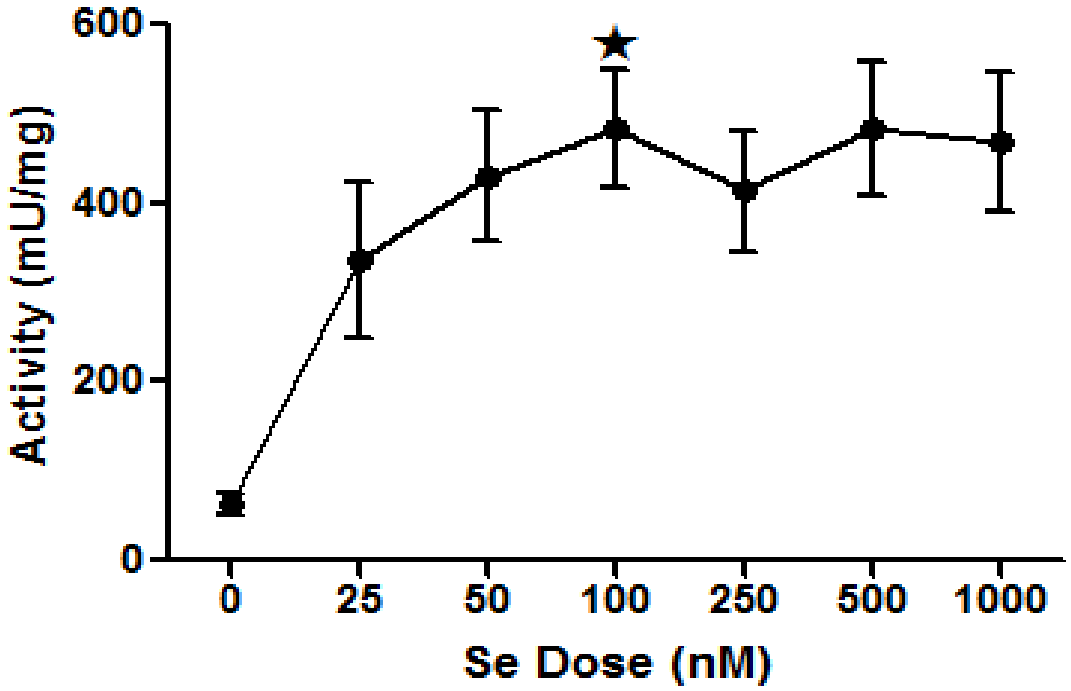
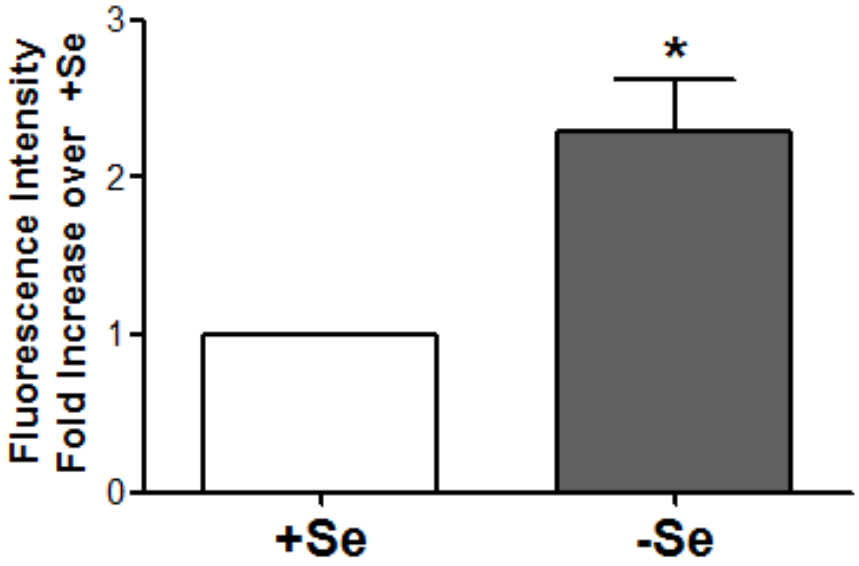



Figure 13: Glutathione peroxidase 1 (GPx1) activity from RAW 264.7 macrophages cultured with various doses of selenium.

RAW 264.7 cells were seeded in 6-well plates and cultured for 3d with various doses of Se provided as sodium selenite. Following 3 d culture, cells were harvested an analyzed for GPx1 activity as described in the Materials & Methods section of Chapter 2.

**A)**




**B)**

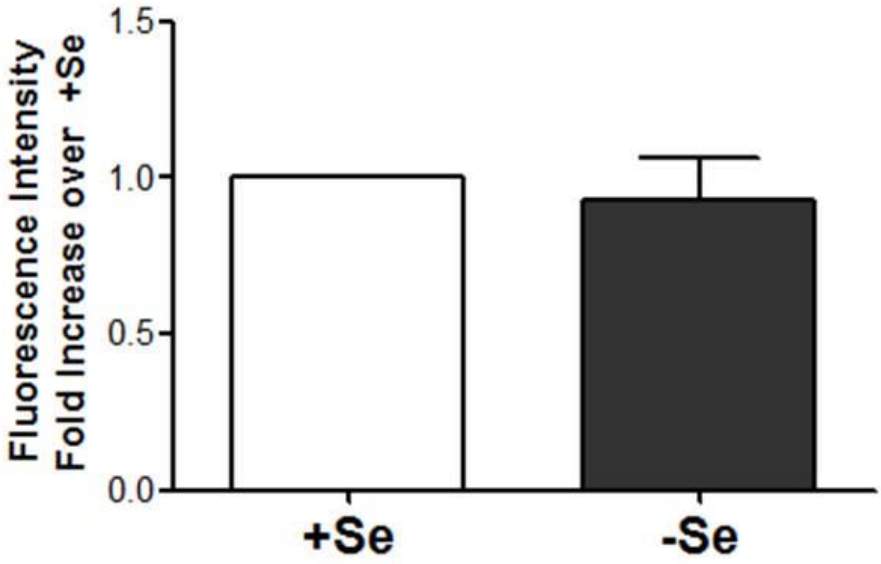
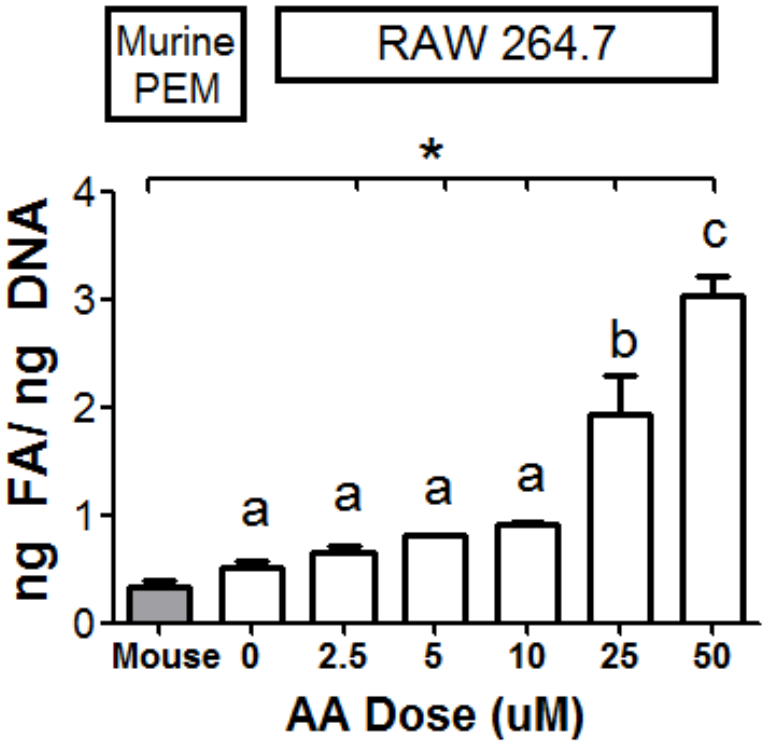



Figure 14. Reactive oxygen species production by RAW 264.7 macrophages cultured in 5% (A) or 10% (B) FBS, n=4.

RAW 264.7 macrophages were cultured in 5% or 10% FBS, stained with the redox-sensitive dye, $H_2$-DCFDA for 30min, and analyzed for ROS production using flow cytometry. (A) A significant increase in ROS production is observed from –Se compared to +Se macrophages cultured in 5% FBS, whereas (B) there is no difference when macrophages are cultured in 10% FBS. *Significance $p \leq 0.05$.

**A)**




**B)**

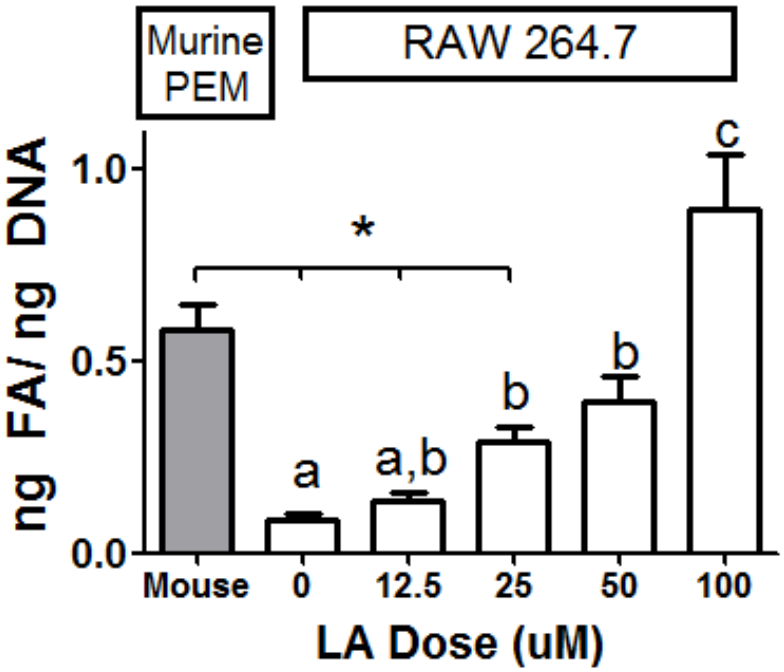



Figure 15. Total fatty acid analysis of arachidonic (A) or linoleic (B) acid from murine peritoneal macrophages (n=3) or RAW 264.7 macrophages (n=6).

**Figure 15 (cont'd)**
RAW 264.7 fatty acid profiles (following 24hr culture with various doses of AA or LA) were compared to PEM from control mice using GC/MS. (A) AA content of RAW 264.7 macrophages significantly increased in a dose-dependent manner. Any dose of AA added to RAW 264.7 macrophages significantly increased the AA content compared to control mouse PEM. (B) LA content of RAW 264.7 macrophages significantly increased in a dose-dependent manner. RAW 264.7 macrophages cultured with 0, 12.5, or 25μM LA was significantly decreased compared to control mouse PEM. RAW 264.7 macrophages cultured with doses of 50 or 100μM LA significantly matched control mouse PEM. *Significance $p \leq 0.05$.

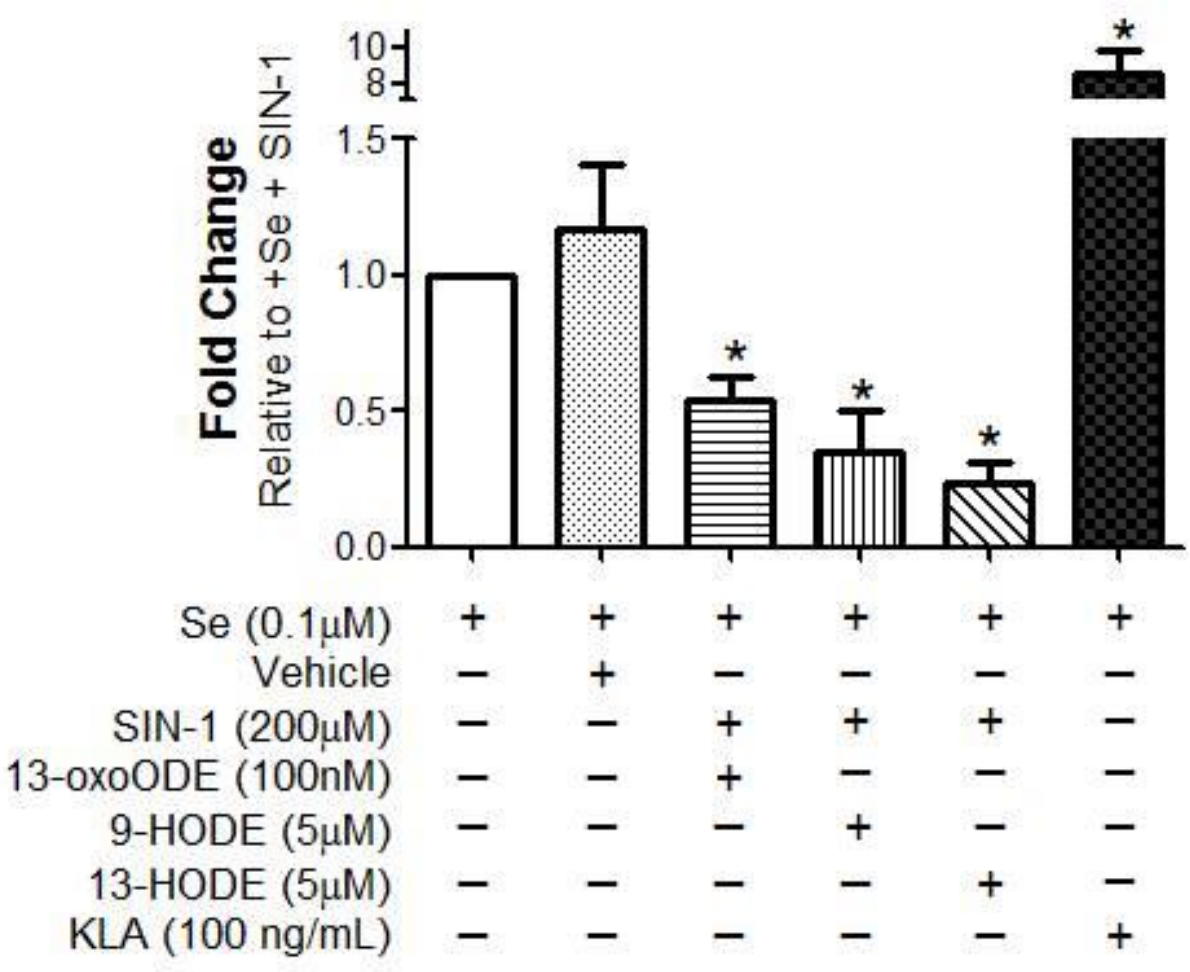



Figure 16. Effect of LA-derived Oxylipid Stimulation on RAW 264.7 Macrophage TNFα Production.

When macrophages were stimulated with 100 nM 13-oxoODE, 5 μM 9-HODE, or 5 μM 13-HODE for 2 h, TNFα was significantly decreased compared to controls. Vehicle represents ethanol without any oxylipid and KLA used as positive control. Production of TNFα was quantified as pg of cytokine per ng DNA and expressed as a fold over control cells. DNA was quantified using Quant-iT DNA Assay Kit, Broad Range. *Significance $p \leq 0.05$ compared to controls, n=3.

Sarah Mattmiller
G385 Veterinary Medical Building
Michigan State University
784 Wilson Rd
East Lansing, MI 48824

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