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REDOX REGULATION OF THE NF-κ B PATHWAY BY SELENIUM IN

MACROPHAGES: ROLE OF 15-DEOXY-Δ^{12,14}-PROSTAGLANDIN J_{2}

A Dissertation in
Pathobiology

by

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Redox-based regulation of gene expression has emerged as a fundamental regulatory mechanism in cell biology. It is increasingly clear that over-production of reactive oxygen species (ROS) by immune cells, resulting in oxidative stress, plays a prominent role in several disease states, including cardiovascular diseases, arthritis, cancer, and AIDS. Of particular importance is the oxidative stress induced over-expression of cyclooxygenase (COX)-2 that is characteristic of inadequate selenium (Se) nutrition. Clinical trials and epidemiological studies have strongly suggested the beneficial effects of Se supplementation in prevention and/or treatment of some of these diseases. To understand the molecular mechanisms underlying the anti-inflammatory property of Se, the RAW 264.7 macrophage cell line or bone marrow macrophages (BMDM) isolated from mice on Se-deficient or Se-supplemented diets were cultured in media deficient or supplemented with Se. *In vitro* kinase assays revealed that Se-supplementation decreased the activity of IkappaB kinase (IKK) β in bacterial lipopolysaccharide (LPS)-treated macrophages. Stimulation by LPS of Se-supplemented macrophages resulted in a time-dependent increase in 15-deoxy- Δ¹₂, ¹⁴-prostaglandin J₂ (15d-PGJ₂) formation, an endogenous inhibitor of IKKβ activity. Further analysis revealed that inhibition of IKKβ activity in Se-supplemented cells correlated with the Michael addition product of 15d-PGJ₂ with Cys-179 of IKKβ, while the formation of such an adduct was significantly decreased in the Se-deficient macrophages. In addition, anti-inflammatory activities of Se were also mediated by the 15d-PGJ₂-dependent activation of the PPARγ in macrophages. Experiments using specific COX inhibitors and genetic knockdown approaches indicated
that the COX-1, and not the COX-2 pathway, was responsible for the increased synthesis of 15d-PGJ2 in Se-supplemented macrophages. Se-supplementation also led to the decrease of the expression of mPGES-1 and TXS; while the expression of H-PGDS was significantly increased. The results presented here argue that incorporation of Se into selenoproteins causes the shunting of arachidonic acid towards 15d-PGJ2 production, which further acts in a positive feedback loop via the activation of PPARγ-dependent transcription of H-PGDS to cause a significant shift in the metabolism of the arachidonic acid by the COX pathway towards 15d-PGJ2 rather than the pro-inflammatory PGE2.

In conclusion, these results suggest that Se-supplementation protects macrophages against oxidative stress-induced pro-inflammatory gene expression via shunting arachidonic acid metabolism towards 15d-PGJ2, while COX-1 is an important enzyme in the production of 15d-PGJ2.
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Chapter 1

LITERATURE REVIEW
Macrophages and the Immune system:

The macrophage plays an important role in both the innate and acquired (humoral and cellular) immune responses. Dendritic cells (DCs), are uniquely potent in induction of naive T and B lymphocytes, whereas macrophages influence a range of immune responses through antigen recognition, capture, clearance and transport [1]. During an infection, macrophages have the capacity to become activated by lymphokines and diverse bacterial products such as lipopolysaccharide (LPS), which is a component of the outer membrane of gram-negative bacteria. As one of the most potent activators of monocytes, exposure to LPS activates several signal transduction pathways including toll-like receptors, cAMP-dependent kinase, protein kinase C, tyrosine kinase, PI3 kinase, and the ERK, p38, and JNK mitogen activated protein kinase (MAPK) families [2]. In addition, LPS-activated macrophages synthesize a great number of substances involved in host defense and inflammation including, complement components, prostaglandins (PGs), proinflammatory cytokines such as interleukin (IL)-1α, and tumor necrosis factor (TNF)-α [3, 4]. During activation of macrophages, to enhance their microbicidal functions, a respiratory burst reaction produces such reactive oxygen and nitrogen species (RONS) as O₂⁻, H₂O₂, NO and NOO⁻ [5]. The large amount and broad reactivity of the RONS produced by activated macrophages assist these essential protective duties, but unfortunately also contribute to pathological conditions [6]. Increases in the intracellular levels of RONS, frequently referred to as oxidative stress, represents a potentially toxic insult, which if not counteracted, will lead to membrane dysfunction, DNA damage, and inactivation of proteins. Chronic oxidative stress has numerous pathological consequences including cancer, arthritis and neurodegenerative disease [7-9]. Under
physiological conditions, a low level of RONS is scavenged effectively by the cellular antioxidant defense system. However, an imbalance between the generation of RONS and cellular antioxidant capacity leads to a state of oxidative stress that contributes to various pathological conditions including cancer [10, 11]. Although certain stimuli such as growth factors, hormones, and neurotransmitters use RONS as a second messenger to execute normal physiological response [12, 13], an excessive generation of RONS by external stimuli including redox chemicals, ultraviolet and ionizing radiation, and bacterial or viral infection, has a deleterious health effects [6, 14]. Oxidative stress contributes to inflammation and tumorigenesis via complex mechanism involving production of RONS, cytokines and pro-angiogenic factors, such as PGE2, leading to DNA damage and genetic instability [15, 16]. In contrast to the conventional dogma that RONS are mostly a trigger for oxidative damage of biological structures, it is now increasingly clear that physiologically relevant concentrations of RONS can regulate a variety of key molecular pathways that may be linked with important cell functions, including gene expression [17]. The antioxidant capacity of macrophages is, therefore, very important not only to maintain its own vital function in its defense, but also to tightly control the intracellular oxidative tone. In light of their many crucial functions, it becomes apparent that any condition that disrupts macrophage integrity will exert its consequences on immune function. A pathophysiology that demonstrates one of these principles is the development of atherosclerotic lesions, resulting in foam cell formation [18]. Macrophage-mediated oxidation of LDL plays a key role during early atherogenesis in arterial wall. However, this process depends on the macrophage antioxidant environment, where glutathione (GSH), Se-dependent glutathione peroxidase (Se-
GPX/GPX), thioredoxin (Trx), and thioredoxin reductase (TR) play an important protective role against cell-mediated oxidation of LDL [18]. Oxidative stress contributes to atherogenesis by increasing oxLDL formation and also by regulating the expression of scavenger receptors (SR-A, SR-B1) via a RONS-dependent activation of the nuclear factor (NF) -κB pathway [18-20]. Thus increased vascular oxidative stress, a potential consequence of Se deficiency, contributes to the formation of macrophage foam cells. Macrophages are major cellular source of arachidonic acid (AA) metabolites. These cells contain an unusually high percentage (20-25%) of AA in membrane phospholipids [21]. They respond to a variety of stimuli with the release of large amounts of free AA, and efficiently metabolize the free acid to an array of cyclooxygenase (COX) and lipoxygenase (LOX) products [22]. Important to this discussion is role of differential regulation of PG production in an anti-inflammatory program within the macrophages as a function of cellular Se status.

**Selenium, Selenoproteins and Se-deficiency:**

Selenium is an essential micronutrient that is an integral component of many major metabolic pathways, including antioxidant defense systems. The ability of Se to regulate many biological functions is due, in part, to its incorporation into selenoproteins. There are over thirty characterized selenoproteins that require the presence of Se for their activity and possess a wide range of functions including cellular proliferation, protein structure and antioxidant activity [23]. Se is inserted into the active site of these proteins through a cotranslational method that incorporates the aminoacid residue, selenocysteine (SeCys), at the penultimate UGA codon [23-27]. These selenoproteins have different
physiological functions, but for those where the enzymatic activity is known, SeCys is located at the active center [21, 22]. The well-characterized selenoenzymes include the Se-GPX and TR families, the activities of which are responsible for the recognition of Se as an important dietary antioxidant [21]. These enzymes are involved in the protection against oxidative stress and redox-based regulation of gene expression, respectively [22]. Although the human genome contains >30 genes that encode selenoproteins, very little is known concerning their basic functions. Indeed, until recently, little was known concerning how Se is transported in the serum and subsequently donated to tissues [28]. Previous studies suggested that Selenoprotein P (SelP) was important in serum Se transport, as it may carry 10–17 selenocysteine moieties (depending on the species), and more than 50% of plasma Se exists in the form of SelP [29, 30]. Se deficiency results in a significant decrease in Se-GPX and TR, accompanied by an increase in RONS production [21, 22]. The Se-GPX family of enzymes comprises one of the major families of selenoproteins that are involved in combating RONS by reducing \( \text{H}_2\text{O}_2 \) and reactive fatty acid hydroperoxides (FAHP) to less reactive water and alcohol, utilizing GSH, the most abundant low molecular weight antioxidant in mammalian cells [31], as an electron donor [32]. Five Se-GPX family members exist that contain SeCys in their enzyme active site: cytosolic GPX (GPX-1), gastrointestinal GPX (GPX-2), plasma GPX (GPX-3), phospholipid hydroperoxide GPX (GPX-4) and sperm nucleus GPX [33-35]. During Se-deficiency there is rapid decrease of GPX-1 expression suggesting this isoform has significant physiological importance [36]. The main substrate for all the members of GPX family is GSH [31]. Once reduced by GPX, GSH reduces critical cysteine residue in proteins, thus acting as a redox regulatory molecule [37]. Together, GSH/GPX system is
considered the major cellular redox couple that significantly contributes to the regulation of oxidant stress.

Inadequate Se nutrition and associated RONS production have been linked to increased risk of such diseases as cardiomyopathy [38], rheumatoid arthritis [39], AIDS [40] and, most notably, cancer [41, 42]. Associated with these findings is the fact that Se is important for optimal functioning of the immune system [43-46]. Several studies demonstrate that Se-deficiency leads to impaired immune responsiveness, while Se-supplementation results in increased immunocompetence [46]. For example, Se-supplementation results in a significant increase in tumor cytotoxicity of macrophages and natural killer cells [45]. It has been proposed that Se, in the form of TR, prevents malignant transformation of cells by serving as a “redox switch” in the control of cellular growth factors by catalyzing oxidation-reduction reactions of critical thiol groups [47]. Se also inhibits the activation of oncogenes and replication of tumor viruses by a similar mechanism in immune cells [48]. Emerging evidence from clinical trials and epidemiological studies have strongly suggested the beneficial effects of Se-supplementation in prevention and/or treatment of some of these diseases [49]. For instance, the chemopreventive activity of Se against the development of tumors or cancers in both animal models and humans [50, 51]. Furthermore, the level of Se in cancer patients is lower than that in normal subjects [52]. In fact, an investigation of the relation between forage-crop Se and county levels of cancer mortality in the USA and cancer mortality rates for the major cancer sites were found to be higher in low Se counties [42, 53]. Initial results of the SELECT study funded by NIH suggest that Se and
vitamin-E supplementation has a profound effect on prostate health [54]. Se intakes may be suboptimal with respect to disease risk, notably in population of adults in the UK, Europe, China, New Zealand, and even in the USA [55]. Based on several such studies, the National Academy of Sciences (USA), has recommended the tolerable upper daily intake of Se at 400 µg, with a daily recommended intake of 55 µg/day [55]. However, with the fast changing life style and food habits, the plasma Se levels of the American population appears to be on the downward trend, tilting more towards deficiency. Particularly in cigarette smokers, HIV positive and breast cancer patients, the plasma Se levels have been shown to be significantly reduced [56-58].

Studies in sub-Saharan Africa have shown that Se-supplementation of AIDS patients increased CD4+ cell counts and decreased the viral titer [59]. Under such circumstances, cellular Se lost upon continuous exposure of macrophages to RONS need to be replenished in order to prevent exacerbation of pro-inflammatory reactions, which generally favor the transcription of HIV. Se can also alter the AA metabolism, which results in a differential production of bioactive fatty acids derivatives, termed eicosanoids [60]. For instance, Se-deficiency alters the distribution of eicosanoid profiles and results in increased levels of thromboxane A₂ (TXA₂) and PGE₂, with concomitant decrease in PGI₂ [61, 62]. As described later in this section, TXA₂ and PGE₂ are considered as pro-inflammatory mediators; while PGI₂ is anti-inflammatory. Therefore, alterations in antioxidant function, AA metabolism, and RONS accumulation as a consequence of altered cellular Se status significantly alter cellular homeostasis. **However, the molecular mechanisms contributing to the anti-inflammatory, anticarcinogenic, and**
chemopreventive activities of Se are not very clear and are the current topics of intense investigation.

**Metabolic Pathways for Selenium:**

The metabolism of Se is dynamic and can be likened to *in vivo* combinatorial chemistry, in the sense that wide arrays of products are formed [63, 64]. The form of Se has a profound effect on its bioavailability [65]. Typically Se is available in both inorganic and organic forms. Hydrogen selenide (H$_2$Se) is a key metabolite, formed from both inorganic and organic Se compounds. H$_2$Se is formed from inorganic compound; while sodium selenite (Na$_2$SeO$_3$) via selenodiglutathione (GSSeSG) through reduction by thiols and NADPH-dependent reductases [66, 67]. From organic compounds, selenomethionine and selenocysteine, H$_2$Se is released by lyase action [66, 67]. H$_2$Se provides Se for synthesis of selenoproteins after activation to selenophosphate [68]. The known functions of Se as an essential element in animals are attributed to ~12 well characterized mammalian selenoproteins [69]. But there is little evidence that the chemopreventive activities of Se involve these selenoproteins.
Figure #1: General Se metabolic pathway. Dietary Se metabolites are metabolized by different pathways, ultimately to yield selenide which serves as the selenium source for the synthesis of selenophosphate, an ultimate Se donor for selenoproteins. (SeCys, seleno cysteine; SeMet, seleneomethionene; H2Se, selenide; CH3SeH, methyl selenol; GSH, glutathione). From: Ip and Ganther, Cancer Res. 2000.
**Selenoprotein Biosynthesis:**

Selenocysteine (SeCys) is the 21st amino acid in the genetic code [70, 71] and, unlike other amino acids, the biosynthesis of SeCys occurs on its tRNA [72, 73]. SeCys is coded by UGA. UGA therefore, functions both as a signal for termination and a codon for SeCys. SelenocysteinylRNA is initially aminoacylated with serine by seryl-tRNA synthetase and is therefore designated SeCys tRNA\[^{[\text{Ser}] \text{Sec}}\] [72, 73]. SeCys tRNA\[^{[\text{Ser}] \text{Sec}}\] is the only known tRNA that governs the expression of an entire class of proteins, the selenoproteins [25]. SeCys tRNA\[^{[\text{Ser}] \text{Sec}}\] has therefore been called the key molecule [74] and the central component [75] in selenoprotein biosynthesis. The active donor of Se that makes SeCys tRNA\[^{[\text{Ser}] \text{Sec}}\] from the intermediate generated by SelA in bacteria is monoselenophosphate [76], which is synthesized by selenophosphate synthetase (SPS) from selenite and ATP. Selenophosphate appears to be the universal donor in the biosynthesis of this amino acid [76, 77]. SPS is designated SelD in bacteria and SPS2 in mammals. Interestingly, in mammals, SPS2 is a selenoprotein, which suggests that SPS2 can be an auto-regulator as well as a regulator of selenoprotein synthesis as a whole [78, 79]. Knock down of SPS2 in NIH3T3 cells using small interfering RNA completely inhibited the selenoprotein synthesis [80]. The fact that UGA has a dual role of serving as a stop and a SeCys codon raises an important question of how the cell distinguishes between these two functions. Besides SeCys tRNA\[^{[\text{Ser}] \text{Sec}}\] and the in-frame UGA codon in selenoprotein mRNA, there are several other factors that are required for the donation of SeCys to protein and dictate the specific function of UGA as SeCys. These include (i) the \textit{cis}-acting stem-loop structure, designated the Sec insertion sequence (SECIS) element [81]; (ii) the SECIS-binding protein 2 (SBP2) [82-84]; and (iii) the SeCys-specific
elongation factor (EFsec, also called mSelB) [85, 86]. SECIS elements function by recruiting SBP2 to form a tight SECIS-SBP2 complex (Fig. 2) [82-84]. SBP2 binds to the SECIS quartet and also to sequences directly preceding the quartet, but not to the apical loop [83, 87]. Evidence was also presented that SBP2 is stably associated with ribosomes via 28S rRNA in a manner independent of its SeCys insertion function, suggesting that SBP2 preselects ribosomes for SeCys insertion [83]. An RNA-binding domain in the C-terminal sequence of SBP2, and an additional domain that was required for SeCys insertion, but not for SECIS binding. Besides binding to SECIS elements and ribosomes, SBP2 binds EFsec, which in turn recruits SeCys tRNA[^Ser]Sec and inserts SeCys into nascent polypeptides in response to the UGA codon [85, 86]. EFsec is specific for SeCys and is different from EF1A, which is involved in insertion of the other 20 amino acids. SBP2 and EFsec jointly constitute the functional equivalent of the single SELB factor in bacteria [88]. The occurrence of SBP2 and EFsec as separate proteins in eukaryotes suggests a mechanism for rapid exchange of the SeCys tRNA[^Ser]Sec-EFsec complex (from empty to aminoacyl tRNA bound), following Sec insertion.
**Figure#2: Mechanism of Sec insertion.** Selenocysteyl-tRNA (in orange with Sec in yellow) is shown in a complex with EFsec (in blue) and SBP2 (in green) and the SECIS element (shown as a hairpin loop in black) that is ready for donation to the ribosomal A site to be decoded by UGA (shown in the selenoprotein mRNA in black). Once the SeCys tRNA[^Ser] Sec complex is donated to the A site, SeCys tRNA[^Ser] Sec is transferred to the peptidyl site and SeCys is incorporated into the nascent selenopeptide. The growing selenopeptide is shown as alternating gold and blue balls attached to the tRNA in the peptidyl site. The mRNA (shown in black with its start and stop codons indicated) is attached to the smaller of the two ribosomal subunits, and the unacylated tRNA is shown leaving the ribosomal exit site. *From: Dolph L. Hatfield and Vadim N. Gladyshev, Molecular and Cell Biology, 2002.*
**Cyclooxygenase and their role in inflammation:**

The COX enzymes catalyze the bis-oxygenation of free AA to PGH$_2$, the committed step in PG formation. PGH$_2$ is converted into the other PGs or TXA$_2$ by specific synthases [89]. There are two COX enzymes, referred to as COX-1 and COX-2, which catalyze identical reactions [90, 91]. COX-1 is thought to produce PGs important for homeostasis and certain physiological functions and is expressed constitutively in most tissues and cells [92], although it can be induced in some cell lines under certain conditions [93]. A second, inducible, form of COX was hypothesized to exist on the basis of the finding of a glucocorticoid-regulated increase in COX activity observed *in vitro* and *in vivo* in response to inflammatory stimuli [94, 95]. During inflammation, COX-1 mRNA, protein and activity levels do not change, but COX-2 levels increase dramatically, and, as a result, PG production increases [96-98]. Moreover, when COX-2 specific inhibitors are administered, PG production and subsequent inflammation are significantly reduced [97]. These data have led to the conclusion that COX-2 is involved in inflammation, whereas COX-1 is not [99]. During the inflammation process, COX-1 contributes to “resolution”. In experimental mesangioproliferative glomerulonephritis, COX-1 is expressed in glomeruli during the repair period [100]. In the process of ulcer healing, the COX-1 and COX-2 specific inhibitors delay healing. These results implicate the role of COX-1 in the resolution, but not the progression, of inflammation. In inflammatory cells, the microsomal PGES (mPGES-1) is also induced by these cytokines [101]. The large amount of PGE$_2$ produced at the inflammation site by the coupling of COX-2 and mPGES-1 may be involved in the progression of inflammation [102-104]. In contrast, during resolution of inflammation COX-1 preferentially couples with the hematopoietic
PGD synthase (H-PGDS), producing PGD$_2$, instead of PGE$_2$ [104]. These observations clearly suggest that COX-1 is involved in the resolution of inflammation by regulating the synthesis of pro- and anti-inflammatory PGs and that preferential shunting of COX-derived PGH$_2$ serves as an important determinant of the type of downstream end product.
The role of PG synthases in inflammation:

The PGs are a family of structurally related molecules that are produced by cells in response to a variety of extrinsic stimuli and that regulate cellular growth, differentiation, and homeostasis [105, 106]. PGs are derived from fatty acids, primarily AA, which are released from membrane phospholipids by the action of phospholipases. AA is converted by COX to PGH₂ via a hydroperoxyendoperoxide, PGG₂, which then is subsequently converted to one of the several related products, including PGD₂, PGE₂, PGF₂α, PGI₂, and TXA₂, through the action of specific PG synthases [105]. Studies have shown that in the resolution phase of inflammation AA metabolism is shifted away from PGE₂ to PGD₂ [102-104]. PGD₂ biosynthesis is mediated by PGD synthases (PGDS), which use the COX-derived PGH₂ as substrate. Two-types of PGDS have been characterized. The lipocalin-type (L-PGDS; β-trace) is expressed primarily in the CNS and heart; while H-PGDS is expressed mainly in mast cells, antigen-presenting dendritic cells, and macrophages. The short-lived PGH₂ is also a substrate for PGE synthases, cytosolic PGES (c-PGES) and membrane-bound PGES-1 (mPGES-1). Of these, only mPGES-1 is responsive to pro-inflammatory stimuli; while cPGES is a constitutive cytosolic enzyme [107]. Accordingly, one would predict that the shift from PGE₂ to PGD₂ biosynthesis during inflammation is reflected by the time course of induction of enzymes involved in the respective biosynthetic pathways combined with the preferential interaction with specific COX enzymes. COX-2 functionally couples with mPGES-1 [103]. Using gain-of-function experiments in HEK293 cells, the functional coupling of COX-1 and -2 with H-PGDS is regulated, in part, by the release of AA [108]. Although exhaustive, these
studies do not reflect the physiological status of immune cells, where all terminal PG synthases are expressed and their levels are subject to transcriptional regulation apart from spatio-temporal localization. In a recent study, Schuligoi et al [107] showed that LPS-induced inflammation caused sequential induction of PGE$_2$ and PGD$_2$ synthases in mouse heart. During the course of infection, an immediate and short-lasting induction of mPGES-1 followed by a delayed induction of H-PGDS was seen, representing the resolution phase. Enhanced biosynthesis of PGD$_2$ has been shown to be one of the endogenous mechanisms operative to counteract the inflammatory reaction [109]. For example, in pulmonary A549 cells, the induction of mPGES-1 by LPS and IL-1β proceeds through the activation of NF-κB and addition of PGD$_2$ and its metabolite, 15-deoxy-Δ$^{12,14}$-PGJ$_2$ (15d-PGJ$_2$), inhibits activation of mPGES-1 transcription [110]. PGD$_2$ is a major COX product that has significant effect on biological processes, including platelet aggregation, relaxation of vascular and nonvascular smooth muscles, and nerve cell functions [111]. However, PGD$_2$ is a fairly unstable molecule that undergoes dehydration $In$ vivo and $In$ vitro to yield 15d-PGJ$_2$ [112, 113], which is characterized by the presence of a reactive α,β-unsaturated ketone in the cyclopentenone ring. Shibata et al have elegantly demonstrated that in activated RAW264.7 cells, extracellular production of PGD$_2$ was associated with a significant increase in the extracellular levels of 15d-PGJ$_2$ [114]. The extracellular 15d-PGJ$_2$ production was reproduced by incubating PGD$_2$ in a cell-free medium or in phosphate-buffered saline [114]. The cyclopentenone PG has its own unique spectrum of biological effects, including antitumor activity [115], inhibition of cell cycle progression [115], and
suppression of viral replication [116-118], induction of heat shock protein expression [115], stimulation of osteogenesis [115, 119], and induction of apoptosis [120, 121]. Recent studies have shown that 15d-PGJ₂ directly inhibits the NF-κB dependent gene expression through covalent modification of critical cysteine residues in IκB kinase [122, 123] and the DNA-binding domains of NF-κB subunits [122, 123] and may also exert several antiinflammatory effects through mechanisms involving peroxisome proliferator activated receptor (PPARγ) activation [123]. [124]

**PPARγ activation and regulation of expression of PG synthases:**

Peroxisome proliferator-activated receptors are a family of ligand-activated nuclear transcription factors, which, upon the binding of ligand, form a heterodimer with the retinoic X receptor (RXR). PPARγ is expressed in neutrophils, macrophages, T- and B-cells [125]. The PPARγ-RXR heterodimer complex binds to PPAR-responsive elements (PPREs) in the promoter regions of target genes [126]. Recruitment of PPARγ/RXR heterodimer to the promoters leads to the inhibition of co-repressor dissociation and/or co-activator binding enabling PPARs to positively regulate gene expression [127, 128]. In addition PPARs also act directly to negatively regulate gene expression of pro-inflammatory genes in a ligand-dependent manner by antagonizing the activities of other transcription factors such as members of NF-κB and activator protein-1 (AP-1) families[124, 129] A major mechanism that underlies the ability of PPARs to interfere with the activities of these transcription factors has been termed as transrepression. The transrepression of NF-κB by such a PPARγ-dependent mechanism finally leads to the
negative regulation of transcription of pro-inflammatory cytokine genes. Consistent with this idea, 15d-PGJ$_2$, was very effective in inhibiting the NF-κB dependent production of IL-12 [109]. Similarly, in the case of human mPGES-1, activation of PPAR$_\gamma$ may cause negative interference with Egr-1, which normally drives the transcription of mPGES-1[130]. However, the mouse mPGES-1 gene promoter is different from the human mPGES-1 in that it contains two putative NF-κB sites -3193 and -928, a conserved PPRE at -2605 and an Egr-1 site at -65 relative to the transcription start site. Interestingly, these observations do not agree with the partial characterization of the mouse mPGES-1 promoter by Naraba et al. [131]. Thus, mPGES-1 may be regulated by different mechanisms. The activation of PPAR$_\gamma$ has also been shown to repress the transcription of TXS in rat macrophages via its interaction with the transcription factor, NF-E2-related factor-2 (Nrf-2) [132]. Interestingly, 15d-PGJ$_2$ has been found to activate Nrf-2 by binding to its cytoplasmic retention protein, Keap-1[132]. Examination of the murine H-PGDS promoter indicates the presence of three putative sites for the binding of PPAR$_\gamma$ at -4501, -2172, and -672. Based on these preliminary findings, it is believed that PPAR$_\gamma$ plays an important role in the expression of H-PGDS leading to the enhanced production of 15d-PGJ$_2$. These findings also suggest that activation of PPAR$_\gamma$ by 15d-PGJ$_2$ could reprogram the cellular eicosanoids biosynthetic machinery towards anti-inflammation.

**Selenium and Arachidonic Acid Cascade:**

Since the main function of Selenoproteins such as Se-GPX, is the reduction of reactive hydroperoxides to alcohols, the role of Se in the biological system is extensive. The formation of oxygen radicals occurs continuously in the mammalian body due to use of
oxygen in the metabolic activities of AA cascade and lipid peroxidation. Some of the roles of GPX1 include removing the threat of oxidative damage to cells, the reduction of fatty acids, and modulation of the AA cascade through the control of peroxide tone[133]. AA is a polyenoic fatty acid that serves as the precursor for eicosanoid production. The initial step in eicosanoid production requires the release of AA from membrane phospholipids through the activity of phospholipase A2 (PLA2), which are known to be activated under conditions of oxidative stress [134]. Due to accumulation of peroxides in Se-deficient conditions, Se status has been indirectly implicated in increased PLA2 actively through decreased GPX1 activity [134, 135]. Metabolism of AA occurs through one of two pathways, COX pathway and LOX pathway [136]. The COX pathway is initiated by the conversion of AA to PGG$_2$ by means of the COX activity of the enzyme PGH synthase (PGHS)-1 or PGHS-2 [105]. PGHS through its peroxidase activity converts PGG$_2$ to PGH$_2$, which is metabolized to several PGs, thromboxanes (TXs) and prostacyclin (PGI$_2$) via tissue specific synthases [105]. Similarly AA is metabolized to a variety of hydroperoxyeicosatetraenoic acid (HPETEs) by the LOX enzyme resulting in the production of leukotrienes (LTs) and lipoxins [35]. The abstraction of 13-pro-S hydrogen from AA serves as the rate limiting step in the COX pathway[137]. In order for PGHS to be capable of abstracting the 13-pro-S hydrogen from AA, the protein must first undergo oxidation by hydroperoxides [137]. Interestingly, though hydroperoxides are continuously necessary for COX activity, at minimal concentrations (<1 µM), higher concentrations (>10 µM) have an inhibitory effect [138, 139]. The reduction of hydroperoxides by GPX1 serves to inhibit COX activity of PGHS not only through preventing the oxidation of the enzyme necessary for activity but also through the
prevention of self-catalysis thought to be caused by the generation free radicals during endoperoxidase activity [140]. The delicate balance of hydroperoxides required for the activity and inhibition of PGHS is referred as the peroxide tone of the cell. Studies in Se-deficient tissues exhibited a variety of different effects on the concentrations of products from COX pathway explained by variations in this tone. Se not only affects eicosanoid production through GPX1 control of peroxide tone, but also through the direct reactions via GPX1. GPX1 is capable of reducing PGHS product PGG$_2$ to PGH$_2$ [141]. The reaction in which PGHS reduces PGG$_2$ has the potential to produce high ROS that are not formed with GPX1 reduction of PGG$_2$ [136, 142]. An example of the utilization of GPX1 reduction of PGG$_2$ has been reported in platelets by Marshal et al. [138]. The availability of GPX1 alternative to PGHS reduction of PGG$_2$ allows for control of system that might otherwise have detrimental each time it was initiated. Similar regulation was demonstrated in gastric mucosa where concentrations of COX products can be influenced by Se status[143]. GPX1 will control PG synthesis depending on the ratio between the COX enzyme activity and GPX1 activity, a ratio that varies depending on the type of tissue and amount of Se [135]. GPX1 also possesses the capability to intervene in the COX pathway even after PGG$_2$ has been reduced to PGH$_2$. In instances of Se-deficiency, alternative pathways for the formation of PGF$_{2\alpha}$ have been shown. PGG$_2$ is normally converted to PGH$_2$, which is a precursor for such PGs as, PGE$_2$, PGD$_2$, PGI$_2$, TXA$_2$ and PGF$_{2\alpha}$. During Se-deficiency, it is hypothesized that non-Se-GPX acts on PGG$_2$ converting it into 15-OOH-PGF$_{2\alpha}$, a substrate not only for non-Se-GPX but for Se-GPX as well [142]. PGE$_2$ and 6-keto-PGF$_{2\alpha}$ concentrations are increased in milk in Se-
deficient conditions which could be explained by the activation of PGHS due to increased concentrations of hydroperoxides in Se depleted conditions [144]. Along the same lines recent studies in our laboratory have suggested that concentrations of PGE$_2$ and RONS are increased along with the increase in PGHS-2 expression in Se-deficient RAW264.7 macrophages [145]. All these studies clearly suggest that cellular Se status can alter AA metabolism. Thus most of these studies are focused to understand the effects of cellular Se status on the production of inflammatory PGs. The present study is focused more on a previously underscribed mechanism of preferentially shunting shifting AA metabolism towards anti-inflammatory PGs away from pro-inflammatory PGs by manipulating the cellular Se status.
**Figure 4:** Proposed pathways of arachidonic acid metabolism in macrophages in the presence (Se+) or absence (Se-) of Se.
**NF-κB activation and pro-inflammatory gene expression:**

The nuclear factor κB (NF-κB) family of transcription factors plays a crucial role in the immune and inflammatory responses [146-149]. Because a large variety of bacteria and viruses activate NF-κB and this transcription factor regulates the expression of inflammatory cytokines, chemokines, immunoreceptors, cell adhesion molecules, it has often been termed as a ‘central mediator of the human immune response [146-148]. Binding sites for the NF-κB are present in the promoter regions of many of the proinflammatory enzymes, cytokines and immunoregulatory mediators involved in inducing acute inflammatory responses [150]. Consistent with this notion, LPS stimulation of macrophages results in an enhanced expression of macrophage inflammatory proteins and other chemokines. The role of macrophage inflammatory protein (MIP)-2 seems to be related to production of TNFα, which in-turn regulates the expression of vascular adhesion molecules required for neutrophil influx, thus governing leukocyte migration and activation [151]. The transcriptional activity of NF-κB can be regulated by different mechanisms, including the amount of IκB present, NF-κB subunit composition, and cellular redox status [150]. It is well established that NF-κB is the major redox-sensitive transcription factor that has been characterized to respond to changes in redox homeostasis of cells [150, 152, 153]. The mammalian NF-κB family contains 5 members: NF-κB1 (p105 and p50), NF-κB2 (p100 and p52), c-Rel, RelB, and RelA (p65). NF-κB comprises a family of dimeric transcription factors that regulate the expression of over 150 genes involved in
immune, stress, and antiapoptotic processes [154]. These complexes bind to DNA at specific NF-κB enhancer binding regulatory sites, such as 5’GGGACTTCC-3’, to activate gene transcription [155]. Under normal circumstances, NF-κB is tightly regulated so as to prevent inappropriate inflammation while allowing a rapid response to infection or stress. In unstimulated cells, NF-κB is found predominantly in the cytoplasm in a complex with IκB proteins, which sequester NF-κB and prevent its migration to the nucleus [156]. Diverse stimuli, including cytokines, bacterial and viral products, oxidants, and mitogens, lead to phosphorylation of two regulatory serine residues on IκBs, which targets it for polyubiquitination and proteolytic degradation. This frees NF-κB to move to the nucleus, where it binds to and stimulates the transcription of target genes, including COX-2, iNOS, and many pro-inflammatory cytokines [157]. Along these lines, the recent data in our laboratory has shown that there is increased activation of NF-κB in the unstimulated Se-deficient RAW 264.7 cells compared to Se-supplemented macrophages [145]. Since the phosphorylation and degradation of IκB is the determining step in the NF-κB activation, the kinase cascade preceding it has been the subject of intense study. Emerging evidence suggests that the IKK complex, which consists of 2 highly homologous kinase subunits, IKKα and IKKβ, and a nonenzymatic regulatory component, IKKγ/NEMO is the key regulator of NF-κB activation [158]. IKKα and β are structurally similar and can form homo or heterodimers. Despite their similarity, they appear to have distinct physiological functions, and can be independently
activated by differing upstream signals [158]. IKKα plays an essential role in mediating p100 processing and, thus, the noncanonical pathway of NF-κB activation [159]. On the other hand, IKKβ and IKKγ are required for the canonical NF-κB signaling, specified by rapid degradation of IκBα and nuclear translocation of RelA-containing NF-κB dimers [160]. Genetic studies reveal that inactivation of IKKβ, but not that of IKKα, severely cripples the activation of NF-κB by proinflammatory stimuli [161, 162]. IKKβ has consistently been shown to function as a key regulator of inflammatory responses [163]. In a series of in vitro experiments, Rossi et al.[122] show that cyPGs, containing α,β-unsaturated carbonyls, such as 15d-PGJ2 inhibits activity (but not expression) of IκB kinase (IKK), that this effect is selective for the IKK pathway, and occurs through a modification of the IKKβ subunit requiring a chemically reactive cyclopentenone moiety [122]. However the findings published by Rossi et al., do not demonstrate if high concentrations of cyPGs are generated in cells to exert such an anti-inflammatory effect.
Figure#5: NF-κB Pathway. While in an inactivated state, NF-κB is located in the cytosol complexed with the inhibitory protein IκBα. Through the intermediacy of integral membrane receptors, a variety of extracellular signals can activate the enzyme IκB kinase (IKK). IKK in turn phosphorylates the IκBα protein which results in ubiquitination, dissociation of IκBα from NF-κB, and eventual degradation of IκBα by the proteosome. The activated NF-κB is then translocated into the nucleus where it binds to specific sequences of DNA called response elements (RE). The DNA/NF-κB complex then recruits other proteins such as coactivators and RNA polymerase which transcribe downstream DNA into mRNA which in turn is transcribed into protein which results in a change of cell function. From: Wikipedia
Rationale and Hypothesis

Selenium (Se) is an essential micronutrient that is an integral component of the antioxidant defense systems. An inadequate level of Se in the diet is associated with an increase in oxidant stress and, therefore, presents an increased risk to the initiation of such pathologies as cancer, AIDS, and cardiovascular diseases, where macrophages play a pivotal role. Macrophages are important components of the innate immune system that aid in immune surveillance and homeostasis. During this process, macrophages produce highly reactive and oxidizing species called reactive oxidant species (ROS), which are normally detoxified by cellular enzymes. Of the antioxidant defense proteins, Se-dependent enzymes such as the GPX1 and to some extent TR, have the catalytic ability to detoxify a broad range of ROS. The cellular levels of these important selenoproteins are directly proportional to the nutritional supply of Se. Apart from geophysical factors, decreased serum levels of Se are seen in individuals with HIV-AIDS, a variety of cancers, and cigarette smokers. Also, there is a negative correlation of body Se with increasing age. In such individuals, during any infection, pro-inflammatory products produced by the activated macrophages could become detrimental and the retard healing (resolution) processes. Thus, Se-supplementation, over the minimal nutritional requirements, has gained popularity and there is overwhelming scientific evidence to support benefits of super-supplementation of Se. However, despite the therapeutic potential of Se in many inflammatory diseases, little is known about the mechanism and regulation of inflammation by Se. Thus the main objective of this thesis is to examine the molecular mechanisms underlying the anti-inflammatory role of Se. Recent studies have shown Se to be a potent regulator of transcription pathways. In fact, Se
deficiency in rats increased the binding of NF-κB to DNA, leading to the transcriptional activation of oxidative-stress responsive genes. Along the same lines, studies in our laboratory have shown that Se-supplementation of macrophages decreased the expression of two pro-inflammatory genes, COX-2 and inducible nitric oxide synthase (iNOS), via the inactivation of NF-κB. COX-2 is one of the isozymes involved in the production of Prostaglandins (PGs). COX-2 is an inducible enzyme where as COX-1 produces PGs important for homeostasis and certain physiological functions and is expressed constitutively in most tissues and cells. In inflammatory cells, the membrane bound type of PGE synthase (PGES) is induced, which may couple with COX-2 to produce the large amount of PGE₂ at the inflammation site and be involved in the progression of inflammation. In contrast, during the inflammation process, COX-1 is thought to contribute to “resolution” by preferentially coupling with hematopoietic PGD synthase (H-PGDS) to synthesize PGD₂. Several studies have indicated that, in the resolution phase of inflammation, arachidonic acid metabolism is shifted away from predominant PGE₂ biosynthesis towards increased PGD₂ biosynthesis. PGD₂, in turn, has been suggested to promote the resolution phase of inflammation. These actions seem to be mediated primarily, but not only, by one of its nonenzymatic degradation products, the cyclopentenone compound 15-deoxy-Δ^{12,14}-PGJ₂ (15d-PGJ₂). Research in our laboratory has shown that Se-deficiency leads to the production of pro-inflammatory PGE₂. Based on the literature reports and previous studies in our laboratory, the present studies are based on the hypothesis that the Se supplementation shunts arachidonic acid pathway towards anti-inflammatory metabolites via COX-1 in LPS stimulated macrophages.
An additional hypothesis to be tested is that the ant-inflammatory functions of Se require Se to be incorporated into the selenoproteins.
Chapter 2

The Anti-inflammatory Effects of Selenium are Mediated through 15-Deoxy-Δ^{12,14}-Prostaglandin J_{2} in Macrophages.

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ABSTRACT

Selenium is an essential micronutrient that suppresses the redox-sensitive transcription factor NF-κB-dependent pro-inflammatory gene expression. To understand the molecular mechanisms underlying the anti-inflammatory property of Se, we examined the activity of a key kinase of the NF-κB cascade, IκB-kinase β (IKKβ) subunit, as a function of cellular Se status in murine primary bone marrow-derived macrophages and RAW264.7 macrophage-like cell line. *In vitro* kinase assays revealed that Se-supplementation decreased the activity of IKKβ in lipopolysaccharide (LPS)-treated macrophages. Stimulation by LPS of Se-supplemented macrophages resulted in a time-dependent increase in 15-deoxy-Δ12, 14-prostaglandin J2 (15d-PGJ2) formation, an endogenous inhibitor of IKKβ activity. Further analysis revealed that inhibition of IKKβ activity in Se-supplemented cells correlated with the Michael addition product of 15d-PGJ2 with Cys-179 of IKKβ, while the formation of such an adduct was significantly decreased in the Se-deficient macrophages. Taken together, our results suggest that Se-supplementation increases the production of 15d-PGJ2 as an adaptive response to protect cells against oxidative stress-induced pro-inflammatory gene expression. More specifically, modification of protein thiols by 15d-PGJ2 represents a previously undescribed code for redox regulation of gene expression by Se.
INTRODUCTION

Macrophages play central roles as effector cells in inflammatory reactions and cell-mediated immune responses. While performing these functions, these cells produce such reactive oxygen species (ROS) superoxide anion, hydrogen peroxide (H₂O₂), hydroxyl and lipid peroxyl radicals along with a great number of proinflammatory substances, including complement components, PGs, chemokines, and cytokines like IL-1β and TNFα [164]. Such reactions represent a potentially toxic insult, which if not counteracted, will lead to membrane dysfunction, DNA damage and inactivation of proteins, leading to the onset and/or progression of many disease pathologies [6, 14]. In contrast to the conventional dogma that ROS are mostly triggers for oxidative damage of biological structures, it is now increasingly clear that physiologically relevant concentrations of ROS can regulate a variety of key molecular pathways that may be linked with important cell functions, including gene expression [17]. Thus, the redox regulation of gene expression has become an important aspect of cell biology today. The antioxidant capacity of macrophages is, therefore, very important not only to maintain its own vital function in its defense, but also to tightly control the intracellular oxidative tone. Macrophages are equipped with a multi-tier antioxidant system in which micronutrient Se plays a major role mainly in the form of selenoproteins [23]. TR and GPX1 represent two well-studied class of selenoenzymes that catalyze the reduction of cellular peroxides and also help in maintaining an optimal redox balance in cells [165]. The activity of selenoenzymes is directly proportional to the total plasma Se [21]. However, in many disease states, including HIV and different cancers, the activity of
selenoenzymes is reduced leading to inflammation and decreased immune function [166]. In recent years, the role of Se in preventing human disease has gained new attention following the association of "super-supplementation" with decreased incidences of prostate cancer in a few preliminary studies [167, 168]. It has been proposed that Se prevents malignant transformation of cells by serving as a "redox switch" through its role in catalyzing oxidation-reduction reactions of critical thiol groups or disulfide bonds, possibly through selenoproteins [169, 170].

Recent studies have shown Se to be a potent regulator of transcription pathways. In fact, Se deficiency in human Jurkat T-cells and lung carcinoma cells increased the nuclear binding and transcriptional activation of oxidative-stress responsive genes by nuclear factor-κB (NF-κB) [79]. Because diverse stimuli activate NF-κB and because this transcription factor regulates the expression of pro-inflammatory enzymes such as cyclooxygenase-2 (COX-2) and cytokines like TNF-α, NF-κB has often been termed a "central mediator of the immune response"[147-149]. Thus, activation of NF-κB is involved in inducing acute inflammatory responses. Overexpression of GPX1 was shown to inhibit NF-κB activation, via inhibition of IκB phosphorylation [171]. Along the same lines, studies in our laboratory have shown that Se-supplementation of macrophages decreased the expression of two proinflammatory genes, COX-2 and inducible nitric oxide synthase (iNOS), via the inactivation of NF-κB; while the expression of COX-1 was unaffected [145, 163]. It is well known that signaling pathways activated by ligation of cell surface pattern recognition receptors converge at the IκB kinase (IKK) complex that phosphorylates the inhibitory subunit and cytoplasmic retention protein of the NF-κB
complex, IκBα [171]. The IKK complex includes highly homologous catalytic kinases, IKKα (IKK1), IKKβ (IKK2), and an essential regulatory subunit, also called as IKKγ or NEMO [158]. Although both IKKα and IKKβ can phosphorylate all three IκB proteins *in vitro*, studies in mice that are deficient in IKK subunits show that in most cells, IKKβ has the dominant role in signal-induced phosphorylation and degradation of these proteins [158]. In addition to phosphorylation by upstream kinases, the enzymatic activity of IKKβ is subjected to further control by Michael adduct formation with α, β-unsaturated carbonyl compounds, 4-hydroxynonenal (4-HNE) [172] or cyclopentenone prostaglandins (cyPGs) such as PGA₁ and 15d-PGJ₂, with a critical cysteine (Cys-179) residue in the activation loop [122]. Thus, the inhibition of NF-κB activation, by these endogenous compounds, could play a seminal role in the resolution of immune response. In addition, 15d-PGJ₂ has been shown to directly bind and activate the nuclear hormone receptor PPARγ[173], which can trans-repress the inflammatory responses mediated by NF-κB [128, 129]. Therefore, cellular production of 15d-PGJ₂ may mediate antiinflammatory responses via these pathways. However, there are no reports on the preferential increase in the intracellular levels of 15d-PGJ₂ to support these claims. Here, we show for the first time that Se supplementation of macrophages leads to the preferential shift in the arachidonic acid pathway towards anti-inflammatory prostaglandin, 15d-PGJ₂ away from pro-inflammatory PGs. In macrophages and other immune cells, PGH₂ is further converted to PGD₂ by the hematopoietic PGD₂ synthase (H-PGDS) that undergoes two spontaneous non-enzymatic dehydration reactions to form 15d-PGJ₂ [114]. The present study is based on the hypothesis that the antiinflammatory
role of Se occurs, in part, via 15d-PGJ$_2$-dependent intracellular signaling pathways in macrophages and 15d-PGJ$_2$ plays a pivotal role in the control of NF-$\kappa$B activity.
**Materials and Methods:**

**Reagents:**

Bacterial endotoxin lipopolysaccharide (LPS; Serotype: 0111:B4) and sodium selenite (Na\textsubscript{2}SeO\textsubscript{3}) were from Sigma Chemical Co., St. Louis, MO. Anti-cyclooxygenase (COX)-1 and anti-COX-2 were obtained from Cayman Chemicals, Ann Arbor, MI. Antibodies for IKKa, IKK\textbeta, p\textkappa\textalpha, and GAPDH were purchased from Santa Cruz Biotechnology. Anti-selenoprotein-P was from BD Biosciences Pharmingen, San Diego, CA. Anti-15d-PGJ\textsubscript{2} was from Assay Designs (Ann Arbor, MI). Goat anti-rabbit IgG, anti-mouse IgG conjugated to horseradish peroxidase, polyvinylidene difluoride (PVDF), and West Pico chemiluminescence reagents were purchased from Pierce Chemical Company.

**Cell Culture and Stimulation:**

The murine macrophage cell line RAW264.7 (ATCC) was cultured in DMEM containing 5 % defined fetal bovine serum (Hyclone), 80 \textmu g/ml gentamicin, and 2 mM L-glutamine (Invitrogen) at 37 °C with a 5 % CO\textsubscript{2}/air mixture. Total Se in the fetal bovine serum was quantitated to be 6 pmol/ml. Cells were cultured in DMEM either supplemented with Se (2 nmol/mL of media) or without any added as described from our laboratory [145]. Cell viability and growth rates of Se-supplemented cells were similar to their Se-deficient counterparts. The enzymatic activity of GPX1 was used as markers of cellular Se status [21]. About 1 x 10\textsuperscript{6} Se-deficient and Se-supplemented cells were seeded in a 6-well plate, and then cultured in respective media for about 24 h to allow the cell number to approximately double. Cells were stimulated with LPS (0-1 \textmu g/mL) and/or other compounds for the indicated time periods. Upon treatment, the cells were harvested,
washed with cold sterile PBS and stored at –80 °C until further use. Femoral bone marrow plugs from mice maintained on a Se-deficient or Se-supplemented diets were isolated and adherent cells, hereafter referred to as primary bone marrow-derived macrophages (BMDM), were differentiated in their respective media containing 20 % L929 fibroblast media supernatant (as a source of GM-CSF) for 1 week. The L929 cells were also cultured under Se-deficient or Se-supplemented conditions. The Se-deficient and Se-supplemented diets were formulated based on a American Institute of Nutrition recommended rodent diet containing 0.015 or 0.4 ppm of Se, as described [174]. The diets were purchased from Zeigler (Gardners, PA). The BMDM cultures from Se-deficient and Se-supplemented mice were used in all experiments.

**LPS Treatment of Mice:**

The Se-deficient and Se-supplemented mice (n = 3 in each category) were treated with *E. coli* LPS (at 5 mg/kg body weight) or PBS control by intraperitoneal injection. The mice were euthanized after 6 h of injection. The serum from each mouse was prepared and used in 15d-PGJ2 enzyme-linked immunosorbent assays as described later in this section. All animal protocols were approved by the Institutional Animal Care and Use Committee.

**Isolation of lung macrophages:**

The Se-supplemented and Se-deficient mice were treated intraperitoneally with LPS (5 mg/kg body weight) for 6 h. Subsequently mice were killed by CO₂ inhalation. The lungs were excised, sliced, and digested in 5 ml of 1X collagenase-A in HBSS-1640 with Ca²⁺ and Mg²⁺ by incubating in a 37 °C water bath for 1 h. The digest was centrifuged at 200g for 5 min and the pellet was washed for two times with 1X PBS with 2 % FBS by
centrifuging at 200g for 5 min. The pellet was resuspended in 5 ml media and layered over with 5 ml Lympholyte® (Cedarlane Laboratories, Ontario, Canada) followed by centrifugation at 500g for 20 min. The cells at the interface were collected and used in experiments described below.

**Flow cytometric analysis:**

Lung cells (1 x 10⁶) in PBS containing 2% heat-inactivated FBS were fixed using 1 % paraformaldehyde and were labeled with Cyc-anti F4/80 and PE-anti CD11b antibodies. Antibodies were purchased from CalTag Laboratories (Burlingame, CA) and were used according to the manufacturer's specifications. Infiltrated macrophages within the lung tissue were identified as cells double labeled with Cyc-anti F4/80 and PE-anti CD11b monoclonal antibodies. Analysis was performed on an EPICS XL flow cytometer (Beckman Coulter, Mannheim, Germany), using FlowJo software (Tree Star, San Carlos, CA). The number of macrophages infiltrating the lungs was calculated from the percentage of each population measured by flow cytometry and the total number of viable cells was counted by trypan blue dye exclusion method.

**Preparation of Cell Lysates:**

The frozen cell pellet was resuspended in 50 μl of mammalian protein extraction reagent (M-PER; Pierce) containing 1 mM EDTA, 10 μM leupeptin, and 1 mM phenylmethylsulfonic acid (PMSF) for 30 min on ice with intermittent vortexing. Supernatants were prepared by centrifuging the cell lysate at 10,000g for 15 min at 4 °C
and used for analyses. Protein concentration in the cell supernatants was determined by BCA protein assay (Pierce).

**Glutathione Peroxidase Activity Assay:**

The cytosolic GPX activity was measured according to the published method using H$_2$O$_2$ as substrate [175]. Primary bone marrow-derived macrophages and RAW264.7 macrophages were cultured in the presence or absence of Se. After a week of Se supplementation cells were harvested, lysed and microfuged for 10 minutes. The resulting supernatant was used in enzyme analysis. The oxidation of NADPH was monitored spectrophotometrically at 340 nm following the addition of 3 mM H$_2$O$_2$. For comparison of individual treatments, the GPX1 activities were expressed as nmoles of NADPH oxidized per minute per milligram of protein and the values are given as the mean of three experiments. The total protein levels were measured using the BCA reagent (Pierce, Rockford, IL.).

**Electrophoresis and Immunoblotting:**

Thirty-micrograms of protein from RAW264.7 or BMDM cell lysates were separated on a 12.5 % SDS-polyacrylamide gel, and transblotted onto PVDF membrane as described [176]. The membrane was blocked with Tris-buffered saline containing 0.05 % Tween-20 and 5 % skim milk (w/v). The membrane was probed with primary antibody followed by an appropriate secondary antibody coupled to horseradish peroxidase.
**TNFα secretion assay:**

Bio-Plex Mouse Cytokine 18-Plex Panel was used to quantitate TNFα according to manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA). The concentrations of TNFα in culture media supernatants of BMDM, in the presence or absence of LPS (1 µg/ml) for various time periods, were determined and normalized to total cellular protein.

**Immunoprecipitation:**

Cell lysates (~30-50 µg protein) from LPS-treated and untreated Se-deficient or Se-supplemented RAW264.7 and BMDMs were used with IKKα- or IKKβ-specific IgG (Santa Cruz). The immunoprecipitated IKK-IgG complex was pulled down using ProteinA/G sepharose (Amersham Biosciences) and used in Western blot analysis, as described above, or *in vitro* kinase assays, as described below. The membrane was probed with anti-15d-PGJ2.

**[14C]Arachidonic Acid Treatment of Macrophages:**

The Se-deficient and Se-supplemented RAW264.7 cells were pretreated with [1-14C] arachidonic acid (1 µCi and 30 μM, American Radiochemicals, St. Louis, MO) for 2 h prior to stimulation with LPS for 1 h. The cell lysates were used to isolate IKKβ by immunoprecipitation, as described earlier, and analyzed on an SDS-PAGE followed by autoradiography. The blots were reprobed for IKKβ to confirm near-equal immunoprecipitation.
In-vitro Kinase Assays:

Cell lysates for in vitro kinase assays were prepared using the lysis buffer as described [177]. In case of IKK, the cell lysate (~30-50 µg protein) was incubated with GST-IκBα and ATP for 1h in the kinase wash buffer (50 mM Tris-Cl, pH 8.0, containing 100 µM NaCl, 10 mM MgCl₂, 1 mM DTT, 10 mM β-glycerophosphate, 10 mM NaF, and 1 mM sodium vanadate) at 25 °C. Glutathione-sepharose beads (Amersham) was added to the reaction mixture and centrifuged. The pellet was washed three times with sterile PBS. The phosho-labeled GST-IκBα was separated by SDS-PAGE and immunoblotted onto PVDF membranes. The membrane was probed with anti-phosphoserine (Sigma Chemical Co., St Louis, MO). Furthermore, to quantitate the activity, immunoprecipitated IKKα or IKKβ was incubated with 32P-ATP (2 µCi; Amersham) and GST-IκBα fusion protein as described [177]. The reaction mixture was subjected to gel-filtration chromatography using the Bio-Gel P30 pre-packed columns (Bio-Rad). The flow-through, which contained the GST-[32P]-IκBα, was subjected to liquid scintillation counting.

Quantitation of PGD₂ and 15d-PGJ₂:

A 96-well based EIA kit from Cayman Chemicals and Assay Designs (Ann Arbor, MI) were used to quantitate PGD₂ and 15d-PGJ₂, respectively. The concentrations of 15d-PGJ₂ in culture media supernatants of RAW264.7 and BMDM in the presence or absence of LPS (0-1 µg/ml) for various time periods were determined by EIA according to manufacturer’s instructions and normalized to total cellular protein. PGD₂ was derivatized using methoximyamine-HCl as per the recommendation of the supplier.
Standard calibration curves were prepared using PGD\textsubscript{2}methoxime or 15d-PGJ\textsubscript{2} and fitted to a log-linear, logit, multi-way frequency regression analysis. PGD\textsubscript{2} and 15d-PGJ\textsubscript{2} were quantitated in cell lysates and normalized to total protein in the cell lysates.

**LC-MS Analysis of 15d-PGJ\textsubscript{2} production:**

Culture media supernatants from LPS-stimulated Se-deficient and Se-supplemented macrophages were acidified with 2 N HCl and clarified by centrifugation at 10,000 g for 5 min. Supernatants were processed using a C18-Sep-Pak column cartridge (Waters) and bound 15d-PGJ\textsubscript{2} was eluted with methanol, evaporated, and stored in ethylacetate at -80\degree C until further analysis. As an internal control to calculate extraction efficiency, 200 ng of deuteriated 15d-PGJ\textsubscript{2} (d4) (Cayman Chemicals) was added to the supernatants before extraction. The 15d-PGJ\textsubscript{2} was resolved on a Restek Ultra Aqueous C18 (5 \mu m; 250 mm × 2.1 mm) HPLC column (Bellefonte, PA) on an aqueous acetonitrile gradient with 0.1 % formic acid at flow rate of 0.2 ml/min. MS analysis was performed on a Micromass ZMD mass spectrometer (Waters) set to scan mode (m/z 200-400) for authentic standards; while selective ion monitoring (SIM) set to m/z 317 (M-H+) and 321 (M-H+) for 15d-PGJ\textsubscript{2}(d0) and 15d-PGJ\textsubscript{2}(d4), respectively, was used for quantitation in the samples. Standard calibration curves for 15d-PGJ\textsubscript{2}(d0) and 15d-PGJ\textsubscript{2}(d4) were set up for the quantitation and calculations were performed based on the equations: b(0)=6545.09; b(1)=25.51; r\textsuperscript{2}= 0.99 and b(0)=253.48; b(1)=79.646; r\textsuperscript{2}=0.98 for d0 and d4, respectively.
Mass Spectrometric Analysis of Post-translational Modification of IKKβ:

To further confirm the modification of IKKβ by 15d-PGJ2 and support the immunoprecipitation studies, murine IKKβ peptide-(173–186), LDQGSLCTSFVGTI, was incubated with Me₂SO, authentic 15d-PGJ2 (mol/mol), or total lipid extract from Se-supplemented macrophages (LPS-treated for 2 h) for 30 min at 37 °C in PBS. The samples were analyzed by MALDI-TOF-MS for modification.

Transient Transfection Assays:

Murine COX-2 gene promoter (-2000 to +75) and its NF-κB double mutant in pGL3 luciferase vector was prepared as described [145]. The plasmid constructs were transfected into RAW264.7 cells using Fugene 6 transfection reagent as per the instructions of the supplier (Roche Biotechnology). To normalize the transfection efficiency, β-galactosidase activity from the pSV-βGal plasmid and total protein were used. RAW264.7 cells were stimulated with LPS for 4 h post transfection. Cell lystates were prepared as described above and the luciferase activity was read in a Turner plate luminometer.

Mouse oligonucleotide arrays:

The Mouse Genome Oligo Set Version 1 was purchased from Operon (Alameda, CA) and contains 6800 optimized 70-mers plus 24 controls, melting temperature normalized to 78 °C. Sequences were optimized by the manufacturer using BLAST against all known mouse genes to minimize cross-hybridization. Oligonucleotides were printed onto glass slides using GeneMachines Omnigrid (San Carlos, California) with additional controls
obtained from Stratagene (SpotReport system, La Jolla, CA) at the Penn State University Microarray Core Facility.

**cDNA Microarray analysis:**

To examine the differential expression of genes that were strictly regulated by cellular Se status, RAW264.7 cells cultured in Se-deficient (6 nM) and Se-adequate or physiological Se (50 nM) conditions were used. The total RNA was isolated from the two groups by Trizol reagent (Invitrogen) and further purified with RNAEasy (Qiagen) according the manufacturers’ instructions. Labeling and hybridization was performed as discussed previously [178, 179]. In the present experiments, co-hybridization was performed with a reference cRNA generated from untreated RAW264.7 cells. Statistical analysis was performed using a Student *t*-test with a *p*-value of <0.02 with the additional criteria of being either 3-fold increased or decreased by cellular Se status. Results are given as mean and were performed in GeneSpring (7.3, Agilent Technologies).

**Statistics:**

All enzymatic assays and ELISAs were performed in triplicates unless otherwise noted and values represented are the mean (+SD). Statistical significance is denoted by an asterisk (*) when *p* values are <.05, as calculated by a student’s *t* test. Microarray results were subjected to Statistical analysis using a Student *t*-test with a *p*-value of <0.02.
Results

Differential Se Status in Macrophages:

Culturing of RAW264.7 cells in the presence or absence of Se as well as primary
BMDMs isolated and differentiated from mice maintained on Se-deficient and Se-
supplemented diets, yielded cell populations that exhibited differential Se status as seen
by cytosolic GPX activity levels (Fig. 6). A 6-fold difference in the cytosolic GPX
activity in the Se-deficient and Se-supplemented BMDM was seen. Along the same lines,
liver homogenates from Se-deficient and Se-supplemented mice demonstrated a 7-fold
difference in the enzymatic activity of cytosolic GPX (data not shown). The results of
differences in Se-GPX activity were consistent with those reported in RAW264.7
macrophages previously from our laboratory [145]. Accordingly, the Se-supplemented
and Se-deficient BMDM and RAW264.7 macrophages were used in all the experiments
described below.

Global changes in pro-inflammatory gene expression as a function of Se-status in
macrophages:

Gene expression affected by cellular Se status was examined by gene expression
microarray. Of the 6800 unique genes on the arrays, 5975 gave a reliable signal on all the
arrays. The determination of statistically significant regulated genes was as described
earlier [178, 179]. Using this conservative measure of significance (p<0.02 and a two-
fold change in gene expression), 245 genes were regulated by cellular Se status (Table 1).
Given that cellular Se plays an important role in the promotion of anti-inflammatory responses, the gene expression microarray experiments (See Table 1) demonstrated increase in the expression of Trxrd1 (3.2-fold increase) as a positive control for Se status. Importantly, supplementation of Se-deficient RAW264.7 macrophages with 50 nM Se modulated the expression of fatty acid binding protein 1 (Fabp1) (8.9-fold), IL-4 (3.2-fold increase), RelA (0.21-fold decrease). The results suggest that cellular Se status could play an important role in wound-healing or resolution responses, although this will require formal confirmation using realtime RT-PCR as well as assays quantifying protein expression.

**Infiltration of macrophages into the lungs upon peritoneal exposure to LPS in Se-deficient or Se-supplemented mice:**

Having established a differential Se status in mice, the physiological effects of Se-deficiency were investigated by following the infiltration of macrophages upon challenge of mice with intraperitoneal LPS. Flow cytometric analysis of the alveolar tissue of Se-deficient mice demonstrated an increased infiltration of macrophages after 6h of exposure to LPS when compared to the alveolar tissue extracts from Se-supplemented mice (Fig. 7). The mean percentage of macrophage infiltration into the lungs of Se-deficient mice was increased by at least 50 % more than in the lungs of Se-supplemented mice suggesting that Se status plays a crucial role in the infiltration of macrophages to effector tissues, such as the lungs, in this case.
LPS-induced expression of TNFα in macrophages as a function of cellular Se status:

To further explore if the cellular Se status had an influence on the expression of pro-inflammatory cytokines, which could possibly explain the reason for the increased infiltration into the lungs, we analyzed the ability of macrophages to express the pleiotropic cytokine, TNFα, in the Se-deficient and Se-supplemented macrophages, upon stimulation with LPS. Using the Bio-Plex multiplex protein assay system (Bio-Rad), we observed that, in a time-course experiment of LPS-induced expression of TNFα, Se-supplementation dramatically suppressed TNFα expression, especially at 4-12 h post-LPS treatment in BMDMs (Fig. 8). A similar trend in the Se-dependent repression of TNFα was observed in RAW264.7 macrophages (data not shown). These results provide further evidence that cellular Se status plays an important role in the regulation of inflammation.

Selenium Deficiency Exacerbates COX-2 Expression via Increased Levels of pIkBα leading to the Activation of NF-κB:

Stimulation of Se-deficient RAW264.7 (Fig. 9A) and BMDM (Fig. 9B) cells with LPS for 0-24 h clearly demonstrated exacerbated expression of COX-2 when compared with those cultured in the presence of Se. The differences in expression were obvious as early as 30 min of stimulation of cells (Fig. 9B). Previous reports from our laboratory have indicated that NF-κB is a key transcription factor in the regulation of COX-2 expression in Se-deficient cells [145]. To further understand the role of Se on the NF-κB-dependent
transcription of COX-2, we mutated the two NF-κB sites in the promoter of murine COX-2. As seen in Fig. 9C, the transient transfection studies with wild type mouse COX-2 promoter luciferase reporter exhibited a 4-5 fold increase in activity in Se-deficient RAW264.7 cells; while there was no increase noted in the NF-κB double mutant reporter. The abrogation of luciferase activity upon LPS stimulation in the NF-κB double mutant clearly suggested that NF-κB plays an important role in the regulation of COX-2 expression. These results also indicated an alteration in the upstream signaling mechanisms, such as the activation of IKK family of enzymes by cellular Se status. Although Western immunoblots showed no obvious differences in the expression of IKKβ in LPS-stimulated Se-deficient or Se-supplemented cells, the levels of both IκBα and pIκBα were significantly higher in the former group (Fig. 9D). This sustained increase in IκBα/pIκBα corroborates well with the increased translocation of p65 in Se-deficient cells described earlier from our laboratory [145].

**Selenium Supplementation Decreases the Activity of IKKβ:**

Since Se affected the levels of cytosolic pIκBα, without altering the expression of IKKβ, we monitored the enzymatic activity of IKKβ in both RAW264.7 and BMDMs cultured in the presence or absence of Se. Using *in vitro* kinase assays we found that in Se-supplemented cell lines, IKKβ activity was significantly reduced. The activity further declined upon LPS stimulation of these cells up to 2h. On the other hand, in Se-deficient cells, the activity of IKKβ increased with LPS stimulation (Fig. 10A&B). In addition, *in-vitro* kinase assays of immunoprecipitates containing IKKα and IKKβ from Se-deficient
and Se-supplemented RAW264.7 cells following LPS stimulation demonstrated increased the activity of IKKβ in LPS-treated Se-deficient cells; while in the Se-supplemented cells, there was no such increase in activity upon treatment with LPS (Fig. 10C). On the other hand, activity of IKKα was neither affected by Se status nor LPS stimulation (Fig. 10C).

Enhanced Production of 15d-PGJ2 in Se-supplemented Macrophages:
The above results suggested that the enzymatic activity of IKKβ was severely affected in the Se-supplemented cells and not in the Se-deficient cells. Since cellular Se status did not affect IKKβ expression (Fig. 9D), we hypothesized that post-translational modification of IKKβ was a likely possibility. Previous research in our laboratory has indicated that Se-supplemented cells produced less PGE2 upon LPS stimulation compared to the Se-deficient cells [145]. We reasoned that in Se-supplemented cells, PGH2 from COX-1 or low levels of COX-2 was possibly shunted to H-PGDS to produce PGD2 and subsequently converted to 15d-PGJ2 to affect IKKβ activity. Surprisingly, the levels of 15d-PGJ2 in the culture supernatant of Se-supplemented BMDMs was significantly higher, prior to stimulation with LPS, when compared to those cultured in the absence of Se (Fig. 11A). In RAW264.7 cells, we failed to observe any generation of 15d-PGJ2 in the unstimulated cells (Fig. 11A). Upon LPS stimulation of BMDMs and RAW264.7 cells for 12h, 15d-PGJ2 increased only in the Se-supplemented cells and not in the Se-deficient group (Fig. 11A). Similar results were also obtained in lysates of Se-supplemented cells treated with LPS for 2 h (data not shown). An extended time course
experiment up to 24 h of LPS stimulation revealed further increases in 15d-PGJ\textsubscript{2} only in the Se-supplemented cells (data not shown). Interestingly, the LPS-stimulated Se-supplemented BMDMs produced ~10-fold higher 15d-PGJ\textsubscript{2} than the corresponding Se-supplemented and LPS-stimulated RAW264.7 cells (Fig. 11A). A dose-dependent effect of LPS (0.1–1.0 µg/ml) on 15d-PGJ\textsubscript{2} was also seen in BMDM cells (Fig. 11B). To further rule out any \textit{in vitro} effect, serum of Se-deficient and Se-supplemented mice with or without LPS treatment for 6 h, clearly demonstrated a similar time-dependent increase in 15d-PGJ\textsubscript{2} only in the Se-supplemented mice (Fig. 11C). Supplementation of Se-deficient RAW264.7 cells with 2 µM sodium selenite for 24 h followed by 12 h of LPS stimulation increased the production of 15d-PGJ\textsubscript{2} by 8-10 fold compared to the untreated cells stimulated with LPS (data not shown). The increases in 15d-PGJ\textsubscript{2} in Se-supplemented macrophages were also confirmed by LC-MS (Fig. 11D). Based on this LC-MS method, the quantitation indicated that Se-supplemented RAW264.7 cells produced 7-fold higher 15d-PGJ\textsubscript{2} compared with Se-deficient cells upon 12 h of LPS treatment (data not shown).

**Formation of IKK\textbeta-15d-PGJ\textsubscript{2} Adduct in Se-supplemented Cells Accounts for the Decreased NF-\kappaB Activation and COX-2 Expression:**

As a consequence of production of 15d-PGJ\textsubscript{2} in Se-supplemented cells, an increased interaction of 15d-PGJ\textsubscript{2} with IKK\textbeta subunit was observed in such cells, which increased upon stimulation with LPS. The formation of adduct correlated well with the increased intracellular production of 15d-PGJ\textsubscript{2} (0–2 h, data not shown) and decreased IKK\textbeta activity in Se-supplemented cells (Fig. 12A). To investigate if the IKK\textbeta subunit was differentially modified with an endogenous product of arachidonic acid oxidation, we performed
radiolabeling studies with \(^{14}\text{C}-1\text{C}\) arachidonic acid added to RAW264.7 cells for 2 h prior to stimulation with LPS for various time periods up to 1 h. As shown in Fig. 11B, IKK\(\beta\) immunoprecipitated from Se-supplemented cells showed increased formation of radiolabeled-adduct with LPS stimulation, whereas, relatively lower levels of adduct formation were observed in Se-deficient cells even in the presence of LPS. Furthermore, the interaction of IKK\(\beta\) with 15d-PGJ\(_2\) in the Se-supplemented cell lysates from LPS (2 h) was confirmed using a murine IKK\(\beta\)-(173–186)-peptide in qualitative MALDI-TOF-MS assays. The results clearly demonstrated an increase in the mass of the peptide from 1440.7 to 1757.2, a difference of 316.5 \(m/z\) units, upon incubation with the total cellular lipid extracts similar to that observed with the authentic 15d-PGJ\(_2\) standard (Fig. 12C). Based on these results, it is clear that endogenous 15d-PGJ\(_2\) produced in Se-supplemented cells modulated the activity of IKK\(\beta\) by covalent modification of the essential Cys-179 residue.
Discussion

Epidemiological studies indicate that deficiency in Se, although a micronutrient, is common in cigarette smokers [58], individuals with breast cancer [180], HIV-patients [56], and those living in geographic locations where the soil Se levels are low [181]. Cardiomyopathy and rhabdomyolysis are characteristics of Se-deficiency [182]. Recently, supplementation with Se has been positively correlated with lowered incidences in prostate cancer and HIV replication, where macrophage activation is a crucial step in the inflammatory processes that forms the underlying basis of disease progression [54]. Here we show that Se-supplementation of macrophages down-regulates the expression of pro-inflammatory genes, COX-2 and TNFα, via the inhibition of the enzymatic activity of IKKβ, most likely by covalent modification of an essential Cys residue within its activation loop by 15d-PGJ2. To the best of our knowledge, this is the first ever report that links cellular Se status to the attenuation of NF-κB-dependent proinflammatory gene expression via the synthesis of 15d-PGJ2.

Variation in tissue Se concentrations might influence gene expression by multiple pathways [63, 64]. While studying the effect of Se compounds, cDNA microarray analysis on the premalignant human breast cells revealed 30 genes responsive to methylseleninic acid (MSA) treatment [183, 184]. Some of these genes are responsible to cell cycle arrest whereas others are likely to play a role in apoptosis. A study with high density cDNA microarray has revealed 951 genes, whose expression is regulated by MSA including those involved in cell cycle regulation [185]. This study revealed that MSA may protect against prostate cancer by inhibiting cell proliferation and by
modulating the expression of androgen receptor and their regulated genes. Studies also revealed that low Se status resulted in differential gene expression pattern indicative of activation of genes involved in DNA damage, oxidative stress and a decrease in the expression of genes involved in detoxification [186]. Beck et al observed diminished T-cell proliferation in response to viral antigen in cultured splenocytes from Se-deficient mice. However, gene expression data on macrophages is lacking. Since macrophages are implicated in both initiation and resolution of inflammation, the time seems propitious to understand the regulation of macrophage function by Se status. Towards this end, gene expression microarray experiments in our laboratory, established that Se-supplementation of Se-deficient macrophages caused an increase in IL-4, while decreasing RelA (p65). The differential regulation of IL-4 and RelA are particularly intriguing due to their role in anti-inflammation or resolution phase of inflammation, which could prime the proatherogenic “classically” activated M1 macrophages towards “alternative” M2 macrophage phenotype, expressing Th2-type cytokines, with anti-inflammatory properties [187]. The Se-dependent up-regulation of Fabp1, which is a target gene of all the three PPARs subtypes, depending on the tissue, suggests that Se may play an important role in ligand-delivery and transactivation of PPARs and thus modulate transcription of pro-inflammatory genes [188]. Although promising, these results need to be evaluated by realtime RT-PCR, which will be taken up in the future.

A significant increase in COX-2 protein induction was observed in Se-deficient macrophages as compared to Se-supplemented macrophages, especially at early time points. It is intriguing to note that Se-deficient cells express COX-2 even prior to LPS
stimulation. There is ample evidence to suggest that NF-κB is involved in the regulation of COX-2 induction by stimuli such as LPS [189]. The use of NF-κB double mutant in the present study clearly demonstrates that COX-2 induction by LPS involves this redox sensitive transcription factor. Another inflammatory marker that was significantly affected by Se status was TNFα. A significant decrease in TNFα production by LPS treatment in Se-supplemented macrophages compared to those cultured in Se-deficient media was observed. These findings propose one possible mechanism underlying the anti-inflammatory role of Se through altering the expression of pro-inflammatory genes which could be responsible for the dampened overall inflammation as seen in the macrophage infiltration to the lungs in LPS (ip) treated mice.

Several studies have indicated that in the resolution phase of inflammation, AA metabolism is shifted away from pro-inflammatory PG biosynthesis towards anti-inflammatory PG biosynthesis [102-104]. The biogenesis of 15d-PGJ2 has been examined to a much greater extent than any other PG due to its ability to form Michael addition product [122, 173]. A preferential time-dependent synthesis of 15d-PGJ2 is seen only in the Se-supplemented cells. However, compared with Se-supplemented RAW264.7 cells, BMDMs produced 7- to 10-fold higher 15d-PGJ2 upon stimulation with LPS, which is likely due to higher levels of COX-1 expression in the latter cell type (data not shown). In vivo studies complement the in vitro experiments with regard to increased production of 15d-PGJ2 in the serum of Se-supplemented mice.

Based on these results, it appears that 15d-PGJ2 is only produced in cells that are sufficient in Se and have the ability to convert PGH2 to PGD2, which then undergoes two
dehydration reactions to be converted to 15d-PGJ2. Although the concentration of Se used in this investigation is higher than that found in the plasma, results with lower Se concentrations have also yielded identical results (data not shown). Rossi et al. [122] have shown that such an interaction of 15d-PGJ2 with IKKβ leads to the inhibition of enzymatic activity, without affecting IKKα. An immediate effect of such an inhibition in Se-supplemented cells is the reduction in the levels of pIκBα, which increases in Se-deficient cells. In Se-deficient cells, higher levels of pIκBα could be due to a feed-back activation mechanism of transcription of IκBα by NF-κB followed by phosphorylation. The net effect of such a well regulated process is the enhanced activation of NF-κB in Se-deficient cells. Apart from interacting with IKKβ, 15d-PGJ2 is an endogenous ligand for PPARγ and may perform the role of an insulin sensitizer without any apparent hepatic, cardiovascular, and hematological toxicities that is commonly seen in the case of the synthetic ligand, rosiglitazone [122]. Based on our results, it appears that 15d-PGJ2 is only produced in cells that are sufficient in Se and have the ability to convert PGH2 to 15d-PGJ2 via PGD2. It is not clear as to how 15d-PGJ2 is produced in Se-supplemented cells when there is COX-2 being expressed, albeit at lower levels.

Phospholipase A2 mediated release of AA is followed by immediate or delayed PG production pathways, depending on the presence of COX-1 and COX-2 and expression of various PG synthases [125]. More recently the functional coupling of various PG synthases, such as H-PGDS, and COX-1 have been suggested in literature to produce elevated levels of 15d-PGJ2 [108, 190]. Such an interaction is likely to take place in Se-
supplemented macrophages to produce elevated 15d-PGJ$_2$ upon LPS stimulation. Thus, preferential and functional interaction of COX-1 with H-PGDS in Se-supplemented macrophages, as opposed to COX-2 and microsomal PGE$_2$ synthase-1 (mPGES-1) in Se-deficiency, could also determine the fate of arachidonic acid towards antiinflammatory products as a function of cellular Se status. This observation could have great biological significance given that the balance between the interaction of COX-2 with H-PGDS and mPGES-1 is a major determinant of atherosclerotic plaque instability in humans [191]. Taken together, it is plausible that in Se-supplemented cells, COX-1 mainly serves as an enzyme for the synthesis of 15d-PGJ$_2$, which is further aided by the transcriptional regulation and interaction of downstream PG synthases with specific COX isozymes. The moderate selective effect on the inhibition of COX-1 expression and activity may give new insights for the mechanism of biosynthesis of 15d-PGJ$_2$ during differential cellular Se status.

In conclusion, we have reported the molecular basis of the anti-inflammatory property of Se appears to be driven, in part, by the modulation of arachidonic acid metabolism, particularly towards 15d-PGJ$_2$. This is the first ever report on the preferential increase of an endogenous antiinflammatory product of arachidonic acid metabolism as a function of cellular Se status. As a consequence of increased 15d-PGJ$_2$, the cellular expression of COX-2 in response to inflammatory stimulus was significantly decreased. Given that 15d-PGJ$_2$ can modify many proteins, a broader question as to how such a Se-dependent “switch” in arachidonic acid metabolism may play a role in host immunity and inflammation needed to be examined.
Table1: Genes altered by Se status in RAW264.7 cells (3-fold and p<0.02):

<table>
<thead>
<tr>
<th>Systematic Common</th>
<th>Genbank</th>
<th>Description</th>
<th>Physiological Selenium Ratio</th>
<th>Selenium Deficiency Ratio</th>
<th>Se/Se-</th>
</tr>
</thead>
<tbody>
<tr>
<td>M005575_01 Pdx1</td>
<td>AF229645</td>
<td>PDZ domain containing, X chromosome</td>
<td>4.59</td>
<td>0.10</td>
<td>45.52</td>
</tr>
<tr>
<td>M002544_01 Slc30a1; Znt1</td>
<td>U17132</td>
<td>mus musculus zinc transporter Znt1-1 (Znt1-1) gene, complete cds.</td>
<td>12.29</td>
<td>0.69</td>
<td>17.74</td>
</tr>
<tr>
<td>M003497_01 Ldb2</td>
<td>U88489</td>
<td>LIM domain binding 2</td>
<td>1.96</td>
<td>0.12</td>
<td>15.86</td>
</tr>
<tr>
<td>M001070_01 Aeg1</td>
<td>L05559</td>
<td>acidic epithelial glycoprotein 1</td>
<td>2.29</td>
<td>0.20</td>
<td>11.72</td>
</tr>
<tr>
<td>M003647_01 Fabp1</td>
<td>Y14660</td>
<td>fatty acid binding protein 1, liver</td>
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<td>1.27</td>
<td>8.95</td>
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<tr>
<td>M008007_01 Apip1</td>
<td>L04538</td>
<td>amyloid beta (A4) precursor-like protein 1</td>
<td>2.44</td>
<td>0.32</td>
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<tr>
<td>M004662_01 Pdha1</td>
<td>M76727</td>
<td>pyruvate dehydrogenase E1 alpha 1</td>
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<td>1.28</td>
<td>6.62</td>
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<tr>
<td>M005834_01 Gab1</td>
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<td>growth factor receptor bound protein 2-associated protein 1</td>
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<td>1.27</td>
<td>6.12</td>
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<td>M001159_01 F9</td>
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<td>coagulation factor IX</td>
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<td>5.83</td>
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<td>M003699_01 Tpra40-pending</td>
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<td>M006562_01 Brs3</td>
<td>NM_005786</td>
<td>bombesin-like receptor 3</td>
<td>1.14</td>
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FIGURE#6: Cytosolic GPX activity in BMDM (A) and RAW264.7 (B) macrophages.

Primary bone marrow-derived macrophages and RAW264.7 macrophages were cultured in the presence or absence of Se for ~1 week. Cell extracts were used in GPX assays with H₂O₂ as substrate. The specific activity is described as the amount of protein required to oxidize 1 µmol of NADPH per minute. Representative of n = 3 experiments.
Figure#7: Recruitment of macrophages to the lung. Se-supplemented (0.4 ppm) and Se-deficient (0.01 ppm) mice were treated with PBS or LPS (5 mg/kg) intraperitoneally for 6 h. Lung tissues were collagenase digested and infiltrating macrophages were quantitated using flow cytometry with anti-CD11b and F4/80. Results are averages of n=4 in each category. Copyright: http://www3.interscience.wiley.com/journal/117935711/grouphome
Figure 8: Production of extracellular TNFα by Se-supplemented (0.4 ppm) and Se-deficient (0.01 ppm) BMDM upon stimulation with LPS (1 µg/ml). TNFα was measured using Bio-Plex (Bio-Rad). Results are normalized to cellular protein. Copyright: http://www3.interscience.wiley.com/journal/117935711/grouphome
FIGURE#9: Se-supplementation leads to inhibition of NF-κB-dependent COX-2 expression. A, Se-supplemented and Se-deficient RAW264.7 cells were stimulated with 1 µg/ml LPS for various time periods, and the cell lysates were analyzed for the expression of IKKβ expression, levels of pIkBα, and GAPDH. B, BMDMs isolated from Se-supplemented or Se-deficient mice were stimulated with LPS for 0–4 h, and the cell lysates were used for Western immunoblot analysis for COX-2 and GAPDH expression. C, the murine COX-2 promoter luciferase reporter constructs of the wild-type and NF-κB double mutant were transiently transfected into RAW264.7 macrophages for 24 h
followed by stimulation with LPS for 4 h. To normalize the transfection efficiency, the \( \beta \)-galactosidase activity from the co-transfected plasmid, pSVGal, was used. The luciferase activities of the unstimulated cells were used to calculate the \( \beta \)-fold increase upon stimulation with LPS (*, \( p < 0.05 \)). D, Se-supplemented or Se-deficient RAW264.7 macrophages were also stimulated for 0–2 h with LPS, and the cell lysates were used for Western immunoblot analysis of expression of IKK\( \beta \), total I\( \kappa \)B\( \alpha \), and pI\( \kappa \)B\( \alpha \) as described above. The blots were exposed to the x-ray film for identical times. All experiments were performed in triplicates and representative in each case is shown.
FIGURE#10: Se-supplementation of macrophages represses the activity of IKKβ. A and B, total kinase activities in RAW264.7 (A) and BMDM cells (B), cultured in the presence or absence of Se, and stimulated with LPS for the indicated time periods. Cell lysates were incubated with GST-κBα fusion protein and subjected to GST pulldown assay with GSH-agarose beads. The phosphorylation status of the GST-κBα was analyzed by Western immunoblot with anti-p-Ser monoclonal antibodies. C, RAW264.7 cell lysates (50 µg) stimulated with LPS ($t = 2$ h) were used in immunoprecipitation reactions with anti-IKKα or anti-IKKβ. The immunoprecipitates were used in in vitro kinase assays with $[\gamma-32P]$ATP and GST-κBα. The -fold increases in activity were calculated with respect to unstimulated cells. Representative of $n = 3$ experiments.
FIGURE #11: Se-supplementation leads to increased production of 15d-PGJ₂. A, Se-supplemented or Se-deficient RAW264.7 (left panel) or BMDM macrophages (right panel) were stimulated with LPS for 0 and 12 h. The cell culture supernatants were quantitated by enzyme-linked immunosorbent assay for 15d-PGJ₂. The values were normalized to the total cellular protein levels (n = 3). B, dose-dependent effect of LPS on 15d-PGJ₂ production in BMDM. Media supernatant from Se-supplemented BMDM stimulated with LPS (0–1 µg/ml) for 12 h was used in the quantitation of 15d-PGJ₂. C, 15d-PGJ₂ production in mice. Serum levels of 15d-PGJ₂ in Se-deficient and Se-
supplemented mice (n = 3 in each category) were quantitated by enzyme-linked immunosorbent assay before and after intraperitoneal injection with LPS or PBS for 6 h. 

a and b represent p < 0.005 compared with Se-deficient and Se-supplemented at t = 0, respectively. 

D, LC-MS profiles of 15d-PGJ₂. Top panel: selective ion monitoring (M-H⁺) at 317 m/z in extracellular culture media from Se-supplemented RAW264.7 cells; middle and bottom panels: scan (200–400 m/z) of authentic 15d-PGJ₂(d4) and 15d-PGJ₂(d0) with the spectrum showing the fragmentation of the parent ions 321 m/z (M-H⁺) and 317 m/z (M-H⁺) for 15d-PGJ₂(d4) and 15d-PGJ₂(d0), respectively.
FIGURE#12: Modification of IKKβ subunit in Se-supplemented RAW264.7 cells by 15d-PGJ2. A, lysates from Se-deficient and Se-supplemented RAW264.7 cells, stimulated with LPS for 0–2 h, were used to analyze the modification of IKKβ by 15d-PGJ2 in immunoprecipitation experiments followed by Western immunoblotting. The polyvinylidene difluoride membrane was stripped and reprobed with anti-IKKβ to normalize for protein loading (n = 2). B, the Se-deficient and Se-supplemented RAW264.7 cells were pretreated with [14C]arachidonic acid (1 µCi and 30 µM) for 2 h prior to stimulation with LPS for 1 h. The cell lysates were used to isolate IKKβ by immunoprecipitation and analyzed on an SDS-PAGE followed by autoradiography (n = 2). C, Mass spectrometric analysis of the interaction of Me2SO, authentic 15d-PGJ2, and total lipid extract from LPS-treated Se-supplemented macrophages with the IKKβ peptide.
Chapter 3

The Role of Cyclooxygenase-1 and Hematopoietic Prostaglandin D\textsubscript{2} Synthase in the Biosynthesis of 15-Deoxy-$\Delta^{12}$, 14-Prostaglandin J\textsubscript{2} in Selenium-supplemented Macrophages

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ABSTRACT

Arachidonic acid (AA) is converted via COXs to PGH₂ followed by immediate and delayed synthesis of PGs consistent with the differential expression of COX isoforms and PG synthases. COX-1 activity is constitutive, present in nearly all cell types at a constant level; COX-2 activity is normally absent from cells, and when induced, the protein levels increase and decrease in a matter of hours after a single stimulus. A series of recent findings have highlighted the complexity of the PG biosynthetic machinery, suggesting a segregated functional coupling of distinct PG biosynthetic enzymes to the COX isoforms even when both COX enzymes are coexpressed within the same cell, and when both immediate and delayed inflammatory responses occur. H-PGDS has been shown to exhibit unique COX coupling profiles among various terminal PGSs. Interestingly, results from chapter 2 suggest that Se-supplementation of activated macrophages causes a dramatic shift in AA metabolism from a pro-inflammatory PGE₂ to an anti-inflammatory PG, 15d-PGJ₂, which down-regulates pro-inflammatory cytokine expression by down regulating the activation of NF-κB. To understand the contribution of COX1/2 in the enhanced production of 15d-PGJ₂ in Se-supplemented macrophages, inhibition studies were done. Experiments using specific COX inhibitors and genetic knockdown approaches indicated that COX-1, and not the COX-2 pathway, was responsible for the increased synthesis of 15d-PGJ₂ in Se-supplemented macrophages. Along with COX-1, a terminal prostaglandin synthase, HPGDS, also played in important role in the production of 15d-PGJ₂ in Se-supplemented macrophages. The results also indicated that the anti-inflammatory activities of Se were mediated by the 15d-PGJ₂ dependent activation of peroxisome proliferator activating receptor-γ in macrophages apart from inhibiting IKKβ
activity. Taken together, these results suggest that COX-1 along with H-PGDS act synergistically leading to the enhanced production of 15d-PGJ₂ in Se-supplemented macrophages.
INTRODUCTION

As an essential component of selenoproteins, Se is involved in cellular antioxidant defense by reducing semistable hydroperoxides to less reactive alcohols [31]. The most well characterized selenoenzymes belong to the families of glutathione peroxidases (GPX) and thioredoxin reductase (TR) [21]. The ability of these selenoenzymes to act on a wide variety of ROS has led to the recognition of Se in human health and development as an important dietary antioxidant [22]. In addition to its general antioxidant defense role, Se could play the more specific role of modulating the enzymatic oxidation of arachidonic acid by way of the COX and LOX pathways [22, 142]. Modification of the level of dietary Se has been shown to affect prostaglandin synthesis in human and animal studies [135, 138, 140, 141]. Recent studies in our laboratory have shown that Se-supplementation shifts arachidonic acid away from pro-inflammatory PGE₂ to anti-inflammatory 15d-PGJ₂ [192].

Prostaglandin synthesis is complex with a number of potential rate-limiting steps. First, arachidonic acid is released by the action of one or more phospholipase A₂ (PLA₂) enzymes, and secondly, either COX-1 or COX-2 converts arachidonic acid to prostaglandin H₂ (PGH₂) [105]. PGH₂, is then converted by several terminal synthases to the major active prostanoids produced in vivo, PGD₂, PGE₂, PGF₂α, prostacyclin (PGI₂) and thromboxane (TXA₂) in a cell- and tissue-specific manner [193]. These terminal synthases include PGE synthase (PGES) for PGE₂, PGDS for PGD₂, PGFS for PGF₂α and PGIS for PGI₂, respectively[193]. PGD₂ gives rise to the important derivatives 9α,
11β PGF₂ [194] and the J series PGs including PGJ₂, Δ₁₂PGJ₂ and 15deoxy- Δ₁₂,₁₄PGJ₂ (15d-PGJ₂), the latter through a series of non-enzymatic steps [195]. Based on the data presented in chapter 2, the Se-dependent production of 15d-PGJ₂ in-vitro and in-vivo suggest skewing of the PG synthetic pathway by Se. This could be due to the differential regulation of PGDS and mPGES-1.

Prostaglandin E Synthase, which converts COX-derived prostaglandin PGH₂ to PGE₂, occurs in multiple forms with distinct enzymatic properties, modes of expression, cellular and subcellular localizations and intracellular functions. To date, three different genes with PGES activity have been cloned [196]. The first PGES, mPGES1, was isolated as a microsomal protein and is a member of the MAPEG (for membrane-associated proteins involved in eicosanoid and glutathione metabolism) superfamily [197, 198]. mPGES-1 expression is low in most normal tissues, although abundant and constitutive expression is detected in a limited number of organs, such as the lung, kidney, and reproductive organs. Interestingly, mPGES1 expression is increased in a number of cancers, including lung, gastric, and colorectal tumors, similar to that observed for COX-2 [199, 200]. Additionally, the coordinated inducibility of COX-2 and mPGES-1 by proinflammatory factors and their efficient cooperation in converting AA to PGE₂ in vitro [103] has led to the generally accepted model that these two enzymes are preferentially coupled for PGE₂ biosynthesis.

Prostaglandin D₂ synthase is the key terminal synthase involved in the conversion of PGH₂ to PGD₂. Two distinct forms of PGDS, L-PGDS and H-PGDS have been cloned
and characterized [201]. L-PGDS is mainly involved in central nervous system including sleep induction, body temperature, and analgesia [201]. H-PGDS is widely distributed in peripheral tissues [201] and is a key enzyme for the production of PGD₂.

Each of these prostanoids acts through specific cell surface prostanoid receptors [202]. Multiple receptor subtypes exist for several of these PGs. For instance, there are four cell surface receptor subtypes, EP1–EP4 that can bind PGE₂, whose differential expression can alter tissue-specific responses to the PG. The binding of PGE₂ to the one of the EP receptor leads to the G-protein-dependent signal cascade culminating in a pro-inflammatory response, at least in a macrophage cell type. Similarly, binding of TP receptor by TXA₂ also causes an inflammatory response. Uniquely, 15d-PGJ₂ [173, 203], and possibly ∆₁²PGJ₂ [204], mediate anti-inflammatory and anti-proliferative effects which may be through direct actions on specific nuclear receptor and transcription factors, the peroxisome proliferator activated receptor-gamma (PPAR-γ) [123]. PPARγ ligation by 15d-PGJ₂ may induce PPAR dissociation from co-repressors and permit interaction with co-activators, resulting in translocation from the cytoplasm to the nucleus [122, 129]. This results in the expression and/or repression of a variety of genes whose promoters contain PPAR-response elements (PPRE). Alternatively, 15d-PGJ₂ has been shown to act via PGD₂ receptors (DP₁ and DP₂) [205]. Studies indicate that 15d-PGJ₂ has a weak agonist activity on DP₁ receptor [205]. It is known that 15d-PGJ₂ has higher binding affinity to DP₂ receptor [205]. A third possible interaction of 15d-PGJ₂ in cells is further supported by many studies, including those from our laboratory that indicates the covalent interaction of 15d-PGJ₂ with intracellular proteins like IKKβ and p50
subunit of NF-κB [128]. Thus, while COX isoforms catalyze the first committed step of the prostanoid biosynthesis pathway, terminal PG synthases commit the pathway to effects.

Phospholipase A2 mediated release of AA is followed by immediate or delayed PG production pathways, depending on the presence of COX-1 and COX-2 and expression of various PG synthases [125]. COX-1 activity is constitutive, present in nearly all cell types at a constant level [92, 93]. In contrast, with the exception of some organs such as the kidney, testis, and the central nervous system, COX-2 expression is extremely low in most normal tissues and is induced by growth factors, cytokines, and pro-inflammatory stimuli [92, 93]. High COX-2 expression is also associated with pathological conditions, such as tissue damage and malignant transformation of gastrointestinal and mammary epithelium [206, 207]. The main reason for labeling COX-1 and COX-2 as physiological and pathological, respectively, is that most of the stimuli known to induce COX-2 are those associated with inflammation, for example, bacterial lipopolysaccharide (LPS) and cytokines such as interleukin (IL)-1, IL-2, and TNF-α. The physiological roles of COX-1 have been deduced from the deleterious side effects of of nonselective NSAIDs, which inhibit PG biosynthesis at inflammatory sites, in addition to inhibiting constitutive biosynthesis. Thus, COX-1 provides PGs in the stomach and intestine to maintain the integrity of the mucosal epithelium and its inhibition leads to gastric damage, hemorrhage, and ulceration. Kirtikara et al. demonstrated that cultured lung fibroblasts from mice bearing targeted deletions of either COX enzyme exhibit compensatory increases in the opposite COX isoform activity [208] and with a recent report by Wang et al. also demonstrated compensatory activity of COX-1 in the uteri of CD-1 COX-2−/−
female mice [209]. These studies suggest that COX-1 plays an important role in the
biosynthesis of PGs at the low concentrations or in the absence of COX-2 expression.
A series of recent findings highlighted the complexity of the PG biosynthetic machinery,
suggesting a segregated functional coupling of distinct PG biosynthetic enzymes to the
COX isoforms even when both COX enzymes are coexpressed within the same cell, and
when both immediate and delayed inflammatory responses occur [210]. H-PGDS has
been shown to exhibit unique COX coupling profiles among various terminal PGs.
Preferred utilization of COX-1 by H-PGDS is manifested by the observations that
A23187 stimulation of macrophages resulted in the production of PGD$_2$ in preference to
those of PGE$_2$ and PGI$_2$ [104] and that IgE/antigen-dependent immediate production of
PGD$_2$ by mast cells entirely depended on COX-1 [190]. Consistently, reconstitution
experiments in HEK293 cells confirmed that H-PGDS preferentially utilizes COX-1
[108] and mPGES-1 preferentially utilizes COX-2 [103, 104, 108]. Thus, the COX-1 and
COX-2-dependent pathways may be more selectively linked to the terminal H-PGDS and
the terminal mPGES-1 respectively. The present study is based on the hypothesis that
COX-1 and H-PGDS play an important role in shunting AA pathway towards anti-
inflammatory PG, 15d-PGJ$_2$ away from pro-inflammatory PG, PGE$_2$ in macrophages that
are supplemented with Se. As in the preceding chapters, these studies further strengthen
the hypothesis that the anti-inflammatory properties of Se are mediated through 15d-PGJ$_2$
via the COX-1 dependent pathway.
MATERIALS AND METHODS:

Reagents:

Bacterial endotoxin lipopolysaccharide (LPS; Escherichia coli Serotype 0111:B4), sodium selenite (Na₂SeO₃), and GW9662 were from Sigma. The following inhibitors, indomethacin, SC-560 (for COX-1), CAY10404 (for COX-2) and HQL-79 (for H-PGDS) and anti-COX-1 were obtained from Cayman Chemicals (Ann Arbor, MI). Antibody for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Se-GPX1 was purchased from Abcam, Inc. (Cambridge, MA); while Anti-SelP was purchased from BD Biosciences Pharmingen, San Diego, CA. Goat anti-rabbit IgG and anti-mouse IgG conjugated to horseradish peroxidase, polyvinylidene difluoride (PVDF), and West Pico chemiluminescence reagents were purchased from Thermo-Pierce(Rockford, IL).

Cell Culture and Stimulation:

The murine macrophage cell line RAW264.7 (ATCC) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% defined fetal bovine serum (HyClone), 80 µg/ml gentamicin, and 2 mM L-glutamine (Invitrogen) at 37 °C with a 5% CO₂/air mixture. Total Se in the fetal bovine serum was quantitated to be 6 pmol/ml by absorption photometry using Na₂SeO₃ as a standard. Cells were cultured in DMEM either supplemented with Se (Na₂SeO₃; 0.05-2 nmole/mL as Se) or without any added as described from our laboratory [145]. Cell viability and growth rates of Se-supplemented cells were similar to their Se-deficient counterparts. The enzymatic activity was used as a marker of cellular Se status [21]. About 1 x 10⁶ Se-deficient and Se-supplemented cells
were seeded in a 6-well plate, and then cultured in respective media for ~24 h to allow the cell number to approximately double. Cells were stimulated with LPS (0–1 µg/ml) and/or other compounds for the indicated time periods. Upon treatment, the cells were harvested, washed with cold sterile PBS and stored at −80 ºC until further use. Femoral bone marrow plugs, from mice maintained on a Se-deficient or a Se-supplemented diet, were isolated, and adherent cells, hereafter referred to as primary bone marrow-derived macrophages (BMDMs), were differentiated in their respective media containing 20% L929 fibroblast media supernatant (as a source of granulocyte-macrophage colony-stimulating factor) for 1 week. The L929 cells were also cultured under Se-deficient or Se-supplemented conditions. The Se-deficient and Se-supplemented diets were formulated based on an American Institute of Nutrition recommended rodent diet containing 0.01 or 0.4 ppm of Se, as described [174]. The diets were purchased from Zeigler (Gardners, PA). The BMDM cultures from Se-deficient and Se-supplemented mice were used in all experiments.

**Preparation of Cell Lysates:**

The frozen cell pellet was resuspended in 50 µl of mammalian protein extraction reagent (M-PER, Pierce) containing 1 mM EDTA, 10 µM leupeptin, and 1 mM phenylmethylsulfonic acid for 30 min on ice with intermittent vortexing. Supernatants were prepared by centrifuging the cell lysate at 10,000 x g for 15 min at 4 ºC and used for analyses or stored frozen at -80 ºC. Protein concentration in the cell supernatants was determined by BCA protein assay using bovine serum albumin (125-2000 µg/ml) as standard (Pierce).
**Electrophoresis and Immunoblotting:**

Thirty micrograms of protein from RAW264.7 or BMDM cell lysates was separated on a 12.5% SDS-polyacrylamide gel and transblotted onto PVDF as described [176]. The membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 and 5% skim milk (w/v). The membrane was probed with primary antibody followed by an appropriate secondary antibody coupled to horseradish peroxidase. The bands were visualized by enhanced chemiluminescence (ECL) assay kit (Pierce) according to the manufacturer’s instructions.

**In Vitro Kinase Assays:**

Cell lysates for *in vitro* kinase assays were prepared using the lysis buffer as described [177]. In the case of IKK, the cell lysate (~30–50 µg of protein) was incubated with \[^{32}P\]ATP (2 µCi, Amersham Biosciences) and GST-\(\text{IκB}\alpha\) fusion protein for 1 h in the kinase wash buffer (50 mM Tris-Cl, pH 8.0, containing 100 mM NaCl, 10 mM MgCl\(_2\), 1 mM dithiothreitol, 10 mM β-glycerophosphate, 10 mM NaF, and 1 mM sodium vanadate) at 25 °C as described [177]. The reaction mixture was subjected to gel-filtration chromatography using the Bio-Gel P30 pre-packed columns (Bio-Rad). The flow-through, which contained the GST-[^32P] IκBα, was subjected to liquid scintillation counting.

**Chemical Treatments:**

Selenium-deficient and Se-supplemented RAW264.7 and BMDM cells (1 x 10^6) were seeded into each well of a 6-well plate and treated with 1 mM indomethacin or SC-560 to
inhibit COX-1. 10 nM CAY10404 was used as a COX-2-specific inhibitor. In all cases, the treatment with inhibitors (for 24 h) was followed by stimulation with LPS for the indicated time periods. The cell lysates were prepared as described earlier and subjected to Western immunoblot analyses, in vitro kinase assays, while the media supernatants were used for 15d-PGJ2 assays. An irreversible PPARγ antagonist, 2-chloro-5-nitro-N-phenylbenzamide (GW9662, 1 µM) was used in some studies to determine the specific role of 15d-PGJ2-dependent activation of PPARγ. The cells were pretreated with GW9662 for 12 h prior to LPS stimulation. To inhibit the activity of H-PGDS, Se-supplemented cells were pretreated with 50 µM 4-(diphenylmethoxy)-1-[3-(1H-tetrazol-5-yl)propylpiperidine] (HQL-79, Cayman), prior to LPS stimulation for 12 h. Supernatants were used in 15d-PGJ2 analysis as described below. All chemical treatment studies included an appropriate Me2SO4 vehicle control.

**Glutathione Peroxidase Activity Assay:**

The cytosolic Se-GPx activity was measured according to the published method using H2O2 as substrate [175]. Primary bone marrow-derived macrophages and RAW264.7 macrophages were cultured in the presence or absence of Se. After a week of Se-supplementation cells were harvested, lysed and microfuged for 10 minutes. The resulting supernatant was used in enzyme analysis. The oxidation of NADPH was monitored spectrophotometrically at 340 nm following the addition of 3mM H2O2. For comparison of individual treatments, the Se-GPX activities were expressed as nmoles of NADPH oxidized per minute per milligram of protein and the values are given as the
mean of three experiments. The total protein levels were measured using the BCA reagent (Pierce, Rockford, IL.) as described earlier.

**Quantitation of 15d-PGJ2:**

A 96-well-based enzyme immunoassay kit from Assay Designs (Ann Arbor, MI), was used to quantitate 15d-PGJ2. The concentrations of 15d-PGJ2 in culture media supernatants of RAW264.7 and BMDM in the presence or absence of LPS (0–1 µg/ml) for various time periods were determined by enzyme immunoassay according to the manufacturer's instructions and normalized to total cellular protein. Standard calibration curves were prepared using 15d-PGJ2 and fitted to a log-linear, logit, multiway frequency regression analysis. 15d-PGJ2 were quantitated in cell lysates and normalized to total protein in the cell lysates.

**siRNA Experiments:**

The siRNA target sequence for murine COX-1 mRNA was designed using the Dhharmacon siGENOME™ design tool available online. The 21-base siRNA oligonucleotides were obtained from Dhharmacon and annealed according to the manufacturer's specifications. For transfection, RAW264.7 or BMDM was seeded into 6-well plates (1 x 10^6 cells/well) without gentamicin. After 24 h, cells were transfected with siRNA duplex using TransIT®-siQUEST™ (Mirus Bio Corp.) according to the manufacturer's specifications. siRNA duplexes were used at a final concentration of 200 pmol/well. To ensure maximum effect, a second transfection was performed after 4 h, and the cells were allowed to recover for an additional 8 h before treatment with LPS. Cells
and media supernatants from LPS-treated (12 h) or untreated cells were collected for 15d-PGJ2 quantitation, whereas the corresponding cell lysates were used for IKKβ activity and Western immunoblot analyses.

**Preparation of Nuclear Extracts for Electrophoretic Mobility Shift Assay:**

For electrophoretic mobility shift assay experiments, nuclear proteins were isolated as described previously from our laboratory [176]. The DNA sequences of the sense strand of double-stranded oligonucleotides specific for NF-κB and PPARγ were 5'-GATCCAGTTGAGGGGACTTTCCCAGGC-3' and 5'-GGTGAGGAGGGGAAGGGTCAGTGTG-3', respectively. Complementary strands were annealed, and double-stranded oligonucleotides were labeled with $[\gamma^{32}P]ATP$ (3000 Ci/mmol and 10 mCi/ml) using the T4 polynucleotide kinase (New England Biolabs). Five micrograms of nuclear proteins was incubated for 10 min at 4 °C in a binding buffer for NF-κB (20 mM Tris-HCl, pH 7.9, 5 mM MgCl2, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 20% glycerol) or PPARγ (20 mM HEPES, pH 7.5, 50 mM KCl, 0.175 mM EDTA, and 5% (v/v) glycerol) in the presence of 2 μg of poly(dI-dC). The extracts were then incubated for 30 min at 4 °C with 10,000 cpm of $^{32}$P-labeled NF-κB or PPARγ probes. The samples were analyzed as described earlier from our laboratory. NF-κB or PPARγ bands were confirmed by competition with a 100-fold excess of the respective unlabeled probe.
Statistics:

All enzymatic assays and ELISAs were performed in triplicates unless otherwise noted and values represented as the mean (+SD). Statistical significance is denoted by an asterisk (*) when p values are <.05, as calculated by a student’s t test.
RESULTS

GPX-1 expression in macrophages and in livers of Se-deficient and Se-supplemented mice as a function of cellular Se status:

Culturing of RAW264.7 cells in the presence or absence of Se and primary BMDMs isolated and differentiated from mice maintained on Se-deficient and Se-supplemented diets, yielded cell populations that exhibited differential Se status as seen by SelP expression levels (Fig. 14A&B). The expression of SelP levels were significantly different in the two groups in that the Se-supplemented cells demonstrated a higher level of SelP expression compared to the Se-deficient cells. Furthermore the expression of Se-GPX was measured in cell lysates from RAW 264.7 cells that were supplemented with different concentrations of Se as a marker for Se status. The Se-GPX expression that was almost non-existent in Se-deficient media was significantly increased in the deficient cells upon supplementation with Se (Fig. 15). The expression was saturated at 100 nM of Se and remained that way even at high concentrations. Furthermore, GPX1 enzyme activity assays performed on these cells complemented the immunoblot results in that the activity increased with increase in Se concentrations (Fig. 16). Much like the expression results, the activity also was saturated around 100 nM, beyond which there was hardly any increase. A ~10-fold increase in activity was noticed in cells that were supplemented with 100 nM Se. In addition, the expression of GPX1 was also analyzed in the homogenates of livers isolated from mice that were maintained on Se-supplemented and Se-deficient diets. The results indicated a significantly increased expression of GPX1 in the livers obtained from Se-supplemented mice compared to Se-deficient mice (Fig. 17).
Contribution of COX-1 and COX-2 to the biosynthesis of 15d-PGJ_2 in the Se-supplemented macrophages:

The fact that COX-2 is overexpressed in Se-deficiency, but 15d-PGJ_2 levels are not increased and IKKβ expression is not affected [145] is compelling evidences that COX-1 activity may contribute to the formation of 15d-PGJ_2. To address this, we utilized selective inhibitors of each of these enzymes. The use of CAY10404, a COX-2-selective inhibitor, at 10-fold higher concentration than the reported IC_{50} values, had no effect on the production of 15d-PGJ_2 in LPS-treated Se-supplemented cells (Fig. 18A). In contrast, the use of indomethacin, as a COX-1 inhibitor at 1 µM, lower than the IC_{50} for COX-2, completely inhibited the production of 15d-PGJ_2 in Se-supplemented cells upon treatment with LPS for 12 h. Identical results were also obtained with another COX-1-selective inhibitor, SC-560 (data not shown). These results suggested that COX-1 plays an important role in the production of the anti-inflammatory 15d-PGJ_2. As confirmation, we used siRNA technology to effectively knockdown the expression of COX-1 in Se-deficient and Se-supplemented BMDM cells (Fig. 18B). The knockdown of COX-1 was confirmed by Western immunoblotting using COX-1-specific polyclonal antibodies (Fig. 18B). LPS stimulation of COX-1 siRNA-transfected Se-supplemented cells completely inhibited the production of 15d-PGJ_2, similar to that observed with the Se-deficient cells. Studies performed with RAW264.7 cells also exhibited similar results as that with the BMDM cells (data not shown).
**Contribution of COX-1 to the 15d-PGJ2-dependent regulation of IKKβ activity in Se-supplemented and Se-deficient macrophages:**

To further investigate the contribution of COX-1 to the anti-inflammatory functions of Se, the effect of SC-560 on the activity of IKKβ in Se-supplemented or Se-deficient RAW264.7 cells was tested by *in vitro* kinase assays following the pulldown of IKKβ. As shown in Fig. 19A, the results clearly indicate that IKKβ activity was increased in LPS-stimulated Se-supplemented cells treated with SC-560. In addition, gel mobility shift assays performed in Se-supplemented and Se-deficient cells indicated that NF-κB activity in SC-560-treated Se-supplemented cells was increased when compared with the untreated (or vehicle-treated) Se-supplemented cells (Fig. 19B). These results unequivocally confirm that, in Se-supplemented cells, where the expression of COX-2 is significantly lower, COX-1 plays an important role in the control of gene expression via the generation of 15d-PGJ2, an endogenous activator of anti-inflammatory pathways, targeting such transcription factors as NF-κB and PPARγ.

**Anti-inflammatory effects of 15d-PGJ2 mediated by PPARγ-dependent mechanisms:**

We tested the activation of PPARγ as a possible anti-inflammatory pathway in the context of cellular Se status. Gel mobility shift assays with a consensus double-stranded PPARγ oligonucleotide showed that the Se-supplemented cells demonstrated increased binding activity upon stimulation with LPS; whereas the binding in Se-deficient cells was significantly lower (Fig. 20A). Incubation of Se-supplemented cells with SC-560 followed by stimulation with LPS significantly decreased the translocation and binding activity of PPARγ. This was further confirmed in experiments with GW9662, a PPARγ
antagonist at 1 μM, which clearly demonstrated that the repression of COX-2 expression was also mediated by ligand-dependent activation of PPARγ in Se-supplemented macrophages (Fig. 20B). Taken together, these results indicate that the anti-inflammatory effect of Se is mediated, in part, by 15d-PGJ2 via IKKβ- and PPARγ-dependent mechanisms dependent mechanisms that can impact the transcription of genes by the NF-κB pathway. In addition, activation of PPARγ by 15d-PGJ2, can modulate the expression of terminal PG synthases.

**PGD\(_2\)** production as a function of cellular Se status:

To examine if Se has any effects on the production of PGD\(_2\) and to examine if a substrate-product relationship existed between PGD\(_2\) and 15d-PGJ2 in Se-supplemented macrophages, PGD\(_2\) formed in the culture media supernatant of cells was quantified as a function of time post LPS stimulation (0-12 h). Results clearly demonstrate that, with in the first 6 h of LPS treatment, PGD\(_2\) levels increased significantly with a peak ~6 h and a decline at ~8 h (Fig. 22). In contrast 15d-PGJ2 levels began to increase after 8 h of LPS treatment.

**H-PGDS contributes to the anti-inflammatory effects of Se:**

While the preceding results demonstrated the importance of COX-1 in the decreased expression of pro-inflammatory genes, in 15d-PGJ2 dependent way, we also investigated the role of H-PGDS, a terminal PG synthase, involved in the production of PGD\(_2\), which undergoes two spontaneous non-enzymatic dehydration reactions to form 15d-PGJ2 [112, 113]. Incubation of Se-supplemented macrophages with HQL-79 completely inhibited the
increase in 15d-PGJ₂ production (Fig. 22A). To further investigate the contribution of H-PGDS to the anti-inflammatory functions of Se, the effect of HQL-79 on the expression of COX-2 in Se-supplemented or Se-deficient RAW264.7 cells was tested. The results clearly indicate that COX-2 expression was increased in LPS-stimulated Se-supplemented cells treated with HQL-79 (Fig. 22B). These results indicate that in conjunction with COX-1, H-PGDS is as well very essential for the biosynthesis of 15d-PGJ₂ in Se-supplemented macrophages.
DISCUSSION

Prostaglandins are COX products derived from C20 unsaturated fatty acids such as arachidonic acid. While COX-1 and -2 exhibit very subtle differences in their structure and catalytic activity [211-213], they differ in terms of their transcriptional regulation, expression [213] and activation of the catalytic apparatus by pro-oxidant lipid hydroperoxides [214, 215]. Consistent with these varying expression and activation kinetics, two patterns of PG synthesis, immediate and delayed PG synthesis have been recognized [125, 213]. Recent studies have suggested that both COX-1 and COX-2 may have distinct roles in cellular functions with respect to the secretion of inflammatory cytokines like TNFα [216, 217]. Interestingly, the contribution of COX-1 to delayed PG synthesis has been recently shown to impact the autocrine regulation of TNFα secretion [216]. Se-deficient cells express COX-2 even prior to LPS stimulation, whereas its expression is attenuated in Se-supplemented cells [145]. Under such circumstances, the role of COX-1 in AA metabolism becomes even more important. Using indomethacin, SC-560, and COX-1 siRNA, we have conclusively shown that COX-1 plays an important role in the activation of NF-κB and consequent expression of pro-inflammatory genes. Our results indicate that AA is preferentially utilized by COX-1, even in the presence of reduced levels of COX-2 in Se-supplemented cells, to eventually produce the anti-inflammatory 15d-PGJ2. Based on our results, we believe that 15d-PGJ2 is converted via its precursor, PGD2, which suggests a Se-dependent regulation of the H-PGDS as an important determinant in this process. It has been shown that COX-2 can functionally couple with mPGES-1 to regulate the production of PGE2 [103]. Similarly, gain-of-
function studies in HEK293 cells have shown that COX-1 and -2 can couple with H-PGDS [108]. Studies with HQL-79 clearly indicate that H-PGDS is involved in the increased production of 15d-PGJ2 (Fig. 22A). Thus, preferential and functional interaction of COX-1 with H-PGDS in Se-supplemented macrophages, as opposed to COX-2 and mPGES-1 in Se-deficiency, could also determine the fate of AA toward resolution as a function of cellular Se status. This observation could have great biological significance given that the balance between the interaction of COX-2 with H-PGDS and mPGES-1 is a major determinant of atherosclerotic plaque instability in humans [191]. Taken together, it is plausible that, in Se-supplemented cells, COX-1 mainly serves as an enzyme for the synthesis of 15d-PGJ2, which, perhaps, aids in the transcriptional regulation of downstream PG synthases and their interaction with specific COX isozymes.

Results of the in vitro kinase assays indicate that increased levels of 15d-PGJ2 inhibits the activity of IKK in Se-supplemented macrophages leading to the decreased activation of NF-κB, which could be reversed by inhibition of COX-1. In contrast, treatment of Se-deficient cells with COX-1 inhibitor neither affected the activity of IKK or NF-κB activation nor had any effect on the basal levels of 15d-PGJ2 (Fig. 19A&B). On the other hand, the nuclear translocation of PPARγ clearly showed reduced levels of binding in the absence of COX-1 activity (Fig. 20A). Inhibition of PPARγ using GW9662 increased the expression of COX-2 even in the Se-supplemented cells suggesting that 15d-PGJ2-dependent activation of PPARγ also plays an important role in the modulation of NF-κB-dependent transcription of genes.
Figure#13: A schematic model for the possible functional coupling between cyclooxygenases and PG synthases during differential cellular Se status. In the LPS stimulated Se-deficient macrophages AA rapidly released by cPLA2 is metabolized to PGE\(_2\) via inducible COX-2 and mPGES-1. In Se-supplemented macrophages, induced by pro-inflammatory stimuli, AA gradually released by PLA2 is metabolized to PGD\(_2\) via constitutive COX-1 and H-PGDS. With in the cells, PGD\(_2\) metabolizes to 15d-PGJ\(_2\), which inhibits NF-κB dependent pro-inflammatory gene expression via two pathways: i) By directly inhibiting IKKβ and ii) By activating PPAR\(γ\).
In summary, these data argues that COX-1 along with H-PGDS contributes to the shift in the metabolism of AA from pro-inflammatory PGE$_2$ to anti-inflammatory 15d-PGJ$_2$ in Se-supplemented macrophages. This shift in the PG production profile in part may be regulated by temporal changes in the expression levels of downstream PG synthases, m-PGES and H-PGDS, under circumstances of altered Se-nutrition. In addition we show that apart from interacting with IKKβ, 15d-PGJ$_2$ acts via PPARγ to transrepress NF-κB-dependent pro-inflammatory gene expression as described in the literature.
Figure#14: Expression of SelP in BMDM and RAW264.7 macrophages. Primary bone marrow-derived macrophages and RAW264.7 macrophages were cultured in the presence or absence of Se for ~2 weeks. The cells were lysed and analyzed by Western immunoblotting with anti-SelP or anti-GAPDH. Equal protein was loaded for each cell type. Copyright: http://www3.interscience.wiley.com/journal/117935711/grouphome
Figure#15: Expression of GPX1 in Se-deficient RAW264.7 macrophages as a function of exogenous addition of Se. Se-deficient RAW264.7 cells were treated with indicated concentrations of Se (as Na$_2$SeO$_3$) for 7 days. Anti-GPX1 was from Abcam. Densitometric values normalized to GAPDH are presented below each panel. Representative of $n=2$ shown. Copyright: http://www3.interscience.wiley.com/journal/117935711/grouphome
Figure#16: Effect of repletion of Se-deficient RAW 264.7 macrophages with Se on GPX activity. Se-deficient cells cultured in media containing 6 nM Se were repleted with different amounts of Se (as Na$_2$SeO$_3$) for 7 days. GPX activity, with H$_2$O$_2$ as substrate, was assayed in the cell lysates. The specific activity is expressed as µmole NADPH/min/mg protein. Representative of $n=3$.  

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Figure 17: Expression of GPX1 in the livers of Se-deficient and Se-supplemented mice. C57/BL6 male mice were maintained on Se-deficient (0.01 ppm Se) or Se-supplemented (0.4 ppm Se) diets for ~3 months. The mice were sacrificed and livers were isolated for analysis. Expression of GPX1 in the liver homogenates was performed using the anti-GPX1 antibodies. The expression of GAPDH was used as a control to normalize protein loading. Blots shown are representative of $n=4$ in each category.

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Figure 18: Inhibition of COX-1 in Se-supplemented macrophages reduces 15d-PGJ$_2$ levels. A, Se-deficient and Se-supplemented RAW 264.7 macrophages were pretreated with Me$_2$SO or indomethacin (1 µM) or CAY10404 (10 nM) for 24 h followed by stimulation with LPS for an additional 12 h. B, Se-deficient and Se-supplemented BMDM were transfected with either COX-1 siRNA duplexes or the siQuest reagent alone following which LPS stimulation for 12 h was carried out. The cell lysates were used to determine the levels of COX-1, whereas the cell culture media supernatants were used to quantify 15d-PGJ$_2$. The values were normalized for total cellular protein.
Figure 19: COX-1 inhibition reverses the IKKβ activity in Se-supplemented macrophages: A, Se-supplemented or Se-deficient RAW264.7 cells were pretreated with Me₂SO or SC-560 for 24 h following which they were stimulated for 2 h with LPS. The IKKβ isolated from the cell lysates were used in in vitro kinase assays with [γ-³²P] ATP and GST-IκBα. B, gel mobility shift analysis demonstrating the increased NF-κB translocation and binding in Se-deficiency and SC-560-treated Se-supplemented RAW264.7 cells. Results shown are representative of n = 3 experiments.
Figure#20: Se-supplementation activates PPARγ via the production of 15d-PGJ₂.

A, gel mobility shift analysis demonstrating the PPARγ translocation and binding in Se-supplemented cells and SC-560-treated Se-supplemented RAW264.7 cells. B, Se-supplemented and Se-deficient RAW264.7 cells were pretreated with GW9662 (1 µM) followed by stimulation with LPS for 2 h. The cell lysates were analyzed for the expression of COX-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by Western immunoblot analysis. Results shown are representative of n = 2 experiments.
FIGURE#21: Modulation of PGD$_2$ and 15d-PGJ$_2$ levels in Se-supplemented macrophages. Comparison of the production of PGD$_2$ and 15d-PGJ$_2$ in Se-supplemented BMDM. Culture media supernatants from LPS (1 µg/ml, 12 h)-treated cells were used to quantitate PGD$_2$ and 15d-PGJ$_2$ using enzyme-linked immunosorbent assay. Results were normalized to the amount of total cellular protein. *Inset:* production of PGD$_2$ at early time points (0–4 h).
Figure 22: H-PGDS contributes to the anti-inflammatory effects of Se. A, Effect of HQL-79 on the production of 15d-PGJ$_2$ in RAW264.7 macrophages. Cells were treated with 50 µM HQL-79 for 12 h followed by LPS stimulation for an additional 12 h. Results shown are representative of three independent experiments. B, Effect of H-PGDS inhibition on the expression of COX-2 in RAW264.7 macrophages. Cells were treated with 1 µM HQL-79 for 12 h followed by LPS stimulation for an additional 2 h. The expression levels of COX-2 were assessed by Western blot.
Chapter 4

Differential Regulation of Prostaglandin Synthases by Selenium via the Enhanced Production of 15-Deoxy-\(\Delta^{12,14}\)-PGJ\(_2\) in Macrophages: Contribution of Selenoproteins
ABSTRACT

Selenium is an essential micronutrient that participates in various physiological functions as an integral component of a range of selenoproteins. There is overwhelming evidence that indicates its therapeutic potential in many inflammatory diseases. However, little is known about the mechanism of anti-inflammatory action of Se. Particularly in macrophages, which play a central role in immunity, contributing to both the initiation and resolution of inflammation, extensive transcriptional reprogramming leads to the generation of reparative macrophages. Based on the studies described in the chapter 2, BMDMs isolated from mice supplemented with Se produce an endogenous prostanoid (15d-PGJ$_2$) that likely promotes resolution phase of inflammation. 15d-PGJ$_2$ is a high affinity ligand for the peroxisome proliferator-activated receptor (PPAR) $\gamma$. 15d-PGJ$_2$ represses several pro-inflammatory genes in activated macrophages, including the inducible iNOS and TNF$\alpha$ genes. This repression is suggested to be, in part, mediated through PPAR$\gamma$ activation. In addition, 15d-PGJ$_2$ is also an endogenous inhibitor of IKK$\beta$, a key kinase of the NF-$\kappa$B signaling cascade. Here we demonstrate that Se in the form of selenoproteins is essential for the production of 15d-PGJ$_2$ in cells adequate in selenoproteins. In addition, studies described here indicate a novel mechanism where by Se-supplementation causes the down regulation of the expression of microsomal PGE$_2$ synthase-1 and thromboxane A$_2$ synthase (TXS); while the expression of hematopoietic-PGD$_2$ synthase (H-PGDS) is significantly upregulated. The results presented here argue that incorporation of Se into selenoproteins causes the shunting of arachidonic acid towards 15d-PGJ$_2$ production, which further acts in a positive feedback loop via the
activation of PPARγ-dependent transcription of H-PGDS to cause shunting of the arachidonic acid towards 15d-PGJ2. More importantly, these studies demonstrate the role of PPARγ as an important regulator of transcription of H-PGDS in macrophages. In summary, studies in this chapter suggest the existence of a novel mechanism where selenoproteins play an important role in the resolution of inflammation by shunting the arachidonic acid metabolism towards anti-inflammatory 15d-PGJ2 from pro-inflammatory PGE2.
INTRODUCTION

Selenium is an essential micronutrient for all mammalian species and functions primarily through selenoproteins, which contain Se as selenocysteine (SeCys). SeCys is the 21st amino acid in the genetic code [70, 71] and, unlike other amino acids, the biosynthesis of SeCys occurs on its tRNA [25, 72, 73]. SeCys tRNA is initially aminoacylated with serine by seryl-tRNA synthetase and is therefore designated selenocysteine tRNA$^{[\text{Ser}]\text{Sec}}$. The active donor of Se that makes selenocysteyl-tRNA$^{[\text{Ser}]\text{Sec}}$ from the intermediate generated by SelA in bacteria is monoselenophosphate [76], which is synthesized by selenophosphate synthetase (SPS) from selenide and ATP. Selenophosphate appears to be the universal donor of Se in the biosynthesis of this amino acid [76, 77]. SPS is designated SelD in bacteria and SPS2 in mammals. Studies have shown that knockdown of SPS2 completely inhibits the selenoprotein synthesis [80]. Any chemical form of Se should be transformed metabolically to the assumed common intermediate, hydrogen selenide (H$_2$Se) before being incorporated into selenoproteins [68]. H$_2$Se provides Se for the synthesis of selenoproteins after activation to selenophosphate via SPS2 [68].

Incorporation of Se into selenoproteins is crucial for the important functions in eukaryotic cells, including macrophages [24, 69]. These selenoproteins have different physiological functions, but for those in which the function is known, SeCys is located at the active center [23-26]. The well-characterized selenoenzymes include the Se-dependent glutathione peroxidases (Se-GPXs) and thioredoxin reductase (TR) families, the activities of which are responsible for the recognition of Se as an important dietary antioxidant [21]. These enzymes are involved in many biochemical processes including protection
against oxidative stress and redox-based regulation of gene expression [22]. Se-deficiency results in a significant decrease in Se-GPX and TR, and an increase in reactive oxygen species (ROS) production [21, 22]. Inadequate Se nutrition and associated ROS production have been linked to increased risk of such diseases as cardiomyopathy [38], rheumatoid arthritis [39], and, most notably, cancer [41, 42]. Recent studies have also established Se as a potential regulator of transcription [22]. We have demonstrated that Se can counteract lipopolysaccharide-induced oxidative stress-dependent expression of pro-inflammatory genes, COX-2, TNF-α, and iNOS [145, 163]. The regulation of these genes occurs through the activation of the redox sensitive transcription factor, NF-κB, which is effectively inhibited by Se in macrophages [145]. Overexpression of Se-GPX was shown to inhibit NF-κB activation, via inhibition of IκB phosphorylation [218]. Along these lines, data from our laboratory indicate that cellular Se status can modulate the activity of NF-κB in murine macrophages [145].

Arachidonic acid is present in vivo esterified to cell membrane glycerophospholipids. Activation of phospholipases (e.g. cytosolic phospholipase A₂) releases free AA from the phospholipid (PL) pools and makes it available for oxidative metabolism by several enzyme systems. The prostaglandin endoperoxide H synthases (PGHS-1 and -2) metabolize AA to PGH₂, which serves as the precursor of the prostaglandins, thromboxane and prostacyclin [213]. These eicosanoids have many physiological functions including both pro- and anti-inflammatory [219]. Accumulation of particular eicosanoid in tissues is purely dependent on the expression of specific synthases [220].
PGE₂ and TXA₂ are well known for their pro-inflammatory functions [221], on the other hand PGD₂ is well recognized for its anti-inflammatory functions via its metabolite, 15d-PGJ₂ [113, 221]. Several studies have indicated that in the resolution phase of inflammation, AA metabolism is shifted away from PGE₂ towards increased PGD₂ biosynthesis [102-104]. We have demonstrated in the preceding chapters that the anti-inflammatory effect of Se is mediated by switching the arachidonic acid pathway towards the production of an endogenous lipid mediator and an anti-inflammatory 15d-PGJ₂, instead of a pro-inflammatory PGE₂. PGD₂ biosynthesis is mediated by PGD synthases (PGDS), which use the COX-derived PGH₂ as substrate. Two types of PGDS have been characterized. The L-PGDS is expressed primarily in the CNS and heart; while H-PGDS is expressed mainly in mast cells, antigen-presenting dendritic cells, and macrophages [201]. The short-lived PGH₂ is also a substrate for PGE synthase enzymes, cytosolic PGES (c-PGES) and membrane-bound PGES-1 (mPGES-1). Of these, only mPGES-1 has been shown to be responsive to pro-inflammatory stimuli; while cPGES is a constitutive cytosolic enzyme [107]. Many laboratories, including ours, have demonstrated that 15d-PGJ₂ can impact the regulation of pro-inflammatory pathways, mainly through the modulation of NF-κB- and PPARγ-dependent pathways. [122, 192, 222].

Peroxisome proliferator activated receptors are a family of ligand-activated nuclear transcription factors, which, upon the binding of ligand, form a heterodimer with the retinoic X receptor (RXR). More recently, the expression of PPARγ has been found in neutrophils, macrophages, T- and B-cells [125, 128]. The PPARγ-RXR heterodimer complex then binds to PPAR-responsive elements (PPREs) in the promoter regions of
target genes [128]. Recruitment of PPARγ/RXR heterodimer to the promoters leads to the inhibition of co-repressor dissociation and/or co-activator binding [127, 128]. The transrepression of NF-κB by such a PPARγ-dependent mechanism finally leads to the negative regulation of transcription of pro-inflammatory cytokine genes. Consistent with this idea, 15d-PGJ2, was very effective in inhibiting the NF-κB dependent production of IL-12 [109]. Similarly, in the case of human mPGES-1, activation of PPARγ may interfere with Egr-1 function, which normally drives the transcription of mPGES-1[130]. The activation of PPARγ has been shown to repress the transcription of TXS, which is involved in atherosclerosis, in rat macrophages via its interaction with the transcription factor, NF-E2-related factor-2 (Nrf-2) [132]. Interestingly, 15d-PGJ2 has been found to activate Nrf-2 by binding to its cytoplasmic retention protein, Keap-1[223]. Thus, studies proposed in this Chapter are based on the central hypothesis that Se-supplementation brings about a negative regulation in the expression of mPGES-1 and TXS; while the transcription of H-PGDS is positively modulated by Se via the formation of 15d-PGJ2. This chapter demonstrates that Se, in the form of selenoproteins, regulates the expression of terminal PG synthases, which play an important role in switching PG synthesis from pro-inflammatory PGE2 towards anti-inflammatory 15d-PGJ2.
MATERIALS AND METHODS

Reagents:

Bacterial endotoxin lipopolysaccharide (LPS) and sodium selenite, GW9662 and Troglitazone were from Sigma. Anti-H-PGDS, Anti-TXAS and 15d-PGJ2 were obtained from Cayman Chemicals (Ann Arbor, MI). Antibodies against glyceraldehyde-3-phosphate dehydrogenase was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GPX1 was purchased from Abcam, Inc. (Cambridge, MA). Goat anti-rabbit IgG and anti-mouse IgG conjugated to horseradish peroxidase, polyvinylidene difluoride membrane, and West Pico chemiluminescence reagents were purchased from Pierce. TRIZol reagent was purchased from Invitrogen. Taqman probes for GPX-1, mPGES-1, H-PGDS, GAPDH and 2X Universal Taqman Master Mix were purchased from Applied Biosystems.

Cell Culture and Stimulation:

The murine macrophage cell line RAW264.7 (ATCC) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% defined fetal bovine serum (HyClone), 80 µg/ml gentamicin, and 2 mM L-glutamine (Invitrogen) at 37 °C with a 5% CO2/air mixture. Total Se in the fetal bovine serum was quantitated to be 6 pmol/ml. Cells were cultured in DMEM either supplemented with Se (Na2SeO3; 0.025-1 µM as Se) or without any added as described from our laboratory [145]. Cell viability and growth rates of Se-supplemented cells were similar to their Se-deficient counterparts. About 1 x 10^6 Se-deficient and Se-supplemented cells were seeded in a 6-well plate, and then cultured in respective media for ~24 h to allow the cell number to approximately double. Cells were
stimulated with LPS (0–1 µg/ml) and/or other compounds for the indicated time periods. Upon treatment, the cells were harvested, washed with cold sterile PBS and stored at −80 °C until further use. Femoral bone marrow plugs, from mice maintained on a Se-deficient or a Se-supplemented diet, were isolated, and adherent cells, hereafter referred to as primary bone marrow-derived macrophages (BMDMs), were differentiated in their respective media containing 20% L929 fibroblast media supernatant (as a source of granulocyte-macrophage colony-stimulating factor) for 1 week. The L929 cells were also cultured under Se-deficient or Se-supplemented conditions. The Se-deficient and Se-supplemented diets were formulated based on an American Institute of Nutrition recommended rodent diet containing 0.01, 0.1 or 0.4 ppm of Se, as described [174]. The diets were purchased from Zeigler (Gardners, PA). The BMDM cultures from Se-deficient and Se-supplemented mice were used in all experiments.

**Preparation of Cell Lysates:**

Frozen cell pellets were resuspended in 50 µl of mammalian protein extraction reagent (M-PER, Pierce) containing 1 mM EDTA, 10 µM leupeptin, and 1 mM phenylmethylsulfonic acid for 30 min on ice with intermittent vortexing. Supernatants were prepared by centrifuging the cell lysate at 10,000 × g for 15 min at 4 °C and used for analyses. Protein concentration in the cell supernatants was determined by BCA protein assay (Pierce).
Electrophoresis and Immunoblotting:

Thirty micrograms of protein from RAW264.7 or BMDM cell lysates was separated on a 12.5% SDS-polyacrylamide gel and transblotted onto PVDF as described [176]. The membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 and 5% skim milk (w/v). The membrane was probed with primary antibody followed by an appropriate secondary antibody coupled to horseradish peroxidase.

Chemical Treatments:

Se-deficient and Se-supplemented RAW264.7 and BMDM cells (1 x 10⁶) were seeded into each well of a 6-well plate and treated with GW9662 (200 nM), as an irreversible PPARγ antagonist. Two μM Troglitazone and/or 15d-PGJ₂ (0.5 μM and 2 μM) were used as PPARγ agonists. 1, 4-phenylenebis (methylene) selenocyanate (PxSc) (5 μM), SeMeCys (5 μM), SeMet (5 μM) and Na₂SeO₃ (0.250-2 μM) were used as sources of Se. In all cases, the treatment with chemicals (for 12 h) was followed by stimulation with LPS for the indicated time periods. The cell lysates were prepared as described earlier and subjected to Western immunoblot and realtime RT-PCR analyses, while the media supernatants were used for 15d-PGJ₂ assays. All chemical treatment studies included an appropriate Me₂SO₄ vehicle control.

Quantitation of 15d-PGJ₂:

A 96-well-based enzyme immunoassay kit from Assay Designs (Ann Arbor, MI), was used to quantitate 15d-PGJ₂. The concentrations of 15d-PGJ₂ in culture media supernatants of RAW264.7 and BMDM in the presence or absence of LPS (0–1 μg/ml)
for various time periods were determined by enzyme immunoassay according to the manufacturer's instructions and normalized to total cellular protein. Standard calibration curves were prepared using 15d-PGJ₂ and fitted to a log-linear, logit, multiway frequency regression analysis. 15d-PGJ₂ were quantitated in cell lysates and normalized to total protein in the cell lysates.

**Reverse Transcription and Real-Time PCR Analyses:**

RNA was isolated by using TRIZol (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol followed by RNeasy column cleanup and ethanol precipitation. Reverse transcription was performed by using the High Capacity cDNA Archive kit (Applied Biosystems), following the manufacturer’s protocol and using 1 µg of each RNA sample in a reaction volume of 50 µl. After an initial 10 min at 25°C, reverse transcription was conducted at 37°C for 120 min. The resulting cDNA was stored at -20°C. Quantitative real-time PCR experiments were performed with ABI 7300 real-time system (applied Biosystems) as described [224]. Each assay sample was run in triplicates alongside its reference assay (on the same plate). TaqMan Gene Expression Assays (Applied Biosystems) were used according to the manufacturer’s recommendations. Cycling parameters comprised an initial polymerase activation step (10 min at 95°C), followed by 2-step cycling for 40 cycles (15 sec at 95°C and 60 sec at 60°C). Twenty microliter reactions were composed of TaqMan Universal Master Mix and 1 µl cDNA inputs equivalent to the 1 µg of total RNA. Data was analyzed by comparing threshold cycles and plotting them as an exponential according to the method of Livek et al [225, 226]. The data was normalized to GAPDH.
SPS2 shRNA transfections:

The pU6-TetO4m4 vector containing shRNA target sequence, 5'-GACGTAGAGTTGGCATACC-3' (nucleotides 694–712), for SPS2 and an empty vector were obtained from Dr. Dolph L. Hatfield National Cancer Institute, National Institutes of Health, Bethesda, MD. The control vector, pU6-TetO4m4, was designated pU6Tet control and it consisted of the same construct minus the target sequence. For transfection, RAW264.7 macrophages were seeded into 6-well plates (1 x 10^6 cells/well) without gentamicin. After 24 h, cells were transfected with either shSPS2 or empty vector using TransIT®-LT1 (Mirus Bio Corp.) according to the manufacturer's specifications. The constructs were used at a final concentration of 2.5 µg/well. After 48 h of transfection cells were left in the media with or without 250 nM Na2SeO3 for 48 h followed by 12 h LPS stimulation. Cells and media supernatants from LPS-treated (12 h) or untreated cells were collected for 15d-PGJ2 quantitation, whereas the corresponding cell lysates were used for Western immunoblot analyses.
RESULTS

Expression of GPX-1 in macrophages as a function of cellular Se status:

The expression of Se-GPX-1 was measured in cell lysates from RAW 264.7 cells that were supplemented with different concentrations of Se as a marker for Se status. The Se-GPX-1 expression was almost non-existent in Se-deficient media, but was significantly increased in the deficient cells upon supplementation with Se (Fig. 24A). The expression was saturated at 250 nM of Se and remained that way even at high concentrations (0.5-1 µM). In addition, the expression of GPX-1 was also analyzed in primary macrophages isolated from mice that were maintained on Se-supplemented and Se-deficient diets. The results indicated a significantly increased expression of GPX-1 in the macrophages obtained from Se-supplemented mice compared to Se-deficient mice (Fig. 24B).

Selenium regulates the expression of terminal prostaglandin synthases:

The facts that Se-supplementation can modulate the biosynthesis of prostaglandins [140] and can regulate the activity of transcription factors like NF-κB and PPARγ, which play an important role in the regulation of many genes including PG synthases [131, 132, 223] suggest that Se may be able to regulate the expression of PG synthases. To address this we cultured Se-deficient RAW264.7 cells in the presence of different concentrations of Se as well as primary BMDMs isolated and differentiated from mice maintained on diets containing different Se concentrations (0.0015, 0.1 and 0.4 ppm). Both mRNA and protein levels of H-PGDS were increased with the supplementation of Se, whereas mRNA levels of mPGES were significantly decreased (~45-fold) with the Se-supplementation (Fig. 25A&C). Identical results were observed in BMDM. Consistent
with RAW264.7 macrophages, the mRNA levels of H-PGDS were increased by ~3-fold, while mRNA levels of mPGES-1 were only decreased by ~6-fold in BMDM isolated from mice on 0.4 ppm Se diet when compared to BMDM isolated from mice on diets containing 0.0015 ppm (Fig. 25B&D). The protein levels of mPGES-1 were also decreased in both RAW264.7 cells and BMDM (data not shown). Furthermore, the LPS effect on H-PGDS expression was determined at different time intervals. Stimulation of Se-deficient RAW264.7 macrophages with LPS for 0-1 h clearly demonstrated exacerbated expression of H-PGDS. On the other hand we failed to observe any time dependent increase in H-PGDS expression upon LPS stimulation in Se-supplemented RAW264.7 macrophages (Fig. 25E). It is very likely that a threshold has already been reached at 0 h. Taken together our data show that Se-mediated production of 15d-PGJ2 occurs through up regulation of H-PGDS.

**H-PGDS is a PPARγ dependent gene:**

Examination of murine H-PGDS gene promoter indicates the presence of three putative binding sites for PPARγ at -4501, -2172, and -672. Based on this analysis, we reasoned that PPARγ activation by 15d-PGJ2 could be responsible for the increased expression of H-PGDS. To address this we utilized agonists and antagonists of PPARγ. Incubation of Se-deficient RAW264.7 macrophages with GW9662, an irreversible antagonist of PPARγ followed by 1 h LPS stimulation resulted in decreased expression of H-PGDS, while incubation with PPARγ agonists, troglitazone and 15d-PGJ2, led to the increased expression of H-PGDS (Fig. 26) suggesting that H-PGDS is a PPARγ dependent gene.
Studies are currently being done to confirm this observation using luciferase reporter and gel shift assays. Taken together with the previous results, these studies suggest that a synergistic interplay of cellular Se status and PPARγ is absolutely necessary for the cellular production of 15d-PGJ2.

**Selenoproteins are necessary for the production of 15d-PGJ2 in macrophages:**

To examine if selenoproteins are involved in the biosynthesis of 15d-PGJ2 in Se-supplemented macrophages, we utilized different forms of Se. Based on the fact that only inorganic but not organic forms of Se can be incorporated into selenoproteins in conventional tissue culture methods due to the lack of lyases [227], we treated Se-deficient cells with Na₂SeO₃ (2 μM) and organic forms of Se, PxSc, SeMeCys and SeMet at 5μM. Organic forms of Se had no effect on the production of 15d-PGJ2 in LPS treated macrophages. In contrast, Na₂SeO₃, an inorganic form, significantly increased the production of 15d-PGJ2 upon treatment with LPS for 12 h (Fig. 27A). We reasoned that due to the absence of selenoproteins in the macrophages treated with organic forms of Se, there is no significant production of 15d-PGJ2. This was confirmed by looking at the expression of GPX-1 as a marker of selenoproteins, by Western immunoblotting using GPX-1 specific polyclonal antibodies (Fig. 27A). These results clearly suggested that only Na₂SeO₃ treatment significantly increased the expression of GPX-1 when compared to organic compounds. The GPX-1 expression was undetected in those cells treated with PxSc and SeMeCys. But we observed a slight increase in the expression of GPX-1 in those cells treated with SeMe. These results suggested that selenoproteins play an important role in the production of anti-inflammatory 15d-PGJ2. As confirmation, we
used a shRNA construct to effectively knockdown the expression of SPS2 in Se-deficient macrophages. To assess the effects of SPS2 knockdown on the selenoprotein expression, as well as to examine the role that Se may have on the expression of selenoproteins in the knockdown cells, the Se-deficient macrophages were transfected with either shSPS2 or control constructs and cultured in medium with and without selenite (250 nM) for 2 days followed by 12 h LPS stimulation. The cell lysates were used for the analysis of the expression of GPX-1 and TR were examined by Western immunoblotting. The expression of GPX-1 and TR were significantly knocked down in shSPS2 treated cells and in the cells cultured in the absence of selenite. There did not appear any change in the expression of these proteins in the cells grown in the presence of selenite and control construct (Fig. 27B). Furthermore to confirm that SPS2 knockdown cells can not produce 15d-PGJ$_2$ due to the lack of selenoproteins, the cell supernatants were utilized for the analysis of 15d-PGJ$_2$ production. The production of 15d-PGJ$_2$ was almost completely inhibited in SPS2 knockdown cells even after supplementing with selenite. On the other hand we observed significant increase in the production of 15d-PGJ$_2$ in those cells which were transfected with or without control constructs followed by selenite treatments (Fig. 27B). These results clearly confirm that selenoproteins are involved in the biosynthesis of 15d-PGJ$_2$ via which Se exerts its anti-inflammatory properties.
DISCUSSION

The LPS-induced release of large amounts of 15d-PGJ2 by Se-supplemented cells, compared to the PGE2 produced by Se-deficient cells, suggests that AA is selectively targeted towards PGJ2 synthesis [192]. This is the first ever observation that indicates a switch from pro-inflammatory (PGE2) to anti-inflammatory (15d-PGJ2) mediated by changes in cellular Se (or redox) status. Intriguingly, in Se-deficient cells, where increased COX-2 expression is seen upon LPS stimulation, there is hardly any 15d-PGJ2 produced [192]. The present study is focused on how differential cellular Se status affects such a switch. In the present study we have demonstrated that cellular Se levels regulate the expression of mPGES-1 and H-PGDS, allowing for the switch to occur from PGE2 to 15d-PGJ2.

Recent studies have shown that 15d-PGJ2 is a ligand for PPARγ [222]. 15d-PGJ2 represses several genes in activated macrophages, including the iNOS and TNF-α genes, and this repression is suggested to be at least partly dependent on PPARγ activation [129, 228, 229]. On the other hand, recent studies including those from our laboratory have shown that 15d-PGJ2 directly inhibits the NF-κB dependent gene expression through covalent modifications of critical cysteine residues in IκB kinase [122] and the DNA binding domains of NF-κB subunits [203]. These studies suggest that 15d-PGJ2 can positively or negatively regulates expression of many pro- and anti-inflammatory genes. 15d-PGJ2 has been shown to repress the transcription of mPGES-1 and TXS through PPARγ activation [132]. Based on these studies and the fact that Se-supplementation results in the accumulation of significant amounts of 15d-PGJ2 in activated macrophages
[192], we speculated that Se could differentially regulate the expression of mPGES-1 and H-PGDS. Supplementing Se-deficient macrophages with selenite significantly decreased the expression of pro-inflammatory genes, mPGES-1 and TXS. Mary Lou’s thesis clearly showed a decrease in TXA₂ and PGE₂ production. This result is identical to that observed earlier in our laboratory. However, Se-supplementation increased the expression of H-PGDS (Fig. 25A), suggesting that the increased levels of 15d-PGJ₂ could be differentially regulating the expression of H-PGDS and mPGES-1 positively and negatively via activating PPARγ and inactivating the NF-κB pathway, respectively. LPS stimulation of Se-deficient macrophages expressed increased levels of H-PGDS in time dependent way, where as we failed to observe such a time dependent increase in H-PGDS expression in LPS treated Se-supplemented macrophages (Fig. 25E). Despite this time dependent increase, H-PGDS did not contribute to the significant increased production of 15d-PGJ₂ in Se-deficient macrophages. This could be due to a small increase in the expression of H-PGDS compared to mPGES-1 and TXS, such that the activity of these enzymes overrides the activity of H-PGDS. The H-PGDS expression was saturated at 0 h time point in Se-supplemented macrophages; however the expression was almost non-existent in Se-deficient cells at 0 time point. Eventhough we observed maximum expression of H-PGDS in Se-supplemented macrophages we did not observe any production of 15d-PGJ₂ at 0 h time point. This is because AA has to be released from membrane phospholipids to be metabolized to 15d-PGJ₂ via H-PGDS. This clearly explains why 15d-PGJ₂ production was seen only after LPS stimulation. Examination of the murine H-PGDS promoter indicates the presence of three putative sites for the binding of PPARγ at -4501, -2172, and -672, suggesting that this gene could be regulated by PPARγ. Based on this
information and above results, it could be that 15d-PGJ$_2$ may be a specific positive regulator of H-PGDS in Se-supplemented macrophages suggesting that H-PGDS expression is likely to be regulated by positive feedback loop mediated through PPAR$\gamma$. To test this possibility, the expression of H-PGDS in Se-deficient macrophages in the presence of PPAR$\gamma$ agonist or antagonist was tested by Western immunoblot analysis. Inhibition of PPAR$\gamma$ using GW9662 decreased the expression of H-PGDS, whereas activating PPAR$\gamma$ with troglitazone and 15d-PGJ$_2$ significantly increased the expression of H-PGDS in Se-deficient cells suggesting that H-PGDS is a PPAR$\gamma$ dependent gene (Fig. 26). Such a consistent increase in the PPAR$\gamma$-dependent upregulation of H-PGDS indicates a unique mechanism of anti-inflammatory activity of some of the anti-diabetic drugs that are commonly used in the clinic. Studies in our laboratory are focused on the elucidation of the transcriptional regulation of H-PGDS in a PPAR$\gamma$-dependent pathway. 15d-PGJ$_2$ is 5-30 times more potent than various thiazolidinediones including troglitazone, in activating PPAR$\gamma$[230]. This could possibly explain the higher potency in inducing the expression of H-PGDS by 15d-PGJ$_2$, than by troglitazone, even at equal concentrations (Fig. 26).

Since we reasoned that AA metabolism shifts away from pro-inflammatory PG synthesis towards anti-inflammatory PG synthesis during Se-supplementation by differentially regulating the expression of PG synthases via 15d-PGJ$_2$, one can pose the question what could be the possible mechanism involved in the initial biosynthesis of 15d-PGJ$_2$ during differential cellular Se status. The biological functions of Se are believed to occur via
several selenoproteins, such as GPX and TR [231]. To determine if there are any selenoproteins involved in the biosynthesis of 15d-PGJ$_2$ in Se-supplemented macrophages, we treated Se-deficient macrophages with different Se compounds. Using both organic and inorganic forms of Se and SPS2 shRNA, we have conclusively shown that selenoproteins play an important role in the biosynthesis of 15d-PGJ$_2$ (Fig. 27). From the results obtained in this chapter we speculate that selenoproteins cause an increase in the production of 15d-PGJ$_2$, and that 15d-PGJ$_2$ leads to the upregulation of H-PGDS and downregulation of mPGES-1 and TXS, to complete a well regulated feed forward mechanism.

In conclusion, we have reported the basis for the switch in the AA pathway towards anti-inflammatory PGs away from pro-inflammatory PGs to be driven by 15d-PGJ$_2$, which differentially regulates the expression of PG synthases in a selenoprotein dependent manner.
Figure#23: A schematic model for the possible feedback regulation of mPGES-1 and H-PGDS by 15d-PGJ$_2$ during Se-supplementation: In the LPS stimulated macrophages AA rapidly released by cPLA2 is metabolized to PGE$_2$ and 15d-PGJ$_2$ via mPGES-1 and H-PGDS respectively. During Se-supplementation selenoproteins (SePr) could initiate the production of 15d-PGJ$_2$, which then activates PPAR$\gamma$, a nuclear receptor, which participates in the regulation of transcription of many genes. Thus formed 15d-PGJ$_2$ positively regulates the transcription of H-PGDS through PPAR$\gamma$, while negatively regulating the transcription of mPGES-1.
Figure#24: Expression of GPX1 in Se-deficient RAW264.7 (A) and BMDM (B) macrophages as a function of cellular Se status. The protein expression of GPX-1 was analyzed by Westernblot in Se-deficient RAW264.7 cells which were treated with indicated concentrations of Se (as Na₂SeO₃) for 2 days and BMDM isolated from mice which were on diets with different Se concentrations (0, 0.1 and 0.4ppm). Anti-GPX1 was from Abcam. Protein levels were normalized to GAPDH.
Figure#25: Se-supplementation differentially regulates the expression of H-PGDS, mPGES-1 and TXS. A and B, Protein expression of H-PGDS and TXS in both RAW264.7 cells (A) and BMDM (B) during differential cellular Se status. Protein expressions of H-PGDS and TXS in Se-deficient RAW264.7 macrophages which were supplemented with indicated concentrations of Se (as Na₂SeO₃) for 2 days and in BMDM, isolated from mice which were on diets containing indicated concentrations of
Se, were determined by using Western immunoblotting. H-PGDS and TXS were normalized by GAPDH, which was used as an internal control to assess equal protein loading. C and D, mRNA expression of H-PGDS and mPGES-1 in both RAW264.7 cells (C) and BMDM (D) during differential cellular Se status. mRNA expressions of H-PGDS and mPGES-1 in Se-deficient RAW264.7 macrophages which were supplemented with 250nM Se (as Na$_2$SeO$_3$) for 2 days and in BMDM, isolated from mice which were on diets containing indicated concentrations of Se, were determined by using RT-PCR. H-PGDS and mPGES-1 were normalized by GAPDH, which was used as an internal control. E, Se-supplemented and Se-deficient RAW264.7 cells were treated with 1 µg/mL LPS for various time periods, and the cell lysates were analyzed for H-PGDS expression and GAPDH expression.
Figure 26: PPARγ activation induces the expression of H-PGDS. Se-deficient RAW264.7 cells were treated with GW6992 (200 nM), troglitazone (2 µM) and 15d-PGJ2 (0.5 and 2 µM) for 12 h followed by 2 h LPS (1 µg/mL). Cell lysates were analyzed for the expression of H-PGDS by Western immunoblotting. The protein levels were normalized by GAPDH.
Figure#27: Inhibition of selenoprotein synthesis reduces 15d-PGJ$_2$ levels in Se-supplemented macrophages. A, Effect of organo Se compounds on the production of 15d-PGJ$_2$. Se-deficient RAW264.7 macrophages were treated with 5 µM of pXSc, SeMeCys, SeMet and with 2 µM of Na$_2$SeO$_3$ for 12 h followed by 12 h LPS (1 µg/mL). Media supernatants were used for the analysis of 15d-PGJ$_2$ and the cell lysates were used for the analysis of GPX-1 expression. B, Effect of SPS2 knockdown on the production of 15d-PGJ$_2$. Se-deficient Raw264.7 cells were transfected with either shSPS2 or the empty vector, and the cells grown in the presence or absence of 250 nM of Na$_2$SeO$_3$ for 2 days followed by 12 h LPS stimulation. Media supernatants were used for the analysis of 15d-PGJ$_2$ and the cell lysates were used for the analysis of the expression of GPX-1 and TR.
Chapter 5

SUMMARY AND CONCLUSIONS
Selenium is essential for life, and no doubt exists that adequate amounts of this element are required for optimum human health. Many of its physiological roles are directly attributed to its presence within selenoproteins, in the form of the 21st amino acid, SeCys [21, 22]. Incorporation of SeCys into selenoproteins employs a unique mechanism that involves decoding of the UGA codon [70, 71]. The function of most selenoproteins is currently unknown; however, TR, Se-GPX and DIO are well characterized selenoproteins involved in redox regulation of intracellular signaling, redox homeostasis and thyroid hormone metabolism, respectively [231]. Moderate Se-deficiency has been linked to many conditions, such as increased cancer and infection risk, male infertility, decrease immune and thyroid function, and several neurologic conditions, including Alzheimer’s and Parkinson’s disease [38, 39, 41, 42]. However, for some of these conditions, the evidence is rather scant, lacks consensus, and must be further demonstrated. The purpose of this course of research is to understand the molecular mechanisms underlying the anti-inflammatory effects of Se in macrophages. In particular, to investigate the effect of Se-supplementation on alterations of AA metabolism and changes in the redox status of certain signal transduction pathways. The hypothesis was tested using Na2SeO3 supplemented or deficient RAW264.7 murine like macrophages or primary macrophage cells, BMDM, isolated from mice on Se-supplemented or Se-deficient diets and LPS as the inflammatory stimulant. The rationale for such a model was based on the importance of macrophages in the immune system as high output producers of RONS, as well as key inflammatory mediators. LPS was chosen for its established ability to induce a strong inflammatory response in macrophages, with a well characterized induction of NF-κB-
dependent pro-inflammatory genes. Na$_2$SeO$_3$ was selected over other forms of Se due to its high efficiency of incorporation in vitro cell cultures.

Lipopolysaccharide signaling in murine macrophages is modulated through Toll-like receptor 4 (TLR4) in concert with CD14 and LPS binding protein (LBP) [232]. Engagement of these receptors transduces the cellular response to LPS, resulting in the expression of pro-inflammatory genes, TNF$\alpha$ and IL-1, as well as the production of RONS [232]. Using LPS as a physiological stimulus to initiate oxidative stress in cells, the role of Se has been described on the expression of pro-inflammatory proteins. Much like the expression and activity of GPX1 in cells (Fig. 15), which serves as an indicator of the Se status of macrophages, the expression of Se-GPX1 in the livers provided evidence regarding the differential status of Se in mice (Fig. 17). Our laboratory has previously demonstrated that the macrophages differentiated ex-vivo exhibit a similar difference in Se-GPX1 activity and expression as described here [145, 163]. A recent study suggests that SelP may be a better indicator of Se status than Se-GPX1[233]. Keeping this in mind, SelP expression was also monitored in both Se-supplemented and Se-deficient macrophages as an indicator of Se status in macrophages. The expression of SelP levels were significantly different in the two groups in that the Se-supplemented cells demonstrated a higher level of SelP expression compared to the Se-deficient cells (Fig. 14). Intraperitoneal injection with LPS caused increased infiltration of macrophages into the lungs of Se-deficient mice; while the mice on Se-supplemented diets exhibited a significantly decreased effect (Fig. 7).
Selenium supplementation, over the minimal nutritional requirements, has gained popularity and there is some scientific evidence to support benefits of super-supplementation of Se. However, despite the therapeutic potential of Se in many inflammatory diseases, little is known about the mechanism and regulation of inflammation by Se. To explain the health benefits of Se and define its biochemical role in mitigating oxidative stress-mediated expression of pro-inflammatory genes and initiate the recovery or resolution phase, it is important to identify those genes whose expression is regulated strictly by Se status in macrophages. Following gene expression microarray experiments, it is clear that Se regulates the expression of many pro-and/or anti-inflammatory genes in macrophages (see table 1). These results suggested that Se-supplementation of Se-deficient macrophages caused an increase in IL-4, while decreasing RelA (p65). The Se-dependent up-regulation of the fatty acid binding protein 1 (Fabp1), which is a target gene of all the three PPARs subtypes, depending on the tissue, suggests that Se may play an important role in ligand-delivery and transactivation of PPARs. These results including previous studies in our laboratory [145] clearly suggest that Se could regulate the activity of two major transcription factors, NF-κB and PPARγ. Differential modulation of the activity of these two major transcription factors by Se could, therefore, have a major effect on the expression of pro-inflammatory genes, whose identities are currently unclear.

Previous research in our laboratory led to the investigation of effects of Se on the expression of two NF-κB dependent pro-inflammatory genes, TNFα and COX-2. A
significant decrease in TNFα production by LPS treatment in Se-supplemented macrophages compared to those cultured in Se-deficient media (Fig. 8) further supports the argument that, through the secretion of pro-inflammatory cytokines, Se may change the dynamics of infiltration of other immune cells into the inflamed area. These observations suggest that Se-deficiency may exacerbate inflammatory responses in the lung possibly leading to the airway hyper responsiveness. Increased expression of COX-2 and TNFα are synonymous with inflammation and are used as markers in many inflammatory diseases such as arthritis, atherosclerosis, and cancer. During Se-deficiency, COX-2 was significantly up regulated in both RAW264.7 macrophages and BMDM, where as Se-supplementation suppresses the expression of COX-2 (Fig. 9A & B). These results further supports our previous data that Se-supplementation of macrophages causes a decrease in the activity of COX-2 [145]. In addition, the result also indicates that, at concentrations of ≥ 0.2 µM of Se, there is a significant down-regulation of COX-2 expression (data not shown), which could be due to the inactivation of some of the signal transduction pathways leading the expression of COX-2. Furthermore, to investigate if Se is attenuating the expression of these genes via NF-κB, Se-deficient and Se-supplemented macrophages were transfected with luciferase constructs driven by COX-2 wild type and NF-κB mutated promoters (Fig. 9C). Transcription of luciferase was dramatically reduced when the κB sites were specifically mutated, demonstrating the requirement for κB in transcription of these genes. The supplemented cultures demonstrated consistently lower luciferase activity than the deficient cultures with COX-2 wild type promoter. These results suggest that Se attenuates the expression of pro-
inflammatory genes in NF-κB dependent manner. These results also indicated that cellular Se status might be altering the expression or activity of a few key upstream kinases such as IKK family enzymes. The upstream protein kinases IKKα/β and IκBα were studied for their expression and phosphorylation status in both Se-deficient and Se-supplemented macrophages. The immunoblots showed that Se-supplementation decreased the expression of pIκBα and IκBα (Fig. 9D). This sustained increase in IκBα/pIκBα corroborates well with the increased translocation of p65 in Se-deficient cells described earlier from our laboratory [145]. We observed no obvious differences in the expression of IKKβ in LPS-stimulated Se-deficient or Se-supplemented cells (Fig. 9D). But, IKKβ activity was significantly reduced in Se-supplemented macrophages (Fig. 10A & B). In addition, in-vitro kinase assays of immunoprecipitates containing IKKα and IKKβ from Se-deficient and Se-supplemented RAW264.7 cells following LPS stimulation demonstrated increased activity of IKKβ in LPS-treated Se-deficient cells; while in the Se-supplemented cells, there was no such increase in activity upon treatment with LPS (Fig. 10C). On the other hand, activity of IKKα was neither affected by Se status nor LPS stimulation. These results suggested that the enzymatic activity of IKKβ was severely affected by some mechanism almost exclusively in the Se-supplemented cells and not in the Se-deficient cells. Apart from phosphorylation by upstream kinases, the activity of IKKβ is also regulated by post-translational modification by 15d-PGJ₂, a cyclopentenone prostaglandin [122].
Previous research in our laboratory has indicated that Se-supplementation can alter AA metabolism [140, 145]. Based on this premise we hypothesized that in Se-supplemented cells, PGH₂ from COX-1 or COX-2 may possibly shunted to H-PGDS to produce PGD₂ and subsequently convert to 15d-PGJ₂ to affect IKKβ activity which eventually decrease NF-κB activity. In order to investigate our hypothesis, we examined the effect of Se on the production of 15d-PGJ₂. Upon LPS stimulation of BMDMs and RAW264.7 cells for 12 h, 15d-PGJ₂ increased only in the Se-supplemented cells and not in the Se-deficient group (Fig. 11A). Similar results were also observed in the serum samples obtained from mice (Fig. 11C). The increased production of 15d-PGJ₂ in Se-supplemented macrophages was also confirmed by LC-MS (Fig. 11D). Conclusively, these results suggested that Se-supplementation shifted AA metabolism towards anti-inflammatory PG, 15d-PGJ₂. From the literature we know that 15d-PGJ₂ attenuates NF-κB activities by forming a covalent adduct with IKKβ and by activating PPARγ [122, 222]. To determine if such an adduct can be formed in vivo, effect of Se-supplementation on the adduct formation between 15d-PGJ₂ and IKKβ in macrophages was examined. As a consequence of production of 15d-PGJ₂ in Se-supplemented cells, an increased interaction of 15d-PGJ₂ with IKKβ subunit was observed in Se supplemented cells, which increased upon stimulation with LPS (Fig. 12). Based on these results it is clear that endogenous 15d-PGJ₂ produced in Se-supplemented macrophages modulated the activity of IKKβ by covalent modification of the essential Cys-179 residue. Apart from interacting with IKKβ, 15d-PGJ₂ is a high affinity ligand for the nuclear receptor PPARγ and modulates gene transcription by binding to this receptor [122, 132, 222]. Antagonistic studies with GW9662 clearly
demonstrated that the repression of COX-2 expression was also mediated by ligand-dependent activation of PPARγ in Se-supplemented macrophages (Fig. 20B). Although GW9662 also antagonizes PPARβ at the concentrations used (200 nM and 1 µM), based on literature we speculate that PPARγ, but not PPARβ is involved in the anti-inflammatory functions of Se. However, further studies are needed to examine the functions of PPARγ and PPARβ during differential cellular Se status.

Based on our data, it is clear that only macrophages supplemented with sufficient amounts of Se produce significant levels of 15d-PGJ2, which then attenuates NF-κB transcription of pro-inflammatory genes via two pathways; a) by covalent modification of IKKβ and b) by activating PPARγ.

Peroxisome proliferator activating receptors bind to cognate DNA elements called peroxisome proliferator response elements (PPRE) as obligate heterodimers with the retinoid X receptor (RXR). After ligand activation, they work as transcription factors [127, 128]. PPARγ has been associated with control of inflammation by inhibiting cytokine stimulation of COX-2 and iNOS expression in different cell types, [234, 235] decreasing release of proinflammatory cytokines [228] and inhibiting vascular smooth cell migration [229, 236]. PPARγ has also been shown to have antineoplastic and antigrowth properties [236]. The activation of PPARγ

15deoxy-Prostaglandin J2-dependent activation of PPARγ repressed the transcription of PG isomerases such as TXS and mPGES-1 [130, 132]. Along these lines, data from chapter 3, suggest a Se-dependent down-regulation of TXS and mPGES-1, whereas H-PGDS expression is increased in Se-supplemented cells (Fig. 25). These results could
explain the possible mechanism underlying the shunting of AA to either pro-inflammatory or anti-inflammatory PGs leading to preferential increase in 15d-PGJ2. This phenomenon may likely involve preferential recruitment of transcriptional coactivators or corepressors at the promoter of these target genes in a Se-dependent manner. Further studies are needed to examine the regulation of transcription of these target genes by cellular Se status.

Segregated utilization of COX-1 and COX-2 in the PG biosynthetic events has been demonstrated by a number of cell biological, pharmacological, and genetic studies [237-239]. It has been suggested that the two COX isozymes are differently coupled with specific terminal PG synthases. For instance, rat peritoneal macrophages produce PGD2 through COX-1 coupled with H-PGDS in the A23187-induced immediate response and PGE2 and PGI2 through COX-2 coupled with mPGES-1 and PGIS respectively in the LPS-induced delayed response [190]. Studies with HQL-79 clearly indicate that H-PGDS is involved in the production of 15d-PGJ2 (Fig. 22A) and is also involved in the 15d-PGJ2 dependent repression of COX-2 in Se-supplemented cells (Fig. 22B). Thus preferential utilization of H-PGDS either by COX-1 or COX-2 in Se-supplemented macrophages could determine the fate of AA. Since Se-supplementation repressed the expression of COX-2 and Se status has no effect on the expression of COX-1, it is very much possible that COX-1 has an important role in AA metabolism, particularly in Se-supplemented cells. The use of CAY10404, a COX-2 selective inhibitor had no effect on the production of 15d-PGJ2 in LPS-treated Se-supplemented cells. In contrast, the use of indomethacin, as a COX-1 inhibitor completely inhibited the production of 15d-PGJ2 in Se-
supplemented cells upon treatment with LPS for 12 h (Fig. 18A). These results suggested that COX-1 plays an important role in the production of the anti-inflammatory 15d-PGJ2. LPS-stimulation of COX-1 siRNA transfected Se-supplemented cells completely inhibited the production of 15d-PGJ2 (Fig. 18B) similar to that observed with the Se-deficient cells. In-vitro kinase assays indicated that IKKβ activity was repressed in Se-supplemented macrophages by increased production of 15d-PGJ2, which is derepressed in cells treated with SC-560, COX-1 specific inhibitor at 50 µM (Fig. 19A). In addition, gel mobility shift assays performed in Se-supplemented and Se-deficient cells indicated that NF-κB activity was increased (Fig. 19B) where as PPARγ activity is decreased (Fig. 20A) in SC-560-treated Se-supplemented cells when compared to the untreated (or vehicle-treated) Se-supplemented cells. Conclusively these results suggested that COX-1 could preferentially interact with H-PGDS in Se-supplemented macrophages to produce 15d-PGJ2, while COX-2 could be preferring mPGES-1 in Se-deficient cells. Although colocalization studies were done to show the functional coupling between these enzymes, further examination is required to characterize the functional interacting domains of each partner.

The mechanism by which Se affects the differential regulation of PG synthases needs to be further investigated. From the data presented here, it appears that the build-up of 15d-PGJ2 in the cells seems to be crucial in the control of several genes, including PGS enzymes, via NF-κB and PPARγ. The 15d-PGJ2 induced gene regulation constitutes a feedback loop, which augments the switch in AA metabolism from PGE2 to PGJ2. Since H-PGDS is an important enzyme in the biosynthesis of 15d-PGJ2 and its gene has three
putative binding sites for PPARγ, it is possible that H-PGDS is a PPARγ-dependent gene. To determine this, the effects of PPARγ activation and/or inactivation on the expression of H-PGDS were studied. Inhibition of PPARγ using GW9662 decreased the expression of H-PGDS, whereas activating PPARγ with troglitazone and 15d-PGJ₂ significantly increased the expression of H-PGDS in Se-deficient cells suggesting that H-PGDS expression is likely to be regulated by positive feedback loop mediated through PPARγ in Se-supplemented macrophages (Fig. 26). Thus, it appears that Se causes an initial increase in the production of 15d-PGJ₂, which then acts via the PPARγ-dependent mechanism to activate transcription of H-PGDS. Such a concerted mechanism leads to an overall increased production of 15d-PGJ₂. On the other hand, the 5′UTR of the mouse PGES-1 gene has two putative binding sites for NF-κB (-3193 and -928), suggesting that NF-κB might regulate the expression of mPGES-1 during Se-deficiency, leading to increased production of PGE₂. These and various other possibilities are currently being tested in our laboratory.

A final hypothesis tested was that the ant-inflammatory functions of Se require Se to be incorporated into the selenoproteins. The role of Se as an antioxidant may be primarily mediated by the expression of selenoproteins [231]. However, anticarcinogenic effects of Se may be mediated by other mechanisms other than activating selenoproteins [240]. Different selenoproteins require different concentrations of Se for their maximum expression [241, 242]. A few selenoproteins like Se-GPX and TR are very sensitive and the maximum expression of these genes is seen even at low concentrations of Se [233, 243], whereas, SelP requires higher concentrations of Se for its maximum expression.
Se-supplementation at high levels (20-50 times higher than the minimum dietary requirement level (0.1ppm) of Se for animals) reduces the incidence of cancer in animals whereas the low Se at lower concentrations has no effect on cancer [244]. At low levels of Se, there could be the lack of expression of a few important selenoproteins, which may be involved in the anticarcinogenic effects of Se. Concentration dependent effects of Se were examined on the levels of 15d-PGJ$_2$ (data not shown). The results clearly suggested that the production of 15d-PGJ$_2$ increases as a function of Se (0-1 $\mu$M), reaching the maximum at 250 nM. Depending on these results we examined if Se-supplemented macrophages produce 15d-PGJ$_2$ in a selenoprotein dependent manner. Treating RAW264.7 macrophages with organic and inorganic compounds of Se, showed that only cells treated with Na$_2$SeO$_3$ produced significant amounts of 15d-PGJ$_2$ (Fig. 27A) and were able to express selenoproteins (Fig. 27A). Along the same lines, cells transfected with SPS2 shRNA, were unable to produce 15d-PGJ$_2$ (Fig. 27B) and did not express selenoproteins, GPX and TR (Fig. 27B). These results suggest that Se has to be incorporated into selenoproteins to biosynthesize 15d-PGJ$_2$ in macrophages. Further studies are required to examine if the same holds true in-vivo, particularly in Se-deficient mice supplemented with various forms of Se. Also, these results make a compelling case to elucidate the identity of the selenoprotein(s) that is pivotal for the production of 15d-PGJ$_2$. These experiments are currently in progress in our laboratory.

In conclusion, this research has demonstrated that Se suppresses the expression of NF-$\kappa$B-dependent pro-inflammatory genes, such as COX-2, through the enhanced production of 15d-PGJ$_2$ in macrophages. Moreover, COX-1 but not COX-2, is responsible for the
shift in AA metabolism toward anti-inflammatory PG, 15d-PGJ₂ in Se-supplemented macrophages. Furthermore, these results suggested that Se status could differentially regulate terminal PGS enzymes either by PPARγ or by NF-κB in a selenoprotein dependent manner, which may in turn affect AA cascade positively or negatively.
Figure#28: A schematic model for the possible fate of Arachidonic acid during Se-supplementation: In Se-supplemented macrophages AA is rapidly metabolized to anti-inflammatory, 15d-PGJ$_2$, via COX-1 and H-PGDS in selenoprotein dependent manner. 15d-PGJ$_2$ then represses NF-$\kappa$B activation and NF-$\kappa$B dependent gene expression by 1) forming a covalent adduct with IKK$\beta$ and 2) activating PPAR$\gamma$, which then inhibits NF-$\kappa$B activity.
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