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UP REGULATION OF CYLOOXYGENASE-2 AND INDUCIBLE NITRIC OXIDE SYNTHASE IN MACROPHAGES DURING SELENIUM DEFICIENCY: ROLE OF NUCLEAR FACTOR-KAPPA B

A Thesis in

Pathobiology

by

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ABSTRACT

While all cells produce reactive oxygen and nitrogen species (ROS/RNS) such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) nitric oxide (NO) and peroxynitrite (NOO^-) during normal respiration, activated macrophages produce elevated levels of these species as components of the immune respiratory burst. In addition, ROS/RNS are important members of cellular signaling pathways, particularly those leading to the inducible activation of nuclear factor-kappa B (NF-κB). NF-κB is a redox sensitive transcription factor, which is associated with the expression of various immediate-early genes including cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). The products of these enzymes, prostaglandins and NO, together with the acute phase cytokines regulate the initiation and duration of the inflammatory response. Thus, the excess production of ROS/RNS by macrophages may contribute significantly to the pathophysiology of inflammatory disorders if it is not carefully controlled by cellular antioxidant defense systems.

To further explore the role of selenium (Se) status in macrophage immune function, the RAW 264.7 macrophage cell line was cultured in media deficient of or supplemented with sodium selenite. The activity of Se –dependent glutathione peroxidase (Se-GPx) was monitored to confirm deficiency. At least an 8-fold difference in the enzyme activity was confirmed and maintained throughout several passages. Fluorescent

labeling and flow cytometric analysis confirmed a higher degree of oxidative stress in the Se deficient group prior to any stimulus.

Following stimulation with lipopolysaccharide, Se deficient macrophages were shown to express higher levels of the key inflammatory enzymes, COX-2 and iNOS as assessed by mRNA, protein and enzyme activity. To determine the role of NF-κB in the over expression of these enzymes, it was assessed at the level of nuclear translocation, binding and initiation of transcription. At three levels were NF-κB was up regulated in the Se-deficient macrophages as compared to the Se-supplemented cells, suggesting that it is involved in the up regulation of key enzymes associated with inflammatory response. To confirm NF-κB involvement, luciferase reporter constructs for COX-2 and iNOS were generated and assessed via transient transfection in both Se-deficient and Se-supplemented group. Deletion or truncation of NF-κB binding sites from the promoters resulted in significant decreases in luciferase activity. Furthermore, upstream mediators, MEKK1, NIK, IKK_{β} and $I\kappa B_{\alpha}$ were detected at higher levels in the Se-deficient macrophages. A greater degree of serine phosphorylation of MEKK1 and also serine and tyrosine phosphorylation of $I\kappa B_{\alpha}$ was observed in deficient conditions, as well. When the Se deficient macrophages were repleted with Se, restoration of Se-GPx activity was observed, along with decreased levels of MEKK-1, p-$I\kappa B_{\alpha}$ and COX-2, comparable to that of the Se-supplemented cultures.

In conclusion, these results suggest a role for Se in the regulation of NF-κB and subsequent expression of COX-2 and iNOS in a macrophage cell line. While the exact

target or mechanism of Se regulation has not been fully identified in this model, the data suggest that it may be at the level of kinases upstream of NF-κB, notably MEKK1. These findings support the data of other studies in this field and highlight the importance of Se status in cellular redox signaling.

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Chapter 1

Literature Review

I. Oxidative stress, antioxidant defense and the role of selenium:

It is now well recognized that normal oxidative metabolism and certain physiological stimuli can give rise to various forms of free radicals such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (OH^-), nitric oxide (NO) and peroxynitrite (NOO-), most of which appear to be interconvertible. Additionally, normal metabolic products such as hydrogen peroxide (H_20_2), heme and free iron can act as strong pro-oxidants, because of their ability to generate extremely reactive hydroxyl radical through non-enzymatic reactions. Although sub cellular sources of oxidants and pro-oxidants may vary in different cell types, four sources can be assigned as major contributing components of reactive oxygen species (ROS) and reactive nitrogen species (RNS). These include mitochondrial and peroxisomal oxidative reactions, microsomal cytochrome P450 enzymes and membrane associated enzymes such as NADPH oxidase and nitric oxide synthase (1). Under normal circumstances all of these oxidants and pro oxidants are either detoxified by interaction with various reducing and sequestering agents including thioredoxin, glutathione (GSH), and tocopherol (vitamin E), or by enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and thioredoxin reductase.(1) The term “oxidative stress” is used to describe conditions in which the levels of reactive species produced outweigh the capacity of the combined cellular antioxidant defense; due to either excessive, unregulated generation of oxidant species and/or the compromise or depletion of one or more cellular antioxidant enzymes.

Since the recent discovery of selenocysteine (SeCys) as the 21st amino acid in proteins, the field of Se (Se) biology has rapidly expanded (2). Selenoproteins are present in every cell type and out of over 30 that have been identified, at least 12 mammalian selenoproteins have been characterized to date (2). These selenoproteins have different cellular functions, but in those selenoproteins for which the function is known, SeCys is incorporated into the active center via a specific in-frame UGA codon (3).

The most well characterized selenoenzymes are the glutathione peroxidases (GPxs) and thioredoxin reductase (TrxR); of which their detoxification of harmful organic hydroperoxides, lipid peroxides and hydrogen peroxide are responsible for the recognition of Se as an important dietary antioxidant (4). Se deficiency results in significant decrease in the expression of Se-GPxs / TrxRs and an increase in ROS production (4;5). Furthermore, Se deficiency increases the sensitivity of cells to oxidative stress (6). As discussed before, examples of biologically relevant ROS/RNS are $O_2^{\bullet -}$, H_2O_2 , NO, and NOO^-. Selenium-dependent glutathione peroxidase is essential for the reduction of H_2O_2 and lipid hydroperoxides as well as peroxynitrite and, more importantly, GSH/Se-GPx system represents the major antioxidant enzyme mechanism to reduce H_2O_2 and fatty acid hydroperoxides in macrophages (7). Thioredoxin reductases (TrxR) are a family of Se-containing oxidoreductases with mechanistic and sequence identity similar to GSH reductases; however, they exhibit broader substrate specificity than the later enzymes (8). This is mainly due to a second redox-active site, a C-terminal –Cys-SeCys–, which is not found in GSH reductases (8;9). This enzyme functions to

reduce thioredoxin (Trx), which, in turn, controls various cellular redox-related processes such as transcription, protein-DNA interactions and DNA synthesis (10).

II. NF-κB activation and Reactive Oxygen Species:

The NF-κB family of transcription factors plays a crucial role in the immune and inflammatory responses. Because a large variety of bacteria and viruses activate NF-κB, and because this transcription factor regulates the expression of inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules, NF-κB has often been termed a 'central mediator of the human immune response' (11-13). The Rel/ NF-κB family of transcription factors comprise one of the most highly studied groups of eukaryotic regulatory proteins (14). The expression of genes involved in the critical physiological responses of immune and acute phase inflammation, cell adhesion, differentiation, redox metabolism and apoptosis are all controlled by these transcriptional activators (14).

Mammalian Rel/ NF-κB transcription complexes are made up of any of a combination of homo and hetero dimers comprised of the p50, p52, Rel A (p65), Rel B and c-Rel protein subunits (15). These complexes bind to DNA at specific NF-κB enhancer binding regulatory sites, such as 5'GGGACTTTCC-3', to activate gene transcription (15). The target gene specificity and degree of transcriptional activation is thought to be a result of the nature and binding properties of the complexes formed in

various cell types (15). In resting cells, NF-κB (typically consisting of p50 and p65 subunits) is bound to a member of the IκB group of cytoplasmic retention proteins (IκB

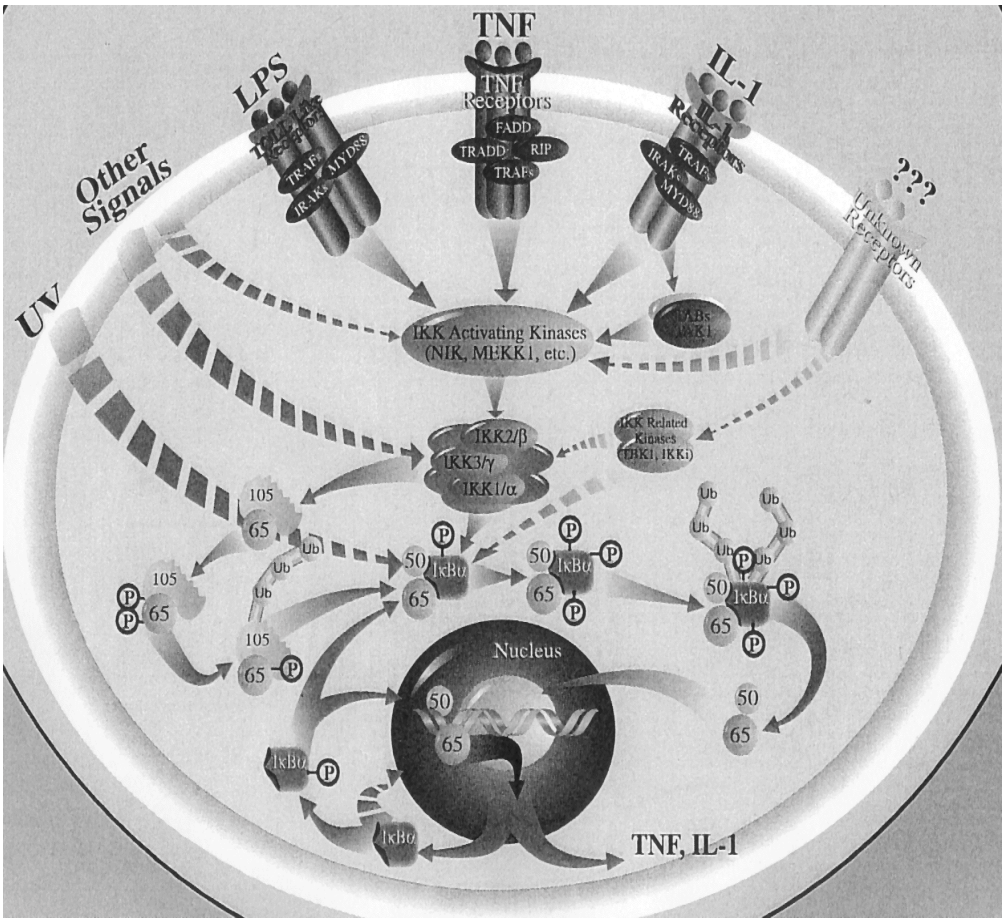

Fig.1 Pathways of NFκB Activation

α, β, γ). Proteins p105, p100 and Bcl-3 also serve an inhibitory function. In response to cell stimulation, a kinase cascade is initiated, IκB is phosphorylated and hence targeted

for ubiquination by the HOS-SCF E3 ubiquitin ligase complex, followed by degradation by the 26S proteosome. Once the inhibitory protein is degraded, NF-κB is free to enter the nucleus and bind to DNA (16) (Fig. 1).

Since the phosphorylation and degradation of IκB is the determining step in NF-κB activation, the kinase cascade preceding it has been the subject of intense study. The IκB kinase (IKK) complex consists of multiple subunits, with IKK α and β being responsible for the kinase activity. The 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 (17). Upon stimulation by growth factors, cytokines or microbes, upstream kinases are activated and recruited to the IKK complex via IKKγ, which results in the phosphorylation of IKKβ, followed by activation of the remainder of the complex through autophosphorylation, with the final result being phosphorylation of IκB at two serine residues (32 and 36 for IκB α, 19 and 23 for IκBβ) (18).

Several upstream kinases have been implicated in the activation of the IKK complex. These include NF-κB-activating kinase (NIK), mitogen-activated protein 3 kinase (MEKK1), ribosome inactivating protein (RIP), and IL-1 receptor-associated kinase (IRAK) (17). The specific kinases that are involved and the resulting phosphorylation of specific residues on IKKα and/or β seem to be dependent on the stimuli and cell type. For example, the NF-κB signaling cascade initiated by the inflammatory cytokine TNFα in monocytes is one of the most characterized and is

mediated by TNF receptor-associated factor (TRAFs), followed by the induction of NIK and the IKK complex (19). To further contribute to the complexity of activation, alternative pathways not involving the IKK complex exist. Examples are ultraviolet radiation, which recruits NF-κB, without the activity of the IKKs (20), and phosphorylation of IκB at a tyrosine residue, rather than serine, have also been reported (21;22).

The activation of NF-κB can be achieved by several different mechanisms, including the amount of IκB present, the activity of upstream kinases, and binding to the DNA site, all of which appear to be influenced by the cellular redox status (14). Recent studies have indicated that NF-κB is the major redox-sensitive transcription factor (23-26). Antioxidants have been shown to block activation of NF-κB and subsequent expression of genes under its control (25). Of particular interest is the elucidation of redox sensitive elements along the NF-κB induction pathway. There is an increasing body of evidence to suggest that the IKK complex is the key regulator of NF-κB-mediated immune and inflammatory responses (27). This is one of the most critical kinase cascade pathways involved in NFκB activation that also appears to be regulated by intracellular redox state (28). Current research suggests that IKK activity is influenced by oxidant tone (22), and there is also evidence pointing towards other upstream kinases, such as the SAP Kinases (SAPK)/JUN kinases (JNK) (13). Various ROS are known to activate NIK to initiate the phosphorylation process and proteolytic degradation of IκB (23-26). Also, the antioxidant thioredoxin is required to maintain the κB site in a reduced

state for binding and activation, and plays a role in modulating the JNK pathway at the level of MEKK1 (29).

Because NF-κB is involved in the regulation of a diverse group of genes implicated in cellular inflammatory responses and tissue damage, it has become a potential therapeutic target for the treatment of human inflammatory diseases. The importance of maintaining optimal oxidant-antioxidant balance, therefore, becomes even more magnified. A dysfunction or deficiency in cellular antioxidant function not only results in direct free radical damage such as the peroxidation of membranes, but also can stimulate or repress genes critical in initiation and resolution of a healthy inflammatory response (1). Relevant to this discussion is the fact that Se plays an important role in the modulation of many protein functions involved in signal transduction, particularly pathways involving NF-κB (30-32); however, the picture on precise molecular events associated with Se regulation is far from clear. As mentioned earlier, thioredoxin is key in regulating DNA binding and obtains its reducing capacity through the action of selenoprotein, thioredoxin reductase (28). Studies with the human HIV-1 LTR promoter demonstrated that NF-κB up regulation is associated with decreased GPx activity, which may be reversed by Se supplementation (33). It was also reported that selenite supplementation to cultured cells leads to protection against peroxynitrite-induced MAP kinase activation and against nitration of protein bound tyrosine residues (34). Since selenite itself does not interact with peroxynitrite, this suggests a protective effect of cellular Se containing proteins, such as GPx, and the ability to modulate upstream kinase pathways that may lead to NF-κB activation.

III. Macrophages and the immune system.

Macrophages play a central role in immune response as phagocytic cells capable of targeting and ingesting invading organisms. During infection, macrophages have the capacity to become "activated" by lymphokines and different bacterial products. Lipopolysaccharide (LPS) is a component of the outer membrane of gram-negative bacteria. As one of the most potent activators of monocytes, exposure to LPS results in the activation of several signal transduction pathways through induction of G-proteins, cAMP dependent kinase, protein kinase C, tyrosine kinase, PI3 kinase, and the ERK, p38, and JNK mitogen activated kinase (MAPK) families (35). In addition, LPS stimulated macrophages produce TNFα, which initiates a cascade of gene transcription and transcriptional de-repression {159). The culmination of this broad signaling response is the synthesis and secretion of inflammatory mediators such as complement components, prostaglandins, interleukins, and pro-inflammatory cytokines.

To enhance their microbicidal and tumoridical functions, "activated" macrophages undergo a respiratory burst that produces such reactive oxygen and nitrogen species as $O_2^{\bullet-}$, H_2O_2, NO and NOO^- {146}. It is important to note that stimulated macrophages release these reactive species in quantities that are orders of magnitude greater than non-phagocytic cells. The large amount and broad reactivity of the ROS produced by activated macrophages assist these cells with their essential protective duties, but unfortunately also contribute to the onset of pathological conditions like bacterial sepsis, ischemia/reperfusion injury, and chronic inflammatory disease (36). The

capacity of the macrophage to regulate and defend itself against oxidant-induced damage is therefore very important, not only to protect and preserve its own function, but also to protect the surrounding tissue parenchyma from oxidative damage. As protection from the deleterious effects of reactive species, macrophages are equipped with a multi-tier antioxidant enzyme system, consisting of catalase, superoxide dismutase, and selenoproteins TrxR and GPx, to name a few (37). Without optimal antioxidant protection, the macrophage would be exposed to excessive levels of reactive oxidant species, which may prolong the activated state and contribute to the development of DNA mutations, tissue damage and a state of chronic inflammatory conditions (38).

IV. Mediators of Inflammation:

The redox regulation of transcription factors, their target genes and gene products are important processes in the normal inflammatory response, as well as in the development of immune disorders. As previously discussed, NF-κB is the major redox responsive transcription factor and it binds with a consensus sequence located in the promoters of nearly all genes involved in the initiation of an inflammatory response (1). Cyclooxygenase-2 (COX-2) and the inducible isoform of nitric oxide synthase (iNOS) are two such genes, whose products exert broad effects in inflammation and the development of inflammatory disease (39).

Prostaglandins, the products of the COX-2 pathway, promote inflammation by increasing vascular permeability, vasodilation, and the secretion of proinflammatory cytokines that direct cellular migration to the site of injury (40). Sustained, elevated

prostaglandin levels are connected with the onset of chronic inflammation and cancer (39). Cyclooxygenase catalyzes the first committed step in the formation of prostaglandins and thromboxane from free arachidonic acid (41). It exists in two isoforms: COX-1 and COX-2. COX-1, for the most part, is constitutively expressed in all cell types and is involved in normal kidney, gastrointestinal and reproductive function (40;42;43). COX-2 may be basally expressed in some tissues, but is largely inducible by a wide variety of mitogens, hormones, cytokines and other stimuli and is thus associated with inflammation and disease (41). Macrophages secrete prostaglandins following stimulation with LPS, primarily due to the induced transcription of the COX-2 gene and production of COX-2 enzyme (44;45).

In addition to responding to stimulation from LPS and cytokines, COX-2 is also subject to redox regulation at two levels. First, low concentrations of peroxides are required for activation, while higher amounts may affect enzyme activity (46;47). In addition, the gene encoding for COX-2 protein is under the regulation of NF-κB, which, again is influenced by oxidant status. The COX-2 promoter in both human and mouse genes contains two κB enhancer elements, to which NF-κB binds to initiate transcription (48;49).

Nitric oxide (NO), produced by the activity of nitric oxide synthases (NOS), is physiologically significant for its role in regulating vascular tone and as a signaling molecule in neurotransmission (50). Nitric oxide is also an important component of the antineoplastic and antimicrobial armament of macrophages (51;52). This highly labile

and noxious gas is produced in large and sustained quantities by macrophages following exposure to a variety of immunologic and inflammatory mediators. The high-output production of nitric oxide is dependent on induction and expression of the inducible isoform of nitric oxide synthase (iNOS) expressed in macrophages (51). Endothelial cells and neurons also express unique forms of nitric oxide synthase; however, the expression is constitutive and nitric oxide is produced at lower steady levels (53). It is this low level production that is of biological significance, while overproduction may lead to circulatory shock, chronic inflammation and carcinogenesis (39).

Similar to COX-2, transcriptional activation of iNOS is considered to be the major means for regulating nitric oxide production and is also subject to redox modulation (51). Antioxidant enzymes inhibit iNOS mRNA synthesis, while hydrogen peroxide generated by LPS activated macrophages up regulates iNOS protein expression (54). Several transcription factors have been identified as important in the activation of the murine iNOS gene, including NF-κB (51;55). Similar to the COX-2 promoter, the human and mouse iNOS gene promoters both contain two NF-κB binding sites (55).

Excessive ROS production, as seen in conditions of Se deficiency, is often associated with chronic inflammation (52) and the uncontrolled sustained production of both prostaglandins and nitric oxide following LPS exposure is a major factor in the onset of septic shock, systemic inflammatory response syndrome (SIRS), and ischemia/reperfusion injury conditions (56;57). Selenium supplementation and restoration of GPx activity has been shown to be therapeutic for patients in the early stages of these

conditions which suggests that Se status may be important in regulating the production of key biological mediators in the initiation and resolution of the inflammatory response (58).

V. Selenium nutrition and related diseases:

The ultimate source of Se is the soil, and it is incorporated into vegetable protein as the amino acids selenocysteine and selenomethionine. It follows that the issue of human Se deficiency is mainly relevant in areas with low Se soil. This is evidenced in regions of China, where "Keshan's Disease" a severe cardiomyopathy is prevalent, as well as in Finland, where the low Se content and resulting heart disease and tumor incidences led to the need for Se supplemented fertilizer (59). Although Se deficiency is not a universal nutritional concern, the presence of selenocysteine at critical sites in naturally occurring enzymes provides an explanation for the important role of Se in human health and development (2;3) As an essential micronutrient, Se has been shown to effect nearly every aspect of the immune system (59;60). *In vivo*, Se supplementation results in enhanced natural killer and cytotoxic T cell activity (61), and enhanced antibody response and killing ability of macrophages *in vitro* (62). Conversely, Se deficiency is associated with increased platelet aggregation and leukotriene synthesis and decreased antibody production (59). Se-GPX activity is reduced in several disease states, most notably HIV infection and AIDS (63-65) and in turn, reactive oxygen and nitrogen species are implicated in atherosclerosis, rheumatoid arthritis, osteoporosis, asthma, renal

failure, Alzheimer's disease and other pathologies where an inflammatory component is involved (56;57;66;67). In recent years, the role of Se in preventing human disease has gained new attention following the association of "super- supplementation" (above the daily recommended dosage) with decreased incidences of numerous forms of cancer (68). It has been proposed that Se 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 or disulfide bonds, possibly through TrxR (69). Also noteworthy is that Ebselen, a biologically active selenoorganic compound with Se-GPX-like activity has been used successfully to scavenge free radicals and inhibit proinflammatory enzymes (70).

Recent studies have also established Se as a potential regulator of oxidant induced transcription pathways (71). Increasing the level of selenoproteins by Se supplementation attenuated mitogen activated protein kinase (p38, JNK, ERK) activation by peroxynitrite in cultured rat liver cells (34). On the other hand, Se deficiency in rats increased the binding of liver nuclear proteins to DNA regulatory elements NF-κB, NF-1, and OCT, which activate transcription in response to oxidative stress (71). In addition, Se-GPx over expression was shown to inhibit NF-κB activation, via inhibition of IκB phosphorylation (32).

Summary and statement of the research question

The following research is based on the premise that an oxidant-antioxidant imbalance, a consequence of inadequate Se nutrition and low GPx activity, leads to increased production of key proinflammatory mediators by macrophages. It is also thought that this increase is a result of enhanced signal transduction mediated by ROS or RNS. Macrophages play a central role in the immune regulation through a number of functions including antigen presentation to lymphocytes and their participation in ingestion and killing of various invading microorganisms. While all cells produce ROS during normal respiration, activated macrophages produce increased levels of various ROS/RNS such as $O_2^{\bullet-}$, H_2O_2 and NO-. In addition, such ROS are important parts of signal transduction and target interactions, particularly the signal-induced activation of NF-κB. NF-κB is a redox sensitive transcription factor, which is associated with the expression of different immediate-early genes including COX-2 and iNOS, whose products, prostaglandins and NO, together with the acute phase cytokines regulate the initiation and duration of the inflammatory response. Thus, the excess production of ROS by macrophages may contribute significantly to the pathophysiology of inflammatory disorders.

Under normal conditions, however, these cells employ multiple defense systems in protecting themselves from cytotoxic effects of ROS. Among the various defense mechanisms, selenoproteins, particularly Se-GPx, are a crucial component in maintaining optimal cellular oxidant/ antioxidant balance.

Thus, the research question seeks to confirm the hypothesis that inadequate Se status in macrophages leads to an oxidant-antioxidant imbalance thereby increasing their synthesis and secretion of biological mediators. **The specific hypothesis to be tested is that oxidant stress, a consequence of Se deficiency, is responsible for the activation of redox-active transcriptional factor, NF-κB , through a complex kinase-mediated pathway(s) and that the activated NF-κB is in turn responsible for the altered transcription of COX-2 and iNOS, finally resulting in increased macrophage release of the proinflammatory mediators, PGE_2 and nitric oxide.**

Chapter 2

Nuclear Factor-κB mediates Over-Expression of Cyclooxygenase-2 during Activation of RAW 264.7 Macrophages in Selenium Deficiency

Abstract

Selenium (Se) is an essential micronutrient for all mammalian species and is associated with a variety of physiological functions, notably immune system, in the form of selenoproteins. Inadequate Se nutrition has been linked to various diseases, including rheumatoid arthritis, cardiomyopathy and cancer. Important to this discussion is that cyclooxygenase-2 (COX-2) is over-expressed in all the aforesaid pathologies; however, a casual relationship between Se status and COX-2 expression remains to be established. The present study is based on the hypothesis that oxidant stress, a consequence of Se deficiency, lowers the activation potential of the redox-sensitive transcription factor, NF-κB, and that the activated NF-κB is required for the altered expression of COX-2. To test this hypothesis, the relationship between Se status and COX-2 expression in response to LPS stimulation was investigated in RAW 264.7, a macrophage-like cell line. In Se-deficient cells, the Se-dependent glutathione peroxidase activity (Se-GPx), a measure of Se status, was markedly reduced and the overall oxidative stress was significantly higher than Se-supplemented cells. Upon lipopolysaccharide (LPS) stimulation, 2-3 fold higher COX-2 protein expression as well as higher PGE_2 levels were observed in Se-deficient cells than Se-supplemented cells. In comparison, COX-1 protein expression was not affected by either LPS stimulation or Se status. Following LPS stimulation, the nuclear localization and DNA binding of NF−κB was increased in Se-deficient macrophages, thereby leading to increased expression of COX-2. Examination of the signaling cascade upstream of NF-κB revealed the increased presence of MEKK1, NIK, and IKK_β during

Se deficiency, as well as enhanced phosphorylation of MEKK1 and IκB$_\alpha$, suggesting that the inhibition of NF-κB activation following selenium supplementation may be modulated at the level of upstream kinase activity. In addition, the effects of selenium deficiency on the reduction in Se-GPx activity and associated up regulation of MEKK1, pIκB$_\alpha$ and COX-2 expression were determined to be reversible when the culture medium was replenished with selenite. These findings are of particular relevance to *in vitro* cell culture conditions, as traditional cell culture media and reagents are lacking in selenium and thus, may be adversely affected.

Introduction

Selenium (Se) is an essential micronutrient for all mammalian species and functions primarily through selenoproteins, which contain Se as selenocysteine (SeCys). These selenoproteins have different physiological functions, but for those in which the function is known, SeCys is located at the active center (72;73). The most well characterized selenoenzymes are the Se-GPxs and TrxR families, the activities of which are responsible for the recognition of Se as an important dietary antioxidant (72). These enzymes are involved in many biochemical processes supporting life. The most important processes include protection against oxidative stress and redox-based regulation of gene expression (73). Selenium deficiency results in a significant decrease in Se-GPx and TrxR, and an increase in ROS production (72;73). Inadequate Se nutrition and associated ROS production have been linked to increased risk of such diseases as cardiomyopathy (74), rheumatoid arthritis (67) and, most notably, cancer (68;75). Emerging evidence from clinical trials and epidemiological studies has shown the beneficial effects of Se supplementation in prevention and/or treatment of some of these diseases (76). For instance, studies have demonstrated the chemo-preventive activity of Se against the development of tumors or cancers in both animal models and humans (77-79). Furthermore, it has been documented that the level of Se in cancer patients is lower than that in normal subjects (80). However, the molecular mechanisms contributing to the anti-carcinogenic and chemo-preventive activity of Se are not very clear and are the current topics of intense investigation.

COX, also known as prostaglandin H synthase (PGHS), is the rate-limiting enzyme in prostaglandin (PG) biosynthesis (81). There are two isoforms of this enzyme: the constitutive form, COX-1, which is implicated in housekeeping or homeostatic functions, and COX-2, which is an inducible form in response to injury or inflammation (81). The discovery of COX-2 in early 90s was followed by an unprecedented search for drugs, which specifically inhibit COX-2, with no influence on COX-1. Intensive research on these isozymes has revealed distinct differences in their function and regulation (81). Cyclooxygenase-2 can be induced by various pathogenic stimuli, including proinflammatory cytokines, mitogens, bacteria and ROS (46). In addition, there is a growing body of evidence to suggest that the NF-κB, a well-known redox-sensitive transcription factor, is involved in the regulation of COX-2 induction by various stimuli (82;83). Important to this discussion is the fact that COX-2 is over-expressed in inflammatory diseases as well as several types of human cancers and COX inhibitors have been shown to be anti-inflammatory and chemo-preventive (84;85).

In the present study the relationship between Se status and COX-2 expression in response to LPS stimulation in RAW 264.7, a murine macrophage-like cell line has been investigated. These cells have been previously established as a model for the study of COX-2 regulation (86). Furthermore, these studies are based on the hypothesis that inadequate Se status in macrophages leads to an oxidant-antioxidant imbalance, and this imbalance leads to a lowered activation potential for the redox-sensitive transcription factor, NF-κB, which, in turn, induces the expression of COX-2. The data shows that COX-2 expression is induced sooner and to a greater extent in LPS stimulated RAW

264.7 cells during Se deficiency. This correlates with subsequent PGE_2 production; however, Se status has no affect on COX-1 expression. This is the first report to demonstrate an inverse relationship between Se status and COX-2 expression. The data also suggests that the level of Se mediation involves upstream kinases in the NF-κB activation pathway.

Materials and Methods

Cell culture conditions

RAW 264.7 cells, a murine macrophage cell line, were cultured at 37 ^{o}C in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM), which contained 2mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies, Inc., Gaithersburg, MD) and 5% defined fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT). The Se-deficient RAW 264.7 cells were grown under these conditions. The only Se present in the medium was derived from the FBS and was determined to be 6 pmoles/ml according to company quality control. The Se-supplemented RAW 264.7 cells were grown in the same medium, containing sodium selenite (Sigma, St. Louis, MO) at a final concentration of 2 nmoles/ml. For the analysis of COX-2 and COX-1 expression and PGE_2 production, as well as NF−κB translocation, the cells were plated at a density of 1 x 10^6 cells per well in 6-well culture plates, allowed to adhere overnight, and used for these experiments the following day. Thereafter the cells were stimulated with LPS (*Escherichia coli* Serotype 0111:B4) for different time periods. The rationale for using LPS as a stimulus was to investigate some of the

molecular details on how Se-deficient macrophages respond to oxidative stress as compared to Se-supplemented cells. In some experiments, N α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (100 μM), a serine protease inhibitor of IκB degradation, was added to the cells 30 minutes prior to LPS challenge.

For luciferase reporter assays involving NF-κB mutants, cells were seeded at 1 x 10^5 cells per well in a 96 well plate and transfected with 750 ng of plasmid DNA using Superfect (Qiagen, Los Angeles, CA) for 2 h. Cells were allowed to grow for 18 h prior to stimulation with LPS for 5 h. Following stimulation, cells were washed with PBS containing calcium and magnesium (1 mM each) and overlaid with 100 μl PBS. Luciferase activity was determined using LucLite Plus Assay kit (Packard, Hartford, CT). The average of 4 transfections was used to determine the relative luciferase values for both LPS-stimulated and unstimulated cells.

Glutathione Peroxidase activity

The cellular Se-GPx activity was measured according to the published method using H_2O_2 as substrate (87). CHP was used as substrate in measurement of the Phospholipid-GPx activity. Selenium-deficient or Se-supplemented RAW 264.7 cells were harvested, lysed and microfuged for 10 minutes. The resulting supernatant was used for enzyme analysis. The oxidation of NADPH was monitored spectrophotometrically at 340 nm following the addition of 1.5 mM H_2O_2. 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).

Measurement of oxidative tone and lipid peroxidation

RAW 264.7 macrophage cells were grown in Se-deficient and Se-supplemented media as described in the previous section. For a sensitive measurement of total ROS generation during Se deficiency, cells were harvested, washed once with PBS and incubated with 2.5 μM of 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Molecular Probes, Eugene, OR) for 15 minutes at 25 ^{o}C. The number of cells exhibiting increased fluorescence of DCF, generated by ROS, was analyzed by a Beckman-Coulter XL-MCL single laser flow cytometer. These studies were performed at the Life Science Consortium's Flow Cytometry Facility, The Pennsylvania State University, University Park. Similarly, the fluorescent label, diphenyl-1-pyrenylphosphine (DPPP) (DOJINDO laboratory, Kumamoto, Japan) was utilized to assess lipid peroxidation the Se-deficient and Se-supplemented cultures according to the protocol of Takahashi, et al (88). Both groups were incubated in the dark with DPPP at a final concentration of 167μM for 5 minutes at 37 ^{o}C. Cells were washed with PBS and placed in fresh Se- deficient or Se-supplemented media and stimulated with LPS (1μg/ml) for the indicated times prior to harvest by scraping. DPPP labeled cells were exposed to room light for 30 minutes and the suspension subjected to fluorescence measurement with excitation of 351nm and emission of 380nm on a Farrand Optical System 3 spectrofluorometer.

Isolation of nuclei

For the analysis of NF−κB translocation and for use in EMSA, nuclear extracts were prepared from Se-supplemented and deficient RAW 264.7 cells (2 x 10^6 cells) grown in 6 well culture plates. Fresh media was added to cells prior to stimulation with LPS (1 μg/ml) for the indicated times. Following stimulation, cells were washed and harvested in ice-cold PBS. Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce), according to the manufacturer's instructions. Total protein was measured using the BCA reagent (Pierce).

Western Blots

The harvested cells were washed three times in ice-cold PBS and centrifuged at 500 x *g* for 10 minutes at 4 ^{o}C. The cell pellets were resuspended in ice-cold lysis buffer (10 mM Tris pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaF, 10 mM NaPi, 10 mM NaPPi) for 15 minutes and then centrifuged at 10,000 x *g* for 15 minutes. The supernatant was collected and total protein concentration was determined using the BCA reagent (Pierce). Samples of 50 μg total protein or 20 μg of nuclear protein, were electrophoresed on a 10 % SDS-polyacrylamide gel and transblotted to nitrocellulose membrane (Bio-Rad, Hercules, CA) and blocked for 1 h at 25 ^{o}C in 10 mM Tris, pH 8.0, containing 150 mM NaCl, 0.1%Tween 20, 5% skim milk. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. Detection of COX-2 and GAPDH or COX-1 and GAPDH were performed after incubation with their specific antibodies overnight at 4 ^{o}C, while NF−κB p65, MEKK1, NIK, $IKK_{\alpha/\beta}$, $IKK_{\alpha,}$ phospho-$I\kappa B_{\alpha}$ and

phospho-serine, phospho-tyrosine were detected after 1 h incubation with specific antibody at 25 ^{o}C. Rabbit anti-mouse COX-2 polyclonal antibody and anti-mouse COX-1 monoclonal antibody were from Cayman Chemicals (Ann Harbor, MI). NF−κB p65, MEKK1, NIK, $IKK_{\alpha/\beta}$ (97% specific for IKK_{β}) and IKK_{α} polyclonals and monoclonal phospho-$I\kappa\beta_{\alpha}$ specific antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-serine and phospho-tyrosine monoclonals were from Sigma and monoclonal antibody for mouse anti- (GAPDH) was from Chemicon International Inc (Temecula, CA). A horseradish peroxidase-conjugated anti-IgG antibody was used as the secondary antibody. The bands were visualized by enhanced chemiluminescence (ECL) assay kit (Pierce) according to the manufacturer's instructions.

Measurements of PGE_2

Aliquots (100 μl) of culture medium were collected from the same cells grown and stimulated with LPS under identical conditions as described for Western blot analysis. PGE_2 was measured using the PGE_2 enzyme immunoassay kit (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions.

Generation of NF-κB mutants

The wild type COX-2 promoter DNA was PCR amplified from genomic DNA isolated from RAW 264.7 cells. The promoter sequence amplified was from –966 to +8 with respect to the RNA start site. The wild type promoter was cloned into pGL3 promoter vector (Promega, Madison, WI) at the Bgl II and Nco I sites (which removes

the SV40 promoter sequence). The NF-κB binding site at position –668 to –659 was changed from GGGAAATACC to TCGATATGAC to generate NF-κBM-666. The NF-κB binding site at position –401 to –392 was changed from GGGGATTCCC to GGTGTGTATC to generate NF-κBM-400, identical to a previously described alteration (89). Both mutations were generated using the GeneEditor Site-directed mutagenesis system (Promega), and verified by restriction enzyme as well as sequence analyses. A double mutant construct was generated by restriction digestion of NF-κBM-666 with Bgl II and Sac I, while NF-κBM-400 was digested with Sac I and Nco I. The purified DNA fragments were sub cloned into pGL3 promoter vector digested with Bgl II and Nco I.

Luciferase assays

The p-NF-κB luciferase reporter plasmid (Stratgene, La Jolla, CA) contains 5 κB responsive elements. This luciferase vector was transiently co-transfected into Se deficient and Se supplemented cells along with the pCMVβ β-galactosidase reporter (gift from Dr. Kelvin Davies, Albert Einstein College of Medicine) via electroporation according the previously described protocol (90). Following transfections, cells were washed in PBS and returned to Se deficient or Se supplemented media for 24 hours prior to stimulation with 1 μg/ml LPS for 4 hours. Luciferase activity was determined using an assay kit with passive lysis buffer (Promega, Madison, WC) and normalized by β-galactosidase activity using Galacto-star reagents (Tropix Applied Biosystems, Bedford, MA).

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins (3 μg) were incubated with 15,000 cpm ^{32}P-labeled NF-κB double-stranded oligonucleotide (Promega), 1 μg poly (dI-dC), and 1.75 pmoles of unlabeled AP-1 double stranded oligonucleotide (Promega), as a non-specific competitor, for 30 minutes and subjected to polyacrylamide gel electrophoresis. To confirm the specificity of NF-κB binding, unlabeled oligonucleotide in five-fold excess was used as a specific competitor (SC).

Immunoprecipitation (IP)

Cells were scraped, washed once in PBS and the cell pellet lysed in radioimmune precipitation (RIPA) buffer (10 mM Tris-Cl, pH 8.0, 140 mM X-100, 1 mM PMSF, 5 mM iodoacetamide, 1% sodium deoxycholate, 0.1% SDS and 0.025% NaN_3). The lysate was pre cleared and the specific primary antibody added. The immune-complex was precipitated using Protein A/G (Pierce, Rockford, IL) and the precipitate washed in PBS buffer. The precipitate was then analyzed by Western blot as described with phospho-serine or phospho-tyrosine specific antibodies.

Selenium Repletion

Deficient cultures were incubated in selenite- supplemented media (identical to that described for the Se- supplemented cultures) for the equivalent of one passage (72 hours), then harvested and assayed for GPx activity or washed with PBS and stimulated

with 1μg/ml LPS for the indicated times. Following stimulation, cells were lysed with M-PER reagent (Pierce) according to the manufacturer's instructions. 50 μg of total lysate was loaded for Western blot analysis with the indicated antibodies.

Statistics

All enzymatic assays 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 (SigmaPlot software, SPSS, Inc.). The SDS-PAGE, Western immunoblotting, immunoprecipitation and EMSA experiments were performed at least in duplicate and typical representative gels or autoradiograms are shown.

Results

Cellular Se-GPx activity differentiates the Se-deficient and Se-supplemented RAW 264.7 cells

The cellular Se-GPx activity was measured in cell lysates from Se-deficient and Se-supplemented RAW 264.7 cells as a marker for Se status. The Se-GPx activity was determined to be significantly higher (> 10 fold) in Se-supplemented cells than that found in Se-deficient cells (Fig. 2A,B). Similar trends were observed with phospholipid GPx (PhGPx) activity (Fig. 2B). The Se-GPx activity was analyzed at 8, 12, and 16 passages after initial Se-supplementation as well as after 6 months. The analyses confirm the differences between the Se-deficient and Se-supplemented cells were stable for six months (6 month data not shown). Selenium-dependent GPx activity was also analyzed in the cells treated with LPS in both experimental cell lines, and the results indicated no change in GPx activity due to LPS treatment (data not shown). Furthermore, incubation of RAW 264.7 cells with 2.5 μM of 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA), a fluorescent label for oxidative tone (Fig. 2C) showed that the overall level of oxidative stress was significantly greater ($p=.029$) in the deficient Se-deficient RAW 264.7 cells prior to being stimulated with LPS. Similarly, fluorescent labeling with Diphenyl-1-pyrenylphosphine (DPPP), also resulted in a visible increase in lipid peroxidation pre- and post LPS challenge in the Se-deficient cultures(Fig. 2D).

Collectively, these data indicate that the cellular oxidant tone has been affected by the Se status within the culture conditions and prior to LPS stimulation.

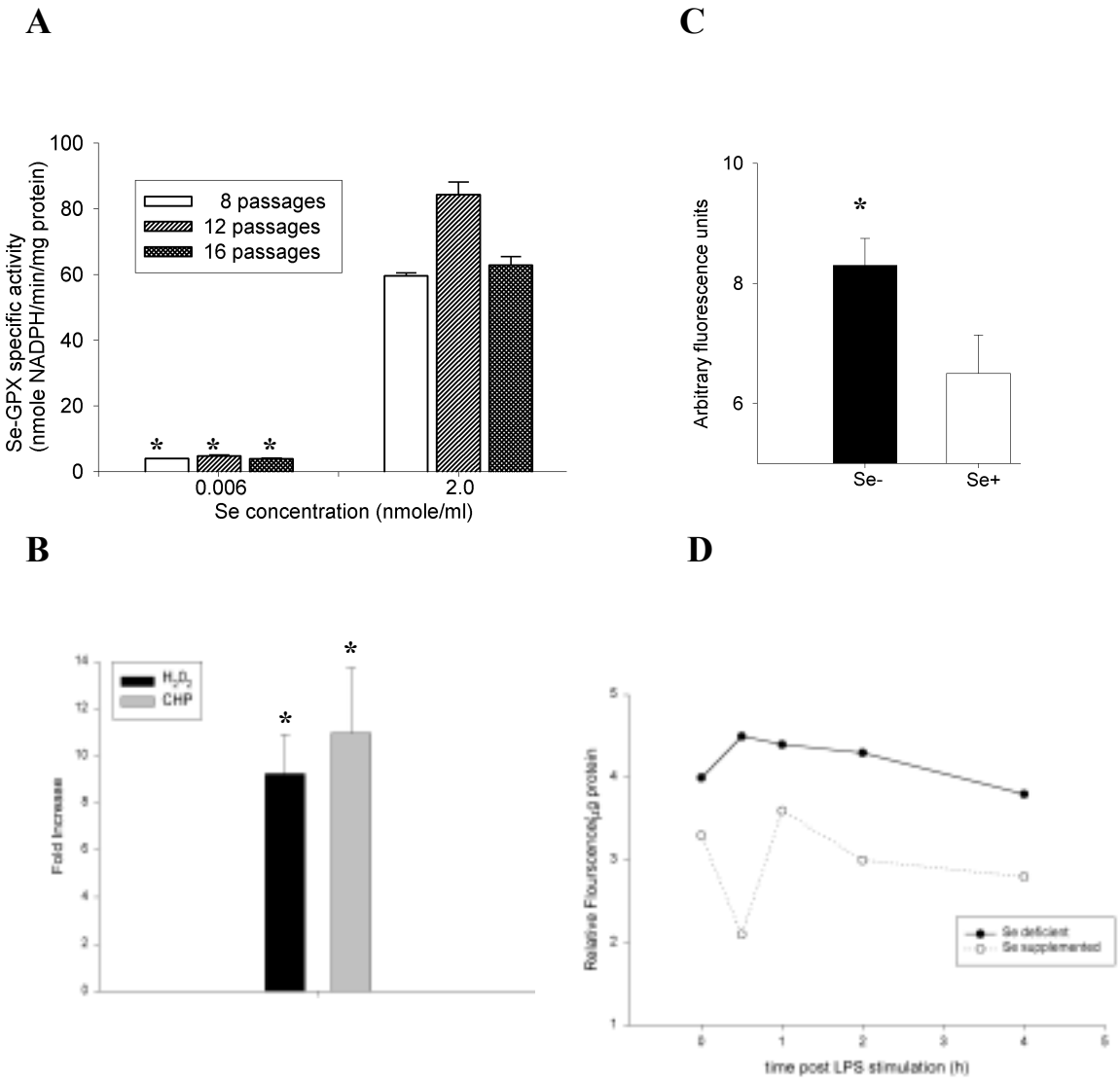

Fig. 2. Effect of Se status on Se-GPx activity and overall oxidative stress.

A. Se-GPx activities were measured at 8, 12 and 16 passages in Se-deficient (.006 nmoles/ml selenium from defined FBS) or Se-supplemented (2 nmoles/ml sodium

selenite) RAW 264.7 cells. The results are expressed as nmole NADPH/min/mg protein. **B.** Fold induction of GPx activity toward H_20_2 and CHP with Se supplementation. **C.** Se-deficient and Se-supplemented RAW 264.7 cells were incubated with 2.5 μM of 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) for 15 minutes. Cellular fluorescence, an indication of overall oxidative stress, was measured by a Beckman Coulter flow cytometer. Arbitrary fluorescence units for each sample, representative of 3 independent experiments, are given. **D.** Incubation of Se deficient and Se supplemented cells with DPPP for 10 minutes, followed by LPS (1μg/ml) stimulation for the indicated times. Relative fluorescence was assessed by spectrofluorometer with 251nm excitation and 380nm emission; n=1, no statistical analyses performed.

Selenium deficiency selectively affects the regulation of COX-2 protein expression in LPS-stimulated RAW 264.7 cells

To determine the effect of Se deficiency on the expression of COX isozymes, both Se-deficient and Se-supplemented cells were activated with LPS, and protein levels of COX-1 and COX-2 were analyzed by Western blots. Cyclooxygenase-2 protein levels were shown to increase faster and to a higher degree in Se-deficient cells during the first 4 h as compared to Se-supplemented cells (Fig. 3A and B). In contrast, COX-1 protein levels remained unaffected by either LPS stimulation or Se status during the experimental time periods. The GAPDH signal, used as internal standard, was also detected to be at the same level for all samples.

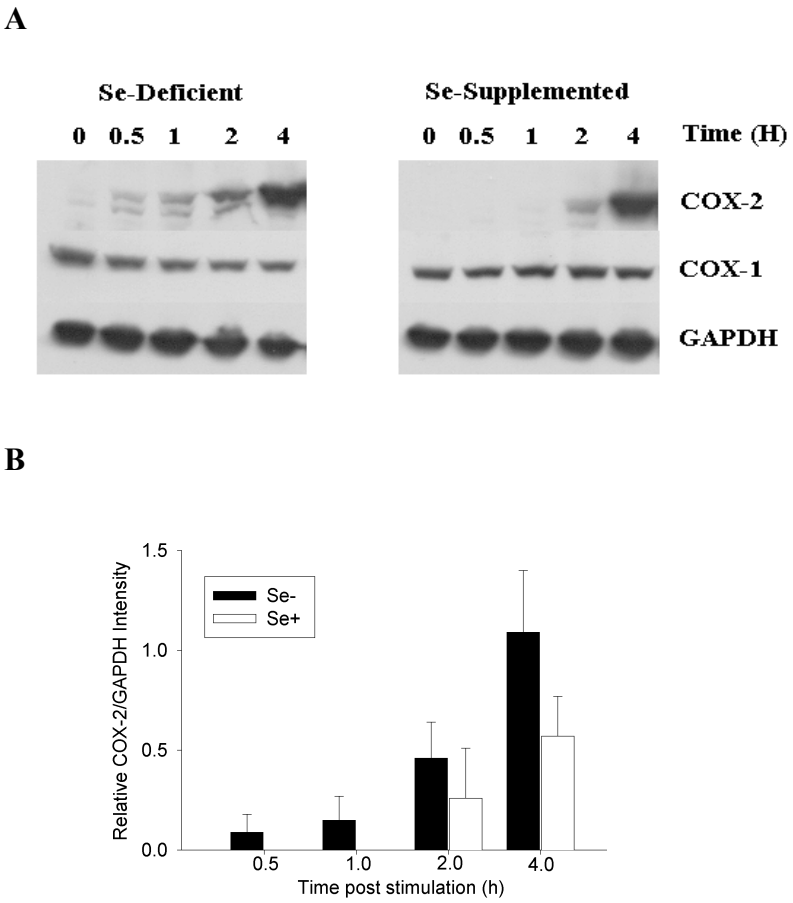

Fig. 3. Effect of Se deficiency on COX-2 protein expression.

A. The time course of COX expression in Se-deficient and Se-supplemented RAW 264.7 cells. Cells were incubated with 1 μg/ml LPS for the indicated time periods. Cell lysates were analyzed by Western blot with the specific antibodies. The data is a representative of four independent experiments. **B.** The densitometric value of the COX-2 and GAPDH

specific bands from four independent Western blots was determined using Scion Image Software. The COX-2 to GAPDH ratio was determined and plotted with standard error.

Selenium status affects PGE_2 production in LPS stimulated RAW 264.7 cells

To demonstrate that the different levels of COX-2 protein expression seen by Western blot analysis, affected the overall COX-2 activity, PGE_2 production, an indicator of COX-2 activity, was analyzed by EIA (Fig. 4).

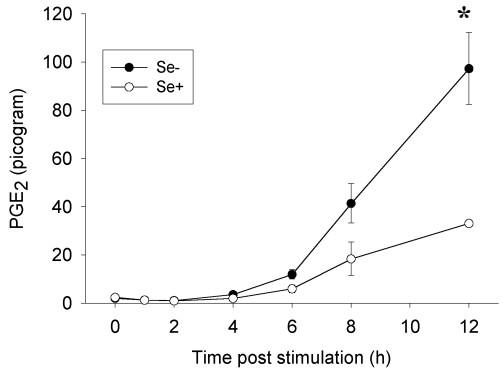

Fig. 4. The effect of Se status on PGE_2 production in LPS-stimulated Se-deficient and Se-supplemented RAW 264.7 cells. Cells were stimulated with 1 μg/ml LPS for the indicated time intervals (0, 2, 4, 6 and 12 h) and supernatants of the cell culture media were collected and assayed by EIA. It should be noted that error bars are present at all time points, although smaller variations cannot be seen due to figure scale.

Cells were stimulated with 1 μg/ml LPS and aliquots of the cell culture supernatant were taken at various time points. Analysis of PGE_2 production demonstrated an increase in PGE_2 levels in stimulated Se-deficient cells as compared to Se-supplemented cells (Fig.

4). The increase in PGE_2 was shown to correlate with the increase in COX-2 protein level; however, with a delayed time phase.

Cyclooxygenase-2 expression is dependent upon NF-κB activation

Recently, Wadleigh *et al*. reported that transcriptional activation of COX-2 in LPS stimulated macrophages was independent of NF-κB sites but rather required C/EBPβ sites [20]. Others have reported that activation of COX-2 involves NF-κB activation [21]. To determine the role of this redox-sensitive transcription factor in LPS activation of COX-2, we generated a COX-2/luciferase construct using the murine COX-2 promoter sequence from RAW 264.7 cells and then mutated the two NF-κB binding sites contained within. These constructs were transfected into RAW 264.7 cells. Following stimulation with LPS, the luciferase activity was determined. The wild type COX-2 promoter was stimulated over 2 fold with LPS when compared to the unstimulated cells (Fig. 5). Almost all the basal activity was lost when the NF-κB sites were mutated. Furthermore, the double mutant showed little activation with LPS. This data indicates that LPS activation of COX-2 is mediated through NF-κB. The role of additional factors, such as C/EBPβ may still be important, as well.

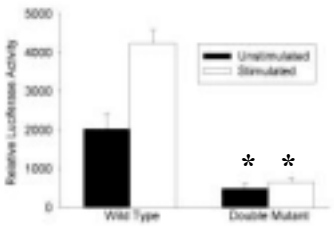

Fig. 5 Mutation of κB sites alters COX-2 promoter activity.

Both the wild type and the double mutant promoter/reporter constructs were transfected into RAW 264.7 cells. After 18 h, one set of

transfected cells was stimulated with LPS (1 μg/ml) for 4 h. Cells were washed and the luciferase activity determined.

Effects of Se status on NF-κB activation

To gain insight into the potential mechanism by which Se status affects COX-2 expression, we investigated the activation of NF-κB. TLCK is a serine protease inhibitor that has been shown to block NF-κB activation (91). Incubation of RAW 264.7 cells with 100μM TLCK prior to LPS stimulation blocked COX-2 expression regardless of Se status (Fig. 6A). The presence of TLCK inhibited COX-2 protein expression to basal levels suggesting that a TLCK-sensitive factor (i.e. NF-κB) is involved in the LPS activation of COX-2 expression. To further investigate the role of NF-κB, Se-supplemented and Se-deficient RAW 264.7 cells were stimulated with 1 μg/ml LPS. Nuclei were isolated from cells at various times post stimulation and nuclear extracts were analyzed by EMSA and Western blot using a NF-κB p65 specific antibody (Fig. 6B). These results indicate a significant increase in nuclear translocation of NF-κB (p65) in Se-deficient cells which is sustained longer than in the Se-supplemented cells following stimulation with LPS.

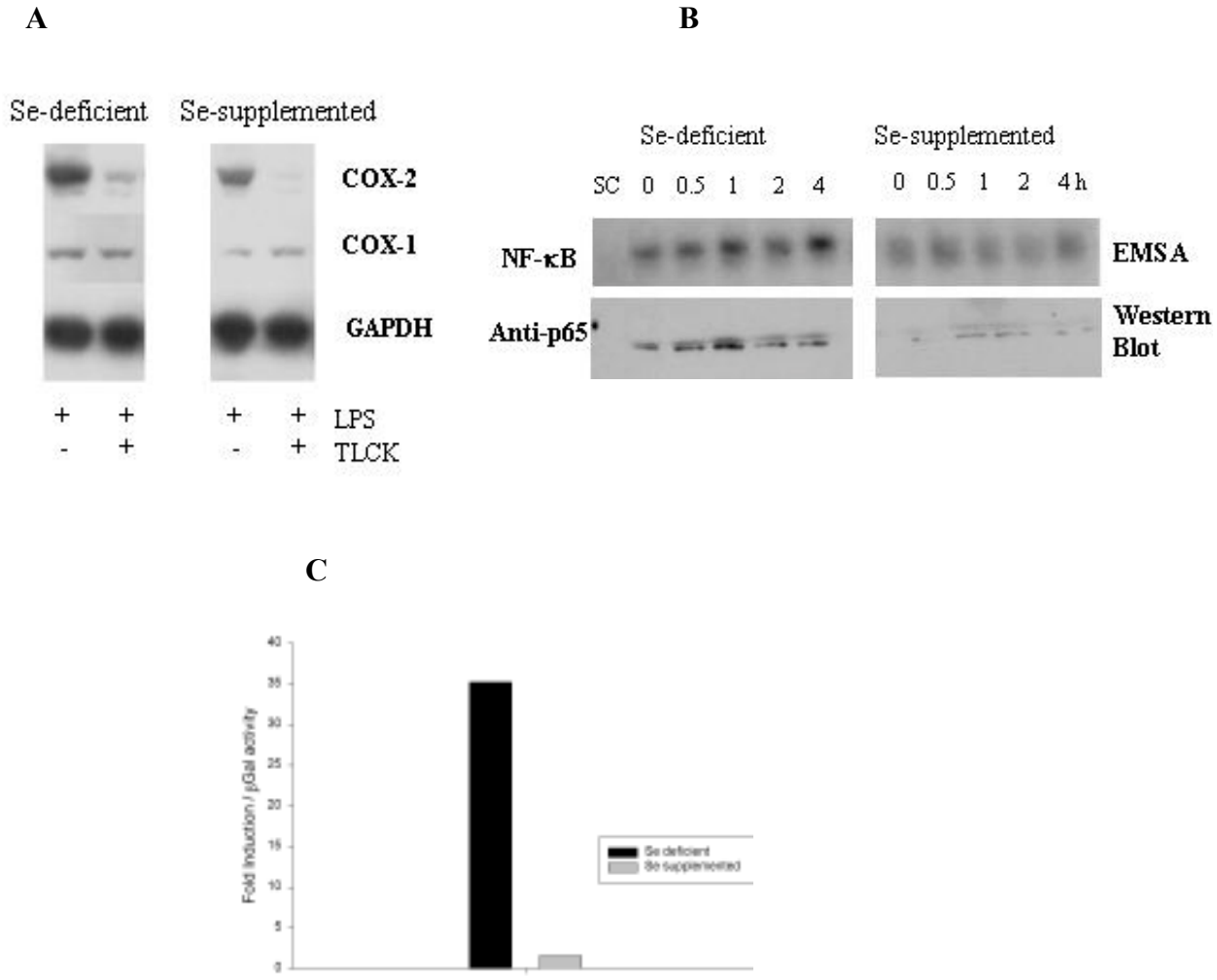

Fig. 6. **NF-κB involvement in COX-2 over-expression during Se deficiency.**

A. Se-deficient or Se-supplemented RAW 264.7 cells were stimulated with LPS (1 μg/ml) with or without TLCK (100 μM) for 2 h. Cell lysates were analyzed by Western blot using the indicated antibodies. **B.** Se-deficient or Se-supplemented RAW 264.7 cells were stimulated with LPS (1 μg/ml) for increasing amounts of time. Cells were harvested at the indicated time and nuclei isolated. Nuclear extracts were analyzed by electrophoretic mobility shift (EMSA) (upper panel) with a ^{32}P –radiolabeled consensus κB enhancer oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3') as well as

Western blot for the presence NF-κB (lower panel). Resolved gels and blotting membranes were stained to confirm equal protein loading and uniform transfer. SC, specific competitor was a two-fold excess of unlabeled κB enhancer oligonucleotide. **C.** Fold induction of a NF-κB driven luciferase reporter following transient transfection into Se supplemented and Se deficient cells and LPS stimulation for 4 hours; n=2, no statistical analyses were performed.

Effects of selenium status on upstream kinases

LPS activation of signaling pathways leading to NF-κB activation in macrophages is well established. In addition, NF-κB is known to respond to cellular oxidant tone, however the model by which this occurs is not universal and is not characterized in all cell types or lines. Since the key step in NF-κB activation is known to be IκB phosphorylation by IKK complexes, kinases or other signaling molecules leading to this step are likely targets of oxidant control. Two members of the MAP kinase family, NIK and MEKK1, have been shown to directly interact with IKK (83).

The data previously shown demonstrate the involvement of NF-κB in COX-2 transcription, as well as a role for selenium at two levels of NF-κB activation: nuclear translocation and DNA binding. The goal of the current studies was to examine the effect of selenium on upstream activators of NF-κB: NIK, MEKK1, $IKK_{\alpha/\beta}$ and $I\kappa B_{\alpha}$. Supplemented and deficient cultures were grown to confluency before stimulation with LPS and preparation of total lysate. Equal amounts of protein were analyzed by Western blot with specific antibodies. The results show increased expression of NIK, and MEKK1

(Fig. 7), IKK_β and $pI\kappa B_\alpha$ (Fig.8) in the deficient group, while differences in IKK_α (Fig.7) are not as obvious. Following immunoprecipitation (Fig. 9), a substantial increase in serine and tyrosine phosphorylation of $I\kappa B_\alpha$ in the deficient group is observed, as well as greater serine phosphorylation of MEKK1. Phosphorylation of IKKs or NIK could not be detected following IP. The results provide evidence for activation of NF-κB via enhanced activation of MEKK1 and IKK_β, leading to the increased phosphorylation and degradation of $I\kappa B_\alpha$ during Se deficiency.

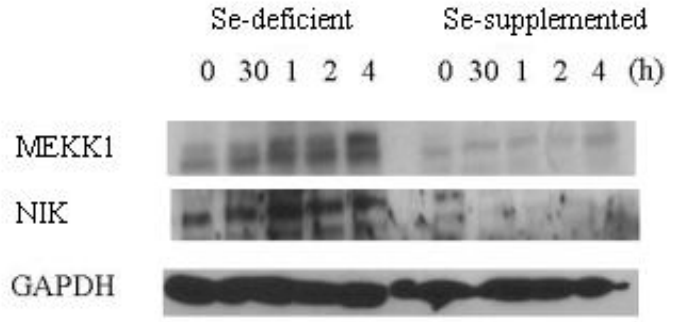

Fig.7 MEKK1 and NIK expression are up regulated during Se deficiency.
Deficient (SE-) and supplemented (SE+) cultures were incubated with LPS for the incubated times, harvested and analyzed for the presence of MEKK, NIK and GAPDH (loading control). The expression of both upstream kinases appears to be responsive to LPS and is also up regulated during deficiency.

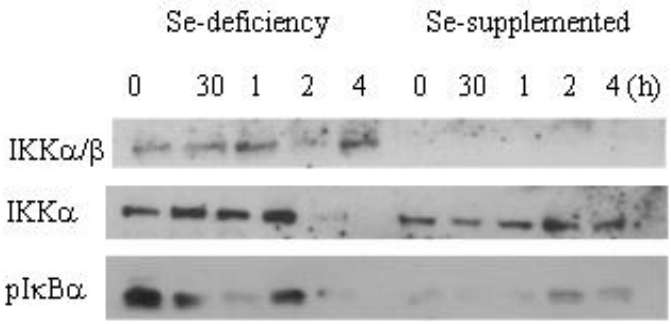

Fig. 8 Expression of IKKs and p-IκB$_\alpha$ is up regulated in deficient cultures.

Se-deficient and Se-supplemented cells were prepared as in Fig. 7 and analyzed by Western blot for the presence of IKK$_\alpha$, IKK$_{\alpha/\beta}$ (97% specific for IKK$_\beta$) , or p-IκB$_\alpha$. Enhanced expression of IKK$_\beta$ and subsequent phosphorylation of IκB$_\alpha$ are greater in the Se-deficient group.

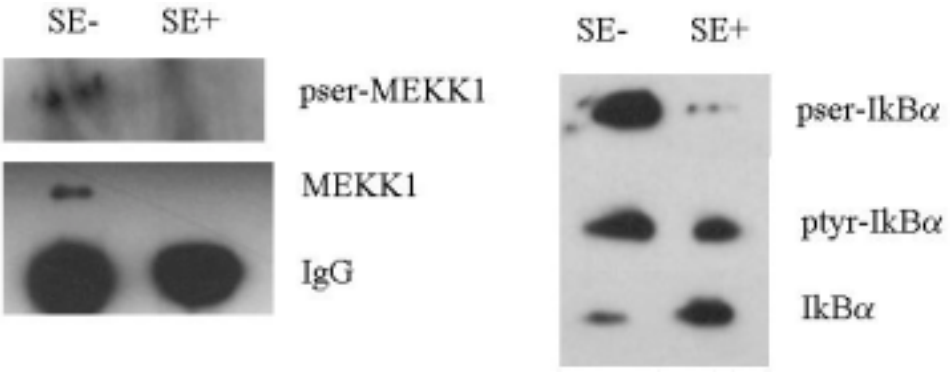

Fig. 9 Enhanced phosphorylation of MEKK1 and IκB$_\alpha$ during Se deficiency.

Immunoprecipitation of MEKK1 and IκB$_\alpha$ from LPS stimulated (2h) Se-deficient (SE-) and Se-supplemented (SE+) cells followed by Western blot with phospho-specific antibodies was performed. Phospho-tyrosine= (ptyr), Phospho-serine= (pser),

MEKK1 =total MEKK1, IκB$_\alpha$ = total IκB$_\alpha$. IgG shown as a loading reference. Phosphorylation could not be detected on NIK or IKKs following IP (results not shown).

Selenium repletion restores GPx activity, and decreases the expression of MEKK1 and COX-2.

Based on our hypothesis, the up regulation of COX-2 during Se deficiency is in part due to activation of the NF-κB pathway by oxidant species. In such a scenario, the reduction of oxidant species or enhancement of the antioxidant defense should have the opposite effect. To confirm this theory and the involvement of Se, deficient cultures were incubated in selenite supplemented media for one passage (72 hours) in an attempt to restore GPx activity and antioxidant function. Cultures were assayed for GPx activity and then stimulated with LPS for the indicated times prior to harvest and Western blot analysis. One passage in supplemented media restores 80% of GPx activity (Fig. 10A), and decreases the expression of MEKK1 and COX-2 (Fig. 10B). Phosphorylation of IκB$_\alpha$ was also decreased to a lesser extent (Fig. 10B). This data further indicates a role for Se in the upstream events leading to transcriptional up regulation of COX-2.

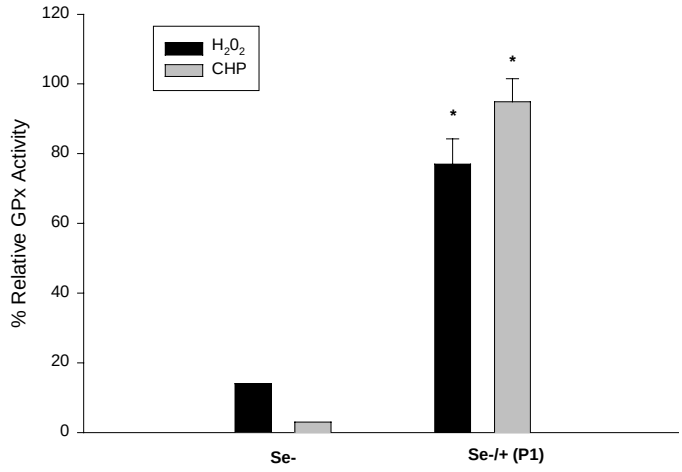

Fig.10A Selenium repletion restores GPx activity. Se-deficient cultures (Se-) were incubated for 72 hours in Se-supplemented media (Se-/+) prior to harvest and GPx assay.

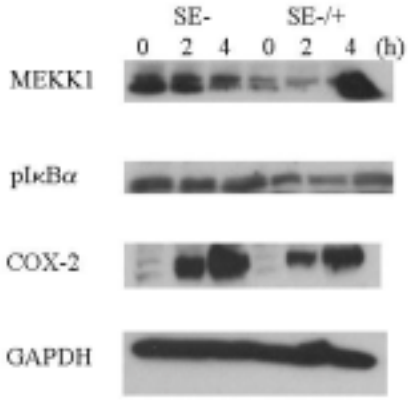

Fig. 10B Selenium repletion down regulates MEKK1, pIκB$_\alpha$ and COX-2 expression. Deficient cultures were stimulated with LPS for the indicated times before (SE-) and after (SE-/+) a 72-hour passage in supplemented media, and analyzed by Western blot.

Discussion

In the present study, experimental data to support the general hypothesis that Se, as an integral part of seleno-enzymes, may exert its anti-inflammatory and chemopreventive effect, in part, by inhibiting the expression of COX-2, which has been widely implicated in inflammation and tumorigenesis of many organs, is provided. The results demonstrate that the cellular Se status has a selective regulatory effect on COX-2 induction and subsequent PGE_2 production, without affecting COX-1 expression. The data was generated using an established RAW 264.7 macrophage model with cells grown in media deficient in Se or supplemented with Se. A concentration of 2 nmoles/ml (0. 3 μg/ml) sodium selenite was found to influence the activity of selenoproteins (such as GPx) without affecting cell viability and growth rate of RAW264.7 macrophages. Cellular GPx is a functional parameter commonly used for the assessment of Se status (73). In this study, a 10-fold decrease in Se-GPx activity was observed in Se-deficient cells when compared to Se-supplemented cells, along with a significant increase in overall oxidant stress levels in the Se-deficient cells.

The data suggests that cellular Se-GPX activity plays an important role in COX-2 expression. A significant increase in COX-2 protein induction was observed in RAW 264.7 macrophage cells grown in Se-deficient media as compared to cells cultured in Se-supplemented medium upon LPS stimulation, especially at early time points. In parallel to the results with COX-2 expression, the differences in PGE_2 production between Se-

deficient and Se-supplemented cells increased with time post-stimulation. The observed lag phase in PGE_2 formation as compared to COX-2 protein expression is most likely due, in part, to the time delay in the release of arachidonic acid necessary for the prostaglandin synthesis. The above results clearly suggest that Se status affects the regulation of COX-2 protein expression, most likely through influencing the signaling pathways to COX-2 induction.

Reactive oxygen species are important for macrophage functions such as phagocytosis and subsequent degradation of phagocytosed material; however, accumulation of excessive ROS will result in oxidative stress leading to pathophysiological events (92) Normally, macrophages employ multiple defense strategies in tightly controlling the intracellular redox balance. For example, selenoproteins, particularly Se-GPxs and TrxRs are important members of this antioxidant defense system (73). Therefore, an oxidant-antioxidant imbalance, a potential consequence of inadequate Se nutrition, in macrophages can lead to important cellular changes derived, in part, from a modification of gene expression. This modification relies on transcriptional factors whose normal activities are modulated by the cellular redox environment. Among these transcriptional factors, NF-κB plays a pivotal role in inducing genes involved in the control of the immune system as well as in the response to injury and infection (83). In addition, there is ample evidence to suggest that NF−κB is involved in the regulation of COX-2 induction by stimuli such as LPS (82). The use of the NF-κB double mutant in the present study definitively demonstrates

that COX-2 induction by LPS involves this redox sensitive transcription factor, which is in contrast to that reported by Wadleigh *et al.* (89). However, they did not mutate both NF-κB sites contained within the promoter sequence, which may explain why it was reported that NF-κB was not necessary. One point of interest is that both data show only a two fold increase in reporter activity in LPS stimulated wild type construct (89). Since the COX-2 protein expression following LPS stimulation is greater than 2 fold, it is conceivable that sequences not contained within the reporter construct influence overall expression levels or that the luciferase expression is not as efficient as the expression of COX-2.

The experiments using TLCK to inhibit NF−κB activation indicate that Se affects the COX-2 expression through a TLCK sensitive pathway like NF−κB, since COX-2 induction in both cell lines, Se-deficient and Se-supplemented, is blocked. While TLCK is a general serine protease inhibitor, more conclusive data can be seen in the EMSA and Western analysis of nuclear extracts from LPS-stimulated cells (Fig 6B), which shows the enhanced presence and binding of NF−κB in Se-deficient cells. Furthermore, the increased presence and phosphorylation of MEKK1, and subsequent phosphorylation of $I\kappa B_\alpha$ in the deficient group provides additional mechanistic details to earlier events of this redox-signaling pathway. Thus, Se status affects the cellular antioxidant system, which, in turn, can alter the cellular ROS levels and the sensitivity of the cells to respond to ROS. These Se-deficiency induced changes in ROS affect signaling molecules important for initiating and activating subsequent gene induction. In particular, the

increased level of ROS as a result of Se deficiency affects the phosphorylation state of upstream kinases such as MEKK1, NIK, and IKKs, leading NF−κB activation and subsequent COX-2 gene-expression and PGE_2 production. Selenium-deficient cells are more sensitive to the changes in cellular redox status following LPS stimulation, which results in higher and faster COX-2 protein induction. This increased induction is partly due to the sustained activation of NF−κB and the preceding kinase cascade. MAPKs are thought to be implicated in the oxidant-induced activation of NF−κB (93), and MEKK1 and NIK have been shown to interact directly with IKKs (94;95). This sets the stage for redox modulation of $I\kappa B_\alpha$ phosphorylation and degradation (96). Of even greater relevance to this hypothesis are the studies demonstrating a role for Se's modulation of MAPKs (34)and influence on the phosphorylation and degradation of IκB (97). In the latter case, Se's effects were attributed to Se-GPx activity (97). The data presented here are in further support for the ability of Se to activate NF−κB in a redox responsive manner. It seems that Se-supplemented cells are less sensitive to the changes of cellular redox status induced by LPS, most likely due to the increased levels of seleno-enzymes, like Se-GPx. This helps to explain the effect of Se as a modifier of ROS levels induced by inflammation as well as Se's chemopreventive effects. The selenium repletion experiments showing restoration of glutathione peroxidase activity, an in turn, decreased expression of MEKK1and COX-2 indicate that the redox-modulated effects are indeed reversible when the oxidant tone is more favorable, and provide insight into the capability of the macrophage to respond to an ever-changing cellular environment.

In summary, these data demonstrate that Se-supplementation selectively down-regulates COX-2 expression and the enzyme activity in activated RAW 264.7 cells without altering COX-1 expression. One possible mechanism is through scavenging ROS, thereby lowering potential COX-2 activity and subsequent PGE_2 production. In agreement with this hypothesis is the observation that the redox sensitive transcription factor, NF−κB, translocates into the nucleus more rapidly and to a higher degree in Se-deficient cells. These findings propose one mechanism of Se's chemopreventive effects through altering COX-2 and PGE_2 synthesis, which in turn, alters the process of carcinogenesis as PGE_2 regulates cytokine profiles and could suppress host immunity. In addition, the moderate selective effect on the inhibition of COX-2 expression may give new insights for the design of a new family of selective COX-2 inhibitors. Collectively, such information provides additional evidence for dietary Se supplementation in the prevention and/or treatment of inflammatory diseases or cancer.

Chapter 3

Selenium Deficiency Increases the Expression of Inducible Nitric Oxide Synthase in RAW264.7 Macrophages: Role of Nuclear Factor-kappa B in Up Regulation

Abstract

Inducible isoform of nitric oxide synthase (iNOS) is implicated in atherosclerosis, malignancy, rheumatoid arthritis, tissue and reperfusion injuries. A key determinant of the pro-oxidant versus protective effects of NO is the underlying redox status of the tissue. Selenoproteins, such as glutathione peroxidases (Se-GPx) and thioredoxin reductases (TrxR), are key components of cellular defense and promote optimal antioxidant–oxidant balance. This section deals with the investigation of the relationship between selenium (Se) status, iNOS expression and nitric oxide (NO) production in Se-deficient and Se-supplemented RAW 264.7 macrophage cell lines. Upon lippopolysaccharide (LPS) stimulation of these cell lines, significantly higher iNOS transcript and protein expression levels were observed with a concomitant increase in NO production in Se-deficient RAW 264.7 cells than the Se-supplemented cells. Electrophoretic mobility gel shift assays, NF-κB-luciferase reporter assays and Western blot analysis indicate that the increased expression of iNOS in Se deficiency could be due to an increased activation and consequent nuclear localization of the redox-sensitive transcription factor NF−κB. These results further help to explain the hypothesis of an inverse relationship between cellular Se status and iNOS expression in LPS-stimulated RAW 264.7 cells and provide evidence for the beneficial effects of dietary Se supplementation in the prevention and / or treatment of oxidative stress-mediated inflammatory diseases.

Introduction

Selenium (Se) is an essential trace element for all mammalian species and functions primarily through selenoproteins, which contain Se as selenocysteine (SeCys)(72;73;98). The best characterized selenoenzymes are the Se-dependent glutathione peroxidase (Se-GPx) and thioredoxin reductase (TrxR) families, the activities of which are responsible for the recognition of Se as an important dietary antioxidant (72). These enzymes are involved in many biochemical processes such as protection against oxidative stress and redox-based regulation of gene expression (73). Selenium deficiency results in a significant decrease in Se-GPx, and an increase in reactive oxygen species (ROS) production (72;73;98). In addition, inadequate Se nutrition is associated with an increase in RNS such as NO- and NOO- production, which have been linked to increased risk of such diseases as cardiomyopathy (74), rheumatoid arthritis (67), cancer (68;75) Alzheimer's (99) and multiple sclerosis (100). Thus cellular Se status plays an important role in the reduction of oxidative stress in the body.

Many immunologic cell types, including macrophages, synthesize NO that regulates their cellular function (101). In addition, NO has also been found to be a major intercellular messenger involved in such diverse activities as neural signaling and vasorelaxation (102). Sustained production of NO endows macrophages with cytostatic or cytotoxic activity against viruses, bacteria, fungi, protozoa, helminthes, and tumor cells (103). However, due to its highly reactive nature, NO can also be destructive to the body's healthy cells when over-produced (104). Furthermore, NO is rapidly oxidized to

RNS that can S-nitrosylate thiols to modify key signaling molecules such as kinases and transcription factors (101).

Nitric oxide synthase (NOS) catalyzes the breakdown of L-arginine to NO and citrulline (53). Three different isoforms of NOS are present in mammals, two constitutive enzymes (i.e., neuronal nNOS, and endothelial eNOS) and one inducible enzyme (iNOS) (104). A variety of stimulants, such as silica, UV light, cytokines, and lipopolysaccharide (LPS), have been shown to up-regulate the expression of iNOS in macrophages (105). In addition, there is evidence to show that up regulation of iNOS expression and nitrite production involve the activation of NF-κB and subsequent binding of the κB enhancer elements in the promoter of the iNOS gene (106;106;107). The present study deals with the investigation of a relationship between Se status, as an important modulator of cellular oxidative stress, and iNOS expression in response to LPS stimulation in RAW 264.7 murine macrophage-like cell line. These cells have been previously established as a model for the study of iNOS expression (106) (86). As in the preceding chapters, these studies further strengthen the hypothesis that inadequate Se status in macrophages leads to an oxidant-antioxidant imbalance, and is responsible for the activation of redox-active transcription factor, NF-κB, which, in turn, induces the expression of iNOS.

Methods

Cell culture conditions

RAW 264.7 cells, a murine macrophage cell line, were cultured as previously described (See Chapter 2). The Se-deficient RAW 264.7 cells were grown under the above conditions and the Se deficiency of cells was documented by the measurement of Se-GPx activity. The Se-supplemented RAW 264.7 cells were grown in the same medium with the addition of sodium selenite (Sigma, St. Louis, MO) to a final concentration of 2 nmoles/ml.

For luciferase reporter assays, cells were seeded at 1 x 10^5 cells per well in a 96 well plate and transfected with 750 ng of plasmid DNA using Superfect (Qiagen, Los Angeles, CA) for 2 h. Cells were allowed to grow for 72 h prior to stimulation with LPS for 6 h. Following stimulation, cells were washed with PBS containing calcium and magnesium (1 mM each) and overlaid with 100 µl PBS. Luciferase activity was determined using LucLite Plus Assay kit (Packard, Hartford, CT).

Cellular Se-GPx activity assay

The harvested cells were washed three times in ice-cold PBS and centrifuged at 500 x *g* for 10 minutes at 4 ^{o}C. Cell pellets were resuspended in mammalian protein extraction reagent (M-PER, Pierce, Rockford, IL) for 30 minutes on ice and then centrifuged at 10,000x *g* for 15 minutes. The supernatant was collected and total protein was determined using the BCA reagent (Pierce). The cellular Se-GPx activity in Se-

deficient and supplemented RAW 264.7 cell lysates, which were either stimulated with LPS (1 μg/ml) or un stimulated, was measured according to standard methods using H_2O_2 as substrate (87). The reaction was initiated by the addition of 1.5 mM H_2O_2 and the oxidation of NADPH was monitored spectrophotometerically at 340 nm. The specific activity is expressed as nmoles of NADPH oxidized per minute per milligram of protein.

Determination of oxidative tone

RAW 264.7 macrophage cells were grown in Se-deficient and Se-supplemented media as described earlier. For a sensitive measurement of total ROS generation during Se deficiency, cells were harvested, washed once with PBS and incubated with 2.5 μM of 2', 7'-dichlorodihydro-fluorescein diacetate (H_2DCF-DA) (Molecular Probes, Eugene, OR) for 15 minutes at 25 ^oC. The number of cells exhibiting increased fluorescence of DCF, generated by ROS, was analyzed by a Beckman-Coulter XL-MCL single laser flow cytometer. These studies were performed at the Life Science Consortium's Flow Cytometry Facility, The Pennsylvania State University, University Park.

Quantitative RT-PCR analyses

Total RNA was isolated from Se-deficient and supplemented RAW 264.7 cells, stimulated at various times with LPS, using Trizol reagent (Life Technologies) and quantitated based on the absorbance at $A_{260\ nm}$. The RNA samples were treated with RNase free DNase for 30 minutes to avoid any genomic DNA contamination as per the manufacturer's instructions (Promega, Madison, WI). Equal amounts of RNA (50 ng)

from each of the samples were used in RT-PCR with iNOS specific sense (5'-AATGGCAACATCAGGT-CGGCCATCACT-3') and antisense (5'-GCTGTGTGTCACAGAAGTCTC-3') primers, respectively. β-Actin was used as an internal standard with the following sense and antisense primers, 5'-TGGAATCCTGTGGC-ATCCATGAAAC-3' and 5'-TAAAACGCAGCT-CAGTAACAGTCCG-3', respectively. The PCR products were analyzed on a 2 % agarose gel and the DNA bands were quantitated on an Eagle Eye system (Stratagene) using the Scion Image software program (Frederick, MD).

Preparation of nuclei

For the analysis of NF-□κB translocation, nuclear extracts were prepared from Se-supplemented and deficient RAW 264.7 cells (2 x 10^6 cells) grown in 6-well cluster dishes essentially as described earlier (See Chapter 2). Fresh media was added to cells prior to stimulation with LPS (1 μg/ml) for the indicated times. Following stimulation, cells were washed and harvested in ice-cold PBS. Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce), according to the manufacturer's instructions. Total protein was measured using the BCA reagent (Pierce).

Western blot analyses

Samples of 50 μg total protein or 20 μg of nuclear protein, were separated on a 12.5 % SDS-polyacrylamide gel and transblotted to nitrocellulose membrane (Bio-Rad, Hercules, CA) and blocked for 1 h at 25 ^oC in 10 mM Tris-Cl, pH 8, containing 150 mM

NaCl, 0.1 % Tween-20, 5 % skim milk. Detection of iNOS and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the whole cell lysates, obtained with M-PER, was performed after incubation with their specific antibodies for 1 h at 25 ^{o}C, while NF−κB was detected in the nuclear fraction after 1 h incubation with a p65-specific antibody at 25 ^{o}C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control to normalize protein loading. Rabbit anti-mouse iNOS polyclonal antibody, rabbit polyclonal NF−κB (p65) and mouse anti-GAPDH monoclonal antibody were from Cayman Chemicals (Ann Arbor, MI), Santa Cruz Biotechnology (Santa Cruz, CA) and Chemicon International Inc (Temecula, CA), respectively. A horseradish peroxidase-conjugated anti-IgG antibody was used as the secondary antibody. The bands were visualized as described previously.

EMSA

Nuclear proteins (6 μg) were incubated with 15,000 cpm ^{32}P-labeled NF-κB double-stranded oligonucleotide (Promega), 1 μg poly (dI-dC), and 1.75 pmoles of unlabeled AP-1 double stranded oligonucleotide (Promega), as a non-specific competitor, for 30 minutes and subjected to electrophoresis on a 6 % polyacrylamide gel under non-denaturing conditions. To confirm the specificity of NF-κB binding, unlabeled oligonucleotide in five-fold excess was used as a specific competitor (SC).

Nitric oxide assay

Aliquots (100 µl) of culture medium were collected from the same cells grown and stimulated with LPS under identical conditions as used for Western blot analysis. Nitric oxide production was measured using the Griess Reagent (Sigma, St. Louis, MO) following the manufacturer's instruction.

Generation of luciferase constructs

The wild type iNOS promoter DNA was PCR amplified from genomic DNA isolated from RAW 264.7 cells. The promoter sequence containing all the *cis*-acting elements, including the TATA box, was amplified from -1589 to -15, and cloned into pGEM-T (Promega, Madison, WI). This sequence contained two NF-κB sites; one from positions –972 to –962 and the other from positions –86 to -76. The wild type iNOS promoter was sub-cloned into pGL3-luciferase vector (Promega) linearized with *Nco* I and *Xho* I. A deletion mutant, NF-κB80pGL3, was generated by restriction digestion of the wild type iNOS-pGL3 construct with *Sac* I. NF-κB80pGL3 contains the iNOS promoter sequence from –329 to –15 and is devoid of the –972 to -962 NF-κB site. The luciferase assays were performed as mentioned earlier (See Chapter 2).

Statistics

All enzymatic assays 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 (SigmaPlot software, SPSS, Inc.). The SDS-PAGE, Western immunoblotting, immunoprecipitation and EMSA experiments were performed at least in duplicate and typical representative gels or autoradiograms are shown.

Results

Selenium deficiency induces oxidative stress in RAW 264.7 cells

The cellular Se-GPx activity was measured in cell lysates prepared from Se-deficient and supplemented RAW 264.7 cells, after 10 passages in the respective media, to be 4.7 ± 0.34 nmoles of NADPH oxidized/min/mg protein and 84.2 ± 3.9 nmoles of NADPH oxidized/min/mg protein, respectively. The 17-fold difference in Se-GPx activity was stable over six months and the Se-GPx activities in Se-deficient and Se-supplemented RAW 264.7 cells treated with LPS did not change either (data not shown). Furthermore, incubation of RAW 264.7 cells with 2.5 μM of 2', 7'-dichlorodihydro-fluorescein diacetate (H_2DCF-DA), a fluorescent label of total oxidative tone, clearly showed that Se-deficient RAW 264.7 cells were already under twice the oxidative stress as Se-supplemented cells prior to stimulation with LPS (Figure 2C, Chapter 2). Following stimulation, the oxidative stress was increased in both cell lines; however, to a greater extent in Se-deficient cells and very significant at 8 h post stimulation (data not shown). Collectively, these data indicate that Se status inversely affects the general oxidative tone of the cells, which is exacerbated by LPS stimulation.

Selenium deficiency increases iNOS expression and NO production in LPS-stimulated RAW 264.7 cells

To determine the effect of Se deficiency on the expression of iNOS, both Se-deficient and supplemented RAW264.7 macrophage cells were activated with LPS, and mRNA was analyzed by RT-PCR, while protein levels were analyzed by Western immunoblot analysis (Figure 12). The RNA samples were processed in RT-PCR reactions for both iNOS and β-actin. The level of iNOS mRNA was compared to the level of β-actin for each time point (Figure 12A). As seen from Figure 12A, the ratio of iNOS to β-actin was 3-fold higher in Se-deficient cells even before LPS stimulation. Following LPS stimulation, the iNOS transcript levels were found to increase in both cell-types; however, the increase in iNOS mRNA in Se-deficient cells was significantly higher than in Se-supplemented cells. The transcript levels were found to plateau around 6 h post stimulation in both cell-types (Figure 11A). Furthermore, the increase in iNOS mRNA levels was accompanied by a corresponding increase in iNOS protein levels in Se-deficient cells (Figure 11B). Small amounts of iNOS protein could be seen in unstimulated Se-deficient cells and, by 4 h post stimulation with LPS, there was a significant increase in iNOS protein levels in Se-deficient cells compared to Se-supplemented cells (Figure 11B). The GAPDH signal, used as internal standard, was also detected to be at the same level for all samples (Figure 11B).

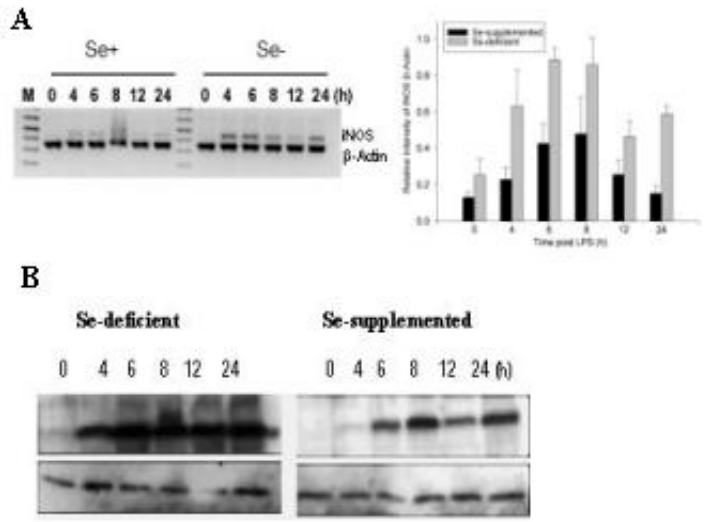

Figure 11. iNOS mRNA (A) and protein (B) levels are increased in Se deficiency. The time course of iNOS expression in Se-deficient and Se-supplemented RAW 264.7 cells was performed by incubating the cells with 1 μg/ml LPS for 0-8 h. Total mRNA was isolated and used in quantitative RT-PCR reactions as described under "Experimental Procedures". A typical gel profile is shown in the left panel. Densitometric evaluation of the bands was performed and the intensities were compared with that of β-actin (right panel). Western immunoblot analysis was performed on the cell lysates (20 μg) with the iNOS specific antibodies. In order to normalize protein loading, GAPDH was used as an internal control. The data is a representative of three independent experiments.

To demonstrate that the different levels of iNOS protein expression seen by Western blot analysis, affected the overall iNOS activity, NO production was analyzed in the culture media over a time period of 24 h post LPS stimulation. Cells were stimulated

with 1 μg/ml LPS and aliquots of the cell culture supernatant were taken at various time points. The extracellular NO levels increased in culture media of both Se-supplemented and Se-deficient cells following with LPS (Figure 12); however, the Se-deficient cells had a larger increase in NO production.

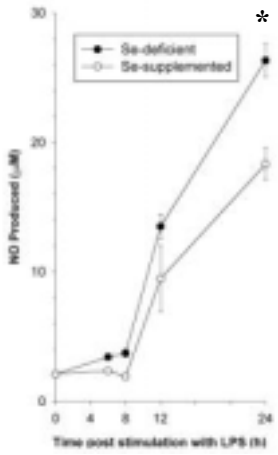

Figure 12. Effect of Se status on NO production in RAW 264.7 cells. Cells were stimulated with 1 μg/ml LPS for the indicated time intervals and supernatants of the cell culture media were collected and nitrite levels were measured by the method using the Griess Reagent.

NF-κB is involved in the over expression of iNOS in LPS-stimulated RAW 264.7 cells during Se deficiency

To gain insight into the potential mechanism by which Se status affects iNOS expression, the activation of NF-κB in these cell lines was investigated. Nuclei were isolated from Se-supplemented and deficient RAW 264.7 cells at various times post stimulation with 1 μg/ml LPS. Nuclear extracts were analyzed by Western blot and EMSA using NF-κB p65 specific antibody and a κB enhancer oligonucleotide, respectively (Figure 13A, B). Western analysis for the p65 subunit of NF-κB indicates a significant increase in nuclear translocation of NF-κB (p65) in Se-deficient cells which is

sustained longer than in the Se-supplemented cells following stimulation with LPS. In addition, EMSA data also indicate an increased presence of NF-κB in nuclear extracts from Se-deficient cells as compared to extracts isolated from Se-supplemented cells.

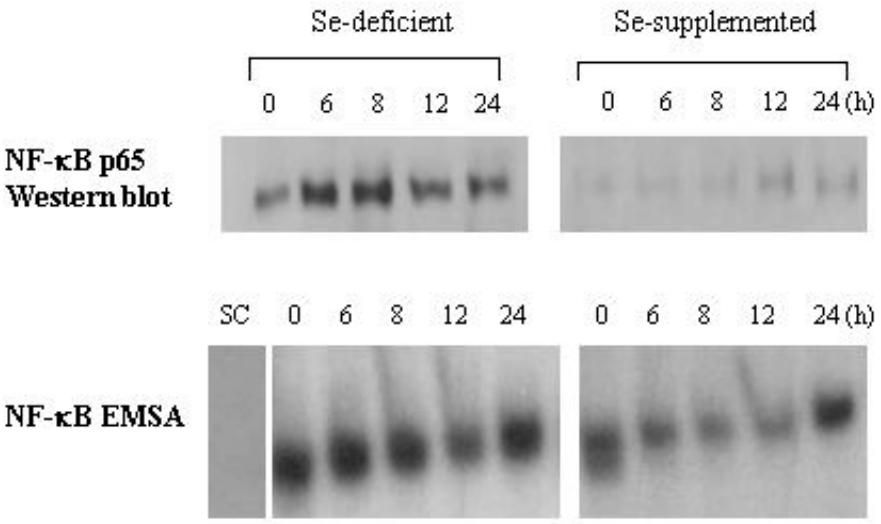

Figure 13. Nuclear translocation of NF-κB in Se-deficiency by Western immunoblot (A) and EMSA (B). Se-deficient or Se-supplemented RAW 264.7 cells were stimulated with LPS (1 μg/ml) for increasing amounts of time. Cells were harvested at the indicated time intervals and nuclei isolated. Nuclear extracts (10μg/ lane) were analyzed by Western immunoblot for the presence of NF-κB (upper panel) and also by electrophoretic mobility shift (EMSA) with a ^{32}P –radiolabeled consensus κB enhancer oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3'). Resolved gels and blotting membranes were stained to confirm equal protein loading and uniform transfer. A five-fold excess of unlabeled κB oligo was used as SC, specific competitor .

To determine the role of this redox-sensitive transcription factor in LPS activation of iNOS, a iNOS promoter-luciferase construct was generated using the murine iNOS promoter sequence from RAW 264.7 cells and then a truncated promoter with only one of the NF-κB binding sites intact was prepared from the former reporter-construct. These constructs were transfected into Se-deficient and supplemented RAW 264.7 cells. Following stimulation of such cells with LPS, the luciferase activity was determined. The wild type iNOS promoter was stimulated over 2 fold with LPS in Se-deficient cells than the supplemented cells (Figure 14). A similar trend was also observed upon using a truncated promoter construct in Se-deficient cells; however, the luciferase activity of the truncated promoter in Se-deficient cells was 2 fold less than that observed with the wild type promoter and the activity with Se-supplemented cells was barely visible (Figure 14). Interestingly, the fold induction in activity of the Se-deficient transfectants over that of the Se-supplemented ones was greater with the truncated promoter. This suggests a role for the regulation of additional transcription factors within the iNOS promoter during Se-deficiency. Collectively, the results of these reporter assays indicate that activation of the murine iNOS promoter is exacerbated in Se deficiency and that κB binding sites may play a role in this up regulation. Specific κB deletion mutants of the iNOS promoter are currently being constructed for further characterization in our lab.

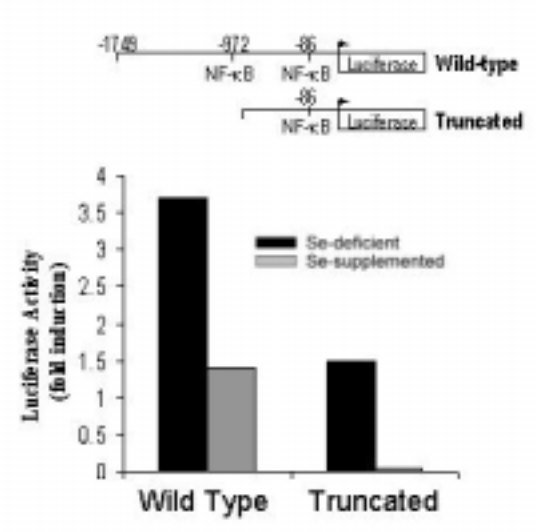

Figure 14. Role of NF-κB binding sites in the over-expression of iNOS during Se-deficiency. Both the wild type and the truncated iNOS promoter/reporter constructs were transfected into Se-deficient and supplemented RAW 264.7 cells. The cells were stimulated with LPS for 6 h and the luciferase activities were determined; n=2, no statistical analyses performed.

Discussion

Macrophages express significant amounts of iNOS upon stimulation by a variety of substances, including LPS, a bacterial endotoxin, leading to increase in NO production (55). One of the biochemical mechanisms by which NO affects cellular processes is through direct interaction with cellular proteins by nitrosylation and nitrosation reactions. In addition, excess nitric oxide can react with superoxide to form peroxynitrite, capable of inducing oxidative damage and initiating cell signaling pathways. Our studies investigating the role of selenium in LPS induced expression of iNOS and NO production suggest that cellular redox status and NF-κB activation play a major role in the over-production of free radicals such as NO and other reactive forms derived from it.

RAW 264.7 macrophage cell lines were grown under Se-deficient and supplemented conditions, as described in the previous chapter. In the current model system, a 17-fold decrease in Se-GPx activity with Se-deficient RAW 264.7 cells has been consistently observed when compared with those values observed with Se-supplemented cells. In addition, elevated levels of ROS indicated by flow cytometry are considerably higher in Se-deficient cells than in Se-supplemented cells and this difference is exacerbated after stimulation with LPS. The results of the experiments performed clearly indicate a compromise in antioxidant defense mechanisms in Se-deficient macrophages and such an impairment has a regulatory effect on iNOS induction and subsequent NO production.

The increase in iNOS protein expression during deficient conditions was accompanied by an increase in activity as evidenced by NO production. The levels of NO produced in Se-deficient cells upon stimulation was significantly higher than the Se-supplemented cells. These results are consistent with the results of flow cytometry experiments in Se-deficient cells that demonstrate an increase in overall levels of ROS, which are likely functioning to signal the production of a pro inflammatory response. The results clearly suggest that Se status affects the regulation of iNOS expression, most likely through influencing the signaling pathways to iNOS induction.

There is ample evidence to suggest that the transcription factor NF−κB is involved in the regulation of iNOS induction by such stimuli as LPS (82) as well as in the control of the immune system in response to injury and infection (83). The use of wild type and truncated iNOS promoter-luciferase constructs in our studies provides clues that iNOS activation by LPS may involve one or both NF-κB binding sites. Furthermore, Western immunoblots and electrophoretic mobility shift assays also confirm the hypothesis that iNOS expression is possibly a result of activation of NF-κB and consequent nuclear localization. The extent of activation of NF-κB is significantly higher and prolonged in the case of Se-deficient cells when compared to that with Se-supplemented cells.

This is the first report to demonstrate the inverse correlation of iNOS expression and activity with Se status. The increased ROS levels seen in the Se-deficient RAW 264.7 macrophage model cell culture system can be attributed, in part, to significantly lowered levels of antioxidant enzymes such as Se-GPx. In fact, Han *et al* have recently

shown that H_2O_2, among other ROS, is mainly produced upon LPS stimulation of RAW 264.7 macrophages, which participates in the up regulation of iNOS gene expression via the NF-κB pathway (54). It is, therefore, conceivable that in Se-deficiency, the over-production of H_2O_2 resulting in overall cellular oxidative stress, which leads to an increased expression of proinflammatory genes such as COX-2 and iNOS (56). The results clearly demonstrate that the increased oxidant stress in Se-deficient cells triggers the over expression of iNOS via the activation of redox-transcription factor, NF-κB. These results are in close agreement with those of Hutter and Greene (108) who have demonstrated that the cellular redox environment plays a singular role in regulating signaling events operating through the control of gene expression by transcription factors, particularly the NF-κB. Se has been shown to modulate the activity of NF-κB in LPS treated human T cells and lung adenocarcinoma cells via a different mechanism involving the redox state of specific cysteine residues of NF-κB and selenoproteins like thioredoxin (107); however, the current study differs from those of Kim and Stadtman (107) in that the cells are continuously cultured in Se-deficient media rather than treating just the nuclear fraction with selenite as reported by them.

Collectively, these results suggest that strategies, such as dietary supplementation of Se, to inhibit NO generation or to scavenge RNS may prove useful in decreasing the risk of cancer development and chronic inflammatory diseases.

Chapter 4

Summary and Conclusions

The purpose of this course of research was to establish the role of Se in the redox-mediated activation of NF-κB, and the subsequent expression and activity of inflammatory enzymes COX-2 and iNOS by macrophages. The hypothesis was tested using selenite supplemented or deficient RAW 264.7 transformed mouse macrophage cells and LPS as the agonist. The rationale for such a model was based on the importance of macrophages in the immune system as high output producers of reactive oxygen and nitrogen species, as well as key inflammatory mediators. The RAW 264.7 cell line was ideal for their ease of culture and compatibility with commercially available mouse antibodies. LPS was chosen for its established ability to induce a strong inflammatory response in macrophages, with a well-characterized induction of both COX-2 and iNOS. Sodium selenite was selected over other forms of Se due to its high efficiency of incorporation.

LPS signaling in murine macrophages is modulated through Toll-like Receptor 4 (TLR4) in concert with CD14 and LBP (LPS binding protein) (36). Engagement of these receptors transduces the cellular response to LPS, resulting in induction of proinflammatory cytokines TNF_{α} and IL-1, as well as the production of ROS/RNS. Therefore, the resulting activation of MAPK cascades and transcription factors may be in response to cytokines, free radical species, or a combination of the two. In either case, exposure of cells in culture to LPS will allow the cells to produce physiological quantities of these biological mediators via a receptor mediated pathway; a point of physiological relevance that does not apply when stimulating directly with oxygen radicals or peroxide species, as there is no known receptor for these mediators and physiological quantities are

uncharacterized and difficult to replicate in vitro. Even if physiological amounts can be estimated, there is no way of determining how much would actually make it into the cell. Furthermore, while ROS/RNS have been implicated in several disease states, LPS exposure is specifically connected to bacterial pathogenesis and the development of septic shock- a condition that is also associated with low plasma selenium levels (58). These studies suggest that selenium supplementation may help control the inflammatory response by regulating the generation of ROS and curbing the induction of key inflammatory enzymes by macrophage cells following LPS stimulation. Our findings support the potential for selenium based therapeutic strategies for the treatment of LPS induced sepsis. An examination of cytokine production during selenium deficiency, specifically TNF_{α} and IL-1 would also be beneficial in developing this model.

The data also confirmed that the Se-deficient cultures had significantly impaired classical, as well as phospholipid GPx activity, and higher levels of oxidative stress and lipid peroxidation prior to LPS challenge. These findings establish the foundation for a redox-based model of transcriptional regulation as a result of Se deficiency and compromised GPx activity, independent of inflammatory stimuli. This theory was evidenced by the increased basal activation observed at several points along the NF-κB activation pathway that was examined. Increased basal activation during selenium deficiency also raises curiosity about the source of radical species involved. Compromised GPx activity is expected to result in an increase in peroxide species. While H_2O_2 may accumulate in the cell during the depletion of GPx, it may not be directly involved in cell signaling due to its high reactivity with membranes as well as other

radicals such as nitric oxide. Secondary species formed via reaction with H_2O_2 such as peroxynitrite and lipid peroxides are more likely to serve as second messengers leading to gene induction. This concept is supported by the fact that MAPKs are activated by peroxynitrite, possibly through nitrosylation of tyrosine residues (34). Peroxynitrite readily reacts with selenoproteins and GPx mimics, and supplementation with selenite has been shown to reverse both MAPK activation and nitrotyrosine formation *in vitro* (109). Our studies demonstrating compromised GPx activity, in conjuction with activation of MAPKs, and increased production of nitric oxide during Se deficiency further support a role for peroxynitrite signaling as part of a redox regulated pathway. Current studies are ongoing in our lab to further characterize peroxynitrite formation in the Se-deficient macrophage model.

While non-enzymatic formation of lipid peroxide species can result from oxidant induced membrane damage and have been shown to influence irregular signaling from membrane receptors, another potential source of lipid peroxide intermediates is the 5-lipoxygenase (LOX) pathway. Products of this pathway such as hydroperoxyeicosatetraenoic acids (HPETES) have been shown to activate NF-κB (110), and of particular relevance to this model are studies showing an increase in LOX metabolites produced by leukocytes isolated from Se-deficient rats (111). These studies, in conjuction with our findings of increased lipid peroxidation during selenium deficiency in macrophages, provide support for selenium mediated lipid-peroxide signaling in two different cell models. Furthermore, selenium supplementation may aid in the prevention of atherosclerosis, as oxidant induced lipid peroxidation (112), NF-κB

activation and apoptosis (1) are major contributors to the development of atherosclerotic lesions .Co-culture studies with vascular endothelial cells are needed to address the role of extracellular lipid peroxides in redox regulated signal transduction and provide a more physiological context.

A key objective of this research was to investigate the activation of transcription factor, NF-κB, as it has been established to respond to cellular oxidant tone. Activation was examined in the Se-supplemented and Se-deficient groups at the levels of nuclear translocation, DNA binding and ability to initiate transcription. All three levels were enhanced during Se deficiency as evidenced by the presence of p65 in nuclear extracts, EMSA binding, and κB driven luciferase reporter activity. Of particular interest is the increased binding of what appear to be p50 homodimers in the supplemented cells, suggesting that Se may down regulate NF-κB activation via the formation and binding of homodimers, which cannot activate the transcription machinery. Another possibility is that the control of thioredoxin in reducing κB binding elements may be altered during deficiency, through the activity of thioredoxin reductase, another selenoprotein. A role for additional uncharacterized selenoproteins or redox responsive transcriptional elements such as AP-1, AREs, or Nrf 2 cannot be ruled out at this time. In any case, the current data highlight the ability of selenium status to influence several steps leading to NF-κB activation; and due to the connection of this transcription factor to the development of multiple inflammatory pathologies, magnifies selenium as a preventive or therapeutic medium for chronic inflammation induced by up regulation of NF-κB.

The data supporting increased NF-κB activation at multiple levels in Se deficiency led to the investigation of upstream events in this signaling pathway. The upstream protein kinases such as MEKK1 and NIK, as well as IKK α and β were studied for their expression and phosphorylation status in both groups. Selenium depletion resulted in increased expression of MEKK1, NIK, IKK_{β} and IκB with LPS stimulation. Immunoprecipitation of these up steam kinases revealed greater total and serine phosphorylated MEKK1 in the Se- deficient group, as well as a higher degree of serine and tyrosine phosphorylated $I\kappa B_{\alpha}$. This data not only identifies upstream molecular targets that are sensitive to oxidative stress as a result of selenium deficiency, but also supports the non-inflammatory role of NF-κB. Beneficial functions of NF-κB include Ig isotope switching, protection from apoptosis and support of cell cycle progression (12). Studies have already shown different physiological roles for IKK α and β, with the former being critical for early dermal and skeletal development, and the latter being a more potent activator of NF-κB with greater kinase activity toward $I\kappa B_{\alpha}$ {84}. Our research demonstrated that IKK_{α} expression was not affected by selenium status or associated oxidant tone, while IKK_{β} was up regulated in the deficient cultures, suggesting that expression of this component of the IKK complex may be critical in redox modulation of NF-κB.

Having established a role for Se in the events leading to NF-κB activation, the effects on molecular gene targets of NF-κB induced transcription and their products were examined. During deficiency two inflammatory enzymes COX-2 and iNOS were up regulated in message, protein and activity, as seen by production of PGE_2 and NO.

Furthermore, both groups of cells were transfected with luciferase constructs driven by COX-2 or iNOS wild type and NF-κB mutated or truncated promoters. Transcription of luciferase was dramatically reduced when the κB sites were specifically mutated (COX-2), or truncated (iNOS), demonstrating the requirement for κB in transcription of these genes. The supplemented cultures demonstrated consistently lower luciferase activity than the deficient cultures with both COX-2 and iNOS wild type promoters. These results fulfilled the final objective of the hypothesis with evidence that inflammatory enzymes are up regulated in Se deficiency, and that NF-κB mediates this process. This course of investigation also provides a novel explanation for the beneficial effect of selenium supplementation in cancer prevention. In recent years, several experimental and clinical studies have documented the ability of selenium to decrease incidences of prostate, colon, breast and lung cancers (113;114). While the mechanism is still being examined, it is thought to be due to regulation of oxidant species, as well as the influence of selenium on cell growth and enzymes involved in detoxification of carcinogens. An alternative pathway for chemo prevention by selenium may be via regulation of COX-2. COX-2, and subsequent PGE_2 production is up regulated in several cancers (115;116) and our data provides evidence that selenium supplementation decreases both COX-2 expression and activity. The chemopreventive benefits of selenium appear to be multi tiered, with one previously uncharacterized level being the regulation of COX-2.

As a final look at the effects of altered Se status on the model, the repletion of Se to the Se-deficient cultures was done to determine if the alterations in NF-κB activated gene expression during Se deficiency could be reversed. Following a 72 hour incubation

in Se- supplemented media, both classical as well as phospholipid GPx activities were restored. MEKK1 expression was lower than that originally seen during deficiency, there was a slight decrease in phosphorylated IκB_{α}, and COX-2 expression was decreased, as well. This suggests that the alterations during Se deficiency can be reversed upon restoration of GPx activity, and assumed decrease in oxidative stress. This further supports the therapeutic potential for selenium supplementation in pathologies such as sepsis and HIV, which are associated with a marked depletion of GPx activity (58;117). If identified early enough, selenium supplementation may be able to reverse or control the over expression of mediators that exacerbate these conditions.

Overall, the results of this research are consistent with the findings that support the redox regulation of gene expression through NFκB. In addition, a novel insight to the role of Se in this process has been discovered, that may be beneficial in the development of therapeutic treatments for inflammatory disease. It is also important to note the relevance of this data for conventional cell culture methodology. The Se "deficient" cultures were grown in commercial media and received Se only from 5% FBS. The data clearly showed that this treatment increased oxidative stress and resulted in up regulation of key kinase pathways, as compared to the supplemented cultures. Considering that the majority of cell lines used in experimental procedures today utilize commercial media supplemented with 10% serum, it is certain that these cultures will have very low GPx activity, and in essence are oxidatively challenged and therefore subject to up regulation of kinase pathways, transcription factor activation and increased production of inflammatory mediators. Any one of these factors or the cross talk and feedback

mechanisms between mediators, kinase cascades and gene expression may directly or indirectly impact numerous areas of investigation. As such, our studies together with others , strongly support the need for Se supplementation to traditional cell culture media to insure that the data collected *in vitro* conditions more accurately reflect that of the physiological environment.

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EXPERIENCE

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Radiology Assistant 1995 – 1996

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Medical Office Assistant 1989 – 1995

EDUCATION

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Ph.D., Pathobiology
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AWARDS AND HONORS

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PUBLICATIONS

F.A. Zamamiri-Davis, Y. Lu, J. Thompson ,S. Prabhu, V.R. Padala, L. Sordillo, C.C. Reddy. Nuclear factor kappa B mediates overexpression of Cyclooxygenase-2 during activation of RAW 264.7 macrophages in selenium deficiency. Free Radic. Biol. Med. (In press).

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