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Research Article

Selenium attenuates pro-inflammatory gene expression in macrophages

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Selenium (Se) is an important element required for the optimal functioning of the immune system. Particularly in macrophages, which play a pivotal role in immune regulation, Se acts as a major antioxidant in the form of selenoproteins to mitigate the cytotoxic effects of reactive oxygen species. Here we describe the role of Se as an anti-inflammatory agent and its effect on the macrophage signal transduction pathways elicited by bacterial endotoxin, LPS. Our studies demonstrate that supplementation of Se to macrophages (Se-deficient) leads to a significant decrease in the LPS-induced expression of two important pro-inflammatory genes, cyclooxygenase-2 (COX-2) and tumor necrosis factor-α (TNF-α) *via* the inhibition of MAP kinase pathways. Furthermore, Se-deficiency in mice exacerbated the LPS-mediated infiltration of macrophages into the lungs suggesting that Se status is a crucial host factor that regulates inflammation. In summary, our results indicate that Se plays an important role as an anti-inflammatory agent by tightly regulating the expression of pro-inflammatory genes in immune cells.

Keywords: Antioxidant / Gene expression / Inflammation / Pro-inflammatory / Signal transduction

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1 Introduction

Macrophages, along with dendritic cells, play a central role in immune regulation by presenting antigen to T-lymphocytes and ingestion and killing of various invading microorganisms. The cellular response to invading microorganisms is multifaceted with the recruitment and subsequent activation of inflammatory cell populations. Macrophages are critical cellular participants in this process and they are activated by diverse stimuli like bacterial endotoxin LPS, to synthesize and secrete pro-inflammatory enzymes, cytokines, and chemokines, which initiate and control inflammatory and immune functions [1]. While fulfilling these functions, "activated" macrophages undergo a respiratory burst that produces such reactive oxygen species (ROS) as superoxide, hydrogen peroxide (H_2O_2), hydroxyl, and lipid peroxyl radicals [2]. Increases in the intracellular levels of ROS represent a potentially toxic insult, which if not counteracted, will lead to membrane dysfunction, DNA damage, and inactivation of proteins [3]. ROS have been implicated in many disease pathologies where an inflammatory component is involved [4]. The antioxidant capacity of macrophages is, therefore, very important not only to maintain its own vital function in its defense, but also to tightly control the intracellular oxidative tone. In light of their many crucial functions, it becomes apparent that any condition that disrupts macrophage integrity will exert its consequences on immune function.

Incorporation of selenium (Se) into selenoproteins is crucial for the important functions in eukaryotic cells, including macrophages [5]. The most well-characterized selenoenzymes belong to the families of glutathione peroxidases (GPXs) and thioredoxin reductase (Txnrd; TR) [6]. The ability of GPXs to act on a wide variety of ROS has led to the recognition of Se in human health and development as an important dietary antioxidant [7, 8]. Recent studies have also established Se as a potential regulator of transcription [9]. Se-deficiency in rats increased the binding of liver nuclear proteins to DNA regulatory elements NF-κB,

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Abbreviations: BMDM, bone marrow-derived macrophages; **COX,** cyclooxygenase; **GPX,** glutathione peroxidase; **PGE_2,** prostaglandin E_2; **ROS,** reactive oxygen species; **TNF-α,** tumor necrosis factor-α

NF-1, and OCT, which activate transcription of many pro-inflammatory target genes in response to oxidative stress [9]. Because diverse stimuli activate NF-κB and because this transcription factor regulates the expression of pro-inflammatory enzymes such as cyclooxygenase-2 (COX-2) and cytokines like tumor necrosis factor (TNF)-α, NF-κB has often been termed a "central mediator of the immune response" [10–13]. Thus, activation of NF-κB is involved in inducing acute inflammatory responses. Overexpression of GPX was shown to inhibit NF-κB activation, *via* inhibition of IκB phosphorylation [14]. Along these lines, data from our laboratory indicate that cellular Se status can modulate the activity of NF-κB in murine macrophages [15–17].

Recently we have demonstrated that the anti-inflammatory effect of Se is mediated by switching the arachidonic acid pathway from the pro-inflammatory prostaglandin E_2 (PGE_2) to anti-inflammatory 15d-PGJ_2, which is a potent inhibitor of the NF-κB pathway [17]. PGE_2 is one of the end-products of the arachidonic acid metabolism by COX family of enzymes. Of the two well studied isozymes, COX-2 expression and consequent PGE_2 production is highly induced by a wide variety of cellular mediators and is used as a prognostic marker of inflammation in cancer [18], arthritis [19], and cardiovascular diseases [20]. Intriguingly, COX-2 expression is responsive to changes in Se status of macrophages [15]. Here we have sought to identify those upstream signal transduction pathways that respond to changes in cellular Se status leading to the modulation of COX-2 expression. The MAP kinase pathways of signal transduction, involving Erk, JNK, and p38 kinases, are known to activate COX-2 and other pro-inflammatory genes [21]. In addition to Erk pathway, which mediates the activation of the NF-κB pathway, activation of the JNK and p38 pathways has also been demonstrated to be sensitive to changes in cellular redox status and plays an important role in the transcription of many immune response genes [22]. Thus, in continuation of our analysis of the anti-inflammatory properties of Se, we describe the effect of cellular Se status on the expression of COX-2 and TNF-α, infiltration of macrophages to the lungs, activation of upstream signal pathways such as Erk, JNK, and p38 MAP kinase pathways in macrophages in response to differential Se status upon treatment with LPS.

2 Materials and methods

2.1 Animal experiments

Three-week-old male C57/BL6 mice purchased from Charles River Laboratories were maintained on a torula yeast-based diet that was either Se-deficient (0.01 ppm) or Se-supplemented (0.4 ppm) for 100 days. The Se-deficient and Se-supplemented diets were formulated based on an American Institute of Nutrition recommended rodent diet containing 0.01 or 0.4 ppm of Se, as described by Moskovitz and Stadtman [23]. The diets were purchased from Zeigler (Gardners, PA). All animal experiments were preapproved by the institutional animal care and use committee.

2.2 Cell culture

The murine macrophage cell line RAW264.7 (ATCC) was cultured in DMEM containing 5% defined fetal bovine serum (Hyclone), 80 μg/mL gentamicin, and 2 mM L-glutamine (Invitrogen) at 37°C with a 5% CO_2/air mixture. Total Se in the fetal bovine serum was quantitated by atomic absorption spectrophotometry, using sodium selenite as a calibration standard, to be 6 pmol/mL in the laboratory of Dr. Karam El-Bayoumy at Penn State University College of Medicine, Hershey. Cells were cultured in DMEM either supplemented with sodium selenite (Na_2SeO_3; 0.05–1.5 nmol/mL as Se) or without any added as described earlier from our laboratory [17]. Se-deficient cells were supplemented by culturing in a Se-supplemented media for 1 wk with three media changes in between. Cell viability and growth rates of Se-supplemented cells were similar to their Se-deficient counterparts. The activity of GPX was used as marker of cellular Se status as described earlier from our laboratory [17]. About 1×10^6 Se-deficient and Se-supplemented (with 0.05–1.5 μM Se) cells were seeded in a six-well plate, and then cultured in respective media for about 24 h to allow the cell number to approximately double. Cells were stimulated with LPS (1 μg/mL) and/or other compounds for the indicated time periods. Upon treatment, the cells were harvested, washed with cold sterile PBS, and stored at –80°C until further use. Femoral bone marrow plugs from Se-deficient or Se-supplemented mice were isolated and adherent cells, hereafter referred to as primary bone marrow-derived macrophages (BMDMs), were differentiated in their respective media containing 20% L929 fibroblast media supernatant (as a source of M-CSF) for 1 wk. The L929 cells were also cultured under Se-deficient or Se-supplemented conditions.

2.3 Isolation of lung macrophages

The Se-supplemented and Se-deficient mice were treated intraperitoneally with LPS (5 mg/kg body weight) for 6 h. Subsequently mice were killed by CO_2 inhalation. The lungs were excised, sliced, and digested in 5 mL of 1X collagenase-A in HBSS-1640 with Ca^{2+} and Mg^{2+} by incubating in a 37°C water bath for 1 h. The digest was centrifuged at $200 \times g$ for 5 min and the pellet was washed for two times with 1X PBS with 2% FBS by centrifuging at $200 \times g$ for 5 min. The pellet was resuspended in 5 mL media and layered over with 5 mL Lympholyte® (Cedarlane Laboratories, Ontario, Canada) followed by centrifugation at $500 \times g$ for 20 min. The cells at the interface were collected, counted

using a hemocytometer, and used in experiments described below.

2.4 Flow cytometric analysis

Lung cells (1×10^6) in PBS containing 2% heat-inactivated FBS were fixed using 1% paraformaldehyde and were labeled with Cyc-anti F4/80 and PE-anti CD11b antibodies. Antibodies were purchased from CalTag Laboratories (Burlingame, CA) and were used according to the manufacturer's specifications. Infiltrated macrophages within the lung tissue were identified as cells double labeled with Cyc-anti F4/80 and PE-anti CD11b monoclonal antibodies. Analysis was performed on an EPICS XL flow cytometer (Beckman Coulter, Mannheim, Germany), using FlowJo software (Tree Star, San Carlos, CA). The number of macrophages infiltrating the lungs was calculated from the percentage of each population measured by flow cytometry and the total number of cells was counted by trypan blue dye exclusion method using a hemocytometer.

2.5 Preparation of cell lysates

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

2.6 Western immunoblot

Thirty micrograms of protein from cell lysates were separated on a 12.5% SDS-polyacrylamide gel, and transblotted onto PVDF membrane as described. The membrane was blocked with Tris-buffered saline containing 0.05% Tween-20 and 5% skim milk w/v. The membrane was probed with primary antibody followed by an appropriate secondary antibody coupled to horseradish peroxidase. Anti-GPX1 was purchased from Abcam (Cambridge, MA); while anti-pErk, anti-Erk1/2, anti-pJNK, anti-JNK, anti-phospho-p38, and anti-p38 were purchased from Cell Signaling Technology (Danvers, MA). To normalize protein loading, blots were stripped and reprobed with the specific antitotal kinase or anti-GAPDH antibody. The immunoreactive bands were subjected to densitometry using ImageQuant™ software program (GE Amersham).

2.7 Treatment with inhibitors

Se-deficient and Se-supplemented RAW264.7 (1×10^6) were seeded into each well of a six-well plate and treated with inhibitors such as PD98509 (Erk inhibitor; 40 μM), SP600125 (JNK inhibitor; 40 μM), SB203580 (p38 inhibitor; 40 μM), or MG132 (proteasome inhibitor; 25 μM) for 30 min prior to stimulation with LPS for 2 h. Cells were harvested and lysates were prepared for analysis of expression of COX-2 by Western immunoblot analysis. The inhibitors were purchased from EMD Chemicals (San Diego, CA).

2.8 TNF-α secretion assay

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

3 Results

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

The expression of Se-GPX was measured in cell lysates from RAW 264.7 cells that were supplemented with different concentrations of Se as a marker for Se status. The Se-GPX expression that was almost nonexistent in Se-deficient media was significantly increased in the deficient cells upon supplementation with Se (Fig. 1). The expression was saturated at 100 nM of Se and remained that way even at high concentrations. Furthermore, GPX1 enzyme activity assays performed on these cells complemented the immunoblot results in that the activity increased with increase in Se concentrations (Fig. 2). Much like the expression results, the activity also was saturated around 100 nM, beyond which there was hardly any increase. A ~ten-fold increase in activity was noticed in cells that were supplemented with 100 nM Se. In addition, the expression of GPX1 was also analyzed in the homogenates of livers isolated from mice that were maintained on Se-supplemented and Se-deficient diets. The results indicated a significantly increased expression of GPX1 in the livers obtained from Se-supplemented mice compared to Se-deficient mice (Fig. 3).

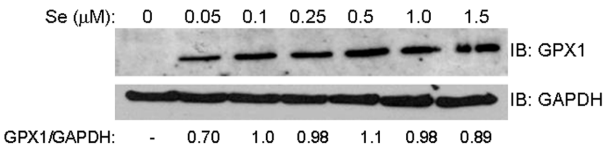

Figure 1. Expression of GPX1 in Se-deficient RAW264.7 macrophages as a function of exogenous addition of Se. Se-deficient RAW264.7 cells were treated with indicated concentrations of Se (as Na_2SeO_3) for 7 days. Anti-GPX1 was from Abcam. Densitometric values normalized to GAPDH are presented below each panel. Representative of $n = 2$ shown.

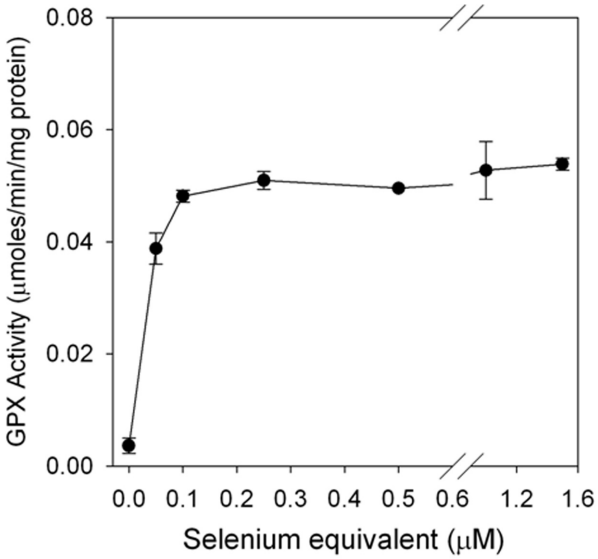

Figure 2. Effect of repletion of Se-deficient RAW 264.7 macrophages with Se on GPX activity. Se-deficient cells cultured in media containing 6 nM Se were repleted with different amounts of Se (as Na_2SeO_3) for 7 days. GPX activity, with H_2O_2 as substrate, was assayed in the cell lysates. The specific activity is expressed as μmol NADPH/min/mg protein. Data represent the mean ± SE of triplicate samples.

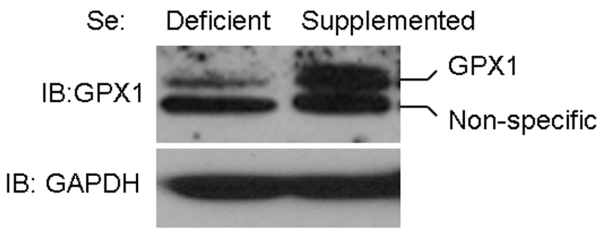

Figure 3. Expression of GPX1 in the livers of Se-deficient and Se-supplemented mice. C57/BL6 male mice were maintained on Se-deficient (0.01 ppm Se) or Se-supplemented (0.4 ppm Se) diets for ~3 months. The mice were sacrificed and livers were isolated for analysis. Expression of GPX1 in the liver homogenates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

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

Having established a differential Se status in mice, the physiological effects of Se-deficiency were investigated by following the infiltration of macrophages upon challenge of mice with intraperitoneal LPS. Flow cytometric analysis of the alveolar tissue of Se-deficient mice demonstrated an increased infiltration of macrophages after 6 h of exposure to LPS when compared to the alveolar tissue extracts from Se-supplemented mice (Fig. 4). The mean percentage of

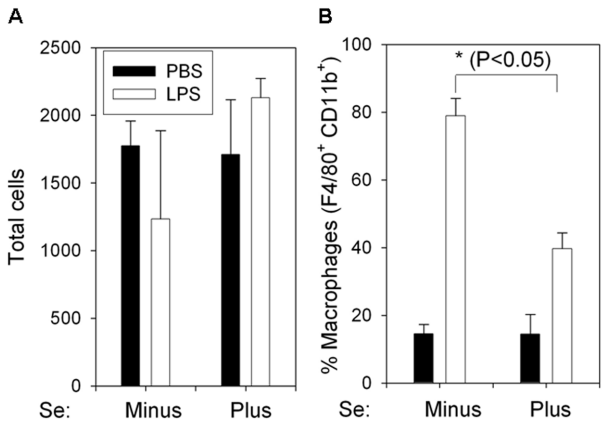

Figure 4. Recruitment of macrophages to the lung. Se-supplemented (0.4 ppm) and Se-deficient (0.01 ppm) mice were treated with PBS or LPS (5 mg/kg) intraperitoneally for 6 h. Lung tissues were collagenase digested and infiltrating total cells (A) and macrophages (B) were quantitated using flow cytometry with anti-CD11b and F4/80. Results are mean ± SE of $n = 4$ mice in each category.

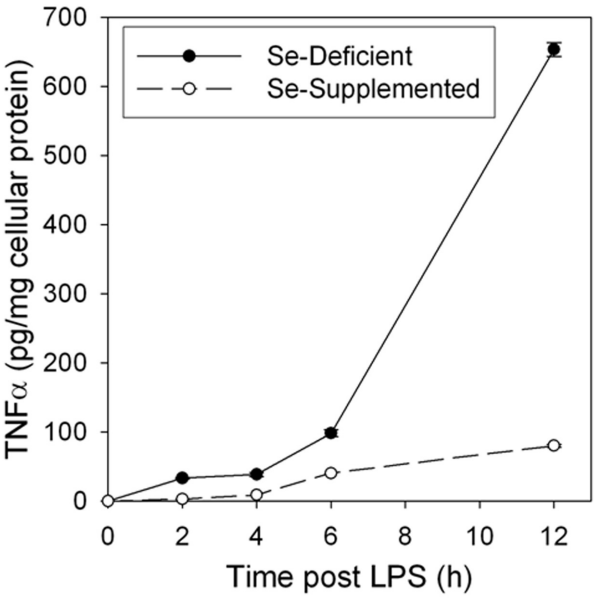

Figure 5. Production of extracellular TNF-α by Se-supplemented (0.4 ppm) and Se-deficient (0.01 ppm) BMDM upon stimulation with LPS (1 μg/mL). TNF-α was measured using Bio-Plex (BioRad). Results are normalized to cellular protein. Results shown are mean ± SE of triplicate samples.

macrophage infiltration into the lungs of Se-deficient mice was increased by at least 50% than in the lungs of Se-supplemented mice; while Se status had no major effect on total number of cells (Fig. 4A). These results suggest that Se status plays a crucial role in the infiltration of macrophages to effector tissues, such as the lungs, in this case.

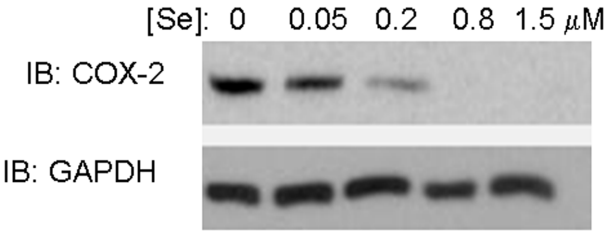

Figure 6. LPS-induced expression of COX-2 as a function of cellular Se status. RAW264.7 macrophages were cultured in the presence of indicated concentrations of Se for 7 days. Such cells were stimulated with LPS (1 mg/mL) for 2 h and the expression of COX-2 was examined using Western immunoblot. The membrane was stripped and reprobed for GAPDH as a measure of uniform protein loading. Representative of $n = 2$ shown.

3.3 LPS-induced expression of TNF-α in macrophages as a function of cellular Se status

To further explore if the cellular Se status had an influence on the expression of pro-inflammatory cytokines, which could possibly explain the reason for the increased infiltration into the lungs, we analyzed the ability of macrophages to express the pleiotropic cytokine, TNF-α, in the Se-deficient and Se-supplemented macrophages, upon stimulation with LPS. Using the Bio-Plex multiplex protein assay system (BioRad), we observed that, in a time-course experiment of LPS-induced expression of TNF-α, Se-supplementation dramatically suppressed TNF-α expression, especially at 4–12 h post-LPS treatment in BMDMs (Fig. 5). A similar trend in the Se-dependent repression of TNF-α was observed in RAW264.7 macrophages (data not shown). These results provide further evidence that cellular Se status plays an important role in the regulation of inflammation.

3.4 LPS-induced expression of COX-2 as a function of cellular Se status

One of the prototypical markers of inflammation, considered for therapeutic intervention, is COX-2 and its downstream product, PGE_2. Using the macrophage cell system, the effect of increasing concentrations of Se on the LPS-induced expression of COX-2 was determined. Results in Fig. 6 clearly indicate the exacerbated expression of COX-2 in Se-deficient cells upon stimulation with LPS. However, in RAW264.7 macrophages cultured in the presence of 0.05 μM Se for a week, the expression of COX-2 was decreased by ~40% compared to the control. Further increasing the concentration of Se, particularly at 0.2 μM and above, led to a significant decrease in COX-2 expression (Fig. 6). At concentrations >0.05 μM, we did not notice any change in cell viability or morphology. These results suggested that Se impacted the regulation of COX-2 expression by possibly modulating the activation of some of the upstream kinases in the TLR4 signal transduction pathway leading to the modulation of COX-2 expression.

3.5 Upstream kinases and their regulation by cellular Se status

Given the upregulation of COX-2 and TNF-α in Se-deficient cells by LPS, the effect of cellular Se status on the regulation of some of the upstream kinases that are critical in

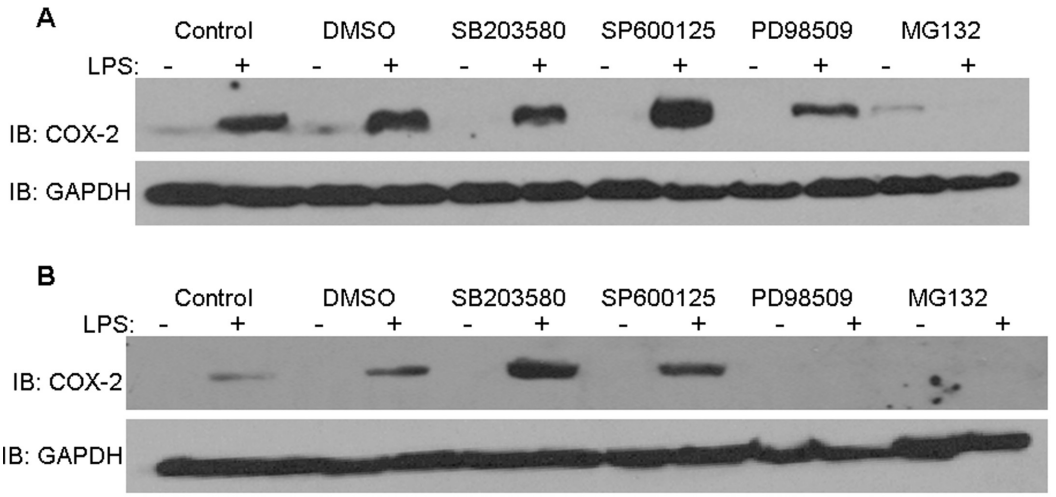

Figure 7. Effect of MAP kinase inhibitors on COX-2 expression. RAW264.7 cells were incubated with 40 mM of PD98509 (Erk inhibitor), SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), or MG132 (proteasome inhibitor) each inhibitor or vehicle control 30 min prior to stimulation with LPS (1 μg/mL) for 2 h. Cells were harvested and lysates were analyzed for COX-2 expression by Western blot. The blots were reprobed for GAPDH. Panels A and B represent Se-deficient and Se-supplemented (0.2 μM) cells, respectively. Representative of $n = 2$ shown.

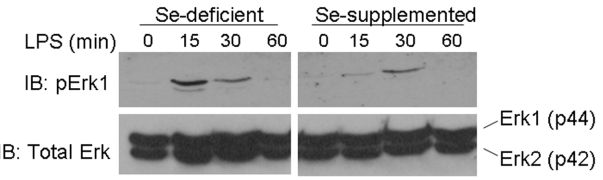

Figure 8. Activation of Erk1 during Se-deficiency. Se-deficient and Se-supplemented RAW 264.7cells were stimulated with LPS (1 mg/mL) for the specified time periods. Following detection of pErk1, the membrane was stripped and reprobed for total Erk1/2 protein levels. Representative of $n = 2$ shown.

the signal transduction pathways initiated by the ligation of the TLR4 receptor was explored. The MAP kinase signal transduction pathways, Erk, p38, and JNK, activated by LPS in macrophages were selected for inhibition in Se-deficient and Se-supplemented RAW264.7 macrophages. These experiments were performed using small molecule inhibitors and the effect on the expression of COX-2, as a downstream gene, was determined as function of cellular Se status (Fig. 7). As reported earlier from our laboratory, cellular Se status, by itself, had an important bearing on the expression of COX-2 [15]. As shown in the Western immunoblots, the proteasome-dependent, and Erk-MAP kinase pathways were most sensitive to cellular Se status; while the p38 MAP kinase pathway did not appear to be affected by changes in cellular Se status (Fig. 7). In addition, treatment of Se-supplemented cells with SP600125, a JNK inhibitor, caused a significant downregulation of COX-2 (Fig. 7). These data necessitated more experiments to analyze the activation of Erk and JNK in the context of cellular Se status by LPS. Western immunoblot analysis of the Erk activation in LPS-treated (0–1 h) indicated that Se supplementation not only decreased the activation of Erk-1, but also shifted the peak activation time from 15 to 30 min (Fig. 8). Along the same lines, higher JNK-1 activation was seen in Se-deficient RAW264.7 macrophages compared to those cultured in the presence of Se-supplemented cells at all times before and after LPS stimulation (Fig. 9).

4 Discussion

Oxidative stress, which is caused by increased production of ROS, appears to be one of the key regulators of inflammatory signaling pathways [24]. Enzymes, such as Se-dependent GPXs, TRs, and other selenoproteins are pivotal in the defense against these ROS [25]. In fact, epidemiological studies have suggested an inverse causal relationship between Se status and increased oxidative stress at the organismal and cellular levels, where inflammation associated with a number of disease pathologies is a common end result [26]. However, there is insufficient data to link inflammation, mediated by bacterial endotoxin, directly to cellular Se status. With that in mind, we have explored the anti-inflammatory effects of Se on macrophages, which are key immune cells that are important regulators of inflammation.

Much like the expression and activity of GPX1 in cells, which serves as an indicator of the Se status of macrophages, the expression of GPX1 in the livers provided evidence regarding the differential status of Se in mice. We have previously demonstrated that the macrophages differentiated *ex vivo* exhibit a similar difference in GPX1 activity and expression as described here [17]. Using LPS, a bacterial endotoxin, as a physiological stimulus to initiate oxidative stress in cells, we have described the role of Se on the expression of pro-inflammatory proteins. Intraperitoneal injection with LPS caused increased infiltration of macrophages into the lungs of Se-deficient mice; while the mice on Se-supplemented diets exhibited a significantly decreased effect. A significant decrease in TNF-α production by LPS treatment in Se-supplemented macrophages compared to those cultured in Se-deficient media further supports the argument that, through the secretion of pro-inflammatory cytokines, Se may change the dynamics of infiltration of other immune cells into the inflamed area. These observations suggest that Se-deficiency may exacerbate inflammatory responses in the lung, leading to airway hyper-responsiveness, as in asthma.

The expression of COX-2 and TNF-α are synonymous with inflammation and are used as markers in many inflammatory diseases such as arthritis, atherosclerosis, and can-

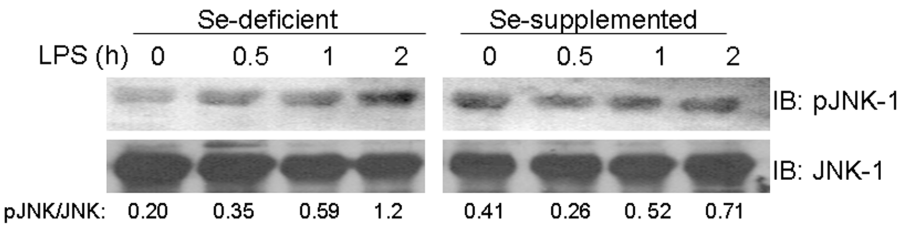

Figure 9. Activation of JNK during Se-deficiency. Se-deficient and Se-supplemented RAW 264.7 cells were stimulated with LPS (1 mg/mL) for the specified time periods. Following detection of pJNK levels, the membrane was stripped and reprobed for total JNK protein levels. Densitometric values of pJNK-1 normalized to total JNK are presented below each panel. Representative of $n = 2$ shown.

cer. The data showing a dose-dependent effect of Se on LPS-induced COX-2 expression further supports our previous data that Se-supplementation of macrophages causes a decrease in the activity of COX-2 [15]. In addition, the result also indicates that, at concentrations of ≥0.2 μM of Se, there is a significant downregulation of COX-2 expression, which could be due to the inactivation of some of the signal transduction pathways leading the expression of COX-2. The mechanisms of downregulation of pro-inflammatory genes by Se are not well understood. However, one of the likely mechanisms is that the arachidonic acid that is produced in cells adequate in Se (≥0.2 mM) is preferentially metabolized to 15d-PGJ_2, a cyclopentenone prostaglandin, which negatively affects pro-inflammatory signal transduction pathways [27]. Based on these results, we believe that the activation of Erk-MAP kinase pathway of activation, which is lowered by Se supplementation, is subject to further control by 15d-PGJ_2 at IKKβ [17]. In addition, activation of PPARγ by 15d-PGJ_2 also inhibits the activation of NF-κB dependent gene expression by a tethering mechanism [28]. It is possible that the activity of Erk-1 is regulated by an upstream kinase that is sensitive to Se status, which is currently unclear. Thus, it appears that the Erk-MAP kinase pathway is regulated at multiple points by cellular Se status. Our studies also indicate that cellular Se status appears to downregulate the JNK pathway, which can possibly modulate activation of genes *via* the inactivation of c-Fos and c-Jun transcription factors that are activated by oxidative stress. Even in this case, there appear multiple points of control, where Se controls upstream kinases or phosphatases that regulate the activation of JNK. Based on the earlier work of Perez-Sala *et al.* [29], 15d-PGJ_2 could further regulate the function of c-Fos and c-Jun leading to the inhibition of expression of pro-inflammatory mediators. We are currently examining the modulation of oxidative stress-response pathways by Se *via* the preferential shunting of the arachidonic acid pathway toward 15d-PGJ_2.

In conclusion, our results clearly demonstrate that optimal Se status is a key regulator of pro-inflammatory gene expression in macrophages, which plays an important role in the regulation of their infiltration into organs such as lungs, possibly contributing to an overall modulation of inflammation. Studies are currently in progress to correlate expression of pro-inflammatory genes to pathological lesions in mice as a function of Se status.

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The authors have declared no conflict of interst.

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