Sodium selenite-induced apoptosis in murine B-lymphoma cells is associated with inhibition of protein kinase C-δ, nuclear factor κB and inhibitor of apoptosis protein

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Selenium (Se) is an essential trace element possessing anticarcinogenic properties and other biological functions. This study determined the role sodium selenite plays on intracellular cellular signaling, including protein kinase C (PKC), nuclear factor-kappa B (NF-κB) and inhibitor of apoptosis protein (IAP) in murine B lymphoma (A20) cells. In vitro supplementation of A20 cells with low concentrations of sodium selenite (0.005–5 µM) caused a significant increase in cellular proliferation exclusively at 72 h. Proliferation and cell viability were decreased in response to selenium concentrations of ≥25 µM and ≥5 µM at 72 and 96 h, respectively. Flow cytometric analysis of A20 cells exposed to 5 µM Se at 72 and 96 h indicated G₂-M phase arrest and increased cell death at higher concentrations. Se-induced cytotoxicity was associated with apoptosis indicated by nuclear fragmentation and DNA laddering. Se concentrations, which induced cell cycle arrest and apoptosis, were associated with inhibition of cytosol to membrane translocation of PKCδ and PKC activity at 72 h. Coincubation of cultures with 0.5 µM phorbol 12-myristate 13-acetate (PMA) and Se (5 and 25 µM) reversed the Se-induced cell death at 72 h. The nuclear NF-κB translocation and NF-κB DNA-binding were inhibited by increasing concentrations of Se (5 and 25 µM) at 72 h. After 72 h exposure to 5 and 25 µM Se, cIAP-2 concentration was decreased. Differential inhibition of PKCδ, NF-κB and cIAP-2 by Se may represent important intracellular signaling processes through which Se induces apoptosis and subsequently exert its anticarcinogenic potential.

Key Words: Selenium; phorbol 12-myristate 13-acetate; protein kinase C-δ; nuclear factor-κB; cellular inhibitor of apoptosis protein-2.
Selenium (Se) is an essential trace element for most living organisms. Epidemiological and experimental studies have suggested that Se possesses anticarcinogenic properties (Combs and Gray 1998; El-Bayoumy et al., 1991; Stadtman, 1996). The mechanisms by which Se exerts its chemopreventive activity is unknown; however, several plausible explanations have been put forward including the role of Se in inducing cell cycle arrest (Sinha et al., 1996), DNA strand breaks (Lu et al., 1994; Wilson et al., 1992) and apoptosis (Lanfear et al., 1994; Lu et al., 1994).

The control of cell cycle progression plays a key role in cellular growth and differentiation (Pines, 1994; 1995). Se induces cell cycle arrest at different phases depending on the chemical form of Se and the cell type (Menter et al., 2000; Zeng, 2002). The inhibitory effect on cell proliferation, with a preference for tumor cells vs. nontransformed cells, is considered to be a mechanism for the anticarcinogenic capability of Se (Ip et al., 2000). Se-induced apoptosis in cancer cells was related to its chemopreventive activity (Kim et al., 2001) and several groups have shown that selenocompounds induce apoptosis in cell culture systems (Cho et al., 1999; Lu et al., 1994; Shen et al., 1999). Apoptosis is a suicide process essential for development, maintenance of tissue homeostasis, and elimination of unwanted or damaged cells (Thompson 1995) with characteristic morphological features that include nuclear membrane breakdown, chromatin condensation and fragmentation, cell membrane blebbing, and the formation of apoptotic bodies (Cryns and Yuan 1998).

Se modulates cellular activities presumably by acting on the functions of many intracellular proteins important for signal transduction (Allan et al., 1999; Gopalakrishna et al., 1997; Spyrou et al., 1995). However, the modulatory functions of selenite and other Se compounds on intracellular signaling pathways are not fully characterized. Protein kinase C (PKC) is a multigene family enzyme that includes at least 12 phospholipid-dependent serine/threonine
kinases, composed of 3 major classes. The PKC family consists of Ca$^{2+}$-dependent conventional (cPKCα, β$\text{II}$ and γ), Ca$^{2+}$-independent or novel (nPKCδ, ε, θ, η and µ), and Ca$^{2+}$ and dioleoyl-sn-glycerol (DAG)-independent or atypical (aPKCζ, ι and λ) isoforms (Mellor and Parker 1998). In response to DAG or the tumor promoter, phorbol 12-myristate 13-acetate (PMA), PKC translocates from the cytosol to the membrane; following redistribution and subsequent activation the enzyme is rapidly cleaved and is proteolytically degraded. PKC was initially characterized as a major high-affinity intracellular receptor for phorbol esters, a class of potent tumor promoters (Buchner, 2000); the enzyme mediates a variety of intracellular signaling establishing the basis of its involvement in multistage carcinogenesis (Blumberg, 1988). PKC plays a role in proliferation (Kiley et al., 1999), growth arrest (Fukumoto et al., 1997; Watanabe et al., 1992) and apoptosis (Cross et al., 2000; Zhong et al., 2002).

Activation of PKC results in the activation of downstream effectors; including the transcription factor nuclear factor-κB (NF-κB, Kontny et al., 2000; Vertegaal et al., 2000). In contrast to most transcription factors that are activated by a restricted number of extracellular stimuli, NF-κB is activated by over 150 different stimuli (Pahl, 1999), including but not limited to inflammatory cytokines, mitogens, bacterial products, protein synthesis inhibitors, reactive oxygen species, UV light, and phorbol esters (Baueurle, 1991; Grilli et al., 1993; Pahl, 1999; Schulze-Osthoff et al., 1995). The actual activation of NF-κB involves a cascade of events including phosphorylation by protein kinases, release and proteolysis of a bound inhibitor, IκB, translocation of NF-κB from the cytosol to the nucleus, maintenance of essential cysteine residues in the protein in the active thiol forms by the thioredoxin and thioredoxin reductase system and binding of NF-κB to the promoter region or a target gene (Kim and Stadtman 1997). NF-κB is ubiquitously expressed and serves as a critical regulator of the inducible expression of
many genes (Sen and Baltimore 1986). Among the genes modulated by NF-κB is the inhibitor of apoptosis protein (IAP) family that functions in a cooperative fashion to suppress apoptosis (Wang et al., 1998).

The A20 is a murine B-lymphoma cell line, which predominantly expresses the PKCδ isoform (Shih and Floyd-Smith 1995), is a good model to investigate the anticarcinogenic properties of Se since it has been demonstrated that selenite is potent at inhibiting the growth of the human B cell line 3B6 (Spyrou et al., 1996), Murphy lymphosarcoma tumor cells (Weisberger and Suhrland 1956) and L1210 leukemic cells (Milner and Hsu 1981). Therefore we investigated the possible signal transduction pathway involved in sodium selenite-induced cell cycle arrest and apoptosis. We examined the effects of sodium selenite on PKCδ, NF-κB and cIAP-2 in association with growth, cell cycle arrest, and apoptosis of murine A20 B-lymphoma cells. We hereby show that sodium selenite in concentrations that mediate cell cycle arrest and apoptosis are associated with inhibition of PKCδ, NF-κB and cIAP-2. In addition, incubation of A20 cells with PMA reversed the Se-induced cell death, suggesting a critical role of PKCδ in Se cytotoxicity in A20 cells. Thus, the ability of Se to induce cell cycle arrest and apoptosis via repression of key intracellular signaling molecules, PKCδ, NF-κB and IAP, may be involved in its anticarcinogenic properties.
MATERIALS AND METHODS

Materials. Anhydrous sodium selenite (98%+ purity) and PMA were purchased from Sigma Chemical Company (St. Louis, MO). Primary rabbit polyclonal anti-PKCδ, primary mouse anti-NF-κB p65 subunit, rabbit polyclonal anti-cIAP-2 and horseradish peroxidase (HRP)-conjugated donkey anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). NF-κB consensus sequences (the sense and anti-sense oligonucleotides, 5’-AGT TGA GGG GAC TTT CCC AGG C- 3’ and 3’ - TCA ACT CCC CTG AAA GGG TCC G- 5’, respectively) were procured from Promega (Madison, WI).

Cell culture s. The American Type Culture collection (ATCC, Rockville, MD) A20 cell line (TIB-208), a murine B-lymphoma culture line originating from the BALB/c strain, was employed. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and incubated at 37°C containing 5% CO₂.

Trypan blue exclusion for cell viability. A20 cells (1×10⁵/ well), exposed to sodium selenite (0.005–50 µM), were incubated in 24 well plates for 24, 72 and 96 h. Cell viability was determined by staining with 0.4% Trypan blue and visualized using a light microscope. Viable cells remained unstained while the cytoplasm of non-viable cells was stained blue. The number of viable cells and total number of cells were recorded and percent viability calculated.

Measurement of cell proliferation by [³H]thymidine incorporation. Cells (1.2×10³ per well) were added to 96-well flat-bottom plates and exposed to sodium selenite (0.005–100 µM) for 24, 72 and 96 h. Each well was pulsed with 20 µl of [methyl-³H]thymidine (25 µCi/ml, 6.7 Ci/mmol, DuPont NEN products, Boston, MA) for 18 h prior to collecting cells. Following incubation, cells were harvested onto glass fiber filter strips using a cell harvester (PHD, Cambridge Technology, Watertown, MA) and radioactivity was counted using a liquid
Proliferative responses were represented as disintegrations per minute (DPM).

**Flow cytometric analysis of cell cycle.** A20 cells (1×10^5 per well) were incubated with sodium selenite (0.5–25 µM) for 24, 72 and 96 h in 24-well plates. Cells were washed twice in cold phosphate buffered saline (PBS) and then resuspended in nuclear isolation media containing 1% Triton X-100:50 µg/ml propidium iodide:1 mg.ml⁻¹ RNaseA. Cells were acquired (50,000 events) using an Epics XL/MCL flow cytometer equipped with a 488 nm argon-ion laser.

**Nuclear morphology by Hoechst 33258 staining.** Nuclear morphology for detection of apoptotic bodies was determined using epifluorescence following staining with Hoechst 33258 (H33258, Sigma). Cells (1×10^5 per well) were treated in 24-well plates with 0, 5 and 25 µM Se, in the absence or presence of 0.5 µM PMA. Following 72 h exposure, cells were stained with H33258 (10 µg/ml in PBS) for 5 min and fluorescence microscopy performed using the Olympus IX71 inverted microscope (Olympus America Inc., Melville, NY). Digital images were acquired using a Magnafire SP® (Olympus) digital camera.

**DNA fragmentation.** DNA from A20 cells (1×10^5 per well) exposed to 5 and 25 µM Se for 72 h was extracted as described by Sellins and Cohen (Sellins and Cohen 1987). Cells were washed in PBS and suspended in 0.1 ml hypotonic lysis buffer [10 mM Tris, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100, pH 8.0]. Cells were incubated for 10 min at 4°C and the lysate was centrifuged for 30 min at 13,000×g. The supernatant containing fragmented DNA was treated with RNase (0.4 µg/µl) for 1 h at 37°C and then incubated with proteinase K (0.4 µg/µl) for 1 h. DNA was precipitated overnight at –20°C in 50% isopropanol and 0.5 M NaCl. The DNA precipitate was pelleted by centrifugation at 13,000×g for 30 min, dissolved in distilled water and electrophoresed in a 2% agarose gel.
containing ethidium bromide (0.5 µg/ml) in 0.5× Tris-Borate-EDTA buffer. The bands were visualized by a UV transilluminator (Ultra Lum Inc., Carson, CA), photographed and quantified by using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT).

**Subcellular fractionation for PKC, NF-κB and cIAP-2.** A20 cells were treated with 0, 5 and 25 µM Se for 72 h prior to appropriate subcellular fractionation. The procedure for cytosol and membrane protein extraction for PKC was performed as described by Clarke et al. (2000), with centrifugation at 42,000×g for 2 h at 4°C as a modification. Detailed procedure has been described elsewhere (Gopee and Sharma, 2003). For NFκB, the nuclear fraction was obtained as described earlier (Weber et al. 2000). To obtain total protein for cIAP-2, cells were solubilized in lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 0.2 mM leupeptin, and 10 µg/mL aprotinin), the lysates centrifuged for 5 min at 600×g and supernatants stored at -80°C.

**Western blot analysis.** Protein concentrations in each sample were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin (Sigma, St. Louis, MO) as the standard. Proteins were fractionated on 8% gels and then electrophoretically transferred to a nitrocellulose membrane followed by blocking in 5% milk protein for 1 h prior to incubating with appropriate primary and secondary antibodies. Efficiency of equal protein loading and transfer to membrane was determined by reversible Ponceau S stain (Sigma). Protein bands were visualized utilizing Pierce Super Signal® chemiluminescent substrate (Pierce, Rockford, IL) on X-ray film (Kodak X-OMAT AR, Rochester, NY). Images were acquired with a scanner and analyzed with UN-SCAN-IT software.

**PKC activity assay in total cell lysates.** Cells were washed three times in cold PBS and scraped in 100 µl lysis buffer to obtain total protein as described elsewhere (Gopee et al., 2003).
The lysates were sonicated on ice and centrifuged for 5 min at 600×g and the supernatants used for the PepTag® assay for non-radioactive detection of protein kinase C activity (Promega Corp., Madison, WI) according to the manufacturer’s instructions. The amount of protein added to the kinase assay was standardized using the Bio-Rad assay described above.

**Fluorescent electrophoretic mobility shift assay.** For the fluorescent electrophoretic mobility shift assays (EMSA), oligonucleotides were annealed by heating for 10 min at 70°C in 10× annealing buffer (200 mM Tris-HCl, pH 7.6, 50 mM MgCl₂, 1mM DTT and 0.1 mM EDTA) followed by incubation at 36.5°C for 30 min. The DNA binding procedure involved coincubating 4 µg of protein with the oligonucleotide duplex for 0.5 h on ice. Bound DNA was separated on a 6% polyacrylamide nondenaturing gel for 1.5 h at 120 V at 4°C. Specificity for the binding reaction was confirmed by addition of NF-κB p65 antibody to one of the binding reactions. Following electrophoresis, the gel sandwich was separated and stained with SYBR Gold (Molecular Probes, Eugene, OR). The gel was rinsed and bands were detected on a UV transilluminator and photographed.

**Statistical analysis.** The results were expressed as mean ± standard error (SE). Differences among various treatment groups were determined by analysis of variance followed by Duncan’s multiple range test utilizing Version 8 of the Statistical Analysis Software (SAS, Cary, NC). The level of \( p \leq 0.05 \) was taken to denote statistical significance. Results are representative of experiments repeated several (generally three) times with similar results.
RESULTS

Viability and proliferative capacity of A20 cells were altered by increasing concentrations of Se

Sodium selenite at 0–50 µM did not affect cell viability after 24 h exposure (Fig. 1A). However, at 72 h exposure, there was a significant decrease in cell viability to approximately 40% and 18% of control levels following exposure to 25 and 50 µM Se, respectively. At 96 h, the cell viability significantly decreased at 5, 25 and 50 µM Se by 46%, 74% and 94%, respectively. Exposure of A20 cells to ≥ 5 µM resulted in significantly greater viability at 24 h than at 96 h. Only at Se concentrations of 25 and 50 µM was viability significantly greater at 24 h than at 72 h. Following 72 h treatment with 5 and 25 µM Se, viability was significantly higher than at 96 h.

In agreement with above findings, no significant alterations in cellular proliferation were observed following 24 h treatment with sodium selenite (Fig. 1B). Cellular proliferation was significantly increased at Se concentrations ≤ 5 µM at 72 h. In contrast, following exposure to ≥ 25 µM Se at 72 h and ≥ 5 µM Se at 96 h, cellular proliferation was significantly decreased. Both proliferation and viability were significantly inhibited in response to ≥ 25 µM Se at 72 h and ≥ 5 µM Se at 96 h. The proliferative capacity of A20 cells exposed to no selenium at 24 h was significantly higher as compared to cells at 72 and 96 h. Cells treated with 0.005- 5 µM Se at 72 and 96 h, showed persistently greater proliferation at 72 h as compared to cells at 24 h. However, Se concentrations of ≥ 50 µM Se at 96 h and 100 µM at 72 h decreased the proliferative capacity of A20 cells as compared to the same Se treatments at 24 h. In addition, proliferation of cells exposed 0.05, 5, 25 and 50 µM Se at 72 h was significantly greater than cells exposed to the identical treatment at 96 h.
Se induced G2-M arrest and cell death in A20 cells

The effect of sodium selenite on the cell cycle progression of A20 cells was examined by flow cytofluorometry. Cells treated with increasing concentrations of selenite were collected at 24, 72 and 96 h. At 24 h in both treated and untreated cells, approximately 47% of the cells were present in the G1 phase, 18% in S phase and 18% in G2-M phase (Table 1). However, at 72 and 96 h post-treatment with 5 µM Se the distribution of DNA content revealed a reduction to 30% and 38% positive cells in G1, and a concurrent increase to 36% and 26% cells positive in the G2-M phase. The population of cells in the S-phase was not significantly altered on exposure to 5 µM Se at 72 h. Treatment with ≥ 5 µM sodium selenite gave a profile with cells accumulating in the G2-M phase at 72 and 96 h. An increase in cell death was observed following exposure to >5 µM selenium at 72 and 96 h. A concentration-dependent (0.5–10 µM Se) increase in the population of cells in the G2-M phase at 72 h is shown in Fig. 2A, with a significant increase in cell death with 10-25 µM Se. Se at 5 µM increased the population of cells in the G2-M phase of the cell cycle in A20 cells at 72 and 96 h (Fig. 2B).

Se induced nuclear and DNA fragmentation in A20 cells at 72 h

A20 cells exposed to 5 µM Se showed diffuse intact nuclear staining, with little or no fragmented nuclei, similar to untreated cells at 72 h (Fig. 3A and 3B). Cells exposed to 5 µM Se showed little fragmented nuclei; however, cells exposed to 25 µM Se showed increased number of apoptotic cells stained by H33258 and numerous fragmented nuclei in a higher magnification (Fig. 3C). Se at 5 µM induced some DNA fragmentation/laddering; however, Se concentration of 25 µM induced extensive DNA fragmentation (Fig. 3D), indicative of apoptosis.
Se inhibited PKCδ membrane translocation and PKC activity in a concentration-dependent manner at 72 h

For the following experiments, A20 cells were exposed to Se concentrations of 5 and 25 µM for 72 h, concentrations that induced cell cycle arrest and apoptosis, respectively. Since PKC is a known modulator of cell cycle arrest and apoptosis, PKC-δ was evaluated because this is the predominant isoform expressed in A20 cells (Shih and Floyd-Smith 1995). A20 cells exposed to 5 and 25 µM Se at 72 h showed a significant concentration-dependent decrease in membrane PKCδ protein concentration (Fig. 4A). Cytosol PKCδ protein levels were not significantly altered following exposure to Se. Total PKC activity was also significantly decreased in a concentration-dependent manner on exposure to Se concentrations of 5 and 25 µM at 72 h (Fig. 4B), correlating decreased cytosol to membrane redistribution with activity.

PMA prevented Se-induced cell death

A20 cells exposed to 25 µM Se showed a significant increase in cell death at 72 h compared to untreated and PMA (0.5 µM) treated cells. However, co-incubation of A20 cells with Se (5 and 25 µM) and 500 nM PMA at 72 h prevented the Se-induced cell death (Fig. 5), suggesting the PKC-dependence of Se-induced cytotoxicity in A20 cells.

Se inhibited NF-κB in a concentration-dependent manner at 72 h

A20 cells exposed to 5 and 25 µM Se at 72 h showed a significant concentration-dependent decrease in nuclear protein concentrations of NF-κB (Fig. 6A). Cytosol NF-κB protein levels were relatively unaffected after exposure to Se. These data suggest a decrease in NF-κB cytosol to nuclear translocation and hence NF-κB DNA-binding was determined by fluorescent EMSA.
A concentration-dependent decrease in NF-κB DNA-binding in cells treated with 5 and 25 μM Se at 72 h was observed (Fig. 6B).

*Se inhibited cIAP-2 in a concentration-dependent manner at 72 h*

NF-κB is known to transcribe for the anti-apoptotic protein, cIAP-2, therefore this protein was evaluated. A20 cells exposed to 5 and 25 μM Se at 72 h showed a significant concentration-dependent decrease in protein concentrations of cIAP-2 (Fig. 7). Since IAP expression is controlled by NF-κB, the decrease in IAP protein concentrations on exposure to Se positively correlates with the decrease in NF-κB DNA-binding suggesting a causal relationship.
DISCUSSION

Selenium has been suggested as an anticarcinogenic agent; the mechanism of this effect is not fully understood. This study examined selenite-induced cell cycle arrest and apoptosis in A20 murine B-lymphoma cells as a model for cancer. Firstly, the cytotoxic effect of selenite was demonstrated by Trypan blue exclusion and $[^3]$H]thymidine incorporation at high Se concentrations. In contrast, we found that at low Se concentrations (0.005–5 µM) at 72 h increased cellular proliferation in A20 cells. Secondly, Se concentrations of ≤ 5 µM induced G2-M arrest followed by an increase in the population of dead cells at Se concentrations > 5 µM. Apoptosis was the predominant form of cell death as determined by H33258 staining and DNA fragmentation. Thirdly, the concentrations of Se that induced G2M arrest and apoptosis were associated with inhibition of PKCδ, NF-κB and cIAP-2 in this model. Importantly, activation of PKC by PMA protected cells from Se-induced cytotoxicity suggesting a PKC-dependent mode of action of Se.

Se stimulates or inhibits cell growth depending on the concentration and chemical form (Ip et al., 2000). We hereby demonstrate that Se at ≤ 5 µM significantly increased the proliferative capacity of A20 B-lymphoma cells without significantly affecting viability at 72 h. In agreement with our findings, Zeng (2002) demonstrated that 0.06 and 0.25 µM Se enhanced the growth of HL-60 cells. Lower doses of Se, e.g., 1-2 ppm, exerted a stimulatory effect on a variety of biologic functions, including stimulated immunoglobulin synthesis (Spallholz et al., 1973), proliferation of lymphoblasts (Turner et al., 1985), and interferon production (Watson et al., 1986). Selenite at low (0.1-2 µM) concentrations inhibited in vitro transformation without inducing toxicity (Zhu et al., 1992). Our data further reiterate that Se supplementation at low concentrations may have a beneficial and immunostimulatory effect to healthy systems.
The present investigation demonstrated that 3-5 µM sodium selenite at 72 and 96 h arrested cells in the G2-M phase of the A20 cell cycle, followed by cells entering apoptosis at 25 µM Se. Similarly, 3–5 µM selenite was the lowest concentration to inhibit cell growth in most cases (Ip et al., 2000). G2-M arrest was documented following sodium selenite treatment in asynchronously dividing mouse mammary epithelial cells (Sinha et al., 1996) and in dividing prostate cancer cells (Menter et al., 2000). Cell cycle arrest at the G2-M transition phase is one mechanism that cells exert on proliferation. Apparently, G2-M arrest protects cells from the lethality that results from undergoing cell division before repairing DNA damage (Leach et al., 1998), it can be induced by cell-cell contact inhibition as cells reach confluency (Blank et al., 1988), and reactive oxygen species can produce free radicals that promote cells to arrest in G2-M (Bijur et al., 1999).

Selenite is well-documented as an inhibitor of cell growth in vitro in many cell lines (Medina and Oborn 1984; Spyrou et al., 1996; Stewart et al., 1997) and appears to be a phenomenon when Se exceeds 5 µM (Stewart et al., 1997); also demonstrated in the present study. In agreement with the present findings, selenite at a concentration of 10 µM was found to significantly decrease growth and viability in the 3B6 human B lymphocyte cell line via a mechanism involving the inhibition of the ribonucleotide reductase activity (Spyrou et al., 1996). Wilson et al. (1992) found that sodium selenite reduced the cell viability and cell proliferation of a murine leukemia cell line. Similar results were obtained in multiple cell models treated with Se in vitro (Thompson et al., 1994). Higher doses of inorganic selenium compounds have strong inhibitory effects on the growth of cultured mammalian cells (Nano et al., 1989) by a cytotoxic mechanism possibly by promoting reductive activation of selenite to selenodiglutathione with
subsequent depletion of cellular glutathione (GSH) and reducing equivalents (Gopalakrishna et al., 1997).

In addition to inducing mitotic arrest and inhibiting cell cycle progression, Se (≥ 25 µM) induced apoptotic cell death in A20 cells. The marked increase in apoptosis and cell growth inhibition following G2-M arrest by high doses of selenium (≥ 25 µM) supports the use of sodium selenite as a chemopreventive agent provided that it is selective for initiated or transformed cells. Se induced apoptosis in various other cancer cells (Lanfear et al., 1994; Menter et al., 2000; Spyrou et al., 1996; Stewart et al., 1997; Thompson, 1995). Sodium selenite was shown to feed into the hydrogen selenide (H₂Se) pool (Ganther, 1986), induce DNA single strand breaks (i.e. genotoxic), and subsequently induce cell death by a composite of acute lysis and apoptosis (Lu et al., 1995). Although selenocompounds could induce apoptosis through mitochondrial change mediated by production of reactive oxygen species within the cells (Li et al., 2003), the selenite-induced apoptotic DNA fragmentation is not associated with activation of caspases or mitochondrial release of cytochrome c, but may be associated with the phosphorylation of c-Jun-NH₂-terminal kinase and p38 mitogen-activated protein kinase/stress activated protein kinases (Jiang et al., 2001).

An objective of current study was to determine the involvement of PKC in Se-induced cell death as a possible mediator of Se anticarcinogenic effect. PKCδ has been shown to be involved in the regulation of apoptosis (Pongracz et al., 1999; Shizukuda et al., 2002) and cell cycle arrest (Fukumoto et al., 1997; Watanabe et al., 1992). The results showed that Se (5 and 25 µM) repressed PKCδ membrane translocation and inhibited PKC activity at 72 h in A20 cells. Alternatively, Se may cause a decrease in the expression of total PKCδ thus leading to a reduced distribution of the active membrane bound enzyme. A decrease in PKC enzyme
activity by selenocompounds was reported earlier (Gopalakrishna et al., 1997; Sinha et al., 1999). Selenite was shown to block phorbol ester-stimulated transformation of cells in both in vivo and in vitro models (Sharma et al., 1994; Zhu et al., 1992). The unique and accessible redox-active cysteine-rich regions present within the catalytic domain of PKC (Gopalakrishna et al., 1995; Quest et al., 1992) can react with certain specificity with redox-active selenocompounds including selenite (Gopalakrishna et al., 1997) leading to its inhibition. In addition, activation of PKC by PMA prevented the Se-induced cell death observed in A20 cells. Induction of apoptosis by Se may be linked to its repression of PKC activity, since PKC inhibition can induce apoptosis in a number of cell lines (Chmura et al., 1996; Reyland et al., 1999). Redox-active seleno-compounds can inactivate PKC by reacting with the cysteine-rich regions present within the catalytic domain or to a lesser extent with the cysteine residues present within the zinc-fingers of the regulatory domain (Gopalakrishna and Gundimeda 2001). It is possible that PMA may reverse the Se–induced toxicity by means of the selenoprotein thioredoxin reductase acting through thioredoxin or through the direct interaction involving the selenosulfur center of thioredoxin reductase with the zinc-thiolates of PKC domain (Gopalakrishna and Gundimeda 2001).

Since the caspase cascade is not always involved in Se-induced apoptosis (Jiang et al., 2001) and inhibition of NF-κB-mediated gene expression can result in apoptosis, it was useful to determine the role of this transcription factor in the present system. It has been suggested that Se-induced inhibition of PKC may also play a role in the Se-mediated inhibition of NF-κB transactivation in intact cells (Gopalakrishna and Gundimeda 2002). We found that Se (5 and 25 μM) inhibited NF-κB nuclear translocation and DNA-binding in A20 cells. Yoon et al. (2001) demonstrated that NF-κB activity was inhibited by selenite in HT1080 fibrosarcoma cells.
Binding of NF-κB to nuclear responsive elements was decreased progressively by increasing selenite levels, and at 7 µM selenite, DNA-binding activity was completely inhibited in human T cells and lung adenocarcinoma cells (Kim and Stadtman 1997). Recent studies in a variety of cell types, including B-cells suggest that NF-κB controls cell cycle progression and the induction of apoptosis (Bellas et al., 1997; Woo et al., 1996). Selenite and other selenium compounds were shown to inhibit the functions of NF-κB by reacting with critical sulfhydryl groups of this protein (Park et al., 2000). Through its modification of glutathione peroxidase activity Se can inhibit NF-κB activation and up-regulate IκB normal half life (Kretz-Remy and Arrigo 2001).

We have also recently reported that selenite inhibited the lipopolysaccharide-induced activation of NF-κB in a murine macrophage cell line (Kim et al., 2004).

IAP is one of the NF-κB responsive genes that are critical to cell survival by inhibiting the active process of apoptosis. Subsequent to NF-κB inhibition, we demonstrated inhibition of cIAP-2 at 72 h after exposure to 5 and 25 µM Se in A20 cells. Inhibition of NF-κB by SN50, an inhibitor of NF-κB, on multiple myeloma cell lines and normal B lymphocytes induced apoptosis and downregulated cIAP-1 and cIAP-2 (Mitsiades et al., 2002). IAP is thought to play a role in carcinogenesis by protecting the tumor cell from apoptosis and promoting tumor development, is expressed by multiple types of tumor cell lines (Tamm et al., 2000). Therefore, our demonstration of the inhibitory action on IAP in Se-treated A20 cells furthers our understanding of the mechanisms of its anticarcinogenesis.

In conclusion, low doses of selenium are capable of stimulating murine B-lymphoma cells to proliferate. In contrast, inhibition of PKCδ, NF-κB and cIAP-2 by higher Se concentrations were associated with G2-M arrest and apoptosis in A20 cells. Activation of PKC reversed the Se-induced cell death in A20 cells. The ability of Se to induce cell cycle arrest by affecting
critical cell signaling molecules may represent one mechanism of the anticarcinogenic potential of this element. Further studies are needed to determine whether these molecular intracellular mediators are sequentially related in this cell system. Additionally the effect of selenite should be investigated using in vitro models. The data presented here may not be extrapolated at this time to human situations because the difference between the desirable intake of selenium and that of toxic levels is relatively small.

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TABLE 1. The effect of increasing concentrations of sodium selenite on cell cycle in A20 cells at 24, 72 and 96 h. Data are presented as the mean (± SEM) percentage of cells in different cell cycle phases (n =3, experiments were done in triplicate and repeated three times).

<table>
<thead>
<tr>
<th>Cell cycle phase</th>
<th>Distribution of cells at different time points (%)</th>
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<tr>
<td></td>
<td>24 h</td>
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<tr>
<td>Control</td>
<td></td>
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<tr>
<td>G₀/G₁</td>
<td>46.97 ± 0.52</td>
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<tr>
<td>S</td>
<td>18.30 ± 0.86</td>
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<tr>
<td>G₂/M</td>
<td>18.43 ± 0.87</td>
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<tr>
<td>Death</td>
<td>6.05 ± 0.11</td>
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<td>Sodium Selenite (0.5 µM)</td>
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<tr>
<td>G₀/G₁</td>
<td>46.46 ± 0.54</td>
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<tr>
<td>S</td>
<td>17.27 ± 0.38</td>
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<tr>
<td>G₂/M</td>
<td>18.93 ± 0.41</td>
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<tr>
<td>Death</td>
<td>6.45 ± 0.10</td>
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<td>Sodium Selenite (5 µM)</td>
<td></td>
</tr>
<tr>
<td>G₀/G₁</td>
<td>46.37 ± 0.12</td>
</tr>
<tr>
<td>S</td>
<td>17.77 ± 0.63</td>
</tr>
<tr>
<td>G₂/M</td>
<td>18.20 ± 0.29</td>
</tr>
<tr>
<td>Death</td>
<td>6.33 ± 0.23</td>
</tr>
<tr>
<td>Sodium Selenite (25 µM)</td>
<td></td>
</tr>
<tr>
<td>G₀/G₁</td>
<td>46.93 ± 0.26</td>
</tr>
<tr>
<td>S</td>
<td>18.53 ± 0.54</td>
</tr>
<tr>
<td>G₂/M</td>
<td>17.27 ± 0.43</td>
</tr>
<tr>
<td>Death</td>
<td>6.49 ± 0.11</td>
</tr>
</tbody>
</table>

* Significantly different from the respective control cultures of same time at p < 0.05.
Legends to figures

FIG. 1. The effects of sodium selenite on cell viability and proliferation. (A) A20 B lymphoma cells at a density of $1 \times 10^5$ cells/ml were incubated with media containing 0, 0.5, 5, 25 or 50 µM sodium selenite. At 24, 72 and 96 h, the number of viable cells was determined by Trypan blue exclusion. (B) Proliferative responses of A20 B-lymphoma cells exposed to sodium selenite as determined by [3H]thymidine incorporation. Cell suspensions (1.2$\times$10³/well) were exposed to increasing concentrations of sodium selenite (0.005-100 µM) for 24, 72 and 96 h and pulsed with [methyl-³H]thymidine for 18 h. Results are expressed as the mean ± SE (n=3) from a representative of three independent experiments. * Significantly different than the respective control at $p \leq 0.05$. “a” Significantly different from 24 h at the same Se concentration at $p \leq 0.05$. “b” Significantly different from 72 h at the same Se concentration at $p \leq 0.05$.

FIG. 2. The effect of increasing concentrations of sodium selenite on cell cycle progression in A20 cells. (A) Cell cycle analysis of A20 cells following 72 h exposure to 0, 0.5, 1, 3, 5, 10 and 25 µM sodium selenite. (B) Cell cycle analysis of A20 cells following 24, 72 and 96 h exposure to 0 and 5 µM sodium selenite. The effect of sodium selenite in cell cycle progression was determined by measuring the DNA content distribution using fluorocytometry after different treatments. Data are representative of two independent experiments.

FIG. 3. Effect of increasing concentrations of sodium selenite on nuclear morphology and DNA fragmentation at 72 h. A20 cells were exposed to 5 and 25 µM Se 72 h, stained with H33258 and visualized via fluorescence microscopy. Representative photographs show
increased nuclear staining with H33258 at 72 h in cells exposed to 0 µM Se (A), 5 µM Se (B) and 25 µM Se (C). Insets show intact nuclei in untreated cells (A) and fragmented nuclei in cells exposed to 25 µM Se (C) at 72 h at a higher magnification. (D) A20 cells were exposed to 5 and 25 µM Se prior to visualization of fragmented DNA via gel electrophoresis. Results are expressed as the mean ± standard error (n=3). *Significantly different than the control at $p \leq 0.05$. Data are representative of three independent experiments. Order of bands in the inset corresponds to bars of the graph.

**FIG. 4.** Sodium selenite inhibited cytosol to membrane translocation of PKCδ and PKC activity in A20 cells at 72 h. (A) Cells exposed to sodium selenite (5 and 25 µM) at 72 h. Cell lysates were resolved on a gel, electroblotted, incubated with antibody and visualized with chemiluminescent reagents. (B) Cells were exposed to 5 and 25 µM sodium selenite for 72 h and PKC activity assay was performed on total cell lysates. Results are representative of 3 independent experiments, the mean ± SE of a representative experiment (n=3) are presented. *Significantly different from the respective control at $p \leq 0.05$.

**FIG. 5.** PMA prevented the selenite-induced cell death at 72 h. Following 72 h exposure to either 5 or 25 µM Se in the presence or absence of 0.5 µM PMA, cells were stained with H33258 and visualized via fluorescence microscopy. Results are expressed as the mean ± SE (n=3) of a representative experiment. *Significantly different from the respective control at $p \leq 0.05$. 
FIG. 6. Sodium selenite inhibited NF-κB cytosol to nuclear translocation and DNA binding in A20 cells at 72 h. (A) A20 cells were exposed to selenite, 5 or 25 μM, for 72 h. Cytosol and nuclear proteins were resolved on gel, electroblotted, hybridized to antibody, and visualized autoradiographically. (B) Cells were treated with sodium selenite (5 or 25 μM) for 72 h prior to isolation of nuclear proteins and fluorescent EMSA performed. The supershift after addition of antibody to p65 is also shown. Results are expressed as the mean ± SE (n=3). * Significantly different from the respective control at $p \leq 0.05$. Experiment was repeated three times with similar results.

FIG. 7. Sodium selenite inhibited cIAP-2 protein concentrations in A20 cells at 72 h. Following 72 h min exposure to 5 or 25 μM Se, A20 cells were lysed, and cIAP-2 protein concentrations were determined by western blot analysis. Results are expressed as the mean ± SE (n=3). * Significantly different from the respective control at $p \leq 0.05$. 
Figure 1

A

Viability (%)

Selenium selenite, μM

B

Proliferation Index (DPM × 10^5)

Selenium selenite, μM
Figure 2

A

Selenium concentration (µM) at 72 h

Relative Frequency

PI fluorescence

B

Control 5 µM Se

Relative Frequency

PI fluorescence

24 h

72 h

96 h
Figure 4
Figure 5