AP-1 DNA-binding activity is inhibited by selenite and selenodiglutathione

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Abstract The binding of the transcription factor AP-1 to DNA has been shown to be modulated by redox control mechanisms. Selenite and selenodiglutathione (GS-Se-SG), inhibit mammalian cell growth and are efficient oxidants of reduced thioredoxin and reduced thioredoxin reductase. Here, we report that selenite and GS-Se-SG efficiently inhibited AP-1 DNA-binding in nuclear extracts from 3B6 lymphocytes. A GS-Se-SG concentration of 0.75 μM resulted in 50% inhibition of AP-1 DNA-binding, whereas the same effect was achieved with 7.5 μM selenite. Nuclear extracts prepared from human 3B6 lymphocytes exposed for 4 h to 10 μM selenite showed a 50% reduction of AP-1 binding. These data suggest that selenite and selenodiglutathione inactivate the AP-1 factor and provide a mechanism by which selenium compounds inhibit cell growth.

Key words: Selenium; Selenodiglutathione; Redox regulation; AP-1

1. Introduction

The protein products, Fos and Jun, of the proto-oncogene families *c-fos* and *c-jun* are components of the transcription factor AP-1 [1]. AP-1 binds to and activates transcription from the TPA-responsive element (TRE) (TGAC/GTCA). Although the AP-1 binding site was originally defined as a phorbol ester-responsive element, it is present in the promoter of genes implicated in a number of signal transduction cascades associated with growth, neuronal excitation, cellular stress, transformation and differentiation suggesting a central role for AP-1 in signal transduction [2,3]. Jun proteins can form homodimers or heterodimers among themselves, but more readily dimerize with the Fos proteins (including c-Fos, Fos-B, Fra-1, and Fra-2) which do not form dimers among themselves. Dimers are formed through a coiled-coil interaction involving leucine zipper domais in each protein [4]. All of the Jun/Jun and Jun/Fos complexes probably have identical DNA binding specificities but different binding affinities as measured in vitro [5]. DNA-binding is mediated through juxtaposition of conserved basic regions of each protein in the heterodimer. DNA binding of endogenous AP-1 complexes is regulated through post-translational modifications including both phosphorylation and dephosphorylation [6–8]. In addition to phosphorylation, reduction-oxidation (redox control) also regulates the DNA binding activity of AP-1 in vitro [9,10]. The redox-status of two conserved cysteine residues (Fos-Cys-154 and Jun-Cys-272) in the heterodimer is important for DNA binding although they do not directly participate in the binding. Reduced cysteine residues leads to enhanced DNA-binding whereas oxidation inhibits binding [9]. A protein (Ref-1) has been isolated that enhances binding via reduction of the oxidized cysteine residues [10].

Thioredoxin (12 kDa), in its oxidized form, has a redox-active disulfide on a protrusion in the three-dimensional structure, and is reduced by thioredoxin reductase and NADPH (the thioredoxin system) [11–13]. The dithiol in reduced thioredoxin is a powerful general protein disulfide reductase [14]. ADF (adult T-cell leukemia derived factor) is secreted human thioredoxin and operates as a cytokine [15]. The thioredoxin system enhances the binding of Fos/Jun to DNA via reduction of Ref-1 [10].

Selenium is an essential trace element in higher eukaryotes [16] but also very toxic. Selenium compounds have well-documented inhibitory effects on mammalian cell-growth [17,18]. Low levels (nM) of sodium selenite stimulate cell-growth and selenite is an essential component of serum-free media [19]. The most established function of selenium is its presence as a selenocysteine residue in glutathione peroxidase. This enzyme is important for the detoxification of hydrogen peroxide, lipid hydroperoxides and organic hydroperoxides [20]. However, changes in the function and levels of glutathione peroxidase do not seem to be directly involved in the effects of selenium compounds on cell growth [21]. Selenite is a potent oxidant of thiols and a direct substrate for mammalian thioredoxin and thioredoxin reductase [22]. In mammalian tissues selenite is believed to react with reduced glutathione (GSH) to form selenodiglutathione GS-Se-SG [23]. This compound can undergo further reduction by the thioredoxin system [24] or glutathione reductase [25]. GS-Se-SG is an oxidant of thiols and kinetically one of the most efficient substrates known for reduced thioredoxin [24]. Furthermore, GS-Se-SG efficiently inhibits human thioredoxin through oxidation of Cys-72 [26], a structural cysteine residue located close to the active site [13]. Incubation of GS-Se-SG or selenite with mammalian thioredoxin reductase leads to redox-cycling with oxygen and a large nonstoichiometric oxidation of NADPH [22,24].

The aim of the present study was to investigate whether selenite and GS-Se-SG could inhibit Fos/Jun-binding via oxidation. An inhibitory effect of selenium on the activity of oncogene products could be an important mechanism in explaning the inhibitory effects of selenium compounds on cell-growth.

2. Experimental

2.1. Materials

GS-Se-SG was prepared as described by Björnstedt, et al. [24]. Recombinant human thioredoxin was prepared as described by Ren et al. [26] and human thioredoxin reductase was prepared from placenta

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Abbreviations: AP-1, activator protein-1, GS-Se-SG, selenodiglutathione; Trx, thioredoxin; TR, thioredoxin reductase; SeO_3^{2-}, selenite; SeO_4^{2-}, selenate; DTT, dithiothreitol.

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essentially as described by Luthman and Holmgren [27]. The preparation was of better than 90% purity. RPMI medium was from Flow Laboratories (Ayshire, UK) and fetal calf serum, glutamine and antibiotics were from GIBCO Ltd., UK. The double strand AP-1 oligonucleotide and the CytoTox 96 non-radioactive cytotoxicity assay were obtained from Promega. All other chemicals were from Sigma. Specific anti-Jun rabbit polyclonal antibodies were generated by immunization with synthetic peptides coupled to keyhole limpet hemocyanin. The peptides used were: c-Jun, SYGAAGLAFPSQPQQ; JunB,ISYLPHAPPFAGG; and JunD, GCQLLPQHQVPAY. No cross-reactivity was observed between the different antisera [28].

2.2. Cell culture conditions

The EBV-transformed lymphoblastoid cell-line 3B6 was kindly provided by Professor T. Tursz, Inst. Gustave-Roussy, Paris, France. The cells were grown in RPMI supplemented with 10% (v/v) fetal calf serum, glutamine and antibiotics, in 5% CO_2 atmosphere. Cultures with a density of 0.5×10^6 cells/ml were used for nuclear extract preparation.

2.3. Preparation of nuclear extracts

Nuclear extracts were prepared according to a modified version of a previously described method [29]. Briefly, cells were washed twice with ice-cold phosphate-buffered saline, pelleted, and then gently resuspended in 200 μl of a buffer composed of 15 mM Tris-HCl, pH 7.5, 0.5 M sucrose, 60 mM KCl, 0.25 mM EDTA, 0.5 mM spermidine, 2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg/ml aprotinin, and 1 μg/ml pepstatin. Cells were allowed to swell on ice for 20 min, then 10 μl of a 10% solution of NP-40 was added, and the tube was vigorously vortexed for 10 s. After centrifugation for 5 min at 6,500 rpm in an Eppendorf centrifuge, the supernatant was removed and the nuclear pellet was resuspended in 150 μl of 20 mM HEPES, pH 7.9, 25% glycerol, 0.4 M NaCl, 0.25 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin. The samples were shaken vigorously at 4°C for 25 min, then centrifuged for 30 min and the supernatant was frozen in aliquots. Protein was determined by the method of Bradford.

2.4. In vitro transcription and translation

Mouse JunB/c-Fos was synthesized in vitro by transcription of mouse junB and c-fos cDNA by using T7 RNA polymerase and subsequent cotranslation in a rabbit reticulocyte translation system (Amersham). In vitro translation was performed according to the manufacturer's instructions.

2.5. Gel retardation analysis

Gel shift analysis was performed with 4 μg of extract protein. Extracts were preincubated in the presence or absence of selenium compounds for 10 min at room temperature. A binding buffer, (0.2 μg of poly(dI-dC) and 1.5 μg of sonicated salmon sperm DNA in 20 mM HEPES, 0.1 mM EDTA, 12% glycerol, 4 mM $MgCl_2$, 4 mM spermidine, and 0.1 mg/ml bovine serum albumin) was added to each sample and the samples were stored on ice for 15 min. The labelled probe was added and the samples were incubated at room temperature for 15 min. The resulting protein-DNA complexes were resolved from unbound oligonucleotides on a pre-electrophoresed polyacrylamide gel (29:1) with 0.25 × TBE (1 × TBE: 90 mM Tris-borate pH 8, 1 mM EDTA) as running buffer. Gels were fixed in 10% acetic acid, 10% methanol, dryed and visualized by autoradiography. Quantification was done with the Molecular Dynamics Phosphoimager. The double-stranded AP-1 oligonucleotide used as probe (factor binding site underlined) (5′-CGCTTGATGAGTCAGCCGGAA-3′/3′-GCGAACT ACTCAGTCGGCCTT-5′) was labelled with [^{32}P]ATP by T4 polynucleotide kinase from Pharmacia.

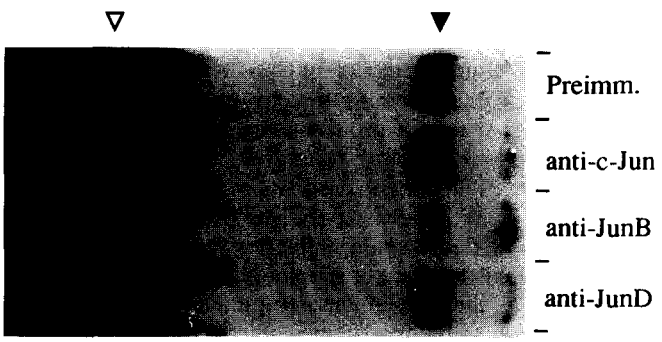

Fig. 1. Gel shift analysis of AP-1 DNA-binding activity in 3B6 nuclear extracts. The ^{32}P-labelled AP-1 specific oligonucleotide was incubated with 4 μg of 3B6 nuclear extracts which had been preincubated with different antisera for 30 min at 4°C. The generation and the specificity of the antisera to c-Jun, JunB and JunD are described in section 2. Preimm denotes extracts preincubated with rabbit preimmune serum. The positions of the free oligonucleotide (open arrowheads) and the retarded protein–DNA complex (filled arrowheads) are indicated.

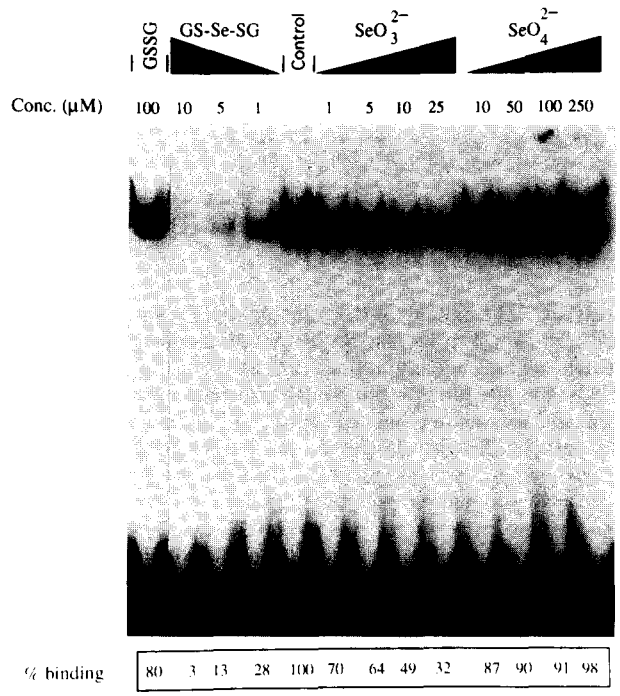

Fig. 2. Inhibition of AP-1 DNA-binding by selenite and selenodiglutathione. Nuclear extracts (4 μg) of 3B6 cells were incubated in the presense of oxidized glutathione (GSSG), selenodiglutathione (GS-Se-SG), selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) at the indicated concentrations in a total volume of 10 μl for 10 min at room temperature. Binding buffer (25 μl) was added for 15 min before the addition of the ^{32}P-labelled AP-1 oligonucleotide. The values at the bottom of the figure are percent of counts in the AP-1 complex, compared to control extracts and were obtained by phosphoimager analysis.

3. Results and discussion

We examined the binding of factors present in the nuclear extracts from human 3B6 lymphocytes to a labeled synthetic double stranded AP-1 oligonucleotide probe using gel shift analysis. Specific antibodies were used to determine the type of Jun protein present in the AP-1 complexes that gave binding to the AP-1 consensus element (Fig. 1). A clear decrease in the intensity of the retarded band was observed when the antiserum specific to JunB was added (Fig. 1). In contrast, the anti-c-Jun and anti-JunD antibodies did not significantly alter the intensity of the bound complex. Thus, in the 3B6 cells, JunB was the predominant Jun component.

Treatment of nuclear extracts with selenite and GS-Se-SG for

10 min at room temperature, before addition of binding buffer and the ^{32}P-labelled AP-1 oligonucleotide, inhibited AP-1 DNA binding (Fig. 2). The concentration of selenite required for 50% inhibition of binding was 7.5 μM. The natural selenium metabolite GS-Se-SG was far more efficient at inhibition. Only 0.75 μM was required to obtain the same result as with 7.5 μM selenite. In contrast selenate at a much higher concentration (250 μM) had only a small inhibitory effect. Also, oxidized glutathione (GSSG), at 100 μM inhibited to a very limited extent (Fig. 2) demonstrating the dramatic effect by the presence of a Se atom between the sulfurs in glutathione disulfide.

Since JunB was the major Jun component of the AP-1 factor in the 3B6 cells, we used a rabbit reticulocyte lysate system to cotranslate JunB and c-Fos. Addition of selenite and GS-Se-SG to in vitro translated JunB/c-Fos also resulted in efficient inhibition (Fig. 3). Total inhibition was obtained by 1 μM of selenite or 1 μM of GS-Se-SG. In accordance with previous reports, DTT stimulated AP-1 DNA binding of nuclear extracts [9]. The presence of DTT also prevented the effect of selenite in a concentration dependent manner (Fig. 4). This clearly showed that

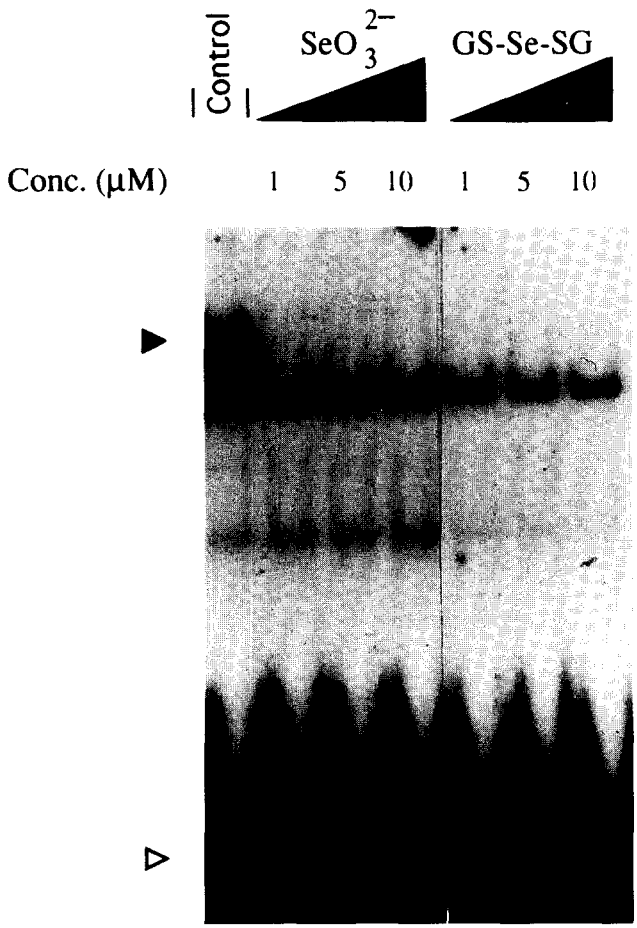

Fig. 3. The DNA-binding activity of in vitro translated JunB/c-Fos is inhibited by selenite and selenodiglutathione. In vitro co-translation of JunB and c-Fos in rabbit reticulocyte lysate was carried out in the presense of in vitro synthesized junB and c-fos mRNAs. In vitro translated JunB/c-Fos (1 μl) was incubated with selenite, selenodiglutathione and binding buffer for 10 min at room temperature in a total volume of 30 μl. The ^{32}P-labelled AP-1 oligonucleotide was added for 15 min at room temperature and the samples were analyzed by gel electrophoresis.

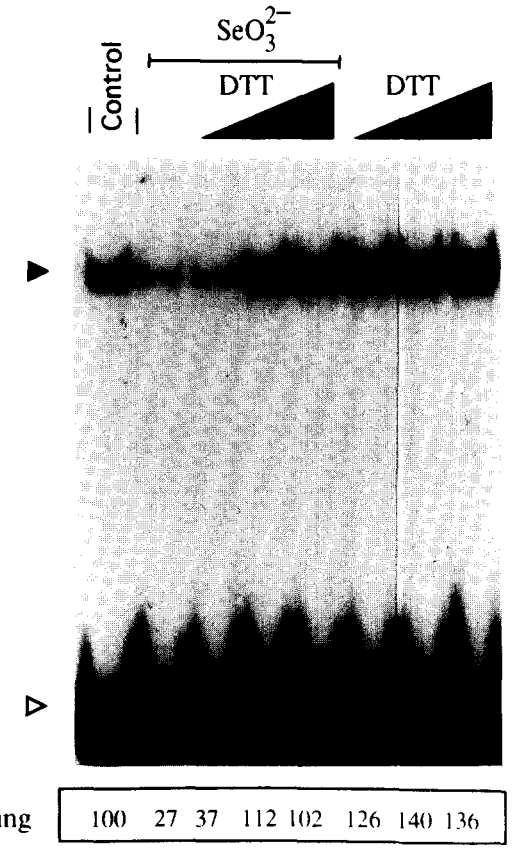

Fig. 4. Effect of DTT on selenite inhibition of DNA binding. Nuclear extracts were incubated for 10 min at room temperature with 50 μM selenite or without (control) using DTT at 50, 250, and 500 μM, respectively. The gel retardation assay was performed as outlined in the legend to Fig. 2.

the inhibitory effects of selenite was mediated by a redox mechanism. Thioredoxin is known to stimulate AP-1 DNA binding through reduction of the Ref-1 protein present in the nuclear extracts [10]. We found that addition of thioredoxin, thioredoxin reductase and NADPH (the thioredoxin system) stimulated binding by 60% (Fig. 5). The presence of the thioredoxin-system could only partly prevent the inhibitory effect of selenite (Fig. 5).

Fos and Jun are regulated by the redox status of a single conserved cysteine residue. Reduction enhances binding whereas oxidation inhibits binding. The mechanism behind the oxidative inhibition is so far not certain, since mutagenesis of the cysteines has revealed that oxidation inhibits binding also when one of the cysteine residues in the heterodimer has been replaced by a serine [9]. This shows that inhibition is not dependent on the ability to form a disulfide bond within the heterodimer as well as with other thiols. However, the cysteines can form disulfide bonds with SH groups of other cysteines from other heterodimers giving rise to slower migrating complexes upon oxidation. It has also been suggested that the formation of a cysteine sulfenic acid may play a role as shown in the case of OxyR [30].

To investigate if treatment of intact human lymphocytes with selenium compounds altered the AP-1 DNA binding activity, selenite or selenate was added to 3B6 cells. After incubation of the cells with selenite (10 μM) or selenate (250 μM) for 4 h,

Fig. 5. Effect of human thioredoxin reductase and thioredoxin on inhibition of AP-1 DNA-binding by selenite. Nuclear extracts were treated with selenite (50 μM) in the absence (−) or presence (+) of 0.04 μM human placenta TR, 5 μM Trx and 0.5 mM NADPH for 15 min at room temperature in a total volume of 15 μl. Binding buffer was added and the samples were treated as described in the legend to Fig. 2.

nuclear extracts were prepared and AP-1 DNA binding was analyzed by gel-shift assays. The viability of the cells treated with selenite and selenate was comparable to controls as determined by trypan blue exclusion and LDH releasing assays. The AP-1 DNA binding was decreased to 50% in the extracts from cells treated with selenite compared to control (Fig. 6). Selenate treatment did not inhibit AP-1 DNA-binding. The different concentrations used were based on unpublished observations (Spyrou, G., Björnstedt, M., Skoog, S. and Holmgren, A., manuscript in preparation) where cell-proliferation was completely inhibited by treatment with 10 μM sodium selenite and 250 μM sodium selenate for 24 hours.

Selenite and GS-Se-SG are efficient oxidizing substrates of the thiols in thioredoxin or thioredoxin reductase and also operate as inhibitors of the mammalian thioredoxin system proteins [22–26]. Low concentrations of GS-Se-SG efficiently inhibit human thioredoxin by oxidation involving the structural Cys-72 [26]. Thus, interaction with the Cys-72 in thioredoxin could be one important mechanism for the inhibition of AP-1 DNA binding in cells treated with selenite. By inhibition of thioredoxin, the Ref-1 protein cannot be reduced and oxidized Fos and Jun proteins cannot be reactivated. Direct oxidation of the sulfhydryl groups of the reduced Ref-1 protein is also an alternative inhibitory mechanism of the selenium compounds.

Selenite in high concentrations has been shown to react with thiols in proteins to form both -S-S- and -S-Se-S- bridges [31]. Upon exposure to selenite, Fos and Jun may form complexes connected by disulfides or -S-Se-S- bridges. Crosslinking of Fos and Jun with other protein thiols in the nuclear extract would also be possible. GS-Se-SG should contribute to complex formation more easily than selenite since only two electrons are needed to form the highly reactive selenopersulfide, GS-Se^-. Further two electron reduction, will generate hydrogen selenide, which is known to redox-cycle with oxygen in the presence of thiols. This is the most likely explaination of the more efficent inhibition by GS-Se-SG compared to selenite.

We have shown that selenite and GS-Se-SG are potent inhibitors of the AP-1 DNA binding activity. The AP-1 complex is an important transcription factor for the regulation of cell-growth and differentiation. Furthermore, many extracellular stimuli activate the AP-1 complex and mediate their stimulatory effects via the AP-1 transcriptional activation. Selenium compounds have well documented inhibitory effects on cell growth and preventive effects for tumor promoter and carcinogenic substances [32,33]. The efficient inhibition of the AP-1 DNA binding activity reported here should be an important

Fig. 6. AP-1 DNA binding in 3B6 cells treated with selenite and selenate. Nuclear extracts were prepared from 3B6 cells treated for 4 h with 10 μM selenite or 250 μM selenate. Equal amounts of protein (4 μg) were incubated with binding buffer for 15 min on ice before the addition of the ^{32}P-labelled AP-1 oligonucleotide.

mechanism for explaining the inhibition of tumor cell growth and possibly blocking the effects of tumor promoters.

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