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Inhibition of Selenite-Catalyzed Superoxide Generation and Formation of Elemental Selenium (Se°) by Copper, Zinc, and Aurintricarboxylic Acid (ATA)*

Randall L. Davis and Julian E. Spallholz†

CENTER FOR FOOD AND NUTRITION AND INSTITUTE FOR NUTRITIONAL SCIENCES, TEXAS TECH UNIVERSITY, LUBBOCK, TX 79409, U.S.A.

ABSTRACT. Selenite catalyzes the oxidation of glutathione (GSH) with the subsequent generation of superoxide ($O_2^{\bullet -}$). Copper, zinc, and aurintricarboxylic acid (ATA) were tested for their ability to inhibit both the selenite-catalyzed generation of superoxide and the conversion of selenite to elemental selenium (Se°). As measured by lucigenin-dependent chemiluminescence (CL), copper, zinc, and ATA were shown to inhibit significantly ($P < 0.05$) selenite-catalyzed CL in a concentration-dependent manner. The inhibition of the selenium-catalyzed generation of superoxide by copper(II) was greater than by either zinc or ATA. In addition, Copper, zinc, and ATA all inhibited the conversion of selenite to Se°. Inhibition of selenite-catalyzed CL by copper, zinc, and ATA is believed to occur as the result of inhibition of Se^{2-} and/or $GSSe^-$, the catalytic selenopersulfide anion of GSH. BIOCHEM PHARMACOL 51;8:1015–1020, 1996.

KEY WORDS. selenite; superoxide; copper; zinc; aurintricarboxylic acid; toxicity

In vitro, selenite has redox catalytic activity. As early as 1947, Feigl and West [1] showed that selenite catalytically oxidized labile sulfides and reduced a variety of electron acceptors, including methylene blue. Levander *et al.* [2] demonstrated that selenite was a reductive catalyst for oxidized cytochrome *c* in the presence of GSH. It has been suggested that $RSSe^-$‡ is the active species in the reduction of methylene blue, cytochrome *c*, and methemoglobin by thiols [2, 3].

Catalytic selenium compounds (selenite and disulfides) oxidize most thiols *in vitro*, in cells and cell membranes generating superoxide and other ROS [4–7]. Seko *et al.* [5] demonstrated that selenite reacted with GSH to produce H_2Se and ultimately superoxide and Se°. Kramer and Ames [8] had earlier suggested that the reaction of selenite with intracellular thiols generated ROS, which they inferred were the principal causal agents of selenite mutagenicity and toxicity in *Salmonella typhimurium*.

Selenite and its metabolites have been shown to cause DNA fragmentation [9, 10] and, more recently, cellular apoptosis [11–13]. Apoptosis is characterized by nuclear fragmentation, chromatin condensation, and plasma membrane blebbing [14]. ROS have been implicated recently as playing an active role in producing DNA fragmentation [15] and cellular apoptosis [16]. Lu *et al.* [11] have found that selenite-induced DNA double-strand breaks and cellular apoptosis could be inhibited by Zn^{2+} or ATA.

In light of ROS generated in selenium toxicity and inhibition of cellular apoptosis by Zn^{2+} and ATA, we sought in the present study to examine the ability of the metal ions Cu^{2+} and Zn^{2+} as sulfates and ATA to inhibit selenite-catalyzed superoxide generation as measured by CL. Inhibition of selenite-catalyzed superoxide generation by metals and ATA would provide a plausible connection between selenite toxicity, free radical generation, and cellular apoptosis.

MATERIALS AND METHODS

Time–Course of Concentration-Dependent Selenite-Catalyzed CL

Selenite-induced CL was measured using the lucigenin (bis-*N*-methylaridinium nitrate)-dependent CL assay [17] as modified by Yan and Spallholz [7]. All reagents were purchased from the Sigma Chemical Co. (St. Louis, MO) and prepared in 0.02 M potassium phosphate buffer (pH 7.0). Sodium selenite (0.06, 0.13, 0.19, and 0.25 mM Se) was added to 1.0 mL of a GSH–lucigenin mixture that contained 0.2 mg GSH/mL and 1.0 mg lucigenin/mL in a 0.02 M potassium phosphate buffer. The pH of the GSH–lucigenin mixture was adjusted with KOH so that the final pH

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† Corresponding author. Tel. (806) 742-3068; FAX (806) 742-3042.

‡ *Abbreviations:* ATA, aurintricarboxylic acid; CL, chemiluminescence; GSH, glutathione; $GSSe^-$, the selenopersulfide anion of glutathione; $^{\bullet}OH$, hydroxyl radical; $O_2^{\bullet -}$, superoxide; ROS, reactive oxygen species; $RSSe^-$, selenopersulfide anion; Se°, elemental selenium; Se^{2-}, selenide anion; and SOD, superoxide dismutase.

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of the solution was 7.2 to 7.4. CL, indicative of superoxide generation, was determined using a model 1215 luminescence photometer (Los Alamos Diagnostics, Los Alamos, NM) at 25°. CL was measured continuously in 2-min intervals until the CL of the reaction equaled the background CL of the GSH–lucigenin mixture alone.

Time–Course of Concurrent Selenite-Catalyzed CL and the Conversion of Selenite to Se°

Selenite-induced CL was measured using the luminescence photometer as described above. Selenite concentrations tested were 0.06 and 0.13 mM Se, and CL was measured over a 24-min period. GSH reduction of selenite to H_2Se and its oxidation to Se° was assessed spectrophotometrically over a 20-min period in the presence and absence of lucigenin (1.0 mg/mL) using a Shimadzu UV-VIS model 160 recording spectrophotometer at 400 nm.

Inhibition of Selenite-Catalyzed CL

The ability of Cu^{2+}, Zn^{2+} or ATA to inhibit selenite-catalyzed CL was assessed in 1 mL of the GSH–lucigenin mixture using the luminescence photometer. ATA or metal ions were tested for inhibition of CL at metal ion or ATA to Se molar ratios of 1:1, 5:1 and 10:1. Following the addition of selenite (0.03 mM Se) and the metal ion or ATA to the GSH–lucigenin mixture ([GSH] = 0.5 mg/mL), CL was measured as described above. Percent inhibition of the selenite-catalyzed reaction was calculated as:

$$\frac{\text{CL of selenite + metal ion (ATA)}}{\text{CL of selenite alone}} \times 100$$

$$= \% \text{ inhibition}$$

Comparison of the Inhibition of Selenite-Catalyzed CL and the Inhibition of the Conversion of Selenite to Se°

We compared the ability of Cu^{2+} (0.04 mM, 0.13 mM), Zn^{2+} (0.13 mM, 0.64 mM) or ATA (0.10 mM, 0.20 mM) to inhibit the selenite-catalyzed CL as well as their ability to inhibit the conversion of selenite to Se°. Inhibition of selenite-catalyzed CL and the inhibition of the conversion of selenite to Se° were assessed as described above with 0.11 mM Se as selenite. The test conditions for the two procedures were identical, except that lucigenin was not used in the assessment of the conversion of selenite to Se°. Controls contained metal sulfates, ATA, or selenite alone.

Statistical Analysis

All data are expressed as means ± SEM. Analysis of variance was used to determine if the treatments were significantly ($P < 0.05$) different. Least significant means was used to separate means when significant treatment effects were observed. All statistical analyses were done using the Statistical Analysis System (SAS) software [18].

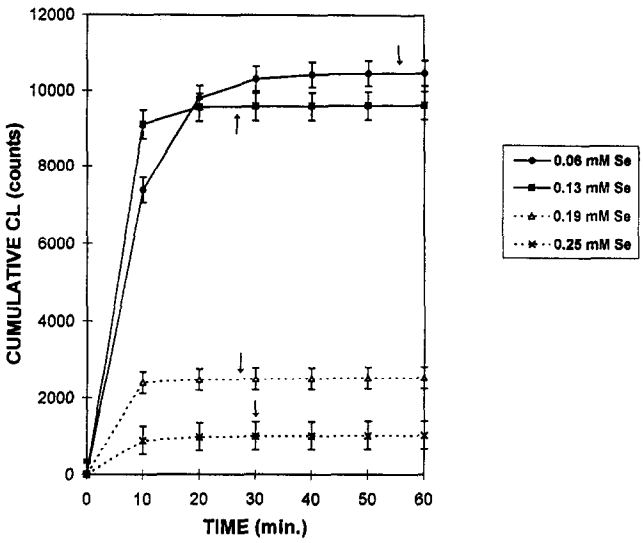

FIG. 1. Time–course of concentration-dependent selenite-catalyzed CL. CL was determined in the presence of 1 mL of a GSH–lucigenin mixture (0.2 mg GSH/mL and 1.0 mg lucigenin/mL in 0.02 M potassium phosphate buffer) using lucigenin-dependent CL. Each point represents mean ± SEM; N = 3–5. Arrows indicate time at which CL equaled the background CL of the GSH–lucigenin mixture alone.

RESULTS

Total selenite-catalyzed CL (over 60 min) decreased significantly ($P < 0.01$) with increasing Se concentration, ranging from a high of 10,472 counts (0.06 mM Se) to a low of 1,032 counts (0.25 mM Se) (Fig. 1). However, total selenite-catalyzed CL was similar ($P = 0.12$) at both the 0.06 and 0.13 mM Se levels. The time for the selenite-catalyzed CL to reach background CL was significantly ($P < 0.05$) greater for 0.06 mM Se than for 0.13, 0.19 or 0.25 mM Se, which were all similar (Fig. 1).

Both total selenite-catalyzed CL and the conversion of selenite to Se° were significantly ($P < 0.05$) greater with 0.13 mM Se than with 0.06 mM Se over the first 6 min of the reactions (Fig. 2, a and b). At the end of the first 6 min, the reduction of selenite to Se° was complete, whereas the CL reaction continued for 24 min before reaching background CL. Over the first 6 min, the addition of lucigenin to either level of Se resulted in a negative absorbance at 400 nm (Fig. 2b). However, after 6 min, the conversion of selenite to Se° was similar at both the 0.06 and 0.13 mM Se levels in both the presence and absence of lucigenin (Fig. 2b).

Copper(II), Zn^{2+} and ATA all significantly ($P < 0.0001$) inhibited selenite-catalyzed CL (Fig. 3). Copper inhibited selenite catalyzed CL ≥ 95% at Cu^{2+}:Se molar ratios between 1:1 and 10:1. Zinc sulfate did not inhibit selenite-catalyzed CL at the Zn^{2+}:Se molar ratio of 1:1. However, at

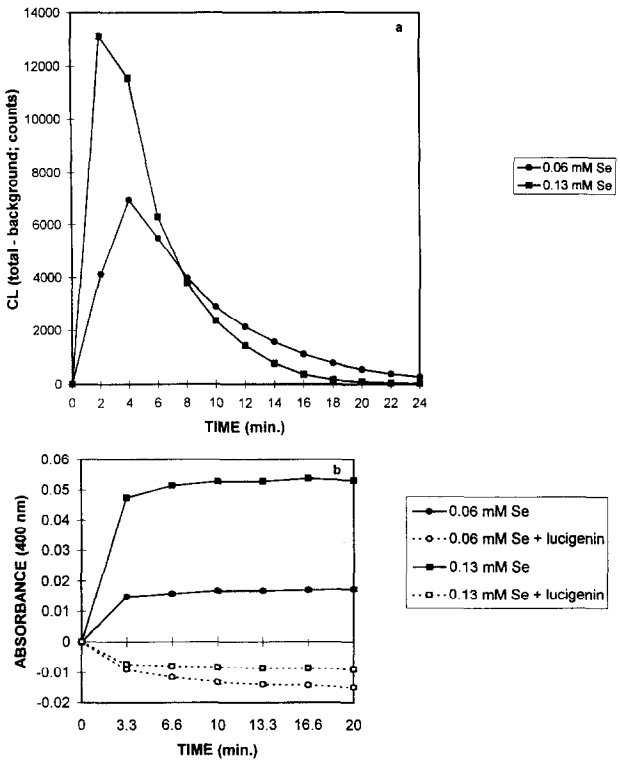

FIG. 2. Time–course of concurrent selenite-catalyzed CL (a) and conversion of selenite to Se° (b). CL was determined in the presence of 1 mL of a GSH–lucigenin mixture (0.5 mg GSH/mL and 1.0 mg lucigenin/mL in 0.02 M potassium phosphate buffer) using lucigenin-dependent CL. Se° levels were determined in the presence of 1 mL of GSH–lucigenin mixture (with and without 1.0 mg lucigenin/mL) at 400 nm. Each point represents mean ± SEM, N = 3.

molar ratios of 5:1 and 10:1, $ZnSO_4$ inhibited selenite-catalyzed CL by 52 and 86%, respectively. ATA inhibited selenite-catalyzed CL between 50 and 86% at ATA:Se molar ratios between 1:1 and 10:1. Sodium (Na^+) as Na_2SO_4 did not inhibit selenite-catalyzed CL at Na^+:Se molar ratios > 10:1 (data not shown), which indicated that the metal ions (Cu^{2+} and Zn^{2+}), and not the sulfate, were responsible for the inhibition of the selenite-catalyzed superoxide CL.

Further analysis of the inhibition by Cu^{2+} at the lowest concentration tested indicated that Cu^{2+} inhibited the selenite-catalyzed CL by >87% at a Cu^{2+}:Se molar ratio of 0.24:1 (Fig. 4). Inhibition of the selenite-catalyzed CL decreased when the Cu^{2+}:Se molar ratio was reduced to 0.02:1 at which point only 4% inhibition (NS, $P > 0.3$) was observed (Fig. 4).

Selenium-catalyzed CL was significantly ($P < 0.002$) inhibited by Cu^{2+} in a concentration-dependent manner over time (Fig. 5a). Over the same 10-min period, Cu^{2+} also significantly ($P < 0.05$) inhibited the generation of Se° (Fig. 5b). Similarly, Zn^{2+} significantly inhibited the selenite-catalyzed CL (Fig. 5c). Both 0.13 and 0.64 mM Zn^{2+} significantly ($P = 0.0001$) inhibited the generation of Se° over the 10-min period (Fig. 5d). Selenite-catalyzed CL and the generation of Se° were also significantly ($P < 0.001$) inhibited by both 0.10 and 0.20 mM ATA over the 10-min period (Fig. 5, e and f).

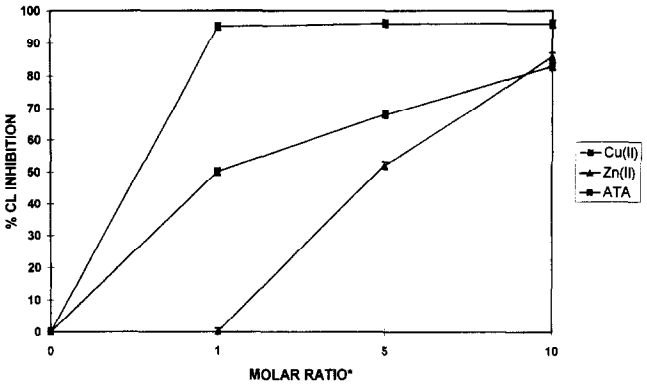

FIG. 3. Percent inhibition (mean ± SEM, N = 4) of selenite-catalyzed CL by metal (Cu^{2+}, Zn^{2+}) sulfates and ATA. The assays were performed using 0.03 mM Se as sodium selenite in the presence of 1 mL of GSH–lucigenin mixture (0.5 mg GSH/mL and 1.0 mg lucigenin/mL in 0.02 M potassium phosphate buffer). CL was determined using lucigenin-dependent CL. CL (counts/10 min) was 639 ± 2 (SEM) and 17,591 ± 283 (SEM), respectively, for the background CL of the GSH–lucigenin mixture and selenite in the presence of the GSH-lucigenin mixture. Key: (*) Metal ion (or ATA) to Se molar ratio.

DISCUSSION

Selenite has been shown to catalyze the oxidation of thiols *in vitro* and *in vivo*, which results in production of superoxide and likely other ROS [7, 19]. Seko *et al.* [5] first showed that selenite catalyzed the oxidation of GSH with the subsequent generation of superoxide and Se° as proposed in Equation 1.

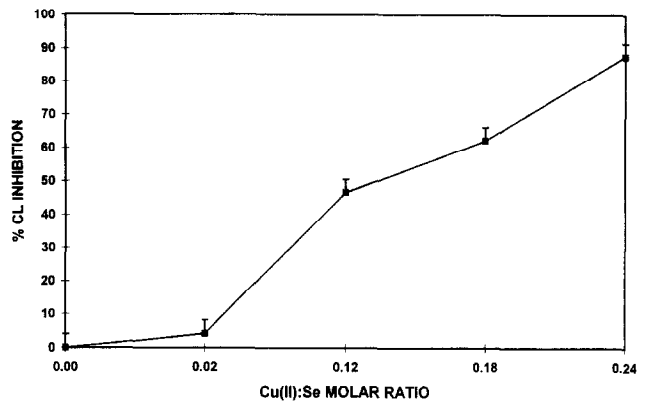

FIG. 4. Percent inhibition (mean ± SEM, N = 4) of selenium-catalyzed CL by Cu^{2+} (as $CuSO_4$) at different Cu^{2+}:Se molar ratios. The assays were performed using 0.03 mM Se as sodium selenite. CL was determined in the presence of 1 mL of GSH–lucigenin mixture (0.5 mg GSH/mL and 1.0 mg lucigenin/mL in 0.02 M potassium phosphate buffer) using lucigenin-dependent CL. CL (counts/10 min) was 608 ± 1 (SEM) and 17,181 ± 861 (SEM) respectively, for the background CL of the GSH–lucigenin mixture and selenite in the presence of the GSH–lucigenin mixture.

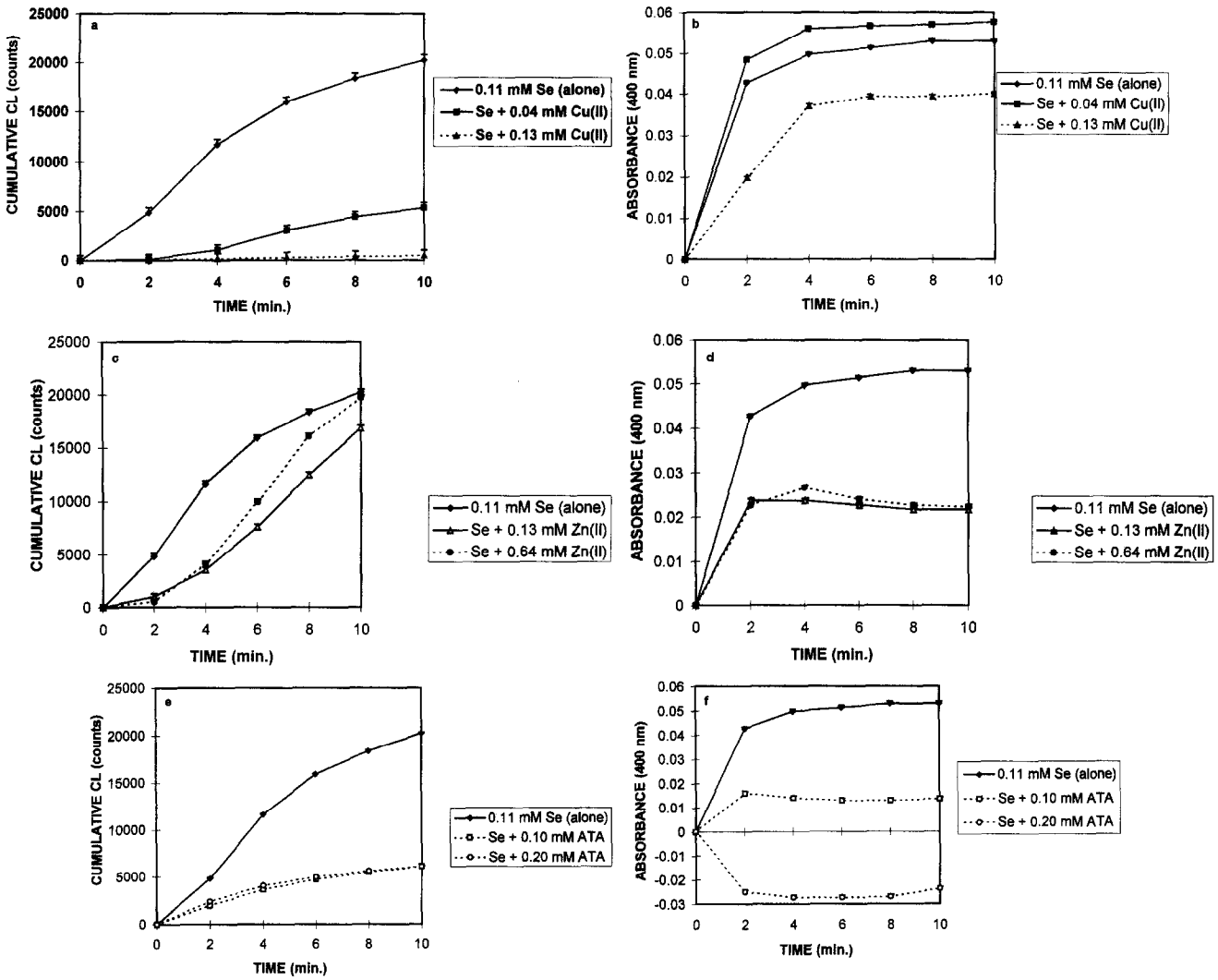

FIG. 5. Effect of 0.04 and 0.13 mM Cu^{2+} (a, b), 0.13 and 0.64 mM Zn^{2+} (c, d) and 0.10 and 0.20 mM ATA (e, f) on selenite-catalyzed CL and the conversion of selenite to Se°. Assays were performed using 0.11 mM Se as sodium selenite in the presence of 1 mL of glutathione (0.5 mg/mL). CL was determined in the presence of 1.0 mg lucigenin/mL using lucigenin-dependent CL, and Se° levels were determined using a spectrophotometer. Each point represents mean ± SEM, N = 3.

$$\underset{\text{4GSH GSSH}}{SeO_3^{2-}} \xrightarrow{\text{GSH GSSG}} \underset{}{GSSeSG} \xrightarrow{\text{GSH GSSG}} \underset{}{GSSeH} \xrightarrow{O_2\ O_2^{\cdot-}} \underset{}{H_2Se} \longrightarrow Se^0 \qquad (1)$$

Figures 1 and 2 suggest that the oxidation of GSH by selenite is not necessarily stoichiometric (GSH:Se, 4:1) as originally proposed by Seko *et al.* (Eq. 1) but rather that low levels of selenite continuously catalyze the oxidation of GSH at GSH:Se ratios >4:1 without the formation of Se° (Fig. 2b). However, the observations of Seko *et al.* [5] do suggest that selenite-catalyzed superoxide generation *in vitro* might be a cause of selenite toxicity and free radical stress *in vivo*. Numerous authors have now shown that selenite toxicity is likely a result of thiol oxidation and free radical generation [19–21]. Kramer and Ames [8] provided evidence that the reaction of selenite with intracellular thiols generated ROS, which they suggested were the principal causal agents of selenite mutagenicity and toxicity in *Salmonella typhimurium*. Additionally, Kitahara *et al.* [21] suggested that the superoxide anion and its reactive metabolites such as the hydroxyl radical (•OH) may be involved in the cytotoxicity of selenite. Lu *et al.* [11] recently found that selenite-induced apoptosis of mouse leukemic L1210 cells could be inhibited by treatment with Zn^{2+} or ATA.

The present findings that Cu^{2+}, Zn^{2+} and ATA inhibit both selenite-catalyzed CL and the conversion of selenite to Se° may lend insight into the mechanism(s) by which Zn^{2+} and ATA inhibit selenite-induced endonuclease activity and apoptosis as previously reported by Lu *et al.* [11]. That selenite generates free radicals *in vivo* from the oxidation of GSH seems plausible, in that the appearance of single-strand breaks in DNA is a common consequence of the interaction of oxygen radicals with DNA [15]. Furthermore, the generation of ROS has been suggested recently to serve as a mediator of apoptosis [16].

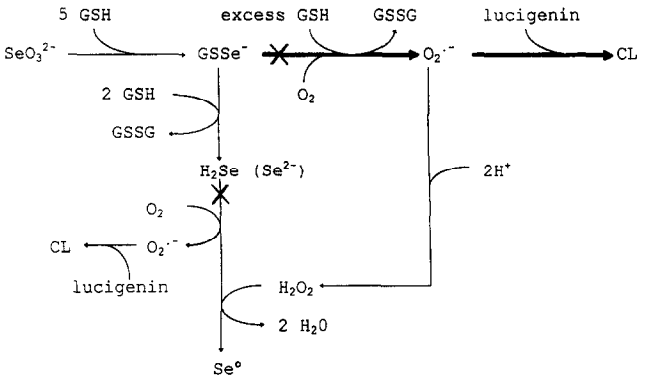

FIG. 6. Proposed mechanism for the inhibition of selenite-catalyzed superoxide generation and conversion of selenite to Se° by Zn^{2+}, Cu^{2+} or ATA. An "X" indicates the points of possible inhibition by metal ion or ATA. In this proposed mechanism, lucigenin oxidizes superoxide, preventing the formation of H_2O_2 by spontaneous superoxide dismutation and the oxidation of H_2Se to Se°. This occurs at low Se:GSH ratios accounting for the results in Fig. 1, showing that total CL is greater at the low Se concentration.

The $GSSe^-$ anion is known to be the catalytic species of the selenite-catalyzed reduction of methylene blue and ferric cytochrome *c* [3]. Diselenides, such as selenocystamine, are reduced by thiols that produce a selenide anion, RSe^- [22], which oxidizes GSH, producing superoxide. Cu^{2+}, Zn^{2+}, and ATA also inhibit lucigenin CL from the generation of superoxide that results from the reaction between GSH and reduced selenocystamine (data not shown).

In animals, heavy metal toxicity is known to be inhibited by the formation of insoluble selenides with mercury, lead, cadmium, copper, and silver rendering both the heavy metal and the selenium less toxic or non-toxic [23, 24]. We suggest that Cu^{2+}, Zn^{2+} and ATA all inhibit selenite-catalyzed CL and the conversion of selenite to Se° by GSH because of their complexation with the selenide anion of GSH forming GSSe–Cu^{2+}–SeSG or GSSe–Zn^{2+}–SeSG coordination complexes, or possibly by complexing with Se^{2-} to form Cu^{2+}- or Zn^{2+}-selenides. ATA, like Cu^{2+} and Zn^{2+}, has a strong electrophilic (carbon) center, which likely also binds to $GSSe^-$ or Se^{2-}.

Copper(II), Zn^{2+}, and ATA were effective in inhibiting selenite-catalyzed CL; however, Cu^{2+} was able to inhibit selenite-catalyzed CL at extremely low concentrations (Cu:Se molar ratio of 0.12:1). At these very low Cu^{2+} concentrations, Cu^{2+} may also act as a SOD mimic because CL inhibition was observed at Cu:Se molar ratios of less than 1:1. Chelates of transition metals have been found to have SOD-like activity and protect various biological systems from oxidative damage [25–27]. Schrauzer [28] had earlier suggested that Zn^{2+} inhibits the selenium-catalyzed oxidation of thiol groups of growth factors, likely as described here. Figure 6 illustrates our proposed mechanism that likely accounts for the experimental results discussed herein as well as the inhibition of both superoxide generation and the conversion of selenite to Se°.

The experimental results herein suggest that Cu^{2+}, Zn^{2+}, and ATA, like iodoacetate and mercaptosuccinate [29], are inhibitors of the selenopersulfide anion which further oxidizes GSH, causing free radical generation.

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