0026-895X/87/060643-04$02.00/0

MOLECULAR PHARMACOLOGY, 31:643-646

Selenite-Induced NAD(P)H Oxidation and Calcium Release in Isolated Mitochondria: Relationship to *In Vivo* Toxicity

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Received July 22, 1986; Accepted March 18, 1987

SUMMARY

The effects of selenite on the mitochondrial NAD(P)H/NAD(P) ratio and calcium pool are described. Small quantities of selenite can 1) oxidize mitochondrial NAD(P)H and 2) induce calcium release from isolated mitochondria. Reduced NAD(P)H within intact mitochondria was monitored kinetically using the wavelength pair, 340-375 nm. NAD(P)H oxidation rates at various concentrations of selenite were calculated. Mitochondria from older animals can oxidize NAD(P)H faster than those of younger animals; maximum selenite-induced oxidation rates correlate well with age of the animal in both kidney ($r = 0.920$) and liver ($r = 0.839$) mitochondria, the oxidation rates in the adult (liver 15.4, kidney 34.8 nmol/min/mg of protein) being 3–5 times the rates in the 1- to 2-day-old newborn (liver 2.8, kidney 10.3 nmol/min/mg protein). Calcium fluxes within mitochondrial suspensions were monitored kinetically using the calcium indicator, Arsenazo III, and the wavelength pair, 660-685 nm. Susceptibility to selenite-induced calcium release is age dependent, the mitochondria of older animals being more susceptible. Incubation time required to induce calcium release was 77 ± 30 sec in the adult compared to 406 ± 25 sec at the age of 0–4 days in the newborn. The bimodal toxic manifestations of selenite *in vivo* are discussed in view of the age-dependent differences in selenite metabolism at the cellular level.

The cellular metabolism of selenite has been extensively studied. As illustrated by the diagram in Fig. 1 (1–7), selenite is reduced spontaneously by glutathione and other sulfhydryl compounds in the cell (1, 2). Oxidized sulfhydryl groups are reduced at the expense of NAD(P)H via GSSG reductase (2–4). GSSG reductase can catalyze reactions II and III as well as the reduction of GSSG (4, 5). Large quantities of cellular NAD(P)H may be consumed when metabolites of selenite enter redox cycles. Once formed, selenide, a very toxic metabolite (8), is methylated under physiological conditions to the nontoxic, volatile dimethyl selenide which is excreted through the lungs (5, 9, 10).

Curiously, the toxic manifestations of selenite in whole animals are dependent on age. Subcutaneous aqueous injection of 20 μmol of selenite/kg body weight in newborn rats causes bilateral nuclear cataracts within 3–4 days without any other toxic effects (11). When rats are injected after 15 days of age, the frequency of cataract formation falls rapidly while mortality rate increases. Mortality approaches 100% at the adult age of 50 days (12). The underlying mechanisms for selenite toxicity and cataractogenesis are unknown.

Cellular calcium homeostasis can be profoundly affected by selenite-oxidized sulfhydryl compounds. In most mammalian cells, intracellular free calcium concentrations are controlled by several transport mechanisms which exist in the endoplasmic reticulum and in mitochondrial and plasma membranes. Oxidation of GSH releases calcium from endoplasmic reticulum of isolated hepatocytes (13, 14). Lehninger and others (15–17) have demonstrated that decreases in the mitochondrial NAD(P)H/NAD(P) ratio can elicit release of mitochondrial calcium. Since selenite can both 1) oxidize reduced glutathione and 2) deplete NAD(P)H via redox cycling, it probably has profound effects on cellular calcium homeostasis.

This paper describes experiments conducted to assess the effects of selenite on the mitochondrial calcium pool. Evidence is provided to show that: 1) mitochondrial NAD(P)H is oxidized in the presence of small quantities of selenite, 2) selenite causes calcium release from liver mitochondria, and 3) the rates of selenite-induced NAD(P)H oxidation and calcium release differ between the newborn and the adult.

Materials and Methods

Topeka guinea pigs were used as experimental animals. Guinea pigs were chosen since the size of newborn animals is sufficient to allow isolation of mitochondrial preparations from one animal at a time. Adult animals were more than 40 days of age. All animals were maintained on *ad libitum* guinea pig chow and water. Newborns were kept with their mothers and allowed to nurse until the time of sacrifice.

These studies were supported in part by National Institutes of Health Grant GM 33267. A. A. V. is an M.D./Ph.D. scholar supported by the Medical Research Foundation of Oregon.

ABBREVIATION: GSSG, oxidized glutathione.

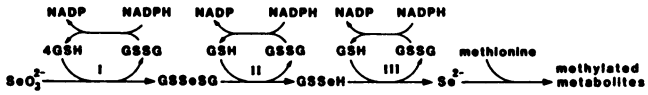

Fig. 1. The metabolism of selenite (SeO_3^{2-}). *GSH*, reduced glutathione.

Liver and kidney mitochondria were chosen as objects of the study because of the important role of these organs in glutathione metabolism (18, 19). Mitochondria were isolated according to the method of Widener and Mela-Riker (20). Protein content of isolated mitochondria was determined according to the method of Lowry *et al.* (21).

NAD(P)H oxidation and calcium measurements were made kinetically using a Hitachi 557 dual wavelength spectrophotometer. NAD(P)H/NAD(P) redox changes of the mitochondrial suspensions were monitored at 340–375 nm. Calcium uptake and release from mitochondria were monitored kinetically with the calcium indicator, Arsenazo III (50 μM) (22), using the wavelength pair 660-685 nm as described by Lehninger *et al.* (15). Calcium was added as calcium chloride. Mitochondria for both determinations were suspended in a medium containing 65 mM KCl, 125 mM sucrose, 8 mM Tris-Cl, 0.2 mM Tris-PO_4, 5 mM $MgCl_2$, 5 μM rotenone, and 2 mM succinate at pH 7.2.

Respiratory activities of mitochondrial suspensions in state 3 and state 4 were determined using a Clark oxygen electrode (5 mM succinate, 1 mM ADP). Respiratory control ratios greater than 10 were obtained in all preparations.

Results

The rate of NAD(P)H oxidation was determined at various concentrations of selenite in liver and kidney mitochondria isolated from animals of different ages. Isolated mitochondria were suspended in the medium described above at 1.7–2.3 mg of protein/ml. Reduced NAD(P)H was monitored kinetically. Rotenone, a site I inhibitor present in the medium, prevents entry of electrons from NAD(P)H into the respiratory chain while allowing ATP synthesis via succinate and $FADH_2$. Examples of tracings obtained from 3.5-day-old and adult liver mitochondria (both at 1.75 mg of protein/ml) are shown superimposed in Fig. 2. NAD(P)H oxidation was initiated by an addition of 10 μM selenite. The rate of mitochondrial NAD(P)H oxidation is more rapid in the adult than in the newborn. Similar results were obtained with kidney mitochondria (data not shown). The total amount of NAD(P)H oxidized by 10 nmol of selenite was calculated from the change in absorbance at 340 nm. Further calculation shows that 2.5–3.2 nmol of NAD(P)H are oxidized per nmol of selenite which agrees well with the stoichiometry of selenite reduction shown in Fig. 1.

The rates of NAD(P)H oxidation were plotted as a function of selenite concentrations for each animal after standardization as nmol of NAD(P)H per min per mg of mitochondrial protein. Fig. 3 depicts titration curves constructed using liver mitochondria from animals of different ages. As the selenite concentration increases, the rate of NAD(P)H oxidation in all animals initially increases rapidly and then plateaus at about 300 μM selenite.

The maximal rates of NAD(P)H oxidation (at 500 μM selenite) in both liver and kidney mitochondria were plotted as a function of the age of the animal and are shown in Fig. 4. The maximal rate of NAD(P)H oxidation in liver mitochondria at 500 μM selenite increases from 2.8 (nmol/mg of protein × min) in the 1.5-day-old to 15.4 in the adult: a 5.5-fold increase. The maximal rate of NAD(P)H oxidation in kidney mitochondria similarly increased from 10.3 in the 1.5-day-old to 34.8 in the adult: a 3.4-fold increase.

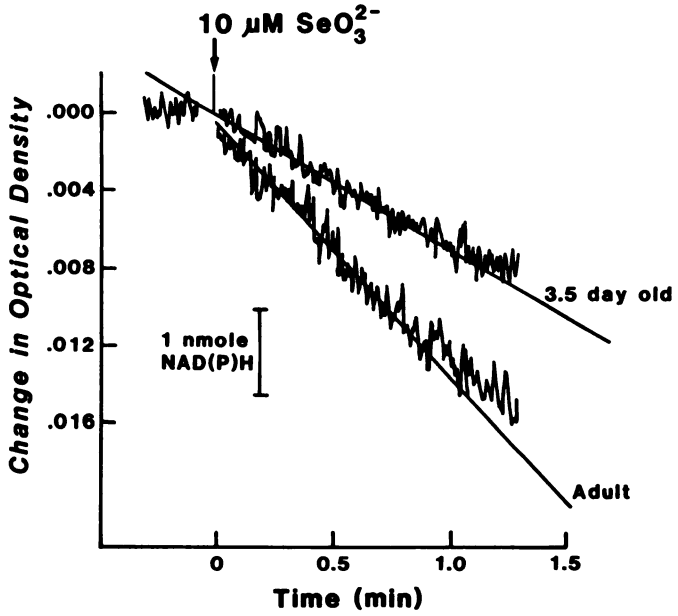

Fig. 2. Change in absorbance at 340–375 nm versus time. Tracings show the oxidation of NAD(P)H in isolated liver mitochondria following the addition of selenite. Tracings from 3.5-day-old and adult are superimposed (both cuvettes contained 1.75 mg of mitochondrial protein/ml).

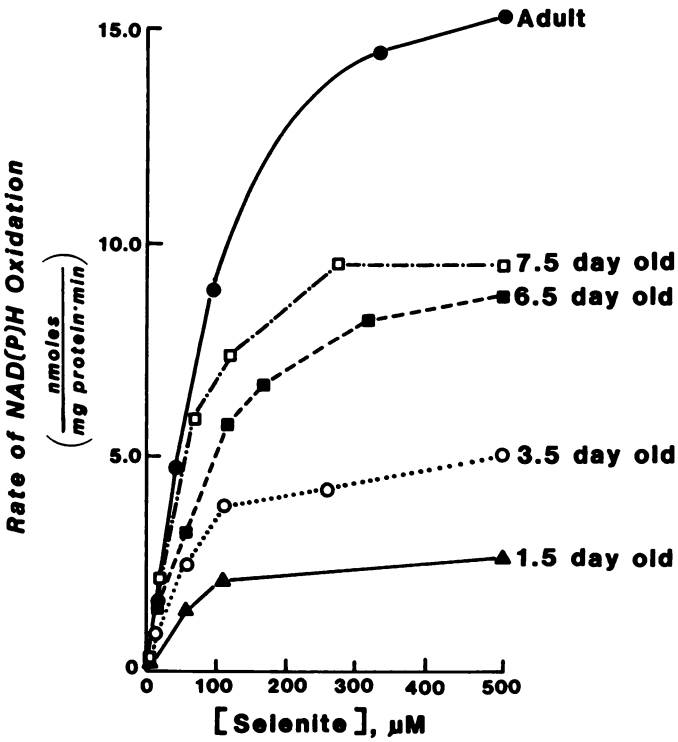

Fig. 3. Rate of NAD(P)H oxidation by isolated liver mitochondria versus selenite concentration from animals of different ages. As the age of the animal increases, the maximum rate at which NAD(P)H is oxidized also increases. All rates are standardized per mg of mitochondrial protein.

Results from calcium uptake and release experiments using liver mitochondria from animals at different ages are displayed in Figs. 5 and 6 and Table 1. The medium contained reducing equivalents in the form of succinate (2 mM) in the presence of 5 μM rotenone. Calcium release was induced by selenite after preloading the mitochondria with 5–70 μM calcium. Mitochondria from the younger animals (Fig. 5, Table 1) were less

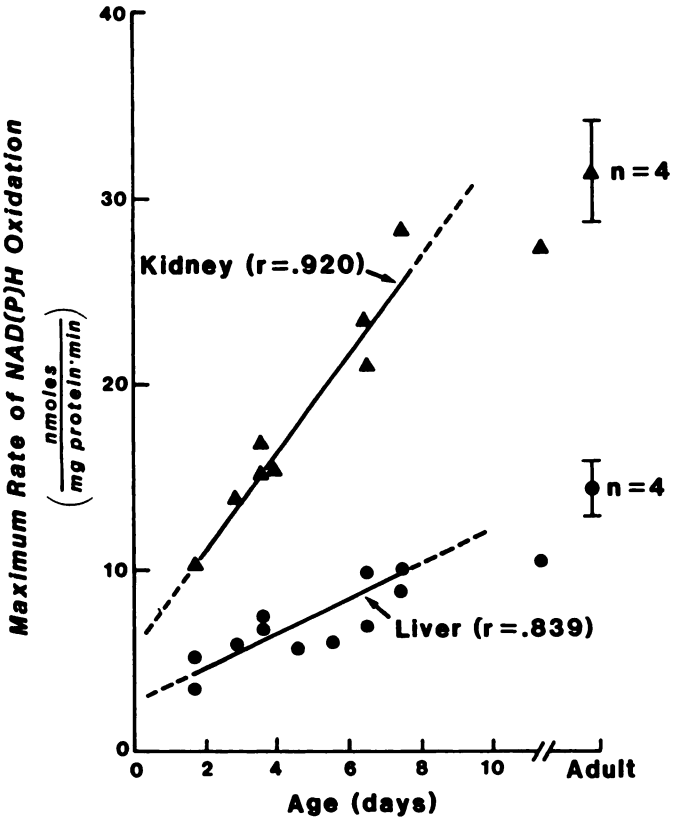

Fig. 4. Maximum rate of selenite-induced NAD(P)H oxidation in isolated liver and kidney mitochondria versus the age of the animal. Each *symbol* depicts the maximum rate obtained from a single animal. Adult rates are expressed as means ± 1 SD.

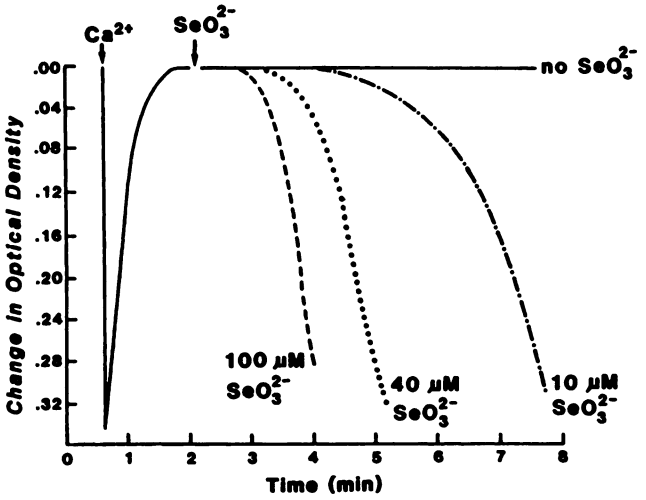

Fig. 5. Selenite-induced release of calcium from the isolated liver mitochondria of a 7.5-day-old animal. Ca^{2+} (70 μM) is added to cuvettes containing 1.25 mg of mitochondrial protein/ml. Once Ca^{2+} uptake by the mitochondria is complete, selenite is added. Four tracings are shown superimposed which demonstrate the Ca^{2+}-releasing effect of various concentrations of selenite.

susceptible to selenite-induced calcium release than the mitochondria from the older animals (Fig. 6, Table 1). The preloading dose of calcium had no effect on this age-dependent calcium release (data not shown).

Similar calcium uptake and release experiments were done using pyruvate (2 mM) and malate (0.7 mM) as substrates in the absence of rotenone. Calcium responses of liver mitochondria from 1.5-, 2.75-, and 6.5-day-old animals showed similar age dependencies. Specifically, mitochondria from the 1.5-day-old retained calcium in the presence of 500 μM selenite for longer than 8 min. In the presence of 50 μM selenite, mitochondria from 2.75- and 6.5-day-old animals began releasing calcium after 2–2.5 min and 40–60 seconds, respectively. Susceptibility to selenite-induced calcium release from liver mitochondria shows an age dependency, the older animals being more susceptible.

The effect of selenite on the integrity of the mitochondrial inner membrane was assessed by measuring state 3 respiratory rates (nmol of O_2 consumed/mg of protein/min) in the presence and absence of selenite (500 μM), using succinate (5 mM) with rotenone (5 μM) as substrate. Maximum state 3 respiratory rates with selenite (49.4 ± 0.74) are reduced by only 10.7% when compared to control values (55.3 ± 1.06). The small reduction in respiratory rate may be explained by incomplete rotenone block (5 μM), allowing reversed electron flow to NAD(P)H.

Discussion

Small quantities of selenite can oxidize liver and kidney mitochondrial NAD(P)H (Fig. 2), resulting in the release of mitochondrial calcium. In adult liver mitochondria, the rate of NAD(P)H oxidation in the presence of just 10 μM selenite is approximately 2.5–3.2 nmol of NAD(P)H per nmol of selenite (per mg of protein × min). Similarly, small quantities of selenite can cause calcium release from the liver mitochondria of older animals (Fig. 5, Table 1). Calcium release is known to occur in response to decreases in the NAD(P)H/NAD(P) ratio induced by normal metabolites (15–17). Selenite-induced oxidation of NAD(P)H is most likely responsible for the calcium release observed in the isolated liver mitochondria depicted in Figs. 5 and 6 and Table 1. Selenite concentrations which induce calcium release do not significantly impair respiration. Therefore, calcium release cannot be explained by generalized membrane damage.

Age-dependent differences in the NAD(P)H oxidation rates of liver and kidney mitochondria may help explain the bimodal toxic manifestations of selenite. As described above, the cataractogenic effect of selenite is seen only in newborns less than 15 days of age. After 15 days, the same dose becomes noncataractogenic and increasingly lethal (12). Mortality in the adult may be explained by two mechanisms. First, the rapid reduction of selenite by adult mitochondria favors the complete three-step reduction of selenite to selenide. Selenide, once formed, is very toxic (18), and methylation pathways become an important means of detoxification (5, 10). Second, cellular calcium homeostasis can be altered by NAD(P)H consuming redox cycles (15–17) and GSSG (13, 14), leading to cell dysfunction and death (23). Conversely, newborn liver and kidney mitochondria have a decreased capacity to reduce selenite; therefore, the formation of toxic selenide is predictably much lower than that of the adult. Seemingly, a smaller percentage of the selenite dose becomes detoxified (i.e., methylated) by the newborn liver and kidney per unit time, and plasma selenite concentrations remain elevated for longer periods. Subsequently, other sulfhydryl-rich areas of the body which receive a small portion of the cardiac output, like the eye, gain the opportunity to sequester plasma selenite. In support of this hypothesis, Babicky *et al.* (24) have recently shown that newborn rat lenses contain

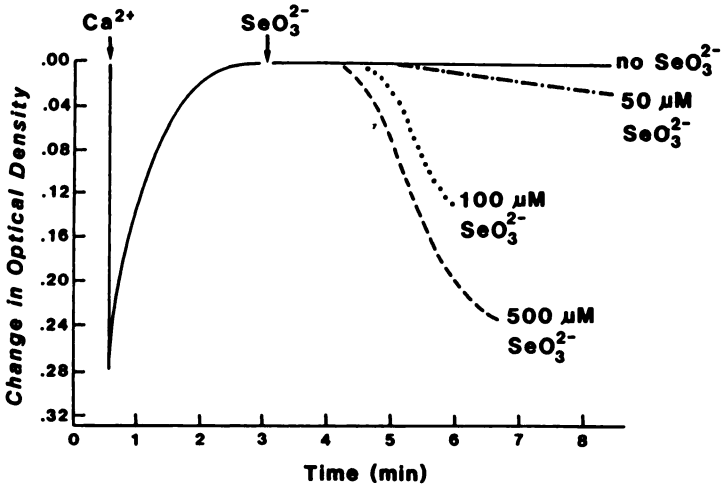

Fig. 6. Selenite-induced release of calcium from the isolated liver mitochondria of a 4.5-day-old animal. Four tracings are shown superimposed. Ca^{2+} (70 µM) is added to cuvettes containing 1.05 mg of mitochondrial protein/ml. Compare with Fig. 5 (7.5-day-old). The 4.5-day-old liver mitochondria are less susceptible to selenite-induced Ca^{2+} release.

TABLE 1

Age-dependent calcium release from isolated liver mitochondria

Mitochondria were incubated under conditions similar to those described for Fig. 5. The time period between the addition of 500 µM selenite and the initiation of calcium release was recorded and standardized for µmol of selenite per mg of mitochondrial protein. Data are presented as mean ± standard deviation (n = a minimum of 3 in each group). Isolated mitochondria from the older animals are more susceptible to selenite-induced calcium release.

Age (days)	Time (sec)
Birth–4	406 ± 25
4–6	193 ± 36
6–12	75 ± 12
Adult	77 ± 30

almost 500 times more radioselenium than do adult lenses (per unit weight) at 2 hr after a subcutaneous injection of 30 µmol of selenite/kg body weight. Bunce *et al.* (11) and Shearer and David (25) also emphasized the role of Ca^{2+} dyshomeostasis in selenium-induced cataracts. No previous evidence has been presented on the possible role of mitochondria in this response, although selenite-induced alterations of glutathione metabolism in the lens have been confirmed (26, 27).

In summary, selenite-induced NAD(P)H oxidation rates of liver and kidney mitochondria at different ages are reported and correlated to the age-dependent susceptibility of selenite-induced mitochondrial calcium release. A possible mechanism for the observed increase in lens selenium of the newborn and the increased lethality in the adult is proposed.

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