Microsomal Ca\(^{2+}\) flux modulation as an indicator of heavy metal toxicity

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Inositol 1,4,5-trisphosphatase (IP3), an intracellular messenger, releases Ca\(^{2+}\) from microsomes. Ca\(^{2+}\) plays a major role in regulating various cellular events like neural transmission and regulation of hormones and growth factors. Aluminum (Al), lead (Pb) and mercury (Hg) were reported to alter Ca\(^{2+}\)-regulated events thereby causing neurotoxicity. Hence, an attempt was made characterize IP3 mediated Ca\(^{2+}\) release from rat brain microsomes under the influence of Al, Pb and Hg. Different concentrations of metals were tested over a designated time scale and their effects on IP3 mediated Ca\(^{2+}\) release from microsomes were monitored using Fura-2 technique. All the three metals inhibited IP3 mediated Ca\(^{2+}\) release, Pb being more potent. The order of potency of these three metals was Pb>Hg>Al. Except for Al, both Hg and Pb independently released Ca\(^{2+}\) from microsomes. Re-uptake of Ca\(^{2+}\) into microsomes was inhibited by all the three metals, Pb being more potent. Microsomal Ca\(^{2+}\)-ATPase activity was also inhibited by all the three metals. These results suggest that neurotoxicity exerted by Al, Pb and Hg may be due to the interference of these metals with IP3 mediated calcium release and also interfering with the microsomal Ca\(^{2+}\) sequestration mechanism. Differential effects of heavy metal induced changes in Ca\(^{2+}\) flux can be used as an index of relative toxicity.

**Keywords:** Ca\(^{2+}\) flux, Heavy metal toxicity, Inositol 1,4,5-trisphosphate, Microsomes.

Several metals have been known to be toxic to man for centuries. Numerous hazardous heavy metals are inhaled and absorbed by humans and animals every day. The major concern with heavy metals is their ability to accumulate in the environment and thereby passing up the food chain. Though acute heavy metal toxicity is limited to exposures in specific occupations, chronic low-grade toxicity is more damaging long-term, contributing to chronic illness. Heavy metals and other toxins may alter, remove, or impair the production of specific molecules needed in the body. They may alter the structure of various entities such as the mitochondria or a cell nucleus and also have a profound impact on membrane integrity. Heavy metals can also create disturbances in the cell-to-cell communication occurring between inflammatory mediators, nerve cells, or hormones. Metals target sites such as membrane or structural proteins, enzymes, or DNA molecules. Although other metals can be toxic under certain circumstances, aluminum, lead and mercury are generally considered to be foremost in public health concerns.

Aluminum is a relative newcomer to the list of toxic metals, long having been considered harmless. Aluminum comes from foods cooked or stored in aluminum pans and aluminum foil. Leafy vegetables, rhubarb, and apples cooked in aluminum pans are prone to leach the metal from the pan. Pressure cookers are especially likely to impart metal into the food. Tap water may contain aluminum when it is used in water reservoirs to flocculate silt from the water. Other sources include antacids taken for stomach trouble, some antiperspirants, food additives, and milk substitutes. Accumulation of aluminum in man and animals have been implicated in diseases like Alzheimer’s and dementia, impaired visual motor coordination and cause decreased visual motor performance, poor long-term memory. Numerous reports have shown that lead exposures in children can result in intelligence quotient deficits. The incidence of documented lead toxicity has declined substantially since leaded gasoline was phased out in the early 1980s. Some of the potential sources for lead exposure are flaking lead paint in older buildings, tap water running through lead pipes, outdoor soil contamination as fallout from earlier usage of leaded gasoline, lead soldering and even art supplies. The well documented affects of lead in smelting and storage battery production include hemopoietic alterations, paralysis and colic.
Mercury is a potent neurotoxin that tends to accumulate in the body and is not easily expelled. Perhaps the most controversial and lively issue concerning mercury today surrounds the silver/mercury dental fillings. Though new composite, porcelain, or ceramic fillings are being used for dental work, still mercury constitutes about 50% of these fillings. Also, the wide use of mercury as fungicide for seed treatment is a potential hazard for human and animal life.

The underlying mechanism(s) of neurological signs caused by heavy metals involve the impairment of neural transmission and these heavy metals are known to interact with cell membrane and alter its function. Maintenance of the integrity of membrane to prevent cell injury and loss of cell viability is invariably linked with the change in Ca$^{2+}$ homeostasis. Intracellular Ca$^{2+}$-homeostasis is maintained by the concerted operation of cellular transport and compartmentalization systems. Phosphoinositide derived second messengers play an important role in the propagation of signal transduction within the cell. Binding of the agonist to the receptor site on the membrane releases inositol phosphates generated from the hydrolysis of membrane bound phosphoinositol bisphosphate. Among the many inositol phosphates formed, Inositol 1, 4, 5-trisphosphate (IP3) is the mediator of transient Ca$^{2+}$ release from intracellular stores. This release of Ca$^{2+}$ is responsible for the regulation of coherent metabolic pathways and Ca$^{2+}$ also act as a messenger in propagating signal transduction within the cell. Comparing the effects induced by heavy metals in regulating/modulating microsomal Ca$^{2+}$ flux can aid as an indicator of how potent the metal will be as a toxicant. The present study was undertaken to assess an indicator of how potent the metal will be as a regulating/modulating microsomal Ca$^{2+}$.

**Materials and Methods**

**Animals**— Male Sprague-Dawley rats (200-250 g each) were obtained from Charles River Laboratories, Wilmington, MA and maintained in central animal facilities under a 12 h photoperiod at 21°C ± 1°C and 50 to 80% RH. The animals were provided with water and Purina Rat Chow ad libitum, and acclimatized for one week before experimentation.

**Chemicals**— Ca$^{2+}$ fluorescent probe Fura-2 was purchased from Molecular Probes, Inc., Eugene, OR. All other chemicals used in the present study were obtained from Sigma Chemical Company, St. Louis, MO. Stock solutions of metallic salts were prepared in distilled water and 1 ul of the test solutions were added to the assay to obtain final concentrations.

**Preparation of microsomes**— Microsomal fraction from rat brain was prepared as described previously. All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of UMC, USA. Furthermore, all procedures conformed to the Guidelines for the Care and Use of Laboratory Animals, American Physiologic Society, USA. Rats were killed by decapitation and the brains were quickly removed and chilled on ice. Subsequent procedures were performed at 4°C. The tissue was homogenized with a glass-Teflon homogenizer in 9 vol. of a microsomal preparation buffer (MPB) containing (mM); sucrose 250, Hepes 5, KCl 10, dithiothreitol 1 and MgCl$_2$ 1, pH 7.05. The homogenate was centrifuged at 1000 g for 10 min and the resultant supernatant fraction was centrifuged at 100,000 g for 1 h to obtain the microsomal pellet.

**Measuring Ca$^{2+}$ movements by fura-2 technique**— Ca$^{2+}$ movements across the microsomal membrane were measured as described previously. The microsomal pellet was resuspended in a buffer containing 150 mM KCl, 5 mM Hepes (pH 7.05), 2 mM MgCl$_2$. Ca$^{2+}$ uptake and release were measured by adding 100 µl microsomal suspension (1.2 mg protein) into a quartz cuvette containing 1.9 ml of a medium composed of (final concentrations in mM); KCl 150, Hepes 5 (pH 7.05), MgCl$_2$ 2, ATP 0.5, phosphocreatine 10, Na$_2$ATP 1, 10 units/ml creatine kinase and 1 mg/ml oligomycin. The cuvette was then placed into a thermostatically controlled sample compartment of a cation measurement fluorescence spectrophotometer (Spex Model, Fluorolog-2). The tissue mixture was incubated at 37°C with constant stirring for 15 min before data were recorded. Changes in the extramicrosomal free Ca$^{2+}$ concentration were monitored with Ca$^{2+}$-sensitive dye fura-2 (2 mM) which was added 1 min before recording. Fura-2 fluorescence was measured at an emission wavelength of 505 nm with excitation alternating between 340 and 380 nm, so that a 340/380 nm ratio could be obtained 30 times a second. To determine the effect of metals and IP3, Ca$^{2+}$-release responses were measured by the difference between the peak signal after, and the steady-state signal just before the addition of the test chemicals. Re-uptake of Ca$^{2+}$ into microsomes was measured by taking the difference between the peak...
signal obtained after IP3 was added and the steady state signal at a period of 75 sec, as IP3 releasable Ca\(^{2+}\) was taken up into the microsomes by 75 sec in control assays. The ratio signal was proportional to the free Ca\(^{2+}\) concentration in the cuvette and the 340/380 ratio was taken as a direct index of the change in extramicrosomal Ca\(^{2+}\).

**Ca\(^{2+}\)-ATPase assay**— Ca\(^{2+}\)-ATPase was assayed in rat brain microsomes as described earlier. Briefly, Ca\(^{2+}\), Mg\(^{2+}\)-ATPase was determined by measuring the inorganic phosphate liberated from the hydrolysis of ATP. The reaction medium contained 135 mM imidazole buffer (pH 7.5), 5 mM MgCl\(_2\), 0.05 mM CaCl\(_2\), 4 mM ATP, different concentrations of metals that were tested and 40 µg of microsomal membrane protein. The reaction mixture was incubated at 37°C for 20 min and the reaction was terminated by adding 0.1 ml of 50% trichloroacetic acid. The inorganic phosphate (Pi) liberated during hydrolysis was measured. The Mg\(^{2+}\)-ATPase activity determined in the presence of 0.5 mM EGTA was subtracted from the total ATPase activity in order to obtain Ca\(^{2+}\)-ATPase activity.

**Statistical analysis**— Data are expressed as the mean + SE of minimum of three experiments assayed in duplicate. Statistical significance was calculated between the control and the activity observed in the presence of test chemical by using Student’s T-test. A value of \(P < 0.05\) was accepted as statistically significant.

**Results**

Microsomes accumulated Ca\(^{2+}\) in presence of ATP until the extramicrosomal free Ca\(^{2+}\) was decreased to 300-500 nM, giving a uniform spectral graph with the fluorescent probe fura-2. On addition of IP3, a rapid release of Ca\(^{2+}\) was observed, that reached a peak by 1-2 sec after the onset, followed by a slower rate of Ca\(^{2+}\) re-accumulation until steady state values were re-established (Fig. 1 – control). Preincubation of microsomes with varying concentrations of heavy metals has differential effects on IP3 mediated Ca\(^{2+}\) release from microsomes (Fig. 2). Lead and mercury at lower concentrations decreased IP3 mediated Ca\(^{2+}\) release whereas, aluminum showed less effect even at higher concentrations (Fig. 2). IP3 triggered an instantaneous release of Ca\(^{2+}\) from microsomes at a concentration as low as 1 µM. (Fig. 1 – control). In absence of IP3, metals themselves showed an increase in the 340/380 ratio indicating the release of Ca\(^{2+}\) from microsomes (Fig. 3). Lead and mercury independently released Ca\(^{2+}\) from microsomes. However, aluminum showed no effect which correlates well with the IP3 mediated Ca\(^{2+}\) release. Also, the threshold concentration to release Ca\(^{2+}\) is much higher for metals when compared to IP3 which is 1 µM. Re-uptake of IP3 releasable Ca\(^{2+}\) into microsomes was significantly altered by metals (Fig. 4). Of all the three metals tested, aluminum is less potent even at higher concentrations. Decrease in Ca\(^{2+}\)-ATPase activity (Fig. 5) of rat brain microsomes by selected concentrations of the metals (concentrations that show significant effect on re-uptake process as shown by fluorescent measurements) confirms the inhibition of Ca\(^{2+}\)-reuptake into the microsomes.

**Discussion**

In excitable cells like neurons, Ca\(^{2+}\) is a ubiquitous first and second messenger and plays a pivotal role in the regulation of cellular metabolic processes. It is now evident that, when Ca\(^{2+}\) homeostasis fails,
as seen in anoxic conditions as well as in chemical insult, the production of peroxy radicals threatens the cell viability. Such states are probably accompanied by the regulation of Ca$^{2+}$ flux in intracellular organelles. It is well known that there is an existence of an IP3 sensitive Ca$^{2+}$ pool located in microsomes. By monitoring the Ca$^{2+}$ movements with fura-2 technique, we observed that heavy metals perturb Ca$^{2+}$ flux mechanisms that are operated through microsomes. As IP3 releasable Ca$^{2+}$ from microsomes is responsible for the initial rapid elevation of [Ca$^{2+}$]i which is involved in the propagation of signal transduction, the additive increase in the metal mediated Ca$^{2+}$ release alters Ca$^{2+}$ homeostasis which may lead to neuronal dysfunction.

It was recognized for many years that toxin mediated cell death is accompanied by large amounts of Ca$^{2+}$ accumulation in the cells. The causative factors responsible for increase in [Ca$^{2+}$]i could be increased influx or release from intracellular stores. The present results showed that, lead and mercury independently release Ca$^{2+}$ from microsomes. Interestingly, aluminum did not have any effect directly on release of Ca$^{2+}$ from microsomes. The rapid release of Ca$^{2+}$ by lead and mercury may directly contribute to the rise in the extramicrosomal Ca$^{2+}$ and can explain the relative toxicity of these metals compared to aluminum.

Metal exposure decreased the IP3 releasable Ca$^{2+}$ pool release from microsomes suggesting that metals interact with the membrane components altering its properties which in turn leads to the disruption of Ca$^{2+}$ homeostasis. Metals alter the membrane integrity, thereby making the microsomal membrane more leaky facilitating Ca$^{2+}$ release. This may be due to the altered membrane permeability thereby facilitating Ca$^{2+}$ flux. The decrease in IP3 mediated Ca$^{2+}$ release

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**Fig. 2**— Effect of (a) aluminum, (b) lead and (c) mercury on IP3 mediated Ca$^{2+}$ release from rat brain microsomes. Microsomes were incubated with varying concentrations of metals for 2 min before addition of IP3. Each value is the mean ± SE of three independent observations. *P<0.05.

**Fig. 3**— Independent effect of heavy metals, (a) lead and (b) mercury on rat brain microsomal Ca$^{2+}$ release. The 340/380 ratio was recorded immediately (for 25 sec) after the addition of different concentrations of metals. Each value is mean ± SE of three independent observations. *P<0.05.
in the presence of lead and mercury may be due to the metal effect on blocking the calcium channel on microsomal membrane. However, aluminum showed no effect which correlates well with the relatively small effect on IP3 mediated Ca\(^{2+}\) release suggesting that aluminum has less effect on the membrane permeability.

Re-uptake of IP3 releasable Ca\(^{2+}\) was significantly affected by metals as seen with fluorescent measurements (Figs 1, 4). Lead and mercury significantly inhibited reuptake of IP3 releasable Ca\(^{2+}\) into microsomes. Mercury completely inhibited the re-uptake mechanisms as seen in the continuous rise in Ca\(^{2+}\) signal in fluorescent measurements, suggesting that mercury permanently damages the membrane and makes the microsomal leaky allowing the intracellular calcium to rise leading to cell death. Again, aluminum only at higher concentrations (80 µM), showed some effect in inhibiting the re-uptake of Ca\(^{2+}\). Correspondingly, Ca\(^{2+}\)-ATPase activity was also decreased by lead and mercury at very low concentrations compared to that of the effect of aluminum suggesting either a direct interaction with the Ca\(^{2+}\) pump or by permanently destroying the membrane integrity as shown by mercury. Re-uptake of Ca\(^{2+}\) into the microsomes is also decreased suggesting the effect of aluminum, lead and mercury on active transport process involving Ca\(^{2+}\) pump. This is in accordance with the studies of ours and others where metals have been shown to be directly interacted with membrane Ca\(^{2+}\) pump.

The present results on brain microsomes showed differential effects on Ca\(^{2+}\) flux mechanisms across microsomal membrane, however characterization of IP3 dependent and independent Ca\(^{2+}\) pools showed that re-uptake mechanism of IP3 dependent Ca\(^{2+}\) pool is more affected by metals. These findings suggest that an augmentation of Ca\(^{2+}\) release from brain microsomes may cause intracellular Ca\(^{2+}\) overload which can activate cytotoxic mechanisms. The release of Ca\(^{2+}\) from microsomes suggests the action of metals on altering the microsomal membrane integrity.

Considering these chains of events involving the inhibition of re-uptake of IP3 released Ca\(^{2+}\), associated with rapid release of Ca\(^{2+}\) by heavy metals and alteration of Ca\(^{2+}\) sequestration, metals may obliterate the Ca\(^{2+}\) transients normally evoked by physiological agonists, thereby resulting in an impairment of cell signaling which leads to neuronal
dysfunction. The results of the present show that of all the three heavy metals studied, mercury has the most profound effect on disrupting Ca\(^{2+}\) flux mechanisms compared to lead and aluminum, aluminum being the least effective. Heavy metal toxicity may be a "silent" threat. As people age they accumulate more heavy metal but are also more susceptible to the toxic effects. Toxicity can cause a wide range of subtle symptoms, but the damage is profound. More frequent testing and evaluating the relative toxic effects with exposure may benefit human health in the long term.

References

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